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Review

Recent Bioorganic Studies on Rhodopsin and Visual Transduction

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Rhodopsin, the pigment responsible for vision in animals, insect and fish is a typical G protein (guanyl-nucleotide binding protein) consisting of seven transmembrane alpha helices and their interconnecting extramembrane loops. In the case of bovine rhodopsin, the best studied of the visual pigments, the chromophore is 11-*cis* retinal attached to the terminal amino group of Lys296 through a protonated Schiff base linkage. Photoaffinity labeling with a 3-diazo-4-oxo-retinoid shows that C-3 of the ionone ring moiety is close to Trp265 in helix F (VI) in dark inactivated rhodopsin. Irradiation causes a *cis* to *trans* isomerization of the 11-*cis* double bond giving rise to the highly strained intermediate bathorhodopsin. This undergoes a series of thermal relaxation through lumi-, *meta*-I and *meta*-II intermediates after which the retinal chromophore is expelled from the opsin binding pocket. Photoaffinity labeling performed with 3-diazo-4-oxoretinal at −196 °C for batho-, −80 °C for lumi-, −40 °C for *meta*-I, and 0 °C for *meta*-II rhodopsin showed that in bathorhodopsin the ring is still close to Trp265. However, in lumi-, *meta*-I and *meta*-II intermediates crosslinking occurs unexpectedly at A169 in helix D (IV). This shows that large movements in the helical arrangements and a flip over of the ring moiety accompanies the transduction (or bleaching) process. These changes in retinal/opsin interactions are necessarily accompanied by movements of the extramembrane loops, which in turn lead to activation of the G protein residing in the cycoplasmic side. Of the numerous G protein coupled receptors, this is the first time that the outline of transduction pathway has been clarified.

Key words transduction path; photoaffinity label; rhodopsin; exciton chirality; Trp2; Ala169

Introduction[1]

The cross section of a human eye is shown in Fig. 1. The estimated 7 million cone cells, responsible for color ("photopic") vision are clustered around the fovea where the incoming image is focused most sharply. These cells contain three kinds of rhodopsins (Rh) or visual pigments absorbing around 450 nm (blue light), 530 nm (green light) and 560 nm (red light). When light passes through a yellow solution, it absorbs the blue light at 450 nm so that green and red light enter our eyes, leading to the sensation of yellow. On the other hand, the 100 million rod cells responsible for black and white ("scopotic") vision are distributed in the peripheral area of the retina and absorb at 500 nm. Since solar energy is strongest at 400—700 nm, the human eye is taking full advantage of the solar energy distribution. The rod/cone cell ratio depends on the animal: in nocturnal rats it is 4000, humans 20, goldfish 15, frogs 1, while owls only have rod cells. Although not nocturnal, bovine (4 million rods), horse, and dog only have rods and hence lack color vision.

The outer segments of rod cells (rod outer segment or ROS) consists of about 1500 thin membranes called disks that are formed at the basis of the rod visual cell by pinching off sections of the plasma membrane. The disks, which embed the 40 kDa Rh molecules consisting of 348 amino acids, move towards the tip of the cell in about 15 d where they are phagocytosed. A breakdown in phagocytosis by the retinal pigment epithelium cells leads to accumulation of orange fluorescent pigments named A2E and iso-A2E that impair the vision, and in certain cases loss of vision or AMD, *i.e.*, age-related macular degeneration.

Visual transduction is the process by which visual cells convert light into a neural signal, which in turn is transmitted to the brain *via* the optic nerve. In the dark, Na$^+$ ions flow into ROS. Upon absorption of one photon of light, the flow of >million Na$^+$ ions is blocked. Namely, absorption of a single photon activates 100 molecules of G proteins (guanyl-nucleotide binding protein, in the case of vision the term transducin is used), which *via* activation of phosphodiesterase, hydrolyzes 100000 molecules of cyclic GMP. This drop in cGMP closes the cation-specific channels and leads to a build-up of electric potential that is picked up by the optic nerves. Rh is one of the most thoroughly investigated G-protein coupled receptors[2] consisting of seven hydrophobic transmembrane helices A, B,... G (or 1, 2,... 7) that are interconnected by hydrophilic extramembrane loops (Fig. 1). The sequence of the 348 amino acids of Rh, a triumph of protein sequencing, was achieved by the groups.[3,4]

The Chromophore and Transduction Intermediates

Unless otherwise specified, the following refers to studies performed with bovine Rh since the readily available bovine rods provide an excellent source for Rh. The chromophore in the majority of visual pigments is 11-*cis*-retinal (**1**, Fig. 2). In bovine Rh this is linked to the terminal amino group of Lys296.[5] The fact that absorption maxima of the visual pigments cover the range 350 nm to 680 nm is quite extraordinary considering the fact that this wide range simply arises from the interaction between the retinal chromophore and their receptors. A number of unique aspects of this chromophore makes it ideally suited for controlling the subtle wavelength regulation of visual pigments so as to adjust the vision to suit the environment[6]:

1. It is a protonated Schiff base (PSB) comprising the terminal moiety of a polyene, and hence the positive charge is delocalized throughout the polyene chain. This delocalization is affected by a various factors, *e.g.*, 2 and 3 in the fol-

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Dedicated to the memory of Dr. Kyosuke Tsuda.

lowing.

2. The counter ion of PSB in bovine Rh is Glu113.[7] In different Rhs, the counterions are different and the distance between the PSB positive charge and counter ion negative charge also differ.
3. The local electrostatic field within the binding site. This concept was originally introduced as the external point charge model.[8]
4. The 1-, 5- and 13-methyl groups are all required for maintaining chromophoric nonplanarity, which in turn is governed by the nature of the Rh binding site.

The 9-methyl group is essential for hydrophobic binding to the Rh receptor which is essential for Rh to change its conformation triggered by the *cis* to *trans* isomerization of the 11-*cis* bond. Finally, retinal is readily available from the ubiquitous carotenoids.

Marine and aquatic animals have 3-dehydro-11-*cis*-retinal as the photopigment (Fig. 2, **2**) in order to shift the vision towards the red, wavelengths more suited for dark vision. Salmon and eel lack the extra double bond when living in fresh water, while the tadpole also loses its 3-ene when it becomes the terrestrial frog. Insects contain an extra 3-hydroxy group,[9] while the squid, *Watasenia scintillans* has three pigments, **1**, **2** and (4*R*)-4-hydroxy-11-*cis*-retinal **3** (Fig. 2).[10]

The visual transduction of Rh is outlined in Fig. 3. Irradiation with 500 nm light isomerizes the 11-*cis*-ene to *trans*, leading to the first intermediate photo-Rh which is formed in the low femto-sec time scale.[11] This is the only light-triggered change that occurs in Rh, the subsequent changes to batho-Rh and others being caused by relaxation of the protein to relieve the high strain caused by the "*trans*" 11-ene of the chromophore/protein complex. The strain energy, estimated to be 30—36 kcal/mol,[12] is reflected in the red-shifted absorption maxima of photo-Rh (555 nm) and batho-Rh (535 nm). Batho-Rh was detected as early as in 1958[13] and more properly characterized by absorption spectroscopy

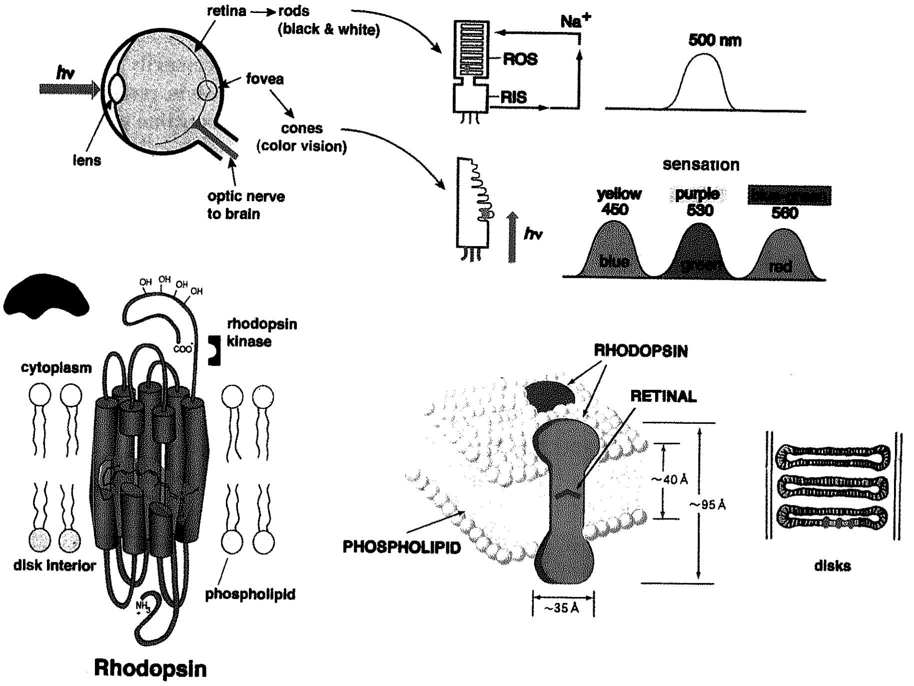
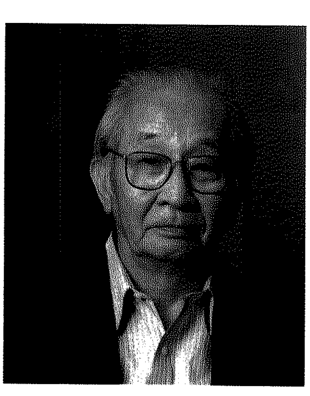


Fig. 1. Cross Section of Eye, Rod and Cone Cells, Disks, and Rhodopsin

Born in Hong Kong, and brought up in Lyon, London, and Alexandria until 11, he received his bachelor's degree in chemistry from Nagoya University, Japan, in 1947 from Fujio Egami. Following two years of post-graduate work with Louis Fieser at Harvard University, he returned to Nagoya University where he completed his Ph.D. in 1954 with Yoshimasa Hirata. He was Assistant Professor at Nagoya until 1958 when he became Professor of Chemistry at Tokyo Kyoiku University. In 1963 he moved to Tohoku University in Sendai and remained there until 1969 when he joined the faculty of Columbia University. In 1980 he became Centennial Professor of Chemistry. He was Chairman of the Chemistry Department, 1987—1990. Dr. Nakanishi was a founding member and a Director of Research at the International Centre of Insect Physiology and Ecology in Kenya from 1969—1977. He also served as the Director of the Suntory Institute for Bioorganic Research in Osaka, 1978—1991. Since 1996 he is assisting the Brazilian government to set up a center of excellence, Institute of Medicinal and Ecological Chemistry centered in São Paulo and Manaus.



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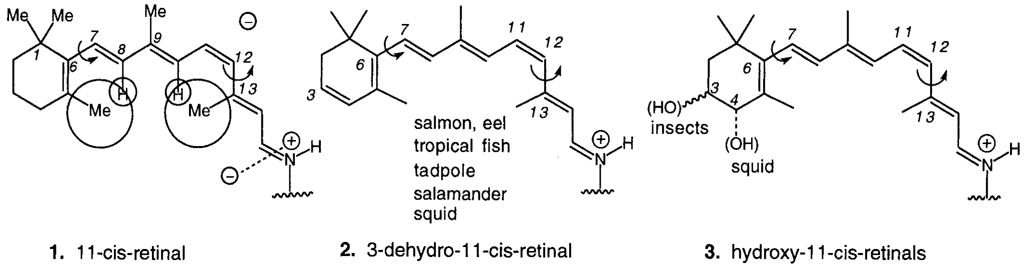


Fig. 2. Rhodopsin Chromophores

They are not planar around 6-*s-cis* and 12-*s-trans* bonds due to steric hindrance.

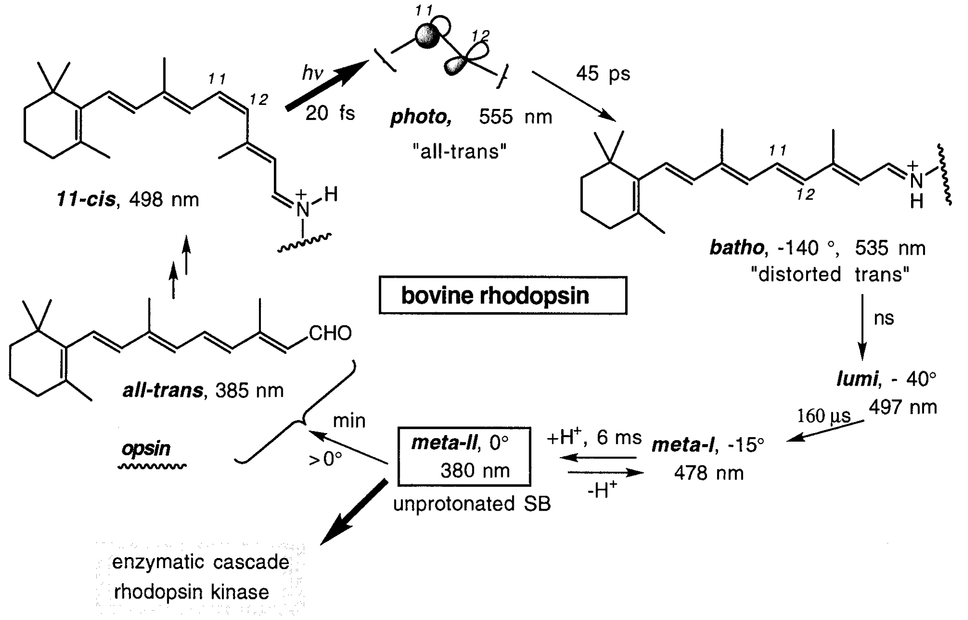


Fig. 3. Rhodopsin Transduction Path

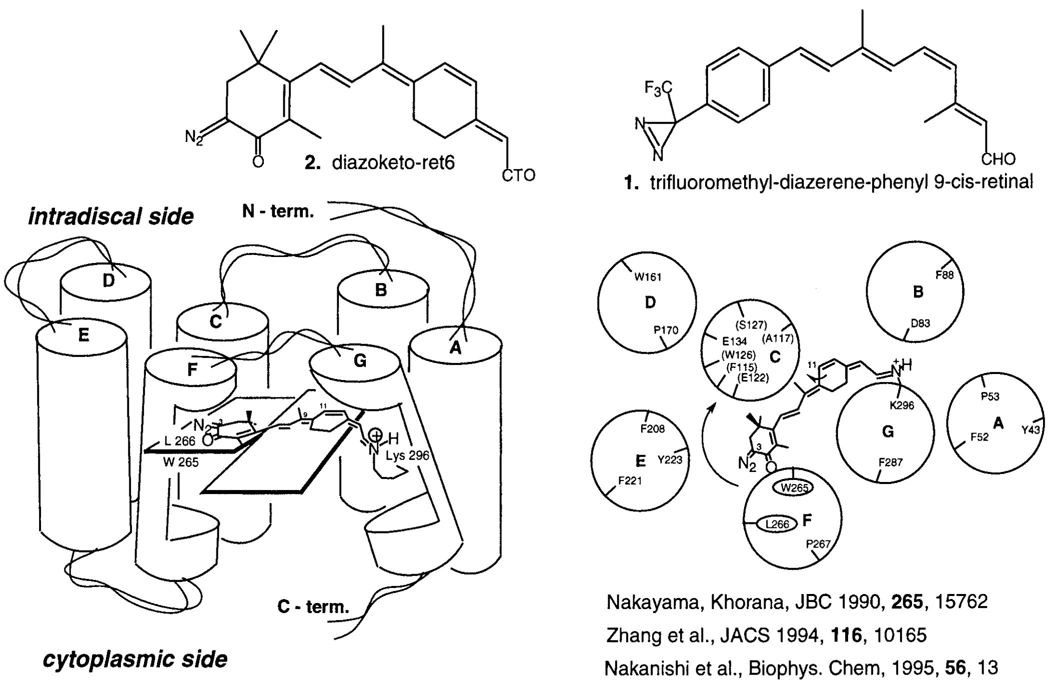
measured in liquid He, a temperature in which it can be sequestered.[14)] Microscopic rate constants coupled with time-resolved spectroscopy led to the finding that a blue-shifted intermediate (BSI) is in equilibrium with batho-Rh (not shown). After batho-Rh the pigment relaxes to lumi-Rh, *meta*-I Rh, and it is at the *meta*-II stage that the conformation of the extramembrane loops on the cytoplasmic side leads to excitation of the G protein, thus initiating a cascade of enzymatic reactions.[15)]

Photoaffinity Labeling of Restive Rh and Absolute Twist around 12-*s* Bond

Photoaffinity labeling of retinal proteins, particularly rhodopsin, has been hindered by serious factors. Namely, the difficulty in separating the peptidic fragments obtained upon enzymatic or chemical cleavages, a factor that caused problems in the sequencing of the protein,[3)] and scrambling of cross-linking upon photoactivation of the photolabel due to isomerization of the *cis*-ene to the *trans*-ene. Although racemic ^{14}C-labeled 9-*cis*-3-diazoacetate[16)] and subsequently the 3*R* and 3*S* enantiomers[17)] were incorporated into Rh and photo-crosslinked, the results were not published except for a Ph. D. thesis[18)] because too many amino acids were affinity labeled. On the other hand, Nakayama and Khorana used the aromatic trifluoromethyldiazirene **1** (Fig. 4) and identified the amino acids in helices C and F to which the diazirene carbon had cross-linked.[19)] Their results show that the ionone ring is in the vicinity of helices C and F, but the multitude of cross-linked amino acids and the large distance between some labeled residues (13.5 Å) was presumably caused by *cis*/*trans* isomerization.[19)]

In view of these difficulties, analog **2**, [15-^{3}H]-3-diazo-4-oxo-10,13-ethano-11-*cis* retinal or diazoketo-ret6 (Fig. 4), where the 11-*cis* to *trans* isomerization is blocked, was employed. Incubation of analog **2** with bovine opsin gave a pigment in 53% reconstitution yield. Irradiation of the pigment at 254 nm resulted in a 23% cross-link as estimated from the radioactivity of the peptide after separation from the unbound retina by size-exclusion HPLC. The cross-linked apoprotein in membrane suspension was cleaved by V8 protease at Glu239/Ser240 on the cytoplasmic side into large (V8-L) and small (V8-S) fragments; cleavage yield *ca.* 50%. After detergent solubilization and carboxymethylation of cysteine residues, the cleaved V8-L and V8-S fragments were separated by SDS-PAGE. All radioactivity resided in V8-S, while *no radioactivity was associated with the V8-L portion of rhodopsin*, indicating that the cross-linking site resided in helix F or G (Fig. 4). It was fortunate that all radioactivity resided in V8-S since this was the only fraction that showed base-line separation. Cyanogen bromide cleavage showed the radioactivity to reside in BrCN peptide-13, which upon Edman degradation disclosed Trp-265 and Leu-266 to be the crosslinked sites.

The helices in Fig. 4 are arranged according to a theoretical assignment of the seven transmembrane helices.[20)] As the current transmembrane model of the rhodopsin helices



Nakayama, Khorana, JBC 1990, **265**, 15762
Zhang et al., JACS 1994, **116**, 10165
Nakanishi et al., Biophys. Chem, 1995, **56**, 13

Fig. 4. Photoaffinity Labeling of Rhodopsin with Retinoids **1** and **2**

places both the labeled Trp-265/Leu-266 residues and the Schiff base linkage to Lys-296 in the middle of the lipid bilayer, this shows that the chromophore in dark Rh has its plane of the polyene essentially parallel to the membrane plane and that the entire chromophore must reside near the center of the bilayer, with the polyene long axis tilted only slightly relative to the membrane plane. The crosslinking of diazoketo **2** to only two amino acids is in sharp contrast to that of phenyl analog **1** which crosslinked to multiple residues in helices F and C. Although implication of this difference was not fully realized at the time, our recent results demonstrate that the binding of the ring in **2** is caused by a flip-over of the ionone moiety when the 11-ene isomerizes.

The electron density map[21)] of rhodopsin reveals a binding pocket that lacks a straight portion to accommodate the all-*trans* chromophore (Fig. 4, helical wheel); thus only *cis* isomers (except for 13-*cis*) can be incorporated into the protein core which is formed predominantly by helices B, C, F, and G. Therefore, the *cis* to *trans* isomerization of the chromophore could push one or more helices away to generate a more accommodating transoid cavity. This could be the origin of the protein conformational changes leading to *meta*-II stage and which are reversed upon release of all-*trans* retinal.[22)] Indeed 11-*cis*-retinal acts as an inverse agonist that retains the pigment in its inactive form that does not activate the G-protein.[23)]

As mentioned above, the 11-*cis*-retinal chromophore is twisted around the 6-*s*- and 11-*s*-*cis* bonds due to steric hindrance between the methyl and hydrogen atoms, Fig. 2, (**1**). The UV/Vis and CD spectra of bovine rhodopsin are shown in Fig. 5 (**1**). Native rhodopsin, $\lambda_{\max}$ 500 nm, exhibits two positive CD Cotton effects at 480 nm ($\Delta\varepsilon$+2.8, "α-band") and 337 nm ($\Delta\varepsilon$+9.8, "β-band"). A rhodopsin with a retinal analog in which B/C planes adopt a planar structure *via* a five-membered ring between C-10/C-13 exhibits a CD with a negligible 480 nm α-band. Similarly, the pigment formed from a retinal with planar A/B conformation *via* a five-membered bridge between C-5/C-8 showed a weak 337 nm β-band. Based on these results, Ito and co-workers[24)] assigned the origin of the α- and β-bands to distortions around 12-*s*-*trans* and 6-*s*-*cis* bonds, respectively. What is the absolute sense of twist around the 12-*s*-bond in Rh ? Is it as depicted in Fig. 5 (**2**) or is the twist of the B/C planes positive ?

This was determined by application of the CD exciton chirality method[25)] to pigments incorporating dihydroretinal chromophores.[26)] According to this nonempirical method, a clockwise twist or a positive helicity between two chromophoric electric transition moments interacting through space leads to a positive exciton couplet, *i.e.*, a CD curve with a positive longer wavelength Cotton effect and a negative shorter wavelength Cotton effect (see Fig. 5). Saturation of the retinal 11-ene breaks the chromophore into two independent conjugated systems, *i.e.*, the triene and the protonated Schiff base (PSB) moieties as shown in (**2**) and (**3**) (Fig. 5). If the through-space interaction between these two chromophores in the pigment is sufficiently intense, it could give rise to a couplet, the sign of the split CD reflecting the absolute twist within the pigment binding site. Provided 11,12-dihydroretinal adopts a conformation similar to 11-*cis*-retinal in rhodopsin, the exciton coupling between the triene and PSB should appear as either a positive or negative CD couplet depending on whether planes B/C constitute a positive or negative twist (Fig. 5). Indeed, incorporation of 11,12-dihydroretinal into Rh gave 11,12-2H-Rh that, unlike Rh itself, showed a negative couplet with an amplitude of -4.5, thus showing the twist to be as depicted in (**2**) Fig. 5. Moreover, the pigment with the less flexible 11,12-dihydro-ret7 (retinal with a trimethylene bridge from C-10 to C-13 (**3** in Fig. 5) also showed a more intense negative couplet with A=−11.6. These data define the absolute sense of twist as negative, namely, planes B and C constitute a negative chirality.[26)] It was gratifying to find that the interchromophoric through space interaction in these dihydro-retinoids was reflected in the pigments.

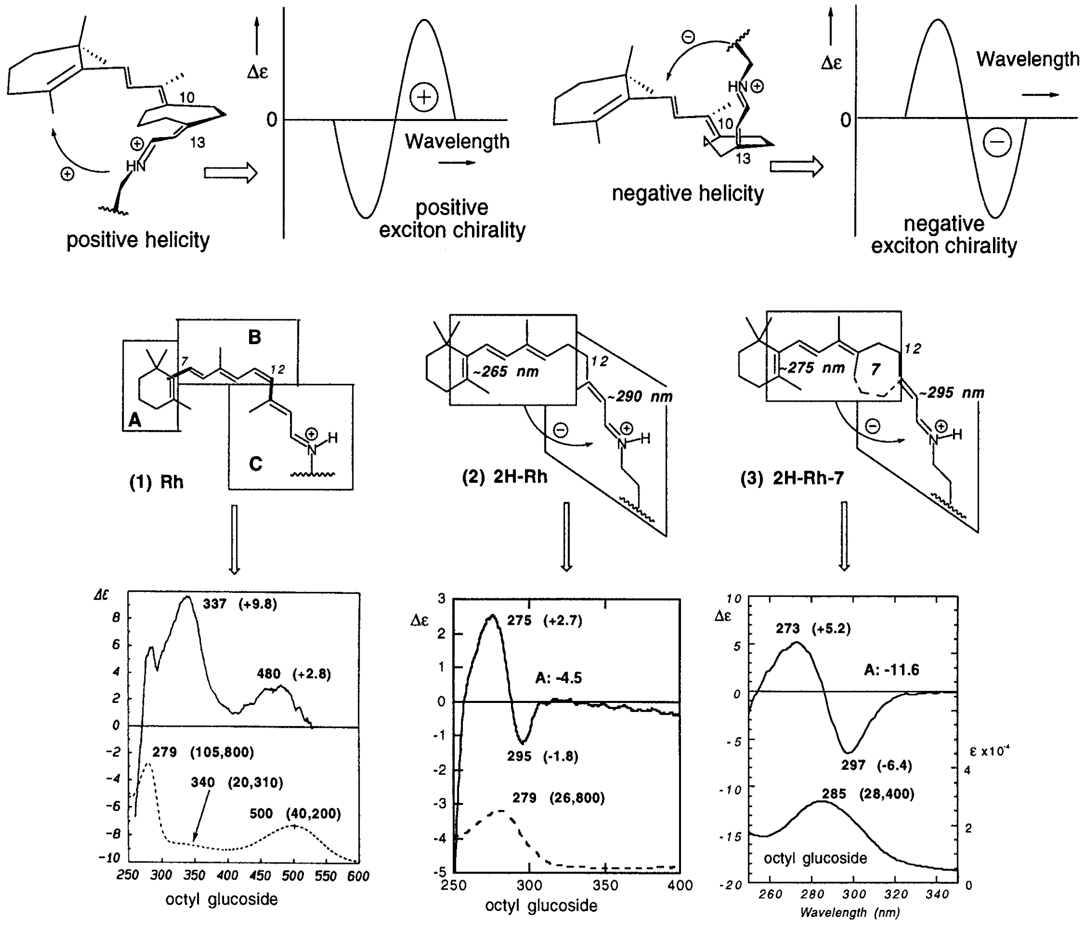
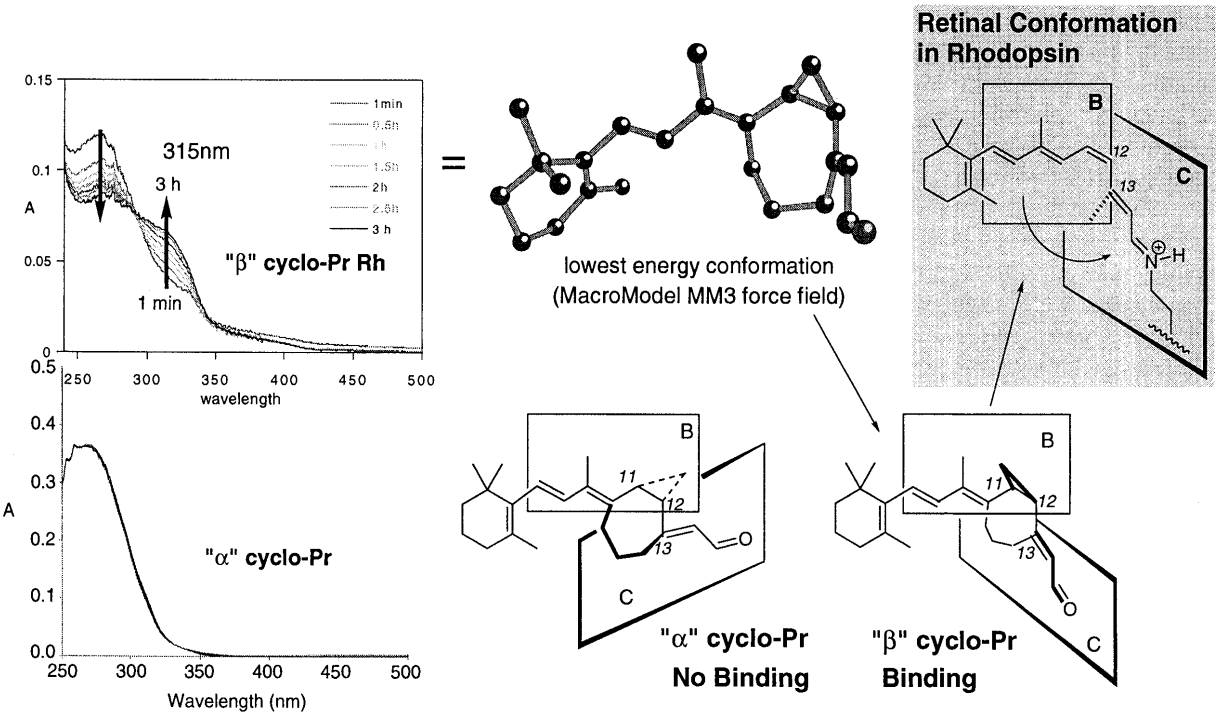


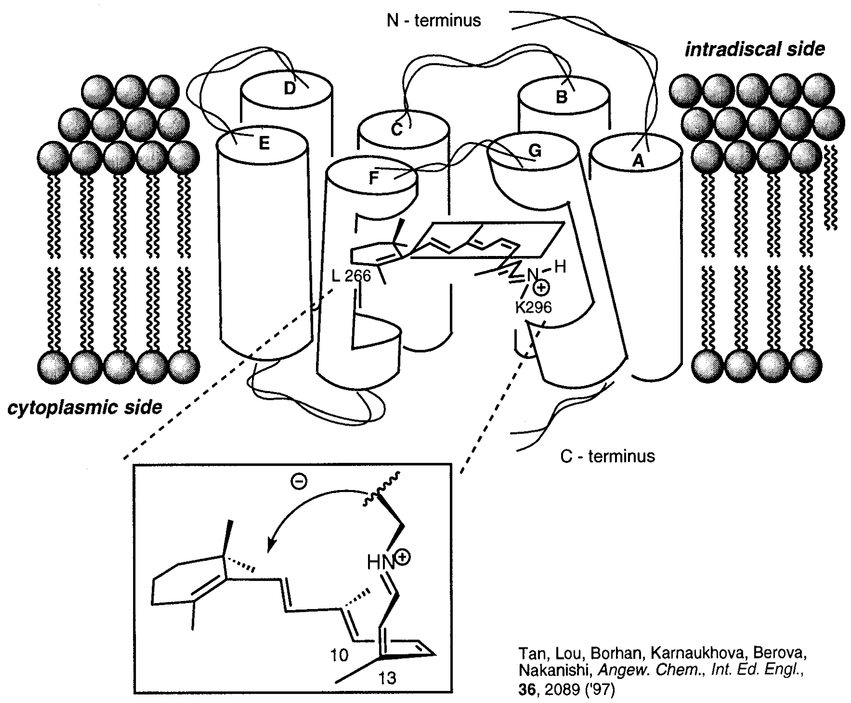
Fig. 5. Exciton Coupled CD Applied to 11,12-2H-Rh and 2H-Rh-7; Also CD of Rh. UV Also Shown



Lou, Hashimoto, Berova, Nakanishi, Organic *Letter*, **1**, 51-54 (1999).

Fig. 6. Stereoselectivity of the Retinal Binding Site in Rh

In addition to the CD studies, an independent bioorganic study to determine the sense of twist, an independent bioorganic study was performed.[27] The two 11-*cis*-locked α-and β-11,12-cyclopropylretinals shown in Fig. 6 were synthesized chemoenzymatically. Molecular modeling showed the lowest energy conformations of the two enantiomers to be as



Tan, Lou, Borhan, Karnaukhova, Berova, Nakanishi, *Angew. Chem., Int. Ed. Engl.*, **36**, 2089 ('97)

Absolute sense of twist of the retinal chromophore in rhodopsin

Fig. 7. Absolute Sense of Twist around the 12-*s*-Bond in the Rh Chromophore

depicted (only one enantiomer shown). It was a matter of checking the selectivity, if any, of the incorporation of the two enantiomers in Rh. It turned out that upon incubation, only the β-cyclopropyl analog bound to give the Rh analog absorbing at 315 nm. The binding experiments with the two enantiomers demonstrate the steric preference of the retinal binding site; as depicted, the twist between planes B and C is in agreement with the results deduced by the CD exciton chirality approach. It is thus concluded that the absolute twist of planes B and C is as shown in Fig. 7.

This negative helicity of the retinal chromophore agrees with theoretical calculations by Kakitani and Kakitani[28)] and solid-state NMR studies by Han and Smith.[29)] However, in a recent *ab initio* study,[30)] the chiroptical data of PSB formed from methylamine and 11-*cis*-retinal with four arbitrarily assigned geometries were calculated, whereupon a positively twisted C12/C13 geometry gave rise to a positive CD which implied a positive helicity around the C12/C13 bond. This is opposite to the results obtained by CD and binding studies. However, the effect of the protein, such as the PSB counter ion and electrostatic field within the retinal binding site was not considered in this calculation.

Preparation of 3-Diazo-4-oxo-11-*cis*-retinal (DK 11-*cis*-Retinal).

The next target was to determine which side of the 11-*cis*-ene flips over upon photoisomerization of the 11-*cis* to all-*trans* chromophore in Rh (see structure at bottom right, Fig. 8). In order to solve this aspect, it became necessary to prepare 3-diazo-4-oxo-11-*cis*-retinal carrying two photolabile moieties. The synthesis of 11-*cis*-retinal analogs in general are complicated by their unusual instability to light and temperature and their facile isomerization under conditions which most *Z* double bonds survive. Moreover, most schemes geared towards introducing the 11-*cis* geometry, especially those close to the end of the synthetic scheme, have either failed or resulted in low yields and formation of complex isomeric mixtures. Also, the more common photoisomerization strategy of all-*trans*-retinoids and HPLC purification of the 11-*cis*-isomer is not compatible with analogs containing light sensitive functional groups. The unusual difficulty encountered in generating the 11-*cis*-geometry is associated with the steric hindrance between 10-H and 13-Me which leads to a nonplanar conformation that could easily isomerize and relieve stress under most experimental conditions.

The title compound was first successfully prepared by a chemoenzymatic synthesis utilizing the unique properties of retinochrome contained in octopus and squid eyes (Fig. 8).[31)] Retinochrome, a protein folded into seven α-helices, is a photo-isomerase that catalyzes the conversion of all-*trans*-retinal to 11-*cis*-retinal. The water-soluble retinal carrier protein incorporates 11-*cis*-retinal upon incubation with metaretinochrome; further incubation with opsin containing membranes yields Rh. Thus the retinal carrier protein appears to act as a light-activated shuttle that transfers the retinal molecules between Rh and retinochrome. Although the conversion of all-*trans* 3-diazo-4-oxoretinal could be carried out in 50% yield, and the enzyme could theoretically be recycled, this approach was not suited for securing sufficient material (*ca.* 10 mg) that would be needed for conducting photo-crosslinking studies.

Numerous attempts to synthesize the title compound by conventional retinoid syntheses all gave unsatisfactory results including semihydrogenation of the 11-yne.[32)] The synthesis was finally achieved in high yield and with 100% *cis* stereoselectivity by performing the yne-reduction with Cu/Ag activated zinc dust in methanol/water at room temperature (Fig. 9).[32)]

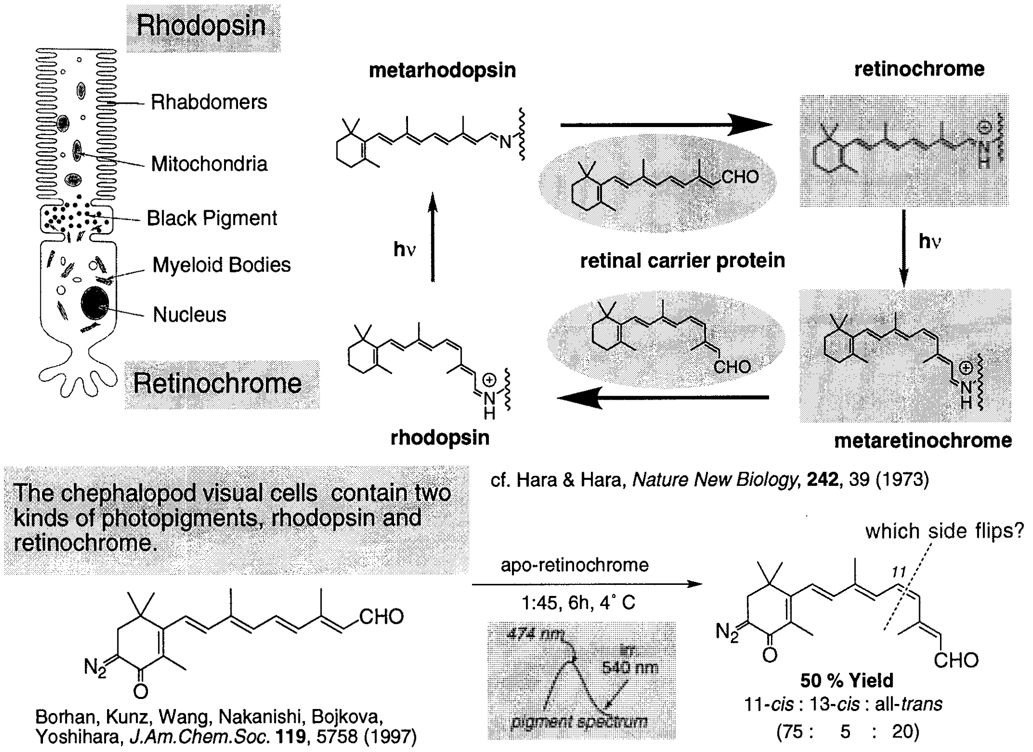


Fig. 8. Retinochrome Catalyzes Isomerization of all-*trans*- to 11-*cis*-Retinal

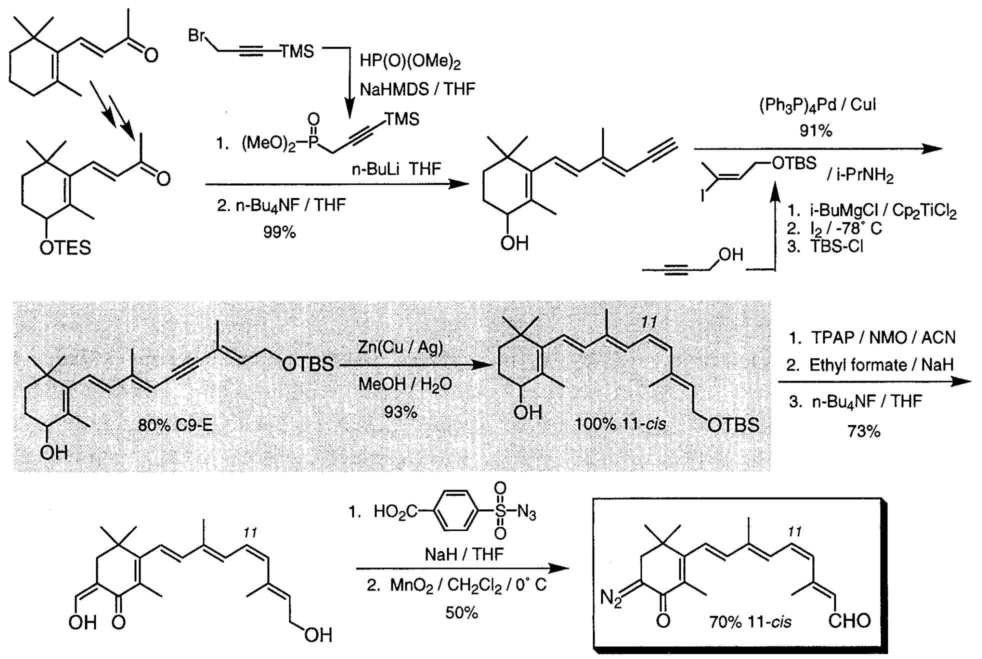


Fig. 9. Synthesis of 11-*cis*-3-Diazo-4-oxoretinal

Changes in Chromophore/Receptor Interactions along the Visual Transduction Path

The photoaffinity labeling experiments with DK-11-*cis* retinal ("DK") was performed using the analog with the tritiated aldehyde function. Incubation of HPLC-purified DK with rod outer segment (ROS) membranes regenerated DK-Rh, λ_{max} 467 nm. Its CD exhibited two positive peaks at 306 nm and 462 nm, closely matching the β and α peaks at 337 and 480 nm, respectively, of native Rh. The rapid regeneration and spectroscopic data suggest that the binding of DK to opsin closely matches that of native Rh. Furthermore, measurement of the photo-intermediates of DK-Rh upon dissolving it in 66% v/v glycerol and irradiation at low temperatures showed that intermediates corresponding to those of native Rh were formed, except at generally lower temperatures (Fig. 10). The changes in chromophore and opsin interactions were then tracked by: (i) performing photoaffinity labeling at the respective optimal temperatures, (ii) pyridiethylate the crosslinked protein prior to separation of the protein from the membrane, (iii) BrCN cleavage, (iv) separate peptidic fragments by HPLC, (v) collect the tritiated HPLC peak and sequence by Edman degradation.[33]

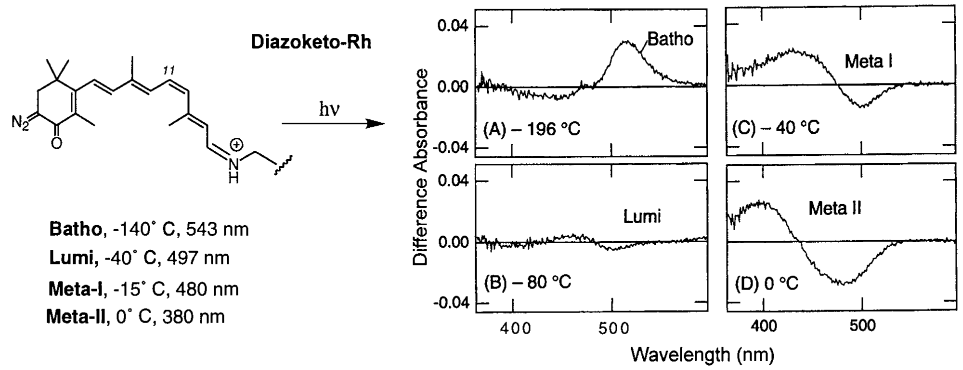


Fig. 10. Photo-intermediates of DK-Rh

The corresponding intermediates of native Rh are also shown together with respective temperatures at which they can be sequestered and absorption maxima.

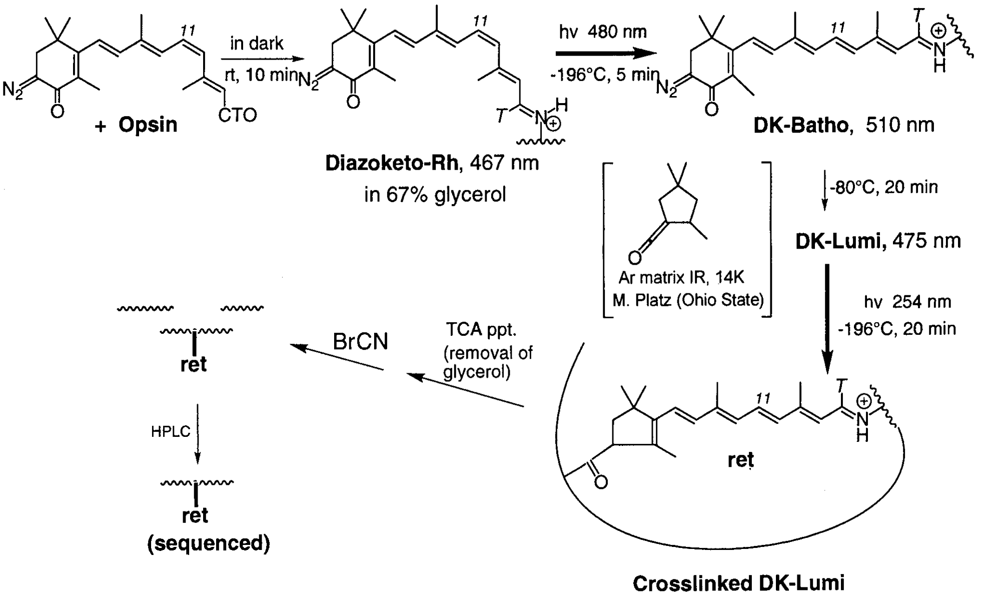


Fig. 11. Photoaffinity Labeling Protocol

DK lumi-Rh with a sequestering temperature of −80°C is taken as example.

The resting state of the pigment was investigated by keeping the DK-Rh glycerol solution at −196 °C in complete darkness and irradiating with 254 nm UV. The labeled amino acid was identified as Trp265 in fragment 13, *i.e.*, CN-13 (Val258 to Phe287) of helix F. In a previous photoaffinity study using non-isomerizable 11-*cis*-retinal analog, DK-ret6 **2** (Fig. 4), Trp265 and Leu266 were the crosslinking sites.[22] The additional crosslinking to Leu266 with DK-ret6 **2** probably is due to difference in the structures of the two DK retinals. Although the 11-*cis*-bond in DK-3 is not locked and the molecule is flexible, clean labeling occurred only at Trp265, indicative of a rigidly bound chromophore. The result shows that Trp265, a highly conserved residue among GPCRs is directly involved in ligand binding. The close proximity of the ionone ring and Trp265 restricts the geometry of 11-*cis*-retinal to a shape that functions as an inverse agonist (antagonist) for opsin activation.

To achieve photoaffinity labeling at the batho-Rh stage, the glycerol DK-Rh solution was irradiated with 480 nm light at −196 °C to populate the batho stage. The photoisomerized sample was then subjected to photo-crosslinking conditions (254 nm, −196 °C) and the labeled amino acid was determined as described above. Crosslinking again occurred only at Trp265. Thus, interaction between the β-ionone ring moiety and the opsin hardly changes at the batho stage.[34] The protocols for photoaffinity labeling of further intermediates were similar except for the temperature at which each state was populated. After cooling the glycerol DK-Rh solution to −196 °C in complete darkness, it was photoisomerized to batho-Rh by 480 nm irradiation. The protein solution was then warmed in darkness to the appropriate temperatures (Fig. 10), −80 °C for lumi, −40 °C for *meta*-I, and 0 °C for *meta*-II, cooled to −196 °C to freeze the protein conformation, and then irradiated with the 254 nm light to achieve photo-crosslinking.

The amino acid labeled in the lumi stage was Ala169 (helix D) in fragment CN-9 (Ala164 to Gly182) (Fig. 12). There was no evidence for any labeling in fragment CN-13, which was labeled in DK-Rh and DK-batho-Rh. At −80°, the temperature at which lumi-Rh crosslinking was performed, the pigment consists of a 1 : 1 mixture of Rh and lumi-Rh and not 100% lumi. The fact that only Ala169 was crosslinked at the lumi intermediate could be due to a more efficient crosslinking to Ala or to a closer proximity of the ionone ring to Ala169 in lumi-Rh than to Trp265 in Rh. Relaxation of the protein/substrate complex accompanies a large movement of the chromophore between the batho and lumi states, leading to a relieve in torsional strain and a sub-

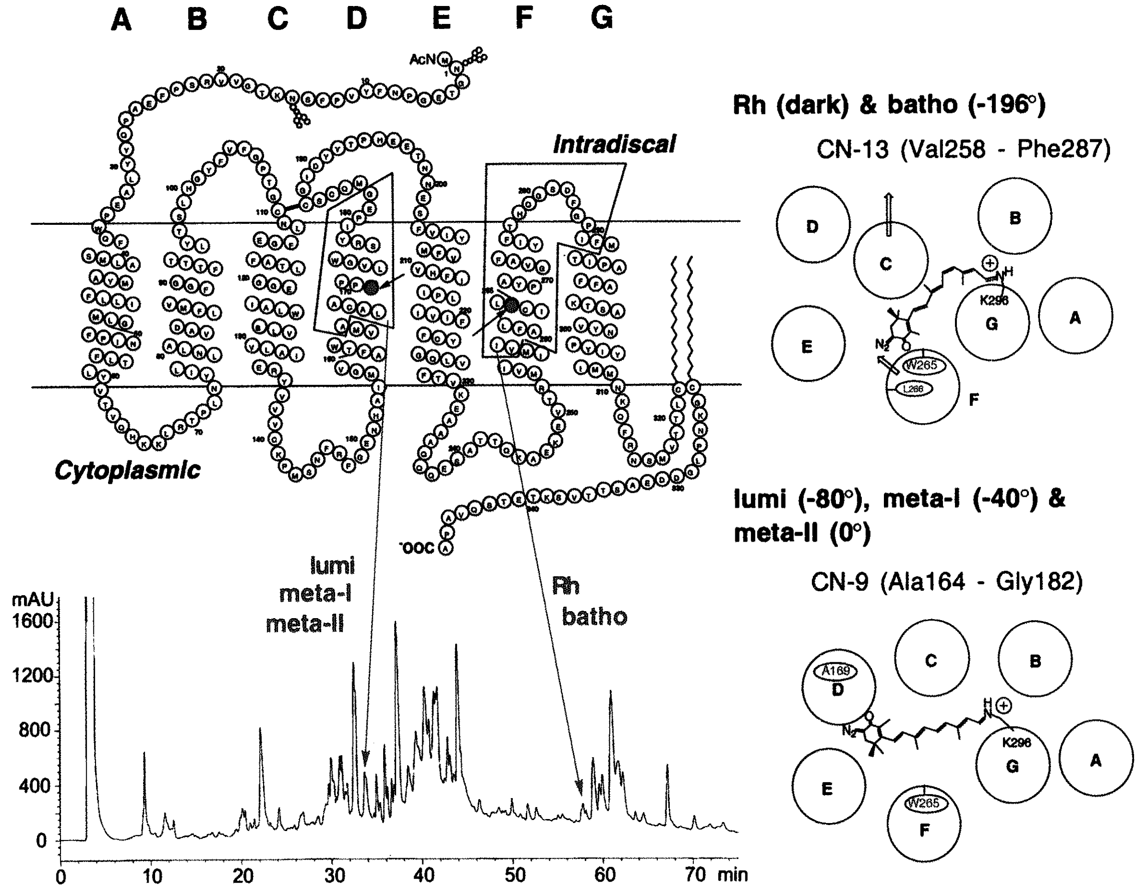
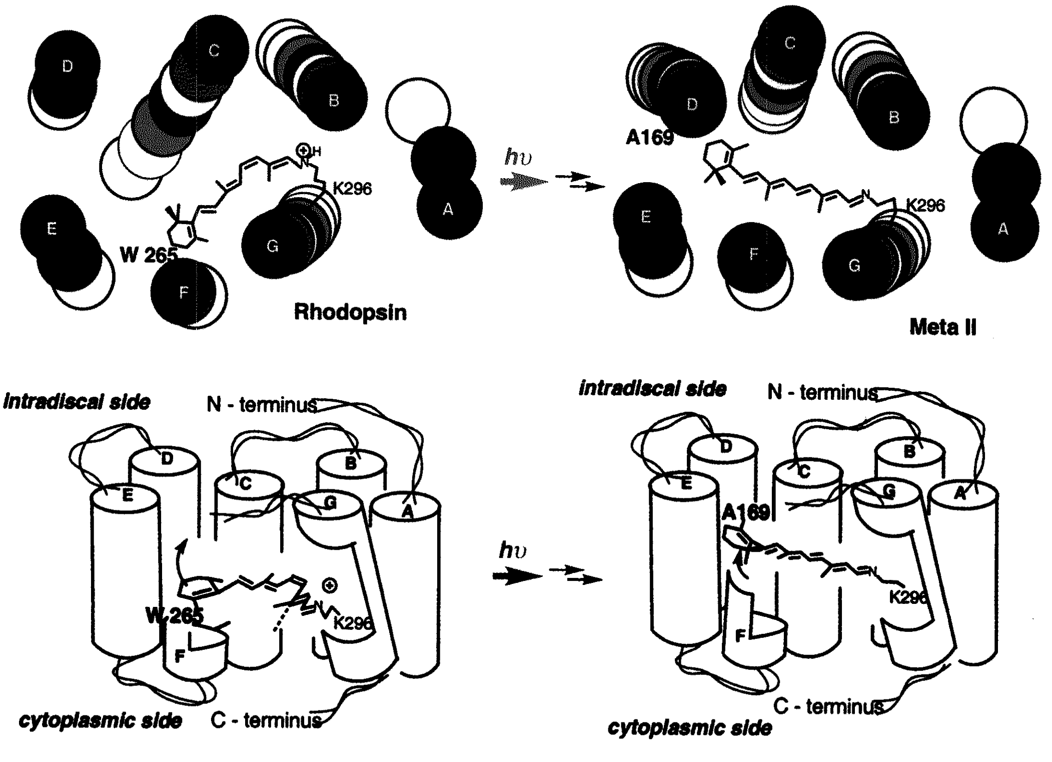


Fig. 12. Photo-crosslinking Sites in Rh (Dark), Batho-, Lumi-, *Meta*-I and *Meta*-II-Rh

The two small HPLC peaks shown by red arrows, the only radioactive peaks in the chromatogram, were sequenced by the Edman method. The crosslinked sites, Trp265 (in dark Rh and batho) and Ala169 (in lumi, *meta*-I and *meta*-II) are highlighted in red in the Rh sequence.



Borhan, Souto, Imai, Shichida, Nakanishi, *Science*, **288**, 2209-2212 (2000)

Fig. 13a. Qualitative Depiction of Opsin Conformational Movements in Visual Transduction

In Rh and batho the ionone ring is close to Trp265 in helix F. In lumi the ring flips over with concomitant changes in helical structure and resides close to Ala169 in helix D. The conformation of the extra membrane loops connecting helices C/D and E/F activates the G protein.

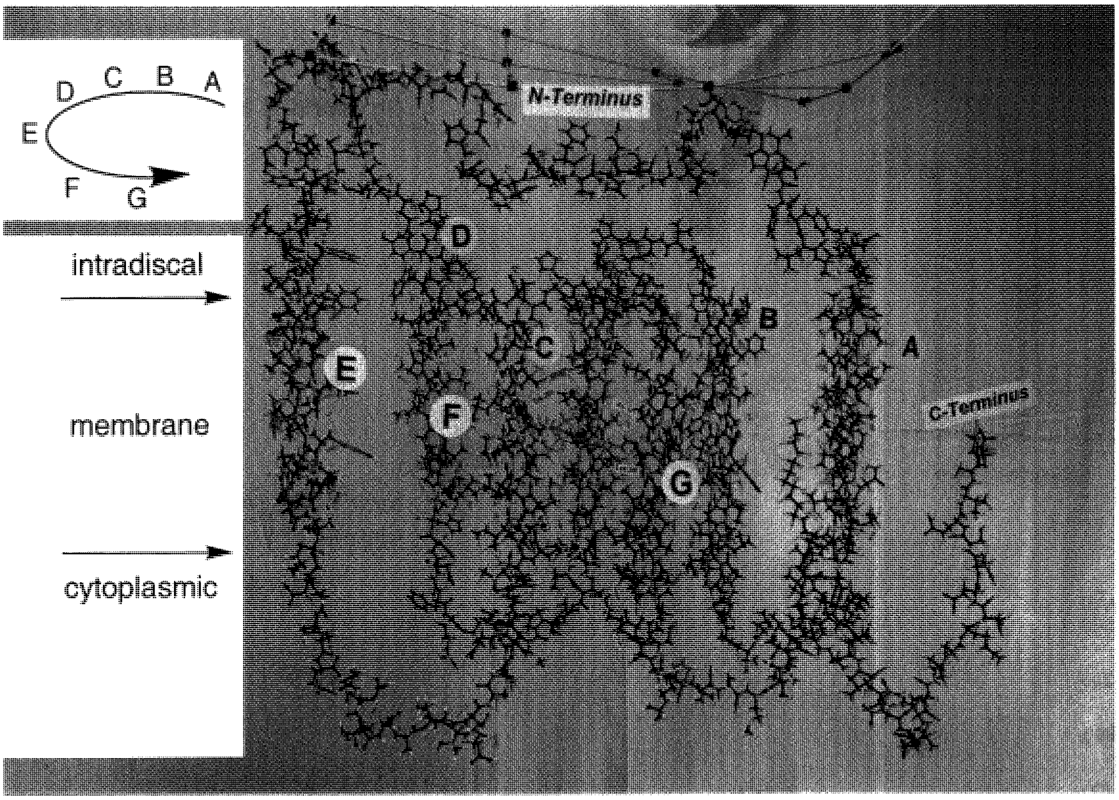


Fig. 13b. A Molecular Model of Rhodopsin Hanging from the Ceiling

The intradiscal side with the N-terminus is at the top as in Fig. 13. The helices are arranged as shown by the circular arrow. The two straight arrows define the boundaries of the membrane.

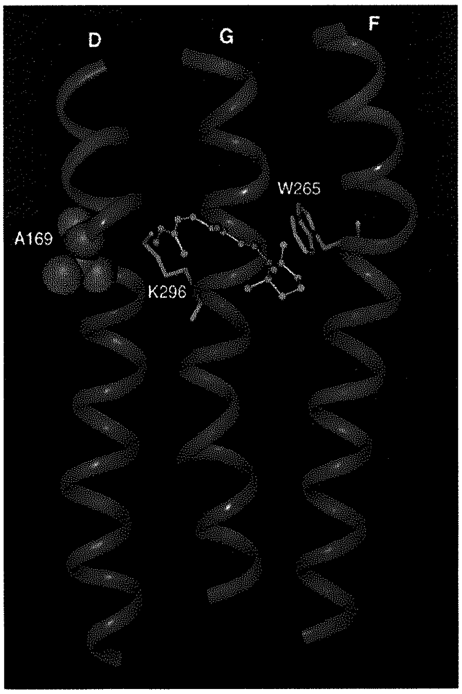


Fig. 14. Side View of a Theoretical 3D Model of Rh by Baldwin *et al.* (1997)

Helices A, B, C and E are omitted. Lys296 anchors the protonated Schiff base. The ionone ring is close to Trp265 in dark-Rh and batho Rh. Upon 11-ene isomerization, the ionone moiety flips over in going from batho to lumi and approaches Ala169. In subsequent *meta*-I and *meta*-II intermediates, the ionone C-3 still crosslinks to Ala169, and hence there is no change in the relative positions of the ionone ring and Ala169. However, the protein conformation changes, as reflected in the CD changes, and the extramembrane loops in *meta*-II lead to activation of the G protein. Note that Lys296, Trp265 and Ala169 are in the same relative heights from the top of the membrane lipid layer.

stantial blue shift (543 nm to 497 nm, Fig. 10). The crosslinked amino acid in *meta*-I and *meta*-II is again only Ala169. The results indicate that photoisomerization, triggered by a flip-over of the chromophoric ring, triggers a gross structural change in the protein, which moves helix D to the vicinity of the photolabel (Fig. 13). Photoisomerization separates the ionone ring from Trp265 in helix F and eliminates the interaction between 9-Me and Phe261, a highly conserved residue in all G protein-coupled receptors one-pitch below Trp265; 9-Me is also close to Gly121 in helix C.[35)] This induces separation of helix C from helix F and approach of the ionone ring to Ala169 in helix D. Although the ionone ring remains close to Ala169 in lumi-, *meta*-I and *meta*-II-Rh, the ligand/opsin interactions are clearly changing as seen by changes in the respective CD.[36)]

Lys296 involved in the protonated Schiff base link (helix G), Trp265 (helix F) and Ala169 (helix D) are all located around equal heights from the top of the membrane in their respective helices (Fig. 14). Therefore, the chromophore that stretches between Trp265 in helix F and Lys296 in helix G runs parallel to the membrane plane. The *cis* to *trans* isomerization of retinal involves a 180° rotation of the chromophore about the 11-ene to the all-*trans*-chromophore, which retains a similar parallel orientation with the membrane plane.

These results demonstrate that the chromophore in batho does not move significantly, and that a large movement occurs during the batho to lumi conversion. Ala169 in helix D is cross-linked in the lumi-, *meta*-I and *meta*-II stages, leading to movements schematically depicted in Fig. 13. This necessarily leads to conformational changes of cytoplasmic loops connecting helices C/D and E/F. It is proposed that

Trp265 keeps 11-*cis*-retinal as an inverse agonist. It is thus not surprising to see Trp265 in close proximity to the ionone ring in both the resting stage Rh and in batho-Rh, the first high-energy intermediate, while the ring moiety has flipped over in the more relaxed lumi and further intermediates.

Current efforts are directed towards defining the absolute sense of twist around the 6-*s* bond (twist between planes A and B) and clarifying the binding between the G protein and the cytoplasmic loops.

Acknowledgement The studies have been supported by NIH GM34509. I am indebted to all colleagues who are quoted in the references, and to Yukari Fujimoto for providing unpublished data.

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A New Gluco Indole Alkaloid, 3, 4-Dehydro-5-carboxystrictosidine, from Peruvian Uña de Gato (*Uncaria tomentosa*)

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A new gluco indole alkaloid, 3,4-dehydro-5-carboxystrictosidine, was obtained from Peruvian Uña de Gato (Cat's Claw, original plant: *Uncaria tomentosa*) together with two known gluco indole alkaloids. This compound was the first example of isolation of a gluco monoterpenoid indole alkaloid having a 3,4-dihydro-*β*-carboline ring system from nature. A characteristic feature of the compound was the quick replacement of the methylene hydrogens on C-14 with deuterium that was observed when it was dissolved in CD_3OD. We demonstrated a similar proton–deuterium exchange on a model compound, 1-methyl-3,4-dihydro-*β*-carboline, in CD_3OD solution.

Key words indole alkaloid; *Uncaria tomentosa*; Uña de Gato (Cat's Claw); *β*-carboline; Rubiaceae

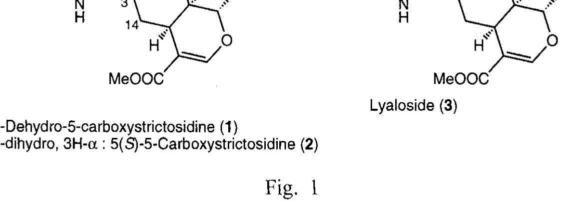
Uña de Gato (Cat's Claw)[1)] has been used as a traditional herbal medicine in Peru for the treatment of various ailments such as arthritics, inflammation and cancer. The original plants of Uña de Gato are known to be *Uncaria tomentosa* and *U. guianensis* belonging to Rubiaceae. The Genus *Uncaria* comprises more than thirty species worldwide, most of which are rich in alkaloidal constituents. We have already studied some *Uncaria* plants distributed in Asia, such as *U. rhynchophylla*[2a,b)] (Japan), *U. kawakamii*,[2b)] *U. florida*[2c)] (Formosa) and *U. attenuata*[2d)] (Thailand). The first two plants also have been used as traditional medicines. We recently obtained Uña de Gato from a Peruvian Market and reported the isolation of new triterpenes.[3)] We report here the isolation and structure elucidation of a new-type gluco indole alkaloid having a 3, 4-dihydro-*β*-carboline ring system.

Results and Discussion

Peruvian Uña de Gato used in this study was the powdered stem of *Uncaria tomentosa*. From the MeOH extract, one new gluco indole alkaloid (**1**) was isolated together with 5(*S*)-5-carboxystrictosidine (**2**),[4)] one of the main constituents of this herbal medicine, and lyaloside (**3**).[4)] The structures of the known compounds were determined by the direct comparison of the spectroscopic data with those of authentic samples.

From a high-resolution FAB-MS (HR-FAB-MS) measurement of a new alkaloid (**1**), a molecular formula of $C_{28}H_{32}N_2O_{11}$, which was two hydrogens less than that of 5(*S*)-5-carboxystrictosidine (**2**), was confirmed. The ^{1}H-NMR spectrum (CD_3OD) showed four aromatic protons due to the A ring of the indole system, and characteristic protons of the secologanin unit, *i.e.*, vinyl protons at δ_H 5.87 (ddd, J=17.4, 10.4, 8.2 Hz, H-19), δ_H 5.36 (br d, J=17.4 Hz, H-18 *trans*) and δ_H 5.32 (d, J=10.4 Hz, H-18 *cis*), one acetal proton at δ_H 5.75 (d, J=7.3 Hz, H-21), one anomeric proton of a *β*-linked glucose at δ_H 4.76 (d, J=7.9 Hz, H-1′), and one olefinic proton of the acrylic ester part at δ_H 7.52 (H-17). The ^{13}C-NMR spectrum (CD_3OD) showed 27 carbons including eight aromatic carbons, one carboxyl carbon (δ_C 173.5), vinyl carbons (δ_C 134.6, 121.0), one acetal carbon (δ_C 97.2), one anomeric carbon (δ_C 100.4), one carbomethoxy group (δ_C 168.7, 51.7), and one glucose unit. Besides one carboxyl and one ester carbonyl carbons, one more low-field shifted carbon at δ_C 168.6 was observed. Carbons due to the secologanin part (C-16—C-21, C-1′—C-6′) were clearly identified with those of 5(*S*)-5-carboxystrictosidine (**2**).[4)] In the heteronuclear multiple bond correlation (HMBC) spectrum, long-range correlations between the carbon at δ_C 168.6 and both protons of H-5 (δ_H 4.48) and H-15 (δ_H 3.38) were observed, indicating that the carbon at δ_C 168.6 was assigned to C-3 and that a double bond existed between C-3 and N-4. This was strongly supported by the UV absorption maxima at 357.5 and 239.0 nm, which were similar to those of 3,4-dihydro-*β*-carboline type compounds. All the above observations strongly demonstrated this compound to be 3,4-dehydro-5-carboxystrictosidine (or 3,4-dehydro-5-carboxyvincoside). The absolute configurations at the C-5 position and the secologanin part of **1** were reasonably assumed from the biogenetic viewpoint to be identical with 5(*S*)-5-carboxystrictosidine (**2**), the main component of this herbal medicine. The *pseudoequatorial* conformation of the carboxyl group of **1** was evidenced by the observed coupling of H-5 signal with the coupling constants of J_1=8.5 and J_2=5.8 Hz.

During the structural study, we encountered a confusing observation; 14-H_2 and C-14 were missing from the corresponding ^{1}H- or ^{13}C-NMR charts measured in CD_3OD. We then found that this characteristic phenomenon can be explained by the proton–deuterium exchange caused by an equilibration between the imine and enamine forms. In our previous work,[5)] we reported a similar deuterium exchange at the C-14 methylene protons of *β*-carbolinium type com-



3, 4-Dehydro-5-carboxystrictosidine (**1**)
3, 4-dihydro, 3H-α : 5(*S*)-5-Carboxystrictosidine (**2**)

Lyaloside (**3**)

Fig. 1

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Dedicated to the memory of Dr. Kyosuke Tsuda.

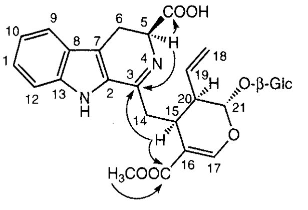

Fig. 2. Selected HMBC Correlation of **1**

Table 1. NMR Data for **1** (in CD_3OD)

	^1H (500 MHz)	^{13}C (125 MHz)
2		127.8
3		168.6
5	4.48 (dd, J=8.5, 5.8)	58.3
6	3.76 (dd, J=17.7, 5.8) 3.46 (dd, J=17.7, 8.5)	24.3
7		125.2
8		125.9
9	7.71 (d, J=8.2)	122.7
10	7.18 (br dd, J=8.2, 8.2)	122.8
11	7.42 (br dd, J=8.2, 8.2)	130.0
12	7.45 (d, J=8.2)	114.1
13		142.8
14	$^{a)}$	$^{a)}$
15	3.38 (m)	34.7
16		108.9
17	7.52 (s)	155.2
18	5.36 (br d, J=17.4) 5.32 (d, J=10.4)	121.0
19	5.87 (ddd, J=17.4, 10.4, 8.2)	134.6
20	2.84 (m)	45.2
21	5.75 (d, J=7.3)	97.2
1′	4.76 (d, J=7.9)	100.4
2′	3.22 (dd, J=9.3, 7.9)	74.7
3′	3.18—3.40 (m)	78.0
4′	3.26 (dd, J=9.3, 9.3)	71.7
5′	3.18—3.40 (m)	78.6
6′	3.94 (dd, J=11.9, 2.1) 3.67 (dd, J=11.9, 6.7)	62.9
5-<u>C</u>OOH		173.5
16-<u>C</u>OOMe		168.7
16-COO<u>Me</u>	$^{b)}$	51.7

a) Not detected. *b*) Under CD_3OD signals.

pounds, ophiorines A and B, in CD_3OD. To study the generality of this interesting phenomenon, we investigated the NMR behavior with a model compound, 1-methyl-3,4-dihydro-*β*-carboline (**4**) (Fig. 3). **4** was prepared from N_b-acetyltryptamine through a Bischler–Napieralski reaction.[6] When **4** was dissolved in dimethyl sulfoxide-d_6 (DMSO-d_6), a methyl group on the C-1 position was detected at δ_H 2.26 (br t, $J_{3,10}$=1.5 Hz) in the ^1H-NMR spectrum and at δ_C 22.0 in the ^{13}C-NMR spectrum. On the other hand, the methyl protons of **4** were observed as a multiplet around δ_H 2.30—2.35 soon after dissolving in CD_3OD. The signals, which contained a triplet peak at δ_H 2.34 (br t, $J_{3,10}$=1.5 Hz) due to CH_3 and peaks due to deuterated methyl groups (CH_2D, CHD_2), decreased and after 2 h, the peaks due to the methyl group of **4** completely disappeared in the ^1H- and ^{13}C-NMR spectra.

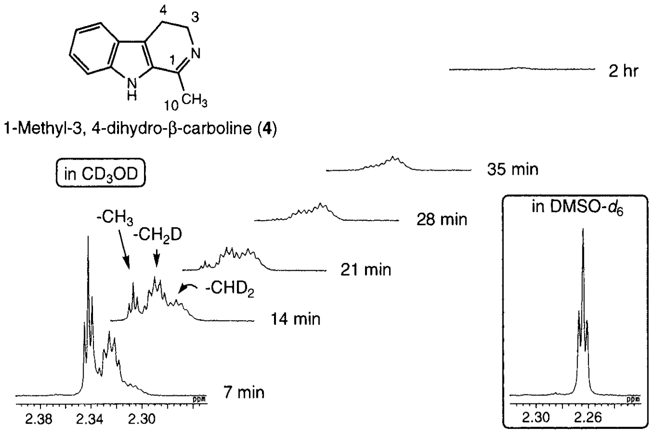

Fig. 3. ^1H-NMR of **4**

In conclusion, a new gluco indole alkaloid, 3,4-dehydro-5-carboxystrictosidine, was isolated from Peruvian Uña de Gato (original plant: *Uncaria tomentosa*). Although some 3,4-dihydro-*β*-carboline-type alkaloids[7] including 1-methyl-3,4-dihydro-*β*-carboline (harmalan) have been isolated from nature to date, this was the first example of a gluco monoterpenoid indole alkaloid having a 3,4-dihydro-*β*-carboline ring system.

Experimental

General UV: JASCO V-560. ^1H- and ^{13}C-NMR spectra: at 500 (^1H-NMR) and 125.65 (^{13}C-NMR) MHz, respectively. JEOL JNM A-500. FAB-MS and HR-FAB-MS: JEOL JMS-HX110. TLC: precoated Kieselgel 60 F_{254} plates (Merck, 0.25 mm thick). Column Chromatography: Kieselgel 60 [Merck, 70—230 mesh (for open column chromatography) and 230—400 mesh (for flash column chromatography)], DIAION HP20 [Mitsubishikasei], Sephadex LH20 [Pharmacia Biotech]. Medium pressure liquid chromatography (MPLC): C. I. G. prepacked column CPS-HS-221-05 (SiO_2) and CPO-HS-221-20 octadecyl silica gel (ODS) [Kusano Kagakukikai], HPLC: YMC Packed Column SH-043-5 S-5 120A SIL (SiO_2) [Yamamura Chemical Lab.] and HPLC Packed Column Inertsil Prep-ODS (ODS) [GL Sciences Inc.].

Plant Material "Uña de Gato" used in this study was imported from Peru through Coperunix Japan Inc. in 1996. The original plant was confirmed to be *Uncaria tomentosa* (stem and stem bark) by that company.

Extraction and Isolation "Uña de Gato" (835 g dried weight) was extracted with hot MeOH six times (1 l×6) to give the MeOH extract (total 95 g). 50 g of the MeOH extract (1st, 2nd and 3rd extracts were combined) was subjected to column chromatography on DIAION HP20 (2.1 cm×85 cm) to give seven fractions; fr. A MeOH : H_2O=3 : 7 (500 ml) 12.80 g, fr. B MeOH : H_2O=1 : 1 (1 l) 8.22 g, fr. C MeOH : H_2O=7 : 3 (1 l) 13.44 g, fr. D MeOH (1 l) 12.44 g, fr. E MeOH : acetone=1 : 1 (1 l) 0.11 g, fr. F acetone (1 l) 0.60 g, insoluble (0.27 g). Fr. B (8.22 g) and fr. C (13.44 g) each were purified by SiO_2 gel open column chromatography, Sephadex LH20 and MPLC (SiO_2) to give 5(*S*)-5-carboxystrictosidine (**2**, 779.4 mg and 231.4 mg, respectively). 45 g of the MeOH extract (4—6th extracts were combined) was dissolved in 30% MeOH–H_2O. After filtration, the filtrate was extracted with $CHCl_3$ and then *n*-BuOH to give the $CHCl_3$ extract (3.4 g) and the *n*-BuOH extract (25 g), respectively. The *n*-BuOH extract was subjected to column chromatography on DIAION HP20 (2.5 cm×90 cm) to give six fractions (each 1 l); fr. G MeOH : H_2O=3 : 7 11.85 g, fr. H MeOH : H_2O=1 : 1 2.46 g, fr. I MeOH : H_2O=7 : 3 5.24 g, fr. J MeOH 9.50 g, fr. K MeOH : acetone=1 : 1 0.02 g, fr. L acetone 0.08 g. Fr. H (2.46 g) was purified by Sephadex LH20, SiO_2 gel flash column chromatography, MPLC (ODS) and HPLC (ODS, SiO_2) to afford a new alkaloid (**1**, 15.1 mg) together with 5(*S*)-5-carboxystrictosidine (**2**, 11.7 mg). Lyaloside (**3**, 5.9 mg) was obtained by the purification of fr. I (5.24 g) with flash column chromatography and MPLC (SiO_2).

3,4-Dehydro-5-carboxystrictosidine (1) UV λ_{max} (MeOH) nm: 357.5, 239.0. FAB-MS (CH_3OH+*m*-Nitrobenzyl alcohol (NBA)) *m/z*: 573 $(MH)^+$. HR-FAB-MS (NBA) m/z: 573.2051 $(MH)^+$ (Calcd for $C_{28}H_{33}N_2O_{11}$: 573.2085). ^1H- and ^{13}C-NMR: Table 1.

1-Methyl-3,4-dihydro-*β*-carboline (4) ^1H-NMR δ ppm 7 min after dissolving in CD_3OD: 7.55 (1H, d like, J=8.1 Hz, H-5), 7.39 (1H, d like, J=8.1 Hz, H-8), 7.22 (1H, dd like, J=8.1, 8.1 Hz, H-7), 7.06 (1H, dd like, J=8.1, 8.1 Hz, H-6), 3.74 (2H, br dd, J=8.1, 8.1 Hz, H_2-3), 2.84 (2H, dd like, J=8.4, 8.4 Hz, H_2-4), 2.34 (br t, J=1.5 Hz), 2.34—2.30 (m) (H_3-10). δ ppm in DMSO-d_6: 11.29 (1H, s, NH), 7.54 (1H, d like, J=8.1 Hz, H-5), 7.39

(1H, d like, J=8.1 Hz, H-8), 7.18 (1H, dd like, J=8.1, 8.1 Hz, H-7), 7.03 (1H, dd like, J=8.1, 8.1 Hz, H-6), 3.68 (2H, br ddd, J=8.4, 8.4, 1.5 Hz, H_2-3), 2.73 (2H, dd like, J=8.4, 8.4 Hz, H_2-4), 2.26 (3H, br t, J=1.5 Hz, H_3-10). ^{13}C-NMR δ ppm 2 h after dissolving in CD_3OD: 161.1 (C-1), 139.0 (C-8a), 130.2 (C-9a), 126.4 (C-4b), 125.5 (C-7), 120.9, 120.8 (C-5, C-6), 117.4 (C-4a), 113.3 (C-8), 20.2 (C-4) and C-3 was buried under CD_3OD signals. δ ppm in DMSO-d_6: 157.0 (C-1), 136.5 (C-8a), 129.1 (C-9a), 124.9 (C-4b), 123.4 (C-7), 119.5 (C-5), 119.3 (C-6), 114.0 (C-4a), 112.3 (C-8), 47.6 (C-3), 22.0 (C-10), 18.9 (C-4).

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Ardisimamillosides C—F, Four New Triterpenoid Saponins from *Ardisia mamillata*

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Four new triterpenoid saponins, ardisimamilloside C (1), 3-*O*-{*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→2)]-*α*-L-arabinopyranosyl}-3*β*,16*α*,28,30-tetrahydroxy-olean-12-en, ardisimamilloside D (2), 3-*O*-{*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→2)]-*α*-L-arabinopyranosyl}-3*β*,15*α*,28,30-tetrahydroxy-olean-12-en, ardisimamilloside E (3), 3-*O*-{*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→2)]-*α*-L-arabinopyranosyl}-13*β*,28-epoxy-3*β*,16*α*,29-oleananetriol, and ardisimamilloside F (4), 3-*O*-{*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→2)]-*α*-L-arabinopyranosyl}-3*β*,16*α*-dihydroxy-13*β*,28-epoxy-oleanan-30-oic acid were isolated from the roots of *Ardisia mamillata* HANCE. Structure assignments were established on the basis of highresolution (HR)-FAB-MS, ^{1}H-, ^{13}C-, and two-dimensional (2D)-NMR spectra, and on the chemical evidence.

Key words *Ardisia mamillata*; Myrsinaceae; triterpenoid saponin; ardisimamilloside C, D, E, F

Ardisia mamillata HANCE (Myrsinaceae) is a widely occurring shrub in southern China. Its roots have been traditionally used to treat respiratory tract infections and menstrual disorders.[1)] Other plants of this genus have also been used for this purpose, and are well documented in traditional medicine in Southeast Asia.[2)] Many saponins have been isolated from *A. crenata*,[3)] *A. crispa*,[4)] and *A. japonica*.[5)] Recently, we isolated two new triterpenoid saponins, ardisimamilloside A, 3-*O*-{*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→2)]-*α*-L-arabinopyranosyl}-3*β*,16*α*,28*α*-trihydroxy-13*β*,28-epoxy-oleanan-30-al and ardisimamilloside B, 3-*O*-{*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→2)]-*α*-L-arabinopyranosyl}-3*β*-hydroxy-13*β*,28-epoxy-oleanan-16-oxo-30-al, from the roots of *A. mamillata*.[6)] In this paper, we report the isolation and structural elucidation of four additional new triterpenoid saponins (**1**—**4**), along with three known ones (**5**—**7**) from this species.

Results and Discussion

The roots of *A. mamillata* were extracted with 95% ethanol, and the ethanol extract was partitioned between water and hexane, ethyl acetate and *n*-butanol, respectively. Chromatography of the *n*-butanol extract on silica gel, Lobar RP-18, and Sephadex LH-20, and then after repeated HPLC purification over octadecyl silica (ODS) gel, furnished four new saponins (**1**—**4**) along with three known ones (**5**—**7**).

Compound **1** was obtained as a white powder. The molecular formula $C_{53}H_{88}O_{22}$ was established by analysis of high resolution (HR)-FAB-MS. The ^{13}C-NMR spectral data of **1** revealed 53 carbon signals, 30 of which were assigned to the aglycone part, while 23 were assigned to the carbohydrate moiety. The six *sp*^{3} quaternary carbon signals at δ 15.8, 16.7, 17.2, 27.4, 28.1, and 28.2, and two *sp*^{2} hybrid carbons at δ 122.5 and 144.9 indicated that the aglycone of **1** was an olean-12-en skeleton. ^{13}C-NMR spectral data of the sapogenin part of **1** were similar to those of the known compound, cyclamiretin D.[7)] As shown in Table 1, there was a signal at δ 67.2 (CH_2 by distortionless enhancement by polarization fransfer (DEPT)) instead of a resonance due to the 30-CHO group. This signal suggested that the –CHO group was reduced to a hydroxymethyl group. The long-range coupling between H-29 and C-30 in the heteronulear multiple bond spectroscopy (HMBC) spectrum supports the same conclusion. As in cyclamiretin D, the existence of hydroxyl groups at C-3 and C-16 was deduced from the ^{13}C-NMR resonances at δ 89.1 and 73.8, respectively. The configuration of the hydroxyl at C-3 was determined using rotating frame Overhauser enhancement spectroscopy (ROESY). The spatial proximities between H_{ax}-3 (δ 3.16) with H-23 (δ 1.17, 3H), H_{ax}-3 with H-5 (δ 0.72), and H-16 (δ 4.95) with H-28 (δ 3.82) indicated a *β*-configuration for the 3-OH and an *α*-configuration for 16-OH. The orientation of 16*α*-OH was determined by comparing the C-16 (δ 73.8) chemical shift on the ^{13}C-NMR spectrum with that in the literature (16*α*-OH: *ca.* δ 77.0; 16*β*-OH: *ca.* δ 64.0).[8)] Based on this evidence, the structure of the new sapogenin of **1** was established to be 3*β*,16*α*,28,30-tetrahydroxy-olean-12-en.

The ^{1}H- and ^{13}C-NMR data of **1** showed four anomeric signals at δ 4.94 (br s), 5.26 (d, J=7.5 Hz), 5.38 (d, J=7.6 Hz), and 6.42 (br s), and δ 104.4, 103.1, 105.5, and 101.6, respectively. Acid hydrolysis of **1** gave three monosaccharides: arabinose, glucose and rhamnose (1 : 2 : 1), which were analyzed by GC as their alditol acetate derivatives. The absolute configurations of the sugars were shown to be D-glucose, L-arabinose and L-rhamnose according to the method reported by Hara and co-workers.[9)] NMR techniques , ^{1}H–^{1}H shift correlation spectroscopy (COSY), homonuclear Hartmann-Hahn spectroscopy (HOHAHA), ^{13}C–^{1}H heteronulear correlated spectroscopy (HETCOR), HMBC, and ROESY, were used to determine the nature of the monosaccharides and sequences of the oligosaccharide chain of **1**. The anomeric configurations and ring sizes of each sugar were obtained following analysis on the H-1 vicinal coupling con-

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Dedicated to the memory of Dr. Kyosuke Tsuda.

Table 1. The ^{13}C-NMR Spectral Data for the Aglycone Moieties of **1**—**4** (125 MHz in Pyridine-d_5)

Carbons	**1**	**2**	**3**	**4**	DEPT
C-1	38.9	39.6	39.3	39.2	CH_2
C-2	26.4	26.4	26.6	26.6	CH_2
C-3	89.1	89.0	89.0	89.1	CH
C-4	39.5	39.4	39.6	39.6	C
C-5	55.8	55.5	55.7	55.6	CH
C-6	18.5	18.8	18.0	18.0	CH_2
C-7	33.3	31.2	34.5	34.5	CH_2
C-8	39.5	41.5	42.5	42.5	C
C-9	47.1	48.1	50.5	50.5	CH
C-10	36.9	37.0	37.0	37.0	C
C-11	23.8	24.0	19.3	19.2	CH_2
C-12	122.5	123.1	32.8 (CH_2)	32.9 (CH_2)	CH
C-13	144.9	146.6	86.6	86.6	C
C-14	42.1	48.2	44.7	44.6	C
C-15	34.7	66.5 (CH)	36.9	36.9	CH_2
C-16	73.8	36.7 (CH_2)	76.4	76.4	CH
C-17	41.3	34.8	44.4	44.3	C
C-18	42.3	43.1	50.1	53.6	CH
C-19	43.1	42.2	36.6	33.3	CH_2
C-20	36.3	36.2	36.8	36.3	C
C-21	28.4	30.1	32.8	32.9	CH_2
C-22	32.0	36.2	31.5	30.0	CH_2
C-23	28.1	28.1	28.0	28.0	CH_3
C-24	16.7	16.7	16.5	16.5	CH_3
C-25	15.8	15.7	16.4	16.5	CH_3
C-26	17.2	17.6	18.6	18.6	CH_3
C-27	27.4	20.9	19.7	19.6	CH_3
C-28	69.6	69.0	78.0	78.1	CH_2
C-29	28.2	28.5	69.8 (CH_2)	29.4	CH_3
C-30	67.2	65.9	32.8 (CH_3)	181.0 (C)	CH_2

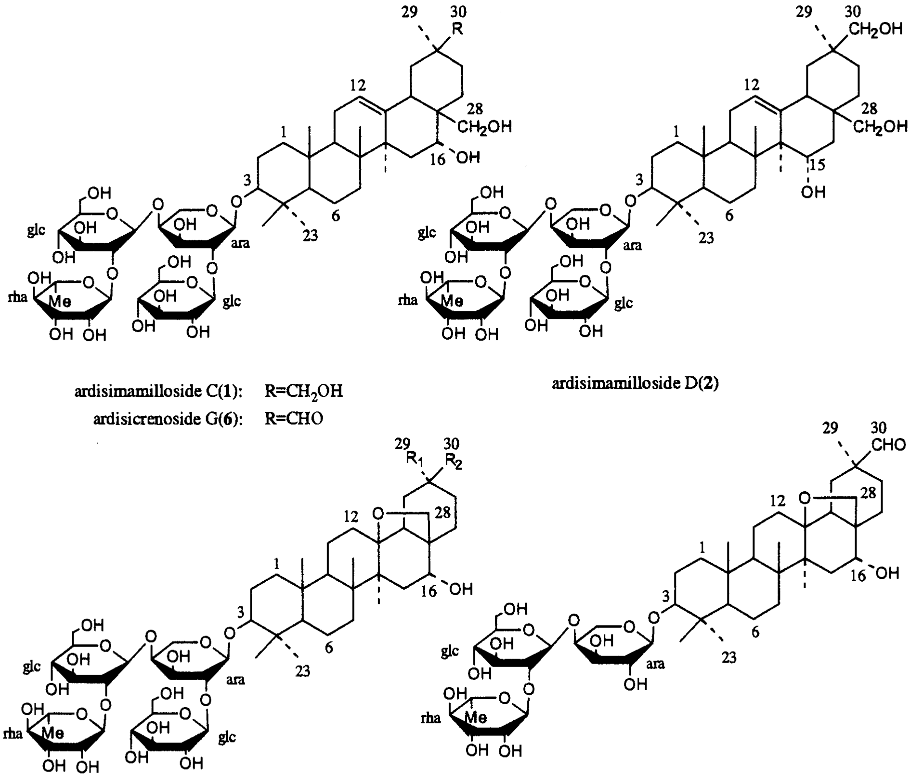
stants ($^3J_{HH}$, $^1J_{CH}$), observing their H-1 chemical shifts, and comparing their ^{13}C-NMR spectral data with those of methyl glycosides.[10] From the relatively large H-1 coupling constants (7.5, 7.6 Hz), the anomeric hydroxyl of both glucose moieties should have a *β*-configuration. In the insensitive nuclei enhanced by polarization transfer (INEPT) spectrum, the CH coupling constant of the signal of C-1 (*δ* 101.6) was 178 Hz, indicating that the glycosidic bond of rhamnose was linked in the *α*-configuration. The small H-1 coupling constant of arabinose, which exhibited a broad anomeric proton singlet in its ^{1}H-NMR spectrum, and the correlation between H-1 with H-3 and H-5 in ROESY indicated that the arabinose should also have an *α*-configuration at its anomeric carbon. Based on these results, the four sugars and their anomeric configurations in **1** were determined to be an *α*-L-arabinopyranose, two *β*-D-glucopyranoses and an *α*-L-rhamnopyranose.

The sequence of the oligosaccharide chain was deduced from ^{13}C shift differences between individual sugar residues and model compounds, and from HMBC and ROESY experiments. The C-1 of arabinose was attached to the 3-OH of aglycone, as indicated by the C-3 chemical shift (*δ* 89.1) of **1**, the correlation between H-1 (*δ* 4.94) of arabinose with C-3 of aglycone in HMBC, and between H-1 of arabinose with H-3 (*δ* 3.16) in ROESY. From the HMBC experiment of **1**, the following correlations were observed: H-1 (*δ* 5.38) of the terminal glucose with C-2 (*δ* 80.8) of arabinose; H-1 (*δ* 5.26) of the inner glucose with C-4 (*δ* 74.9) of arabinose; and H-1 (*δ* 6.42) of rhamnose with C-2 (*δ* 78.1) of the inner glucose. Based on the above findings, the structure of compound **1** was elucidated to be 3-*O*-{*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→2)]-*α*-L-arabinopyranosyl}-3*β*,16*α*,28,30-tetrahydroxy-olean-12-en. This is a new triterpenoid saponin, trivially named ardisimamilloside C.

Compound **2** was obtained as a white powder, and had the molecular formula $C_{53}H_{88}O_{22}$ based on the HR-FAB-MS spectrum. The ^{13}C-NMR spectral data of **2** showed 53 carbon signals, 30 of which were assigned to the aglycone part, while 23 were assigned to the carbohydrate moiety. The ^{13}C-NMR spectral data of the sapogenin part of **2** were similar to those of **1**. Comparing the ^{13}C-NMR spectral data of **1** and **2**, there was a lack of any resonance due to C-16 at *δ* 73.8 in **2**; instead, a signal was observed at *δ* 66.5 (CH by DEPT). From the HMBC experiment of **2**, the long-range coupling of H-27 (*δ* 20.9) with *δ* 66.5 was observed. Therefore, there should be a hydroxyl group at C-15. This assignment was confirmed by the downfield shift at C-13 (+2.7 ppm), C-14 (+6.1 ppm) and at C-15 (+31.8 ppm), and the upfield shift at C-27 (−6.5 ppm) and C-17 (−6.7 ppm). The configuration of the hydroxyl at C-3 and C-15 can be determined using a ROESY experiment. The correlations of H_{ax}-3 (*δ* 3.17) with H-23 (*δ* 1.17) and H_{ax}-3 with H-5 (*δ* 0.69) indicated that the hydroxyl at C-3 should have a *β*-configuration. The correlation of H-15 (*δ* 4.74) with H-28 (*δ* 3.87) indicated that the hydroxyl at C-15 should have an *α*-configuration. Based on these findings, the structure of the new sapogenin of **2** was established to be 3*β*,15*α*,28,30-tetrahydroxy-olean-12-en.

The ^{1}H- and ^{13}C-NMR data of **2** displayed four anomeric signals at *δ* 4.95 (br s), 5.25 (d, J=7.6 Hz), 5.38 (d, J=7.6 Hz), and 6.41 (br s), and *δ* 104.5, 103.2, 105.4, and 101.6, respectively. Acid hydrolysis gave three monosaccharides, arabinose, glucose and rhamnose in a ratio of 1 : 2 : 1, which were analyzed by the same method as with **1**. Using the same methods as with **1**, glucose was determined to have a D-configuration, while arabinose and rhamnose were determined to have an L-configuration. In **2**, the same sequence of the oligosaccharide as in **1** was determined using NMR techniques (^{1}H–^{1}H COSY, HOHAHA, HETCOR, HMBC, ROESY). These above findings indicated that the structure of **2** should be 3-*O*-{*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→2)]-*α*-L-arabinopyranosyl}-3*β*,15*α*,28,30-tetrahydroxy-olean-12-en. This is a new triterpenoid saponin, trivially named ardisimamilloside D.

Compound **3** was obtained as a white powder. The molecular formula $C_{53}H_{88}O_{22}$ was found to be the same as that of **2** by HR-FAB-MS. The ^{13}C-NMR spectral data of **3** showed 53 carbon signals, 30 of which were assigned to the aglycone part, while 23 were assigned to the carbohydrate moiety. The ^{1}H-NMR spectrum (Table 2) showed signals of six tertiary methyl groups at *δ* 0.85, 1.03, 1.17, 1.36, 1.56, 1.59, and two pairs of germinal protons at *δ* 3.45, 3.66 (d, J=7.3 Hz) and *δ* 3.81, 4.08 (d, J=9.2 Hz) corresponding to two –CH_2–O groups. The ^{13}C-NMR spectral data of **3** were similar to those of the known saponin ardisicrenoside A (**5**).[3] Comparing the ^{1}H-, ^{13}C-NMR spectral data of **3** with those of **5** (Tables 1, 2), only the signals due to C-29 and C-30 were completely different. These findings indicated **3** should be the isomer of ardisicrenoside A. The 13*β*,28-epoxy bridge and



ardisimamilloside C(**1**): R=CH_2OH
ardisicrenoside G(**6**): R=CHO

ardisimamilloside D(**2**)

ardisimamilloside E(**3**): R_1=CH_2OH; R_2=CH_3
ardisimamilloside F(**4**): R_1=CH_3; R_2=COOH
ardisicrenoside A(**5**): R_1=CH_3; R_2=CH_2OH

(**7**)

Chart 1

C-16 hydroxyl could be explained by the ^{13}C-NMR resonances at δ 86.6 (C-13), 78.0 (C-28), and 76.4 (C-16), respectively. Furthermore, these assignments could be confirmed through long-range coupling in a HMBC experiment and through spatial interaction in a ROESY experiment. Configurations of hydroxyl at C-3, C-16, and C-20 could be determined using ROESY results. The spatial proximities between H_{ax}-3 (δ 3.14) with H-23 (δ 1.17, 3H), H_{ax}-3 with H-5 (δ 0.66), and H-16 (δ 4.14) with H-28 (δ 3.45, 3.66) indicated a β-configuration for the 3-OH and an α-configuration for 16-OH. A correlation which existed between H-18 and δ 1.56 (3H, s, H-30) indicated that a hydroxymethyl group was located at C-29. From the above evidence, the structure of the new sapogenin of **3** was established to be 13β,28-epoxy-3β,16α,29-oleananetriol.

The ^{1}H- and ^{13}C-NMR data of **3** displayed four anomeric signals at δ 4.95 (br s), 5.25 (d, J=7.6 Hz), 5.38 (d, J=7.6 Hz), and 6.41 (br s), and at δ 104.4, 103.1, 105.4, and 101.6, respectively. Using the same methods as with **1**, the glucose was determined to have a D-configuration, while the arabinose and the rhamnose were determined to have an L-configuration in **3**. The sequence of the oligosaccharide in **3** was established to be the same as in **1** using NMR techniques (^{1}H–^{1}H COSY, HOHAHA, HETCOR, HMBC, ROESY). Therefore, the structure of **3** was determinated to be 3-*O*-{α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranosyl}-13β,28-epoxy-3β,16α,29-oleananetriol. This is a new triterpenoid saponin, trivially named ardisimamilloside E.

Table 2. The ^{13}C-NMR Spectral Data for the Sugar Units of **1**—**4** (125 MHz in Pyridine-d_5)

Carbons	1	2	3	4	DEPT
Arabinose (A)					
A-1	104.4	104.5	104.4	104.4	CH
A-2	80.8	80.8	80.7	80.7	CH
A-3	74.7	74.7	74.7	74.6	CH
A-4	74.9	74.9	74.8	74.8	CH
A-5	62.9	62.8	62.8	62.8	CH_2
Glucose (terminal) (G)					
G-1	105.5	105.4	105.4	105.4	CH
G-2	76.4	76.4	76.4	76.4	CH
G-3	77.3	77.3	77.3	77.1	CH
G-4	71.7	71.7	71.7	71.7	CH
G-5	78.0	78.0	78.0	78.0	CH
G-6	62.9	62.8	62.8	62.8	CH_2
Glucose (inner) (G′)					
G′-1	103.1	103.2	103.1	103.2	CH
G′-2	78.1	78.1	78.1	78.1	CH
G′-3	79.6	79.5	79.5	79.6	CH
G′-4	71.9	71.9	71.8	71.9	CH
G′-5	78.4	78.4	78.4	78.4	CH
G′-6	62.6	62.6	62.6	62.6	CH_2
Rhamnose (R)					
R-1	101.6	101.6	101.6	101.6	CH
R-2	72.4	72.4	72.4	72.4	CH
R-3	72.7	72.7	72.7	72.7	CH
R-4	74.9	74.9	74.9	74.9	CH
R-5	69.4	69.4	69.4	69.4	CH
R-6	19.0	19.0	18.9	18.9	CH_3

Compound **4** was obtained as a white powder. The HR-FAB-MS spectrum of **4** showed quasimolecular ion data at *m/z* 1113.5450, corresponding to the formula $C_{53}H_{86}O_{23}$. The ^{13}C-NMR spectral data of **4** showed 53 carbon signals, 30 of which were assigned to the aglycone part, while 23 were assigned to the carbohydrate moiety. The ^{13}C-NMR spectral data of the sapogenin part of **4** were similar to those of the known triterpene cyclamiretin A.[11] In **4**, there was a lack of any resonance due to the formyl group at C-30 of cyclamiretin A; instead, a signal was observed at δ 181.0 (C by DEPT). This signal suggested that the –CHO group of cyclamiretin A is oxidized to a carboxyl group in **4**. The long-range coupling between H-29 and C-30 in the HMBC experiment supports the same conclusion. As in cyclamiretin A, the 13β,28-epoxy bridge and C-16 hydroxyl could be explained by the ^{13}C-NMR resonances at δ 86.6 (C-13), 78.1 (C-28), and 76.4 (C-16), respectively. Furthermore, these assignments could be confirmed through long-range coupling in the HMBC experiment and through spatial interaction in a ROESY experiment. Configurations of hydroxyl at C-3 and C-16 could be determined using ROESY results. The spatial proximities between H_{ax}-3 (δ 3.15) with H-23 (δ 1.17, 3H), H_{ax}-3 with H-5 (δ 0.65), and H-16 (δ 4.08) with H-28 (δ 3.15, 3.29) indicated a β-configuration for the 3-OH and an α-configuration for 16-OH. From these findings, the structure of the sapogenin of **4** was established to be 3β,16α-dihydroxy-13β,28-epoxy-olean-30-oic acid.

The ^{1}H- and ^{13}C-NMR data of **4** displayed four anomeric signals at δ 4.96 (br s), 5.23 (d, J=7.6 Hz), 5.37 (d, J=7.6 Hz), and 6.41 (br s), and δ 104.4, 103.2, 105.4, and 101.6, respectively. Using the same methods as with **1**, the glucose was determined to have a D-configuration, while the arabinose and the rhamnose were determined to have an L-configuration. In **4**, the same sequence of the oligosaccharide as in **1** was determined using NMR techniques (^{1}H–^{1}H COSY, HOHAHA, HETCOR, HMBC, ROESY). From these findings, the structure of compound **4** was verified to be 3-*O*-{α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranosyl}-3β,16α-dihydroxy-13β,28-epoxy-olean-30-oic acid. This is a new triterpenoid saponin, trivially named ardisimamilloside F.

Compound **5** was obtained as a white powder. The matrix-assisted laser desorption ionization time of fligh mass spectrum (MALDI-TOF-MS) quasimolecular ion data were at *m/z* 1099 $[M+Na]^+$ and 1115 $[M+K]^+$. On acid hydrolysis, **5** gave rhamnose, glucose, and arabinose (1 : 2 : 1), identified by using the same methods as in **1**. On the basis of its ^{13}C-NMR, COSY, HETCOR, and HMBC experiments, the sequence of its oligosaccharide chain was assigned the same structure as that in the established **1**, and in addition, the attachment of this chain to C-3 of the aglycone was also identified. From the above evidence, **5** was assigned to be 3-*O*-{α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranosyl}-13β,28-epoxy-3β,16α,30-oleananetriol (ardisicrenoside A), a compound already isolated from *A. crenata*.[3a]

Compound **6** was obtained as a white powder. The MALDI-TOF-MS quasimolecular ion data were at *m/z* 1097 $[M+Na]^+$ and 1113 $[M+K]^+$. On acid hydrolysis, **6** gave rhamnose, glucose, and arabinose (1 : 2 : 1), identified using the same methods as in **1**. On the basis of its ^{13}C-NMR, COSY, HETCOR, and HMBC experiments, the sequence of its oligosaccharide chain was assigned the same structure as that in the established **1**, and in addition, the attachment of this chain to C-3 of the aglycone was also identified. From the above evidence, **6** was assigned to be be 3-*O*-{α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranosyl}-3β,16α,28-olean-12-en-30-al (ardisicrenoside G), a compound already isolated from *A. crenata*.[3b]

Compound **7** was obtained as a white powder. The MALDI-TOF-MS quasimolecular ion data were at *m/z* 935 $[M+Na]^+$ and 951 $[M+K]^+$. On acid hydrolysis, **7** gave rhamnose, glucose, and arabinose (1 : 1 : 1), identified using the same methods as in **1**. On the basis of its ^{13}C-NMR, COSY, HETCOR, and HMBC experiments, the sequence of its oligosaccharide chain was assigned, and in addition, the attachment of this chain to C-3 of the aglycone was also identified. From the above evidence, **7** was assigned to be 3-*O*-{α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-α-L-arabinopyranosyl}cyclamiretin A, a compound already isolated from *Myrsine pellucida*.[12]

Experimental

General Optical rotations were measured using a DIP-1000 digital polarimeter (JASCO corporation). MALDI-TOF-MS and HR-FAB-MS were conducted using Perseptive Voyager RP and a JMS-700K(JEOL) mass spectrometer, respectively. ^{1}H- and ^{13}C-NMR were recorded using a JEOL FT-NMR JNM A-500 spectrometer (^{1}H at 500 MHz, ^{13}C at 125 MHz). Standard pulse sequences were used for the two-dimensional (2D) NMR experiments. Chemical shifts were expressed in δ (ppm) downfield from internal tetramethylsilane (TMS) as an internal standard, and coupling constants (J) were reported in Hertz (Hz). TLC was carried out on Silica gel 60F$_{254}$, and the spots were visualized by spraying with 10% H_2SO_4 and heating. Silica gel (Silica gel 60—70, 230 mesh, Merck), Lichroprep RP-18 (Lobar, 40—63 μm, Merck) and Sephadex LH-20 were used for column chromatography. Preparative HPLC was performed using an ODS column (PEGASIL ODS, 250×10 mm, Senshu Pak; detector: reflective index and UV 210 nm). GC was run on a Shimadzu GC-14B gas chromatograph (column: Supelco SP-2380 fused silica capillary column; 0.53 mm i.d.×15 m, 0.2 μm film; column temperature: 140 °C→220 °C, 4 °C/min; injection temperature: 250 °C).

Isolation of Saponins The roots of *A. mamillata* HANCE were obtained from Sichuan, China in 1996, and the voucher specimens were identified by Prof. Hao Zhang and deposited with the West China University of Medical Sciences. Dried powder (2.5 kg) of the roots of *A. mamillata* was extracted with 95% EtOH (10 l×2) under reflux conditions. The EtOH extract (249 g) was partitioned successively between water and hexane, ethyl acetate, and *n*-butanol, respectively. After removing the solvent, the *n*-butanol extract (38.8 g) was dissolved in methanol (40 ml) and the methanol solution was dropped into ether (2.5 l) to obtain a precipitate (31.5 g). Ten grams of the ether precipitate were chromatographed on a silica gel column with a solvent system of $CHCl_3$–MeOH–H_2O (7.5 : 2.5 : 0.25). Fractions were combined according their TLC behavior. Fractions 550—670 were chromatographed on a Lichroprep RP-18 column with 30, 50, 70, and 100% MeOH to obtain parts 1—7 (p1—7). Part 1 (412 mg) was chromatographed on a Lichroprep RP-18 column (solvent: CH_3CN : H_2O/1 : 6, 2 : 7) to obtain p1-1—4. P1-1 (23 mg) was chromatographed on a Lichroprep RP-18 column with CH_3CN–H_2O/2 : 8 as an eluate to obtain ardisimamilloside D (**2**, 9.9 mg). P1-2 (41 mg) was purified on a Sephadex LH-20 column with MeOH as an eluate to obtain ardisimamilloside C (**1**, 38.3 mg). P1-3 (44 mg) was isolated on a Lichroprep RP-18 column with CH_3CN : H_2O/2 : 8 as an eluate to obtain ardisimamilloside E (**3**, 29.5 mg). P5 (296 mg) was isolated on a Lichroprep RP-18 column with CH_3CN–H_2O/2 : 8 as an eluate to obtain p5-1—3. Furthermore, p5-2 (118 mg) was isolated on a Lichroprep RP-18 column with CH_3CN–H_2O/2 : 8 as an eluate to obtain ardisicrernoside A (**5**, 19.1 mg). P6 (1 g) was isolated on a Lichroprep RP-18 column with 30—70% MeOH as the gradient eluate to obtain p6-1—7. P6-3 (98 mg) and p6-4 (268 mg) were purified on a Sephadex LH-20 column with MeOH as an eluate to obtain ardisimamilloside F (**4**, 21.3 mg) and ardisicrenoside G (**6**, 248 mg), respectively. P7 (125 mg) was isolated on a silica gel column with $CHCl_3$–MeOH–H_2O/8 : 2 : 0.2 as an eluate to obtain **7** (10 mg).

Ardisimamilloside C (**1**): $[\alpha]_D^{25}$ −28.5° (MeOH; c=0.31); HR-FAB-MS (positive): m/z: 1099.5696 $[M+Na]^+$ (Calcd for $C_{53}H_{88}O_{22}Na$; 1099.5662). ^{1}H-NMR (500 MHz, pyridine-d_5): δ (ppm) 0.72 (1H, H-5), 0.85 (3H, s, H-25), 0.94 (3H, s, H-24), 1.04 (3H, s, H-26), 1.17 (3H, s, H-23), 1.33(3H, s, H-29), 1.77 (3H, d, J=13.2 Hz, H-6 of rhamnose), 1.81 (3H, s, H-27), 2.62 (1H, H-18), 3.16 (1H, dd, J=9.8, 4.2 Hz, H-3), 3.82 (2H, s, H-28), 4.14, 4.31 (2H, H-30), 4.94 (1H, br s, H-1 of arabinose), 4.95 (1H, H-16), 5.26 (1H, d, J=7.5 Hz, H-1 of inner glucose), 5.38 (1H, d, J=7.6 Hz, H-1 of terminal glucose), 6.42 (1H, br s, H-1 of rhamnose). ^{13}C-NMR spectral data are given in Tables 1 and 2.

Ardisimamilloside D (**2**): $[\alpha]_D^{25}$ −21.6° (MeOH; c=0.15); HR-FAB-MS (positive): m/z: 1099.5671 $[M+Na]^+$ (Calcd for $C_{53}H_{88}O_{22}Na$; 1099.5662). ^{1}H-NMR (500 MHz, pyridine-d_5): δ (ppm) 0.69 (1H, H-5), 0.86 (3H, s, H-25), 1.04 (3H, s, H-24), 1.08 (3H, s, H-26), 1.17 (3H, s, H-23), 1.22 (3H, s, H-29), 1.80 (3H, d, J=13.2 Hz, H-6 of rhamnose), 1.64 (3H, s, H-27), 4.74 (1H, H-15), 2.62 (1H, H-18), 3.17 (1H, dd, J=10.0, 4.0 Hz, H-3), 3.87 (2H, H-28), 3.89 (2H, H-30), 4.95 (1H, br s, H-1 of arabinose), 5.25 (1H, d, J=7.6 Hz, H-1 of inner glucose), 5.38 (1H, d, J=7.6 Hz, H-1 of terminal glucose), 6.41 (1H, br s, H-1 of rhamnose). ^{13}C-NMR spectral data are given in Tables 1 and 2.

Ardisimamilloside E (**3**): $[\alpha]_D^{25}$ −25.1° (MeOH; c=0.24); HR-FAB-MS (positive): m/z: 1099.5679 $[M+Na]^+$ (Calcd for $C_{53}H_{88}O_{22}Na$; 1099.5662). ^{1}H-NMR (500 MHz, pyridine-d_5): δ (ppm) 0.66 (1H, H-5), 0.85 (3H, s, H-25), 1.03 (3H, s, H-24), 1.17 (3H, s, H-23), 1.36 (3H, s, H-26), 1.56 (3H, s, H-30), 1.59 (3H, s, H-27), 1.81 (3H, d, J=13.2 Hz, H-6 of rhamnose), 2.62 (1H, H-18), 3.14 (1H, dd, J=9.5, 4.0 Hz, H-3), 3.46, 3.66 (2H, d, J=7.3 Hz, H-28), 3.81, 4.08 (2H, d, J=9.2 Hz, H-29), 4.14 (1H, m, H-16), 4.95 (1H, br s, H-1 of arabinose), 5.25 (1H, d, J=7.6 Hz, H-1 of inner glucose), 5.38 (1H, d, J=7.6 Hz, H-1 of terminal glucose), 6.41 (1H, br s, H-1 of rhamnose). ^{13}C-NMR spectral data are given in Tables 1 and 2.

Ardisimamilloside F (**4**): $[\alpha]_D^{25}$ −18.6° (MeOH; c=0.63); HR-FAB-MS (positive): m/z: 1113.5450 $[M+Na]^+$ (Calcd for $C_{53}H_{86}O_{23}Na$; 113.5455). ^{1}H-NMR (500 MHz, pyridine-d_5): δ (ppm) 0.65 (1H, H-5), 0.84 (3H, s, H-25), 1.02 (3H, s, H-24), 1.17 (3H, s, H-23), 1.33 (3H, s, H-26), 1.54 (3H, s, H-29), 1.81 (3H, d, J=13.2 Hz, H-6 of rhamnose), 1.60 (3H, s, H-27), 2.62 (1H, H-18), 3.15 (1H, dd, J=9.6, 4.2 Hz, H-3), 3.15, 3.29 (2H, d, J=7.3 Hz, H-28), 4.08 (1H, H-16), 4.96 (1H, br s, H-1 of arabinose), 5.23 (1H, d, J=7.6 Hz, H-1 of inner glucose), 5.37 (1H, d, J=7.6 Hz, H-1 of terminal glucose), 6.41 (1H, br s, H-1 of rhamnose). ^{13}C-NMR spectral data are given in Tables 1 and 2.

Ardisicrenoside A (**5**): $[\alpha]_D^{25}$ −12.4° (MeOH; c=0.40); MALDI-TOF-MS m/z: 1099 $[M+Na]^+$.

Ardisicrenoside F (**6**): $[\alpha]_D^{25}$ −22.9° (MeOH; c=0.53); MALDI-TOF-MS m/z: 1097 $[M+Na]^+$.

Compound **7**: $[\alpha]_D^{25}$ −27.1° (MeOH; c=0.24); MALDI-TOF-MS m/z: 935 $[M+Na]^+$.

Compounds **1**—**7** (1 mg each) were hydrolyzed, reduced and acetylated, respectively. The arabitol, glucitol and rhamnitol acetates from compounds **1**—**6** were detected in a ratio of 1 : 2 : 1, and from compound **7** they were detected in a ratio of 1 : 2 : 1 by GC analysis.

The absolute configurations of the sugars were determined according to the method reported by Hara and co-workers[9)] using GC. GC conditions: column: 3% ECNSS-M (2 m×0.3 mm); column temp.: 190 °C; injection temp.: 210 °C; retention times (min): L-rhamnose (8.6), L-arabinose (14.4), D-glucose (49.2).

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Five Lanostane Triterpenoids and Three Saponins from the Fruit Body of *Laetiporus versisporus*

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Five lanostane triterpenes, named versisponic acids A—E and three lanostanoid glycosides, laetiposides E—G, were isolated from the fruit bodies of *Laetiporus versisporus*. Their structures were established by extensive NMR experiments and chemical methods.

Key words *Laetiporus versisporus*; versisponic acid; laetiposide; Polyporaceae; lanostane

We earlier reported the isolation and structure elucidation of four new lanostanoid glucosides, designated as laetiposides A—D from the EtOH extract of the fruit bodies of *Laetiporus versisporus* (LLOYD) IMAZ. (Polyporaceae).[1] Further purification of the resulting fractions by reversed-phase HPLC gave five lanostane triterpenes, termed versisponic acids A (**1**), B (**2**), C (**3**), D (**4**), and E (**5**), and minor glycosides, laetiposides E (**6**), F (**7**), and G (**8**). We describe here the isolation and structure elucidation of **1**—**8** by various NMR techniques, including ^{1}H–^{1}H shift correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple-bond connectivity (HMBC), and rotating-frame nuclear Overhauser effect spectroscopy (ROESY) experiments, and by chemical degradation.

Versisponic acid A (**1**) was obtained as needles and its IR spectrum showed hydroxy (3400 cm^{-1}) and carboxy (1700 cm^{-1}) absorptions. The molecular formula, $C_{30}H_{48}O_5$ for **1** was obtained from the elemental analysis and the observation of quasi-molecular ion at *m/z* 487 $[M-H]^-$ in the negative FAB-MS and DEPT ^{13}C-NMR data. Of the seven degrees of unsaturation implied by $C_{30}H_{48}O_5$, three are accounted for by a carboxy [δ 178.8 (s)] and two double bonds [δ 126.4 (d), 135.0 (s), 135.1 (s), 137.1 (s)]. The ^{1}H-NMR spectrum of **1** exhibited six singlet methyls at δ 1.04, 1.07, 1.17, 1.22, 1.35 and 1.48, two hydroxymethines at δ 3.44 (t, J=8.0 Hz) and 4.62 (dd, J=9.5, 5.6 Hz), a hydroxymethylene group at δ 4.43 and 4.50 (each d, J=12.7 Hz), and one vinyl proton at δ 5.34 (m). The ^{13}C-NMR spectrum of **1** provided evidence of a lanostane skeleton. Therefore, ^{13}C-NMR spectral comparison of **1** with 15α-hydroxytrametenolic acid (**9**),[1,2] of which isolation was already reported from this fungus, showed that **1** differs structurally from **9** only in the side chain, and it was assumed that the C-27 methyl group (δ 18.1) in **9** is replaced by CH_2OH group (δ 60.8) in **1**. The nuclear Overhauser effect (NOE) between H-24 (δ 5.34) and H_3-26 (δ 1.48) evidently established the existence of the OH group on C-27. Thus, the structure of versisponic acid A (**1**) was determined to be 3β,15α,27-trihydroxylanosta-8,24-dien-21-oic acid.

The molecular formula of versisponic acid B (**2**) was established as $C_{32}H_{48}O_5$ by the elemental analysis and an $[M-H]^-$ ion peak at *m/z* 511 in its negative FAB-MS, which suggested two additional degrees of unsaturation as compared with **9**. This was fully supported by ^{13}C-NMR data, which showed the appearance of a ketone function at δ 216.1 (s) and an acetyl group at δ 170.9 and 21.3 (Table 2). The ^{1}H-NMR spectrum of **2** also showed the absence of H-3 observed at δ 3.44 (t, J=8.1 Hz) in **9** and the downfield shift of H-15 from δ 4.63 (dd, J=9.5, 6.0 Hz) in **9** to δ 5.38 (dd, J=9.5, 5.5 Hz). Furthermore, significant cross peaks were observed from methyl groups [δ 0.99 (H_3-28), 1.15 (H_3-29)] at C-4 to C-3 (δ 216.1) in the HMBC experiment and NOE was observed between methyl group [δ 1.10 (H_3-18)] and H-15 (δ 5.38) in the ROESY experiment. Thus, the structure of versisponic acid B (**2**) was determined to be 15α-acetoxy-3-oxolanosta-8,24-dien-21-oic acid.

Versisponic acid C (**3**) showed an $[M-H]^-$ ion peak at *m/z* 525, 14 mass units more than that of **2**. Two coupled broad singlets at δ 4.88 and 4.91 in the ^{1}H-NMR data of **3** are characteristic of 24-methylenelanostane. The ^{13}C-NMR spectrum of **3** was very similar to that of **2**, except for the side chain resonances, indicating the presence of ketone at C-3 and α-acetoxy at C-15. Thus, the side chain resonances of **3** were nearly identical to those of sulphurenic acid (**10**).[1,2] The structure of versisponic acid C (**3**) was determined to be 15α-acetoxy-3-oxo-24-methylenelanost-8-en-21-oic acid.

The features of ^{13}C-NMR spectrum of versisponic acid D (**4**) indicated that **4** is a monoacetate of sulphurenic acid (**10**). The position of the acetyl group was assigned to C-15 on the basis of acylation shift (+0.8 ppm) of H-15 as compared with that of **1**. Alkaline treatment of **4** gave **10**. Thus, the structure of versisponic acid D (**4**) was determined to be 15α-acetoxy-3β-hydroxy-24-methylenelanost-8-en-21-oic acid.

The features of ^{13}C-NMR spectrum of versisponic acid E (**5**) also demonstrated that **5** is a diacetate of 15α-hydroxytrametenolic acid (**9**). The positions of two acetyl groups were assigned on the basis of the downfield chemical shifts of H-3 (δ 4.66) and H-15 (δ 5.40) by acylation shifts as compared with **9**. Alkaline treatment of **5** gave **9**. Thus, the structure of versisponic acid E (**5**) was determined to be 3β,15α-diacetoxylanosta-8,24-dien-21-oic acid.

Laetiposide E (**6**) was obtained as colorless needles, which gave a quasi-molecular ion peak at *m/z* 617 $[M-H]^-$ in its FAB-MS. The ^{13}C-NMR spectrum displayed 36 carbon signals, including the pentose signals [δ 102.0 (d), 78.8 (d), 75.3 (d), 72.4 (d), 67.8 (t)]. Actually, acid hydrolysis of **6** with 5% H_2SO_4 afforded dehydroeburicoic acid[1,3] and D-xylose, which was confirmed by specific rotation using chiral

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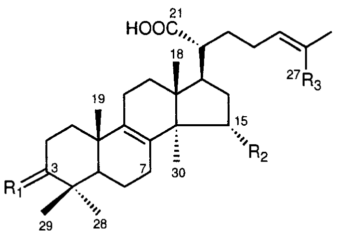
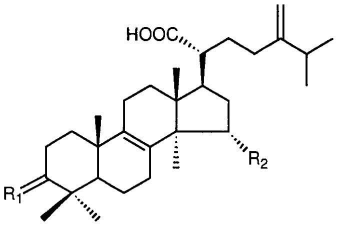
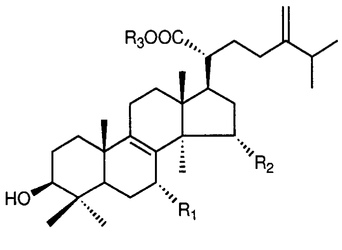
Dedicated to the memory of Dr. Kyosuke Tsuda.



1 $R_1 = {}^{\text{OH}}_{\text{H}}$ R_2= OH, R_3= CH_2OH
2 R_1= O, R_2= OAc, R_3= CH_3
5 $R_1 = {}^{\text{OAc}}_{\text{H}}$ R_2= OAc, R_3=CH_3
9 $R_1 = {}^{\text{OH}}_{\text{H}}$ R_2= OH, R_3=CH_3



3 R_1= O, R_2= OAc
4 $R_1 = {}^{\text{OH}}_{\text{H}}$ R_2= OAc
10 $R_1 = {}^{\text{OH}}_{\text{H}}$ R_2= OH



6 R_1= O-Xyl, R_2, R_3= H
7 R_1= O-Glc, R_2= OH, R_3= H
8 R_1= H, R_2=OH, R_3= Glc
11 R_1= O-Glc, R_2, R_3= H

Chart 1

detection by HPLC analysis.[4a,b] The coupling constant of the anomeric proton observed at δ 4.88 (J=7.7 Hz) in the ^{1}H-NMR spectrum of **6** indicated the xylose having β-configuration. A ^{13}C-NMR spectral comparison of **6** with laetiposide A (**11**)[1] showed that **6** differs structurally from **11** only in the sugar moiety, namely, a xylose instead of a glucose in **11** attached at C-7-α-OH. Indeed, a long-range correlation in the HMBC spectrum of **6** was observed between H-1 (δ 4.88) of xylose and C-7 (δ 71.5). Thus, the structure of laetiposide E (**6**) was determined to be 3β,7α-dihydroxy-24-methylenelanost-8-en-21-oic acid 7-*O*-β-D-xylopyranoside.

Laetiposide B (**7**) gave a quasi-molecular ion at *m/z* 663 $[M-H]^-$, 16 mass units more than that of **11**. Comparison of ^{13}C-NMR data of **7** with those of **11** revealed that the only difference was the appearance of an additional oxymethine carbon signal at δ 72.2 in **7** and suggested the presence of an α-OH at C-15, as in **1**. Acid hydrolysis of **7** also yielded D-glucose which has β-configuration judging from the coupling constant (J=7.4 Hz) of the anomeric proton signal at δ 5.13. Thus, laetiposide F (**7**) was determined to be 3β,7α,15α-trihydroxy-24-methylenelanost-8-en-21-oic acid 7-*O*-β-D-glucopyranoside.

The FAB-MS of laetiposide G (**8**) gave a quasi molecular ion at *m/z* 647 $[M-H]^-$. Acid hydrolysis of **8** gave D-glucose which was confirmed in the same way as for **6** and **7**. The ^{1}H-NMR and ^{13}C-NMR spectra of **8** showed the presence of an ester-linked β-glucopyranosyl moiety suggested by the characteristic chemical shifts of anomeric proton [δ 6.44 (J=8.1 Hz)] and carbon (δ 95.9). Indeed, a ^{13}C-NMR spectral comparison of **8** with **10** showed glycosylation shift[5] by -2.8 ppm at C-21, and the remaining carbon signals were similar. In the HMBC experiment on **8**, the H-1 of glucose at δ 6.44 gave a cross peak with the ester carbon at δ 175.9 (C-21), demonstrating the sugar linkage was located at C-21. Thus, the structure of laetiposide G (**8**) was determined to be 3β,15α-dihydroxy-24-methylenelanost-8-en-21-oic acid 21-*O*-β-D-glucopyranosyl ester.

Triterpenes are ubiquitous in basidiomycetous fungi, but triterpenoid glycosides have rarely been reported.[6] Recently, the triterpernoid xylosides, tsugariosides B and C were isolated from *Ganoderma tsugae* (Polyporaceae).[7] We earlier reported the structures of eight new lanostane glycosides, laetiposides A—G from *L. versisporus*.[1] Two of the compounds, laetiposides C and D were tested for activities against KB and L1210 cells. No activity was observed in these assays (ID_{50} >50 μg/ml).

Experimental

Melting points were measured with a Yanagimoto micromelting point apparatus, without correction. Optical rotations were recorded on a JASCO DIP-140 digital polarimeter. IR spectra were measured on a JASCO FT/IR-5300 instrument. NMR spectra were recorded on a Varian UNITY 600 or JEOL GSX-400 spectrometer in C_5D_5N solution using tetramethylsilane (TMS) as an internal standard. NMR experiments included ^{1}H–^{1}H COSY, DEPT, HMQC, HMBC, and ROESY. Coupling constants (J values) are given in Hertz (Hz). FAB-MS was measured on a JEOL JMS-HX-100 mass spectrometer. Kieselgel 60 (230—400 mesh, Merck) was used for column chromatography, and Silica gel 60F-254 (Merck) for TLC.

Extraction and Isolation The fresh fruit bodies (1.3 kg) of *L. versisporus* were extracted with 70% EtOH at room temperature for 6 weeks. The ethanolic extract (57.5 g) was partitioned between H_2O and EtOAc. The EtOAc soluble portion (9.0 g) was repeatedly subjected to Si gel column chromatography with CH_2Cl_2–MeOH–H_2O (25 : 1 : 0→25 : 4 : 0.1) to afford

Table 1. ^{1}H-NMR Spectral Data for **1**—**9** (in Pyridine-d_5, 400 MHz)

		1$^{a)}$	**2**$^{a)}$	**3**	**4**	**5**	**6**	**7**$^{a)}$	**8**	**9**
Aglycon	3	3.44 (t, 8.0)			3.45 (t, 7.2)	4.66 (dd, 12, 4.5)	3.13 (dd, 11, 4.4)	3.14 (t, 7.5)	3.44 (t, 8.1)	3.44 (t, 8.1)
	7						4.62 (br s)	4.87 (br s)		
	15	4.62 (dd, 9.5, 5.6)	5.38 (dd, 9.5, 5.5)	5.40 (dd, 9.5, 5.0)	5.42 (dd, 9.5, 5.5)	5.40 (dd, 9.5, 5.5)		4.74 (dd, 9.5, 5.5)	4.62 (dd, 9.5, 6.0)	4.63 (dd, 9.5, 6.0)
	18	1.17 (s)	1.10 (s)	1.12 (s)	1.14 (s)	1.08 (s)	0.99 (s)	1.05 (s)	1.21 (s)	1.03 (s)
	19	1.04 (s)	1.04 (s)	1.03 (s)	1.01 (s)	0.91 (s)	0.99 (s)	0.97 (s)	1.01 (s)	1.00 (s)
	24	5.34 (m)	5.30 (m)			5.28 (m)				5.28 (m)
	26	1.48 (s)	1.67 (s)	1.02 (d, 6.8)	1.02 (d, 6.8)	1.67 (s)	1.00 (d, 6.2)	1.00 (d, 6.8)	1.00 (d, 6.8)	1.67 (s)
	27	4.43, 4.50 (d, 12.7)	1.60 (s)	1.02 (d, 6.8)	1.02 (d, 6.8)	1.60 (s)	1.00 (d, 6.2)	1.00 (d, 6.8)	1.00 (d, 6.8)	1.60 (s)
	28	1.22 (s)	0.99 (s)	1.00 (s)	1.23 (s)	0.91 (s)	1.48 (s)	1.50 (s)	1.23 (s)	1.37 (s)
	29	1.07 (s)	1.15 (s)	1.15 (s)	1.06 (s)	0.91 (s)	1.10 (s)	1.09 (s)	1.06 (s)	1.07 (s)
	30	1.35 (s)	1.19 (s)	1.21 (s)	1.24 (s)	1.20 (s)	1.45 (s)	1.49 (s)	1.36 (s)	1.22 (s)
	31			4.88, 4.91 (br s)	4.88, 4.91 (br s)		4.80, 4.86 (br s)	4.91, 4.86 (br s)	4.81, 4.87 (br s)	
Ac			2.18 (s)	2.18 (s)	2.16 (s)	2.03 (s), 2.17 (s)				
Glc or Xyl	1						4.88 (d, 7.7)	5.13 (d, 7.4)	6.44 (d, 8.1)	
	2						3.95 (dd, 8.4,7.7)	3.97 (dd, 8.8, 7.4)	4.23 (dd, 9.1, 8.1)	
	3						4.19 (dd, 8.4, 8.4)	4.27 (dd, 9.0, 8.8)	4.29 (dd, 9.4, 9.1)	
	4						4.23 (m)	4.11 (dd, 9.0, 8.8)	4.35 (dd, 9.4, 8.8)	
	5						3.79 (dd,10.5, 10.5) 4.40 (dd, 10.5, 4.8)	4.15 (m)	4.05 (m)	
	6							4.41 (dd, 11.7, 5.6) 4.88 (dd, 11.7, 2.2)	4.26 (dd,11.3, 4.4) 4.46 (dd,11.3, 2.3)	

a) 600 MHz.

five fractions (Frs. 1—5). Fraction 2 (3.0 g) was further purified by Si gel column chromatography with CH_2Cl_2–MeOH (30 : 1) to give five fractions (Frs. 2.1—5). Fraction 2.2 (1.2 g) was subjected to preparative HPLC (octadecyl silica (ODS), 85—90% CH_3OH) to afford versisponic acids B (**2**, 23 mg), C (**3**, 7 mg), D (**4**, 15 mg), and E (**5**, 22 mg). Fraction 5 (2.0 g) was also subjected to Si gel column chromatography with CH_2Cl_2–MeOH–H_2O (25 : 4 : 0.1) to give four fractions (Frs. 5.1—4). Fraction 5.1 (0.8 g) was subjected to HPLC (ODS, 70—75% MeOH) to afford versisponic acid A (**1**, 45 mg), laetiposides F (**7**, 5 mg) and G (**8**, 5 mg). Fraction 5.2 (0.2 g) was subjected to HPLC (ODS, 68% MeOH) to afford laetiposide E (**6**, 5 mg).

Versisponic Acid A (**1**): Colorless needles, mp 195—197 °C, $[\alpha]_D^{25}$ +34.8° (c=1.0, MeOH). IR (film) cm^{-1}: 3400 (br), 1700 (br), 1060. Negative FAB-MS *m/z*: 487 $[M-H]^-$. *Anal.* Calcd for $C_{30}H_{48}O_5$: C, 73.73; H, 9.90. Found: C, 74.0; H, 9.52. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Versisponic Acid B (**2**): An amorphous powder, $[\alpha]_D^{25}$ +68.8° (c=1.2, MeOH). IR (film) cm^{-1}: 3400 (br), 1730, 1700 (br), 1240, 1060. Negative FAB-MS *m/z*: 511 $[M-H]^-$. *Anal.* Calcd for $C_{32}H_{48}O_5$: C, 74.96; H, 9.44. Found: C, 74.66; H, 9.30. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Versisponic Acid C (**3**): An amorphous powder, $[\alpha]_D^{25}$ +66.3° (c=0.6, MeOH). IR (film) cm^{-1}: 3400 (br), 1730, 1700 (br), 1240, 1050. Negative FAB-MS *m/z*: 525 $[M-H]^-$. *Anal.* Calcd for $C_{33}H_{50}O_5$: C, 75.25; H, 9.57. Found: C, 75.30; H, 9.71. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Versisponic Acid D (**4**): Colorless needles, mp 245—247 °C, $[\alpha]_D^{25}$ +59.0° (c=0.2, MeOH). IR (film) cm^{-1}: 3400 (br), 1730, 1700 (br), 1240, 1060. Negative FAB-MS *m/z*: 527 $[M-H]^-$. *Anal.* Calcd for $C_{33}H_{52}O_5$: C, 74.96; H, 9.91. Found: C, 75.26; H, 10.11. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Versisponic Acid E (**5**): An amorphous powder, $[\alpha]_D^{25}$ +51.0° (c=1.1, MeOH). IR (film) cm^{-1}: 3400 (br), 1735, 1700 (br), 1240, 1060. Negative FAB-MS *m/z*: 555 $[M-H]^-$. *Anal.* Calcd for $C_{34}H_{52}O_6$: C, 73.34; H, 9.41. Found: C, 73.44; H, 9.57. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Laetiposide E (**6**): Colorless needles, mp 218—220 °C, $[\alpha]_D^{25}$ +9.1° (c=0.3, MeOH). IR (film) cm^{-1}: 3400 (br), 1700 (br), 1060. Negative FAB-MS *m/z*: 617 $[M-H]^-$. *Anal.* Calcd for $C_{36}H_{58}O_8 \cdot H_2O$: C, 67.89; H, 9.50. Found: C, 68.01; H, 9.82. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Laetiposide F (**7**): Colorless needles, mp 220—222 °C, $[\alpha]_D^{25}$ +16.7° (c=0.4, MeOH). IR (film) cm^{-1}: 3400 (br), 1700 (br), 1060. Negative FAB-MS *m/z*: 663 $[M-H]^-$. *Anal.* Calcd for $C_{37}H_{60}O_{10} \cdot H_2O$: C, 65.08; H, 9.15. Found: C, 64.85; H, 9.02. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Laetiposide G (**8**): Colorless needles, mp 238—240 °C, $[\alpha]_D^{25}$ +28.8° (c=0.3, MeOH). IR (film) cm^{-1}: 3400, 1730, 1060. Negative FAB-MS *m/z*: 647 $[M-H]^-$. *Anal.* Calcd for $C_{37}H_{60}O_9 \cdot H_2O$: C, 66.64; H, 9.37. Found: C, 66.46; H, 9.12. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Alkaline Methanolysis of Versisponic Acids D (4) and E (5) A solution of each compound (10 mg each) in MeOH (1.0 ml) was treated with 28% sodium methoxide (0.3 ml) under an N_2 atmosphere. The mixture was stirred at room temperature for 2 h. The reaction mixture was acidified with dilute HCl, and extracted with EtOAc. The EtOAc layer afforded sulphurenic acid (**10**, 8 mg) from **4** and 15α-hydroxytrametenolic acid (**9**, 7 mg) from **5**, respectively, which were confirmed by comparison of ^{1}H-NMR data and by co-HPLC (ODS, 80% MeOH) with an authentic sample.

Acid Hydrolysis of Laetiposide E (6) A solution of **6** (3 mg) in 5% H_2SO_4–dioxane (1 : 1) was heated at 100 °C for 2 h. The reaction mixture was diluted with H_2O, and extracted with EtOAc. The EtOAc layer gave an aglycone, which was confirmed by comparison with co-TLC (CH_2Cl_2 : MeOH=25 : 1) with dehydroeburicoic acid. The H_2O layer was neutralized with Amberlite IRA-35 and evaporated *in vacuo* to dryness. The sugar was determined using refractive index (RI) and chiral detection in HPLC (Shodex RSpak NH_2P-50 4D, CH_3CN–H_2O–H_3PO_4, 95 : 5 : 1, 1 ml/min, 47 °C) by comparison with authentic sugar (10 mM of D-Xyl). Compound **6** gave the positive peak of D-(+)-Xyl at 9.10 min.

Identification of Component Sugar of Laetiposides F (7) and G (8) Acid hydrolysis of **7** and **8** was carried out in the same way as for **6**. Each sugar part gave a positive peak of D-(+)-Glc at 20.7 min.

Table 2. ^{13}C-NMR Spectral Data for **1**—**11** (in Pyridine-d_5, 100 MHz)

C	**1**$^{a)}$	**2**	**3**	**4**	**5**	**6**	**7**$^{a)}$	**8**	**9**	**10**	**11**$^{a)}$
1	36.1	35.9	35.7	35.8	36.0	35.6	35.0	36.4	36.1	36.1	35.4
2	28.7	34.6	34.5	28.5	24.3	28.8	28.4	29.0	28.6	28.6	28.5
3	78.0	216.1	216.1	77.8	80.5	78.0	77.7	78.2	78.0	78.1	77.8
4	39.5	47.3	47.2	39.4	37.9	39.6	39.1	39.8	39.4	39.4	39.1
5	50.8	51.2	51.1	50.5	50.4	44.5	43.7	51.0	50.8	50.8	44.3
6	18.8	17.7	18.4	18.6	18.2	23.9	23.0	18.5	18.7	18.8	23.6
7	27.6	26.4	26.4	26.7	26.5	71.5	71.5	28.0	27.6	27.6	72.2
8	135.0	133.8	133.8	132.8	133.1	134.4	133.5	134.8	135.0	135.0	134.5
9	135.1	134.7	134.7	135.1	135.5	142.3	143.9	135.0	135.1	135.1	142.1
10	37.5	37.0	37.0	37.3	37.1	38.9	39.1	37.8	37.4	37.4	38.5
11	21.1	20.9	20.8	20.9	20.9	21.9	21.5	21.6	21.0	21.1	21.6
12	30.1	29.3	29.2	29.4	29.3	30.2	29.9	30.1	30.0	30.0	29.6
13	45.4	45.1	45.0	45.1	45.1	45.9	46.4	45.8	45.3	45.4	45.6
14	52.0	51.0	50.9	51.0	51.0	50.6	53.5	52.3	52.0	52.0	50.4
15	72.4	75.9	75.8	75.8	76.0	29.7	72.2	72.6	72.4	72.5	29.4
16	39.2	35.9	35.9	35.8	35.2	28.0	38.6	39.4	39.2	39.2	27.8
17	46.7	46.4	46.4	46.4	46.5	48.1	47.3	46.8	46.6	46.6	47.8
18	16.8	16.6	16.5	16.5	16.7	17.0	17.0	16.7	16.7	16.7	16.6
19	19.3	19.5	19.4	19.2	19.1	18.3	19.5	19.7	19.3	19.3	18.0
20	49.0	48.7	48.8	48.8	48.7	49.7	49.3	48.6	48.9	49.3	49.5
21	178.8	178.3	178.3	178.3	178.6	178.8	178.5	175.9	178.9	178.7	178.8
22	33.4	33.1	31.6	31.7	33.2	32.1	31.7	32.4	33.2	31.8	31.8
23	26.1	26.3	32.5	32.6	26.7	33.1	32.7	32.4	26.7	32.8	32.7
24	126.4	124.7	155.8	155.5	124.7	155.8	155.8	155.7	124.8	156.0	156.0
25	137.1	131.8	34.1	34.1	131.8	34.5	34.1	34.4	131.7	34.1	34.2
26	21.8	25.7	21.7	21.7	25.7	22.2	21.9	21.6	25.6	21.8	21.9
27	60.8	18.5	21.8	21.9	18.5	22.3	22.0	22.2	18.1	21.9	22.0
28	28.6	26.6	26.2	28.5	28.0	28.9	28.6	29.0	28.5	28.5	28.6
29	16.3	21.2	21.1	16.2	16.5	17.3	17.0	16.7	16.2	16.2	16.9
30	18.1	18.5	18.5	18.5	17.7	25.9	17.8	18.5	18.1	18.0	25.7
31			107.0	106.9		107.2	107.1	107.1		107.1	107.0
Glycosyl moiety											
Glc or 1						102.0	99.8	95.9			101.2
Xyl 2						75.3	74.9	74.2			74.6
3						78.8	77.9	79.2			78.5
4						72.4	71.5	71.5			72.4
5						67.8	78.8	79.4			78.3
6							62.9	62.7			63.6
Ac 1		170.9	170.9	170.7	170.7						
2		21.3	21.2	21.1	21.1						
1					170.9						
2					21.2						

a) 125 MHz.

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A New Pentanorlanostane Derivative, Cladosporide A, as a Characteristic Antifungal Agent against *Aspergillus fumigatus*, Isolated from *Cladosporium* sp.

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In the course of searching for new antifungal agents, a new pentanorlanostane derivative, cladosporide A (1), was isolated along with ergosterol, ergosterol peroxide and 23,24,25,26.27-pentanorlanost-8-ene-3β,22-diol (2) from *Cladosporium* sp. as a characteristic antifungal agent against the human pathogenic filamentous fungus *Aspergillus fumigatus*. The structure of 1 was established as 3β,22-dihydroxy-23,24,25,26, 27-pentanorlanostane-29-al by spectroscopic and chemical investigation and X-ray crystallographic analysis. Inhibitory activity against *A. fumigatus* (IC_{80} 0.5—4.0 μg/ml) was observed for cladosporide A (1), but no activity was observed against pathogenic yeasts, *Candida albicans* and *Cryptococcus neoformans*, and other pathogenic filamentous fungi, *Aspergillus niger* and *A. flavus*. The 4β-aldehyde residue in 1 might be essential for the antifungal activity, since 23,24,25,26,27-pentanorlanost-8-ene-3β,22-diol (2) showed no inhibition against the above four fungi.

Key words *Cladosporium* sp.; pentanorlanostane; antifungal activity; cladosporide A; *Aspergillus fumigatus*

The incidence of life-threatening fungal infections has steadily increased in immunocompromised hosts such as human immunodeficiency virus (HIV) infected persons and cancer patients.1) It is well known that *Aspergillus fumigatus* Fresenius is one of the major opportunistic human pathogens causing aspergillosis. Invasive pulmonary aspergillosis and *Pneumocystis carinii* pneumonia are a leading cause of deaths in bone marrow transplant recipients and in HIV-infected patients, respectively. Moreover, resistance to the azoles which are the most widely used antifungals today is attracting much attention. Therefore, novel antifungal agents are continuously required to overcome the above fungal diseases.

The isolation of 18,22-cyclosterols [Mer-NF8054X (**3**) *etc.*] as an antifungal agent against *A. fumigatus*,2) from *Aspergillus ustus* (Bain.) Thom *et* Church3) and *Emericella heterothallica* (Kwon, Fennell *et* Raper) Malloch *et* Cain4) was recently reported. We also reported the isolation of tetranorditerpenoids [LL-Z1271*α* (**4**) *etc.*], which are antifungal antibiotics against pathogenic yeasts, *i.e.*, *Candida albicans* (Roben) Berkhout *etc.*, isolated from *Oidiodendron* sp.5) Screening for further antifungal substances from fungal sources, the chloroform–methanol (1 : 1) extract of about 140 freshly isolated fungal strains, cultivated on rice at 25 °C for 21 d, were subjected to the antifungal test against pathogenic yeasts, *C. albicans* IFM40009 and *Cryptococcus neoformans* (Sanfelice) Vuillemin ATCC 90112, and pathogenic filamentous fungi, *A. fumigatus* IFM 41243 and *Aspergillus niger* Van Tieghem H7160B. Twenty strains showed antifungal activity against some fungi tested, which is shown in Table 1. Now we were interested in antifungal substances characteristically inhibiting the growth of *A. fumigatus* causing allergenic bronchopulmonary aspergillosis (ABPM) *etc.* The extract of a dematiaceous fungus, *Cladosporium* sp. IFM 49189 prompted us to isolate antifungally active compounds.

Two pentanorlanostane derivatives, **1**, designated as cladosporideA, and **2**, were isolated along with ergosterol and ergosterol peroxide from the dichloromethane–ethanol (1 : 1) extract of this fungus. The structural elucidation and the antifungal activity of **1** and **2** are described in this paper.

The structure of compound **2** was confirmed as 23,24,25,26,27-pentanorlanost-8-ene-3β,22-diol, which was first isolated from an entomopathogenic fungus *Verticillium lecanii* (Zimmermann) Viegas,6) by means of the analysis of the ^{1}H- and ^{13}C-NMR, ^{1}H-detected heteronuclear multiple-quantum coherence *via* direct bond (HMQC), and heteronuclear multiple bond connectivity by 2D multiple quantum NMR (HMBC) spectra. The assignments of the ^{1}H- and ^{13}C-NMR signals of **2** are summarized in Table 2.

The molecular formula of cladosporide A (**1**), colorless plates, mp 206—209 °C (from methanol), was confirmed as $C_{25}H_{40}O_3$ by high resolution electron-impact ionization mass spectrometry (EI-MS). From the analysis of the ^{1}H-NMR spectrum (Table 2), it was clear that **1** had five methyl groups [δ 0.72 (s), 0.90 (s), 0.93 (s), 1.03 (d), and 1.31 (s)], two hydroxyl groups, one of which was a primary alcohol [methylene protons at δ 3.36 (dd), 3.66 (dd)] and the other a secondary alcohol [a methine proton at δ 3.18 (m)], and an aldehyde [δ 9.78 (d)]. The ^{13}C-NMR spectrum of **1** (Table 2) showed 25 carbons: they were assigned as 5 methyl carbons, 9 sp^3 methylene carbons, one of which was bearing an oxygen function (δ 67.9), 4 sp^3 methine carbons, one of which was bearing an oxygen function (δ 77.0), 4 sp^3 quaternary carbons, 2 sp^2 quaternary carbons (δ 133.2, 134.4), and an aldehyde carbon (δ 208.1). The UV absorption maximum at 284 nm (log ε 2.12) showed the presence of an isolated carbonyl group in **1**. From the above results, it was assumed that **1** should be a tetracyclic pentanortriterpenoid or sesterterpenoid derivative. The ^{1}H- and ^{13}C-NMR signals of **1** (Table 2) were similar to those of co-occurring **2**, except for the ap-

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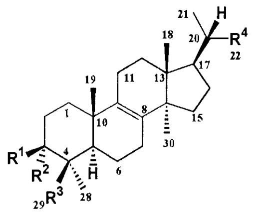
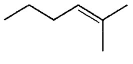
Dedicated to the memory of Dr. Kyosuke Tsuda.

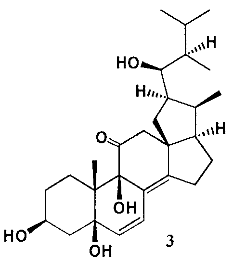
Table 1. Antifungal Activity of Fungal Extracts and Cladosporide A (**1**)

Strain of fungus or compound **1**	Test organisms			
	A. fumigatus	*A. niger*	*C. albicans*	*C. neoformans*
Paecilomyces lilacinus (Jo-1Ab)	16	22	18	(11)
Fusarium sp. (101)	11	(12)	10	—
Unidentified fungus (Mo-15A)	(13)	27	—	—
Unidentified fungus (133C)	17	—	—	—
Cladosporium sp. (Na92=IFM 49189)	(15)	—	—	—
Cladosporium sp. (150B)	(15)	—	—	—
Cladosporium sp. (145B)	(12.5)	—	—	—
Cladosporium sp. (165A)	(12)	—	—	—
Cladosporium sp. (142E)	(11.5)	—	—	—
Unidentified fungus (133A)	(11)	—	—	—
Cladosporium sp. (159A)	(11)	—	—	—
Cladosporium sp. (165B)	(11)	—	—	—
Unidentified fungus (168)	(10)	—	—	—
Tricoderma sp. (75B)	—	12	11	—
Unidentified fungus (Fo-1a)	—	—	(11)	(10)
Asergillus japonicus (80A)	—	—	11	—
Unidentified fungus (152B)	—	—	—	(11)
Penicillium sp. (133D)	—	—	—	(10)
Penicillium sp. (138A)	—	—	—	(10)
100 μg/disc of **1**	(19)	—	—	—
50 μg/disc of **1**	(14)	—	—	—
25 μg/disc of **1**	(13)	—	—	—
12.5 μg/disc of **1**	(12.5)	—	—	—
6.25 μg/disc of **1**	(12)	—	—	—

The diameter of inhibitory zone was measured in mm. The parenthesis means slightly growing in the inhibition circle. The minus (—) means no inhibition.



1 : R^1 = OH, R^2 = H, R^3 = CHO, R^4 = CH$_2$OH
2 : R^1 = OH, R^2 = H, R^3 = CH$_3$, R^4 = CH$_2$OH
5 : R^1, R^2 = O, R^3 = R^4 = CHO
6 : R^1, R^2 = O, R^3 = CHO, R^4 = CO$_2$H
7 : R^1 = OH, R^2 = H, R^3 = CHO,
R^4 = 



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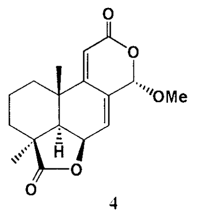

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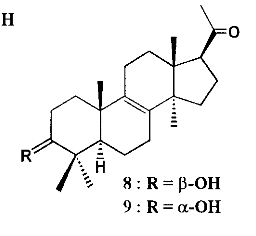
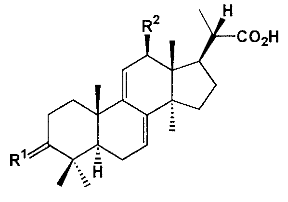


8 : R = β-OH
9 : R = α-OH



10 : R^1 = β-OH, α-H, R^2 = H
11 : R^1 = β-OH, α-H, R^2 = OH
12 : R^1 = O, R^2 = H

pearance of an aldehyde signal [δ 208.1, δ 9.78 (1H, d, J= 1.8 Hz)] in **1** instead of a methyl signal [δ 28.0, δ 1.00 (3H, d, J=1.2 Hz)]. The only other differences observed in the ^{13}C-NMR spectra of **1** and **2** were as follows: the signals observed at δ 79.0 (C-3), 38.9 (C-4), 50.4 (C-5), and 15.4 (C-28) in **2** were shifted to δ 77.0 (C-3), 52.4 (C-4), 52.0 (C-5), and 18.7 (C-28) in **1**, respectively. Therefore, it is concluded that the aldehyde group in **2** was attached at C-4. The analysis of the homonuclear ^{1}H–^{1}H shift correlation (^{1}H–^{1}H COSY), HMQC, and HMBC (Fig. 1) spectra also supported the planar structure of cladosporide A (**1**) as an oxidized derivative of **2** at one of the 4,4-dimethyl groups.

A 6.0 and a 0.7% nuclear Overhauser effect (NOE) was observed on the aldehyde proton at δ 9.78 and the methyl protons at δ 0.72 assigned to C-18, respectively, when the methyl protons at δ 0.93 assigned to C-19 were irradiated. An NOE correlation peak was also observed between the protons at C-3 (δ 3.18) and C-5 (δ 1.32). These results showed that the aldehyde group and the hydroxyl group at C-3 are located on the same side. A 2.7% NOE was observed on the proton at C-17 (δ 1.58) when the methyl protons at δ 0.90 assigned at C-30 were irradiated. Therefore, it is determined that the stereochemistry of the side chain attached at C-17 was β-orientation.

In order to confirm the relative stereochemistry of cladosporide A (**1**), an X-ray crystallographic analysis was undertaken. Compound **1** was crystallized from methanol as colorless plates, suitable for X-ray analysis, in the form of a

Table 2. ^{1}H- and ^{13}C-NMR Chemical Shifts of Pentanorlanostane Derivatives in $CDCl_3$

Carbon No.	**1**		**2**	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	35.3	1.25 ddd (13.3, 13.1, 4.5)	35.6	1.22 m
		1.83 m		1.74 m
2	28.4	1.83 m	27.9[a]	1.57 m
		1.93 m		1.68 m
3	77.0	3.18 m	79.0	3.23 dd (11.6, 4.6)
4	52.4		38.9	
5	52.0	1.32 dd (14.7, 2.2)	50.4	1.05 m
6	18.1	1.69 m	18.3[a]	1.48 m
		2.01 m		1.68 m
7	26.4	2.12 m	26.5[a]	1.90 m
		2.12 m		2.03 m
8	134.4		134.5[a]	
9	133.2		134.3[a]	
10	37.0		37.0	
11	21.1	2.02 m	21.0	2.03 m
		2.10 m		2.03 m
12	30.5	1.71 m	30.9[a]	1.68 m
		1.78 m		1.77 m
13	44.3		44.7	
14	49.3		49.6	
15	30.7	1.23 ddd (11.6, 11.6, 1.8)	31.0[a]	1.22 m
		1.60 m		1.57 m
16	27.3	1.40 m	27.7[a]	1.37 m
		1.93 m		1.68 m
17	46.4	1.58 m	46.8	1.57 m
18	15.7	0.72 s	15.9	0.72 s
19	18.3	0.93 s	19.2	0.99 s
20	39.2	1.58 m	39.4	1.57 m
21	16.5	1.03 d (6.1)	16.8	1.03 d (5.8)
22	67.9	3.36 dd (10.4, 6.4)	68.2	3.36 dd (10.7, 4.6)
		3.66 dd (10.4, 2.4)		3.65 br d (10.7)
28	18.7	1.31 s	15.4[a]	0.81 d (1.5)
29	208.1	9.78 d (1.8)	28.0	1.00 d (1.2)
30	24.1	0.90 d (0.6)	24.3	0.88 s

a) The assignments are revised from those in ref. 6.

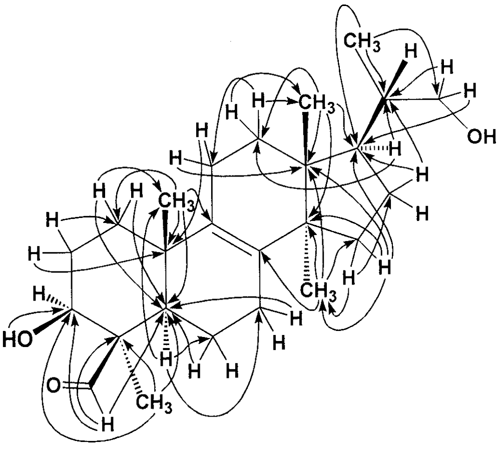


Fig. 1. HMBC Correlations in Cladosporide A (**1**)

methanol solvate. The crystal structure was solved by direct methods and the final R value reached to 0.073. The crystal structure was established to be as shown in Fig. 2. The bond lengths and angles are not significantly different from the expected ones. The molecules of **1** and methanol are mainly packed through hydrogen bonding between 22-OH---3β-OH---H_2O---3β-OH [O2–H---O1 (2.672 Å), O1–H---O4 (2.732 Å), and O4–H---O1 (2.732 Å)].[7] The relative structure of cladosporide A was consequently confirmed as shown in **1**.

We intended to confirm the absolute configuration of cladosporide A (**1**) by the application of the octant rule to a 3-keto derivative of **1**. Compound **1** was at first oxidized by pyridinium dichromate (PDC) at room temperature or at 40 °C to give a ketodialdehyde derivative (**5**) or a ketoaldehyde carboxylic acid (**6**), respectively, the structures of which were confirmed from the analysis of the ^{1}H-HMR spectra. Deformylation of **5** or **6** was attempted with chlorotris(triphenylphosphine)rhodium(I) by the method of Tsuji and Ohno,[8] but the expected deformyl compound could not be obtained. The Cotton effect in the circular dichroism (CD) spectra of ketones or aldehydes is usually observed at 290—300 nm, due to the n→π^* transition. Compound **1** showed a negative Cotton effect [$\Delta\varepsilon$ −0.55 (300 nm)] due to the 4β-aldehyde, whereas **6** exhibited a negative Cotton effect [$\Delta\varepsilon$ −0.84 (291 nm)] by the sum of those due to the 4β-aldehyde and the 3-ketone. Since the amplitude of the Cotton effect of **6** was larger than that of **1**, the negative Cotton effect was expected due to the 3-ketone in **6**. From the application of the octant rule to the 3-ketone, the absolute structure of cladosporide A was consequently determined as 3β,22-dihydroxy-23,24,25,26,27-pentanorlanostane-29-al as shown in **1**.

The antifungal assay was first performed by the paper disc

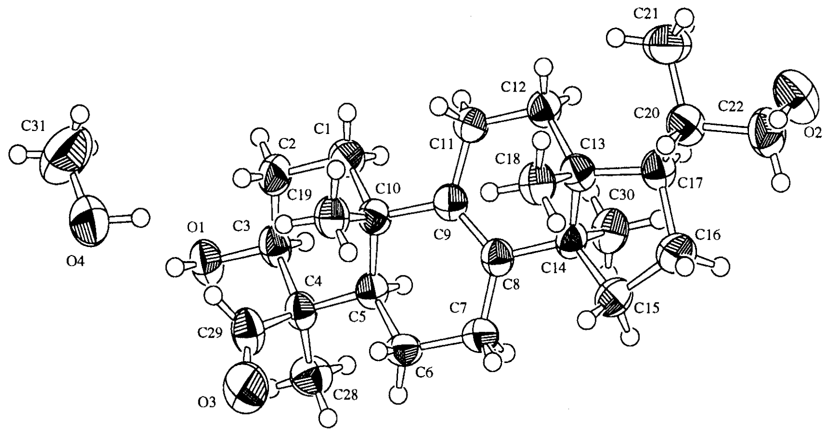


Fig. 2. Perspective View of the Crystal Structure of Cladosporide A (**1**) Methanol Solvate with Thermal Ellipsoids at 30% Possibility

method. Cladosporide A (**1**) showed the characteristic inhibition against a human pathogenic filamentous fungus, *A. fumigatus*, at 6.25 μg/disc, whereas no inhibition was observed against a pathogenic filamentous fungus, *A. niger*, and pathogenic yeasts, *C. albicans* and *C. neoformans* (Table 1). Compound **1** did not completely inhibit the growth of *A. fumigatus*, but **1** apparently reduced the growth of this fungus. Since **2**, the 4β-methyl derivative of **1**, showed no inhibition against the above four fungi, the 4β-aldehyde seems to be essential for this antifungal activity against *A. fumigatus*.

In order to know the antifungal spectrum of cladosporide A (**1**), we intended to determine the minimum inhibition concentrations (MIC) of **1** against 10 strains of filamentous fungi and 8 strains of yeasts, including human pathogens. Since **1** did not show a clear MIC, the 80% inhibition concentrations (IC_{80}) for cladosporide A (**1**) are summarized in Table 3.

The antifungal activity of **1** against all 7 strains of *A. fumigatus* including 5 human pathogens isolated from the patients of ABPM *etc.* (IFM 4942, IFM 40819, IFM 46075, IFM 47064, IFM 47078) showed IC_{80} of 0.5—4.0 μg/ml, but no activity was observed against other filamentous fungi, *A. flavus* LINK: FRIES and *A. niger*, and various pathogenic yeasts, *C. albicans*, *C. dubliniensis* D. J. SULLIVAN, *C. guilliermondii* (CASTELLANI) LANGERON *et* GUERRA, *C. kefyr* (BEIJERINCK) VAN UDEN *et* BUCKLEY, *C. stellatoidea*, *C. tropicalis* (CASTELLANI) BERKHOUT, and *Cryptococcus neoformans* up to a concentration of 128 μg/ml. On the other hand, **2** showed entirely no antifungal activity against all the organisms tested up to a concentration of 128 μg/ml. The antifungal activity of **1** against *A. fumigatus* was not precisely determined and might be stronger than that tested now, because of the low solubility of **1** in dimethylsulfoxide and/or methanol.

Since *A. fumigatus* is one of the major opportunistic pathogens causing various aspergillosis, ABPM *etc.*, it is interesting that cladosporide A (**1**) has considerably strong antifungal activity only against *A. fumigatus*, though this activity might be fungistatic, and not fungicidal.

There are few examples of compounds derived from lanosterol (**7**) with a shortened side chain that are isolated from fungal sources. Hexanorlanosterols **8** and **9**, isolated from *Fomes officinalis* (VILLARS et FRIES) FAULL (Polyporaceae),[9] are the first instance of the occurrence of a lanostane derivative that is degraded on the normal side chain in a microorganism. The first example of a pentanorlanostane derivative is 23,24,25,26,27-pentanorlanost-8-ene-3β,22-diol (**2**), one of the metabolites from our strain of *Cladosporium* sp., which was originally isolated from an entomopathogenic fungus,[6] as described already. Claydon *et al.* also reported several pentanorlanostanecarboxylic acid derivatives, 3β-hydroxy-4,4,14α-trimethyl-5α-pregna-7,9(11)-diene-20*S*-carboxylic acid (**10**),[10] 3β,12β-dihydroxy-4,4,14α-trimethyl-5α-pregna-7,9(11)-diene-20*S*-carboxylic acid (**11**),[11] and 4,4,14α-trimethyl-3-oxo-5α-pregna-7,9(11)-diene-20*S*-carboxylic acid (**12**)[11] *etc.* from the above fungus. Cladosporide A (**1**) is the first example of a new pentanorlanostane derivative having 4β-methyl group oxidized to an aldehyde.

Table 3. Antifungal Activity of Cladosporide A (**1**)

Test organism	Strain	IC_{80} (μg/ml)
A. fumigatus	IFM 4942	1.0
A. fumigatus	IFM 40819	4.0
A. fumigatus	IFM 41375	0.5
A. fumigatus	IFM 41382	1.0
A. fumigatus	IFM 46075	4.0
A. fumigatus	IFM 47064	4.0
A. fumigatus	IFM 47078	0.5
A. flavus	IFM 47031	>128
A. flavus	IFM 47032	>128
A. niger	H7160B	>128
Candida albicans	ATCC 90028	>128
C. albicans	ATCC 90029	>128
C. dubliniensis	CBS 7988	>128
C. gulliermondii	IFM 46823	>128
C. kefyr	IFM 46921	>128
C. stellatoidea	CBS 1905	>128
C. tropicalis	IFM 46816	>128
Cryptoccocus neoformans	IFM 40215	>128

It is interesting that similar steroidal compounds, cladosporide A (**1**) and 18,22-cycloergostane steroid [Mer-NF8054X (**3**)], showed characteristic antifungal activity against *A. fumigatus*, one of the major opportunistic pathogens.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. EI-MS were taken with a JEOL JMS-MS600W spectrometer. UV and IR spectra were recorded on a Hitachi U-3210 spectrophotometer and a JASCO IR-810 spectrophotometer, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL Lambda-500 (^{1}H, 500.00 MHz; ^{13}C, 125.43 MHz) spectrometer, using tetramethylsilane as an internal standard. CD curves were determined on a JASCO J-600 spectropolarimeter. Column chromatography was performed using Kieselgel 60 (Art. 7734, Merck). Low-pressure liquid chromatography (LPLC) was performed with a Chemco Low-Prep 81-M-2 pump and glass column (200×10 mm) packed with Silica gel CQ-3 (30—50 μm, Wako). HPLC was performed with a JASCO PU-980 pump (flow rate, 7 ml/min), equipped with a JASCO RI-930 detector. TLC was conducted on pre-coated Kieselgel 60 F_{254} plates (Art. 5715; Merck). Spots on TLC were detected by UV light on 254 nm and/or by spraying with 5%-H_2SO_4 and then heating.

Antifungal Assay by Paper Disc Method The antifungal assay was performed by the paper disc method against *C. albicans* IFM40009, and *C. neoformans* ATCC 90112, *A. fumigatus* IFM 41243, *A. niger* H7160B, as test organisms. Each source strain was cultured at 25 °C for 21 d in a test-tube containing 10 g of moist rice. The cultivated rice was extracted with $CHCl_3$–MeOH (1 : 1) and the organic layer was evaporated *in vacuo*. The extracts were subjected to the paper discs (8 mm diameter) as 2.5 mg/disc and placed on the assay plates. The fungi were cultivated in Sabouraud's glucose broth (SB) agar at 25 °C. After 48—72 h incubation, zones of inhibition (mm in diameter) were recorded. Compound **1** was also subjected to this assay system as desired weight (μg/disc). The results are summarized in Table 1.

Isolation of Cladosporide A (1) from *Cladosporium* sp. *Cladosporium* sp. strain IFM 49189, was cultured at 25 °C for 21 d in 10 Roux flasks containing 150 g of moist rice in each flask. The cultivated rice was extracted with CH_2Cl_2–EtOH (1 : 1) and the organic layer was evaporated *in vacuo*. The extract (24 g) was suspended in water and extracted with $CHCl_3$, and the organic layer was evaporated *in vacuo*. The residue (14.5 g) was chromatographed on silica gel with $CHCl_3$, $CHCl_3$–EtOH (50 : 1), $CHCl_3$–EtOH (5 : 1), $CHCl_3$–EtOH (1 : 1), and acetone, in turn. Ergosterol was obtained from the fraction eluted with $CHCl_3$ and $CHCl_3$–EtOH (50 : 1) as the main component. The fraction eluting just after ergosterol with $CHCl_3$–EtOH (50 : 1) was purified with LPLC on a silica gel column using the solvent system of CH_2Cl_2–acetone (20 : 1) to give an ergosterol peroxide-rich fraction. This fraction was evaporated and dissolved in MeOH, and then centrifuged to remove the precipitated ergosterol peroxide and fatty acid. The supernatant was evaporated *in vacuo* and the obtained residue was purified repeatedly with LPLC on an ODS column (90% MeOH) followed by purification with HPLC on silica gel [$CHCl_3$–EtOH (50 : 1)] to give cladosporide A (**1**) (13 mg) and a small amount of 23,24,25,26,27-pentanorlanost-8-ene-3β,22-diol (**2**).

Cladosporide A (**1**): Colorless plates, mp 206—209 °C (from MeOH), mp 160—162 °C (from $CHCl_3$–EtOH). EI-MS *m/z* (%): 388.2962 (M^+, 388.2977 for $C_{25}H_{40}O_3$, 61), 373 ($M-CH_3$, 100). UV λ_{max}^{MeOH} nm (log ε): 284 (2.12). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1710 (CHO). CD ($CDCl_3$) (nm): −0.56 (295). CD (MeOH) $\Delta\varepsilon$ (nm): −0.55 (300). The ^1H- and ^{13}C-NMR signal assignments are summarized in Table 2.

Structure Determination of Cladosporide A (1) Methanol Solvate by X-Ray Diffraction Cladosporide A (**1**) was grown from methanol to give crystals of **1** methanol solvate as plates. Diffraction intensities were collected from a crystal of dimensions 0.80×0.20×0.05 mm on a Rigaku AFC-7 FOS four-circle diffractometer. Of the total 1686 reflections (complete for $2\theta<120.1°$), 1514 satisfied the criterion $F>3\sigma(F)$ and only these were used in the solution and refinement of the structure.

Crystal Data: $C_{25}H_{40}O_3 \cdot CH_4O$, M=420.63, triclinic, space group $P1$, a=7.141 (1), b=15.186 (2), c=6.192 (1) Å, α=90.51 (1), β=111.08 (1), γ=93.21 (1)°, V=625.3 (1) Å^3, Z=1, D_c=1.117 g·cm^{-3}, $F(000)$=232, Cu-$K\alpha$ X-radiation (graphite monochromator), λ=1.54178 Å.

Structure Solution and Refinement: The structure was solved by direct methods using MITHRIL 90[12] and in the final refinement by the full-matrix least-squares method; anisotropic thermal parameters were used for all non-hydrogen atoms and isotropic thermal parameter for hydrogens were fixed. The refinement converged to R 0.073 (R_w 0.073).[7]

Oxidation of Cladosporide A (1) by PDC PDC (20 mg) was added to a dimethylformamide solution (1.5 ml) of cladosporide A (**1**) (2.7 mg) and the mixture was stirred at room temperature for 10 h. The reaction mixture was poured into ice-water and extracted with ether. The evaporated residue was purified by LPLC using the solvent system of hexane–acetone (8 : 1) to give 23,24,25,26,27-pentanorlanost-8-ene-3,22,29-trione (**5**) (2.2 mg).

23,24,25,26,27-Pentanorlanost-8-ene-3,22,29-trione (**5**): Colorless amorphous powder. ^1H-NMR ($CDCl_3$) δ: 0.77 (3H, s, 18-H_3), 0.93 (3H, s, 30-H_3), 1.12 (3H, d, J=6.8 Hz, 21-H_3), 1.15 (3H, s, 19-H_3), 1.29 (3H, s, 28-H_3), 2.37 (1H, ddd, J=13.7, 6.1, 3.5 Hz, 2-H), 2.43 (1H, m), 2.50 (1H, dd, J=6.0, 3.3 Hz, 20-H), 2.66 (1H, ddd, J=15.1, 13.7, 5.5 Hz, 2-H), 9.58 (1H, d, J= 3.3 Hz, 22-H), 9.72 (1H, s, 29-H).

PDC (40 mg) was added to a dimethylformamide solution (1.5 ml) of cladosporide A (**1**) (2.0 mg) and the mixture was stirred at 40 °C for 20 h. The reaction mixture was poured into ice-water and extracted with ether. The evaporated residue was purified by LPLC using the solvent system of hexane–acetone (4 : 1) to give 3,29-dioxo-23,24,25,26,27-pentanorlanost-8-ene-22-oic acid (**6**) (1.4 mg).

3,29-Dioxo-23,24,25,26,27-pentanorlanost-8-ene-22-oic Acid (**6**): Colorless amorphous powder. EI-MS *m/z* (%): 400 (M^+, 38), 375 ($M-CH_3$, 20). ^1H-NMR ($CDCl_3$) δ: 0.75 (3H, s, 18-H_3), 0.94 (3H, s, 30-H_3), 1.15 (3H, s, 19-H_3), 1.23 (3H, d, J=6.8 Hz, 21-H_3), 1.29 (3H, s, 28-H_3), 2.49 (2H, 2-H, 20-H), 2.67 (1H, ddd, J=15.1, 13.3, 6.9 Hz, 2-H), 9.72 (1H, s, 29-H). CD (MeOH) $\Delta\varepsilon$ (nm): −0.84 (291).

Antifungal Activity of Cladosporide A (1) The antifungal activities of cladosporide (**1**) were determined by the serial micro-broth dilution method, with incubation in RPMI-1640 agar supplimented with 3-morpholinopropane-sulfonic acid (MOPS) buffer at 30 °C for 72 h for filamentous fungi and at 35 °C for 48 h for yeast, in accordance with the National Commitee for Clinical Laboratory Standard (NCCLS) method.[13] *Aspergillus fumigatus* IFM 4942, IFM 40819, IFM 41375, IFM 41382, IFM 46075, IFM 47064, and IFM 47078, *A. flavus* IFM 47031 and IFM 47032, and *A. niger* H7160B were used as filamentous fungi, and *C. albicans* ATCC 90028 and ATCC 90029, *C. dubliniensis* CBS 7988, *C. guilliermondii* IFM 46823, *C. kefyr* IFM 46921, *C. stellatoidea* CBS 1905, *C. tropicalis* IFM 46816, and *Cryptococcus neoformans* IFM 40215 were used as yeasts. Since cladosporide A (**1**) did not show a clear MIC, though the positive control, amphotericin B, showed a clear MIC, IC_{80} was used instead. The results are summarized in Table 3.

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Oleanane Acid from *Myrica cerifera*

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From the twigs of *Myrica cerifera* L. (Myricaceae), a new oleanane triterpenic acid named myrica acid was isolated along with myricalactone and several other known constituents. The structure of the acid was determined as 3*β*-hydroxy-1-oxoolean-11,13(18)-dien-28-oic acid on the basis of chemical and spectral evidence.

Key words oleanane acid; myrica acid; *Myrica cerifera*; myricalactone; Myricaceae

Myricaceae plants are distributed in the coasts and poor lands of Japan, China, South-East Asia, Europe, America and the southern part of Africa.[1)] Two species, *Myrica rubra* and *M. gale* var. *tomentosa* are domestic to Japan, and from the Japanese Myrica plants we have reported the isolation and structure determination of several diarylheptanoids and triterpenoids.[2)] In a previous paper, we reported on myricalactone, a diketonic lactone of an oleanane type triterpene isolated from *M. gale* var. *tomentosa*.[3)]

Myrica cerifera (bayberry, wax myrtle) is a tall tree distributed in North America.[4)] The chemical constituents of the plant have been investigated, and diarylheptanoids, myricanone and *dl*-myricanol, and triterpenoids, myricadiol, taraxerol and oleanolic acid have been isolated.[5,6)] Recently Fujimoto *et al.*[7)] reported isolation of myriceric acids A, B, C, D from the plant. They also reported that myriceric acid A, a caffeoyl ester at C-27 of 27-hydroxy-3-oxoolean-12-en-28-oic acid, is a strong antagonist of the endothelin receptor.

In the course of our continuing research on the chemical constituents of *Myrica*,[2)] we isolated a new oleanane type triterpene named myrica acid (**1**) from the twigs of *M. cerifera* along with myricalactone (**2**) and several known constituents (Experimental). Myrica acid (**1**), mp 274—277 °C, $[\alpha]_D$ $-57°$, positive Liebermann–Burchard (L.B.) color reaction (reddish violet), was obtained from an acidic fraction of the methanolic extract through repeated chromatography on silica gel, and gave a monomethyl ester (**1b**) on treatment with diazomethane. The molecular formula of **1** was determined to be $C_{30}H_{44}O_4$ by HR-MS.

In the IR spectrum (KBr), **1** showed absorptions of hydroxyls (3500—2600 cm^{-1}), a carboxyl (1710 cm^{-1}) and a carbonyl (1701 cm^{-1}). In the ^{13}C-NMR spectrum of **1**, thirty carbon signals were observed including two carbonyls at δ_C 181.4 (COOH) and 212.2 (ketonic) ppm and two pairs of double bond carbons at 124.5, 130.0, 131.1 and 136.7 ppm. Seven methyl groups of **1** were observed as singlets at δ_H 0.80, 0.82, 0.95, 1.01, 1.035, 1.043 and 1.30 ppm in the ^{1}H-NMR, and so, they are all attached to quaternary carbons.

The molecular formula of **1** shows nine equivalents of unsaturation and four out of the nine were accounted for by two carbonyls and two double bonds. One oxygen atom out of the four in **1** was found to form a secondary alcohol, not an ether, because only one sp^3 carbon bearing an oxygen function was observed as a doublet at δ_C 78.9 ppm in the ^{13}C-NMR spectrum. It follows that **1** is a pentacyclic triterpene acid.

Compound **1** showed UV absorption maxima at 242 nm (log ε 4.32), 249 nm (log ε 4.37), 258 nm (log ε 4.22). The maxima did not show shifts on addition of acid nor alkali; namely **1** has a neutral chromophore. The maxima of the chromophore resemble those due to the olean-11,13(18)-diene system of myricalactone (λ_{max} 254, 262 nm) and especially of papiriogenin D (λ_{max} 243, 251, 260 nm).[8)] In the ^{1}H-NMR of **1**, two protons on double bonds were observed at δ_H 5.63 (J=2, 11 Hz) and 6.41 (J=3, 11 Hz) ppm. These protons are coupled with each other with a J value of 11 Hz to form a *cis* double bond, and are also coupled with a methine (C-9, δ_H 2.61, δ_C 46.2 ppm) from the result of ^{1}H–^{1}H and ^{13}C–^{1}H COSY spectra of **1**. The HMBC spectrum of **1** showed correlations between 9-H/C-11, 12, 13; 11-H/C-9, 13, 18; 12-H/C-9, 13 (Chart 1). On the basis of the above evidence, **1** has a partial structure A (Chart 1).

Compound **1** has a secondary alcoholic function (*vide supra*). The carbon forming the function which was observed at δ_C 78.9 ppm has a methine proton observed as a doublet of doublets (J=5, 12 Hz) at δ_H 3.50 ppm from the ^{13}C–^{1}H COSY spectrum of **1**. Since the ^{1}H–^{1}H COSY spectrum showed that the methine proton was coupled with two protons, which were both bound to a single carbon observed as a triplet at δ_C 44.0 ppm, at δ_H 2.45 (1H, dd, J=5, 12 Hz) and δ_H 3.15 ppm (1H, t, J=12 Hz) to form an ABX coupling pattern, a partial structure, 3*β*-hydroxy-1-one or 1*β*-hydroxy-3-one structure in the A ring of the oleanane skeleton, was assumed for **1**. The 3*β*-hydroxy-1-one structure (B in Chart 1) is reasonable for **1**, because the twin methyl groups (23-H$_3$, 24-H$_3$) have a long-range coupling with the alcoholic methine carbon, while the methyl group (25-H$_3$) is coupled with the ketonic carbon in the HMBC spectrum of **1**. The methylene protons at C-2 were experimentally exchangeable with deuterium atoms to form a dideuteriated **1** (**1a**) on alkaline treatment of **1** in methanol-d_4.

The HMBC spectrum showed that the protons at C-25 (δ_H 1.30 ppm) have a long-range coupling with the methine carbon at C-9 (δ_C 46.2 ppm, δ_H 2.61 ppm, *vide supra*), and each of the two protons at C-9 (δ_H 2.61 ppm) and C-12 (ethylenic proton, δ_H 5.63 ppm), with a quaternary carbon (δ_C 52.1 ppm, C-10) and 2-H$_2$, 25-H$_3$ and 5-H, also with the quaternary carbon of C-10. On the basis of the above HMBC spectral analysis of **1**, the partial structures A and B (Chart 1) were combined together to a new extended partial structure C.

The molecule **1** has an isolated methylene (t at δ_C 40.5

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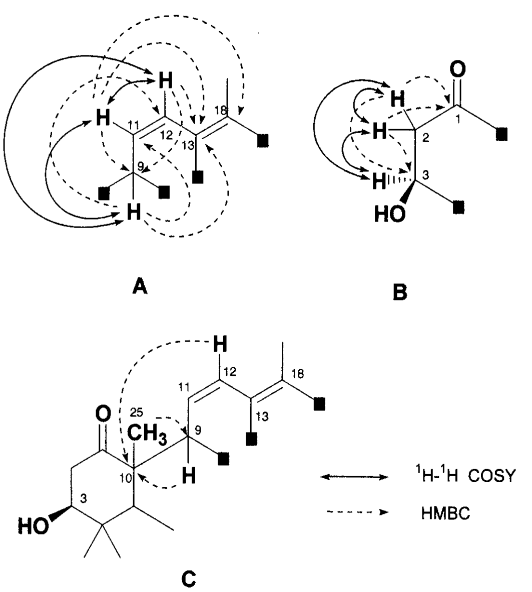
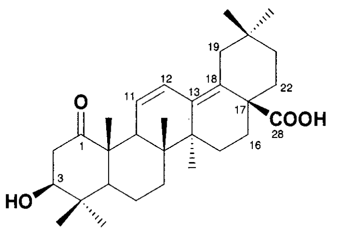


Chart 1



3β-hydroxy-1-oxoolean-11,13(18)-dien-28-oic acid (**1**)

Chart 2

ppm; d at δ_H 1.66 ppm (J=14 Hz) and dd at δ_H 2.54 ppm (J=2, 14 Hz); position C-19). Since the methylene protons exhibited clear correlations with C-13 (double bond carbon), 17, 18 (double bond carbon), 20, 21 and 30 in the HMBC spectrum of **1**, the methylene is reasonably assigned to that at C-19 of an oleanane skeleton. Furthermore, in the spectrum, methylene (16-H_2, δ_H 1.73, 1.99 ppm) has long-range coupling with C-14, 22, 27 and 28 (COOH), and another methylene (22-H_2, δ_H 1.40, 2.27 ppm), with C-17, 18 (double bond carbon), 20, 28 (COOH), and 30. Each of the latter two methylenes was considered to form an isolated ethylene residue, judging from the ^{1}H–^{1}H COSY spectrum of **1**.

On the basis of all above findings, the structure of **1** was elucidated as 3β-hydroxy-1-oxoolean-11,13(18)-dien-28-oic acid (Chart 2).

Experimental

Instruments and TLC procedures used in this work were essentially the same as described in our previous papers.[2,3] NMR spectra (^{1}H with 500 MHz; ^{13}C with 125 MHz) were taken with a JMN GX 500 model, using tetramethylsilane (TMS) as internal standard. Chemical shifts were expressed in ppm and coupling constants (J-values) in Hz. s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet.

Plant Material Dried twigs of *Myrica cerifera* L. were purchased from Shinwa Bussan Co., Ltd., Osaka, Japan in 1995.

Extraction and Isolation The plant material (960 g) was extracted with MeOH (2.3 l), at room temperature three times. The combined MeOH solution (7 l) was concentrated under reduced pressure to afford the MeOH extract (57.4 g). The extract was suspended in EtOAc (200 ml) and filtered. The filtrate was concentrated under reduced pressure as far as possible to give an EtOAc soluble part (6.4 g). The EtOAc soluble part (1.0 g) was extracted with $CHCl_3$. The $CHCl_3$ solution was washed with water, and concentrated. The residue (420 mg) tentatively called "$CHCl_3$ solubles" below, was dissolved in ether, and extracted with a saturated solution (50 ml) of $NaHCO_3$ in water, three times. The combined aq. solution was acidified with *dil*-HCl and extracted with ether. The ether solution, after concentrated to dryness, left a mixture (89 mg) of acidic compounds. The mixture of acidic compounds (total 0.5 g) was chromatographed on silica gel to give myricalactone (**2**, 13 mg) from fractions eluted with $CHCl_3$, myrica acid (**1**, 9.4 mg) from fractions eluted with $CHCl_3$–MeOH (100 : 3) and myriceric acid C (190 mg) from fractions eluted with $CHCl_3$–MeOH (100 : 7). Repeated chromatographic separation of "$CHCl_3$ solubles" above on silica gel provided myricanone, *dl*-myricanol, oleanolic acid, myricadiol, taraxerol and β-sitosterol. All the known compounds including myricalactone (**2**) were directly identified by comparison with authentic samples.

Compound 1 Colorless needles (MeOH), mp 274—277 °C, $[\alpha]_D$ −57° (c=0.8, MeOH), CD (c=2.50×10^{-5}, MeOH) $\Delta\varepsilon$ (nm): −29.05 (257), −46.89 (249), −40.73 (243). L.B. color reaction: positive (reddish violet). EI-MS m/z (%): 468 (33), 450 (41), 422 (63), 404 (100), 339 (94), 293 (41). HR-MS m/z: Calcd for $C_{30}H_{44}O_4$: 468.3239. Found: 468.3248. UV λ_{max} (MeOH) nm (log ε) : 242 (4.32), 249 (4.37), 258 (4.22, s). IR ν_{max} (KBr) cm^{-1}: 3500—2600, 1710, 1701, 1633, 1041, 968. ^{1}H-NMR ($CDCl_3$) δ: 0.80, 0.82, 0.95, 1.01, 1.035, 1.043, 1.30 (each 3H, s), 2.45 (1H, dd, J=5, 12 Hz, 2-Hα), 2.54 (1H, dd, J=2, 14Hz, 19-H), 2.61 (1H, brs, 9-H), 3.15 (1H, t, J=12 Hz, 2-Hβ), 3.50 (1H, dd, J=5, 12 Hz, 3-H), 5.63 (1H, dd, J=2, 11 Hz, 12-H), 6.41 (1H, dd, J=3, 11 Hz, 11-H). ^{13}C-NMR ($CDCl_3$) δ: 212.2 (CO-1), 44.0 (CH_2-2), 78.9 (CH-3), 39.4 (C-4), 53.2 (CH-5), 17.8 (CH_2-6), 31.7 (CH_2-7), 40.5 (C-8), 46.2 (CH-9), 52.1 (C-10), 124.5 (CH-11), 130.0 (CH-12), 136.7 (C-13), 42.3 (C-14), 25.0 (CH_2-15), 32.6 (CH_2-16), 48.0 (C-17), 131.1 (C-18), 40.5 (CH_2-19), 32.6 (C-20), 36.8 (CH_2-21), 35.5 (CH_2-22), 27.9 (CH_3-23), 15.3 (CH_3-24), 17.6 (CH_3-25), 16.7 (CH_3-26), 19.8 (CH_3-27), 181.4 (COOH-28), 32.2 (CH_3-29), 24.0 (CH_3-30).

Deuteriation of 1 A solution (0.75 ml) of 0.1% NaOMe-d_3 in MeOH-d_4 was added to a solution of **1** (2.0 mg) in MeOH-d_4 and kept overnight at room temperature. After removing the solvent under a N_2 stream the residue was acidified with 2.5% HCl and extracted with ether. The ether solution was washed with water, dried over anhydrous Na_2SO_4 and then concentrated to dryness. The residue was purified with chromatography on silica gel and elution with $CHCl_3$–MeOH (50 : 1) gave **1a** (0.7 mg). **1a**, colorless needles (MeOH), mp 273—275 °C. EI-MS m/z (%): 470 ($M^{+\cdot}$, 68), 452 (37), 405 (74), 340 (35), 293 (72), 248 (43), 199 (82).

Methylation of 1 A solution of **1** (2.0 mg) in MeOH (2 ml) was treated with diazomethane in ether in the usual manner. The product was purified by chromatography on silica gel ($CHCl_3$–MeOH (100 : 1)) to afford **1b** (1.2 mg). **1b**, colorless needles (MeOH), mp 196—198 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.79, 0.82, 0.92, 1.016, 1.024, 1.04, 1.31 (each 3H, s), 2.45 (1H, dd, J=5, 12 Hz), 2.52 (1H, dd, J=2, 14 Hz), 2.61 (1H, s), 3.15 (1H, t, J=12 Hz), 3.49 (1H, dd, J=5, 12 Hz), 3.66 (3H, s), 5.61 (1H, d, J=11 Hz), 6.41 (1H, dd, J=2, 11 Hz).

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Medicinal Foodstuffs. XX.[1)] Vasorelaxant Active Constituents from the Roots of *Angelica furcijuga* KITAGAWA: Structures of Hyuganins A, B, C, and D

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From the methanolic extract with vasorelaxant activity obtained from *Angelica furcijuga* KITAGAWA, four new khellactone-type coumarins, hyuganins A, B, C, and D, were isolated together with twelve known coumarins, two known acetylenic compounds, and a known lignan. The structures of hyuganins A, B, C, and D were determined on the basis of chemical and physicochemical evidence. Nine principal coumarins (hyuganin A, anomalin, pteryxin, isopteryxin, isoepoxypteryxin, praerosides II and IV, apiosylskimmin, (*R*)-peucedanol 7-*O*-*β*-D-glucopyranoside), two acetylenic compounds [(−)-falcarinol and falcarindiol], and related compounds were examined for inhibitory activities on high concentration of K^+ (High K^+)- and *dl*-norepinephrine (NE)-induced contractions. The results indicate that the 3′- and 4′-acyl groups of khellactone-type coumarins are essential for the inhibitory activity on the contractions by High K^+. Hyuganin A and anomalin showed inhibitory effects on High K^+-induced contraction, but not on NE-induced contraction. Other active coumarins (pteryxin, isopteryxin, isoepoxypteryxin) and an acetylenic compound (falcarindiol) non-selectively inhibited both contractions by High K^+ and NE.

Key words hyuganin; vasorelaxant activity; khellactone; *Angelica furcijuga*; coumarin; structural requirement

The Umbelliferae plant *Angelica furcijuga* KITAGAWA (Japanese name "Hyugatouki") has been believed to be effective for hepatopathy, allergosis, inflammation, diabetes, and hypertension in Japanese folk medicine and the dried whole plant is consumed as a health food, which is commonly called "Yamaninjin" in Japan.[2)] In the course of our studies on the bioactive constituents of medicinal foodstuffs,[1,3)] we found that the methanolic extract from the fresh roots of *A. furcijuga* showed *in vivo* and *in vitro* hepatoprotective activities. In a previous communication,[4)] we reported the hepatoprotective effect of coumarins and acetylenic compounds isolated from the methanolic extract through a bioassay-guided separation, in addition to their inhibitory effect on nitric oxide production from macrophages. As a continuation of this study, the methanolic extract from the fresh roots of *A. furcijuga* was found to show potent inhibitory activity on the contraction of isolated rat thoracic aorta by high concentration of potassium cation (High K^+). From the methanolic extract, four new khellactone-type coumarins named hyuganins A (**1**), B (**2**), C (**3**), and D (**4**) were isolated together with fifteen known compounds. Furthermore, we examined the inhibitory effects of the principle constituents on the contractions by High K^+ and *dl*-norepinephrine (NE)-induced contractions.

In this paper, we describe the structural elucidation of **1**—**4** and the inhibitory effects of the principal constituents from *A. furcijuga* on High K^+ and NE-induced contractions in isolated thoracic aorta of rats.

The fresh roots of *A. furcijuga* (4.0 kg) were extracted with methanol under reflux. The methanolic extract was subjected to HP-20 ($H_2O \rightarrow MeOH \rightarrow$acetone), silica gel (hexane–AcOEt$\rightarrow CHCl_3 \rightarrow MeOH–H_2O$), and reversed-phase silica gel column chromatography and finally HPLC [YMC-pack R&D-ODS-5-A ($MeOH–H_2O$, $CH_3CN–H_2O$) and YMC-pack CN (*n*-hexane–2-PrOH)] to give four new acylated khellactones [**1** (0.011% from the fresh roots), **2** (0.002%), **3** (0.014%), and **4** (0.002%)] together with the following known compounds, five acylated khellactones [anomalin[5)] (**7**, 0.013%), pteryxin[6)] (**8**, 0.017%), isopteryxin[7)] (**9**, 0.040%), isoepoxypteryxin[8)] (**10**, 0.040%), and suksdorfin[6a,9)] (**15**, 0.003%)], another coumarin [bergapten[10)] (**20**, 0.001%)], six coumarin glycosides [praerosides II[11)] (**11**, 0.012%), IV[11)] (**12**, 0.019%), apiosylskimmin[12)] (**17**, 0.030%), hymexelsin[13)] (**18**, 0.001%), (*R*)-peucedanol 7-*O*-*β*-D-glucopyranoside[14)] (**19**, 0.004%), marmesinin[15)] (**21**, 0.001%)], two acetylenes [(−)-falcarinol[16)] (=panaxynol, **23**, 0.002%), falcarindiol[16c,17)] (**24**, 0.045%)], (+)-pinoresinol *O*-*β*-D-glucopyranoside[18)] (**22**, 0.0001%), and *β*-sitosterol (0.032%).

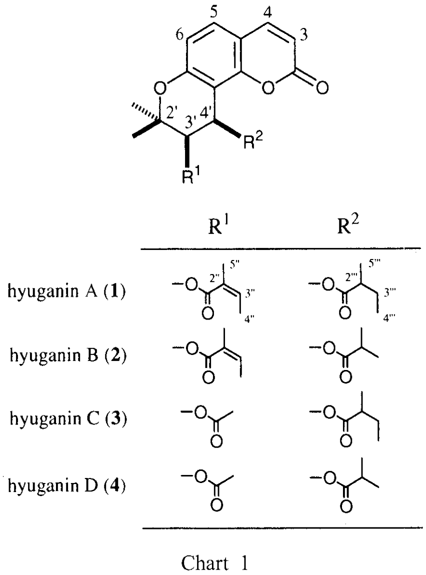

Chart 1

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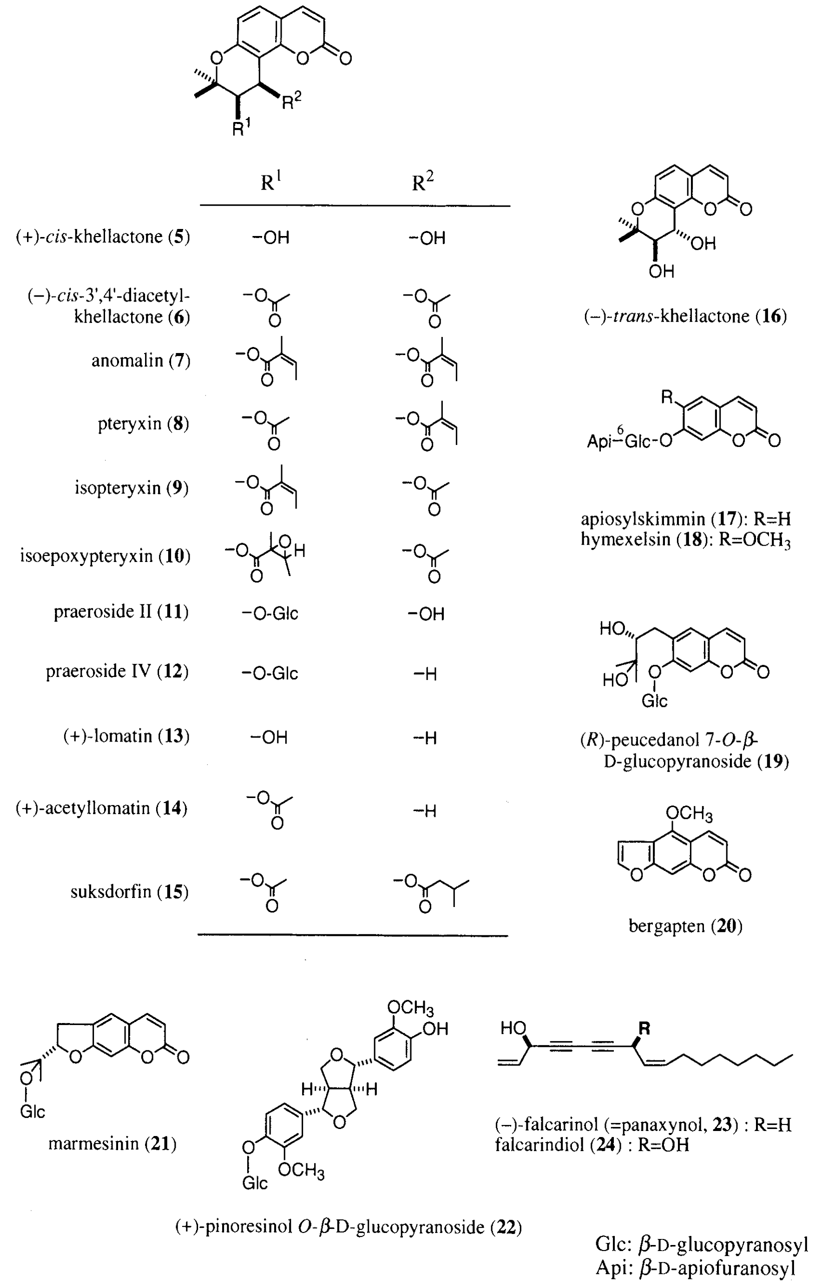


Chart 2

Structures of Hyuganins A (1), B (2), C (3), and D (4) Hyuganin A (**1**) was isolated as a white powder with negative optical rotation ($[\alpha]_D^{27}$ −37.1°). Its molecular formula $C_{24}H_{28}O_7$ was confirmed from the molecular ion peak at *m/z* 428 (M^+) in the EI-MS of **1** and by high resolution MS measurement. The IR spectrum of **1** showed absorption bands due to carbonyl groups at 1736—1717 and 1649 cm^{-1}, and an aromatic ring at 1606, 1570, 1490, and 845 cm^{-1}. In the UV spectrum of **1**, absorption maxima (log ε) were observed at 217 (sh., 4.4), 300 (sh., 4.0), 322 (4.2) nm suggestive of a coumarin skeleton. The ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR (Table 1) spectra of **1**, which were completely assigned by means of NMR analytical methods,[19] showed signals assignable to a coumarin moiety [δ 6.22 (d, J=9.6 Hz, 3-H), 7.61 (d, J=9.6 Hz, 4-H), 7.37 (d, J=8.6 Hz, 5-H), 6.81 (d, J=8.6 Hz, 6-H)], two tertiary methyls [δ 1.45, 1.48 (both s, 2'-*gem*-CH_3)], two methines bearing an ester function [δ 5.40 (d, J=4.9 Hz, 3'-H), 6.61 (d, J=4.9 Hz, 4'-H)], an angeloyl group [δ 6.12 (qq, J=1.3, 7.3 Hz, 3''-H), 1.98 (qd, J=1.7, 7.3 Hz, 4''-H_3), 1.88 (dq, J=1.3, 1.7 Hz, 5''-H_3)], and a 2-methylbutyroyl group [δ 2.38 (ddq, J=6.9, 6.9, 6.9 Hz, 2'''-H), 1.43 (dqd, J=6.9, 7.6, 14.3 Hz), 1.72 (dqd, J=6.9, 7.6, 14.3 Hz) (3'''-H_2), 0.90 (t, J=7.6 Hz, 4'''-H_3), 1.19 (d, J=6.9 Hz, 5'''-H_3)]. The carbon signals in the ^{13}C-NMR spectrum of **1** were shown to be superimposable on those of anomalin (**5**), except for some signals assignable to an acyl group at the 4'-position. In addition, the EI-MS data of **1** showed fragment ion peaks at *m/z* 328 (**i**, 22%), 313 (**iii**, 21%), 244 (**v**, 29%), 229 (**vi**, base peak), 85 (**vii**, 25%) and 83 (**viii**, 96%), which were derived by eliminations of the angeroyl and 2-methylbutyroyl groups.

Alkaline hydrolysis of **1** with 5% aqueous potassium hydroxide (KOH) in dioxane (2 : 1) liberated (+)-*cis*-khellactone (**5**) and (−)-*trans*-khellactone (**16**),[20] which was known to be derived from **8** by the epimerization at the benzylic 4'-position, together with angelic and 2-methylbutyric acids.

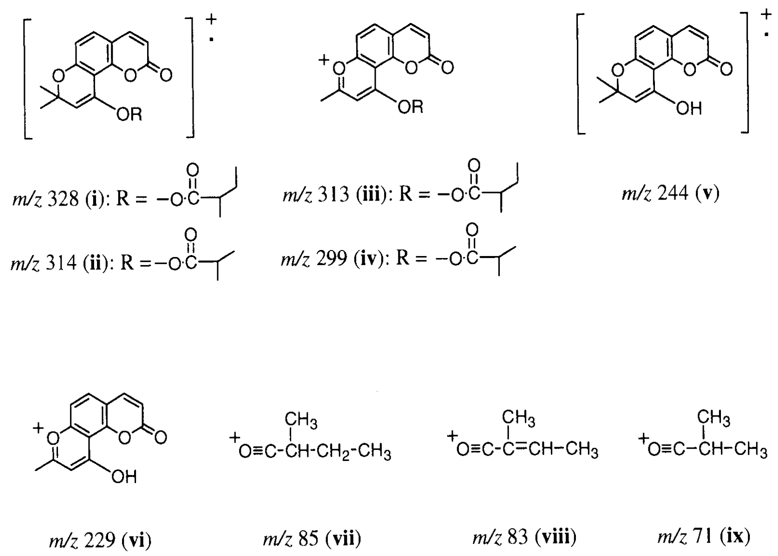
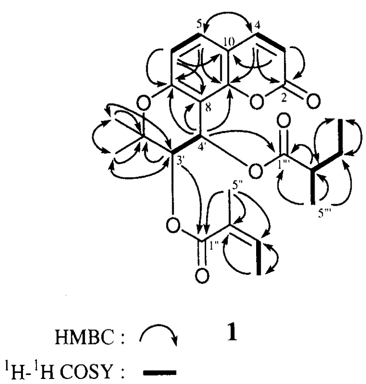


Chart 3



1

Fig. 1. HMBC and ^{1}H–^{1}H COSY Correlations of **1**

These organic acids were converted to the *p*-nitrobenzyl esters,[21] which were identified by HPLC analysis.

The position of the angeloyl and 2-methylbutyroyl groups in **1** was clarified by HMBC experiment, which showed a long-range correlation between the 3′-proton and the 1-carbonyl carbon of the angeloyl group and between the 4′-proton and the 1-carbonyl carbon of the 2-methylbutyroyl group (Fig. 1). The configurations of the 3′ and 4′-positions in **1** were deduced to be *cis* orientation on the basis of the coupling constant (*J*=4.9 Hz) between the 3′-proton and the 4′-proton in the ^{1}H-NMR spectrum of **1**. Furthermore, in a difference nuclear Overhauser effect (NOE) experiment on **1**, an NOE correlation was observed between the 3′-proton and the 4′-proton. On the basis of this evidence, the structure of **1** was elucidated as shown.

Hyuganin B (**2**), isolated as a white powder, was also found to be optically active ($[\alpha]_D^{24}$ −21.5°). Its IR and UV spectra were very similar to those of **1**. In the EI-MS of **2**, a molecular ion peak was observed at *m/z* 414 (M^+) in addition to the fragment ion peaks at *m/z* 314 (**ii**, 37%), 299 (**iv**, 39%), 244 (**v**, 28%), 229 (**vi**, base peak), 83 (**viii**, 71%), and 71 (**ix**, 24%) and the molecular formula was determined to be $C_{23}H_{26}O_7$ by high-resolution MS measurement. The ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR (Table 1) spectra[19] of **2** showed the presence of a coumarin moiety, two tertiary methyls, two methines connecting to an acyl group [δ 5.42 (d, *J*=4.9 Hz, 3′-H), 6.59 (d, *J*=4.9 Hz, 4′-H)], an angeloyl group [δ 6.12 (qq, *J*=1.5, 7.3 Hz, 3″-H), 1.96 (qd, *J*=1.5, 7.3 Hz, 4″-H_3), 1.87 (qd, *J*=1.5, 1.5 Hz, 5″-H_3)], and an isobutyroyl group [δ 2.57 (qq, *J*=7.0, 7.0 Hz, 2‴-H), 1.16, 1.20 (both d, *J*=7.0 Hz) (3‴ and 4‴-H_3)]. The carbon signals in the ^{13}C-NMR spectrum of **2** were shown to be superimposable on those of **1**, except for some signals assignable to an acyl group at the 4′-position. Alkaline hydrolysis of **2** liberated **5** and **16** together with angelic and 2-isobutyric acids, which were identified by HPLC analysis of their *p*-nitrobenzyl ester. In the HMBC experiment on **2**, long-range correlations were observed between the 3′-proton and the angeloyl carbonyl carbon and between the 4′-proton and the 2-isobutyroyl carbonyl carbon. The stereostructures of the 3′ and 4′-positions were characterized on the basis of the coupling constant and NOE observation between the 3′- and 4′-protons. Consequently, the structure of **2** was determined as shown.[22]

Hyuganin C (**3**) was also isolated as a white powder and its IR and UV spectra were very similar to those of **1**. In the EI-MS of **3**, a molecular ion peak was observed at *m/z* 388 (M^+) in addition to fragment ion peaks at *m/z* 328 (**i**, 14%), 313 (**iii**, 19%), 244 (**v**, 27%), 229 (**vi**, base peak), 85 (**vii**, 12%), and 43. The molecular formula was determined to be $C_{21}H_{24}O_7$ by high-resolution MS measurement. Alkaline hydrolysis of **3** liberated **5** and **16** together with 2-methylbutyric and acetic acids. The ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR (Table 1) spectra[19] of **3** showed the presence of a *cis*-khellactone moiety [δ 6.22 (d, *J*=9.5 Hz, 3-H), 7.60 (d, *J*=9.5 Hz, 4-H), 7.36 (d, *J*=8.7 Hz, 5-H), 6.80 (d, *J*=8.7 Hz, 6-H), 1.42, 1.45 (both s, 2′-*gem*-CH_3), 5.31 (d, *J*=4.9 Hz, 3′-H), 6.52 (d, *J*=4.9 Hz, 4′-H)], an acetyl group [δ 2.10 (s, 2″-H_3)], and a 2-methylbutyroyl group [δ 2.42 (ddq, *J*=7.0, 7.0, 7.0 Hz, 2‴-H), 1.48 (dqd, *J*=7.0, 7.6, 14.3 Hz), 1.73 (dqd, *J*=7.0, 7.6, 14.3 Hz) (3‴-H_2), 0.95 (t, *J*=7.6 Hz, 4‴-H_3), 1.22 (d, *J*=7.0 Hz, 5‴-H_3)]. The carbon signals in the ^{13}C-NMR spectrum of **3** were shown to be superimposable on those of **1**, except for some signals assignable to an acyl group at the 3′-position. On the basis of the *J* value and NOE correlation between the 3′ and 4′-protons, the structure of **3** was characterized as shown.

Hyuganin D (**4**) was isolated as a white powder and its IR

Table 1. ^{13}C-NMR Data of Hyuganins A (**1**), B (**2**), C (**3**), and D (**4**)

	1	**2**	**3**	**4**
C-2	159.7	159.7	159.7	159.7
3	113.2	113.2	113.3	113.3
4	143.2	143.2	143.2	143.2
5	129.2	129.1	129.3	129.2
6	114.4	114.4	114.5	114.4
7	156.7	156.8	156.6	156.7
8	107.5	107.5	107.4	107.3
9	154.0	154.1	154.1	154.1
10	112.5	112.5	112.5	112.6
2′	77.3	77.6	77.3	77.4
3′	70.3	70.0	70.8	70.4
4′	60.5	60.8	60.4	60.6
2′-*gem*-CH_3	22.5	22.7	21.9	22.3
	25.3	25.1	25.6	25.1
1″	166.4	166.4	169.8	169.8
2″	127.0	127.0	20.7	20.7
3″	139.6	139.7		
4″	15.8	15.7		
5″	20.5	20.5		
1‴	175.2	175.6	175.6	175.9
2‴	41.2	34.1	41.4	34.1
3‴	26.4	18.8	26.6	18.8
4‴	11.6	18.8	11.6	18.9
5‴	16.3		16.6	

Measured in $CDCl_3$.

Table 2. Inhibitory Effects of Coumarin and Acetylenic Constituents (**1**, **7**—**12**, **17**, **19**, **23**, **24**) from *A. furcijuga* and Related Coumarins (**5**, **6**, **13**, **14**, **16**) on High K$^+$-Induced Contractions in Isolated Thoracic Aorta of Rat

Compounds	IC_{50} (μM)	Inhibition (%) at 100 μM
Hyuganin A (**1**)	17	85.0±2.3**
(+)-*cis*-Khellactone (**5**)	>100	3.3±2.1
(−)-*cis*-3′,4′-Diacetylkhellactone (**6**)	25	100.8±2.4**
Anomalin (**7**)	30	68.1±4.2**
Pteryxin (**8**)	4.1	104.0±0.7**
Isopteryxin (**9**)	9.8	101.3±1.5**
Isoepoxypteryxin (**10**)	21	97.9±9.4**
Praeroside II (**11**)	>100	12.8±5.6
Praeroside IV (**12**)	>100	16.6±3.7
(+)-Lomatin (**13**)	>100	37.1±3.6**
(+)-Acetyllomatin (**14**)	70	72.1±2.0**
(−)-*trans*-Khellactone (**16**)	>100	2.5±1.5
Apiosylskimmin (**17**)	>100	11.2±4.5
(*R*)-Peucedanol 7-*O*-β-D-glucopyranoside (**19**)	>100	12.4±2.8
(−)-Falcarinol (**23**)	>100	39.1±4.7**
Falcarindiol (**24**)	10	96.9±0.6**

Significantly different from the control, $**p<0.01$ (n=4—6).

and UV spectra were very similar to those of **1**. The EI-MS of **4** showed a molecular ion peak at *m/z* 374 (M$^+$) in addition to fragment ion peaks at *m/z* 314 (**ii**, 8%), 299 (**iv**, 13%), 244 (**v**, 18%), 299 (**vi**, base peak), 71 (**ix**, 37%) and 43, and the molecular formula was determined to be $C_{20}H_{22}O_7$ by high-resolution MS measurement. Alkaline hydrolysis of **4** liberated **5** and **16** together with 2-isobutyric and acetic acids. The ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR (Table 1) spectra[19] of **4** showed signals assignable to a *cis*-khellactone moiety, an acetyl group [δ 2.09 (s, 2″-H_3)], and an 2-isobutyroyl group [δ 2.61 (qq, J=7.0, 7.0 Hz, 2‴-H), 1.20, 1.23 (both d, J=7.0 Hz) (3‴ and 4‴-H_3)]. An NOE correlation was observed between the 3′-proton and 4′-proton in a difference NOE experiment on **4**. Finally, comparison of ^{1}H- and ^{13}C-NMR data for **4** with those for **2** and **5** led us to formulate the structure of **4** to be as shown.[22]

Vasorelaxant Effects of Coumarins and Acetylenic Compounds Relaxant effects of the methanolic extract from *A. furcijuga* were examined in contractions of isolated rat thoracic aorta by High K$^+$. Cumulative application of the methanolic extract (25—200 μg/ml) inhibited the sustained contraction induced by K$^+$. Its IC_{50} value was 66 μg/ml, and that of nifedipine as a reference drug was 6.4 nM.

As shown in Table 2, five acylated khellactones (**1**, **7**, **8**, **9**, **10**) potently relaxed the sustained contractions induced by High K$^+$ (IC_{50} values 4.1—30 μM). On the other hand, their desacyl derivatives [(+)-*cis*- and (−)-*trans*-khellactones (**5**, **16**) and a khellactone glucoside (**11**), and three coumarin glycosides (**12**, **17**, **19**) lacked activity. (+)-Lomatin (**13**) showed weak activity, while (−)-*cis*-3′,4′-diacetylkhellactone (**6**) and (+)-acetyllomatin (**14**) exhibited potent activity. These results indicate the following structural requirements of khellactone-type coumarins for activity: 1) the 3′ and/or 4′ acyl groups of *cis*-khellactones are essential for the activity, and 2) the glycoside moiety does not contribute the activity. Figures 2 and 3 show the inhibitory effects of the principal constituents on NE-induced contractions in isolated thracic aorta of rats. Among the coumarins with inhibitory effect on High K$^+$-induced contraction, **6**, **8**, **9**, **10**, and **14** (100 μM) significantly inhibited NE-induced contraction in the presence of nifedipine (1 μM), although these inhibitory effects were less potent than those in High K$^+$-induced contractions. However, **1** and **7** (100 μM) lacked activity. The principal acetylenic compound (**24**) showed potent inhibitory effects on both contractions, while another acetylene (**23**) showed weak activity (Table 2 and Fig. 3).

It is well known that High K$^+$-induced contraction in smooth muscle is the result of an increase in Ca^{2+} influx through a voltage-dependent Ca^{2+} channel, and Ca^{2+}-blockers such as nifedipine selectively inhibit this contraction. Previous studies also demonstrated that several acylated khellactones such as praeruptorin A (Pd-Ia) showed Ca^{2+} blocking activity using ileum or taenia coli of guinea-pig and rat mast cells.[23] The results in the present experiments demonstrated that **1** and **7** relaxed High K$^+$-induced contractions by their Ca^{2+}-blocking activities like nifedipine, and that **6**, **8**, **9**, **10**, and **14** also relaxed the contractions mainly by their Ca^{2+}-blocking activities, but other mechanisms may also be involved.

These acylated khellactones and acetylenic compound with vasorelaxant activity may be important evidence substantiating the traditional use of this plant.

Experimental

The instruments used to obtain physical and experimental conditions for chromatography were described previously.[1]

Extraction and Isolation The fresh roots of *Angelica furcijuga* KITAGAWA (4.0 kg, cultivated in Miyazaki prefecture, Japan) were cut into small pieces and extracted three times with methanol (10 l) under reflux. Removal of the solvent from the MeOH solution under reduced pressure gave the MeOH extract (217.0 g). MeOH ext. (195.2 g) was subjected to Diaion HP-20 column chromatography [(Nippon Rensui Co., 2.0 kg), $H_2O \rightarrow$ MeOH→acetone] to give the H_2O-eluted fraction (140 g) and three fractions [fr. 1 (6.6 g), fr. 2 (17.7 g), and fr. 3 (25.0 g)]. Fraction 2 (15.8 g) was subjected to ordinary-phase silica gel column chromatography [PW-200 (Fuji

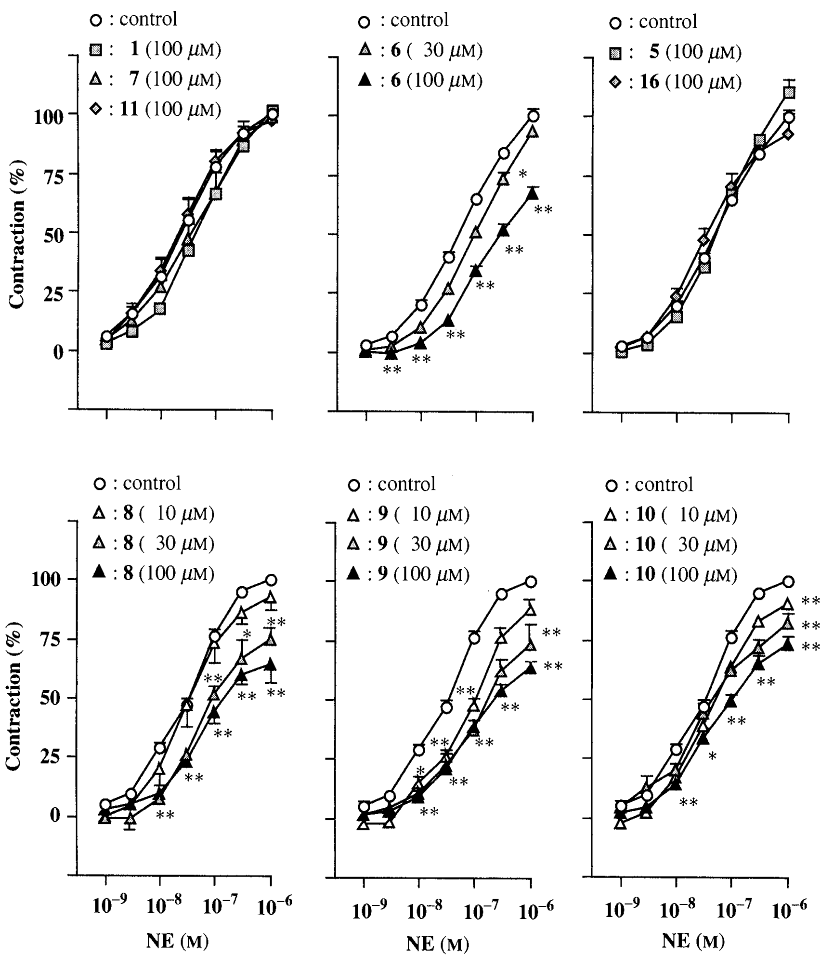


Fig. 2. Inhibitory Effects of Coumarin Constituents (**1**, **7**—**11**) from *A. furcijuga* and Related Compounds (**5**, **6**, **16**) on NE-Induced Contractions in Isolated Thoracic Aorta of Rats

Each point represents the mean with S.E.M. (n=4—6). Significantly different from each control, $*p<0.05$, $**p<0.01$.

Silysia Chemical Ltd.,) 480 g, $CHCl_3$–MeOH–H_2O (15:3:1, lower layer→65:35:10, lower layer, v/v)→MeOH] to give falcarindiol (**24**, 1.3 g) and nine fractions (fr. 2-1—fr. 2-9). Fraction 2-5 (2.4 g) was further separated by reversed-phase silica gel column chromatography [Chromatorex ODS DM 1020T (Fuji Silysia Chemical Ltd.) 96 g, MeOH–H_2O (40:60→60:40, v/v)→MeOH] to give five fractions (fr. 2-5-1—2-5-5). Fractions 2-5-3 (632.5 mg) and 2-5-4 (1.2 g) were purified by HPLC [YMC-pack R&D-ODS-5-A, 250×20 mm i.d. (YMC Co., Ltd), CH_3CN–H_2O (75:25, v/v)] separation to give marmesinin (**21**, 28.6 mg), praerosides II (**11**, 374 mg) and IV (**12**, 612 mg), and (+)-pinoresinol *O*-*β*-D-glucopyranoside (**22**, 3.2 mg). Fraction 2-7 (1.3 g) was further separated by reversed-phase silica gel column chromatography [52 g, MeOH–H_2O (30:70, v/v)→MeOH] to give five fractions (fr. 2-7-1—2-7-5). Fractions 2-7-2 (84.6 mg) and 2-7-3 (554.1 mg) were purified by HPLC [YMC-pack R&D-ODS-5-A, 250×20 mm i.d., MeOH–H_2O (30:70, v/v)] separation to give apiosylskimmin (**17**, 19.8 mg), hymexelsin (**18**, 31.7 mg). (*R*)-peucedanol 7-*O*-*β*-D-glucopyranoside (**19**, 142 mg). Fraction 2-8 (1.9 g) was further separated by reversed-phase silica gel column chromatography [60 g, MeOH–H_2O (30:70, v/v)→MeOH] to give apiosylskimmin (**17**, 952 mg).

Fraction 3 (20.7 g) was subjected to ordinary column chromatography [500 g, *n*-hexane–AcOEt (10:1→5:1→1:1, v/v)→$CHCl_3$–MeOH–H_2O (10:3:1, lower layer, →65:35:10, lower layer, v/v)→MeOH] to give twelve fractions (fr. 3-1—3-12). Fraction 3-2 (571 mg) was purified by reversed-phase column chromatography [17 g, MeOH–H_2O (90:10, v/v)→MeOH] and finally HPLC [YMC-pack R&D-ODS-5A, 250×20 mm i.d., MeOH–H_2O (90:10, v/v)] to give (−)-falcarinol (=panaxynol) (**23**, 64.2 mg). Fractions 3-3 (4.1 g) and 3-4 (4.5 g) were purified by reversed-phase silica gel column chromatography [120 g, MeOH–H_2O (80:20→90:10, v/v)→MeOH] and HPLC [YMC-pack R&D-ODS-5A, 250×20 mm i.d., MeOH–H_2O (75:25)] to give anomalin (**7**, 378 mg), hyuganins A (**1**, 330 mg), B (**2**, 45.1 mg), D (**4**, 47.8 mg), bergapten (**20**, 25.5 mg), pteryxin (**8**, 506 mg), isopteryxin (**9**, 1.19 g), falcarindiol (**24**, 140 mg) and a mixture (631 mg) of **3** and **15**, and *β*-sitosterol (960 mg). The mixture (50.2 mg) of **3** and **15** was separated by HPLC [YMC-pack CN, 250×10 mm i.d., *n*-hexane–2-PrOH (30:1, v/v)] to give hyuganin C (**3**) (33.0 mg) and suksdorfin (**15**, 6.6 mg). Fraction 3-6 (1.24 g) was purified by reversed-phase silica gel column chromatography [36 g, MeOH–H_2O (75:25, v/v)→MeOH] to give isoepoxypteryxin (= isoepoxybuterixin, **10**, 1.18 g).

Three known compounds, **23**, **24**, and *β*-sitosterol, were identified by comparison of TLC behavior and ^{1}H-NMR, and ^{13}C-NMR spectra with those of authentic samples and other known compounds were identified by comparison of their physical data ($[\alpha]_D$, ^{1}H-NMR, and ^{13}C-NMR) with the reported values.[5—18)]

Hyuganin A (**1**): A white powder, $[\alpha]_D^{27}$ −37.1° (c=1.3, $CHCl_3$). High-resolution EI-MS: Calcd for $C_{24}H_{28}O_7$ (M^+): 428.1835. Found: 428.1838. UV λ_{max} (MeOH) nm (log ε): 217 (sh., 4.4), 300 (sh., 4.0), 322 (4.2). IR (KBr): 1736—1717, 1649, 1606, 1570, 1490, 1254, 1144, 845 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.90 (3H, t, J=7.6 Hz, 4‴-H_3), 1.19 (3H, d, J=6.9 Hz, 5‴-H_3), 1.43 (1H, dqd, J=6.9, 7.6, 14.3 Hz, 3‴-H), 1.45, 1.48 (3H each, both s, 2′-*gem*-CH_3), 1.72 (1H, dqd, J=6.9, 7.6, 14.3 Hz, 3‴-H), 1.88 (3H, dq, J=1.3, 1.7 Hz, 5″-H_3), 1.98 (3H, qd, J=1.7, 7.3 Hz, 4″-H_3), 2.38 (1H, ddq, J=6.9, 6.9, 6.9 Hz, 2‴-H), 5.40 (1H, d, J=4.9 Hz, 3′-H), 6.12 (1H, qq, J=1.3, 7.3 Hz, 3″-H), 6.22 (1H, d, J=9.6 Hz, 3-H), 6.61 (1H, d, J=4.9 Hz, 4′-H), 6.81 (1H, d, J=8.6 Hz, 6-H), 7.37 (1H, d, J=8.6 Hz, 5-H), 7.61 (1H, d, J=9.6 Hz, 4-H). ^{13}C-NMR ($CDCl_3$, 68 MHz) δ_C: given in Table 1. EI-MS m/z (%): 428 (M^+, 7), 328 (22), 313 (21), 244 (29), 229 (100), 213 (5), 85 (25), 83 (96).

Hyuganin B (**2**): A white powder, $[\alpha]_D^{24}$ −21.5° (c=1.0, $CHCl_3$). High-resolution EI-MS: Calcd for $C_{23}H_{26}O_7$ (M^+): 414.1679. Found: 414.1687. UV λ_{max} (MeOH) nm (log ε): 217 (sh., 4.4), 300 (sh., 3.9), 322 (4.1). IR (KBr): 1742—1718, 1649, 1608, 1560, 1490, 1232, 1150, 840 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 1.16, 1.20 (3H each, both d, J=7.0 Hz, 3‴, 4‴-H_3), 1.44, 1.47 (3H each, both s, 2′-*gem*-CH_3), 1.87 (3H, qd, J=1.5, 1.5 Hz, 5″-H_3), 1.96 (3H, qd, J=1.5, 7.3 Hz, 4″-H_3), 2.57 (1H, qq, J=7.0, 7.0 Hz, 2‴-

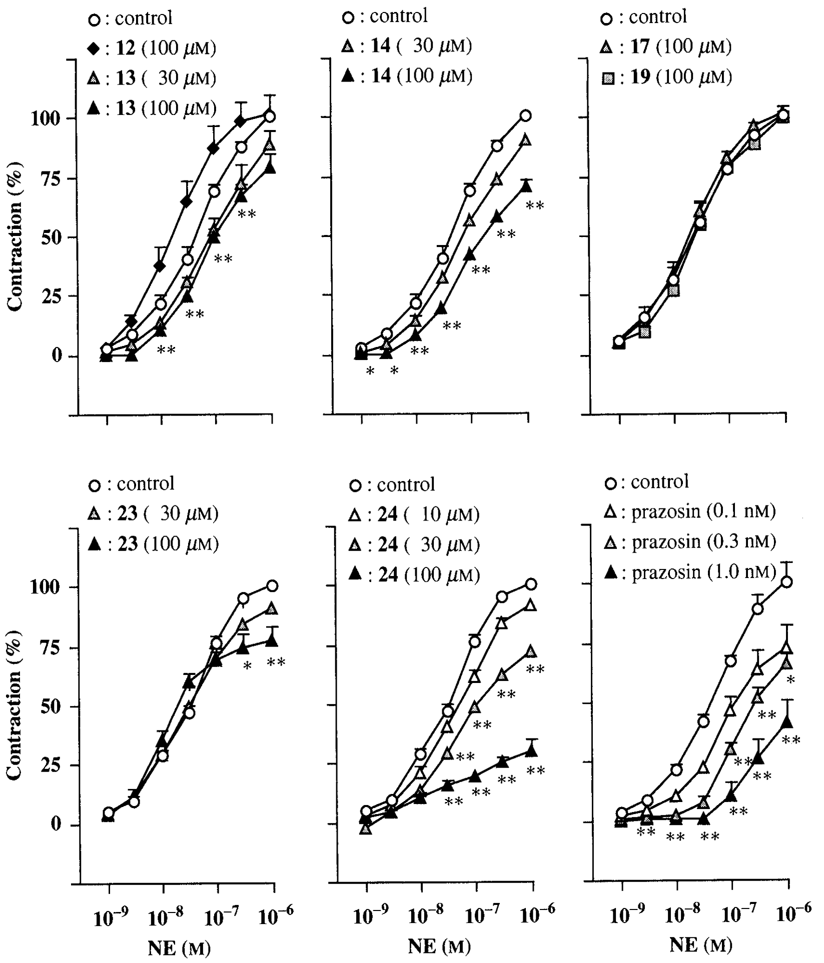


Fig. 3. Inhibitory Effects of Coumarin and Acetylene Constituents (**12**, **17**, **19**, **23**, **24**) from *A. furcijuga* and Related Compounds (**13**, **14**) on NE-Induced Contractions in Isolated Thoracic Aorta of Rats

Each point represents the mean with S.E.M. (n=4—6). Significantly different from each control, $*p<0.05$, $**p<0.01$.

H), 5.42 (1H, d, J=4.9 Hz, 3′-H), 6.12 (1H, qq, J=1.5, 7.3 Hz, 3″-H), 6.21 (1H, d, J=9.5 Hz, 3-H), 6.59 (1H, d, J=4.9 Hz, 4′-H), 6.80 (1H, d, J=8.5 Hz, 6-H), 7.35 (1H, d, J=8.5 Hz, 5-H), 7.59 (1H, d, J=9.5 Hz, 4-H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ_C): given in Table 1. EI-MS m/z (%): 414 (M^+, 10), 314 (37), 299 (39), 244 (28), 229 (100), 83 (71), 71 (24).

Hyuganin C (**3**): A white powder, $[\alpha]_D^{26}$ −2.8° (c=1.1, $CHCl_3$). High-resolution EI-MS: Calcd for $C_{21}H_{24}O_7$ (M^+): 388.1522. Found: 388.1523. UV λ_{max} (MeOH) nm (log ε): 218 (sh., 3.8), 299 (sh., 3.6), 322 (3.8). IR (KBr): 1744—1720, 1649, 1608, 1560, 1490, 1230, 1146, 840 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.95 (3H, t, J=7.6 Hz, 4‴-H_3), 1.22 (3H, d, J=7.0 Hz, 5‴-H_3), 1.42, 1.45 (3H each, both s, 2′-*gem*-CH_3), 1.48 (1H, dqd, J=7.0, 7.6, 14.3 Hz), 1.73 (1H, dqd, J=7.0, 7.6, 14.3 Hz) (3‴-H_2), 2.10 (3H, s, 2″-H_3), 2.42 (1H, ddq, J=7.0, 7.0, 7.0 Hz, 2‴-H), 5.31 (1H, d, J=4.9 Hz, 3′-H), 6.22 (1H, d, J=9.5 Hz, 3-H), 6.52 (1H, d, J=4.9 Hz, 4′-H), 6.80 (1H, d, J=8.7 Hz, 6-H), 7.36 (1H, d, J=8.7 Hz, 5-H), 7.60 (1H, d, J=9.5 Hz, 4-H). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ_C: given in Table 1. EI-MS m/z (%): 388 (M^+, 2), 328 (14), 313 (19), 244 (27), 229 (100), 213 (17), 85 (12), 43 (2).

Hyuganin D (**4**): A white powder, $[\alpha]_D^{27}$ +8.6° (c=0.9, $CHCl_3$). High-resolution EI-MS: Calcd for $C_{20}H_{22}O_7$ (M^+), 374.1365. Found: 374.1362. UV λ_{max} (MeOH) nm (log ε): 217 (sh., 4.2), 299 (sh., 3.9), 322 (4.1). IR (KBr): 1744—1718, 1655, 1608, 1560, 1490, 1230, 1148, 840 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 1.20, 1.23 (3H each, both d, J=7.0 Hz, 3‴, 4‴-H_3), 1.42, 1.45 (3H each, both s, 2′-*gem*-CH_3), 2.09 (3H, s, 2″-H_3), 2.61 (1H, qq, J=7.0, 7.0 Hz, 2‴-H), 5.31 (1H, d, J=5.0 Hz, 3′-H), 6.22 (1H, d, J=9.6 Hz, 3-H), 6.53 (1H, d, J=5.0 Hz, 4′-H), 6.80 (1H, d, J=8.7 Hz, 6-H), 7.36 (1H, d, J=8.7 Hz, 5-H), 7.60 (1H, d, J=9.6 Hz, 4-H). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ_C: given in Table 1. EI-MS m/z (%): 374 (M^+, 3), 314 (8), 299 (13), 261 (7), 244 (18), 229 (100), 213 (12), 71 (37), 43 (39).

Alkaline Hydrolysis of Hyuganins (1—4) The solution of **1** (17 mg) in dioxane (1 ml) was treated with 5% KOH (2 ml) and the mixture was stirred at room temparature for 6 h. The reaction mixture was neutralized with Dowex HCR WX2 (H^+ from) and the resin was removed by filtration. After evaporation of the solvent from the filtrate under reduced pressure, the residue was subjected to reversed-phase silica gel column chromatography [1 g, $H_2O \rightarrow MeOH$] to give the H_2O eluted fraction (7.5 mg) and MeOH eluted fraction (9 mg). The MeOH eluted fraction was purified by ordinary-phase silica gel column chromatography [0.5 g, n-hexane–AcOEt (1 : 1, v/v)] to give (+)-*cis*-khellactone (**5**, 2.5 mg) and (−)-*trans*-khellactone (**16**, 6.2 mg). Compounds **5** and **16** were identified by comparison of their $[\alpha]_D$ and ^{1}H-NMR data with the reported values.[20] Compounds **2** (15 mg), **3** (16 mg), and **4** (14 mg) were also treated with alkaline to obtain **5** and **16** (2.3, 6.2 mg from **2**, 2.5, 7.0 mg from **3**, 2.4, 6.4 mg from **4**). Each H_2O eluted fraction and p-nitrobenzyl-N,N'-diisopropylisourea (20 mg) were dissolved in $(CH_2)_2Cl_2$ (4 ml). Then the solution was stirred at 80 °C for 1.5 h. The reaction mixture was subjected to HPLC analysis [column: YMC-pack R&D ODS-A (250×4.6 mm i.d.); solvent, MeOH–H_2O (7 : 3, v/v), flow rate, 1.0 ml/min] to identify p-nitrobenzylacetate [t_R (7.3 min) from **3** and **4**], p-nitrobenzylisobutylate [t_R (13.2 min) from **2** and **4**], p-nitrobenzylangelate [t_R (19.3 min) from **1** and **2**], p-nitrobenzyl-2-methylbutylate [t_R (19.5 min) from **1** and **3**].

(+)-Lomatin (13) from Enzymatic Hydrolysis of 12 A solution of **12** (80.0 mg) in 0.1 M acetate buffer (pH 3.8, 5 ml) was treated with naringinase (80 mg), and the whole mixture was stirred at 38 °C for 12 h. After treatment of the reaction mixture with EtOH, the whole mixture was evaporated to dryness under reduced pressure and the residue was purified by ordinary-phase silica gel column chromatography [1 g, $CHCl_3$–MeOH–H_2O (10 : 3 : 1, v/v, lower layer)] to give **13**[24] (48.2 mg).

(−)-*cis*-3′,4′-Diacetylkhellactone (6) and (+)-Acetyllomatin (14) from 5 and 13 Each solution of **5** (22.3 mg) and **13** (14.9 mg) in pyridine (0.5 ml) was treated with Ac_2O (0.25 ml) and the reaction mixture was stirred at room temperature (20 °C) for 1 h. The reaction mixture was washed successively with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, then dried over Na_2SO_4, and filtered. Evaporation of the solvent from the filtrate under reduced pressure gave **6**[6b)] (29.4 mg) and **14** (17.4 mg).

6: A white powder, $[\alpha]_D^{25}$ −8.7° (c=0.34, $CHCl_3$). High-resolution EI-MS: Calcd for $C_{18}H_{18}O_7$ (M^+), 346.1052. Found: 346.1055. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.42, 1.45 (3H each, both s, 2′-*gem*-CH_3), 2.11, 2.14 (3H each, both s, $-COCH_3$), 5.31 (1H, d, J=4.9 Hz, 3′-H), 6.24 (1H, d, J=9.4 Hz, 3-H), 6.54 (1H, d, J=4.9 Hz, 4′-H), 6.81 (1H, d, J=8.6 Hz, 6-H), 7.37 (1H, d, J=8.6 Hz, 5-H), 7.61 (1H, d, J=9.4 Hz, 4-H).

14: A white powder, $[\alpha]_D^{25}$ +10.5° (c=0.3, EtOH). High-resolution EI-MS: Calcd for $C_{16}H_{16}O_5$ (M^+), 288.0998. Found: 288.0995. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.35, 1.38 (3H each, both s, 2′-*gem*-CH_3), 2.07 (3H, s, $-COCH_3$), 2.99 (1H, dd, J=4.9, 18.1 Hz), 3.19 (1H, dd, J=5.3, 18.1 Hz) (4′-H_2), 5.14 (1H, d, J=4.9, 5.3 Hz, 3′-H), 6.24 (1H, d, J=9.2 Hz, 3-H), 6.79 (1H, d, J=8.6 Hz, 6-H), 7.27 (1H, d, J=8.6 Hz, 5-H), 7.63 (1H, d, J=9.2 Hz, 4-H).

Bioassay Methods

Tissue Preparation Male Wistar rats weighing 250—350 g were sacrificed by severing both carotid arteries under anesthesia, and the thoracic aorta was isolated and cut into helical strips (2—3 mm × 15—20 mm). Physiological salt solution contained NaCl (118.0 mM), KCl (4.7 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (2.5 mM), $NaHCO_3$ (25.0 mM), and glucose (10.0 mM). The solution was aerated with a 95% O_2–5% CO_2 gas mixture and kept at 37 °C. To investigate the mechanical response, each preparation was suspended in an organ bath (6 ml) and subjected to an initial load of about 1 g. One hour equilibration period was allowed before initiation of the experiments. Contractions were measured isometrically *via* a force-displacement transducer (Nihon Denki Sanei, Tokyo, Japan) and recorded on a polygraph.

High K^+-Induced Contraction After equilibration, 3 M KCl (0.1 ml) was added to the bath (final concentration of K^+: 54 mM). The tissues were washed 3 times and re-equilibrated after the contration had reached the maximum level. Sustained contraction was induced again by the addition of KCl, and then test compound was cumulatively applied at 3—100 μM. The contractile response prior to the application of test sample was taken to be 100%. Nifedipine was used as a reference compound.

NE-Induced Contraction After equilibration, NE was added to the bath (final concentration of NE: 1 μM) in the presense of nifedipine (1 μM, a voltage-dependent Ca^{2+}-channnel blocker). The tissues were washed 3 times and re-equilibrated after the contration had reached the maximum level. This procedure was repeated, and a second contraction was obtained. Tissues were incubated with each test compound for 10 min and then NE was cummulatively applied (1 nM—1 μM). In order to minimize variability between tissues, the contraction ratio of the third response to the second response at 1 μM NE was used. The mean contraction ratio in the control was taken to be 100%. Prazosin hydrochloride was used as a reference compound.

Statistical Analysis Values are expressed as the means ± S.E.M. For statistical analysis, one-way analysis of variance following Dunnett's test was used. IC_{50} values were determined graphically.

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Immunomodulatory Constituents from an Ascomycete, *Emericella aurantio-brunnea*

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Fractionation monitored by the immunomodulatory activity of the AcOEt extract of an Ascomycete, *Emericella aurantio-brunnea*, afforded two known fungal sesterterpenes, variecolin (1) and variecolactone (2), two new variecolin congeners named variecoacetals A (3) and B (4), and a new sesquiterpenetriol diester named emeremophiline (5), as the immunosuppressive constituents of this fungus. The absolute configuration of 1, which was previously not determined, was determined to be (2*S*,3*S*,6*R*,10*S*,11*R*,14*S*,15*R*,16*S*) from the NMR spectral data of the (6*R*,7*R*)-dimethyl-1,3,5-trioxacycloheptyl derivative of 1 (7). The absolute configurations of the other variecolin congeners, 2—4, and variecolol (6) are also proposed from biosynthetic considerations.

Key words fungal metabolite; *Emericella aurantio-brunnea*; immunosuppressant; variecolin congener; absolute configuration; Ascomycete

Recently, several 2-pyrones, multiforisins A—I[1a,b] (from *Gelasinospora multiforis*, *G. heterospora*, and *G. longispora*), macrophin[1c] (from *Diplogelasinospora grovesii*), a macrocyclic sesterterpene, kobiin, three 2-furanones, kobifuranones A—C[1d] (from *Gelasinospora kobi*), a hexaketide, sordarial[1b] (from *G. heterospora* and *G. longispora*), two anthraquinones, questin and rubrocristin, two octaketides, cladosporin and cladosporin 8-*O*-methylether, and two dioxopiperazines, tardioxopiperazines A and B[1e] (from *Microascus tardifaciens*), have been isolated as immunosuppressive constituents in our screening program on immunomodulatory components from fungi. We have also found that the AcOEt extract of an Ascomycete, *Emericella aurantio-brunnea* appreciably suppressed the proliferation (blastogenesis) of mouse splenic lymphocytes stimulated with mitogens, concanavalin A (Con A) and lipopolysaccharide (LPS). Solvent partition followed by repeated chromatographic fractionation of the extract guided by immunosuppressive activity afforded five constituents tentatively named EA-1 (**1**)—-5 (**5**), as the immunosuppressive features of this fungus. This report deals with the structures and immunosuppressive activities of these constituents recently isolated from *E. aurantio-brunnea*.

Results and Discussion

The AcOEt extract of *E.aurantio-brunnea* IFM42008[2] cultivated on sterilized rice medium suppressed the Con A-induced proliferation of mouse splenic lymphocytes by 99% at 50 μg/ml. The AcOEt extract was partitioned with *n*-hexane and water into an *n*-hexane layer and an aqueous suspension. The aqueous suspension was further partitioned with AcOEt and water into an AcOEt layer and an aqueous layer [yields (%) of the *n*-hexane, AcOEt, and aqueous layers, after evaporation of the solvents, from the AcOEt extract: 72.4, 22.1, and 1.0, respectively]. The *n*-hexane, AcOEt, and aqueous layers had immunosuppressive activity of 99, 85, and −9% at 2.5 μg/ml, respectively. Repeated chromatographic fractionation of the *n*-hexane layer guided by the immunosuppressive activity afforded five constituents tentatively named EA-1—-5 (**1**—**5**) as the immunosuppressive constituents of this fungus [yields (%) of **1**—**5** from the AcOEt extract: 3.66, 0.28, 0.79, 0.24, and 0.21, respectively].

EA-1 (**1**), $C_{25}H_{36}O_2$, was obtained as optically active colorless prisms, whose UV spectrum gave absorptions due to >C=C—C=O and >C=C< systems. The ^{1}H- and ^{13}C-NMR data including spin-decoupling ^{1}H-NMR, and two dimensional ^{1}H–^{1}H shift correlation (COSY), ^{1}H-detected heteronuclear correlation through multiple quantum coherence (HMQC), and ^{1}H-detected heteronuclear multiple-bond correlation (HMBC) NMR data showed the similarity of EA-1 to a tetracyclic (rings A—D) fungal sesterterpene, variecolin (**1**), which was isolated from a Fungi Imperfecti *Aspergillus variecolor* as an angiotensin II receptor binding inhibitor by Hensens *et al.*,[3] and later isolated also from an Ascomycete *Emericella purpurea* together with its congeners, valiecolactone (**2**) and valiecolol (**6**), by Kawai and his co-workers.[4] Direct comparison of EA-1 with an authentic sample of variecolin showed that EA-1 was identical with variecolin (**1**).

On the stereostructure of variecolin (**1**) and its congeners variecolactone and variecolol, their relative configurations have already been elucidated,[3,4] but the absolute configurations has not yet been determined. Namely, it is known that the absolute structure of variecolin is expressed as **1** [(2*S*,3*S*,6*R*,10*S*,11*R*,14*S*,15*R*,16*S*)-configuration] or **1a** [(2*R*,3*R*,6*S*,10*R*,11*S*,14*R*,15*S*,16*R*)-configuration] (see Chart 1). In order to decide the absolute configuration, **1** was treated with (2*R*,3*R*)-(−)-butane-2,3-diol[5] to give an optically active product (**7**), $C_{29}H_{44}O_3$, whose IR spectrum suggested the absence of any OH group and UV spectrum gave only the end absorption due to an isolated >C=C< system. Comparison of the ^{1}H- and ^{13}C-NMR data of **7** with those of **1** (in $CDCl_3$) indicated the appearance of six new fragments, $\underline{CH_3}$CH< [δ_H/δ_C, 1.09 (3H, d, 6.6)/18.55 (q)], $\underline{CH_3}$CH< [1.11 (3H, d, 6.6)/18.59 (q)], $CH_3\underline{CH}$(O–)CH< [3.62 (1H, dq, 7.3, 6.6)/73.58 (d)], $CH_3\underline{CH}$(O–)CH< [3.99 (1H, dq, 7.3, 6.6)/77.39 (d)], –O$\underline{CH}$(O–)– [5.69 (1H, s)/106.70 (d)], and –(–O)$\underline{C}$(O–)– [121.72 (s)], instead of just the expected disappearance of –$\underline{CH}$=O at position 20 [9.16 (1H, s)/193.04 (d)] and >$\underline{C}$=O at position 5 [220.74 (s)] (see Table 1). These data suggested that the glycol group of (2*R*,3*R*)-(−)-butane-

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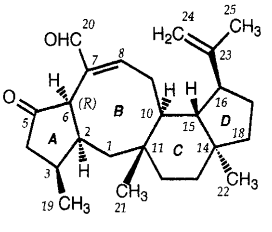
Dedicated to the memory of Dr. Kyosuke Tsuda.

2,3-diol reacted with not only the –CH=O group but also the >C=O group of **1** to form a hexacyclic (rings A—F) product possessing two new rings E (five-membered) and F (seven-membered), that is, a (6*R*,7*R*)-dimethyl-1,3,5-trioxacycloheptyl derivative of **1** (**7**), during this reaction (see Chart 2). From a nuclear Overhauser effect (NOE) experiment, an NOE was significantly observed between H-6 and H-3′ in the (2*R*,3*R*)-2,3-dioxybutane group of **7**, indicating that both H-6 and the (2*R*,3*R*)-2,3-dioxybutane group were present on the same side of the plane of ring E in **7**. Furthermore, the HMBC NMR data supported also the structure of **7**, as shown in Chart 2.

Considering the rings E and F were five- and seven-membered respectively, it was found from careful investigation with molecular models that only four conformations I—IV were possible for rings E and F of **7**. Namely, conformation I or II was possible for **7** if this compound possessed a (6*R*)-type configuration structure in which the configuration at position 6 was (*R*), and conformation III or IV was possible for **7** if this compound possessed a (6*S*)-type configuration structure in which that was (*S*) (see Chart 2). Here, it was expected from molecular models that a significant NOE would be observed between H-6 and H-3′, between H-6 and CH_3-1′, between H-6 and H-2′, and between H-6 and CH_3-4′ in the case of conformations I, II, III, and IV, respectively, as shown in Chart 2. In the NOE experiment on **7**, an NOE was not observed between H-6 and CH_3-1′, between H-6 and H-2′, or between H-6 and CH_3-4′, but was observed of 6—9% between H-6 and H-3′, indicating that **7** had conformation I. Thus, the configuration at 6 in **7** was elucidated to be (*R*). Accordingly, the absolute structure of variecolin was decided not to be **1a**, but to be **1** [(2*S*,3*S*,6*R*,10*S*,11*R*,14*S*,15*R*,16*S*) configuration] (see Chart 1). Considering the biosynthetic fact that **2** was isolated together with **1** from *E. aurantiobrunnea* as mentioned below, and both **2** and **6** were formerly isolated together with **1** from *E. purpurea*,[4] the absolute configuration of the two variecolin congeners, variecolactone (**2**)



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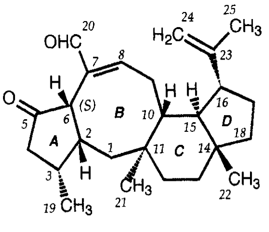


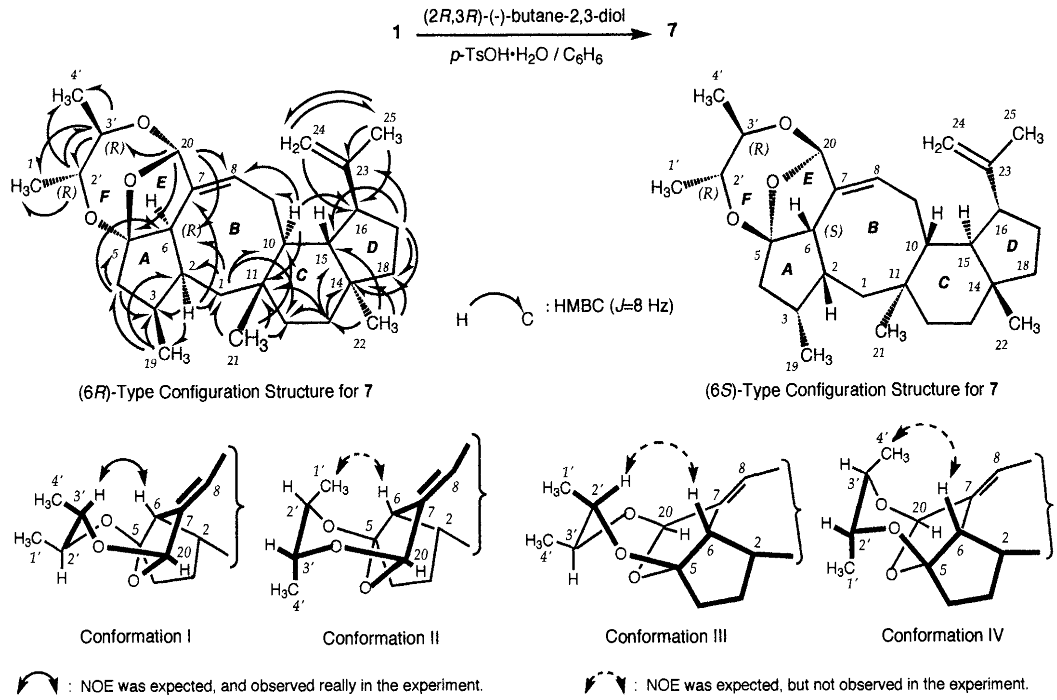
1a

Chart 1

Table 1. ¹H-NMR and ¹³C-NMR Data for EA-1 (**1**), (6*R*,7*R*)-Dimethyl-1,3,5-Trioxacycloheptyl Derivative of **1** (**7**), and Dihydro Derivative of **1** (**6**) in $CDCl_3$

Position	**1**		**7**		**6**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.20 (br d, 14.3), 1.53 (dd, 14.3, 11.9)	42.19 (t)	1.00 (dd, 14.4, 1.2), 1.64 (dd, 14.4, 12.5)	41.37 (t)	1.01 (dd, 14.4, 1.3), 1.76 (dd, 14.4, 12.5)	41.37 (t)
2	2.62 (m)	39.22 (d)	2.70 (br td, 11.8, 5.8)	39.45 (d)	2.86 (m)	39.57 (d)
3	2.38 (m)	34.71 (d)	2.10 (m)	36.72 (d)	2.00 (m)	38.55 (d)
4	2.38 (m), 2.50 (dd, 18.6, 8.3)	46.26 (t)	1.90 (m), 2.10 (m)	43.46 (t)	1.90 (m), 2.40 (m)	45.16 (t)
5		220.74 (s)		121.72 (s)		119.68 (s)
6	3.55 (d, 10.3)	50.34 (d)	3.19 (br d, 11.5)	55.74 (d)	3.38 (br d, 9.5)	53.66 (d)
7		139.82 (s)		135.55 (s)		135.53 (s)
8	6.89 (dd, 5.1, 1.1)	160.76 (d)	5.85 (dd, 6.6, 3.9)	128.11 (d)	5.45 (br s)	120.59 (d)
9	2.19 (m), 2.83 (br d, 17.0)	31.51 (t)	1.90 (m), 2.45 (dt, 19.3, 3.4)	29.11 (t)	2.00 (m), 2.42 (br d, 19.5)	28.85 (t)
10	2.19 (m)	40.62 (d)	2.10 (m)	38.46 (d)	1.93 (m)	38.63 (d)
11		39.10 (s)		38.78 (s)		38.72 (s)
12	1.01 (dt, 13.8, 2.6), 1.85 (td, 13.8, 4.4)	35.42 (t)	0.93 (ddd, 13.7, 3.7, 2.7), 1.95 (m)	34.54 (t)	0.93 (dt, 13.7, 3.4), 2.00 (m)	34.57 (t)
13	1.42 (2H, m)	35.21 (t)	1.50 (2H, m)	35.35 (t)	1.45 (2H, m)	35.36 (t)
14		43.26 (s)		43.48 (s)		43.44 (s)
15	1.42 (m)	48.63 (d)	1.50 (m)	48.38 (d)	1.45 (m)	48.45 (d)
16	2.38 (m)	48.42 (d)	2.41 (td, 11.0, 5.1)	47.98 (d)	2.38 (td, 11.0, 5.5)	48.13 (d)
17	1.20 (m), 1.95 (m)	29.89 (t)	1.35 (m), 2.00 (m)	30.38 (t)	1.34 (m), 2.00 (m)	30.24 (t)
18	1.20 (m), 1.42 (m)	39.81 (t)	1.25 (m), 1.45 (m)	40.10 (t)	1.23 (m), 1.45 (m)	40.01 (t)
19	0.77 (3H, d, 7.3)	15.78 (q)	0.76 (3H, d, 7.6)	15.71 (q)	0.81 (3H, d, 7.3)	15.45 (q)
20	9.16 (s)	193.04 (d)	5.69 (s)	106.70 (d)	4.45 (br d, 11.8), 4.62 (br d, 11.8)	73.44 (t)
21	0.91 (3H, s)	21.80 (q)	0.90 (3H, s)	22.01 (q)	0.90 (3H, s)	21.98 (q)
22	0.81 (3H, s)	18.04 (q)	0.87 (3H, s)	18.15 (q)	0.85 (3H, s)	18.13 (q)
23		150.34 (s)		151.08 (s)		151.11 (s)
24	4.61 (br t, 1.5), 4.70 (br d, 1.5)	110.66 (t)	4.61 (dd, 2.3, 1.3), 4.71 (d, 2.3)	109.64 (t)	4.58 (dd, 2.0, 1.5), 4.69 (br d, 2.0)	109.60 (t)
25	1.69 (3H, br s)	19.05 (q)	1.70 (3H, s)	19.62 (q)	1.68 (3H, br s)	19.40 (q)
1′			1.11 (3H, d, 6.6)	18.59 (q)		
2′			3.99 (dq, 7.3, 6.6)	77.39 (d)		
3′			3.62 (dq, 7.3, 6.6)	73.58 (d)		
4′			1.09 (3H, d, 6.6)	18.55 (q)		

δ (ppm) from TMS as an internal standard [coupling constants (Hz) in parentheses].



(6*R*)-Type Configuration Structure for 7

(6*S*)-Type Configuration Structure for 7

Conformation I

Conformation II

Conformation III

Conformation IV

Chart 2

and variecolol (**6**), are proposed to be (2*S*,3*S*,5*R*,6*R*,10*S*, 11*R*,14*S*,15*R*,16*S*) (Chart 2). Similarly to Kawai and his coworkers,[4] EA-1 (**1**) was treated with $NaBH_4$ to give a dihydro derivative which was identical with an authentic sample of variecolol (**6**) (see Chart 3).

EA-2 (**2**), $C_{25}H_{36}O_3$, was obtained as colorless prisms. The physicochemical and spectral data of EA-2 showed a similarity to variecolactone.[4] Direct comparison of EA-2 with an authentic sample of variecolactone indicated that EA-2 was identical with variecolactone (**2**).

EA-3 (**3**), $C_{27}H_{42}O_3$, was obtained as a colorless amorphous solid, whose UV spectrum gave only end absorption. Comparison of the ^1H- and ^{13}C-NMR data of **3** with those of **2** suggested that the hemiacetal group $-(\underline{H}O)\underline{C}(O-)-$ at position **5** [δ_H, 3.57 (1H, br s), δ_C, 115.24 (s)] was substituted with an acetal group $-(\underline{H_3C}O)\underline{C}(O-)-$ [δ_H/δ_C, 3.38 (3H, s)/50.91 (q), 123.18 (s)], and the lactone $-O-\underline{C}=O$ at position 20 [170.68 (s)] was substituted with an acetal $-O-\underline{CH}-O\underline{CH_3}$ [5.26 (1H, s)/108.91 (d), 3.42 (3H, s)/54.94 (q)] (see Table 2). Significant NOEs of 5% were observed between H-2 and H-6, of 3% between H-6 and OCH_3-5, and of 1% between OCH_3-5 and OCH_3-20 on the NOE experiment of **3**, suggesting the possibility that the configurations at positions 5 and 20 in **3** were (*R*) and (*S*), respectively. Finally, considering the biosynthetic fact that **3** was isolated together with **1** from *E. aurantio-brunnea*, the absolute configuration of EA-3 (**3**) was proposed to be (2*S*,3*S*,5*R*,6*R*,10*S*, 11*R*,14*S*,15*R*,16*S*,20*S*), as shown in Chart 3.

EA-4 (**4**), $C_{28}H_{44}O_3$, was obtained as a colorless amorphous solid, whose UV spectrum gave only end absorption. Comparison of the ^1H- and ^{13}C-NMR data of **4** with those of **3** suggested that the acetal $-(H_3CO)C(O-)-$ at position 5 was substituted with a different acetal $-(\underline{H_3CH_2C}O)\underline{C}(O-)-$ [δ_H/δ_C, 1.24 (3H, t, J=7.3 Hz)/15.63 (q), 3.59, 3.78 (each 1H, dq, J=9.0, 7.3 Hz)/58.87 (t), δ_C, 122.94 (s)] (see Table 2). Significant NOEs were observed of 6% between H-2 and H-6, and of 1—2% between H-6 and $O\underline{CH_2}CH_3$-5 on the NOE experiment of **4**, suggesting the possibility that the configuration at position 5 in **4** was (*R*). Though the NOE between $O\underline{CH_2}CH_3$-5 and OCH_3-20 was not clearly observed in the experiment, the fact that the ^1H- and ^{13}C-NMR signals of $\underline{CH}$-20 [δ_H/δ_C, 5.28 (1H, s)/108.85 (d)] and $O\underline{CH_3}$-20 [3.42 (3H, s)/54.75 (q)] of **4** were quite similar to those of $\underline{CH}$-20 [5.26 (1H, s)/108.91 (d)] and $O\underline{CH_3}$-20 [3.42 (3H, s)/54.94 (q)] of **3** suggested that the configuration at position 20 in **4** was the same to that in **3** (see Table 2). Finally, considering the biosynthetic fact that **4** was isolated together with **1** from *E. aurantio-brunnea*, the absolute configuration of EA-4 (**4**) is also proposed to be (2*S*,3*S*,5*R*,6*R*,10*S*,11*R*,14*S*,15*R*,16*S*, 20*S*), as shown in Chart 3. Now, we propose the names variecoacetals A (**3**) and B (**4**) for EA-3 and EA-4, respectively.

EA-5 (**5**), was obtained as a colorless amorphous solid. The ^{13}C-NMR spectral data showed the presence of six methyls, namely, one $\underline{C}H_3-CH_2-$ (δ_C, 11.88), two $\underline{C}H_3-CH<$ (10.40, 20.09), and three $\underline{C}H_3-C-$ (12.40, 20.89, 22.75), four methylenes, namely, two $C-\underline{C}H_2-C$ (29.94, 44.96), one $C-\underline{C}H_2-O-$ (63.63) and one $\underline{C}H_2=C$ (114.37), nine methines, namely, two $C-\underline{C}H-C$ (34.94, 41.01), one $C-\underline{C}H-O$ (68.67) and six $C-\underline{C}H=C$ (114.89, 124.07, 130.69, 133.88, 149.13, 150.79), and eight quaternary carbons, namely, one $C-\underline{C}-C$ (36.33) and one $C-\underline{C}-O$ (76.48), three $C=\underline{C}-C$ (131.54, 147.58, 163.20), two $-O-\underline{C}=O$ (167.07, 170.40), and one $>\underline{C}=O$ (198.40) in **5**. These ^{13}C-NMR data were quite compatible with the ^1H-NMR data (see Table 3). All of these fragments were connected by the aid of the COSY and the HMBC NMR data to construct an eremophilane-type sesquiterpenetriol diester (**5**). This structure was also supported by the presence of a molecular ion peak at m/z 456 in the electron impact (EI)-MS spectrum. As fungal eremophilane-type metabolites, phomenone (**8**) from *Phoma exigua* var. *non oxydabilis*,[6] and other compounds[7] have already

Table 2. ^{1}H-NMR and ^{13}C-NMR Data for EA-2 (**2**), EA-3 (**3**) and EA-4 (**4**) in CDCl$_3$

Position	**2**		**3**		**4**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.09 (br d, 14.6), 1.52 (m)	40.84 (t)	0.99 (br d, 13.5), 1.66 (m)	41.05 (t)	1.02 (br d, 12.7), 1.69 (br t, 12.7)	41.12 (t)
2	2.78 (m)	39.78 (d)	2.61 (m)	38.94 (d)	2.65 (m)	38.80 (d)
3	2.25 (m)	37.94 (d)	2.20 (m)	38.23 (d)	2.11 (br t, 11.4)	38.27 (d)
4	2.12 (m), 2.24 (m)	44.85 (t)	2.00 (2H, m)	41.69 (t)	2.01 (2H, m)	42.29 (t)
5		115.24 (s)		123.18 (s)		122.94 (s)
5-OH	3.57 (br s)					
5-OMe			3.38 (3H, s)	50.91 (q)		
5-OEt					1.24 (3H, t, 7.3) 3.59 (dq, 9.0, 7.3), 3.78 (dq, 9.0, 7.3)	15.63 (q) 58.87 (t)
6	3.60 (d, 10.7)	51.73 (d)	3.49 (d, 11.4)	52.32 (d)	3.48 (br d, 8.6)	52.36 (d)
7		125.12 (s)		136.23 (s)		136.35 (s)
8	6.98 (dd, 6.7, 4.3)	144.64 (d)	5.77 (dd, 5.8, 2.9)	128.72 (d)	5.79 (dd, 6.0, 3.2)	128.65 (d)
9	2.15 (m), 2.78 (m)	29.74 (t)	1.90 (m), 2.49 (br d, 19.8)	29.08 (t)	1.92 (m), 2.49 (br d, 19.5)	29.13 (t)
10	2.15 (m)	38.65 (d)	2.15 (m)	38.32 (d)	2.16 (m)	38.27 (d)
11		38.96 (s)		38.76 (s)		38.80 (s)
12	1.00 (dt, 12.2, 3.1), 1.97 (m)	34.35 (t)	0.90 (dt, 13.6, 3.3), 1.97 (m)	34.41 (t)	0.93 (m), 1.95 (m)	34.40 (t)
13	1.50 (2H, m)	35.10 (t)	1.50 (2H, m)	35.31 (t)	1.46 (2H, m)	35.35 (t)
14		43.40 (s)		43.42 (s)		43.45 (s)
15	1.52 (m)	47.94 (d)	1.45 (m)	48.29 (d)	1.48 (m)	48.31 (d)
16	2.39 (td, 11.2, 5.5)	48.10 (d)	2.29 (td, 11.2, 5.3)	47.82 (d)	2.35 (td, 11.0, 5.3)	47.62 (d)
17	1.37 (m), 2.00 (m)	29.98 (t)	1.37 (m), 2.00 (m)	30.23 (t)	1.30 (m), 1.96 (m)	30.30 (t)
18	1.25 (m), 1.45 (m)	39.86 (t)	1.20 (m), 1.45 (m)	40.01 (t)	1.20 (m), 1.42 (m)	40.09 (t)
19	0.69 (3H, d, 7.4)	15.92 (q)	0.74 (3H, d, 7.6)	15.58 (q)	0.76 (3H, d, 6.6)	15.63 (q)
20		170.68 (s)	5.26 (s)	108.91 (d)	5.28 (s)	108.85 (d)
20-OMe			3.42 (3H, s)	54.94 (q)	3.42 (3H, s)	54.75 (q)
21	0.91 (3H, s)	21.75 (q)	0.87 (3H, s)	21.92 (q)	0.90 (3H, s)	21.91 (q)
22	0.86 (3H, s)	18.14 (q)	0.78 (3H, s)	18.06 (q)	0.83 (3H, s)	18.15 (q)
23		150.48 (s)		150.64 (s)		150.69 (s)
24	4.63 (br s), 4.71 (br s)	110.42 (t)	4.52 (d, 1.6), 4.62 (d, 1.6)	109.83 (t)	4.54 (d, 2.1), 4.64 (d, 2.1)	109.79 (t)
25	1.70 (3H, s)	19.28 (q)	1.63 (3H, s)	19.46 (q)	1.65 (3H, s)	19.50 (q)

δ (ppm) from TMS as an internal standard [coupling constants (Hz) in parentheses].

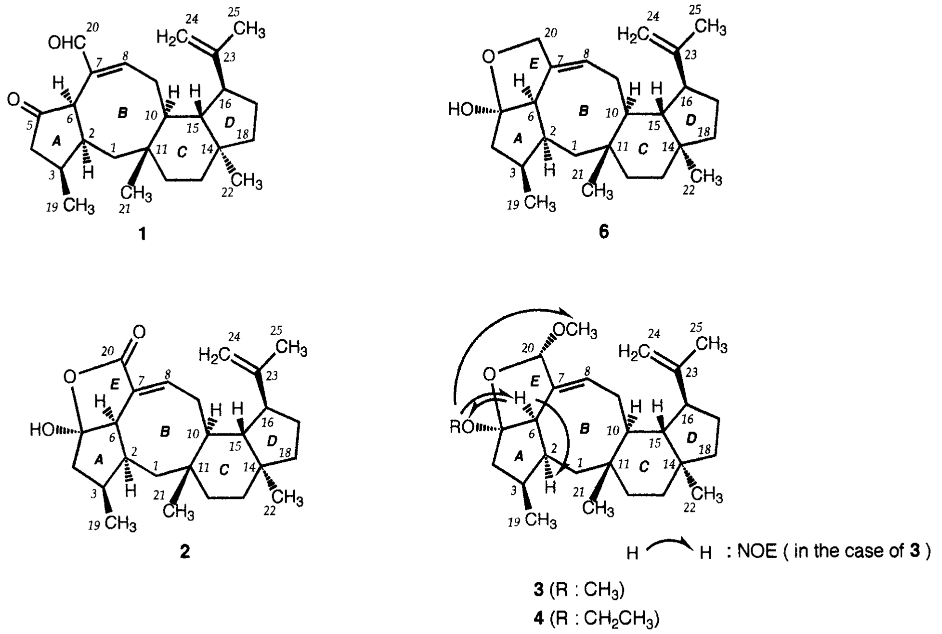


Chart 3

been isolated. The ^{1}H-NMR data of the eremophilane-type skeleton moiety of EA-5 (**5**) were similar to those of **8**$^{6)}$ (see Table 3). To our knowledge, the structure of **5** has hitherto been unknown. But at this time we were not successful in elucidating of the stereostructure of EA-5 because this compound was unstable and rapidly decomposed. We propose to

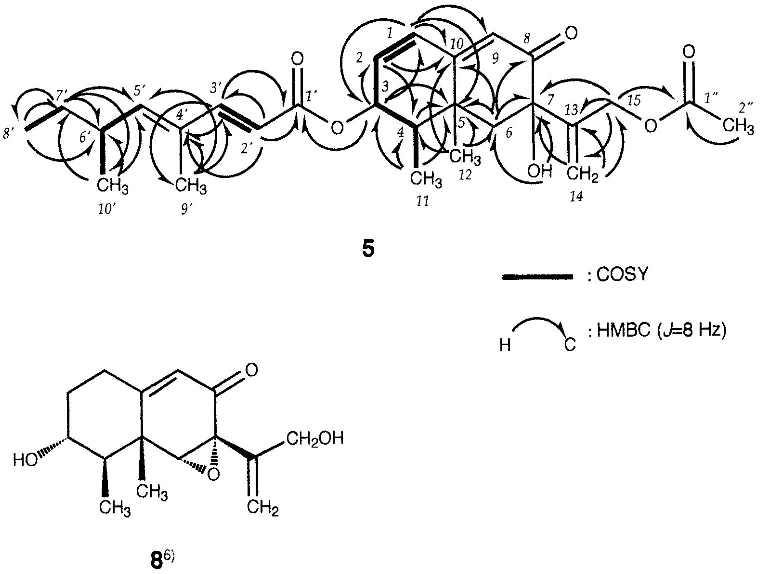


Chart 4

Table 3. ^{1}H-NMR and ^{13}C-NMR Data for EA-5 (**5**)

Position	**5**	
	δ_H	δ_C
1	6.38 (d, 9.7)	130.69 (d)
2	6.35 (dd, 9.7, 5.1)	133.88 (d)
3	5.45 (t, 5.1)	68.67 (d)
4	1.95 (m)	41.01 (d)
5		36.33 (s)
6	2.00 (d, 14.9), 2.25 (d, 14.9)	44.96 (t)
7		76.48 (s)
7-OH	2.70 (br s)	
8		198.40 (s)
9	5.95 (s)	124.07 (d)
10		163.20 (s)
11	1.08 (3H, d, 7.1)	10.40 (q)
12	1.50 (3H, s)	22.75 (q)
13		147.58 (s)
14	5.35 (s), 5.48 (s)	114.37 (t)
15	4.58 (2H, br s)	63.63 (t)
1′		167.07 (s)
2′	5.78 (d, 15.7)	114.89 (d)
3′	7.30 (d, 15.7)	150.79 (d)
4′		131.54 (s)
5′	5.68 (br d, 9.8)	149.13 (d)
6′	2.45 (m)	34.94 (d)
7′	1.35 (m), 1.40 (m)	29.94 (t)
8′	0.85 (3H, t, 7.3)	11.88 (q)
9′	1.80 (3H, d, 1.2)	12.40 (q)
10′	1.00 (3H, d, 6.6)	20.09 (q)
1″		170.40 (s)
2″	2.10 (3H, s)	20.89 (q)

δ (ppm) from TMS as an internal standard in CDCl$_3$ [coupling constants [Hz] in parentheses].

Table 4. Immunosuppressive Effects of EA-1 (**1**)—EA-5 (**5**), Dihydro Derivative of **1** (**6**), and (6*R*,7*R*)-Dimethyl-1,3,5-Trioxacycloheptyl Derivative of **1** (**7**), and Azathioprine, Cyclosporin A, and FK506 on the Con A-Induced and LPS-Induced Proliferation of Mouse Splenic Lymphocytes

Compound	IC$_{50}$ (μg/ml)	
	Con A-induced	LPS-induced
1	0.4	0.1
2	8.0	2.5
3	4.5	1.5
4	6.5	2.2
5	2.0	Not tested
6	1.7	0.6
7	12.0	4.0
Azathioprine	2.7	2.7
Cyclosporin A	0.04	0.07
FK506 (tacrolimus)	1.5×10^{-5}	1.6×10^{-3}

The IC$_{50}$ value of each sample was calculated from the correlation curve between the sample concentration (horizontal axis) and the cell proliferation (vertical axis). The curve of each sample was drawn with 7 points, each of which represented the mean of 3 experiments on each correlation between 7 different concentrations and cell proliferations.

call EA-5 emeremophiline (**5**).

The immunosuppressive activities (IC$_{50}$ values) of **1**—**7** were calculated against Con A-(T cells) and LPS-induced (B-cells) proliferations of mouse splenic lymphocytes, as shown in Table 4. Among these five natural metabolites **1**—**5** from *E. aurantio-brunnea*, **1** which was obtained in the largest quantities from the fungus showed the highest immunosuppressive activity, indicating that **1** was the main immunosuppressive constituent of this fungus. The fact that **1** which possessed both the ketone group at position 5 and the conjugated aldehyde group at position 20 displayed higher immunosuppressive activity than other variecolin related compounds **2**—**4**, **6**, and **7** which lost both the ketone group and the conjugated aldehyde group indicated that the existence of both the ketone group at position 5 and the conjugated aldehyde group at position 20 in **1** might be very important for the appearance of immunosuppressive activity of **1**. The immunosuppressive activity of **1** seemed to be comparatively high, because it was higher than that of azathioprine, though lower than those of cyclosporin A and FK506 (tacrolimus). To our knowledge, this is the first time that **1** and **2** have been isolated as immunosuppressive constituents from a natural source.

Experimental

The general procedures for chemical experiments and other experimental conditions, including those for the evaluation of suppressive activity (IC$_{50}$

values) of samples against the proliferation of mouse splenic lymphocytes stimulated with Con A and LPS, were the same as those described in our previous report [this method is based on the formation ratio of MTT-formazan from exogenous 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) in lymphocytes].[1b] Investigation on the stereostructure of **7** used molecular models, HGS Type-C (Maruzen Co.). Chemical shifts are expressed in δ (ppm) values from tetramethylsilane (TMS) as an internal standard in $CDCl_3$.

Isolation of EA-1 (1)—-5 (5) from *E. aurantio-brunnea* *E. aurantio-brunnea* IFM42008[2] was cultivated on sterilized rice (200 g/flask×3) at 25 °C for 23 d. The moldy rice was extracted with AcOEt (900 ml) with shaking at room temperature for 6 h two times to give an AcOEt solution (1.80 l), which gave, after evaporation *in vacuo*, an AcOEt extract (2.90 g). The AcOEt extract was partitioned with *n*-hexane–H_2O (1 : 1, v/v) (240 ml) into an *n*-hexane layer (after evaporation *in vacuo*, 2.10 g), and an aqueous suspension which was further partitioned with AcOEt (120 ml) into an AcOEt layer (0.64 g) and an aqueous layer (0.03 g). The *n*-hexane, AcOEt, and aqueous layers suppressed the Con A-induced proliferation of mouse splenic lymphocytes by 99, 85, and −9% at 2.5 μg/ml, respectively. The *n*-hexane layer was subjected to chromatography on a silica gel column with *n*-hexane–AcOEt (20 : 1), (15 : 1), (5 : 1), (1 : 1), and MeOH to give seven fractions: I—VII (154, 120, 24, 315, 53, 7, and 29 mg, respectively). Fractions I—VII suppressed the Con A-induced proliferation of the lymphocytes by 2, −6, −3, 100, 10, 1, and −2% at 2.5 μg/ml, respectively. Fraction IV was recrystallized from CH_3CN to give **1** (80 mg), and the mother liquor was chromatographed on an HPLC octadecyl silica gel (ODS) column with 90% CH_3CN at a flow rate of 8 ml/min to afford **1** (26 mg) and **2** (8 mg). Fraction V was chromatographed on a silica gel column with *n*-hexane–AcOEt (5 : 1), (1 : 1), and MeOH to give four fractions: Va—Vd (15, 14, 19, and 13 mg, respectively). Fractions Va—Vd suppressed the Con A-induced proliferation of the lymphocytes by −16, −25, 54, and 31% at 2.5 μg/ml, respectively. Fraction Vc was further chromatographed on an HPLC ODS column with 90% CH_3CN at a flow rate of 8 ml/min to afford **5** (6 mg). Fraction I was also chromatographed on a silica gel column with *n*-hexane–AcOEt (100 : 1) and MeOH to give three fractions: Ia—Ic (49, 45, and 59 mg, respectively). Fraction Ib was further chromatographed on an HPLC ODS column with CH_3CN at a flow rate of 8 ml/min to afford **3** (23 mg) and **4** (7 mg).

EA-1 (**1**) (Variecolin): Colorless prisms from CH_3CN, mp 160.5—161.5 °C. $[\alpha]_D^{19}$ $-110.5°$ (c=0.50, CH_3CN) [lit.[3] $-11.5°$, the authentic sample provided by Dr. Kawai gave $-84.4°$]. High resolution (HR)-FAB-MS *m/z*: 369.2779 (Calcd for $C_{25}H_{37}O_2$ $[(M+H)^+]$: 369.2794). IR (KBr) cm^{-1}: 2940, 1737, 1684, 1626, 1455, 1377, 1227, 887. UV λ_{max} (MeOH) nm (log ε): 200 (end absorp., 3.90), 240 (3.74). Circular dichroism (CD) (0.54 mM, MeOH) $\Delta\varepsilon$ (nm): +4.3 (295), −3.7 (234), −2.2 (220). This compound was identical with an authentic sample of variecolin (**1**)[4] in terms of ^{1}H-NMR spectra and TLC behavior [Kieselgel $60F_{254}$ (Merck), *n*-hexane–AcOEt (2 : 1); RP18 $F_{254}S$ (Merck), CH_3CN].

EA-2 (**2**) (Variecolactone): Colorless prisms from CH_3CN, mp 253—255 °C (lit.[4] 249—251 °C). $[\alpha]_D^{24}$ $+87.4°$ (c=0.19, CH_3CN). Electron impact (EI)-MS *m/z* (%): 384 (20, M^+), 366 (21), 323 (12) [lit.[4] 384 (21, M^+), 369 (7), 366 (8)]. HR-FAB-MS *m/z*: 385.2747 (Calcd for $C_{25}H_{37}O_3$ $[(M+H)^+]$: 385.2743). IR (KBr) cm^{-1}: 3391, 2912, 1729, 1662, 1438, 1384, 1243, 961, 882. UV λ_{max} (MeOH) nm (log ε): 200 (end absorp., 3.97), 231 (3.96) [lit.[4] 232 (3.78)]. This compound was identical with an authentic sample of variecolactone (**2**) in terms of ^{1}H- and ^{13}C-NMR spectra and TLC behavior [Kieselgel $60F_{254}$ (Merck), *n*-hexane–AcOEt (2 : 1)].

EA-3 (**3**) (Variecoacetal A): Colorless amorphous solid, $[\alpha]_D^{25}$ $+101.4°$ (c=0.14, CH_3CN). EI-MS *m/z* (%): 414 (29, M^+), 399 (10), 383 (100), 367 (40), 355 (41). HR-EI-MS *m/z*: 414.3147 (Calcd for $C_{27}H_{42}O_3$: 414.3134). IR (KBr) cm^{-1}: 2938, 1642, 1456, 1379, 1323, 1090, 1019, 969. UV λ_{max} (MeOH) nm (log ε): 205 (end absorp., 4.12).

EA-4 (**4**) (Variecoacetal B): Colorless amorphous solid, $[\alpha]_D^{25}$ $+81.5°$ (c=0.13, CH_3CN). EI-MS *m/z* (%): 428 (18, M^+), 413 (10), 396 (100), 381 (38), 367 (49). HR-EI-MS *m/z*: 428.3270 (Calcd for $C_{28}H_{44}O_3$: 428.3290). IR (KBr) cm^{-1}: 2938, 1638, 1460, 1382, 1320, 1062, 1015, 968. UV λ_{max} (MeOH) nm (log ε): 205 (end absorp., 4.11).

EA-5 (**5**) (Emeremophiline): Colorless amorphous solid, EI-MS *m/z* (%): 456 (2, M^+), 397 (6), 228 (16), 211 (14), 152 (100).

Formation of the (6*R*,7*R*)-Dimethyl-1,3,5-Trioxacycloheptyl Derivative of EA-1 (7) from EA-1 (1) To a solution of **1** (103 mg) and *p*-toluenesulfonic acid monohydrate ($TsOH \cdot H_2O$) (20 mg) in benzene (16 ml), (2*R*,3*R*)-(−)-butane-2,3-diol (800 μl) was added. The reaction mixture was stirred at room temperature for 19 h. The reaction mixture was neutralized with a saturated $NaHCO_3$ solution under ice-cooling. The organic layer was washed with water, and then with a saturated NaCl solution. After evaporation *in vacuo*, the organic layer was subjected to chromatography on a silica gel C-60 column with *n*-hexane–AcOEt (15 : 1) to give **7** (30 mg) as a colorless oil, $[\alpha]_D^{28}$ $+92.0°$ (c=0.2, CH_3CN). EI-MS *m/z* (%): 440 (90, M^+), 425 (4), 396 (8), 368 (14), 352 (100). HR-FAB-MS *m/z*: 441.3356 (Calcd for $C_{29}H_{45}O_3$ $[(M+H)^+]$: 441.3369. IR (KBr) cm^{-1}: 2937, 1646, 1449, 1379, 1305, 1230, 1123, 1056. UV λ_{max} (MeOH) nm (log ε): 205 (end absorp., 4.08).

Formation of Variecolol (6) from EA-1 (1) A suspension of $NaBH_4$ (4 mg) in EtOH (400 μl) was added to a solution of **1** (22 mg) in EtOH (750 μl). The reaction mixture was stirred at room temperature for 30 min. After addition of a saturated NH_4Cl solution (2 ml) under ice-cooling, the reaction mixture was treated as usual to give a crude product which was purified on an HPLC ODS column with CH_3CN–H_2O (9 : 1) to afford **6** (13 mg) as a colorless amorphous powder, $[\alpha]_D^{24}$ $+137.1°$ (c=0.14, CH_3CN), EI-MS *m/z* (%): 370 (9, M^+), 352 (100), 337 (13), 309 (10). UV λ_{max} (MeOH) nm: end absorp. only. This compound was identical with an authentic sample of variecolol (**6**) in terms of ^{1}H- and ^{13}C-NMR spectra.

Acknowledgements We are grateful to Prof. K. Kawai of Hoshi University for the gift of authentic samples of variecolin, variecolactone and variecolol, and the copies of their ^{1}H- and ^{13}C-NMR spectra, and also to Dr. T. Uegaki of Fujisawa Pharmaceutical Co., Ltd. for the gift of FK506.

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2) This strain was deposited earlier at Research Institute for Chemobiodynamics, Chiba University (present name: Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University). Now, the voucher specimen is also on deposit in our laboratory.

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Argadin, a New Chitinase Inhibitor, Produced by *Clonostachys* sp. FO-7314

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A new chitinase inhibitor, designated as argadin (1), was isolated from the cultured broth of a fungal strain FO-7314. The strain was identified as *Clonostachys* sp. from the morphological characteristics. Argadin was purified from the cultured mycelium by a combination of cation exchange, adsorption and gel filtration chromatographic methods. The structure of argadin was elucidated as cyclo(N^{ω}-acetyl-L-arginyl-D-prolyl-homoseryl-histidyl-L-2-aminoadipyl) in which homoseryl γ-methylene bonded to histidyl α-amino residue. The IC_{50} value of argadin against *Lucilia cuprina* (blowfly) chitinase was 150 nM at 37 °C and 3.4 nM at 20 °C. Argadin arrested the moult of cockroach larvae upon injection into the ventral abdominal part.

Key words chitinase inhibitor; cyclic peptide; *Clonostachys*; argadin; microbial metabolite

Chitin occurs in fungi, some algae and many invertebrates including insects, but is not found in vertebrates. Thus chitin synthesis and degradation processes might be expected to be specific targets for fungicides and insecticides.[1,2] Though chitin synthesis inhibitors, such as fungicidal polyoxins and insecticidal benzoylphenylureas, are commercially available, chitin degradation inhibitors have not yet been used. Chitinase (EC 3.2.1.14) is a chitin degradation enzyme and hydrolyzes chitin into oligomer of *N*-acetylglucosamine. Only few inhibitors have been reported, *e.g.* allosamidin,[3] styloguanidine[4] and cyclo(L-arginyl-D-prolyl).[5] An inhibitor of chitinase would be expected to inhibit apolysis during the insect moulting and prevent maturation to the adult reproductive stage.

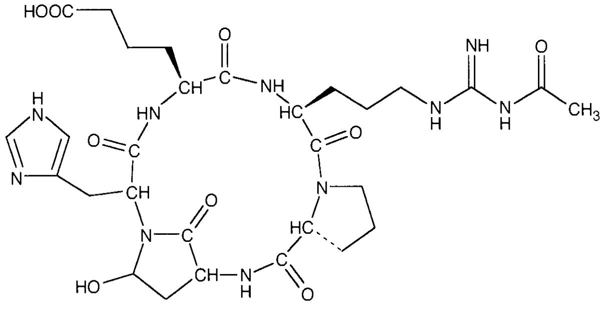
In the course of screening for new insecticides of microbial origin, we previously reported a new cyclic pentapeptide chitinase inhibitor, argifin (**2**), which was the first inhibitor produced by fungi and showed inhibition in a submicromolar range.[6—8] Our continuing screening efforts to find chitinase inhibitors were rewarded by finding of a new compound, argadin (**1**, Fig. 1), from the cultured broth of *Clonostachys* sp. FO-7314.

In this paper, we report on the isolation, structure elucidation and biological properties of **1** as well as the taxonomy of the producing organism.

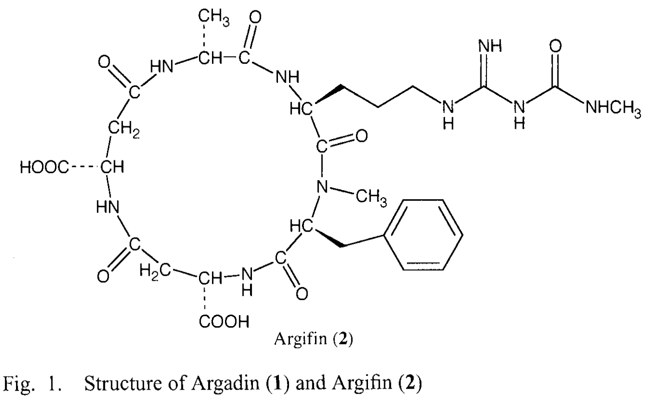
Results and Discussion

Taxonomy of Strain FO-7314 The fungal strain was isolated from a soil sample collected on Oshima Island, Tokyo, Japan. Morphological properties were examined after incubation at 25 °C for 14 d on potato dextrose agar (Difco), cornmeal agar (Difco), Czapek agar (3.0% sucrose, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$ and 2.0% agar, pH 6.0), Miura's medium (0.1% glycerol, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% KCl, 0.2% $NaNO_3$, 0.02% yeast extract and 1.5% agar, pH 6.0), and malt extract agar (2.0% malt extract, 0.1% peptone, 2.0% glucose and 2.0% agar, pH 6.0). The strain grew moderately to form white colonies with diameters of 40—50 mm. Reverse of the colonies was white to pale yellow. The colonies were floccose to powdery on each medium. Microscopic observation showed that conidiophores (45—100×2.5—3.5 μm) were branched two or more times, and their upper portions bore penicillate branches. Phialides that grew on the penicillate branches were enteroblastic, phialidic, obclavate and 10—14×1.5—3.0 μm in size. Phialoconidia were ellipsoidal to cylindrical, 5.5—7.5×1.5—2.0 μm in size, viscous and producing long chains more than 100 μm length in a spicate form (Fig. 2).

From the above characteristics, strain FO-7314 was believed to belong to the genus *Clonostachys* and named



Argadin (**1**)



Argifin (**2**)

Fig. 1. Structure of Argadin (**1**) and Argifin (**2**)

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Dedicated to the memory of Dr. Kyosuke Tsuda.

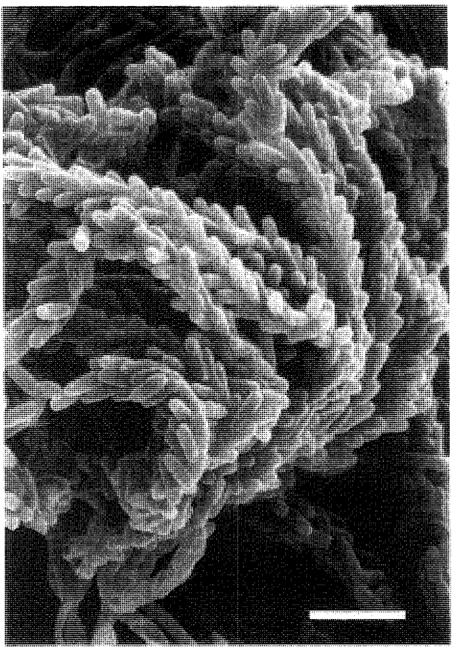

Fig. 2. Electromicrograph of Phialoconidia of Strain FO-7314

Bar represents 10 μm.

Clonostachys sp. FO-7314. The culture was deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology Japan, as FERM P-17200.

Fermentation and Isolation A stock culture of strain FO-7314 grown on potato dextrose agar was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium and incubated on a rotary shaker (210 rpm) at 27 °C for 4 d. One milliliter of the seed culture was transferred into each of eighty 500-ml Erlenmeyer flasks containing 100 ml of a production medium. The fermentation was carried out on a rotary shaker at 27 °C for 6 d. Compound **1** was produced mainly in mycelia.

Compound **1** was extracted with methanol from the mycelia from the cultured broth of FO-7314 (8 l). It was purified successively by a cation exchange column Diaion SK1B (NH_4^+ form, Mitsubishi Chemical Corp.), an adsorption column Diaion HP20 (Mitsubishi Chemical Corp.), a cation exchange column SP-Sephadex C-25 (NH_4^+ form, Amersham Pharmacia Biotech), an octadecyl silica (ODS) column (Pegasil ODS-7515-12A, Senshu Scientific Co.), a Sephadex LH-20 column (Amersham Pharmacia Biotech), and a Bio-Gel P-2 Gel column (fine, Bio-Rad). Twenty-five milligrams of a white powder of **1** was ultimately obtained. It showed a single spot on a silica gel TLC (Merck 1.05715, developed with 80% *n*-propanol) detected by ninhydrin reagent.

Structure Elucidation The molecular formula of **1** was established as $C_{29}H_{42}N_{10}O_9$ based on high resolution (HR)-FAB-MS. Compound **1** was soluble in acidic water and acidic dimethyl sulfoxide (DMSO), slightly soluble in water and DMSO, and insoluble in methanol, acetone and chloroform. Compound **1** gave positive reactions by both Rydon–Smith and ninhydrin reagents. A strong band at 1650 cm^{-1} in the IR spectrum indicated the presence of amide linkages.

Table 1. The ^{1}H-, ^{13}C- and ^{15}N-NMR Data of **1**[a]

Position	^{13}C[b]	^{1}H[b,c]	^{15}N[d]
Acetyl			
C=O	172.7 s		
Me	24.1 q	2.10 s	
Arginine			
NH		8.58 d (6.8)	125.0
C=O	170.1 s		
α	51.6 d	4.10 m	
β	27.4 t	1.66 m, 1.73 m	
γ	24.3 t	1.50 m	
δ	40.7 t	3.25 m	
ε		9.16 t (4.8)	97.4
ζ	153.1 s		
η_1		11.78 s	133.3
η_2		8.68 br s	84.7
Proline			
N			137.4
C=O	171.1 s		
α	59.8 d	4.59 m	
β	27.9 t	1.75 m, 2.11 m	
γ	23.6 t	1.75 m, 3.28 m	
δ	46.0 t	3.31 m, 3.41 ddd (11.4, 11.4, 11.4)	
Homoserine			
NH		7.16 d (9.1)	123.1
C=O	172.9 s		
α	49.0 d	4.33 ddd (4.3, 9.1, 11.6)	
β	35.9 t	1.66 m, 2.63 ddd (6.2, 11.6, 19.4)	
γ	78.7 d	5.31 d (6.2)	
Histidine			
N			150.2
C=O	169.9 s		
α	57.2 d	4.58 m	
β	22.5 t	3.26 dd (9.6, 19.9), 3.36 dd (12.6, 19.9)	
γ	129.4 s		
π			179.1
δ	117.0 d	7.43 s	
ε	134.5 d	8.97 s	
τ		14.19 br s	172.1
2-Aminoadipic acid			
NH		8.27 d (7.9)	122.1
C=O	171.4 s		
α	53.2 d	4.08 m	
β	31.8 t	1.57 m, 1.66 m	
γ	20.7 t	1.43 m	
δ	33.1 t	2.17 ddd (9.1, 16.9, 21.3), 2.21 ddd (9.1, 16.9, 21.3)	
ε	174.3 s		

a) The spectra were obtained with Varian Inova 600 and Unity 400 spectrometers. *b)* Solvent: DMSO-d_6 with 1.7% TFA. *c)* The coupling constants (Hz) are in parentheses. *d)* Solvent: H_2O–D_2O (4 : 1) with 2% TFA.

Chemical shifts in the ^{1}H-, ^{13}C- and ^{15}N-NMR of **1** are shown in Table 1. The HMQC (heteronuclear multiple quantum coherence) experiment revealed the connectivity of each proton and carbon. In the DEPT (distortionless enhancement by polarization transfer) spectra, **1** showed 1 methyl, 11 methylene, 8 methine and 9 quaternary carbon signals. The ^{1}H-NMR spectrum showed 40 proton signals, whereas the remaining 2 hydrogens were not observed in either spectrum. The ^{15}N signals obtained by f_1 projection of the ^{1}H–^{15}N HMBC (heteronuclear multiple bond coherence) indicated 10 nitrogens.

Presence of partial structures **a**—**f** (Fig. 3) were elucidated by ^{1}H–^{1}H COSY (correlation spectroscopoy) and ^{1}H–^{13}C HMBC experiments in DMSO-d_6 containing 1.7% trifluoracetic acid (TFA). The partial structures **a**—**f** were deduced to be arginine (**a**), proline (**b**), homoserine (Hse, **c**), histidine

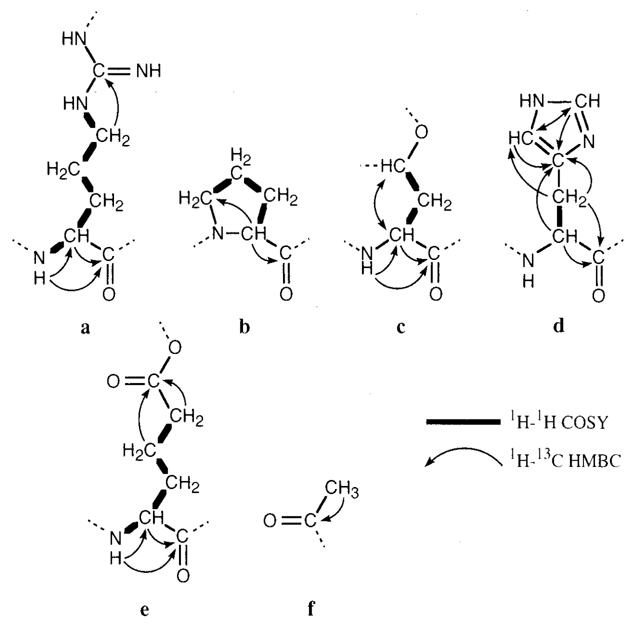

Fig. 3. Partial Structures of **1**

Table 2. Comparison of the ^{13}C Chemical Shifts of **1** Measured in D_2O with 2% TFA and H_2O–D_2O (4 : 1) with 2% TFA

Position	D_2O+TFA	H_2O–D_2O+TFA	Δ (ppm)
Aad ε	177.82	177.96	0.14
Ac C=O	174.64	174.82	0.18
Hse C=O	174.46	174.48	0.02
Pro C=O	173.29	173.36	0.07
Aad C=O	172.83	172.94	0.11
Arg C=O	172.48	172.51	0.03
His C=O	172.20	172.28	0.08
Arg ζ	152.86	153.12	0.26
His ε	133.96	134.19	0.23
His γ	127.82	127.93	0.11
His δ	116.98	117.11	0.13
Hse γ	79.88	79.99	0.11
Pro α	60.83	60.87	0.04
His α	57.68	57.75	0.07
Aad α	53.18	53.28	0.10
Arg α	52.57	52.69	0.11
Hse α	50.27	50.40	0.14

The spectra were obtained with a Varian Unity 400 spectrometer.

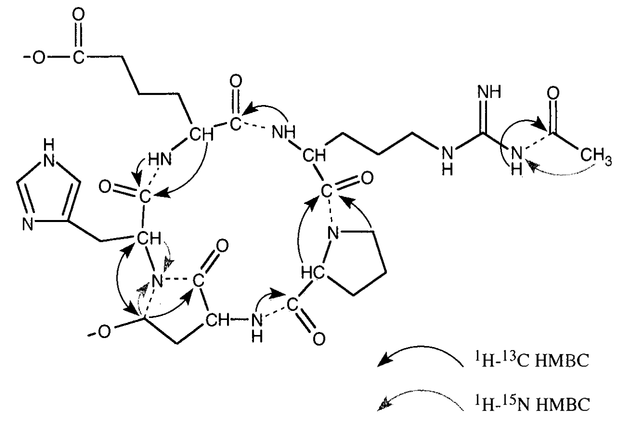

Fig. 4. Structure of **1** Elucidated by ^1H–^{13}C HMBC and ^1H–^{15}N HMBC

(**d**), 2-aminoadipic (Aad, **e**) and acetyl (**f**) residues.

The connectivity of the partial structures **a**—**f** was determined from long-range couplings of ^1H–^{13}C HMBC and ^1H–^{15}N HMBC (Fig. 4). A long-range ^1H–^{13}C coupling was observed from Pro α-CH (δ_H 4.59) to Arg carbonyl (δ_C 170.1), which suggested the linkage of Arg (**a**)–Pro (**b**). A long-range coupling between Hse NH (δ_H 7.16) and Pro carbonyl (δ_C 171.1) suggested the linkage of Pro (**b**)–Hse (**c**). Long-range couplings were also observed from Aad NH (δ_H 8.27) and Aad α-CH (δ_H 4.08) to His carbonyl (δ_C 169.9), which suggested the linkage of His (**d**)–Aad (**e**), and a long-range coupling between Arg α-NH (δ_H 8.58) and Aad carbonyl (δ_C 171.4) suggested the linkage of Aad (**e**)–Arg (**a**).

The homoseryl γ-carbon was shown to be a methine. The amide linkage between Hse (**c**)–His (**d**) and a bond between Hse γ-CH and His α-N were suggested by long-range couplings from Hse γ-CH (δ_H 5.31) to Hse carbonyl (δ_C 172.9) and His α-CH (δ_C 57.2) and from His α-CH (δ_H 4.58) to Hse γ-CH (δ_C 78.7), forming a γ-lactam. This partial structure was supported by long-range ^1H–^{15}N couplings from Hse γ-CH and His α-CH protons to His α-N (δ_N 150.2). The γ-lactam structure was confirmed by the comparison of ^{13}C chemical shifts measured in D_2O with 2% TFA and H_2O–D_2O (4 : 1) with 2% TFA (Table 2). Among seven carbonyl carbons, Hse carbonyl (δ_C 174.46 in D_2O–TFA) and Arg carbonyl (δ_C 172.48 in D_2O–TFA) signals showed small shifts (Δ 0.02—0.03 ppm), and the other carbonyls showed large shifts ($\geq \Delta$ 0.07 ppm). These deuterium isotope effects suggested that the former carbonyl carbons bonded tertiary nitrogens. Hse γ-CH (δ_C 79.88 in D_2O–TFA) also showed a large shift (Δ 0.11 ppm), which suggested that a hydroxyl bonded the methine.

Attachment of acetyl residue (**f**) to ω-terminal of Arg (**a**) was suggested from the ^1H–^{13}C and ^1H–^{15}N long-range couplings between Arg η_1-NH (δ_H 11.78) and acetyl carbonyl (δ_C 172.7) and between acetyl CH_3 (δ_H 2.10) and Arg η_1-NH (δ_N 133.3), respectively. The skeletal structure of **1** was thus elucidated. The two hydrogens missing in the ^1H-NMR were deduced to be hydrogens of Hse γ-OH and Aad ε-COOH from the above result.

The optical activity of each amino acid was elucidated by chiral HPLC of acid hydrolysate of **1**, which revealed the presence of 1 mol of L-arginine, D-proline and L-2-aminoadipic acid. Histidine and homoserine were not detected since they formed a γ-lactam complex that was not hydrolyzed to respective amino acid residues.

Combining the above results, the structure of **1** was elucidated as cyclo(N^{ω}-acetyl-L-arginyl-D-prolyl-homoseryl-histidyl-L-2-aminoadipyl) in which homoseryl γ-methylene bonded to histidyl α-amino residue.

Chitinase Inhibitory Activity Compound **1** inhibited chitinase from *Lucilia cuprina* (blowfly) dose dependently (Fig. 5). Inhibitory activity of **1** against *L. cuprina* chitinase was studied compared with that of argifin (**2**) and allosamidin[3)] (Table 3). Compound **1** inhibited the chitinase with IC_{50} values of 150 nM at 37 °C and 3.4 nM at 20 °C, and the inhibitory activities were about thirty times more potent than those of **2**. Allosamidin showed inhibition with IC_{50} values of 2.3 nM at 37 °C and 0.40 nM at 20 °C. Therefore, **1** was only nine times weaker than allosamidin at 20 °C.

Cockroach Larvae Mortality by Injection Assay Compound **1** (20 or 2 μg/1 μl of 0.1 N AcOH) was injected into twenty american cockroach (*Periplaneta americana*) larvae

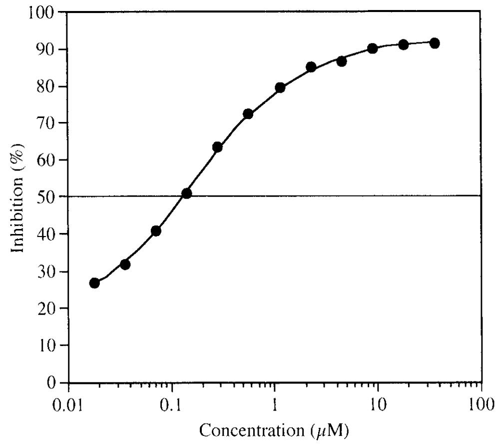

Fig. 5. Inhibition of *Lucilia cuprina* Chitinase by **1**

The enzyme assay was carried out at 37 °C.

Table 3. *Lucilia cuprina* Chitinase Inhibition by Argadin, Argifin and Allosamidin

Compound	IC_{50} (nM)	
	37 °C	20 °C
Argadin (**1**)	150	3.4
Argifin (**2**)	3700	100
Allosamidin	2.3	0.40

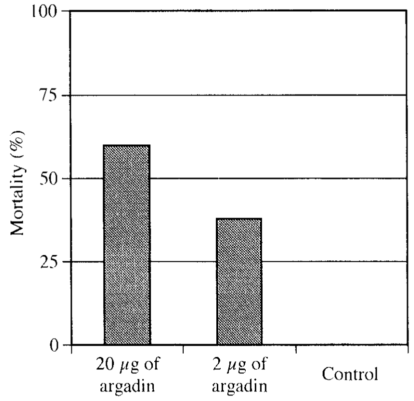

Fig. 6. Efficacy of **1** against *Periplaneta americana* Cockroach Larvae

and compared with mock injected controls (1 μl of 0.1 N AcOH). Mortality was assessed at 1, 5 and 23 d after injection. There was unexpectedly high control mortality (35%) at day 1. Therefore, the numbers of dead larvae at day 5 and day 23 were summed and are shown in Fig. 6. The efficacy of **1** was shown against the larval stage of cockroaches dose dependently. No mortality was observed for control cases after day 1, while 60 and 38% mortality was observed for cockroaches injected with 20 μg and 2 μg of **1**, respectively.

Mortality at day 1 may be due to the use of an acidic solvent to dissolve **1** as well as to mechanical disruption of the cockroaches by the injection. We have reported a cockroach assay of argifin (**2**) using water as a solvent[7]; at that time, the control mortality at day 1 was only 12%. Therefore, the acidic solvent may have enhanced the control death.

Other Biological Activities Argadin did not inhibit the growth of the following microorganisms at 10 μg/disc: *Bacillus subtilis* PCI 219, *Micrococcus luteus* PCI 1001, *Mycobacterium smegmatis* ATCC 607, *Staphylococcus aureus* FDA 209P, *Acholeplasma laidlawii* PG 8, *Bacteroides fragilis* ATCC 23745, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* P 3, *Xanthomonas campestris* pv. *oryzae* KB 88, *Candida albicans* KF 1, *Saccharomyces cerevisiae* KF 26, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581 or *Pyricularia oryzae* KF 180. Argadin also did not inhibit the growth of P388, KB or HL-60 cells at 25 μg/ml.

We isolated a new chitinase inhibitor, argadin (**1**), from the cultured broth of *Clonostachys* sp. FO-7314, after the isolation of **2** from the broth of *Gliocladium* sp. FTD-0668. Both **1** and **2** are cyclic pentapeptides containing N^{ω}-substituted-L-arginine. The guadinino group may be important for chitinase inhibition because known inhibitors, styloguanidines,[4] cyclo(L-arginyl-D-prolyl)[5] and compound A,[9] also have one or more guadinino groups. The producing strain of **2** was *Gliocladium* sp. which is close to the genus *Clonostachys*. Though there have so far been only two examples, the production of cyclic peptide chitinase inhibitors may be a genus specific event.

Chitinase is widely distributed in the group of bacteria, fungi, plants and arthropods, but it is not essential for mammals. Chitinase inhibitors would be expected to interfere with the life cycle of the former group, but not that of the latter. Therefore, cyclic pentapeptide containing N^{ω}-substituted-L-arginine could be an interesting lead for the development of novel insecticides.

Experimental

General NMR spectra were obtained with Varian Inova 600 and Unity 400 spectrometers at room temperature using DMSO-d_6 with 1.7% TFA, H_2O–D_2O (4 : 1) with 2% TFA and D_2O with 2% TFA as solvents.

^{1}H–^{15}N HMBC was recorded on a Varian Unity 400 spectrometer equipped with pulse field gradients and an inverse detection 5 mm probe. Other conditions were as follows: sample 8.0 mg, solvent 0.6 ml of H_2O–D_2O (4 : 1) with 2% TFA, $f_1 \times f_2 = 13037 \times 3507$ Hz, $t_1 \times t_2 = 64 \times 1024$ points, transients=2032, repetition time=1.5 s, relaxation delay optimized for $J = 6$ Hz.

Mass spectrometry was conducted on a JEOL JMS-AX505 HA spectrometer. UV and IR spectra were measured with a Shimadzu UV-240 spectrophotometer and a Horiba FT-210 Fourier transform IR spectrometer, respectively. Optical rotation was recorded on a JASCO model DIP-181 polarimeter. Melting point was measured with a Yanaco micro melting point apparatus MP-S3.

4-Methylumbelliferone and 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotriose were obtained from Sigma Chemical Co.

Fermentation and Isolation The seed medium was composed of 2.0% glucose, 0.5% Polypepton (Nihon Pharmaceutical Co.), 0.1% agar, 0.2% yeast extract (Oriental Yeast Co.), 0.05% $MgSO_4 \cdot 7H_2O$ and 0.1% KH_2PO_4 (pH 5.7 prior to sterilization). The production medium was composed of 5.0% maltose, 3.0% dry yeast (Gist-brocades), 1.0% KBr, 0.5% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 6.0 prior to sterilization).

The cultured broth of FO-7314 (8 l) was filtered, and the mycelia were extracted with methanol (8 l) and concentrated *in vacuo*. The extract was diluted to 8 l with water, neutralized, and charged on a Diaion SK1B cation exchange column (NH_4^+ form, 800 ml). After washing with water, the active substance was eluted with 0.5—2.0 N NH_4OH. The eluate was neutralized and applied on a Diaion HP20 column (800 ml). The column was washed with water and eluted with 10—80% methanol. The eluate was concentrated *in vacuo* and dissolved in a small amount of 15 mM ammonium acetate. The solution was charged on an SP-Sephadex C-25 cation exchange column (NH_4^+ form, 800 ml). After washing with 50 mM ammonium acetate, the active substance was eluted with 100 mM ammonium acetate. The active fractions were collected and applied on an ODS column (Pegasil ODS-7515-12A, 200 ml). After washing with water, the active substance was eluted with 100 ml of 40% methanol. The eluate was concentrated *in vacuo* and dissolved in a small amount of 65% methanol. The solution was charged on a Sephadex LH-20 column (800 ml) and eluted with 65 % methanol. The active fractions were collected and concentrated *in vacuo*, then dissolved in a small amount of 0.05% acetic acid, charged on a Bio-Gel P-2 Gel column (fine, 800 ml), and eluted with 0.05% acetic acid. The active fractions were collected and concentrated *in vacuo* to yield a white powder of pure **1** (25 mg).

Argadin (**1**): White powder, $[\alpha]_D^{25}$ +52.1° (c=0.1, H_2O). $C_{29}H_{43}N_{10}O_9$ (m/z 675.3214 $(M+H)^+$; Calcd for $C_{29}H_{43}N_{10}O_9$, 675.3214) by HR-FAB-MS. mp 270 °C (dec.). UV λ_{max} (H_2O) nm (ε): 200 (31400). IR ν_{max} (KBr) cm^{-1}: 3310, 3220, 1710, 1650, 1575, 1530, 1435, 1390, 1245.

Acid Hydrolysis of 1 Compound **1** (100 μg) was hydrolyzed with 6 N HCl–1% phenol vapor at 120 °C for 11 h. The reaction mixture was concentrated to dryness, dissolved in a small amount of water, and charged on a chiral HPLC: column, Sumichiral OA-5000 (Sumika Chemical Analysis Service, i.d. 4.6×150 mm); mobile phase, 1 mM $CuSO_4$; flow rate 1.0 ml/min; detection, UV 254 nm.

The amino acid peaks were identified by co-injection of each authentic optically pure sample. The molar ratios of amino acids were calculated from the peak area.

Assay Methods for Biological Activities Chitinase inhibitory activity was measured as described previously.[7] In brief, 20 μl of 100 mM sodium phosphate buffer, pH 7.0, 30 μl of crude chitinase solution (prepared from late pupae of the blowfly, *Lucilia cuprina*), and 50 μl of 80 μM 4-methylumbelliferyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose in the buffer were placed in each well of a microplate, and incubated with or without the sample at 37 °C for 10 min or 20 °C for 15 min. Fluorescence (excitation at 355 nm, emission at 460 nm) was measured by a fluorometer (Fluoroscan II, Labsystems), and the rate of 4-methylumbelliferone production was corrected by calibrating the quenching ratio of each sample using the mixture of the sample and 4-methylumbelliferone.

Assay methods for antimicrobial activity and cockroach larvae injection were also described previously.[7]

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Colopsinols D and E, New Polyhydroxyl Linear Carbon Chain Compounds from Marine Dinoflagellate *Amphidinium* sp.

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Colopsinols D (1) and E (2), two new polyhydroxyl linear carbon chain compounds, have been isolated from the cultured marine dinoflagellate *Amphidinium* sp. The structures of 1 and 2 were elucidated on the basis of two-dimensional NMR and FAB-MS/MS data.

Key words dinoflagellate; *Amphidinium* sp.; polyhydroxyl compound; FAB-MS/MS

Marine dinoflagellates are an important source of bioactive natural products. During our search for bioactive metabolites from marine dinoflagellates,[1—3] we found three new polyhydroxyl linear carbon chain compounds with a glucoside moiety, colopsinols A, B, and C, from laboratory cultured marine dinoflagellate *Amphidinium* sp.[4,5] Further investigation of the extract resulted in the isolation of two minor polyhydroxyl linear carbon chain compounds, colopsinols D (**1**) and E (**2**). This paper describes the isolation and structure elucidation of **1** and **2**.

The harvested cells of the dinoflagellate were extracted with MeOH/toluene (3 : 1). The toluene-soluble materials of the extract were subjected to a silica gel column ($CHCl_3$/MeOH, 95 : 5→MeOH). The fraction eluted with MeOH was separated by gel filtration on Sephadex LH-20 ($CHCl_3$/MeOH, 1 : 1) and centrifugal partition chromatography (CPC, ascended mode, $CHCl_3/MeOH/H_2O$, 5 : 6 : 4) followed by C_{18} column chromatography (iso-PrOH/H_2O, 45 : 55) to yield colopsinols D (**1**, 0.0001%, wet weight) and E (**2**, 0.0003%) together with colopsinols A[4] (**3**), B, and C.[5]

The physico-chemical data of colopsinol D (**1**) were similar to those of colopsinol A[4] (**3**). The IR spectrum was indicative of the presence of hydroxyl group(s) (ν_{max} 3430 cm^{-1}), ketone carbonyl(s) (ν_{max} 1710 cm^{-1}), and sulfate ester (ν_{max} 1250 cm^{-1}).[6] The presence of sulfate ester was also suggested by characteristic fragment ions observed in the negative ion fast atom bombardment (FAB) MS [*m/z* 80 (SO_3^-), 97 (HSO_4^-)]. Positive ion high resolution electron spray ionization (HR-ESI) MS data [*m/z* 1431.7490, $(M+Na)^+$, Δ+3.9 mmu] of colopsinol D (**1**) indicated the molecular formula, $C_{71}H_{117}NaO_{24}S$, corresponding to that of a dehydrated form of **3**. The ^{13}C-NMR data including distortionless enhancement by polarization transfer (DEPT) experiments revealed that **1** contained two ketones, one sp^2 quaternary carbon, seven sp^2 methines, two sp^2 methylenes, twenty-three oxymethines, two oxymethylenes, two sp^3 methines, twenty-nine sp^3 methylenes, and three methyl groups, thus accounting for total 71 carbons. The number of hydroxyl groups was deduced from the following ^{13}C-NMR deuterium-induced shift experiment using CD_3OH and CD_3OD as solvents. Of 25 signals observed for oxygenated carbons (δ_C 59—106), 15 oxygenated carbons (14 oxymethines and one oxymethylene) did not show deuterium-induced upfield shifts, while colopsinol A (**3**) included 13 unchanged oxygenated carbons in the ^{13}C-NMR deuterium-induced shift experiment.[4] Of the unchanged oxygenated carbons for **1**, two low-field methine carbons at δ_C 104.86 (C-1′) and 105.81 (C-1″) in CD_3OH were attributed to anomeric carbons of sugar units, while four high-field ones at δ_C 61.14 (C-45), 60.58 (C-46), 59.84 (C-51), and 60.26 (C-52) were involved in two epoxide rings. The remaining unchanged oxygenated carbons were assigned as those bearing ether-oxygen (C-4, δ_C 73.85; C-8, δ_C 74.64, C-9, δ_C 82.86, C-12, δ_C 82.07), glycoside linkages (C-18, δ_C 78.19; C-6′; δ_C 71.08), and a sulfate ester[7] (C-5, δ_C 80.10) from the NMR and MS data described below. Thus, colopsinol D (**1**) was indicated to have an ether ring more than colopsinol A (**3**).

Interpretation of ^1H–^1H correlation spectoscopy (COSY), total correlation spectoscopy (TOCSY), and ^{1}H-detected heteronuclear single quantam coherence (HSQC) spectra led to the following six partial structures: **a**) from C-1 to C-13, **b**) from C-15 to C-21, **c**) from C-28 to C-48, **d**) from C-50 to C-56, **e**) from C-1′ to C-6′, and **f**) from C-1″ to C-6″ (Fig. 1). ^{1}H-Detected heteronuclear multiplebond correlation (HMBC) cross-peaks for H_2-13/C-14 (δ_C 212.20), H_2-15/C-14, H_2-21/C-22 (δ_C 215.37), and H_2-23/C-22 suggested that the partial structures **a**, **b**, and **c** were connected through two ketone carbonyls (C-14, C-22). The presence of an *exo*-methylene group at C-49 was deduced from HMBC correlations for H_2-59/C-48 (δ_C 34.57), H_2-59/C-49 (δ_C 147.37), and H2-59/C-50 (δ_C 40.42). The relative stereochemistry of the two epoxide rings at C-45—C-46 and C-51—C-52 in the partial structure **c** was assigned as both *trans* by ^1H-^1H coupling constants ($J_{45/46}$=2.5 Hz, $J_{51/52}$=2.0 Hz).[8] The *E*-geometries of three disubstituted double bonds at C-31—C-32, C-35—C-36, and C-39—C-40 in the partial structure **c** were deduced from the carbon chemical shifts of allylic carbons (C-30, δ_C 41.97[9]; C-33, δ_C 38.41[10]; C-34, δ_C 42.26[9]; C-37, δ_C 34.65; C-38, δ_C 34.65; C-41, δ_C 34.26). The linear aliphatic chain at C-23—C-28 was revealed by the substantial product ion peaks at *m/z* 931, 917, 903, 889, 875, and 861 observed in the FAB-MS/MS spectrum of **1** [precursor ion *m/z* 1385 $(M-Na)^-$] (Fig. 2). Two sugar units (**e**, **f**) were both assigned as β-glucose on the basis of the ^1H-^1H coupling constants ($J_{1'/2'}$=7.5 Hz, $J_{1''/2''}$=8.1 Hz). Both sugars were elucidated to be D-form by chiral HPLC analyses of 1-*O*-methyl-2,3,4,6-tetra-*O*-benzoyl derivatives of the methanolysis products of **1**.[11] Nuclear Overhauser effects

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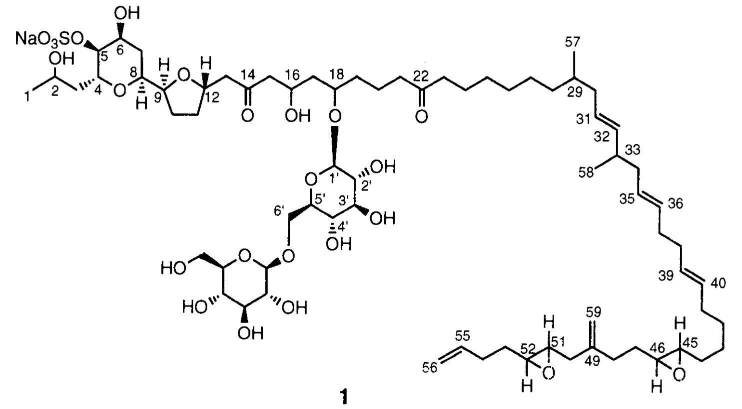
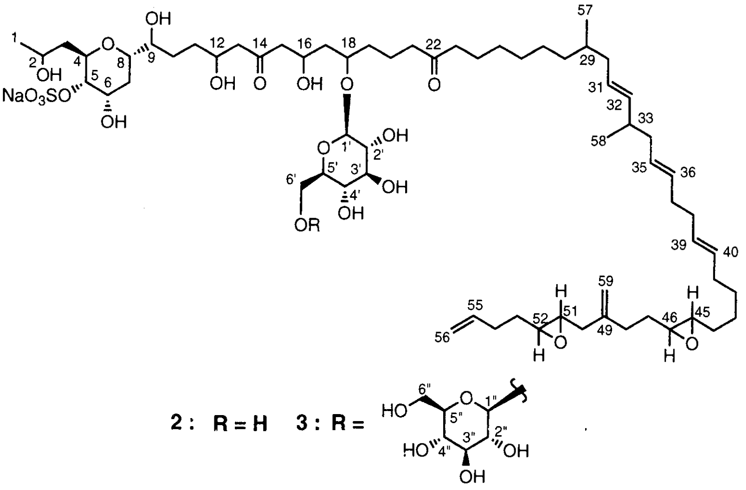
Dedicated to the memory of Dr. Kyosuke Tsuda.



1

Stereochemistry of the tetrahydropyran and tetrahydrofuran rings are relative, and two epoxide rings are *trans*.



2 : R = H **3** : R =

Stereochemistry of the tetrahydropyran ring is relative, and two epoxide rings are *trans*.

Chart 1. Structures of **1**—**3**

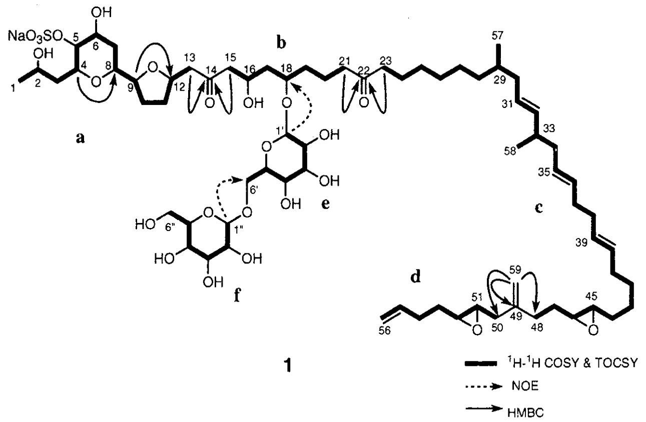


Fig. 1. 2D-NMR Correlations of Colopsinol D (**1**)

(NOE) were observed from H-1′ to H-18 and from H-1″ to H-6′, indicating that a β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside was connected to C-18. The presence of a tetrahydropyran ring in the partial structure **a** was implied by HMBC cross-peaks from H-4 to C-8 (δ_C 74.64). The relative stereochemistry of the tetrahydropyran ring at C-4—C-8 was deduced as a chair form from ^{1}H–^{1}H coupling constants ($J_{4/5}$=3.5 Hz; $J_{5/6}$=3.0 Hz; $J_{6/7\alpha}$=10.0 Hz; $J_{6/7\beta}$=4.3 Hz; $J_{7\alpha/8}$=9.0 Hz; $J_{7\beta/8}$=3.3 Hz). The carbon signal (δ_C 80.10) of C-5 in **1** was located at a lower-field than those (*ca.* δ_C 70) of the axially-oriented hydroxy-bearing carbons on two tetrahydropyran rings of luteophanol A,[12] suggesting that the sulfate ester was attached to C-5. The existence of a tetrahydrofuran ring at C-9—C-12 was deduced from HMBC correlation from H-9 to C-12 (δ_C 82.07). The oxymethine carbon signals at C-9 (δ_C 82.86) and C-12 (δ_C 82.07) of **1** were located at a relatively lower-field than those of **3** (C-9, δ_C 74.75; C-12, δ_C 69.54), the chemical shift differences of geminal methylene protons of C-10 (δ_H 2.05, 1.73) and C-11 (δ_H 2.19, 1.60) were relatively large,[13] and the FAB-MS/MS spectrum (Fig. 2) of **1** showed product ion peaks at *m/z* 283 and 281, supporting the presence of a tetrahydrofuran ring at C-9—C-12. The relative stereochemistry of the tetrahydrofuran ring was elucidated to be *trans* by NOE to H$_2$-13 obtained from the irradiation of H-9, while the relative stereochemistry between C-8 and C-9 was not defined. Thus, the structure of colopsinol D was elucidated to be **1**.

Colopsinol E (**2**) had the molecular formula of $C_{65}H_{109}NaO_{20}S$ as revealed by the HR-ESI-MS [*m/z* 1287.7058, $(M+Na)^+$, Δ+3.0 mmu]. The ^{1}H- and ^{13}C-NMR data (Table 1) of **2** were similar to those of colopsinol A (**3**), except for lack of resonances for one glucose in **2**. Two-dimensional (2D)-NMR data including the ^{1}H–^{1}H COSY, TOCSY, and HSQC spectra as well as negative ion FAB-

Table 1. ^{1}H- and ^{13}C-NMR Data of Colopsinols D (**1**) and E (**2**)

Position	**1**				**2**			
	δ_{H}[a]	(m, Hz)	δ_{C}[b]	m	δ_{H}[a]	(m, Hz)	δ_{C}[b]	m
1	1.25[c]	(d, 6.2)	24.72	q	1.25[c]	(d, 6.3)	24.86	q
2	3.97	(m)	65.81	d	3.97	(m)	65.83	d
3	1.86	(m)	39.98	t	1.89	(m)	39.98	t
	1.61	(m)			1.59	(m)		
4	4.50	(dt, 10.4, 3.5)	73.85	d	4.52	(dt, 10.6, 3.5)	73.76	d
5	4.36	(br t, 3.1)	80.10	d	4.36	(br t, 3.1)	80.33	d
6	4.12	(ddd, 3.0, 4.3, 10.0)	67.34	d	4.10	(ddd, 3.0, 4.4, 10.0)	67.47	d
7	1.86	(m)	33.23	t	1.88	(m)	39.98	t
	1.77	(m)			1.78	(m)		
8	3.60	(ddd, 9.0, 6.0, 3.3)	74.64	d	3.55	(ddd, 9.3, 5.8, 3.5)	74.94	d
9	4.23	(br dt, 1.3, 6.0)	82.86	d	3.68	(m)	74.75	d
10	2.05	(m)	41.97	t	1.66	(m)	35.29	t
	1.73	(m)			1.60	(m)		
11	2.19	(m)	40.28	t	1.65	(m)	30.57	t
	1.60	(m)			1.60	(m)		
12	4.40	(m)	82.07	d	4.12	(m)	69.54	d
13	2.81[d]	(m)	53.17[a]	t	2.67[d]	(m)	53.34[a]	t
14			212.20	s			212.18	s
15	2.71[d]	(m)	52.56[a]	t	2.66[d]	(m)	52.69[a]	t
16	4.44	(m)	66.11	d	4.44	(m)	66.17	d
17	1.71	(m)	44.51	t	1.71	(m)	44.37	t
	1.61	(m)			1.58	(m)		
18	3.99	(m)	78.19	d	3.98	(m)	78.45	d
19	1.86[d]	(m)	31.78	t	1.85[d]	(m)	31.79	t
20	1.86[d]	(m)	31.78	t	1.87[d]	(m)	31.49	t
21	2.69[d]	(m)	40.08	t	2.68[d]	(m)	40.12	t
22			215.37	s			215.47	s
23	2.53[d]	(m)	44.51	t	2.53[d]	(t, 7.4)	44.42	t
24	1.59[d]	(m)	25.82	t	1.58[d]	(m)	25.73	t
25	1.33[d]	(m)	28.98	t	1.33[d]	(m)	28.97	t
26	1.35[d]	(m)	31.23	t	1.35[d]	(m)	31.21	t
27	1.33[d]	(m)	31.51	t	1.33[d]	(m)	31.49	t
28	1.37	(m)	35.21	t	1.37	(m)	38.39	t
	1.13	(m)			1.13	(m)		
29	1.48	(m)	39.13	d	1.48	(m)	35.23	d
30	2.02	(m)	41.97	t	2.00	(m)	42.01	t
	1.87	(m)			1.88	(m)		
31	5.37	(m)	128.94	d	5.36	(m)	128.98	d
32	5.35	(m)	139.21	d	5.34	(m)	139.25	d
33	2.16	(m)	38.41	d	2.16	(m)	39.20	d
34	2.01[d]	(m)	42.26	t	2.00[d]	(m)	42.30	t
35	5.42	(m)	132.92	d	5.42	(m)	132.96	d
36	5.42	(m)	130.91	d	5.42	(m)	130.96	d
37	2.08[d]	(br s)	34.65	t	2.07[d]	(br s)	34.69	t
38	2.08[d]	(br s)	34.65	t	2.07[d]	(br s)	34.67	t
39	5.45	(m)	132.22	d	5.45	(m)	132.26	d
40	5.45	(m)	132.05	d	5.45	(m)	132.08	d
41	2.04[d]	(m)	34.26	t	2.04[d]	(m)	34.31	t
42	1.46[d]	(m)	27.25	t	1.46[d]	(m)	27.30	t
43	1.46[d]	(m)	31.23	t	1.45[d]	(m)	31.27	t
44	1.55[d]	(m)	33.73	t	1.54[d]	(m)	33.78	t
45	2.77	(ddd, 9.3, 5.0, 2.5)	61.14	d	2.77	(ddd, 9.3, 5.0, 2.5)	61.18	d
46	2.77	(ddd, 9.3, 5.0, 2.5)	60.58	d	2.77	(ddd, 9.3, 5.0, 2.5)	60.62	d
47	1.74	(m)	32.06	t	1.74	(m)	32.10	t
	1.69	(m)			1.69	(m)		
48	2.28[d]	(m)	34.57	t	2.27[d]	(m)	34.61	t
49			147.37	s			147.41	s
50	2.28[d]	(m)	40.42	t	2.29[d]	(m)	40.45	t
51	2.88	(ddd, 6.2, 5.0, 2.0)	59.84	d	2.88	(ddd, 6.2, 5.0, 2.0)	59.87	d
52	2.81	(ddd, 6.2, 5.0, 2.0)	60.26	d	2.80	(ddd, 6.2, 5.0, 2.0)	60.29	d
53	1.65[d]	(m)	33.23	t	1.68[d]	(m)	33.27	t
54	2.25[d]	(m)	32.06	t	2.24[d]	(m)	32.07	t
55	5.90	(ddt, 17.1, 10.3, 6.9)	139.73	d	5.90	(ddt, 17.0, 10.3, 6.7)	139.77	d
56	5.10	(m)	116.35	t	5.10	(m)	116.40	t
	5.02	(m)			5.03	(m)		
57	0.90[c]	(d, 6.9)	20.77	q	0.90[c]	(d, 6.7)	20.81	q
58	1.00[c]	(d, 6.2)	21.56	q	0.99[c]	(d, 6.7)	21.62	q

Table 1. (Continued)

Position	1				2			
	δ_H[a]	(m, Hz)	δ_C[b]	m	δ_H[a]	(m, Hz)	δ_C[b]	m
59	4.95	(br s)	113.02	t	4.95	(br s)	113.06	t
	4.92	(br s)			4.92	(br s)		
1′	4.44	(d, 7.5)	104.86	d	4.43	(d, 7.7)	105.22	d
2′	3.22	(m)	76.37	d	3.21	(dd, 8.8, 7.7)	76.40	d
3′	3.41	(m)	78.99	d	3.40	(m)	79.12	d
4′	3.55	(m)	72.63	d	3.35	(m)	72.65	d
5′	3.50	(m)	77.66	d	3.31	(m)	78.71	d
6′	4.19	(dd, 11.5, 1.9)	71.08	t	3.89	(br d, 11.5)	63.86	t
	3.79	(dd, 11.5, 5.6)			3.71	(dd, 11.8, 5.0)		
1″	4.40	(d, 8.1)	105.81	d				
2″	3.26	(dd, 9.3, 8.1)	76.09	d				
3″	3.40	(m)	78.99	d				
4″	3.32	(m)	72.63	d				
5″	3.32	(m)	78.83	d				
6″	3.91	(br d, 11.8)	63.77	t				
	3.71	(11.8, 5.6)						

a) In CD_3OD. *b*) In CD_3OH. *c*) 3H. *d*) 2H.

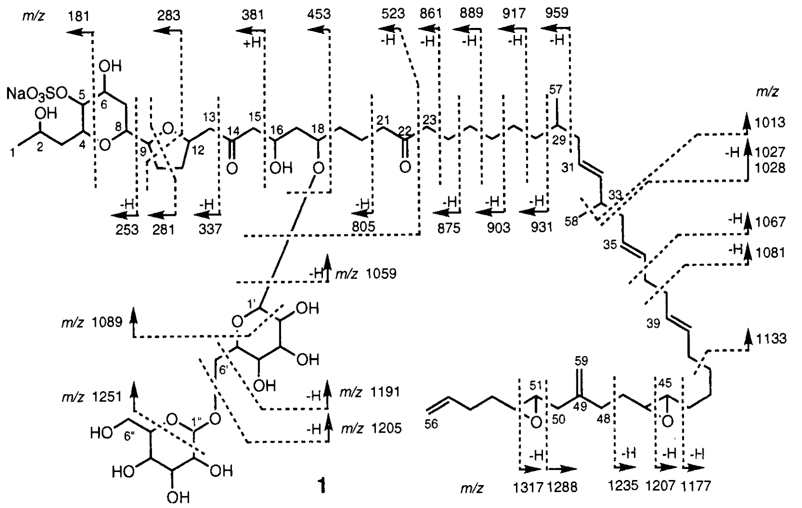


Fig. 2. Fragmentation Patterns Observed in Negative FAB-MS/MS Spectrum of Colopsinol D (**1**) in MeOH (Precursor Ion, *m*/*z* 1385)

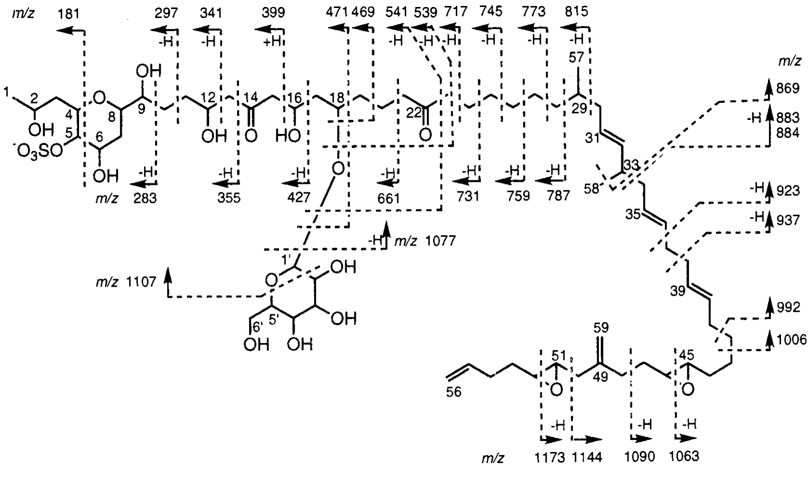


Fig. 3. Fragmentation Patterns Observed in Negative FAB-MS/MS Spectrum of Colopsinol E (**2**) in MeOH (Precursor Ion, *m*/*z* 1241]

MS/MS data (Fig. 3) of **2** [precursor ion, *m*/*z* 1241 $(M-Na)^-$] revealed that the C-1—C-59 part of **2** possessed the same overall structure as that of **3**. The product ion peaks at *m*/*z* 469 and 471 in the FAB-MS/MS spectrum indicated that one glucose was attached to C-18. Thus, colopsinol E (**2**) was elucidated to be the mono-deglucosyl form of colopsinol A (**3**).

Colopsinols D (**1**) and E (**2**) are new congeners of colopsinol A (**3**) possessing a C_{53}-linear aliphatic chain with three C_1 branches, a tetrahydropyran ring, two epoxides, five hydroxyl groups, a glucoside moiety, and a sulfate ester. Colopsinol D (**1**) has a tetrahydrofuran ring at C-9—C-12, while colopsinol E (**2**) is the mono-deglucosyl form of colopsinol A (**3**). Colopsinol E (**2**) exhibited cytotoxicity against L1210 murine leukemia cells (IC$_{50}$ value: 7 μg/ml), while colopsinol D (**1**) did not show such cytotoxicity (IC$_{50}$ $>$20 μg/ml).

Experimental

IR and UV spectra were recorded on JASCO FT/IR-5300 and JASCO Ubest-35 spectrophotometers, respectively. ESI mass spectra were obtained on a JEOL JMS-700TZ, JEOL SX-102A, or Shimadzu LCMS-QP8000 spectrometer using a sample dissolved in MeOH with a flow rate of 200 μl/min. The MS/MS spectra were obtained on a JEOL JMS-700TZ tandem mass spectrometer equipped with a charge coupled device (CCD) array detector. The mass spectrometer was operated at an accelerating voltage of 3 kV and in the negative mode. Argon collision gas was used with the pressure to reduce the selected precursor ion intensity by 30%. A 1 : 1 mixture of glycerol and nitrobenzyl alcohol was used as a matrix of FAB-MS/MS.

Cultivation and Isolation The dinoflagellate *Amphidinium* sp. (strain number Y-5) was unialgally cultured at 25 °C for two weeks in seawater medium enriched with 1% Erd-Schreiber (ES) supplement. The harvested cells of the cultured dinoflagellate (1205 g wet weight, from 4960 l of culture) were extracted with MeOH/toluene (3 : 1, 3 l×3). After addition of 1 M NaCl (1 l), the mixture was extracted with toluene (4 l×3). The toluene soluble fraction was evaporated under reduced pressure to give a residue (44.4 g), which was partially (26.7 g) subjected to a silica gel column eluted with $CHCl_3$/MeOH and then MeOH. Part (2.33 g) of the fraction (3.3 g) eluted with MeOH was purified by gel filtration on Sephadex LH-20 ($CHCl_3$/MeOH, 1 : 1), CPC (ascended mode, $CHCl_3$/MeOH/H_2O, 5 : 6 : 4), and C_{18} column (iso-PrOH/H_2O, 45 : 55) to afford colopsinols D (**1**, 0.8 mg, 0.0001%, wet weight) and E (**2**, 1.9 mg, 0.0003%) together with colopsinols A (**3**, 0.0008%), B (0.001%), and C (0.0002%).

Colopsinol D (**1**): IR (KBr) cm^{-1}: 3430, 1710, 1250. ^{1}H- and ^{13}C-NMR (Table 1); ESI-MS (negative mode) *m/z*: 1385 [M−Na]$^-$. HR-ESI-MS (positive mode) *m/z* (M+Na)$^+$: Calcd for $C_{71}H_{117}O_{24}SNa_2$: 1431.7451. Found 1431.7490.

Colopsinol E (**2**): IR (KBr) cm^{-1}: 3430, 1705, 1250. ^{1}H- and ^{13}C-NMR (Table 1); ESI-MS (negative mode) *m/z*: 1241 [M−Na]$^-$; HR-ESI-MS (positive mode) *m/z* (M+Na)$^+$: Calcd for $C_{65}H_{109}O_{20}SNa_2$ 1287.7028. Found 1287.7058.

Determination of Stereochemistry of the Sugar Units of Colopsinols D (1) and E (2) by Chiral HPLC Colopsinol D (**1**, 0.1 mg) or E (**2**, 0.1 mg) was treated with 3% HCl/MeOH (300 μl) at 110 °C for 1 h. After the solvent was removed by nitrogen stream, to the residue was added $CHCl_3$ (100 μl) and the $CHCl_3$ solution was extracted with H_2O (100 μl×3). The aqueous fraction evaporated *in vacuo* was treated with pyridine (100 μl), triethylamine (15 μl), and benzoyl chloride (15 μl) at room temperature for 21 h. After addition of MeOH (100 μl), the reaction mixture was extracted with hexane (100 μl×3). The hexane-soluble fraction was evaporated *in vacuo* to afford the 1-*O*-methyl-2,3,4,6-tetra-*O*-benzoyl derivative of each sugar unit. Authentic D- and L-glucose were treated with benzoyl chloride as described above to give the 1-*O*-methyl-2,3,4,6-tetra-*O*-benzoyl derivatives of D- and L-glucose, respectively. The 1-*O*-methyl-2,3,4,6-tetra-*O*-benzoyl derivatives were subjected to chiral HPLC analyses using Chiralpak OP(+) (Daicel Chemical Industry, Ltd., 4.6×250 mm; MeOH; flow rate, 0.5 ml/min; UV detection at 254 nm). The retention time of the 1-*O*-methyl-2,3,4,6-tetra-*O*-benzoyl derivative of the methanolysis product of **1** or **2** was found to be both 23.8 min, while the retention times of the 1-*O*-methyl-2,3,4,6-tetra-*O*-benzoyl derivatives of authentic D- and L-glucose were found to be 23.8 and 25.8 min, respectively.

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9) These chemical shifts were closer to that of the methine-bearing allylic methylene carbon (C-24; δ_C 41.9) on the *E*-double bond of (22*E*)-cholesta-5,22-diene-3β,7β,19-triol [Aiello A., Fattorusso E., Mienna M., *J. Nat. Prod.*, 1992, **55**, 321—325 (1992)] than that (C-13; δ_C 32.8) on the Z-double bond of patellazole B (Corley D. G., Moore R. E., Paul V. J., *J. Am. Chem. Soc.*, **110**, 7920—7922 (1988)].

10) The chemical shift corresponded to that of the allylic methine carbon (C-19; δ_C 36.2) on the *E*-double bond of theonezolide A[7] rather than that (C-10; δ_C 48.5) on the *Z*-double bond of patellazole B.[9]

11) Kobayashi J., Doi Y., Ishibashi M., *J. Org. Chem.*, **59**, 255—257 (1994).

12) The axially-oriented hydroxy-bearing positions (C-27; δ_C 69.7, C-40; 69.5) on two tetrahydropyran rings of luteophanol A [Doi Y., Ishibashi M., Nakamichi H., Kosaka T., Ishikawa T., Kobayashi J., *J. Org. Chem.*, **62**, 3820—3823 (1997)].

13) Geminal proton signals of C-21 (δ_H 2.10, 1.50) and C-22 (δ_H 1.95, 1.65) on the tetrahydrofuran ring in amphidinolide C; Kobayashi J., Ishibashi M., Wälchli M. R., Nakamura H., Hirata Y., Sasaki T., Ohizumi Y., *J. Am. Chem. Soc.*, **110**, 490—494 (1988).

Isolation and Characterization of Bioactive Metabolites from Marine-Derived Filamentous Fungi Collected from Tropical and Sub-Tropical Coral Reefs

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Two new compounds, paecilospirone (1) and phomopsidin (2), and seven known compounds, chaetoglobosin A (3), griseofulvin (4), fusarielin A (5), fusapyrone (6), deoxyfusapyrone (7), and verrucarins J (8) and L acetate (9), have been isolated and characterized from marine-derived fungi collected in tropical and sub-tropical coral reef environments. The utility of marine-derived fungi as a source of bioactive secondary metabolites is discussed.

Key words bioactive metabolite; paecilospirone; marine fungi; isolation; phomopsidin; marine natural product

Marine-derived fungi have recently been utilized as a new source of novel bioactive secondary metabolites.[2)] We are currently involved in a research project to search for bioactive compounds from culture broths of marine-derived filamentous fungi.[3—5)] The fungi were isolated from marine organisms and submerged materials collected in tropical and sub-tropical coral reef environments of Okinawa, Yap, Pohnpei, and Palau. Secondary metabolites were isolated by the cytotoxicity assay against human (HL-60) and murine (L1210) leukemia cell lines and by the bioassay method we recently developed to search for antimitotic and antifungal compounds using conidia of *Pyricularia oryzae* P-2b.[3)] Two new compounds, paecilospirone (**1**)[4)] and phomopsidin (**2**),[5)] and seven known compounds, previously reported as terrestrial fungal metabolites, chaetoglobosin A (**3**), griseofulvin (**4**), fusarielin A (**5**), fusapyrone (**6**), deoxyfusapyrone (**7**), and verrucarins J (**8**) and L acetate (**9**), have been isolated and characterized from culture broths of nine marine-derived filamentous fungi. We describe here the detail of isolation and fermentation of the fungi and structures of the secondary metabolites, and discuss the utility of marine-derived fungi as a prolific source of biologically active natural products.

Isolation and Fermentation of Fungi Marine organisms (sponges, tunicates, soft corals, and algae) and submerged materials (leaves, branches, and dead algae) were collected by Scuba diving, and a few small pieces of substrates were homogenized with sterile seawater. The liquid portion was applied on an agar plate, and two to three pieces of substrate, if the structure remained after homogenization, were also applied on an agar plate. Mycelia grown on the plates were individually isolated and inoculated onto a slant.

Fungi were separately cultured on a plastic plate with a half nutrient potato-dextrose medium (1/2 PD), and the broth was added with MeOH and stored in a freezer.

Bioassay 1) Conidia Assay: The broth was evaporated to remove MeOH and adjusted to the original volume with water for bioassay. The conidia assay was performed as reported previously[3)] and as briefly mentioned in the Experimental section. Deformations of mycelia germinated from conidia of *P. oryzae* P-2b were observed.[3)]

2) Cytotoxicity: The broth was evaporated to dryness, and the residue was extracted with EtOH to remove the salt. The EtOH extract was used for bioassay. The human (HL-60) and murine (L1210) leukemia cell lines were each cultured in a 24-well assay plate with samples. The shape of cells was observed after 24, 48, and 72 h, and the inhibitory activity of proliferation was observed after 72 h by comparing the number of cells in sample wells with those in control wells.

Paecilospirone (1)[4)] The culture broth of *Paecilomyces* sp., isolated from the coral reef at Yap, showed a curing effect on mycelia in the conidia assay. Paecilospirone (**1**) was separated from the MeOH extract of mycelia. The molecular formula of **1**, $C_{32}H_{44}O_5$, was deduced from high-resolution (HR) FAB-MS and NMR data. ^{1}H- and ^{13}C-NMR data for **1** (Table 1) showed the presence of two benzene rings, one ketone and a secondary alcohol, and two hydrocarbon chains. The ^{1}H–^{1}H correlated spectroscopy (COSY) spectra of **1** revealed the connection of carbon bonds at 4–5–6, 1′–9–10–11, 15–16, 4′–5′, 9′–10′–11′, and 15′–16′ (Table 1). The connectivity of carbons 1 through 11 and 1′ through 11′ were elucidated by heteronuclear multiple-bond correlation (HMBC) experiments (Table 1).

The lengths of the two hydrocarbon chains were assigned from ^{13}C chemical shifts of C-10 to C-16 and C-10′ to C-16′ and confirmed by electrospray-ionization MS/collisionally induced dissociation (ESI-MS/CID)/mass spectra of **1** (Fig. 1).

The relative stereochemistry of **1** was determined from ^{1}H-NMR and rotating frame nuclear Overhauser and exchange spectroscopy (ROESY) data. The coupling constants between H-1′ and H-9, H-9 and H-10a, and H-9 and H-10b were 4.0, 4.0, and 12.0 Hz, respectively, which showed H-9 and 1′-OH group are pseudaxial and H-1′ is pseudequatorial orientation. The ROESY experiment revealed the nuclear Overhauser effect (NOE) correlations (Fig. 2) and confirmed the relative stereochemistry of **1**.

Paecilospirone (**1**) has a unique skeletal structure, spiro[chroman-2,1′(3′*H*)-isobenzofuran], which has only been reported as chemical reaction products. Two hemiacetal derivatives of spiro[chroman-2,1′(3′*H*)-isobenzofuran]-3,4-

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Dedicated to the memory of Dr. Kyosuke Tsuda.

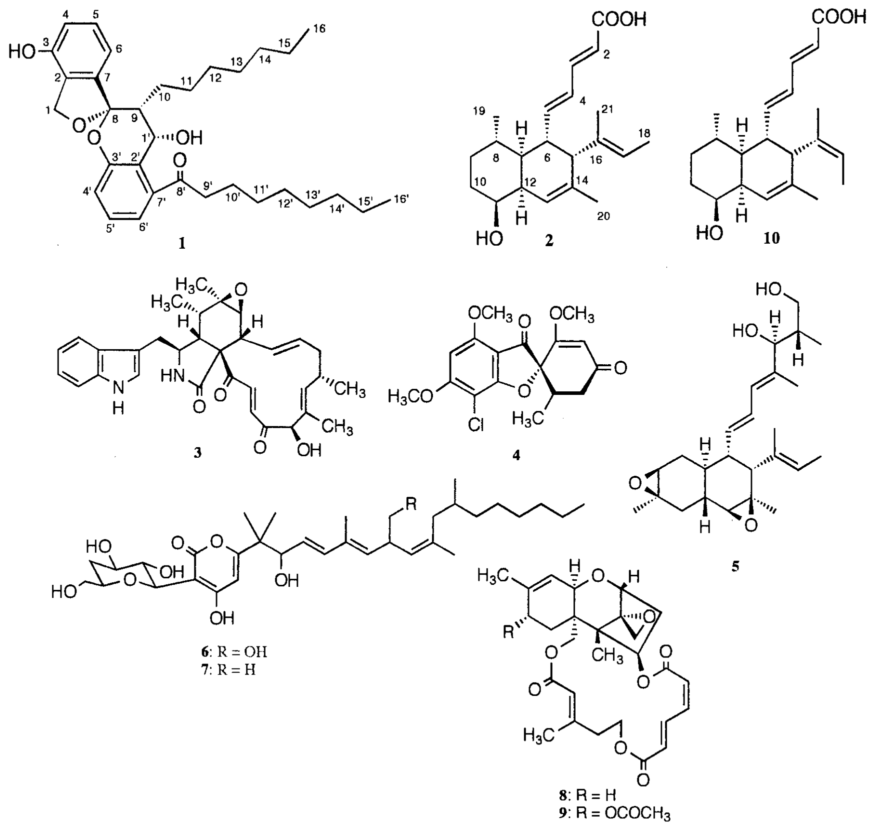

Chart 1

dione were synthesized by oxidation with periodic acid from 2′-hydroxymethylflavonol.[6] Two diastereomers of 4-hydroxy derivatives were prepared from phthalide with the mixture of metallic samarium and bromine (4 : 3).[7] Paecilospirone (**1**) is, therefore, the first example of this unique skeletal structure obtained as a biosynthetic product.

Paecilospirone (**1**) may be biosynthesized from two units of an octaketide, 2-hydroxy-6-(1-oxononyl)benzaldehyde *via* aldol condensation (Fig. 3). This would be a new route to dimeric derivatives biosynthesized from two polyketide units.[8]

The inhibitory activity of **1** to purified porcine brain microtubule proteins[9] was weak (20% inhibition at 50 μM), and accordingly, **1** was not antifungal at 25 μg/disc (disc diffusion assay) nor was it cytotoxic at 20 μg/ml to several tumor cell lines. The biosynthesis and biological activity of **1** are interesting subjects for future studies.

Phomopsidin (2)[5] *Phomopsis* sp. was isolated from a fallen mangrove branch on the bottom (−3 m) of a coral reef in Pohnpei. Phomopsidin (**2**), isolated from the broth filtrate, did not give an $(M+H)^+$ ion but showed the $(M+Na)^+$ ion at *m*/*z* 353 in the FAB and ESI mass spectra. The $(M-H)^-$ ion was detected at *m*/*z* 329 in the negative FAB-MS.

The molecular formula, $C_{21}H_{30}O_3$, was assigned from the negative HR-FAB-MS and NMR data. The ^{1}H–^{1}H COSY spectra of **2** revealed the connectivity of carbons 2 to 6, 19–8–7–12–11–10, and 17–18. ^{1}H couplings between H-12 and 13, and H-6 and 15 were very small (J=*ca.* 0 Hz). The analysis of HMBC (Fig. 4) and nuclear Overhauser and exchange spectroscopy (NOESY) (Fig. 5) data finally elucidated the skeletal structure of **2**.

The relative stereochemistry of **2** was assigned from ^{1}H-NMR and NOESY data (Fig. 5). Two double bonds at the 2 and 4 positions were determined to have *E*-orientations from the ^{1}H coupling constants, 15.2 and 15.0 Hz, respectively, and *E*-orientation at Δ^{16} was assigned from the NOE observed between H-17 and 15. The signal at δ 3.57 (H-11) showed coupling constants of 11.2 Hz with H-10*a*, 4.6 Hz with H-10*e*, and 4.6 Hz with H-12, which revealed that the configurations of H-11 and 10*a* are axial and that H-10*e* and 12 are equatorial. Signals due to H-10*e* (δ 1.63) and H-12 (δ 2.65) showed a W-shaped long-range coupling. NOEs were observed between H-11/7, H-11/9*a*, H-7/9*a*, and H-7/H$_3$-19. These data showed that H-7, 9*a*, and 11 are axial, and the configuration of C-19 is equatorial, that is, H-8 is axial. The dihedral angle of H-12 and 13 was assumed to be *ca.* 90° ($J_{12,13}$=*ca.* 0 Hz), and H-13 showed an NOE with H-10*a* (δ 1.38) and a long-range coupling with H-15 (δ 2.76). An NOE was observed between H-15 and 8 (δ 1.42). The configuration of H-15 was, therefore, deduced to be pseudaxial. The dihedral angle of H-15 and H-6 was shown to be *ca.* 90°, since the coupling constant was small ($J_{6,15}$=*ca.* 0 Hz). NOEs observed between H-6/H-4 and H-6/H$_3$-19 revealed the stereochemistry at C-6. Consequently, the relative stereochemistry of phomopsidin (**2**) was assigned as shown.

The structure of **2** resembled that of MK8383 (**10**) isolated from terrestrial *Phoma* sp. as an antifungal component to several phytopathogens.[10] Although **10** could not be separated from **2** chromatographically, ^{1}H-NMR spectra of **2** and **12** measured by the same instrument (500 MHz) clearly distinguished the two compounds.[11] Reported ^{13}C-NMR data for **10**[10,11] were very similar to those of **2**, except for C-15 and 21, which are consistent with the different stereochemistry at the C-18 methyl group.

Table 1. ^1H- and ^{13}C-NMR Data for Paecilospirone (**1**)

C#	^{13}C	^1H mult. (J in Hz)	COSY (H#)	HMBC (C#)[a]	ROESY (H#)
1a	71.3	5.11 d (13.0)		2, 3, 4, 5, 6, 7, 9	1b
1b		5.21 d (13.0)		2, 3, 4, 5, 6, 7, 9	1a
2	126.4				
3	140.8				
4	115.9	6.80 d (8.0)	5	1, 2, 3, 6, 7	5
5	129.8	7.24 dd (7.5, 8.0)	4, 6	2, 3, 4, 6, 7, 8	4, 6
6	113.9	6.87 d (7.5)	5	2, 3, 4, 5, 7, 8	5, 9, 10a, 11a
7	150.5				
8	112.2				
9	42.4	2.49 ddd (12.0, 4.0, 4.0)	10b, 10a, 1′	8, 10, 11	6, 10a, 11a, 1′
10a	25.2	1.07 m	9, 10b, 11a, 11b	8, 9, 11, (12), 1′	6, 9, 10b, 11b
10b		1.80 m	9, 10a, 11a, 11b	8, 9, 11, (12), 1′	10a, 11a, 11b, 1′, 1′-OH
11a	26.7	1.20 m	10a, 10b, 11b	9	6, 9, 10b, 11b, 1′
11b		1.57 m	10a, 10b, 11a	9, (12), (13)	10a, 10b, 11a, 1′
12	29.3[b]	1.25—1.35 m			
13	29.2[b]	1.25—1.35 m			
14	31.8	1.2—1.3 m			
15	22.6	1.2—1.3 m	16		
16	14.1	0.84 t (7.0)[c]	15	14, 15	
1′	60.7	5.11 dd (7.0, 4.0)	9, 1′-OH	8, 9, 2′, 3′, 7′	9, 10b, 11a, 11b, 1′-OH
2′	123.5				
3′	139.7				
4′	121.4	6.97 dd (7.0, 2.0)	5′	2′, 3′, 6′, 7′	5′
5′	128.9	7.28 m	4′	2′, 3′, 4′, 7′	4′
6′	121.5	7.27 m		2′, 3′, 4′, 7′, 8′	9′
7′	152.2				
8′	206.8				
9′	42.3	2.97 m	10′	8′, 10′, (11′)	6′, 10′
10′	24.4	1.74 m	9′, 11′	8′, 9′, (11′), (12′)	9′
11′	29.6[b]	1.25—1.35 m	10′		
12′	29.4[b]	1.25—1.35 m			
13′	29.2[b]	1.25—1.35 m			
14′	31.8	1.2—1.3 m			
15′	22.6	1.2—1.3 m	16′		
16′	14.1	0.88 t (7.0)[c]	15′	14′, 15′	
1′-OH		4.36 d (7.0)	1′	9, 10, 1′	10b, 1′

C#: carbon number. H#: proton number. *a*) Carbon number in the parenthesis for HMBC data indicates the most probable signal giving the correlation among the five overlapped signals at δ_C 29.2—29.6. *b*, *c*) These signals may be exchanged within the same mark.

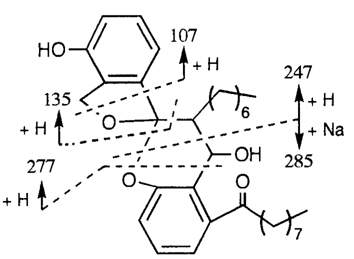

Fig. 1. Fragment Ions Detected in the ESI-MS/CID/Mass Spectra of Paecilospirone (**1**)

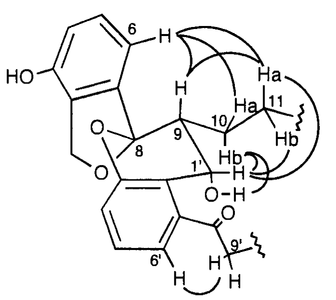

Fig. 2. NOE Correlations Assigned from the ROESY Spectrum of Paecilospirone (**1**)

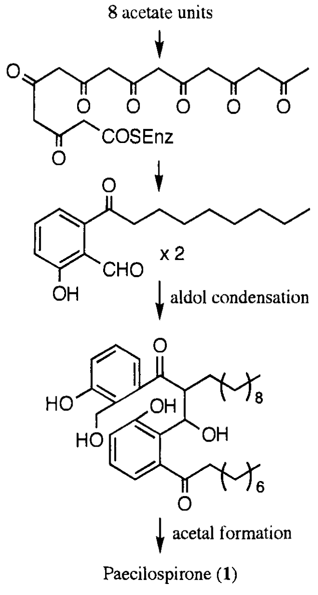

Fig. 3. A Probable Biosynthetic Pathway to Paecilospirone (**1**)

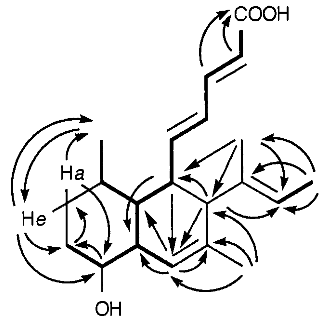

Fig. 4. COSY (—) and Selected HMBC Date for Phomopsidin (**2**)

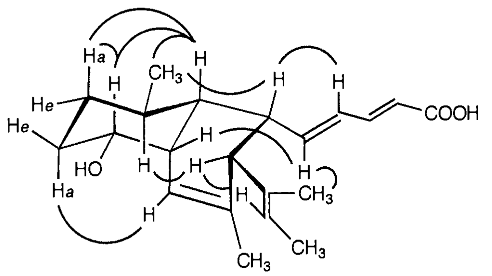

Fig. 5. NOE Correlations Assigned from the NOESY Spectrum of Phomopsidin (**2**)

The microtubule assembly assay using purified porcine brain microtubule proteins was performed as reported previously,[9] and **2** showed strong inhibitory activity (IC_{50}, 5.7 μM). Colchicine, rhizoxin, and compound **10** showed IC_{50} of 10, 4, and 8 μM, respectively, in the same experiment. Structurally similar compounds, fusarielins, possessing a *trans*-decaline moiety, did not show any inhibitory activity.[9] The *cis*-decaline structure may, therefore, be important to the activity.

Phomopsidin (**2**) would biogenetically be synthesized *via* a biological Diels–Alder reaction similar to the biosynthesis of solanapyrones and other decaline derivatives.[12]

Characterization of Known Compounds The culture broth of *Chaetomium* sp., isolated from a green alga at Yap, showed bead shape deformation on mycelia of *P. oryzae* in the conidia assay.[3] Bioassay guided separation of the broth filtrate as mentioned in the Experimental section gave chaetoglobosin A (**3**).[13] The structure of **3** was elucidated based on the spectral data (^{1}H- and ^{13}C-NMR, 2-dimensional (2D) NMR, mass, IR, and UV spectra) and confirmed by direct comparison with the authentic sample.

The characteristic curling effect on mycelia of *P. oryzae* in the conidia assay was observed by broths of *Penicillium* sp. and *Fusarium* sp., both isolated at Pohnpei. TLC analysis of the EtOAc extracts from the broths of *Penicillium* sp. and *Fusarium* sp. suggested the presence of griseofulvin (**4**)[14] and fusarielin A (**5**),[9] respectively. The compounds were separated by TLC and compared directly with the authentic samples.

Acremonium sp. from Yap and *Colletotrichum* sp. from Pohnpei showed a strange deformation (lumpy mycelia) in the conidia assay. The MeOH extract of mycelia of *Acremonium* sp. gave fusapyrone (**6**)[15,16] and a very small amount of deoxyfusapyrone (**7**).[16] The mycelia extract of *Colletotrichum* sp. afforded deoxyfusapyrone (**7**) as the only bioactive component. The structures of **6** and **7** were assigned by mass, ^{1}H- and ^{13}C-NMR, and 2D NMR spectra.

The EtOAc extract of whole broth of an unidentified strain (97F95), isolated from a sponge body collected at Aka Island, Okinawa, showed strong cytotoxicity to HL-60 and L1210. Verrucarin J (**8**)[17,18] was obtained as the only cytotoxic component of this strain. The structures of these compounds were elucidated based on ^{1}H-, ^{13}C-NMR, and 2D NMR spectral data. The fungus is not identified, since no conidium has been generated on several agar plates. The IC_{50} values of **8** to HL-60 and L1210 were both 2.5 ng/ml.

An unidentified strain (95F137), isolated from a sponge body at Pohnpei, showed cytotoxicity to HL-60. The bioactive compound was isolated by similar separation procedures as above and the structure characterized by ^{1}H-, ^{13}C-NMR, and 2D NMR spectral data as verrucarin L acetate (**9**).[19] The IC_{50} value of **9** to HL-60 was 1.0 ng/ml.

Discussion

Fungi living in marine environments are expected to have different biosynthetic pathways than their terrestrial counterparts. The differences are thought to result from the adaptation to the higher pressure and especially to the presence of salt. Therefore, the marine-derived fungi will be important sources of unique components. Although most of the secondary metabolites obtained from marine-derived fungi are parallel, if not identical, to those from terrestrial fungi, a few marine-derived fungi produced compounds having unique carbon skeletons.[2] Paecilospirone (**1**) is a remarkable example of a novel structure isolated for the first time from a natural source and also of a new biosynthetic pathway. Dimerization of two octaketide units, probably *via* aldol condensation (Fig. 3), has not been observed in terrestrial fungi. Another example of marine-derived fungus produced a chlorinated polyketide derivative which required seawater for the biosynthesis, while other metabolites were produced in freshwater culture medium by the same fungus.[20] We observed a similar phenomenon of salt being required in the culture of two fungi for the production of secondary metabolites (unpublished data).

Marine-derived fungi also show different ratios of biosynthetic products as observed in the present study. *Colletotrichum* sp. and unidentified strains 97F95 and 95F137 produced, respectively, deoxyfusapyrone (**7**), verrucarin J (**8**), and verrucarin L acetate (**9**) as the main components, while these compounds were isolated as minor products from terrestrial fungi *Fusarium semitectum*,[16] *Myrothecium* spp.,[18] and *Myrithecium verrucaria*,[18] respectively.

This study also showed that the same compounds were isolated from different genera of marine-derived fungi to those of terrestrial fungi. The marine-derived *Acremonium* sp. and *Colletotrichum* sp. produced fusapyrone (**6**) and deoxyfusapyrone (**7**), but these compounds were first isolated from the terrestrial *Fusarium* spp.[15,16]

The results obtained in this study suggest that marine-derived fungi are useful not only for a source of novel bioactive substances but also for new producers of known compounds which were isolated from terrestrial fungi as minor components with insufficient amounts

Experimental

NMR spectra were measured either on a JEOL JNM A-500 NMR spectrometer or a Varian UNITY INOVA-500 spectrometer. Mass spectra were obtained by either a JEOL HX-110 mass spectrometer (FAB mode) or a

Finnigan TSQ 700 triple quadrupole mass spectrometer (ESI mode). UV and IR spectra were recorded on a Shimadzu UV-300 and on a JASCO A-102, respectively.

Isolation of Fungi Marine organisms (sponges, tunicates, soft corals, and algae) and submerged material (leaves, branches, and dead algae) were collected by Scuba diving in coral reefs at Yap (1994), Pohnpei (1995), Palau (1995, 1998), and Aka Island, Okinawa (1996—1998). The substrates were sealed in sterile plastic bags in the water and stored in a cooler box with coolant. Treatment of the substrates was done within 3 h in a laboratory of the research vessel Sohgen-maru (Yap, Pohnpei, Palau 1995), the training vessel Umitaka-maru (Palau 1998), or in the Akajima Marine Science Laboratory (Aka Island). Three to five small pieces of each substrate were placed in a sterile mortar with 1—2 ml of sterile seawater and homogenized with a pestle. One- to two-hundred microliters of the liquid portion was placed on an agar plate (0.02% yeast extract, 0.1% soluble starch, 2% agar, and 200 ppm chloramphenicol in 90% natural seawater). The substrate remaining in the mortar was pressed by the pestle to remove liquid and further dried with sterile paper towels, and two to three pieces were applied on an agar plate. The plates were placed in the research room (25—26 °C) of the ship and then incubated at 20 °C in incubators after returning to the university.

The mycelia grown on an agar plate or on a substrate were inoculated on a slant in a culture tube. The cultures are maintained in the Tokyo University of Fisheries as TUF strain numbers designated in the individual description.

Fermentation of Fungi Each fungus was cultured on a plastic plate for screening bioassays with 15 ml of 1/2 PD (hot water (500 ml) extract of potato (100 g), 10 g dextrose, 500 ml natural seawater) for three weeks at 20 °C. MeOH (8 ml) was added and the broth was stored in a freezer (−30 °C).

This sample was filtered, and 1.0 ml of filtrate was evaporated to about half the volume, and then the volume adjusted to 1.0 ml with water. This solution was used for the conidia assay.

For cytotoxicity assays, 1.0 ml of the above filtrate was evaporated to dryness, dissolved in EtOH, and filtered. The EtOH extract was evaporated and used for bioassays.

Bioassay 1) Conidia Assay: The assay was performed as described previously.[3] Briefly, a suspension (50 μl) of conidia of *P. oryzae* P-2b in sterile water containing 0.2% yeast extract was placed in each well of a 96-well assay plate, and the sample solution (50 μl) was added to the first well. The suspension was mixed and 50 μl moved to the second well. The procedure was repeated to the last well of the column. The assay plates were incubated for 16 h at 27 °C, and the shape of mycelia germinated from the conidia was observed and compared with controls (negative: water, positive: rhizoxin) under an inverted microscope.

2) Cytotoxicity: The human (HL-60) and murine (L1210) leukemia cell lines were incubated in Roswell Park Memorial Institute 1640 and minimal essential medium (modified Eagle's medium), respectively, using 24-well assay plates. Ten microliters of the EtOH solution of each sample in the required concentration was placed in a well and the solvent evaporated. The suspension (1 ml, 1×10^4 cells/ml) of L1210 or HL-60 was added to each well and incubated at 37 °C for 72 h in a CO_2 incubator (5% CO_2). The shape of cells was observed after 24, 48, and 72 h under an inverted microscope. The number of vital cells after 72 h was compared between sample and control wells using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disufophenyl)-2*H*-tetrazolium, monosodium salt][21] (Cell Counting Kit-8®).

Paecilospirone (1) *Paecilomyces* sp. 94F79 was cultured in ten 500-ml flasks (each 150 ml, 1/2 PD, 50% natural seawater) for three weeks at 20 °C and filtered. The MeOH extract (1.5 g) of mycelia was subjected to solid phase extraction with octadecylsilanated silica gel (ODS), and the bioactive fraction (100% MeOH eluate) was separated by silica gel column chromatography (benzene, benzene–acetone=11 : 1 and 4 : 1, and then MeOH). The fraction eluted with benzene–acetone (11 : 1) was purified with a silica gel column (benzene : acetone=98 : 2) to give 8.5 mg of **1**, $[\alpha]_D^{25}$ +202.5° (c=0.37, MeOH); HR-FAB-MS: $[M+Na]^+$ m/z 531.3087, Calcd for $C_{32}H_{44}O_5Na$, 531.3086; UV λ_{max} nm (MeOH): 206, 218, 251, 278, 299; IR (KBr): 3340, 2930, 2860, 1666, 1607, 1582, 1452, 1096, 1060, 1000, 840 cm^{-1}. ^{1}H- and ^{13}C-NMR data are listed in Table 1.

Phomopsidin (2) *Phomopsis* sp. 95F47 was cultured in ten 500-ml flasks (each 100 ml of 1/2 PD, 50% natural seawater) for three weeks at 20 °C. Acetone was added to the cultured broth and filtered. The filtrate was extracted with benzene, and the extract was separated by a silica gel column (benzene, benzene : acetone=2 : 1, and then MeOH). The bioactive fraction (benzene–acetone eluate) was subjected to silica gel column chromatography (benzene : acetone=4 : 1) followed by HPLC (ODS, $CH_3CN : H_2O$= 1 : 1) to afford 12.0 mg of **2**, $[\alpha]_D^{27}$ +31° (c=0.1, MeOH); HR-FAB-MS [$(M-H)^-$, m/z 329.2121, Calcd for $C_{21}H_{29}O_3$, 329.2125]; UV (MeOH) λ_{max} nm (ε): 260 (23000); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.77 (d, 15.2 Hz, H-2), 7.18 (dd, 15.2, 10.2 Hz, H-3), 6.15 (dd, 15.0, 10.2 Hz, H-4), 6.26 (dd, 15.0, 9.0 Hz, H-5), 2.78 (m, H-6), 1.25 (m, H-7), 1.42 (m, H-8), 1.00 (m, H-9*a*), 1.65 (m, H-9*e*), 1.38 (m, H-10*a*), 1.63 (m, H-10*e*), 3.57 (m, 11.2, 4.6, 4.6 Hz, H-11), 2.65 (m, H-12), 5.73 (s, $J_{12,13}$=*ca.* 0 Hz, H-13), 2.76 (s, $J_{6,15}$=*ca.* 0 Hz, H-15), 5.22 (q, 6.5 Hz, H-17), 1.55 (dd, 6.5, 0.8 Hz, H-18), 0.94 (d, 6.5 Hz, H-19), 1.60 (s, H-20), 1.48 (s, H-21); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 170.9 (C-1), 120.6 (C-2), 146.9 (C-3), 129.0 (C-4), 148.8 (C-5), 45.3 (C-6), 49.3 (C-7), 29.9 (C-8), 34.0 (C-9), 31.1 (C-10), 73.3 (C-11), 39.1 (C-12), 124.3 (C-13), 136.1 (C-14), 51.3 (C-15), 136.4 (C-16), 123.5 (C-17), 13.5 (C-18), 19.4 (C-19), 22.3 (C-20), 16.8 (C-21).

Chaetoglobosin A (3) *Chaetomium* sp. 94F49 was cultured in 10 plates of 1/2 PD (50% seawater, each 25 ml) at 20 °C for three weeks and filtered. The filtrate was extracted with HP-20 (MeOH eluate) and then with the mixture of water, acetone, and benzene. The organic layer was concentrated, and the residue was chromatographed on silica gel with benzene, benzene–acetone and then methanol to afford 7 mg of **3**, UV λ_{max} nm (MeOH): 222; IR (KBr): 3490, 3440, 2990, 1695, 1618, 1460, 1300, 1160, 980, 910 cm^{-1}. The structure was confirmed by direct comparison with the authentic sample using HPLC (ODS, $CH_3CN : H_2O$=55 : 45).

Griseofulvin (4) *Penicillium* sp. 95F629 was cultured in three plates (each 15 ml of 1/2 PD). The broth was added with acetone and extracted with EtOAc. The EtOAc extract was separated by TLC (benzene : acetone=3 : 2) to give **4**, which was compared with the authentic sample of griseofulvin by TLC (*Rf*=0.71).

Fusarielin A (5) The broth of *Fusarium* sp. 95F858 cultured in plates (1/2 PD) was filtered, and mycelia were extracted with MeOH. The solid phase extraction of mycelia extract by ODS (80% MeOH eluate) and of broth filtrate by HP-20 (100% MeOH eluate) showed bioactivity. These fractions gave similar spots on TLC (benzene : acetone=3 : 2), and the bioactive component (**5**) was separated by TLC. TLC analysis (*Rf*=0.65, red spot by sulfuric acid followed by heating) of **5** with the authentic sample revealed that **5** was identical to fusarielin A.

Fusapyrone (6) and Deoxyfusapyrone (**7**) *Acremonium* sp. 94F74 was cultured in ten plates (1/2 PD, each 25 ml). The broth was filtered, and the mycelia were extracted with MeOH, then subjected to solid phase extraction with ODS. The 85% MeOH eluate was separated by HPLC (ODS, 80% MeOH) to give **6** and **7**. Fusapyrone (**6**), HR-FAB-MS: $[M+H]^+$ m/z=607.3827, Calcd for $C_{34}H_{55}O_9$, 607.3846; IR (KBr): 3420, 2940, 1651, 1502, 1451, 970 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.88 (H-35), 0.90 (H-29), 1.17 (H-30), 1.24 (H-31), 1.24 (10H, H-24, 25, 26, 27, 28), 1.61 (H-23), 1.66 (H-34), 1.84 (H-32), 1.89 (H-10), 1.96, 2.06 (H-22), 3.44 (H-33a), 3.5 (3H, H-12, 33b), 3.6 (2H, H-11, 19), 3.7 (H-9), 4.21 (H-8), 4.49 (H-14), 4.67 (H-7), 5.09 (H-20), 5.39 (H-18), 5.69 (H-15), 6.00 (H-5), 6.37 (H-16); ^{13}C-NMR (125 MHz, $CD_3OD : C_6D_6$=2 : 1): δ 169.4 (C-2), 97.7 (C-3), 180.2 (C-4), 106.8 (C-5), 169.2 (C-6), 76.3 (C-7), 73.2 (C-8), 74.3 (C-9), 35.7 (C-10), 77.4 (C-11), 65.0 (C-12), 44.8 (C-13), 77.9 (C-14), 126.5 (C-15), 138.4 (C-16), 134.2 (C-17), 134.6 (C-18), 41.8 (C-19), 126.7 (C-20), 136.7 (C-21), 41.0 (C-22), 32.0 (C-23), 38.0 (C-24), 28.2 (C-25), 30.5 (C-26), 32.8 (C-27), 23.5 (C-28), 14.5 (C-29), 22.6 (C-30), 20.8 (C-31), 13.4 (C-32), 66.7 (C-33), 24.0 (C-34), 20.1 (C-35). Deoxyfusapyrone (**7**), HR-FAB-MS: $[M+H]^+$ m/z=591.3884, Calcd for $C_{34}H_{55}O_8$, 591.3897.

Colletotrichum sp. 95F7 was cultured in 1/2 PD as above. The MeOH extract of mycelia showed a similar deformation on mycelia of *P. oryzae* in the conidia assay to that of the extract from *Acremonium* sp. The bioactive component was separated by ODS (85% MeOH eluate) followed by TLC ($CH_2Cl_2 : MeOH : H_2O$=80 : 19 : 1) and identified as deoxyfusapyrone (**7**) by TLC and HPLC (ODS, 80% MeOH) with compounds **6** and **7** obtained from *Acremonium* sp.

Verrucarin J (8) An unidentified strain 97F95, isolated at Aka Island, was cultured in 1/2 PD (each 150 ml, six 500-ml flasks), and the broth was added with acetone (450 ml) and extracted with EtOAc. The extract was separated by a silica gel column (benzene–EtOAc, benzene–acetone, and then MeOH) followed by HPLC (ODS, 70% MeOH) to afford **8** (0.4 mg), UV λ_{max} nm (MeOH): 263; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.88 (d, 5.0, H-2), 2.16 (m, H-3a), 2.54 (m, H-3b), 6.00 (m, H-4), 1.84 (m, H-7a), 1.98 (m, H-7b), 2.00 (H-8), 5.28 (br d, 5.0 Hz, H-10), 3.68 (d, 5.5 Hz, H-11), 2.83 (d, 4.0 Hz, H-13a), 3.12 (d, 4.0 Hz, H-13b) 0.83 (s, H-14), 3.98 (d, 12.5 Hz, H-15a), 4.42 (d, 12.5 Hz, H-15b), 1.72 (s, H-16), 5.85 (s, H-2′), 2.53 (H-4′), 4.15 (m, H-5′a), 4.44 (m, H-5′b), 6.02 (d, 15.5 Hz, H-2″), 8.09 (dd, 15.5, 11.2 Hz, H-3″), 6.62 (dd, 11.2, 11.0 Hz, H-4″), 6.10 (d, 11.0 Hz, H-5″); ^{13}C-NMR (125 Hz, $CDCl_3$): δ 79.0 (C-2), 35.1 (C-3), 75.3 (C-4), 48.8 (C-5), 43.0 (C-6), 20.8 (C-7), 27.7 (C-8), 140.4 (C-9), 118.6 (C-10), 67.3 (C-11), 65.5 (C-12), 48.1 (C-13), 7.00 (C-14), 63.3 (C-15), 23.3 (C-16), 166.1 (C-1′), 118.1 (C-2′), 156.6 (C-3′), 40.2 (C-4′), 60.4 (C-5′), 17.2 (C-6′), 165.5 (C-1″), 127.4 (C-2″), 139.1 (C-3″), 139.5 (C-4″), 125.5 (C-5″), 165.8 (C-6″).

Verrucarin L Acetate (9) An unidentified strain 95F137, isolated at Palau, was cultured in 1/2 PD (six 500-ml flasks), and the broth was added with acetone (450 ml) and extracted with EtOAc. The EtOAc extract was separated by a silica gel column (benzene, benzene–acetone, and then MeOH) followed by TLC (benzene : acetone=4 : 1) to afford **9**, UV λ_{max} nm (MeOH): 262; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.84 (d, 5.0 Hz, H-2), 2.21 (m, H-3a), 2.49 (m, H-3b), 5.93 (dd, 8.0, 4.0 Hz, H-4), 2.19 (m, H-7), 5.19 (m, H-8), 5.70 (br d, 5.5 Hz, H-10), 3.80 (d, 5.5 Hz, H-11), 2.84 (d, 4.0 Hz, H-13a), 3.11 (d, 4.0 Hz, H-13b), 0.81 (s, H-14), 4.21 (d, 12.5 Hz, H-15a), 4.54 (d, 12.5 Hz, H-15b), 1.76 (s, H-16), 1.95 (s, H-18), 5.77 (br s, H-2′), 2.54 (m, H-4′), 4.13 (ddd, 11.0, 11.0, 3.5 Hz, H-5′a), 4.49 (ddd, 11.0, 4.0, 4.0 Hz, H-5′b), 2.28 (d, 1.0 Hz, H-6′), 6.01 (d, 15.5 Hz, H-2″), 8.00 (dd, 15.5, 11.5 Hz, H-3″), 6.62 (dd, 11.5, 10.5 Hz, H-4″), 6.09 (d, 10.5 Hz, H-5″); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 78.9 (C-2), 34.9 (C-3), 74.8 (C-4), 49.0 (C-5), 42.2 (C-6), 26.5 (C-7), 68.8 (C-8), 136.5 (C-9), 123.8 (C-10), 67.0 (C-11), 65.3 (C-12), 47.9 (C-13), 7.0 (C-14), 64.4 (C-15), 20.5 (C-16), 170.9 (C-17), 21.0 (C-18), 165.6 (C-1′), 117.7 (C-2′), 157.0 (C-3′), 40.2 (C-4′), 60.4 (C-5′), 17.1 (C-6′), 165.5 (C-1″), 127.8 (C-2″), 138.8 (C-3″), 139.9 (C-4″), 125.1 (C-5″), 165.8 (C-6″).

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Biochemical and Partial Molecular Characterization of Bitter and Sweet Forms of *Lupinus angustifolius*, an Experimental Model for Study of Molecular Regulation of Quinolizidine Alkaloid Biosynthesis

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The bitter and sweet forms of a plant species differing with alkaloid contents may provide a model system for investigation of alkaloid biosynthesis at a molecular level. The pattern and concentration of quinolizidine alkaloids were determined by capillary GC-MS in bitter and sweet plants of *Lupinus angustifolius.* Bitter plant contained lupanine, 13α-hydroxylupanine, angustifoline, α-isolupanine, tetrahydrorhombifoline, and ester-derivatives of 13α-hydroxylupanine. In contrast, no alkaloid was detected in sweet plant. The enzymatic activity of acyltransferase for formation of 13α-tigloyloxylupanine was similar or even higher in the cell-free extracts of sweet plant than that in bitter plant. These results suggest that the biosynthetic step(s) of ring closure forming the initial cyclic alkaloid, lupanine, from cadaverine is presumably blocked in sweet plant, and that the later steps for modification of the cyclized alkaloids are not altered. We hypothesized that the gene(s) encoding enzyme(s) for ring-closure step might be repressed in sweet plant, and that the expression might take place only in bitter plant. To isolate the genes specifically expressed in bitter plant, cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis was carried out. However, no bitter-specific gene was isolated, suggesting that alkaloid biosynthesis in sweet plant may be down-regulated at a post-transcriptional level.

Key words quinolizidine alkaloid; biosynthesis; sweet lupin; bitter lupin; *Lupinus angustifolius*

The quinolizidine alkaloids of lupin plants are formed from L-lysine *via* cadaverine as the first detectable intermediate (Chart 1). The crucial steps of ring cyclization of cadaverine units are believed to occur as enzyme-bound intermediates and subsequently to yield the initial bicyclic alkaloids, *e.g.* (−)-lupinine, or the tetracyclic alkaloids such as (+)-lupanine.[2—5] These cyclic alkaloids are subsequently transformed by cellular enzymes through dehydrogenation, oxygenation or esterification.[6,7] For understanding the regulatory mechanism of biosynthesis, the alkaloid-rich 'bitter' form and alkaloid-poor 'sweet' form of lupin plants are useful experimental materials.[8]

Previously we have shown that the contents of total crude alkaloids were *ca.* four-fold higher in bitter plants of *Lupinus luteus* and *L. albus* than those in sweet plants, although the alkaloid patterns were similar in bitter and sweet plants.[8] However, the cellular concentrations of L-lysine and cadaverine did not markedly differ between bitter and sweet plants.[8] The enzymatic activities of acyltransferases for the formation of the ester alkaloids were also the same in the cell-free extracts of bitter and sweet plants.[7] These results suggest that the biosynthetic steps of ring closure forming initial cyclic alkaloids such as (−)-lupinine and (+)-lupanine from cadaverine are presumably blocked in sweet plants, but the steps prior to cadaverine formation and the later steps for modification of the cyclized alkaloids are not altered.

In the present study, we used bitter and sweet forms of *L. angustifolius.* Bitter form (cultivar (cv.) Fest) and sweet form (cv. Uniharvest) were derived from the cv. New Zealand Blue by addition of several domesticating genes.[9] Uniharvest and Fest have a similar genetic background, but are homozygous for the mutant *iuc* (*iucundus*) alleles and wild type alleles, respectively. The *iuc* is a recessive allele causing low alkaloid phenotype. We have determined the accumulation patterns and concentrations of alkaloids and the enzymatic activities of acyltransferase in these bitter and sweet plants of *L. angustifolius.* We tried to isolate genes encoding enzymes for ring cyclization by a molecular biology approach. From these results, we discuss the possible regulation of alkaloid biosynthesis in the bitter and sweet forms.

Results

The accumulation patterns and concentrations of alkaloids in the leaves of bitter and sweet plants of *L. angustifolius* were determined by capillary GC-MS (Table 1, Fig. 1). The identification of known alkaloids by GC-MS was performed by comparison with the standard alkaloids of our laboratory stock and the data in the literature.[3] In bitter plant, the first major alkaloid was lupanine (**1**), followed by 13α-tigloyloxylupanine (**2**). Other components were angustifoline (**3**), α-isolupanine (**4**), 13α-hydroxylupanine (**5**), tetrahydrorhombifoline (**6**), 13α-benzoyloxylupanine (**7**), 13α-caproyloxylupanine (**8**), 13α-cinnamoyloxylupanine (**9**), and the presumed ester derivatives of 13α-hydroxylupanine judged by the pattern of fragment ions in MS. In sweet plants, no alkaloid was detected (Table 1).

The Activity of Tigloyl-CoA: 13α-Hydroxylupanine/13α-hydroxymultiflorine *O*-tigloyltransferase (HLT/HMTase),[7] which catalyses the formation of 13α-tigloyloxylupanine and 13α-tigloyloxymultiflorine, was measured. These two reactions of tigloyltransferase have been proven to be catalyzed by a single enzyme in *Lupinus* plants.[7] The activity in the hypocotyls and roots was two-fold higher in the sweet form than that in bitter form (Table 2).

To clarify the genetic variations between bitter and sweet forms, random amplified polymorphic DNA (RAPD) analysis of total DNA was conducted. DNAs extracted from bitter and sweet plants were amplified using nineteen kinds of ran-

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Dedicated to the memory of Dr. Kyosuke Tsuda.

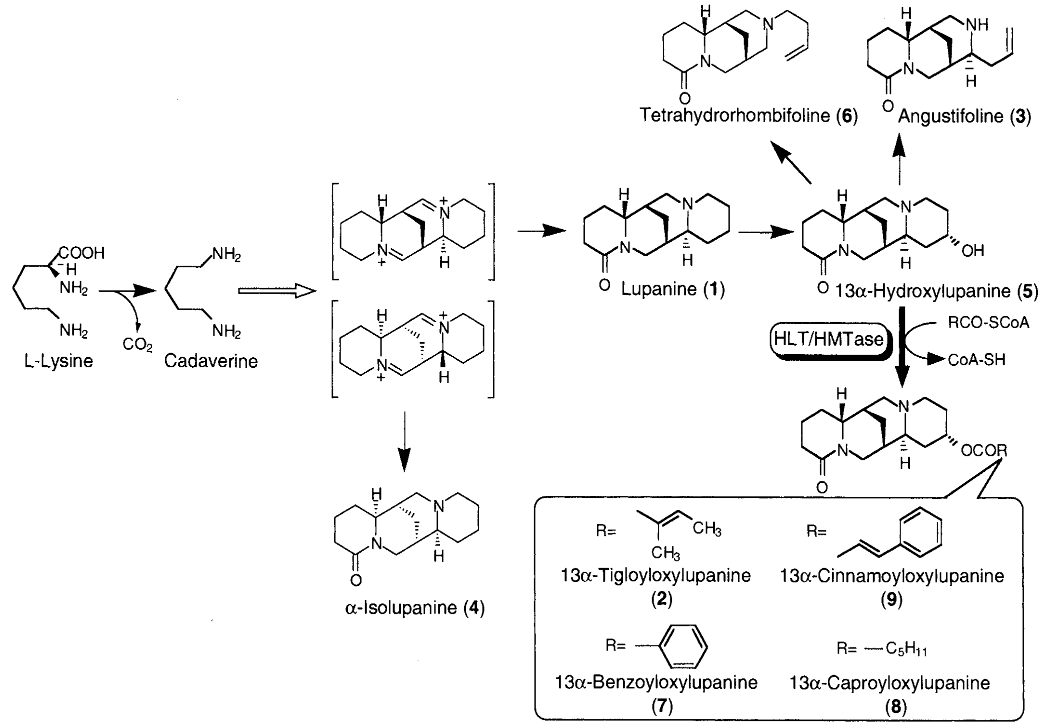

Chart 1. Biosynthetic Pathway of Lupin Alkaloids in Bitter and Sweet Forms of *L. angustifolius*

⇨, Absent in sweet plant; ➡, enzymatic activities detected both in bitter and sweet plants. Structures in parentheses are the proposed intermediates.

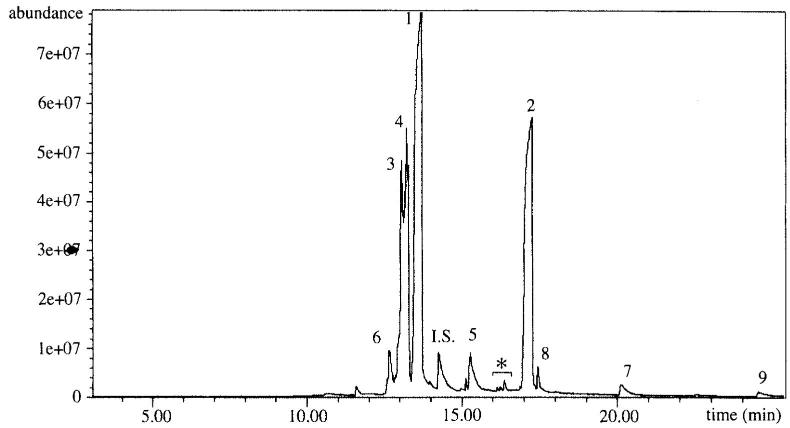

Fig. 1. Representative GC-MS Profile of Alkaloids Accumulated in the Bitter Form of *L. angustifolius*

Separation was carried out by a programmed temperature gradient (100—300 °C). (+)-Matrine was used as an internal standard (I.S.). The numbers in the figure correspond to those in Table 1, Chart 1 and the text. * Indicates the peaks for three presumed ester derivatives of 13α-hydroxylupanine.

dom 10 mer primers.[10] Twenty-five bands were observed by agarose gel electrophoresis stained by ethidium bromide, and only one band was amplified differently between bitter and sweet forms (data not shown). This result indicated that bitter and sweet forms of *L. angustifolius* have only a little polymorphism with total DNA.

Our previous findings[8] and the results in the present study suggested that the biosynthetic steps of ring closure forming the initial cyclic alkaloid lupanine from cadaverine may be blocked in sweet plants of *L. angustifolius* (Chart 1). We hypothesized that expression of the gene(s) encoding the enzyme(s) responsible for ring-closure steps may be repressed only in sweet plant. To isolate the genes specifically expressed in bitter plant, we conducted a cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis. In two hundred and fifty-six primer combinations, *ca.* 13000 bands were amplified with cDNAs from bitter and sweet plants, respectively. Among them, one fragment was reproducibly amplified only in bitter plants. Northern analysis was conducted using this fragment as a probe, but there was no difference between bitter and sweet forms (data not shown).

Discussion

As is the case with *L. albus* and *L. luteus*,[8] the limiting step of the biosynthetic pathway in sweet plant of *L. angustifolius* causing an alkaloid-poor phenotype may be the reaction from cadaverine to lupanine (Chart 1). The same amount of HLT/HMTase activity, or even higher activity, of post-ring-closure steps was detected in sweet plant, while no alkaloid was detected in sweet plant of *L. angustifolius*. It was also the case with *L. albus* and *L. termis*, exhibiting the same level of acyltransferase activities in spite of different levels of alkaloid accumulation.[8] These facts suggest that the genes encoding enzymes for the initial step for cyclic alkaloid for-

Table 1. Lupin Alkaloids in Leaves of Bitter and Sweet Forms of *L. angustifolius*

Alkaloid	Bitter form (cv. Fest) (*iuc*$^+$/*iuc*$^+$, *leu*$^+$/*leu*$^+$)	Sweet form (cv. Uniharvest) (*iuc*/*iuc*, *leu*/*leu*)
Lupanine (**1**)	452±78.1	<1.0
13α-Tigloyloxylupanine (**2**)	337±55.2	<1.0
Angustifoline (**3**)	174±14.4	<1.0
α-Isolupanine (**4**)	160±27.2	<1.0
13α-Hydroxylupanine (**5**)	36.5±6.99	<1.0
Tetrahydrorhombifoline (**6**)	30.3±2.28	<1.0
13α-Benzoyloxylupanine (**7**)	13.7±2.36	<1.0
13α-Caproyloxylupanine (**8**)	7.54±3.01	<1.0
13α-Hydroxylupanine ester?	2.30±1.31	<1.0
13α-Cinnamoyloxylupanine (**9**)	2.10±3.64	<1.0
13α-Hydroxylupanine ester?	1.35±0.13	<1.0
13α-Hydroxylupanine ester?	1.10±0.04	<1.0
Total	1218	<1.0

The average and standard deviation of the alkaloid contents in 3 individual plants are shown in μg g^{-1} fresh wt. The detection limit was below *ca.* 1.0 μg g^{-1} fresh wt. Genotypes of the plants are shown in the parentheses. *iuc*, mutant *incundus* allele; *iuc*$^+$, wild type *incundus* allele; *leu*, mutant *leucospermus* allele; *leu*$^+$, wild type *leucospermus* allele.

Table 2. The Alkaloid Acyltransferase Activities in Bitter and Sweet Forms of *L. angustifolius*

	HLT/HMTase activity (pkat mg^{-1} protein)
Bitter form (cv. Fest)	0.62±0.07
Sweet form (cv. Uniharvest)	1.19±0.12

The HLT/HMTase activity in hypocotyls and roots of 2-week-old seedlings was determined as described in the Experimental. The average and standard deviation of five repetitive assays are shown.

mation from cadaverine are probably controlled independently from the genes encoding enzymes for the later step such as acyltransferases, and that the initial step may be susceptible to be blocked at the transcriptional or translational level. Otherwise, the genes for the ring-closure step may be easily mutated to encode peptides that exhibit no substantial enzymatic activity (discussed later). The HLT/HMTase activity was two-fold higher in sweet plant than that detected in bitter plant. This may be due to the up-regulation of the activity under repressed condition of *de novo* alkaloid biosynthesis. Since the alkaloid acyltransferases are apparently localized in the mitochondria matrix,11) the regulatory mechanism of acyltransferase expression might be different from that for early-step enzymes.

In the case of anthocyanin biosynthesis in *Perilla*, the regulatory mechanism is quite different from alkaloid biosynthesis in *Lupinus*. In a red form of *Perilla frutescens*, anthocyanins are actively biosynthesized in leaves and stems, while in a green form, which has a similar genetic background to the red form, no anthocyanin is accumulated. Almost all genes encoding biosynthetic enzymes of anthocyanins, except for chalcone synthase, are repressed at the transcriptional level in the green form. It is presumed that the expression of these structural genes is controlled by the same transacting factor(s) that are impaired in the green form of *P. frutescens*.12)

Among the three species of genus *Lupinus*, *L. albus*, *L. luteus* and *L. angustifolius*, investigated so far with respect to bitter and sweet forms, *L. angustifolius* seemed to be the most appropriate experimental material, because the chemical phenotype regarding alkaloid accumulation in sweet plant is most drastic. Moreover, bitter and sweet forms of *L. angustifolius* showed only a little polymorphism in RAPD analysis, suggesting small genetic variation. In the case of *L. albus*, bitter and sweet forms showed polymorphism in the restriction fragment length in genomic DNA.13) We conducted cDNA-AFLP to isolate the genes specifically expressed in bitter plant, some of which were expected to encode enzymes for initial ring-closure step. We have extensively analyzed the amplification by cDNA-AFLP but failed to isolate any bitter-specific gene. In the present study, mRNA species carrying more than two *Taq*I sites in their molecules were detectable in cDNA-AFLP analysis (see Experimental). One possibility for failure of specific-gene isolation is that the expression of the genes encoding enzymes for ring-closure step is repressed at the transcriptional level in the sweet plant, but these cDNAs possess one or no *Taq*I site. In this case, the choice of a different restriction enzyme would lead to cloning of the cDNAs. The second possibility is that the ring-closure step may be blocked at the post-transcriptional level, *i.e.*, at translation or enzymatic activity, although mRNAs are accumulated at the same level in bitter and sweet plants. For this possibility, the differential investigation on proteins accumulated in two forms would be necessary to gain more insights on the molecular basis of regulation of quinolizidine alkaloid formation. In addition, further trials to detect cell-free enzymatic activity of post-cadaverine steps would be also needed.

Experimental

Plant Materials Seeds of *L. angustifolius* cv. Fest (bitter form) and cv. Uniharvest (sweet form) were from Drs. T. J. V. Higgins and L. M. Tabe, Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia. Uniharvest and Fest have a similar genetic background, but are homozygous for the mutant *iuc* allele, a recessive allele causing low alkaloid phenotype, and wild type allele, respectively. These cultivars also differ in color of stem, flower and seed, Uniharvest having green stems and white flowers and seed coats due to homozygosity for the *leucospermus* (*leu*) allele, whereas Fest has the wild type allele conferring reddish stems, blue flowers and gray mottled seeds.9) The seeds were germinated and grown in vermiculite under fluorescent light (30 μE/m^2/s, 16 h light/8 h dark) at 25 °C in a plant growth chamber (BIOTRON LH200, Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan).

Determination of Alkaloids Mature leaves were harvested from 3-week-old seedlings of bitter and sweet plants, and homogenized in distilled water with a few drops of 10% HCl (pH *ca.* 2). After centrifuging, the resulting acidic supernatant was extracted with ethyl acetate twice. The aqueous solution was made alkaline by dissolving potassium carbonate to saturation (pH *ca.* 11). The alkaline solution was then extracted with ethyl acetate twice. The organic layers were combined, dried with sodium sulfate (anhydrous) and evaporated *in vacuo*. The resulting residue (basic fraction) was dissolved in methanol and analyzed by a combined GC-MS system 5980II/5971A (Hewlett Packard, U.S.A.) equipped with a fused silica capillary column DB-1 (J & W Scientific, CA, U.S.A., 0.25 mm×30 m). Separation was carried out by a programmed temperature gradient (100—300 °C). (+)-Matrine was used as an internal standard for quantification. Identification of alkaloids was made by comparison with the authentic compounds of our laboratory stock and the published data.3)

Assay of the Alkaloid Acyltransferase Activity The crude enzyme solution was obtained by extraction of the hypocotyls and roots of 2-week-old seedlings with buffer A (0.2 M potassium phosphate, pH 8.0, 10 mM 2-mercaptoethanol, 0.5 mM ethylenediamine tetraacetate (EDTA), 250 mM sucrose). The extracts were centrifuged (6000 ***g***, 10 min) and the supernatant was subjected to ammonium sulfate fractionation. The pellet between 20 and

70% solution was collected and dissolved in 20 mM sodium phosphate, pH 6.3/10 mM 2-mercaptoethanol, followed by desalting with PD-10 column (Amersham Pharmacia Biotech, U.K.). The standard reaction mixture comprised 100 mM potassium phosphate, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 0.15 mM (−)-13α-hydroxymultiflorine,[7] 0.15 mM tigloyl-CoA (Sigma Chemical Co., MO, U.S.A.), and the enzyme protein in a total volume of 240 μl. After incubation for 60 min at 30 °C, the reaction was terminated by the addition of 160 μl of methanol and centrifuged (8000 ***g***, 5 min). One hundred microliters of the supernatant was subjected to HPLC analysis. HPLC analysis was carried out on a Mightysil RP-18 GP150-4.6 (5 μm) column (Kanto Chemical Co., Inc., Tokyo, Japan) by using a solvent system, 15% acetonitrile in 20 mM sodium dihydrogenphosphate, pH 5.5 at a flow rate 1.0 ml/min with monitoring at 327 nm. The acyltransferase activity was expressed as the formation of ester alkaloid, (−)-13α-tigloyloxymultifloline[7] (pkat (pmol/s)/mg of protein).

RAPD Analysis Total DNA was extracted from stems of the seedlings of bitter and sweet forms as described by Dellaporta *et al.*[14] RAPD analysis was conducted as described previously.[10] Briefly, the polymerase chain reaction (PCR) mixture was made as follows: 10 ng of DNA, 0.5 μM random 10-mer primer (Operon 10-mer kits, Operon Technologies, Inc., CA, U.S.A.), 1 mM magnesium chloride, 120 μM deoxyribonucleoside triphosphates, 0.5 units of rTaq DNA polymerase (Toyobe, Co., Ltd., Osaka, Japan) and 1×rTaq buffer (Toyobo, Co., Ltd.) in a total volume of 25 μl. Amplification was carried out under the following temperature conditions: 72 °C for 30 s, 40 cycles of (94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min) and 72 °C for 5 min. Products were subjected to 1% agarose gel electrophoresis and stained by ethidium bromide.

cDNA-AFLP Total RNA was extracted from leaves of 3-week-old seedlings of bitter and sweet plants by a modified guanidine HCl method as described.[15] Poly (A)$^+$ RNA was purified using mRNA Separator Kit (Clontech Laboratories, Inc., CA, U.S.A.). Double-stranded cDNA (ds-cDNA) was synthesized using cDNA Synthesis Module (Amersham Pharmacia Biotech). cDNA-AFLP was conducted according to Habu *et al.*[16] In short, ds-cDNA was digested by *Taq*$^{\alpha}$I (New England Biolabs, Inc. MA, U.S.A.) and ligated to *Taq*I linker (top strand linker, 5′-GACGATGAGTCCTGAG-3′; bottom strand linker, 5′-pCGCTCAGGACTCAT-3′). They were preamplified using T-N primers (5′-GATGAGTCCTGAGCGAN-3′, N=A, G, C or T). Next they were amplified using T-N primer and ^{32}P-labelled T-NNN primer (5′-GATGAGTCCTGAGCGANNN-3′, NNN=AAA to TTT). Then labeled amplified fragments were separated on 5% acrylamide/7.5 M urea sequencing gel. The gel was dried and subjected to autoradiography. cDNA fragment in the bitter form-specific band was extracted from the dried gel, reamplified by PCR, and labeled with ^{32}P using Random Primer DNA Labeling Kit ver. 2 (Takara Shuzo, Shiga, Japan) for a probe in Northern analysis. Northern analysis was carried out as described elsewhere.[12]

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Determination of α-Tocopherol and α-Tocopherylquinone in Rat Tissues and Plasma by High-Performance Liquid Chromatography with Electrochemical Detection

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α-Tocopherol and α-tocopherylquinone in rat tissues and plasma were determined simultaneously by using high-performance liquid chromatography–electrochemical detection (HPLC-ED) with dual electrodes in the series mode. Biological samples were saponified in the presence of a mixture of butylated hydroxytoluene, ascorbic acid, and pyrogallol and then extracted with hexane. The compounds were separated on a C18 column using a mobile phase containing 95% methanol and 0.05 M sodium perchlorate as the supporting electrolyte. After HPLC separation, α-tocopherylquinone was first reduced at an upstream electrode at −500 mV. Both α-tocopherol and the reduction product of α-tocopherylquinone were then oxidized downstream at +600 mV. Only the downstream electrode current was monitored for the determination. Linearity of the standard curves was obtained over the range 5—30 pmol for α-tocopherol and α-tocopherylquinone. Minimum detectable quantities (S/N of 3) were 0.25 pmol for α-tocopherol and 0.31 pmol for α-tocopherylquinone. The method was applied to analysis of the contents of α-tocopherol and α-tocopherylquinone in rat tissues and plasma. By hyperoxia, the content of α-tocopherol was decreased remarkably in lung, and in contrast, the contents of α-tocopherylquinone were increased in all tissues studied with the exception of plasma, though the content of α-tocopherylquinone in normal rats is quite small. The technique is particularly useful in the quantitation of the oxidation of α-tocopherol in biological samples.

Key words α-tocopherol; α-tocopherylquinone; HPLC-ED; rat tissue, hyperoxia

Tocopherols are potent antioxidants and their presence in biological membranes is believed to represent the major defense system against peroxidation of membrane lipids, which are highly susceptible to peroxidative degradation.[1—9] Either antioxidative enzymes, including superoxide dismutase, catalase, and glutathione peroxidase, or antioxidants such as ascorbic acid, tocopherol, and glutathione are presumed to prevent the oxidative deterioration of physiological function in living tissues. Especially, α-tocopherol is now accepted to function as a potent physiological chain breaking antioxidant in biomembranes by scavenging the chain-carrying peroxyl radicals, preventing oxidative damage to polyunsaturated lipids. From kinetic studies, it is known that α-tocopherol can trap one peroxyl radical to generate tocopherol radical, which traps another peroxyl radical to be consumed to α-tocopherylquinone. Since it has been recognized that α-tocopherylquinone is detected in several organs, it is important to analyze the content of α-tocopherylquinone in living tissue for global assessment of antioxidative defense systems. Nevertheless, there have been only few articles concerning determination of tocopherols and their oxidation products, tocopherylquinones, in biological samples.[10—14] One of the causes of uncertainly is artificial oxidation of tocopherols during sample processing. Previously, it has been reported that the addition of a mixture of butylated hydroxytoluene (BHT), ascorbic acid and pyrogallol into biological samples during saponification eliminates the oxidation of α-tocopherol and α-tocopherylquinone.[13] Therefore, we used these experimental conditions, and investigated the influence of oxidative stress on the content of α-tocopherol and α-tocopherylquinone in several organs.

Conventional methods for simultaneous quantitation of α-tocopherol and α-tocopherylquinone involve separation by HPLC with UV detection,[11] however they generally lack sufficient sensitivity for α-tocopherylquinone. Although fluorescence assay is generally sensitive, α-tocopherylquinone is not detectable. Previously, we have developed an electrochemical detector (ED) with a new porous carbon coulometric electrode[15] and investigated analysis of neurotransmitters, such as the catecholamines and its metabolites, in biological fluid.[16—18] In the present study, we report the determination of α-tocopherol and α-tocopherylquinone in rat tissues and plasma using a series dual-electrode detector which consists of the downstream-oxidative detection of reduction products from an upstream electrode (reductive-oxidative detection). The effect of hyperoxia in rat on contents of α-tocopherol and α-tocopherylquinone in homogenates of tissues and plasma were examined by this method. In addition, elimination of oxygen interference during reductive mode HPLC-ED and improvements in detection limits for the compounds were investigated.

Experimental

Chemicals α-, β-, γ- and δ-Tocopherols were purchased from Eisai (Tokyo, Japan); α-tocopherylquinone was purchased from ICN Biochemicals (Cleveland, OH, U.S.A.). Sodium perchlorate was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Methanol was HPLC grade. Water was distilled and passed through a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.). All other chemicals were of analytical-reagent grade.

Processing of Biological Samples Animals: Six male Wistar rats of age of 12 months old were used in this experiment. To induce oxidative stress, three rats were subjected to hyperoxia by maintaining them in an atmosphere of 100% oxygen gas with flow-rate of 1 ml/min for 48 h at 20 °C. The other three rats were used as a control.

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Dedicated to the memory of Dr. Kyosuke Tsuda.

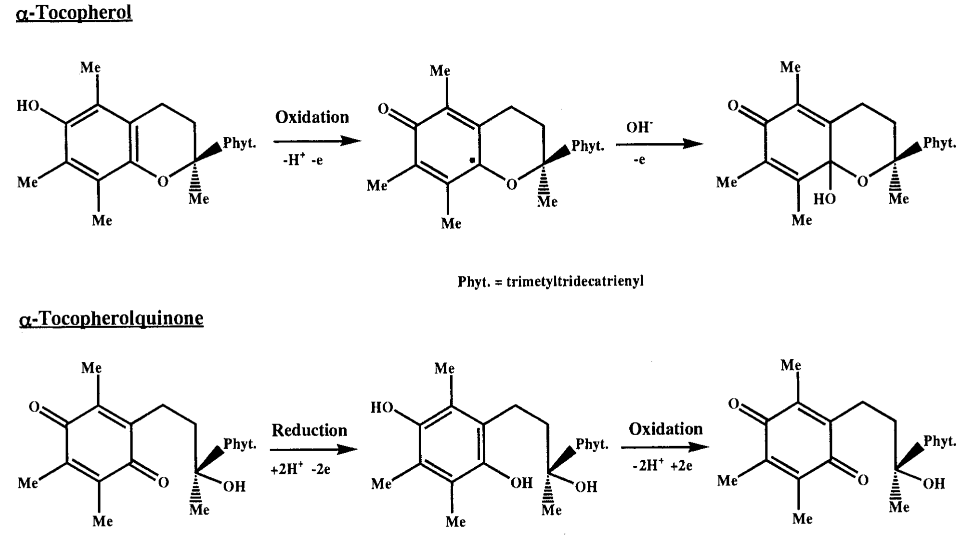

Fig. 1. Electrochemical Reactions of α-Tocopherol and α-Tocopherylquinone at the Electrode

The sample preparation of tissue extracts and plasma were as follows: 0.5 g of fresh rat tissues were accurately weighed and homogenized in 2 ml of 0.1 M Tris buffer (pH 7.3). For plasma sample preparation, 2 ml of rat plasma was obtained from heparinized venous blood by centrifugation. Each sample was placed under vortex-mixing into tubes containing 2 ml of 0.025 w/v% butylated hydroxytoluene (BHT) in ethanol, 0.2 ml of 15 w/v% ascorbic acid, 0.2 ml of 25 w/v% pyrogallol, and 100 pmol of δ-tocopherol (an internal standard). The samples were saponified at 60°C for 30 min with 1 ml of 10 w/v% potassium hydroxide solution. The tubes were cooled and 2 ml of 0.9% NaCl and 2 ml of hexane–ethyl acetate (9 : 1) containing 0.025% BHT were added. Tocopherols and tocopherylquinone were extracted into the hexane phase by vortexing for 1 min. The samples were centrifuged for 10 min at 3000 rpm. The hexane phase was separated and evaporated to dryness under nitrogen gas. The residue was redissolved in 0.5 ml of 95% methanol containing 50 mM sodium perchlorate and filtered prior to injection. All the biological samples were analyzed within 24 h.

HPLC Conditions The HPLC system consisted of a Hitachi Model L-6200 intelligent pump, a dual electrode electrochemical detector (original made) and a D-2500 data processor. The electrodes of the coulometric ED consisted of a working electrode made of glassy carbon (3.0 mm diameter× 3.0 mm thickness; Kurahashi Giken, Kyoto, Japan) in series mode, an Ag/AgCl reference electrode and a counter electrode.[15]

The analytical column was a reversed-phase C18 Neo-pack ODS2 column (5 μm, 150×4.6 mm i.d., Nishio Kogyo, Tokyo, Japan). The mobile phase was 95% methanol containing 50 mM sodium perchlorate as the supporting electrolyte, and it was continuously bubbled with helium gas to maintain an oxygen-free state. In the series mode, the upstream electrodes was set at −500 mV, while the downstream electrode was set at +600 mV.

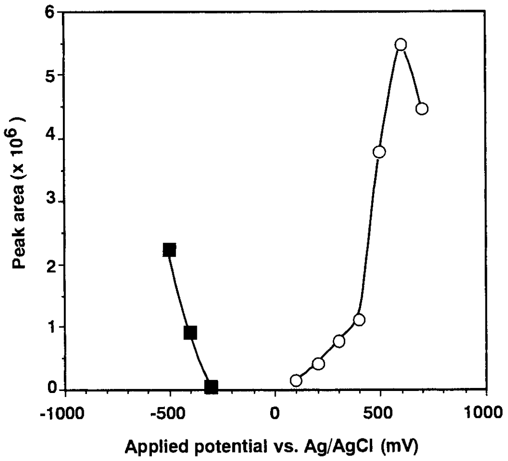

Fig. 2. Hydrodynamic Voltammograms of Standard α-Tocopherol (○) and α-Tocopherylquinone (■)

Samples of α-tocopherol (25 pmol) were monitored at the downstream (detecting) electrode with increasing positive potentials from +100 to +700 mV. Setting the downstream electrode at +600 mV, samples α-tocopherylquinone (25 pmol) were monitored at the downstream electrode while the upstream electrode was varied from −300 to −500 mV.

Results and Discussion

Hydrodynamic Voltammograms The reduction of α-tocopherylquinone at the upstream electrode during flow-cell electrochemistry produced exclusively the hydroquinone, which was oxidized at the downstream electrode to regenerate α-tocopherylquinone (Fig. 1). α-Tocopherol is oxidized at the downstream electrode in the presence of water *via* a carbocation intermediate to 8a-hydroxyl-α-tocopherone.[11] Hydrodynamic voltammograms for oxidation of α-tocopherol and reduction of α-tocopherylquinone, using the dual-series flow cell, are shown in Fig. 2. The potential of the downstream electrode was varied from +100 to +700 mV for oxidation of α-tocopherol. Then by setting the downstream electrode at +600 mV, the potential of the upstream electrode was varied from −300 to −500 mV for reduction of α-tocopherylquinone. From the hydrodynamic voltammograms, a reductive potential of −500 mV for the upstream electrode and an oxidizing potential of +600 mV for the downstream electrode were chosen for further experiments.

Separation of Tocopherols α-, δ-Tocopherol and α-tocopherylquinone were well separated and determined by the dual-series electrode HPLC-ED. Figure 3 shows a chromatogram of a standard mixture containing 25 pmol each of α-tocopherol, β-tocopherol, δ-tocopherol (used as the internal standard in the assay of biological samples), γ-tocopherol, and α-tocopherylquinone. After HPLC separation, α-tocopherylquinone was first reduced at an upstream electrode at −500 mV. Both tocopherols and α-tocopherylquinone were then detected at the downstream electrode at +600 mV.

Calibration Curves Linear relationships between the amounts and the peak area ratio (α-tocopherol, or α-tocopherylquinone/δ-tocopherol: R) were obtained over the range 5—30 pmol as indicated by the following equations:

α-tocopherol; $R/10^{-2}=1.25x+1.16$ ($r=0.999$)

α-tocopherylquinone; $R/10^{-2}=1.06x-0.79$ ($r=1.000$)

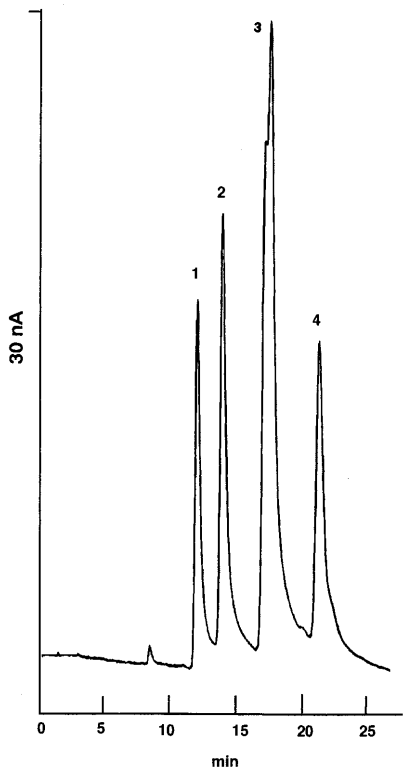

Fig. 3. A Chromatogram of a Standard Mixture of Tocopherol and Tocopherylquinone

Peak No.1: α-tocopherylquinone, 2: δ-tocopherol (i.s.), 3: β-tocopherol+γ-tocopherol, 4: α-tocopherol (25 pmol each). HPLC conditions: column, Neo-pack ODS2 column (5 μm, 150×4.6 mm i.d.); eluent, 95% methanol containing 50 mM sodium perchlorate; flow-rate, 1.0 ml/min. The upstream electrode was set at −500 mV, while the downstream detecting electrode was set at +600 mV.

The precision of the method was established from 5 assays. The variability (coefficients of variation) of the retention times were less than 1% and those of peak areas were less than 4%. Minimum detectable quantities (S/N of 3) were 0.25 pmol for α-tocopherol, 0.33 pmol for β-tocopherol, 0.12 pmol for γ-tocopherol, 0.26 pmol for δ-tocopherol and 0.31 pmol for α-tocopherylquinone. The recoveries of the analytical procedure, calculated by addition of α-tocopherol, δ-tocopherol and α-tocopherylquinone to tissues (25 pmol, $n=5$), were 86.4%, 100% and 94.2%, respectively. UV-monitoring is also possible for the determination of α-tocopherol and α-tocopherylquinone. α-Tocopherol can be quantitated at 295 nm and α-tocopherylquinone at 265 nm. In our laboratory, electrochemical detection is at least 100 times more sensitive than UV detection for tocopherols and α-tocopherylquinone.

Determination of α-Tocopherol and α-Tocopherylquinone in Rat Tissues and Plasma There have been some controversies regarding the presence of tocopherylquinones in biological organs and tissues.[19,20] To shed light on the problem and to study the effects of oxidative stress, the contents of α-tocopherol and α-tocopherylquinone in brain, heart, lung, liver, kidney and plasma of rats were measured by the present method. Tocopherols in biological samples are very susceptible to oxidation during sample processing. Previously, addition of a mixture of BHT, ascorbate and pyrogallol was reported to eliminate the loss of α-tocopherol and α-tocopherylquinone during the assay.[13] To prevent oxidation during the extraction procedure, samples were saponified in the presence of a mixture of BHT, pyrogallol, and ascorbic acid and then extracted with a mixture of hexane and ethyl acetate. Extraction and HPLC-ED analysis of samples of α-tocopherol (0.15 nmol) showed no presence of detectable α-tocopherylquinone, and endogenous δ-tocopherol was not detectable in this sample. From these results, it is suggested that the α-tocopherylquinone detected in samples of tissues and plasma is not an artifact of this sys-

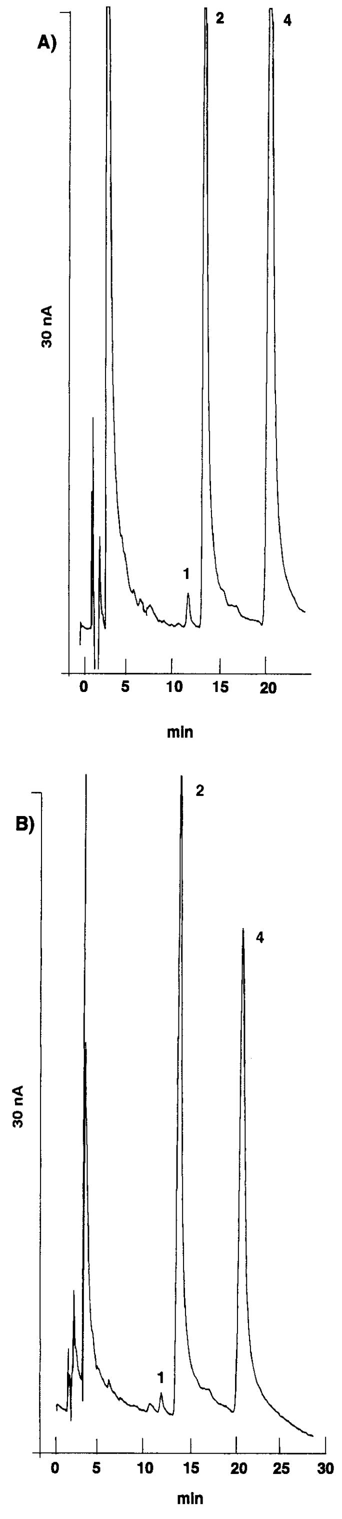

Fig. 4. Chromatograms of Brain (A) and Kidney (B) Tissue Extracts of Rat

Rat tissues were extracted as described in the Experimental section, and 5 μl of the extract was injected for analysis. HPLC conditions and peak numbers were the same as Fig. 3.

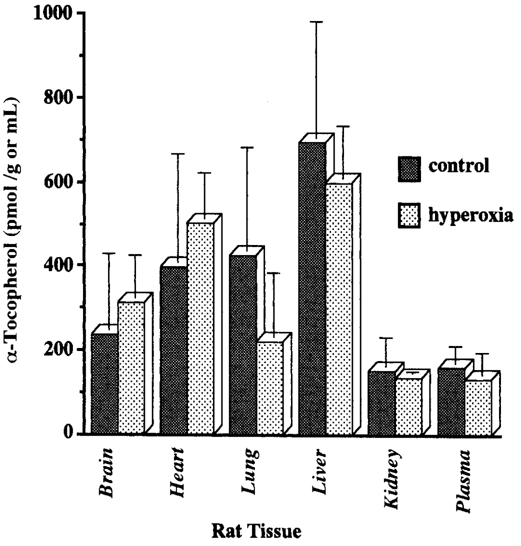

Fig. 5. The Effect of Oxidative Stress on the Contents of α-Tocopherol in Rat Tissues and Plasma

The error bars show standard deviations from triplicate determinations.

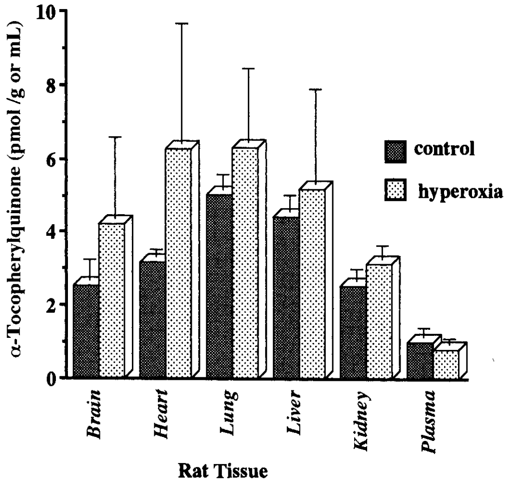

Fig. 6. The Effect of Oxidative Stress on the Contents of α-Tocopherylquinone in Rat Tissues and Plasma

The error bars show standard deviations from triplicate determinations.

tem. Chromatograms of brain and kidney tissue extracts of rat obtained by the procedure described in the Experimental section are shown in Fig. 4. The concentrations of α-tocopherylquinone in tissues and plasma are quite small (less than 1%) compared to α-tocopherol. It should be noted that the coefficients of variation for assays of such low baseline levels of α-tocopherylquinone usually tend to be somewhat high. As the separation between γ- and β-tocopherol was not achieved, the contents of these compounds were not determined. Figure 5 shows the effect of oxidative stress on the contents of α-tocopherol in rat tissues and plasma. Under oxidative stress, decreased contents of α-tocopherol were noted in plasma and tissues except for heart and brain. Especially, α-tocopherol decreased remarkably in lung. The results suggest that the influence of oxidative stress is large in organs with greater oxygen consumption such as lung. Figure 6 shows the effect of oxidative stress on the contents of α-tocopherylquinone. The contents of α-tocopherylquinone were increased in all tissues studied with the exception of plasma. The reason why both α-tocopherol and α-tocopherylquinone were increased in brain and heart is not clear. The α-tocopherol content of mammalian tissue is primarily maintained by dietary intake, but it has been hypothesized to be maintained during an oxidative challenge by redox cycling to regenerate the parent compound.

Table 1. Effect of Oxidative Stress on the Ratio[a] of α-Tocopherol and α-Tocopherylquinone

	Brain	Heart	Lung	Liver	Kidney	Plasma
Control	1.08	0.80	1.18	0.64	1.67	0.63
Hyperoxia	1.34	1.24	2.85	0.86	2.30	0.62

a) α-Tocopherylquinone as % of α-tocopherol; (α-tocopherylquinone/α-tocopherol) ×100.

The ratio of α-tocopherol and α-tocopherylquinone concentrations were increased under oxidative stress with the exception of plasma as shown in Table 1. It is known that losses of tocopherol and tocopherylquinone during sample processing are greater with plasma than with other biological samples. This oxidative loss of tocopherol may be due to the presence of endogenous iron and heme compounds in red cells. The catalytic effect of iron in oxidation reaction[21] and hematin in epoxidation reactions of lipids[22] are well known. Information on oxidation of tocopherol and production of quinone will be useful in understanding the mechanism of membrane damage resulting from oxidative reactions. The ratio of α-tocopherol and α-tocopherylquinone concentrations might serve as a useful indicator of the redox state of tissues and organs.

Conclusion

α-Tocopherol and α-tocopherylquinone in rat tissues and plasma were well separated and determined by HPLC-ED. The influence of oxidative stress on contents of α-tocopherol and α-tocopherylquinone were observed in homogenates of rat tissues. The high selectivity of the electrochemical detection for easily oxidizable compounds such as tocopherols makes it possible to determine these compounds even in highly complex mixtures, *i.e.*, liver extracts. The method described herein is simple and very sensitive, allowing the simultaneous detection of tocopherols in biological tissues, a feature highly desirable for small samples.

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Antioxidant Activity of Eugenol and Related Monomeric and Dimeric Compounds

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Since the inhibitory effect of eugenol (a), which was isolated as an antioxidative component from plant, *Caryopylli flos*, on lipid peroxidation was less than that of α-tocopherol, we synthesized the eugenol-related compounds dieugenol (b), tetrahydrodieugenol (c), and dihydroeugenol (d), to find new strong antioxidants and assessed them for their inhibitory effect on lipid peroxidation and scavenging ability for superoxide and hydroxyl radicals. The antioxidative activities were in the order: (b)>(c)>(d)>(a) for the thiobarbituric acid reactive substance (TBARS) formation. These results suggest that the dimerized compounds have higher antioxidant activities than that of the monomers. Electron spin resonance (ESR) spin trapping experiments revealed that eugenol and its dimer, having allyl groups in the structure, scavenged superoxide, and that only eugenol trapped hydroxyl radicals under the conditions used. These finding suggest that eugenol and dieugenol have a different mechanism of antioxidation, *i.e.* eugenol may inhibit lipid peroxidation at the level of initiation, however, the related dimeric compounds may inhibit lipid peroxidation at the level of propagation of free radical chain reaction like α-tocopherol.

Key words eugenol; dimer; lipid peroxidation; electron spin resonance; dieugenol

Peroxidation of cell membrane lipid results in membrane destabilization and change of transport characteristics, which eventually leads to cell death. Many studies have demonstrated that lipid peroxidation, caused by free radicals, may be related to aging and several diseases, such as atherosclerosis, diabetes, cancer, and ischemia-reperfusion injury.[1—5] The polyunsaturated fatty acids in the cell membrane are protected against lipid peroxidation through endogenous antioxidants such as α-tocopherol.[6] Consequently, it is important to find compounds that prevent oxidation. Many attempts have been made to find highly active antioxidants that prevent oxidation *in vivo*. A large number of naturally occurring molecules having antioxidant properties are known to be phenolic compounds: acid-phenols or flavonoids and their esters.[7] The antioxidative potency of phenolic compound depends on the chemical structure, in particular, electron delocalization on the aromatic nucleus.[8] However, the mechanism of their scavenging reaction *in vivo* is still obscure.

In order to find antioxidants from Chinese drugs (Kampo drugs or crude drugs), we have recently showed that $CHCl_3$: EtOH (3 : 1) extracts from the bark of *Magnolia cortex* have potent antioxidant activity, and demonstrated that the antioxidative components are magnolol and honokiol.[9] In the present work, the identification of the antioxidative components from *Caryophylli Flos* and the characterization of their inhibitory effects on lipid peroxidation are described.

Materials and Methods

Chemicals *Caryophylli Flos* was kindly supplied by Dr. T. Nishimura of Teikyo University, Tokyo, Japan. Eugenol was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). α-Tocopherol was purchased from E. Merck (Darmstast, Germany). Thiobarbituric acid (TBA), L-ascorbic acid, and $FeSO_4$ were purchased from Tokyo Kasei Kogyo Co., Daiichi Kagaku Yakuhin Co., and Kanto Chemical Co. Inc. (Tokyo, Japan), respectively. Hypoxanthine (HX), xanthine oxidase (XO), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ethylenediaminetetraacetic acid disodium salt (EDTA) were from Wako Pure Chemical Co. (Osaka, Japan). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was from Labotec Co. (Tokyo, Japan). $CHCl_3$, EtOH, and BuOH were from Yoneyama Yakuhin Kogyo Co. (Osaka, Japan).

Fe^{2+} and Ascorbic acid Induced Oxidation of Egg Phosphatidylcholine (Egg PC) and Assessment of Antioxidant Activity Lipid peroxidation was assayed as formation of thiobarbituric acid reactive substance (TBARS).[10] Mixtures of a solution of egg PC in chloroform (200 μl; 10 mg/ml) and each sample (100 μl) were evaporated to dryness under nitrogen gas. Control and reference substrate with 2 mM α-tocopherol were also tested for comparison. Lipid peroxidation was initiated by the addition of 0.2 mM $FeSO_4$, and 2 mM ascorbic acid. After incubation at 37 °C for 30 min, the reaction was terminated by addition of 5 mM EDTA. The oxidation mixture was then mixed with 3 ml of 1% phosphoric acid and 1 ml of aqueous 0.7% thiobarbituric acid solution and heated at 98 °C for 45 min. The mixture was extracted with 4 ml of *n*-butanol and the absorbance was measured at 535 nm with a Hitachi 220A spectrophotometer (Hitachi Seisakusho Co., Ltd., Japan). Antioxidant activity was calculated as follows:

$$\text{inhibition (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where A represents absorbance.

Measurement of Superoxide, Hydroxyl Radical and DPPH Scavenging Activities Superoxide and hydroxyl radical scavenging activities were measured by electron spin resonance (ESR) with DMPO as a spin trapping reagent.[11] The HX–XO system was used as a superoxide generating system, and hydroxyl radicals were generated by the Fenton reaction (Fe^{2+}–H_2O_2). In superoxide trapping, DMPO produces DMPO–OOH spin adducts, and DMPO–OH spin adducts are obtained when hydroxyl radicals are trapped, so these radical scavenging activities of samples could be calculated as the decreasing rate of the DMPO–OOH or DMPO–OH signal intensities.[9] DPPH (2 mM) was dissolved in ethyl alcohol. Each sample (50 μl) in chloroform was evaporated to dryness under nitrogen gas. The DMPO–OOH, DMPO–OH and DPPH spin adducts were measured after exactly 60 seconds. ESR spectra were recorded on a JEOL-JES-RE1X spectrometer (JEOL Co. Ltd., Tokyo, Japan). The ESR conditions are shown in Table 1.

Isolation of Eugenol *Caryophyllia Flos* (50 g) was suspended in 800 ml of $CHCl_3$/EtOH (3 : 1) at room temperature overnight. After filtration, the extract was concentrated to dryness *in vacuo*. The residue was dissolved in $CHCl_3$ and applied to a silica gel column (3×23.5 cm). The column was eluted with $CHCl_3$, and fractions were combined and evaporated to give a yellow oil (1 g). The yellow oil was further purified by Sephadex LH-20 column (3×15 cm) (Pharmacia Biotech, Sweden). FAB-MS *m/z*: 165 $(M+H)^+$. HR-FAB-MS *m/z*: 165.1061 (Calcd for $C_{10}H_{13}O_2$: 165.1130). The column was developed with MeOH, and active fractions (320 mg) were identified as eugenol, based on physico-chemical date reported previously.[12]

Synthesis of Dieugenol Eugenol (10 g) was dissolved in pyridine (10 g) and mixed with $FeSO_4$ (100 mg) and 31% H_2O_2 (20 g×3) at 60 °C for 24 h. The reaction product was extracted with AcOEt and distilled by steam distil-

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Table 1. Electron Spin Resonance (ESR): Reaction Mixtures and Conditions

DMPO–OOH		DMPO–OH		DPPH	
2 mM hypoxanthine	50 μl	10 mM $FeSO_4$	75 μl	2 mM DPPH	200 μl
5.5 mM DETAPAC	50 μl	1 mM H_2O_2	75 μl	Sample[a] or H_2O	50 μl
92 mM DMPO	100 μl	92 mM DMPO	75 μl		
0.02 U/ml Xanthin oxidase (phosphate buffer, pH 7.2)	50 μl	Sample[a] or H_2O	75 μl		
Sample[a] or H_2O	50 μl				
Temperature	25 °C		25 °C		25 °C
Power	0.8 mW		8 mW		0.8 mW
Field	335.1+5 mT/G		335.1+5 mT/G		335.1+5 mT/G
Modification	100 kHz		100 kHz		100 kHz
Time constant	0.03 s		0.03 s		0.03 s

Abbbrevitions: DMPO: 5,5-dimethyl-1-pyrroline *N*-oxide; DETAPAC: diethylenetriamine pentaacetic acid. a) Each sample in $CHCl_3$ was evaporated to dryness under nitrogen gas. H_2O was used as a control.

lation. The residue was recrystallized from ethanol to give dieugnol (3.56 g, 35.6%) as a white solid, mp 93 °C.[13] FAB-MS *m/z*: 327 $(M+H)^+$. HR-FAB-MS *m/z*: 327.1985 (Calcd for $C_{20}H_{23}O_4$: 327.2022).

Synthesis of Dihydroeugenol Eugenol (100 mg) was dissolved in EtOH and hydrogenated on 10% Pd–C at room temperature for 5 h. After filtration, the reaction mixture was evaporated to dryness *in vacuo*. The residue was distilled by steam distillation to give dihydroeugenol as a yellow oil (98 mg, 98%). NMR ($CDCl_3$) δ: 0.93 (t, 3H, J=7.3 Hz, 9-H), 1.61 (m, 2H, J=7.3, 7.9 Hz, 8-H), 2.51 (t, 2H, J=7.9 Hz, 7-H). FAB-MS *m/z*: 167 $(M+H)^+$. HR-FAB-MS *m/z*: 167.1320 (Calcd for $C_{10}H_{15}O_2$: 167.1289).

Synthesis of Tetrahydrodieugenol Dieugenol (300 mg) was dissolved in EtOH and hydrogenated on 10% Pd–C at room temperature for 5 h. After filtration, the reaction mixture was evaporated to dryness *in vacuo*. The residue was recrystallized from EtOH to give tetrahydrodieugenol as a white solid (289 mg, 96%), mp 113 °C. NMR ($CDCl_3$) δ: 0.93 (t, 3H, J=7.3 Hz, 9,9′-H), 1.62 (m, 2H, J=7.3, 7.9 Hz, 8,8′-H), 2.52 (t, 2H, J=7.9 Hz, 7,7′-H). FAB-MS *m/z*: 331 $(M+H)^+$. HR-FAB-MS *m/z*: 331.2385 (Calcd for $C_{10}H_{25}O_2$: 331.2340).

Results and Discussion

Extract from *Caryophylli flos* with organic solvents showed excellent antioxidant activity (Table 2). Especially, the extract with a mixture of chloroform : ethanol (3 : 1) had potent antioxidant activity, *i.e.*, an inhibition of TBARS formation, and scavenging ability for superoxide and hydroxyl radicals. In order to identify the active compound in the extract, the purification was carried out using column chromatography, and we identified the potent compounds as eugenol based on its physico-chemical properties, IR, UV, ^{1}H- and ^{13}C-NMR spectra. Eugenol is a major component of the oils from clove, cinnamon, basil and nutmeg.[14,15] Since Nagababu *et al.* revealed that eugenol might inhibit lipid peroxidation at the stage of the initiation, propagation, or both, many attempts for elucidation of the mechanism of antioxidant activity of eugenol have been carried out.[16] Nevertheless, the mechanism details are still obscure. In order to define the mechanism, and to search for related compounds having a stronger antioxidant activity, we synthesized some related compounds; dieugenol, which is an oxidized compound of eugenol,[13] dihydroeugenol and tetrahydrodieugenol, which were synthesized to understand the antioxidant effects of the allyl group (Fig. 1). As shown in Table 3 and Fig. 2, Fe^{2+}-ascorbic acid induced lipid peroxidation was inhibited significantly in a dose-dependent manner by these compounds. Antioxidant potency decreased in the order: dieugenol>tetrahydrodieugenol>dihydroeugenol>eugenol. The inhibitory effect of dimerized compounds is higher than that of monomeric compounds, eugenol itself. These results suggest that bulky substituents are necessary at the neighboring position of the hydroxyl group in eugenol. An organic radical as a model of a lipid carbon-centered radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) was trapped remarkably by the dimerized compounds, dieugenol and tetrahydrodieugenol (Table 3). The trapping potency of dieugenol was almost 5 times higher than that of eugenol. Interestingly, the order of the potency was similar to that of the inhibitory effect on TBARS formation. Although it is not obvious at present why the trapping effect of the monomeric dihydroeugenol is two times higher than that of eugenol, it is likely that the allyl group at the *para* position of the phenolic hydroxyl moiety is not critical for inhibition of lipid peroxidation.

Table 2. Inhibition of Lipid Peroxidation, Superoxide and Hydroxyl Radicals by Extract with Various Solvents

Solvent	Inhibition of lipid peroxidation (%)	DMPO–OH IC_{50} (mg/ml)	DMPO–OOH IC_{50} (mg/ml)
$CHCl_3$	93.2±0.1	1.0	>2.0
EtOH	93.8±0.6	>2.0	0.6
$CHCl_3$: EtOH (3:1)	94.3±0.1	1.0	1.0
H_2O	50.4±2.0	>2.0	>2.0

Mean±S.E., n=4.

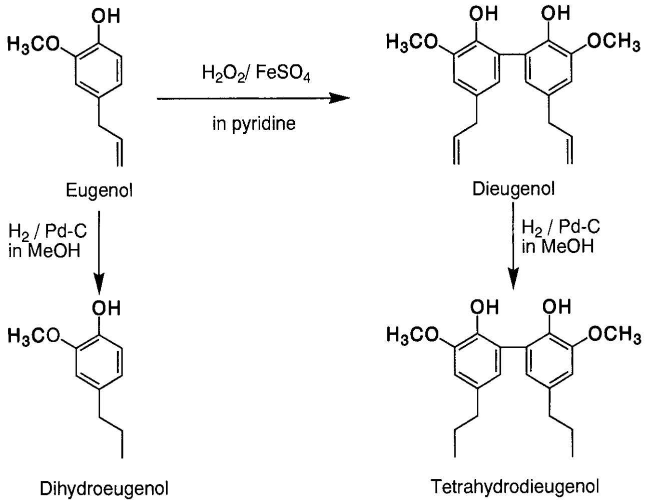

Fig. 1. Structures of Eugenol and Related Assessed Compounds

To understand the antioxidant mechanism of these phenolic compounds, we analyzed *in vitro* lipid peroxidation using ESR spectrometry to see whether they scavenged active oxygen species directly or whether they acted as free-radical chain breaking antioxidants, such as α-tocopherol. Relative signal intensities for both the spin adduct DMPO–OOH, which is produced by O_2^- generated from HX–XO systems, and the spin adduct DMPO–OH, produced by hydroxyl radical through the Fenton reaction, decreased upon the addition of phenolic compounds with antioxidant activity (Table 3). The scavenging values are expressed as the concentration of phenolic compounds that causes a 50% decrease in the level of O_2^- or hydroxyl radicals (50% inhibitory concentration (IC_{50}) values). Eugenol and dieugenol were able to trap O_2^-, and no decrease in the signal was observed following addition of other phenolic compounds under the experimental conditions used (Table 3). An allyl moiety at the *ortho* or *para* position of the phenols increased the O_2^- scavenging activity.[9] Among the four phenols, only eugenol scavenged hydroxyl radicals under the conditions used. Our results suggest that eugenol may inhibit lipid peroxidation by trapping active oxygen species, such as O_2^- or hydroxyl radicals, rather than by breaking the free radical chain reaction.[9]

In summary, it is indicated that the inhibition mechanism of eugenol to lipid peroxidation has two steps; 1) it interferes with the chain reactions by trapping the active oxygen, 2) it is metabolized to dimer, and the dimeric compound (dieugenol) inhibits lipid peroxidation at the level of propagation of free radical chain reaction like α-tocopherol. Although the activities of eugenol itself are less than that of α-tocopherol *in vitro*, it is anticipated that the protective effect of eugenol is comparative to α-tocopherol in organisms injured by many oxidative stresses, because of its inhibition mechanism of monomeric and dimeric stage (Fig. 3).

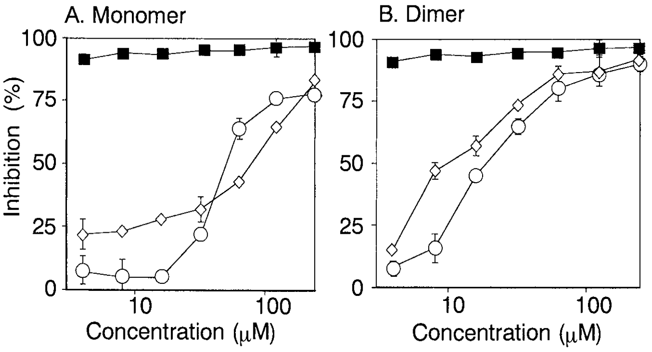

Fig. 2. Inhibition of Lipid Peroxidation by Phenolic Compounds through TBARS Measurement

(A) ■, α-tocopherol; ◇, dihydroeugenol; ○, eugenol. (B) ■, α-tocopherol; ◇, tetrahydrodieugenol; ○, dieugenol. Each point and bar represent the mean±S.E., n=4.

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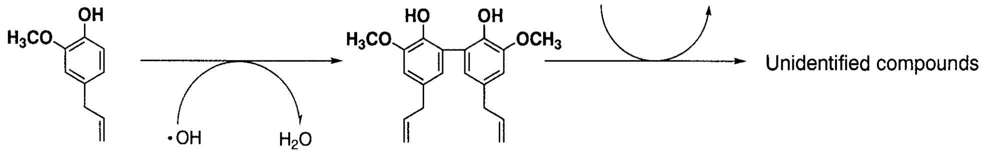

Fig. 3. Antioxidant Mechanisum of Eugenol

Table 3. Inhibition of Lipid Peroxidation, DPPH, Superoxide and Hydroxyl Radicals by Eugenol and Its Related Compounds

Samples	TBA method 62.5 μM	TBA method 250 μM	DPPH IC_{50} (μM)	DMPO–OOH IC_{50} (mM)	DMPO–OH IC_{50} (mM)
Eugenol	43.0±1.2	76.0±0.5	>800	5.3	1.88
Dihydroeugenol	63.7±0.1	77.0±3.2	421	>10	>10
Dieugenol	86.0±0.2	92.5±0.1	160	3.3	>10
Tetrahydrodieugenol	78.9±0.1	89.8±0.6	164	>10	>10
α-Tocopherol	97.2±0.1	97.4±0.1	80	>10	>10

Mean±S.E., n=4.

Synthesis of NG-061 and Its Analogs, and Their Biological Evaluation as an Enhancer of Nerve Growth Factor

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A novel potentiator of nerve growth factor (NGF), NG-061, which had been isolated from the fermentation broth of *Penicillium minioluteum* F-4627, was synthesized from methoxybenzoquinone and phenylacetylhydrazine in a single step. A series of acyl hydrazone derivatives were also synthesized and their potentiator activity of neurotrophic effect of NGF on neurite outgrowth was evaluated by assay with a rat pheochromocytoma cell line PC12.

Key words NG-061; nerve growth factor; biological activity

Nerve growth factor (NGF) is a polypeptide which acts as a prototypical neurotrophic factor essential for growth and development of neurons in the central as well as peripheral nervous systems.[1,2] Survival and growth of cultured neuronal cells are also affected by such biological polypeptides as fibroblast growth factor. These neurotrophic factors have been shown to protect the neuron from dysfunction and death *in vivo* in animal models of injury and neurologic diseases. It was reported that NGF treatment could ameliorate age-related memory impairment and prevent lesion-induced loss of septal cholinergic neurons in rats.[3—5] Also known is that NGF can prevent neuronal loss of the hippocampus in the cerebral ischemia model in the Mongolian gerbil.[6] These findings suggest that NGF can be effective for the treatment of dementia and cerebral paralysis. However, it is very difficult to use NGF as a medicine since it must be administered to a patient intraventricularly because of its impermeability across the blood-brain barrier. To resolve these difficulties, it is anticipated that low molecular-weight compounds exhibiting and/or enhancing neurotrophic actions may have significant potential for development as therapeutic agenst to prevent neuronal cell death.

During the course of our screening program for low molecular-weight natural products with ability to potentiate and/or mimic the neurotrophic effect of NGF, we isolated a novel fungal metabolite, NG-061, from the fermentation broth of *Penicillium minioluteum* F-4627, and determined the structure by spectroscopic means and X-ray diffraction method as shown in Fig. 1.[7,8] In this paper, we wish to describe the synthesis of NG-061 and its analogs as well as their biological evaluation as a potentiator of NGF.

Results and Discussion

The synthesis of NG-061 was straightforward. Simple treatment of 2-methoxy-1,4-benzoquinone with phenylacetylhydrazide in $CHCl_3$ at room temperature for 2 d as shown in Fig. 2 gave, after recrystallization, **4a** in 12% yield. Spectroscopic data of synthesized **4a** was completely identical with those of natural NG-061. Further, a series of analogs of NG-061 were synthesized similarly as also shown in the figure. The compounds **4a**—**e**, **4g**, **4h**, **4j**, **4l**, **4m**, **4o**, **4q**—**u**, **4x**, and **4y** were obtained as a mixture of isomers (*ca.* 1 : 1—10 : 1). Because these compounds were derived from asymmetrical quinones, the presence of isomer appeared to be due to the geometrical isomer of hydrazone moiety. As reported previously,[8] natural NG-061 exists in equilibrium between the geometrical isomer in solution depending on the solvent: 1) *ca.* 4 : 1 in $CDCl_3$, 2) 8 : 1—10 : 1 in dimethyl sulfoxide (DMSO)-d_6, or 3) 1 : 1 in pyridine-d_5. Although not absolutely comfirmed, the above mentioned analogs should also be in equilibrium in solution.

The biological activity of the synthesized NG-061 and its analogs (**4a**—**y**) for the enhancement activity of neurite outgrowth effect of NGF was examined in PC12 cells, and the results are shown in Fig. 3. Unfortunately, since compounds **4c**—**e**, **4m**, **4r**—**u**, **4w**, and **4y** showed cytotoxicity against PC12 cells at the dose of 10 μg/ml, the activities of these compounds were not determined. The synthesized NG-061 **4a** showed the same level of activity as natural NG-061 in enhancing neurite outgrowth effect by NGF, therefore, the active principle from the fermentation broth of *Penicillium minioluteum* F-4627, has now been proven to be NG-061.

The structure–activity relationship of NG-061 was studied by structurally modifing it. Modification of the phenylacetyl moiety into an acetyl group compound **4b** significantly reduced the activity and slightly inhibited the effect of NGF. This result suggested that the presence of an aromatic ring in the acyl hydrazone moiety plays an important role in the biological acitivity. The replacement of the methoxy group of NG-061 into a hydrogen atom compound **4f** also reduced the activity. Introduction of alkyl groups into the quinone moiety generally diminished the activity of NG-061. Particularly, the bulky di-*tert*-butylated compounds **4k** and **4p** were found to

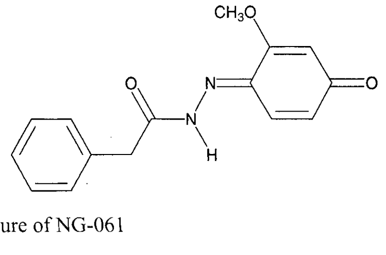

Fig. 1. Structure of NG-061

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have an inhibitory effect against NGF. As far as tested, the synthetic analogs **4b**—**y** turned out to be less active or toxic, and the compound **4a** (NG-061) showed the highest activity.

During the synthetic studies of NG-061 and its analogs, we noticed that the low product yields were due to the formation of several by-products, and a major by-product was found to be the Michael adduct of acylhydrazide to the quinone-type structure. It was speculated that the Michael addition to NG-061 by a nucleophilic residue of a targeted biological polymer leading to a covalent bond formation might be a plausible mechanism of action of NG-061. In fact, the treatment of NG-061 with *N*-acetylcysteamine in the presence of triethylamine in a CH_2Cl_2 solution afforded, after auto-oxidation, the Michael addtion product **5** in moderate yield as shown in Fig. 4. Further detail of the mechanism of NG-061 action is intriguing and is to be investigated.

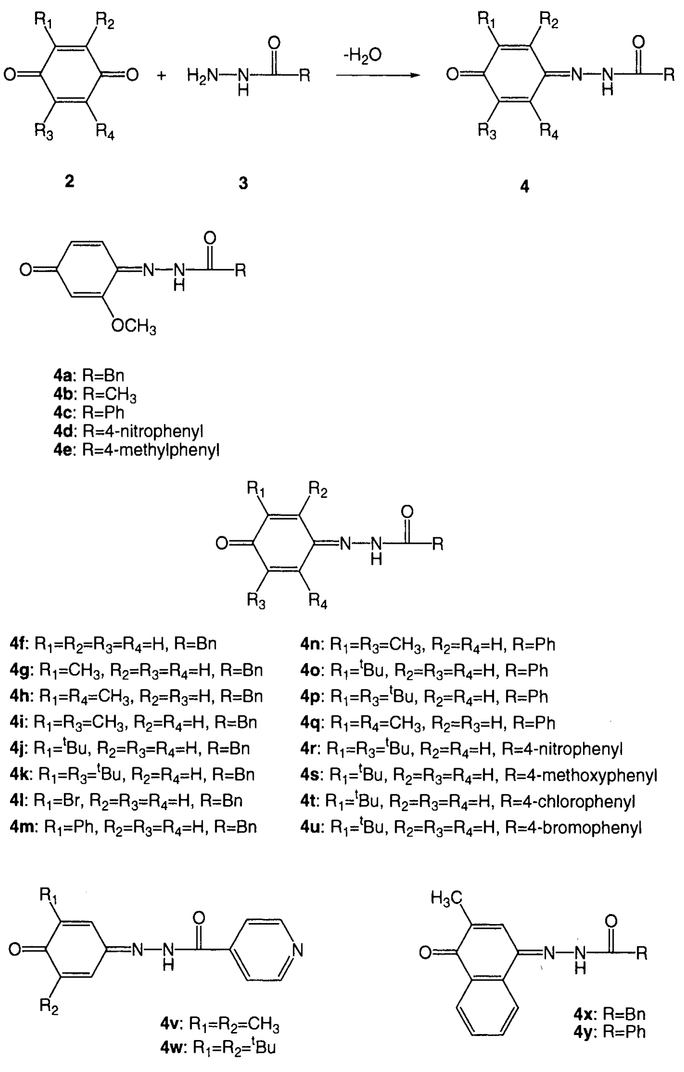

Fig. 2. Synthesis of NG-061 and Its Analogs

Experimental

General Melting points were determined with a Yanagimoto micromelting point apparatus and are uncorrected. IR spectra were taken on a Horiba FT-710 Fourier transform IR spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL LA-300 spectrometer. Deuteriochloroform (99.8% atom ^{2}H, Merck) was used as the solvent for NMR spectra, unless otherwise stated. Chemical shifts were reported in δ values based on the internal tetramethylsilane ($\delta_H=0$) or the solvent signal of $CDCl_3$ ($\delta_C=77.0$) as the reference. Mass spectrum was obtained on a Finnigan LCQ LC/MS spectrometer in atmospheric pressure chemical ionization mode. Elemental analyses were performed with a Perkin Elmer 2400 apparatus.

Compound 4a (NG-061) A mixture of 2-methoxy-1,4-benzoquinone (1.32 g, 9.54 mmol) and phenylacetic hydrazide (1.42 g, 9.46 mmol) in $CHCl_3$ (150 ml) was stirred at room temperature for 2 d. After evaporation of the solvent, the residual solid was recrystallized from $CHCl_3$–ether to afford NG-061 (310 mg, 12%) as yellow powder; mp 189—190 °C (from $CHCl_3$–ether); IR (KBr) cm^{-1}: 1685, 1639, 1567, 1529; ^{1}H-NMR (major : minor=4 : 1) δ (major): 3.90 (s, 3H), 4.10 (s, 2H), 5.90 (s, 1H), 6.44 (d, 1H, $J=9.7$ Hz), 7.09 (d, 1H, $J=9.7$ Hz), 7.19—7.50 (m, 5H), 11.36 (br, 1H); ^{13}C-NMR δ: 29.65, 38.72, 43.27, 55.62, 56.24, 106.91, 127.13, 128.10 (br), 128.50, 128.62, 128.87 (br), 129.47, 130.10 (br), 132.81, 133.75, 138.70, 139.13, 159.87, 174.04, 186.60. *Anal.* Calcd for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.66; H, 5.35; N, 10.35.

Compound 4b In the same manner as described in the synthesis of **4a**, the compound **4b** was obtained from the corresponding quinone and hydrazide in 22% yield; yellow powder; mp 220—221 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1722, 1689, 1639, 1621, 1565, 1542; ^{1}H-NMR (major : minor=4 : 1) δ (major): 2.38 (s, 3H), 3.92 (s, 3H), 5.91 (d, 1H, $J=1.7$ Hz), 6.42 (dd, 1H, $J=1.7$, 9.8 Hz), 7.04 (d, 1H, $J=9.8$ Hz), 11.32 (br, 1H); ^{13}C-NMR δ: 19.64, 56.23, 105.01, 106.88, 128.38, 138.84, 159.90, 174.01, 186.64. *Anal.* Calcd for $C_9H_{10}N_2O_3$: C, 55.67; H, 5.19; N, 14.43. Found: C, 55.44; H, 5.32; N, 14.30.

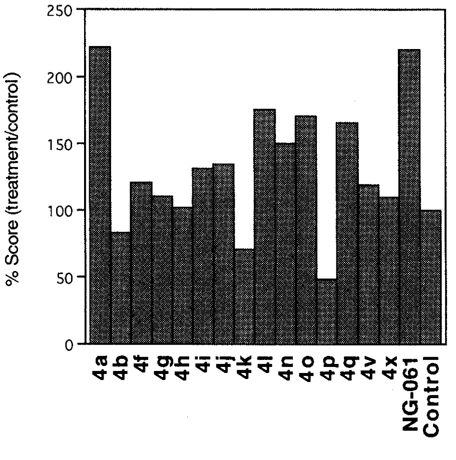

Fig. 3. The Activity of Synthesized NG-061 and Its Analogs (**4a**—**y**) in Enhancing the Neurite Outgrowth Effect of NGF in PC12 Cells

Compounds **4c**—**e**, **4r**—**u**, and **4y** showed toxicity against PC12 cells at doses of 10 μg/ml, therefore, the activities of these compounds were not determined.

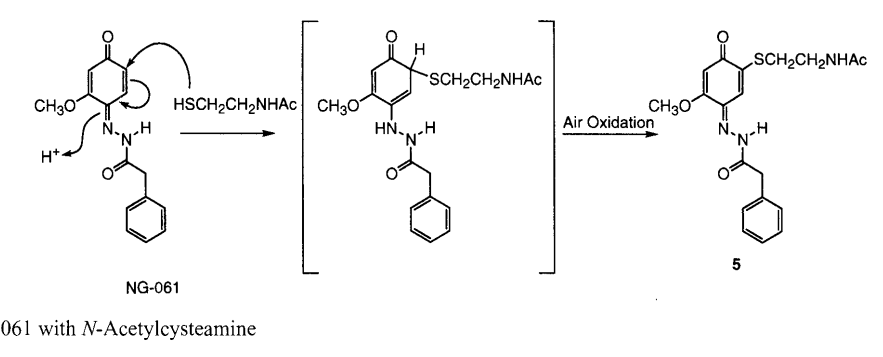

Fig. 4. Reaction of NG-061 with *N*-Acetylcysteamine

Compound 4c In the same manner as described in the synthesis of **4a**, the compound **4c** was obtained from the corresponding quinone and hydrazide in 27% yield; yellow powder; mp 187—188 °C (from $CHCl_3$–hexane); IR (KBr) cm^{-1}: 1700, 1644, 1612, 1573, 1529; ^{1}H-NMR (major : minor=8 : 1) δ (major): 4.01 (s, 3H), 5.95 (d, 1H, *J*=1.7 Hz), 6.44 (dd, 1H, *J*=1.7, 10.0 Hz), 7.25 (d, 1H, *J*=10.0 Hz), 7.53 (m, 2H), 7.63 (m, 1H), 7.87 (m, 2H); ^{13}C-NMR δ: 56.56, 107.21, 127.19, 127.89, 128.65, 128.92, 132.00, 132.95, 132.95, 135.77 (br), 139.17, 159.70, 186.40. *Anal.* Calcd for $C_{14}H_{12}N_2O_3$: C, 65.62; H, 4.72; N, 10.93. Found: C, 65.45; H, 4.90; N, 10.93.

Compound 4d In the same manner as described in the synthesis of **4a**, the compound **4d** was obtained from the corresponding quinone and hydrazide in 16% yield; pale yellow powder; mp 232—233 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1710, 1675, 1644, 1571, 1525; ^{1}H-NMR (major : minor=9 : 1) δ (major): 4.00 (s, 3H), 5.97 (d, 1H, *J*=1.5 Hz), 6.46 (dd, 1H, *J*=1.5, 9.8 Hz), 7.14 (d, 1H, *J*=9.8 Hz), 8.03 (m, 2H), 8.36 (m, 2H); ^{13}C-NMR (DMSO-d_6) δ: 56.08, 104.72 (br), 106.63, 123.26, 123.93, 124.33, 128.72, 129.28 (br), 129.65, 129.96, 130.64 (br), 137.75, 138.54, 138.73, 149.38, 149.62, 159.71, 162.95, 185.94. *Anal.* Calcd for $C_{14}H_{11}N_3O_5$: C, 55.82; H, 3.68; N, 13.95. Found: C, 55.56; H, 3.41; N, 13.80.

Compound 4e In the same manner as described in the synthesis of **4a**, the compound **4e** was obtained from the corresponding quinone and hydrazide in 16% yield; yellow powder; mp 214—215 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1698, 1644, 1608, 1571, 1525; ^{1}H-NMR (major : minor=8 : 1) δ (major): 2.46 (s, 3H), 4.00 (s, 3H), 5.95 (d, 1H, *J*=2.0 Hz), 6.45 (dd, 1H, *J*=1.7, 10.0 Hz), 7.28 (d, 1H, *J*=10.0 Hz), 7.33 (m, 2H), 7.77 (m, 2H); ^{13}C-NMR δ: 21.61, 56.55, 107.17, 127.16, 127.95, 128.55, 129.15, 129.39, 129.63, 135.54, 139.26, 143.89, 159.76, 186.46. *Anal.* Calcd for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found C, 66.37; H, 5.12; N, 10.07.

Compound 4f In the same manner as described in the synthesis of **4a**, the compound **4f** was obtained from the corresponding quinone and hydrazide in 13% yield; yellow powder; mp 134—135 °C (from $CHCl_3$–hexane); IR (KBr) cm^{-1}: 1677, 1641, 1533; ^{1}H-NMR δ: 4.13 (s, 2H), 6.46 (dd, 1H, *J*=2.2, 10.2 Hz), 6.55 (dd, 1H, *J*=2.2, 10.0 Hz), 7.24—7.36 (m, 6H), 7.68 (dd, 1H, *J*=2.7, 10.5 Hz); ^{13}C-NMR δ: 39.17, 123.53, 127.28, 128.56, 129.62, 130.33, 132.72, 133.60, 140.31, 140.97, 176.34, 187.03. *Anal.* Calcd for $C_{14}H_{12}N_2O_2$: C, 69.99; H, 5.03; N, 11.66. Found: C, 70.17; H, 5.19; N, 11.78.

Compound 4g In the same manner as described in the synthesis of **4a**, the compound **4g** was obtained from the corresponding quinone and hydrazide in 14% yield; brown powder; mp 185—186 °C (from $CHCl_3$–hexane); IR (KBr) cm^{-1}: 1691, 1675, 1639, 1616, 1533; ^{1}H-NMR (major : minor=3 : 1) δ (major): 1.91 (d, 3H, *J*=1.2 Hz), 4.14 (s, 2H), 6.53 (d, 1H, *J*=10.0 Hz), 7.19 (dd, 1H, *J*=2.9, 10.0 Hz), 7.25—7.35 (m, 5H), 7.57 (br, 1H), 11.35 (br, 1H); ^{13}C-NMR δ: 15.74, 16.65, 39.23, 120.56, 123.04, 127.16, 128.57, 129.66, 130.11, 132.57, 133.83, 136.63, 138.40, 139.92, 141.52, 141.57, 176.04, 176.14, 187.25, 187.36. *Anal.* Calcd for $C_{15}H_{14}N_2O_2$: C, 70.85; H, 5.55; N, 11.02. Found: C, 70.62; H, 5.83, N, 10.93.

Compound 4h In the same manner as described in the synthesis of **4a**, the compound **4h** was obtained from the corresponding quinone and hydrazide in 10% yield; yellow powder; mp 245—247 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1680, 1623, 1537; ^{1}H-NMR (DMSO-d_6) (major : minor=2 : 1) δ (major): 1.89 (s, 3H), 2.18 (s, 3H), 4.05 (s, 2H), 6.36 (s, 1H), 7.2—7.35 (m, 5H), 7.85 (s, 1H), 10.11 (s, 1H); ^{13}C-NMR (DMSO-d_6) δ: 15.92, 17.34, 122.22, 126.49, 126.62, 127.97, 128.21, 128.35, 129.01, 129.37, 135.01, 135.72, 138.45, 168.91, 186.34. *Anal.* Calcd for $C_{16}H_{16}N_2O_2$: C, 71.62; H, 6.01; N, 10.44. Found: C, 71.46; H, 6.19; N, 10.23.

Compound 4i In the same manner as described in the synthesis of **4a**, the compound **4i** was obtained from the corresponding quinone and hydrazide in 63% yield; yellow powder; mp 191—192 °C (from $CHCl_3$–ether); IR (KBr) cm^{-1}: 1677, 1623, 1535; ^{1}H-NMR δ: 1.91 (d, 3H, *J*=1.5 Hz), 2.05 (d, 3H, *J*=1.2 Hz), 4.13 (s, 2H), 7.02 (m, 1H), 7.24—7.33 (m, 5H), 7.52 (m, 1H), 11.24 (br, 1H); ^{13}C-NMR δ: 15.95, 16.85, 39.22, 120.22, 127.07, 128.55, 129.69, 134.06, 136.28, 138.02, 141.25, 141.68, 176.00, 187.54. *Anal.* Calcd for $C_{16}H_{16}N_2O_2$: C, 71.62; H, 6.01; N, 10.44. Found: C, 71.34; H, 6.13; N, 10.40.

Compound 4j In the same manner as described in the synthesis of **4a**, the compound **4j** was obtained from the corresponding quinone and hydrazide in 16% yield; pale yellow powder; mp 186—188 °C (from $CHCl_3$–ether); IR (KBr) cm^{-1}: 1670, 1643, 1590, 1540; ^{1}H-NMR (major : minor=5 : 4) δ (major): 1.27 (s, 9H), 4.13 (s, 2H), 6.47 (d, 1H, *J*=10.0 Hz), 7.14 (dd, 1H, *J*=2.7, 10.0 Hz), 7.22—7.34 (m, 5H), 7.40 (d, 1H, *J*=2.7 Hz), 10.93 (br, 1H); ^{13}C-NMR δ: 29.07, 29.23, 34.86, 36.02, 39.16, 39.41, 117.69, 121.31, 127.17, 128.61, 129.57, 132.03, 133.92, 134.69, 138.33, 141.41, 141.79, 145.60, 149.11, 152.25, 175.54, 175.62, 186.91. *Anal.* Calcd for $C_{18}H_{20}N_2O_2$: C, 72.95; H, 6.80; N, 9.45. Found: C, 72.66; H, 6.92; N, 9.41.

Compound 4k In the same manner as described in the synthesis of **4a**, the compound **4k** was obtained from the corresponding quinone and hydrazide in 26% yield; yellow powder; mp 193—194 °C (from $CHCl_3$); IR (KBr) cm^{-1}: 1683, 1621, 1583, 1533; ^{1}H-NMR δ: 1.26 (s, 9H), 1.31 (s, 9H), 4.13 (s, 2H), 6.96 (d, 1H, *J*=2.7 Hz), 7.25—7.33 (m, 5H), 7.38 (d, 1H, *J*=2.7 Hz), 11.20 (br, 1H); ^{13}C-NMR δ: 29.38, 29.54, 35.05, 36.25, 39.35, 116.74, 127.00, 128.57, 129.53, 133.07, 134.33, 142.13, 150.10, 153.45, 175.67, 186.83. *Anal.* Calcd for $C_{22}H_{28}N_2O_2$: C, 74.53; H, 7.74; N, 8.28. Found: C: 74.48, H: 7.65, N: 8.06.

Compound 4l In the same manner as described in the synthesis of **4a**, the compound **4l** was obtained from the corresponding quinone and hydrazide in 12% yield; reddish powder; mp 160—161 °C (from $CHCl_3$); IR (KBr) cm^{-1}: 1689, 1637, 1529; ^{1}H-NMR (major : minor=5 : 4) δ (major): 4.14 (s, 2H), 6.70 (d, 1H, *J*=9.8 Hz), 7.23—7.35 (m, 5H), 7.29 (dd, 1H, *J*=2.2, 9.8 Hz), 8.29 (d, 1H, *J*=2.2 Hz), 11.30 (br, 1H); ^{13}C-NMR δ: 39.20, 125.60, 127.39, 128.73, 129.16, 129.60, 131.59, 133.00, 133,25, 139.79, 140,24, 141.38, 176.04, 179.24, 182.73. *Anal.* Calcd for $C_{14}H_{11}BrN_2O_2$: C, 52.69; H, 3.47; N, 8.78. Found: C, 52.90; H, 3.65; N, 8.85.

Compound 4m In the same manner as described in the synthesis of **4a**, the compound **4m** was obtained from the corresponding quinone and hydrazide in 36% yield; brown powder; mp 154—155 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1677, 1635, 1525; ^{1}H-NMR (major : minor=4 : 3) δ (major): 4.12 (s, 2H), 6.67 (d, 1H, *J*=9.8 Hz), 7.27 (m, 5H), 7.30 (dd, 1H, *J*=2.7, 9.8 Hz), 7.40 (m, 3H), 7.60 (m, 2H), 7.88 (d, 1H, *J*=2.7 Hz), 11.43 (br, 1H); ^{13}C-NMR δ: 39.29, 39.34, 120.71, 122.84, 127.21, 128.22, 128.28, 128.61, 128.85, 129.21, 129.41, 129.47, 130.94, 133.33, 133.71, 134.70, 134.91, 137.38, 139,45, 140.22, 141.48, 141.54, 142.08, 176.03, 176.05, 185.66, 185.88. *Anal.* Calcd for $C_{20}H_{16}N_2O_2$: C, 75.93; H, 5.10; N, 8.86. Found: C, 75.94; H, 5.33; N, 8.57.

Compound 4n In the same manner as described in the synthesis of **4a**, the compound **4n** was obtained from the corresponding quinone and hydrazide in 51% yield; yellow powder; mp 210—211 °C (from $CHCl_3$–hexane); IR (KBr) cm^{-1}: 1646, 1627, 1538; ^{1}H-NMR δ: 2.03 (d, 3H, *J*=1.2 Hz), 2.11 (d, 3H, *J*=1.2 Hz), 7.11 (brs, 2H), 7.45—7.65 (m, 3H), 7.92 (m, 2H); ^{13}C-NMR δ: 15.95, 17.18, 118.64 (br), 128.52 (br), 128.77, 132.16 (br), 132.62, 136.51, 138.30, 141.58 (br), 187.47. *Anal.* Calcd for $C_{15}H_{14}N_2O_2$, C, 70.85; H: 5.55, N: 11.02. Found: C, 71.01; H, 5.71; N, 11.27.

Compound 4o In the same manner as described in the synthesis of **4a**, the compound **4o** was obtained from the corresponding quinone and hydrazide in 14% yield; orange powder; mp 160 °C (from ether); IR (KBr) cm^{-1}: 1681, 1664, 1533; ^{1}H-NMR (major : minor=2 : 1) δ (major): 1.34 (s, 9H), 6.46 (d, 1H, *J*=9.7 Hz), 7.1—7.4 (br, 2H), 7.52 (m, 2H), 7.62 (m, 1H), 7.90 (m, 2H); ^{13}C-NMR δ: 29.03, 29.21, 34.97, 35.96, 117.21, 120.71, 127.30, 128.51 (br), 128.74, 132.00, 132.53, 132.67, 134.61, 135.07, 138.56, 149.35, 152.19, 186.92. *Anal.* Calcd for $C_{17}H_{18}N_2O_2$: C, 72.32; H, 6.43; N, 9.92. Found: C, 72.28; H, 6.71; N, 9.84.

Compound 4p In the same manner as described in the synthesis of **4a**, the compound **4p** was obtained from the corresponding quinone and hydrazide in 25% yield; yellow powder; mp 210—211 °C (from $CHCl_3$); IR (KBr) cm^{-1}: 1658, 1637, 1581, 1535; ^{1}H-NMR δ: 1.28 (s, 9H), 1.33 (s, 9H), 7.05 (br, 1H), 7.29 (br, 1H), 7.51 (m, 2H), 7.60 (m, 1H), 7.89 (m, 2H); ^{13}C-NMR δ: 29.37, 29.53, 35.20, 36.22, 115.42 (br), 128.37 (br), 132.37, 133.27, 150.43, 153.72, 186.81. *Anal.* Calcd for $C_{21}H_{26}N_2O_2$: C, 74.70; H, 7.90; N, 8.28. Found, C, 74.53; H, 7.74; N, 8.38.

Compound 4q In the same manner as described in the synthesis of **4a**, the compound **4q** was obtained from the corresponding quinone and hydrazide in 25% yield; pale yellow powder; mp 206—207 °C (from $CHCl_3$–ether); IR (KBr) cm^{-1}: 1643, 1619, 1575, 1535; ^{1}H-NMR (DMSO-d_6) (major : minor=2 : 1) δ (major): 1.95 (s, 3H), 2.16 (s, 3H), 6.40 (s, 1H), 7.53 (m, 2H), 7.60 (m, 1H), 7.91 (m, 2H), 8.31 (s, 1H), 10.52 (br, 1H); ^{13}C-NMR (DMSO-d_6) δ: 15.78, 17.25, 122.36, 127.46, 128.26, 128.53, 129.04, 131.88, 132.19, 132.56, 133.13, 148.09, 165.83. *Anal.* Calcd for $C_{15}H_{14}N_2O_2$: C, 70.85; H, 5.55; N, 11.02. Found: C, 70.70; H, 5.68; N, 10.95.

Compound 4r In the same manner as described in the synthesis of **4a**, the compound **4r** was obtained from the corresponding quinone and hydrazide in 23% yield; yellow powder; mp 229—230 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1675, 1658, 1625, 1536; ^{1}H-NMR δ: 1.27 (s, 9H), 1.32 (s, 9H), 6.81 (br, 1H), 7.43 (br, 1H), 8.04 (d, 2H, J=8.8 Hz), 8.36 (d, 2H, J=8.8 Hz), 11.11 (br, 1H); ^{13}C-NMR δ: 29.37, 29.58, 35.57, 36.39, 115.46 (br), 123.20 (br), 131.19 (br), 132.53, 138.13, 149.65, 151.13, 154.50, 186.63. *Anal.* Calcd for $C_{21}H_{25}N_3O_4$: C, 65.78; H, 6.57; N, 10.96. Found: C, 65.59; H, 6.68; N, 10.81.

Compound 4s In the same manner as described in the synthesis of **4a**, the compound **4s** was obtained from the corresponding quinone and hydrazide in 30% yield; yellow powder; mp 199—200 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1650, 1633, 1606, 1540; ^{1}H-NMR (major : minor=1.1 : 1) δ (major): 1.30 (s, 9H), 3.89 (s, 3H), 6.52 (d, 1H, J=10.0 Hz), 6.99 (m, 2H), 7.22 (br, 1H), 7.46 (br, 1H), 7.94 (m, 2H), 10.14 (br, 1H); ^{13}C-NMR δ: 29.09, 29.26, 34.98, 35.96, 55.50, 113.70, 113.87, 116.68, 116.90, 120.33, 124.06, 131.10 (br), 131.61 (br), 131.90, 134.62, 135.08, 138.59, 142.32 (br), 143.48, 149.24, 152.18, 163.16, 163.24, 186.92. *Anal.* Calcd for $C_{18}H_{20}N_2O_3$: C, 69.21; H, 6.45; N, 8.97. Found: C, 68.92; H, 6.36; N, 8.87.

Compound 4t In the same manner as described in the synthesis of **4a**, the compound **4t** was obtained from the corresponding quinone and hydrazide in 5% yield; reddish powder; mp 140—141 °C (from ether–hexane); IR (KBr) cm^{-1}: 1654, 1635, 1533; ^{1}H-NMR (major : minor=2 : 1) δ (major): 1.29 (s, 9H), 6.46 (d, 1H, J=9.7 Hz), 7.14 (br, 1H), 7.35 (br, 1H), 7.48 (m, 2H), 7.87 (m, 2H), 10.19 (br, 1H); ^{13}C-NMR δ: 29.17, 35.56 (br), 120.64 (br), 128.65, 130.39, 130.93 (br), 134.57 (br), 138.96, 143.38 (br), 186.56. *Anal.* Calcd for $C_{17}H_{17}ClN_2O_2$: C, 64.46; H, 5.41; N, 8.84. Found: C, 64.69; H, 5.37; N, 8.88.

Compound 4u In the same manner as described in the synthesis of **4a**, the compound **4u** was obtained from the corresponding quinone and hydrazide in 6% yield; yellow powder; mp 160—161 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1654, 1635, 1589, 1533; ^{1}H-NMR (major : minor=1.1 : 1) δ (major): 1.29 (s, 9H), 6.52 (d, 1H, J=10.0 Hz), 7.13 (br, 1H), 7.40 (br, 1H), 7.65 (m, 2H), 7.79 (m, 2H), 10.54 (br, 1H); ^{13}C-NMR δ: 29.07, 29.29, 35.01, 36.06, 117.05, 120.52, 127.35, 127.44, 127.58, 130.82, 131.53, 131.75, 132.21, 134.76, 134.90, 138.32, 149.66, 152.58, 186.82. *Anal.* Calcd for $C_{17}H_{17}BrN_2O_2$: C, 56.52; H, 4.74; N, 7.75. Found: C, 56.39; H, 5.04; N, 7.49.

Compound 4v In the same manner as described in the synthesis of **4a**, the compound **4v** was obtained from the corresponding quinone and hydrazide in 17% yield; yellow powder; mp 205—206 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1683, 1625, 1546; ^{1}H-NMR δ: 2.04 (s, 3H), 2.11 (s, 3H), 6.96 (br, 1H), 7.64 (br, 1H), 7.74 (d, 2H, J=5.7 Hz), 8.80 (d, 2H, J=5.7 Hz), 11.17 (br, 1H); ^{13}C-NMR δ: 15.90, 17.19, 118.93 (br), 123.63 (br), 135.97, 138.88, 139.61, 142.03, 150.09 (br), 187.30. *Anal.* Calcd for $C_{14}H_{13}N_3O_2$: C, 65.87; H, 5.13; N, 16.46. Found: C, 65.84; H, 5.34; N, 16.34.

Compound 4w In the same manner as described in the synthesis of **4a**, the compound **4w** was obtained from the corresponding quinone and hydrazide in 45% yield; yellow powder; mp 193—194 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1662, 1627, 1533; ^{1}H-NMR δ: 1.28 (s, 9H), 1.30 (s, 9H), 6.82 (br, 1H), 7.54 (br, 1H), 7.69 (d, 2H, J=5.5 Hz), 8.78 (d, 2H, J=5.5 Hz), 11.63 (br, 1H); ^{13}C-NMR δ: 29.35, 29.57, 35.22, 36.35, 115.54 (br), 116.14 (br), 123.55 (br), 132.58, 140.01, 143.84 (br), 149.85 (br), 151.02 (br), 154.28, 170.48 (br), 186.69. *Anal.* Calcd for $C_{20}H_{25}N_3O_2$: C, 70.77; H, 7.42; N, 12.38. Found: C, 70.98; H, 7.62; N, 12.49.

Compound 4x In the same manner as described in the synthesis of **4a**, the compound **4x** was obtained from the corresponding quinone and hydrazide in 26% yield; pale yellow powder; mp 234—235 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1681, 1639, 1600, 1546; ^{1}H-NMR (major : minor=10 : 1) δ (major): 2.07 (s, 3H), 4.19 (s, 2H), 7.20—7.33 (m, 5H), 7.60 (t, 1H, J=7.8 Hz), 7.73 (t, 1H, J=7.8 Hz), 8.02 (d, 1H, J=7.8 Hz), 8.14 (s, 1H), 8.31 (d, 1H, J=7.8 Hz), 10.10 (s, 1H); ^{13}C-NMR (DMSO-d_6) δ: 16.60, 79.14, 123.25, 124.10, 125.59, 126.45, 126.54, 128.17, 128.30, 128.98, 129.39, 129.97, 132.66, 135.26, 135.69, 168.87, 184.23. *Anal.* Calcd for $C_{19}H_{16}N_2O_2$: C, 74.98; H, 5.30; N, 9.20. Found: C, 74.70; H, 5.56; N, 9.40.

Compound 4y In the same manner as described in the synthesis of **4a**, the compound **4y** was obtained from the corresponding quinone and hydrazide in 42% yield; yellow powder; mp 220—222 °C (from $CHCl_3$–MeOH); IR (KBr) cm^{-1}: 1666, 1644, 1598, 1577, 1544; ^{1}H-NMR (major : minor=10 : 1) δ (major): 2.26 (s, 3H), 7.46—7.65 (m, 6H), 7.90 (d, 1H, J=8.0 Hz), 7.98 (m, 2H), 8.16 (d, 1H, J=7.8 Hz), 10.59 (br, 1H); ^{13}C-NMR (DMSO-d_6) δ: 16.46, 123.36, 124.70, 125.61, 127.44, 128.25, 128.49, 128.72 (br), 129.53, 130.04, 131.93 (br), 132.62, 133.40, 134.70, 138.80, 165.83, 184.37. *Anal.* Calcd for $C_{18}H_{14}N_2O_2$: C, 74.47; H, 4.86; N, 9.65. Found: C, 74.17; H, 5.02; N, 9.70.

Reaction of NG-061 with *N*-Acetylcysteamine A mixture of compound **4a** (5.5 mg, 0.020 mmol), *N*-acetylcysteamine (2.5 μl, 1.1 eq), and triethylamine (11 μl, 4 eq) in $CHCl_3$ (0.5 ml) was stirred at room temperature for 2 d. After evaporation of the solvent, the residue was purified by preparative TLC to give a product (3.3 mg, 42%) as yellow amorphous; ^{1}H-NMR δ: 2.05 (s, 3H), 2.95 (m, 2H), 3.45 (m, 2H), 3.80 (s, 3H), 4.15 (br s, 2H), 5.85 (s, 1H), 7.14—7.42 (m, 5H), 7.82 (s, 1H), 11.40 (br, 2H); MS: *m/z* [rel. intensity]; 388 [100%, $(M+H)^+$], 345 [7%, $(M+H)^+-CH_3CO$], 270 [72%, $(M+H)^+-SCH_2CH_2NHAc$].

Assay for Neurotrophic Activity in PC12 Cells PC12 cells were obtained from the RIKEN Cell Bank and maintained as monolayer culture in DULBECCO's modified Eagle's medium (DMEM, GIBCO) with 10% heat-inactivated fetal bovine serum (FBS), 5% horse serum (HS) 50U penicillin G and 50 μg/ml streptomycin. The cells were kept in a humidified incubator at 37 °C and 5% CO_2, then plated on collagen-coated 24-well plates (Corning) at a density of 1×10^4 per well. After 24 h of culture, NG-061 and its analogs at the concentration of 10 μg/ml were added to the medium in the presence of 0.5 ng/ml of NGF. The cells were further incubated for 48 h. To evaluate activity, 100 cells were observed under a phase-contrast microscope with scoring (round cells: 0, morphologically changed cells without neurite: 1, cells with neurites shorter than the diameter of the cell body: 2, cells with neurites longer than the diameter of the cell body: 3). One hundred cells were scored from a randomly chosen field and this was repeated 3 times (300 cells scored in total).

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Synthesis of 15α-Fluoro-24,25-dihydrolanosterol as a Potential Inhibitor and/or Mechanistic Probe for Lanosterol 14α-Demethylase

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As a potential inhibitor and/or mechanistic probe for lanosterol 14α-demethylase, 15α-fluoro-24,25-dihydrolanosterol was prepared by fluorination of 15α-hydroxy-24,25-dihydrolanost-7-en-3β-yl benzoate with diethylaminosulfur trifluoride, followed by hydrogen chloride-catalyzed isomerization of the Δ^7 to Δ^8 and reductive cleavage of the benzoate.

Key words lanosterol; 14α-demethylase; fluorination; diethylaminosulfur trifluoride; cholesterol; lanost-9-en-3β-ol

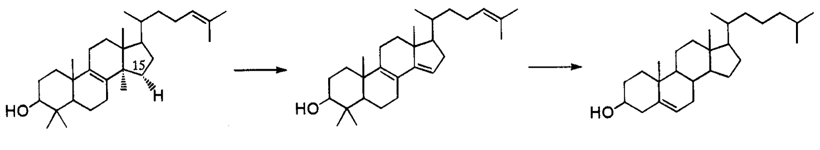
One of the key steps of cholesterol biosynthesis is 14α-demethylation of lanosterol. During this carbon–carbon bond cleavage reaction by cytochrome P-450$_{14DM}$, the 15α-hydrogen is concomitantly eliminated to yield 8,14-lanostadien-3β-ol (Chart 1).[1] It is reasonably assumed therefore, that 15α-fluorinated analogs of lanosterol, *e.g.* Δ^7- and Δ^8-15α-fluoro-24,25-dihydrolanosterol (**2**, **1**, Chart 2) would be inhibitor and/or mechanistic probes of lanosterol 14α-demethylase, in view of a similar steric bulkiness of fluorine to hydrogen atoms and yet the stronger bond of carbon–fluorine compared to carbon–hydrogen.[2] Complexation of 14α-demethylase with these fluorinated compounds would allow structure determination by X-ray crystallography.

Chart 2 represents our retrosynthetic route to the target compounds **1** and **2**. The 15-fluorine would be introduced by fluorination with diethylaminosulfur trifluoride (DAST) on the corresponding 15-hydroxy compounds **3** and **4**, which should be obtained by hydride reduction of 15-ketones **5** and **6** prepared from the commercially available 7-dehydrocholesterol (**7**) in several steps. Acid-catalyzed isomerization of the Δ^7-bond in **2** into the Δ^8-bond would be an alternative way leading to **1**.

Results and Discussion

Several synthetic routes including Woodward's classical method have been reported for Δ^7- and Δ^8-4,4,14-trimethyl-15-ketone **5** and **6**.[3] Our presently used route is depicted in Chart 3.

Oppenauer oxidation of 7-dehydrocholesterol (**7**) gave 4,7-dien-3-one **8**, and the subsequent methylation with iodomethane–potassium *tert*-butoxide yielded 4,4-dimethylketone **9** in 84% yield from **7**.[4] Hydride reduction of the latter gave the 3β-ol **10**, which was then hydrogenated in the presence of 10% Pd–carbon in ethyl acetate to afford $\Delta^{8(14)}$-olefin **11a**[4] as a single product in 80% yield. Although acid-catalyzed isomerization of various Δ^7- and $\Delta^{8(14)}$-olefins into the Δ^{14}-isomer has been well documented,[5] we reexamined this reaction by treatment of a 6.5 mM dichloromethane solution of the $\Delta^{8(14)}$-benzoate **11b** with hydrogen chloride at −20 °C, followed by HPLC and ^{1}H-NMR analysis. It can be seen from Fig. 1 that at least three compounds corresponding to peaks 1, 2 and 3, appeared in the course of reaction. The expected Δ^{14}-compound **14** (peak 1) was indistinguishable from the starting $\Delta^{8(14)}$-olefin **11b** on HPLC,[6] but it was easily discerned by the C-15 olefinic signal at 5.17 ppm. This Δ^{14}-olefin **14** was the major product during the initial 1 h, and was isolated by recrystallization (60—70% yield). The second product (peak 2) was assigned to the 17β(H)-isomer **13** of Δ^{14}-olefin **14** from the characteristic ^{1}H-NMR signals at 5.08 (15-H).[5c] The last appearing compound **12** (peak 3), showed no olefinic proton in the ^{1}H-NMR and was transformed cleanly into the 17β(H)-olefin **13** by treatment with triethylamine. These behaviors are in accord with those reported for the 4-demethyl analog having 14β-chloro-17β(H)-skeleton.[5c] In conjunction with the published discussion on the analogous reaction,[5c] Chart 4 depicts for the course of the HCl-catalyzed reaction of $\Delta^{8(14)}$-olefin **11b**. In this scheme, formation of the intriguing 17β(H)-compounds **12** and **13** would be explained by protonation of the postulated spiro intermediate in parentheses at the C-17 position from the front side (β).[5c] Conversion of Δ^{14}-olefin **14** to Δ^8-14β(H)-15-one **19** was carried out according to the previously described procedures.[7] Thus, epoxidation of **14** with *m*-chloroperbenzoic acid (MCPBA) gave 14α,15α-epoxide **15** in 64% yield, which was then treated with BF$_3$-etherate in benzene at 25 °C to give Δ^8-14β(H)-15α-ol **16** in 78% yield, together with the conjugated dienic compounds (6% yield) including $\Delta^{8,14}$-diene **17**. Oxidation of **16** with pyridinium chlorochromate (PCC) gave 15-ketone **19** in 86% yield. The same ketone **19** was also prepared in 40% yield from



lanosterol

cholesterol

Chart 1. Elimination of 15α-Hydrogen during 14-Demethylation of Lanosterol

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Dedicated to the memory of Dr. Kyosuke Tsuda.

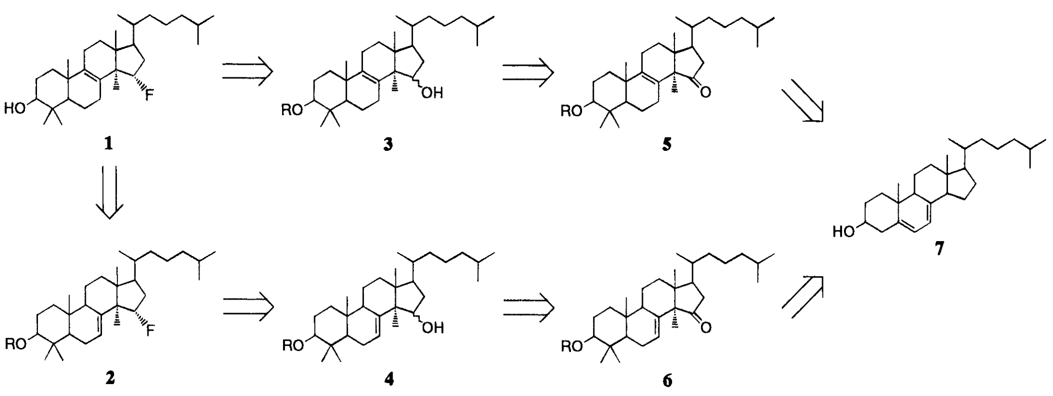

Chart 2. Retrosynthesis of 15α-Fluoro-24,25-dihydrolanosterol (**1**)

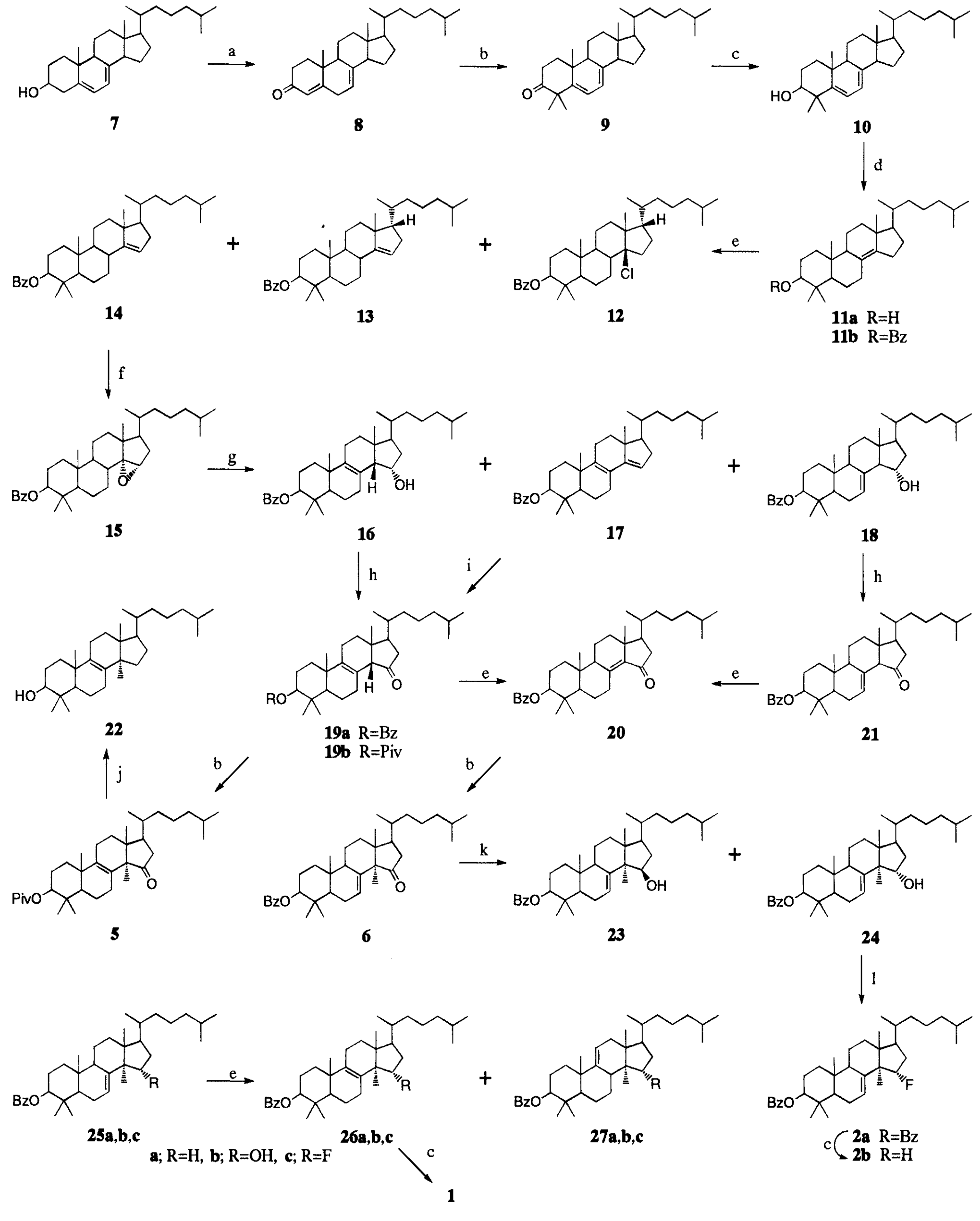

Chart 3. Synthesis of 15α-Fluoro-24,25-dihydrolanosterol (**1**)

Reagents: a, cyclohexanone–Al(iso-PrO)$_3$; b, CH$_3$I-*tert*-BuOK; c, LiAlH$_4$; d, H$_2$–10%Pd–C; e, HCl; f, MCPBA; g, BF$_3$–etherate; h, PCC; i, 1) MCPBA; 2) BF$_3$–etherate; j, NH$_2$NH$_2$–KOH; k, NaBH$_4$; l, DAST.

the 8,14-diene **17** by epoxidation with MCPBA followed by treatment with BF_3-etherate. The intermediary $\Delta^{8(9)}$-$14\alpha,15\alpha$-epoxide was thought to be opened by the action of the Lewis acid with a concomitant migration of the C-15β hydrogen to the C-14 position. In the case of the epoxide **15**, epoxide opening should be followed by the C-8β hydrogen migration to the C-14 position and elimination of the C-9 hydrogen. When the reaction of the epoxide **15** with BF_3-etherate was conducted at 5—7 °C, instead of 25 °C, an isomeric alcohol **18** was also produced in addition to the above mentioned **16** (**16** : **18**, *ca.* 1 : 1). That this compound is Δ^7-15α-ol **18**, was deduced from NMR analysis (Experimental) and its facile transformation into $\Delta^{8(14)}$-15-ketone **20** by PCC oxidation followed by acidic isomerization. In comparison, isomerization of the $\Delta^{8(9)}$-ketone **19** into **20** required a prolonged acidic treatment at a higher temperature (Experimental).

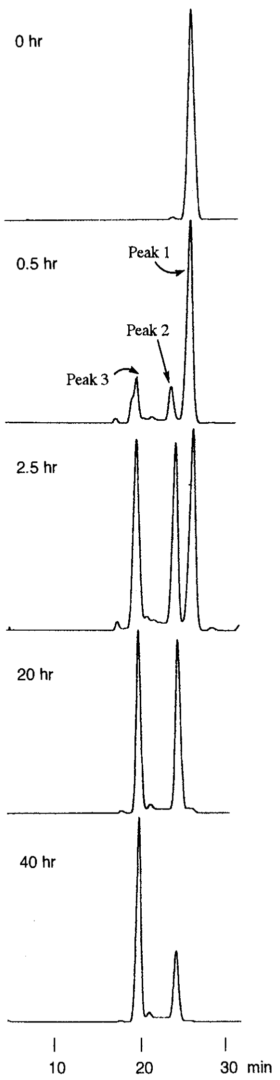

Fig. 1. Time Course of HCl-Catalyzed Isomerization of $\Delta^{8(14)}$-Olefin **11b** as Analyzed by HPLC

Peaks 1, 2 and 3 are due to Δ^{14}-olefin **14**, 17β(H)-Δ^{14}-olefin **13**, and 17β(H)-14β-chloride **12**, respectively.

Having synthesized the $\Delta^{8(9)}$- and $\Delta^{8(14)}$-15-ketones **19** and **20**, we turned our attention to introduction of the 14α-methyl and 15α-fluoro groups. Methylation of the $\Delta^{8(14)}$-15-one **20** with iodomethane and potassium *tert*-butoxide in *tert*-butanol gave the Δ^7-14α-methylketone **6** in 79% yield. Similar reaction on the $\Delta^{8(9)}$-15-one **19b** derived from the 3-pivaloate analog of **14**,[7] afforded the Δ^8-14α-methylketone **5** in 63% yield. The orientation at the C-14 position of **5** was established as 14α-methyl by its transformation to 24,25-dihydrolanosterol (**22**) as described previously.[7] Reduction of the 15-ketone **6** with sodium borohydride did not go to completion even by the use of a large excess of reagent, but the requisite 15β- and 15α-ols **23** and **24** were obtained by silica gel column chromatography *via* tetrahydropyranyl ether of 15β-ol **23** (Experimental). Fluorination with DAST at the C-15 position was at first studied with the 4-demethyl analogs of **3** and **4**.[8] It was found that only the Δ^7-15α-ol derivative gave the expected 15α-fuorinated product, whereas the other three alcohols, *i.e.* Δ^7-15β-ol, Δ^8-15α-ol and Δ^8-15β-ol yielded a complex mixture of olefinic compounds. When Δ^7-15α-ol **24** was treated with DAST, 15α-fluoride **2a** was obtained in 36% yield. Reduction of **2a** with $LiAlH_4$ afforded **2b**. The stereochemistry of **2a** was determined as 15α-fluoro by NMR analysis. Thus, the H–F coupling (J=3.7 Hz) and C–F coupling (J=8.1 Hz) were observed between 14-Me and 15-F, while no such a coupling was observed between 13-Me and 15-F.[9] The vicinal coupling pattern of 15-H (dd, J=9.5, 5.2 Hz) was similar to that of 15α-hydroxy compound **24** (dd, J=9.5, 6.5 Hz) but distinct from that of the 15β-hydroxyl isomer **23** (d, J=4.5 Hz). Further, an Nuclear Overhauser effect (NOE) was observed between 15-H and 13-Me in compounds **2a** and **24**, and between 15-H and 14-Me in compound **23**. It is therefore concluded that the fluorine substitution reaction of 15α-ol **24** occurred with retention of the configuration. This behavior of stereochemical course is not unprecedented in the reaction of some steroidal alcohols with DAST.[10]

Because the expected Δ^8-15α-fluoro compound **26c** was not obtained by substitution reaction of 15-hydroxyl group, we were obliged to depend on the isomerization reaction of Δ^7-15α-fluoride **2a**=**25c** to reach the final target **1**. It was revealed in the present experiment that hydrogen chloride-catalyzed isomerization reaction of Δ^7-olefins[3] is highly dependent both on reaction temperature and the substituent at the

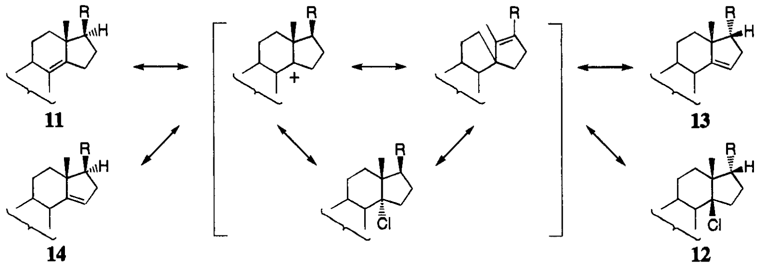

Chart 4. HCl-Catalyzed Isomerization of $\Delta^{8(14)}$-Olefin **11b**

C-15 position. When the 3.9 mM dichloromethane solution of lanost-7-en-3β-yl benzoate (**25a**) was bubbled with hydrogen chloride at -78 °C for 5 min, a 4 : 3 mixture of Δ^7-olefin **25a** and Δ^8-isomer **26a** was obtained, as revealed by HPLC and ^{1}H-NMR. However, prolonged (1.5 h or longer) reaction time produced another olefin **27a** (**25a** : **26a** : **27a** = 1 : 1 : 9). ^{1}H-NMR of this crude product indicated characteristic signals at 5.25, 0.76 and 0.65 ppm which coincided with the published[11] chemical shifts of 11-H, and 14-Me and 13-Me, respectively of $\Delta^{9(11)}$-14α-methylsterols. Although **27a** has not yet been isolated in a pure state, it can safely be assigned to 24,25-dihydroparkeol benzoate. Reaction at a higher reaction temperature, *e.g.* -10 or -30 °C for 2 h yielded **25a** and **26a** in a 1 : 1 ratio, with negligible formation of **27a**. These results suggested that the isomerization reaction occurs more efficiently at a lower temperature, and the equilibrium between Δ^7 and Δ^8 is rapidly attained and then is gradually biased toward probably the most stable Δ^9-isomer. On the other hand, the 15α-hydroxyl compound **25b** (and also its acetate derivative) isomerized more reluctantly, and almost no reaction occurred at -10 °C, while at -78 °C, a 2 : 3 mixture of Δ^7- and Δ^8-olefin (**25b**, **26b**) was produced. The reactivity of the 15α-fluoride **25c** appeared to be intermediate between **25a** and **25b**, and the reaction at -78 °C for 2 h gave a mixture of Δ^7-, Δ^8- and Δ^9-olefin (**25c**, **26c**, **27c**) in a 4 : 6 : 3 ratio. The Δ^8-isomer **26c** was isolated by preparative HPLC (10% yield from **25c**), and subsequent treatment with LiAlH$_4$ afforded **1**.

Moderate inhibitory activity of Δ^7-15α-fluoride **2b** on hepatic microsomal lanosterol 14α-demethylase has been already demonstrated by us and others.[12,13]

Experimental

^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-A500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer in CDCl$_3$ solutions and chemical shifts (δ) are reported in ppm downfield from tetramethylsilane (used as an internal reference), while ^{13}C chemical shifts are expressed relative to CDCl$_3$ (δ 77.0). MS (70 eV) were obtained on a JEOL JMS-700 or JMS-DX-303. HPLC was carried out on a Shimadzu LC-6A instrument with an SPD-6A UV detector using Shim-pack octadecyl dimethylsiloxylated silica (ODS) column (15 cm×4 mm i.d.). Merck Kieselgel F$_{254}$ plates and Merck Kieselgel 60 were used for thin layer chromatography (TLC) and column chromatography, respectively. SGC refers to silicagel column chromatography. Extractive work-up refers to dilution of a reaction mixture with water, extraction with the given organic solvent, washing of the extract to neutrality, drying over MgSO$_4$, decantation, and removal of the solvent under reduced pressure. All recrystallization in this work was carried out by the use of methanol–CH$_2$Cl$_2$.

4,4-Dimethyl-8(14)-cholesten-3β-yl Benzoate (11b) A mixture of 7-dehydrocholesterol (**7**) (100 g, 0.26 mol, VITAMINS-INC), toluene (1 l), cyclohexanone (200 ml) and aluminum isopropoxide (28 g, 0.14 mol) was refluxed with the aid of a Dean–Stark trap for 2 h. Extractive work-up (AcOEt) and SGC with hexane–AcOEt 20 : 1 gave 4,7-dien-3-one **8** (*ca.* 100 g), contaminated with cyclohexanol. This was, without further purification, dissolved in tetrahydrofuran (THF) (120 ml) and was added dropwise into the potassium *tert*-butoxide solution prepared from potassium (28 g, 0.72 mol) and *tert*-butanol (1.5 l) during 40 min. Then iodomethane (220 ml, 3.5 mol) was added dropwise during 40 min, and the mixture was stirred for 30 min. Extractive work-up (hexane) and recrystallization gave 4,4-dimethyldienone **9**[4] (91 g, 84% from **7**). To a solution of **9** (49 g, 0.12 mol) in THF (500 ml) was added slowly LiAlH$_4$ (6.7 g, 0.18 mol)), and the mixture was stirred for 1.5 h. Extractive work-up (AcOEt, hexane) and recrystallization gave 3β-ol **10** (31 g, 63%). This compound **10** (210 mg, 0.51 mmol) was stirred with 10% Pd–C (50 mg) in AcOEt (12 ml) under H$_2$. Filtration and solvent evaporation gave 8(14)-en-3β-ol **11a**, which was treated with benzoyl chloride–pyridine to give benzoate **11b** (200 mg, 76%), mp 132—133 °C. ^{1}H-NMR δ: 0.82, 0.85, 0.96, 1.05 (3H×4, s, 4α-, 4β-, 10-, 13-Me), 4.92 (1H, m, 3-H). *Anal.* Calcd for C$_{36}$H$_{54}$O$_2$: C, 83.34; H, 10.49. Found: C, 83.22; H, 10.29.

Treatment of 8(14)-Olefin 11b with HCl To a solution of **11b** (50 mg, 0.097 mmol) in CH$_2$Cl$_2$ (15 ml) was treated with a stream of HCl evolved by mixing conc. HCl with conc. H$_2$SO$_4$ at -20 °C. An aliquot (*ca.* 1 ml) was taken out from the reaction mixture at 0.25, 0.5, 1, 2.5, 20 and 45 h and shaken with NaHCO$_3$ solution. Drying (MgSO$_4$) and solvent evaporation gave the crude product, which was analyzed with HPLC (Fig. 1) and ^{1}H-NMR.

4,4-Dimethyl-14-cholesten-3β-yl Benzoate (14) The 0.5 h-reaction product above was recrystallized to give 14-olefin **14** (60—70% yields on preparative scale reactions), mp 153—155 °C. ^{1}H-NMR δ: 0.90, 0.93, 0.96, 1.05 (3H×4, s, 4α-, 4β-, 10-, 13-Me), 4.73 (1H, m, 3-H), 5.17 (1H, m, 15-H). *Anal.* Calcd for C$_{38}$H$_{54}$O$_2$: C, 83.34; H. 10.49. Found: C, 83.39; H, 10.50.

Conversion of 14β-Chloride 12 into 17β(H)-14-Olefin (13) The 45 h-reaction product (45 mg) above, the major component of which should be 14β-chloride **12** (Fig. 1) showing ^{1}H-NMR δ: 0.93, 0.94, 1.03, 1.20 (3H×4, s, 4α-, 4β-, 10-, 13-Me),[5c] was treated with triethylamine (50 μl) in methanol (1 ml)–CH$_2$Cl$_2$ (0.5 ml) at room temperature for 1 h. Solvent evaporation and SGC with hexane–AcOEt 80 : 1 gave an oil (40 mg). HPLC revealed almost a single peak at the retention time corresponding to peak 2 (compound **13**) in Fig. 1. ^{1}H-NMR δ: 0.93, 0.99, 1.05, 1.11 (3H×4, s, 4α-, 4β-, 10-, 13-Me), 4.72 (1H, m, 3-H), 5.10 (1H, m, 15-H).

14α,15α-Oxido-4,4-dimethylcholestan-3β-yl Benzoate (15) To a solution of 14-olefin **14** (1.12 g, 2.1 mmol) in CH$_2$Cl$_2$ was added MCPBA (0.7 g, 4.1 mmol) at 0 °C. One hour later the precipitate was removed by filtration and the filtrate was diluted with CH$_2$Cl$_2$. Extractive work-up (CH$_2$Cl$_2$) gave solid (1.3 g), which was purified by SGC with hexane–AcOEt 50 : 1 and recrystallization to give epoxide **15** (740 mg, 64%), mp 184—185 °C. ^{1}H-NMR δ: 0.85, 0.91, 0.97, 1.03 (3H×4, s, 4α-, 4β-, 10-, 13-Me), 3.32 (1H, s, 15-H), 4.72 (1H, m, 3-H). *Anal.* Calcd for C$_{36}$H$_{54}$O$_3$: C, 80.85; H, 10.18. Found: C, 80.94; H, 10.27.

Reaction of Epoxide 15 with BF$_3$-Etherate a) At 25 °C: To a solution of epoxide **15** (140 mg, 0.26 mmol) in benzene (1.0 ml) was added BF$_3$–etherate (40 μl, 0.3 mmol) at 25 °C and the mixture was allowed to stand at 25 °C for 0.5 h. Extractive work-up (AcOEt) gave solid (140 mg), and then SGC with hexane–AcOEt 80 : 1 and 10 : 1 gave a 1 : 4 mixture (8 mg, 6%) of 7- and 8-ene **17** and 14β(H)-15α-hydroxy-4,4-dimethyl-8(9)-cholesten-3β-yl benzoate (**16**), (109 mg, 78%), mp 155—156 °C. ^{1}H-NMR δ: 0.92, 0.97, 1.06, 1.08 (3H×4, s, 4α-, 4β-, 10-, 13-Me), 4.10 (1H, m, 15-H), 4.75 (1H, m, 3-H). *Anal.* Calcd for C$_{36}$H$_{54}$O$_3$: C, 80.85; H, 10.18. Found: C, 80.56; H, 10.30.

b) At 5 °C: To a solution of epoxide **15** (550 mg, 1.0 mmol) in benzene (7 ml) was added BF$_3$-etherate (0.14 ml, 1.1 mmol) at 5 °C for 8 min. Extractive work-up (AcOEt) gave solid (540 mg), which was subjected to SGC with hexane–AcOEt 80 : 1 to give the olefinic compounds (54 mg, 9%), 8(9)-en-15α-ol **16** (210 mg, 38%) and 15α-hydroxy-4,4-dimethyl-7-cholesten-3β-yl benzoate (**18**) (254 mg, 46%), mp 155—156 °C. ^{1}H-NMR δ: 0.87, 0.93, 0.96, 1.12 (3H×4, s, 14α-, 4β-, 10-, 13-Me), 4.05 (1H, m, 15-H), 4.78 (1H, m, 3-H), 5.54 (1H, m, 7-H). *Anal.* Calcd for C$_{36}$H$_{54}$O$_3$: C, 80.55; H, 10.18. Found: C, 80.57; H, 10.32.

14β(H)-15-Oxo-4,4-dimethyl-8(9)-cholesten-3β-yl Benzoate (19a) a) To a stirred suspension of PCC (24 g, 110 mmol) in CH$_2$Cl$_2$ (200 ml) was added a solution of 8(9)-en-15α-ol **16** (38 g, 71 mmol) in CH$_2$Cl$_2$ (100 ml). Two hours later the reaction mixture was filtered through Florisil and SGC with hexane–AcOEt 30 : 1 gave 8(9)-en-15-one **19a** (33 g, 86%), mp 169—170 °C. ^{1}H-NMR δ: 0.95, 0.98, 1.04, 1.06 (3H×4, s, 4α-, 4β-, 10-, 13-Me), 4.73 (1H, m, 3-H). *Anal.* Calcd for C$_{36}$H$_{52}$O$_3$: C, 81.15; H, 9.84. Found: C, 80.87; H, 10.03.

b) To a stirred solution of 8,14-diene **17** (200 mg, 0.39 mmol) in diethyl ether (5.4 ml) was added MCPBA (155 mg, 0.90 mmol) under cooling with an ice-water bath. Ten minutes later the precipitates were removed by filtration, and the filtrate was diluted with benzene (10 ml). Most of the ether was evaporated off under reduced pressure, and to the resultant epoxide solution in benzene (*ca.* 2 ml) was added BF$_3$-etherate (50 ml, 0.38 mmol) at 5 °C. Extractive work-up (AcOEt) 15 min later, gave an oil (210 mg), which was subjected to SGC with hexane–AcOEt 15 : 1 to give **19a** (83 mg, 40%), identical (HPLC, ^{1}H-NMR) with the sample obtained from **16** (*vide supra*).

15-Oxo-8-lanosten-3β-yl Pivaloate (5) To the potassium *tert*-butoxide solution prepared from potassium (3.5 g, 90 mmol) and *tert*-butanol (200 ml) was added dropwise a solution of 14β(H)-15-oxo-4,4-dimethyl-8(14)-cholesten-3β-yl pivaloate[7] (**19b**) (12.6 g, 25 mmol) in THF (50 ml) at 30—40 °C during 10 min. After cooling to room temperature, iodomethane

(16 ml, 0.26 mol) was added dropwise at 10—15 °C. The mixture was stirred at room temperature overnight and then concentrated to *ca.* 60 ml by vacuum distillation. Extractive work-up (diethyl ether) gave a solid (11.8 g) which was recrystallized to give 14α-methyl-15-one **5** (8.2 g, 63%), mp 196—198 °C. ^{1}H-NMR δ: 0.76, 0.88, 0.90, 1.01, 1.09 (3H×5, s, 4α-, 4β-, 10-, 13, 14-Me), 1.57 (6H, s, pivaloyl Me). MS *m/z*: 526.4408 (Calcd for $C_{35}H_{58}O_3$: 526.4386).

15-Oxo-7-lanosten-3β-yl Benzoate (6) To the stirred potassium *tert*-butoxide solution prepared from potassium (7.0 g, 0.18 mol) and *tert*-butanol (400 ml) was added dropwise a solution of 8(14)-en-15-one **20** (27 g, 52 mmol) in THF (70 ml) over 10 min. After 15 min iodomethane (32 ml, 0.52 mol) was added dropwise, and the mixture was stirred at room temperature for 1.2 h. Extractive work-up (diethyl ether) and recrystallization gave 14α-methyl-15-one **6** (22 g, 79%), mp 205—207 °C. ^{1}H-NMR δ: 0.76, 0.95, 0.95, 1.13, 1.20 (3H×5, s, 4α-, 4β-, 10-, 13-, 14-Me), 4.76 (1H, m, 3-H), 6.53 (1H, d, J=6.0 Hz, 7-H). MS *m/z*: 546.4120 (Calcd for $C_{37}H_{54}O_3$: 546.4073).

15-Oxo-4,4-dimethyl-8(14)-cholesten-3β-yl Benzoate (20) a) A solution of 8(9)-en-15-one **19a** (140 mg, 0.26 mmol) in ethanol (4.5 ml)–CH_2Cl_2 (1.5 ml) containing conc. HCl (0.2 ml) was heated at 70 °C for 8 h. Extractive work-up (AcOEt) gave an oil (130 mg), which was subjected to SGC with hexane–AcOEt 15 : 1 to give 8(14)-en-15-one **20** (60 mg, 42%), mp 155—157 °C. ^{1}H-NMR δ: 0.85, 0.98, 0.99, 1.06 (3H×4, s, 4α-, 4β-, 10- and 13-Me), 4.21 (1H, m, 7b-H), 4.80 (1H, m, 3-H). MS *m/z*: 532.3873 (Calcd for $C_{36}H_{52}O_3$: 532.3916).

b) A solution of 7-en-15-one **21** (18 mg, 0.034 mmol) in ethanol (0.9 ml)–CH_2Cl_2 (0.3 ml) containing conc. HCl (50 μl) was heated at 70 °C for 1 h. Extractive work-up (AcOEt) gave **20** (17 mg, 94%) identical (^{1}H-NMR) with the substance derived from **19a**.

15-Oxo-4,4-dimethyl-7-cholesten-3β-yl Benzoate (21) To a stirred suspension of PCC (100 mg, 0.46 mmol) in CH_2Cl_2 (1.5 ml) was added 7-en-15α-ol **18** (120 mg, 0.22 mmol) in CH_2Cl_2 (0.7 ml). Three hours later the reaction mixture was filtered through Florisil and then subjected to SGC with hexane–AcOEt 50 : 1 to give 7-en-15-one **21** (81 mg, 67%), mp 205—205 °C. ^{1}H-NMR δ: 0.89, 0.98, 1.13, 1.13 (3H×4, s, 4α-, 4β-, 10-, 13-Me), 4.75 (1H, m, 3-H), 5.49 (1H, m, 7-H). *Anal.* Calcd for $C_{36}H_{52}O_2$: C, 81.15; H, 9.84. Found: C, 81.26; H, 9.97.

24,25-Dihydrolanosterol (22) A mixture of 8-en-15-one **5** (180 mg, 0.34 mmol), diethyleneglycol (15 ml) and hydrazine hydrate (80%, 7.2 ml) was heated at 150 °C under N_2 for 4 h. After cooling to room temperature, KOH (600 mg) was added and the mixture was heated at 230 °C for 5 h. Extractive work-up (CH_2Cl_2) and then SGC with hexane–AcOEt 30 : 1 gave 24,25-dihydrolanosterol (**22**, 71 mg, 48%), mp 148 °C identical with a natural sample given by Prof. S. Sato and Y. Sonoda of this college.

Reduction of 7-En-15-one 6 with $NaBH_4$ To a stirred solution of 7-en-15-one **6** (400 mg, 0.73 mmol) in THF (16 ml)–methanol (16 ml) was added $NaBH_4$ (400 mg, 11 mmol) and the mixture was stirred at room temperature for 2 h. Further $NaBH_4$ (300 mg, 7.9 mmol) was added and stirring was continued overnight. Extractive work-up (AcOEt) gave solid (400 mg). ^{1}H-NMR and HPLC of the crude product revealed the presence of 15-ketone **6**, 15β-ol **23** and 15α-ol **24** in a 2 : 3 : 10 ratio. SGC with hexane–AcOEt 30 : 1 gave 15α-ol **24** (190 mg, 47%), mp 227—228 °C. ^{1}H-NMR δ: 0.72, 0.94, 0.95, 1.05, 1.13 (3H×5, s, 4α-, 4β-, 10-, 13-, 14-Me), 4.25 (1H, dd, J=9.5, 6.5 Hz, 15-H), 4.77 (1H, m, 3-H), 5.49 (1H, m, 7-H). MS *m/z*: 548.4275 (Calcd for $C_{37}H_{56}O_3$: 548.4229). Separation of 15β-ol **23** from the recovered ketone **6** needed derivatization of **23** to the tetrahydropyranyl ether (dihydropyran/*p*-toluenesulfonic acid in CH_2Cl_2) followed by SGC with hexane–AcOEt 80 : 1. Acidic treatment (HCl/methanol–CH_2Cl_2) afforded 15β-ol **23** (45 mg, 11%), mp 204—205 °C. ^{1}H-NMR δ: 0.95, 0.98, 0.98, 1.00, 1.13 (3H×5, s, 4α-, 4β-, 10-, 13-, 14-Me), 4.05 (1H, d, J=4.5 Hz, 15-H), 4.77 (1H, m, 3-H), 5.59 (1H, d, J=5.0 Hz, 7-H). MS *m/z*: 548.4181 (Calcd for $C_{37}H_{56}O_3$: 548.4229).

15α-Fluoro-7-lanosten-3β-yl Benzoate (2a) To a solution of 15α-ol **24** (50 mg, 0.09 mmol) in CH_2Cl_2 (3 ml) was added DAST (24 ml, 0.18 mmol) at −10 °C under N_2, and the mixture was allowed to stand at −10 °C for 0.5 h. Extractive work-up (CH_2Cl_2) gave solid (51 mg), whose HPLC with methanol (1.8 ml/min) revealed prominent peaks at 12.0 and 13.5 min. SGC with hexane–AcOEt 50 : 1 followed by recrystallization gave 15α-fluoride **2a** (18 mg, 36%), mp 231—232 °C (lit.[13] mp 226—227 °C). ^{1}H-NMR δ: 0.70, 0.94, 0.95, 1.13 (3H×4, s, 4α-, 4β-, 10-, 13-Me), 1.10 (3H, d, J=3.0 Hz, 14-Me), 4.77 (1H, m, 3-H), 5.02 (1H, m, 15-H), 5.52 (1H,m, 7-H). *Anal.* Calcd for $C_{37}H_{55}FO_2$: C, 80.68: H, 10.06. Found: C, 80.40; H, 10.14.

15α-Fluoro-7-lanosten-3β-ol (2b) To a stirred solution of the benzoate **2a** (100 mg, 0.18 mmol) in THF (3 ml) was added $LiAlH_4$ (50 mg, 1.3 mmol) at 0 °C and the mixture was stirred at 0 °C for 10 min and then at room temperature for 1 h. Extractive work-up (AcOEt) and recrystallization gave 3β-ol **2b** (56 mg, 70%), mp 175—176 °C (lit.[13] mp 172.5—173 °C). ^{1}H-NMR δ: 0.69, 0.87, 0.89, 0.99, (3H×4, s, 4α-, 4β-, 10-, 13-Me), 1.07 (1H, d, J=3.0 Hz, 14-Me), 3.25 (1H, m, 3-H), 5.02 (1H, m, 15-H), 5.49 (1H, m, 7-H). *Anal.* Calcd for $C_{30}H_{51}FO$: C, 80.66; H, 11.51. Found: C, 80.64; H, 11.44.

Isomerization of 7-Lanostene Derivatives (25a, b, c) with HCl HCl gas was evolved by mixing conc. HCl with conc. H_2SO_4 and introduced into a solution of 7-lanostene derivatives (**25a**, **b**, **c**, 27 mmol) in CH_2Cl_2 (7 ml) at −10, −30 or −78 °C for the given time. Excess HCl was removed by bubbling with Ar, and the CH_2Cl_2 solution was shaken with $NaHCO_3$ solution and H_2O, dried ($MgSO_4$) and evaporated to dryness. The residue was analyzed by HPLC and/or ^{1}H-NMR. Diagnostic signals are: 0.66 (13-Me) and 5.22 (7-H) for 7-ene **25a**, 0.70 (13-Me) for 8-ene **26a**, and 0.65 (13-Me), 0.76 (14-Me) and 5.25 (11-H) for 9-ene **27a**. Increasing order of retention time on HPLC with CH_3CN–CH_3OH 2 : 1 was **25a**<**27a**<**26a**, but the three isomers were not completely resolved. Reaction of **25a** at −78 °C for 5 min gave **25a** and **26a** in a 4 : 3 ratio; at −78 °C for 15 min gave **25a**, **26a** and **27a** in a 1 : 1 : 3 ratio; at −78 °C for 2h gave **25a**, **26a** and **27a** in a 1 : 1 : 9 ratio; at −30 and −10 °C for 2 h gave **25a** and **25b** in a 1 : 1 ratio. Reaction of 15α-hydroxyl compound **25b** at −78 °C for 2 h gave **25b** and **26b** in a 2 : 3 ratio, while the corresponding 15α-acetoxyl derivative gave 7-ene and 8-ene in a 1 : 2 ratio. Reaction of 15α-fluoride **25c** at −78 °C for 2 h gave **25c**, **26c** and **27c** in a 4 : 6 : 3 ratio.

15α-Fluoro-8-lanosten-3β-yl Benzoate (26c) For isolation of **26c**, the reaction products of several runs of **25c** were combined to give solid (200 mg) and this was subjected to preparative HPLC using Shim-pack PREP-ODS (0.25 m×20 mm i.d.) and CH_3CN–CH_3OH–THF 4 : 2 : 1 (8.0 ml/min). Subsequent recrystallization gave **26c** (54 mg), mp 208—209 °C. ^{1}H-NMR δ: 0.73, 0.97, 1.05, 1.06 (3H×4, s, 4α-, 4β-, 10-, 13-Me), 0.99 (3H, d, J=3.5 Hz, 14-Me), 4.75 (1H, m, 3-H), 5.01 (1H, m, 15-H). *Anal.* Calcd for $C_{37}H_{55}FO_2$: C, 80.68; H, 10.06. Found: C, 80.71; H, 10.18.

15α-Fluoro-8-lanosten-3β-ol (1) The benzoate **26c** (54 mg, 0.10 mmol) was treated with $LiAlH_4$ (50 mg, 1.3 mmol) in THF (3 ml) at 0 °C for 5 min and then at room temperature overnight. Extractive work-up (AcOEt) and SGC with hexane–AcOEt 10 : 1 gave 3β-ol **1** (32 mg, 73%), mp 164—165 °C. ^{1}H-NMR δ: 0.71 (3H, s, 13-Me), 0.82 (3H, s, 4β-Me), 0.97 (3H, d, J=3.5 Hz, 14-Me), 0.98 (3H, s, 10-Me), 1.01 (3H, s, 4α-Me), 3.24 (1H, m, 3-H), 5.00 (1H, ddd, J=57.0, 9.5, 5.0 Hz, 15-H). ^{13}C-NMR δ: 15.42 (29-C), 15.93 (18-C), 17.45 (d, J=12.4 Hz, 30-C), 18.19 (6-C), 18.42 (21-C), 19.14 (19-C), 20.79 (11-C), 22.53 (26-C), 22.79 (27-C), 24.01 (23-C), 26.76 (7-C), 27.80 (2-C), 27.94 (28-C), 27.99 (25-C), 31.12 (12-C), 35.49 (1-C), 36.04 (d, J=20.6 Hz, 16-C), 36.23 (22-C), 36.32 (20-C), 37.09 (10-C), 38.90 (4-C), 39.43 (24-C), 44.61 (13-C), 48.90 (17-C), 50.27 (5-C), 51.89 (d, J=14.4 Hz, 14-C), 95.70 (d, J=183 Hz, 15-C), 132.77 (8-C), 135.39 (9-C). *Anal.* Calcd for $C_{30}H_{51}FO$: C.80.66; H, 11.51. Found: C, 80.39; H, 11.38.

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Synthesis of 3-Epi-6,7-dideoxyxestobergsterol A

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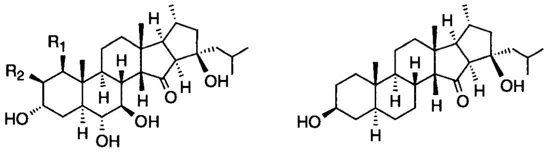
3-Epi-6,7-dideoxyxestobergsterol A (2), an analogue of xestobergsterol A, has been synthesized from dehydroepiandrosterone (3) in 15 steps. The key synthetic intermediate, 15β,16α-dioxypregn-17(20)*E*-ene derivative 8, was prepared from the corresponding 15β,16β-epoxide 6 by treating with acetic acid and titanium tetraisopropoxide. The 23-oxo side chain was constructed stereoselectively by orthoester Claisen rearrangement of 8 followed by introduction of an isobutyl group. Basic treatment of the 15,23-diketone 12 followed by deprotection gave the title compound 2.

Key words xestobergsterol A; steroid; synthesis; epoxide; orthoester Claisen rearrangement

Xestobergsterols A (**1a**) and B (**1b**) were isolated in 1992 from the sponge *Xestospongia bergquistia*, as unique steroids having a *cis* C/D ring junction and an additional carbocyclic E-ring.[1] Subsequently, xestobergsterol C (**1c**) was isolated, and the full structures of these xestobergsterols were established (Fig. 1).[2] Xestobergsterols are strong inhibitors of the release of histamine from rat mast cells induced by anti-immunogloblin E (anti-IgE).[1] It has been reported that the mechanism of action of xestobergsterol A is through strong inhibition of phosphatidylinositol phospholipase C.[3] Several structurally related steroidal compounds, but devoid of the E-ring, *i.e.*, contignasterol, have been isolated from marine sources.[4] Synthetic approaches toward xestobergsterol A have been reported by Krafft's[5] and Jung's[6] groups, and recently the first synthesis of **1a** has been completed by the latter group using Breslow's remote functionalization for the introduction of C-15 functionality.[7] We report herein our approach to the 15-oxo functionalization and the side chain construction starting from dehydroepiandrosterone (**3**) which led to the synthesis of 3-epi-6,7-dideoxyxestobergsterol A (**2**), an analogue of xestobergsterol. The route described herein can be applied to the synthesis of xestobergsterols by employing a 17-oxoandrostane derivative having the requisite hydroxy groups in the A,B-ring.

Our retrosynthetic analysis is outlined in Chart 1. Since we decided to use commercially available dehydroepiandrosterone (**3**) as a starting material for the synthesis of all xestobergsterols, the inversion of 14α-H to 14β-H is required at a certain stage of the synthesis. Based on a series of molecular mechanics (MM2) calculations on various candidates, this inversion appeared possible either at a very early stage, *i.e.*, a 15-en-17-one compound or at the last stage, *i.e.*, an E-ring containing compound.[8] The C-14 epimerization was indeed embodied by Jung's group, on the basis of a similar consideration of the stability of E-ring containing compounds.[6] They demonstrated that a basic treatment of a 14α-H-15,23-dioxo compound effected epimerization–aldol condensation to yield a cyclized product having the thermodynamically favorable and correct configurations at C-14, C-16 and C-23. Thus, a 15,23-diketone **A** was confidently chosen as a precursor of **2**. Intentional addition of Δ^{16} to the structure **A** gave a synthetic precursor, structure **B**. Hydrogenation of the steroidal Δ^{16} is known to occur from the α-face to yield the requisite 17β-orientation of the side chain.[9—12] The 16-ene **B** was expected to be available from a 15-oxygenated allylic alcohol (16-hydroxy-17(20)-ene) **C** through a 3,3-sigmatropic reaction, since such an alcohol without 15-oxygen function, was reported to undergo Carroll[11] rearrangement to the product with the correct C-20 configuration. It is known that the stereochemistries, 16α-alcohol and *E* configuration of $\Delta^{17(20)}$, are essential to obtain the rearranged product **B** with the correct C-20 configuration. It is clear from the foregoing analysis that a simple preparation of the key intermediate **C** is crucial for the success of this retrosynthesis. Fortunately, the corresponding epoxide **D**, a possible precursor of **C**, is known and its synthesis from **3** is well documented.[9,10,12] It should be noted the 15-oxy function originates from the epoxide oxygen.

The synthesis of **2** was carried out according to Chart 2. Dehydroepiandrosterone was protected as methoxymethyl ether **4** and the resulting ether was converted to the conjugated enone **5** in a two-step sequence *via* the enol silyl ether by the Saegusa method.[13] Epoxidation of the enone with *tert*-butyl hydroperoxide (TBHP) and benzyltrimethylammonium hydroxide (Triton-B) afforded, as reported previously, the 15β,16β-epoxide stereoselectively.[10] Wittig olefination of the epoxide using ethyl triphenylphosphonium iodide and *n*-BuLi gave a *ca.* 9 : 1 mixture of $\Delta^{17(20)}$-olefin favoring the *E*-isomer as reported previously.[5c] Change of the base from *n*-BuLi to potassium hexamethyldisilazide (KHMDS) gave $\Delta^{17(20)E}$-olefin **6** free of the *Z*-isomer. We attempted to open the epoxide ring by attack of a hydroxyl or acetoxy group to obtain a 15,16-dioxy derivative. Although acidic reaction conditions afforded more than several products, treatment of **6** with CsOAc in hexamethylphosphoric triamide (HMPA)[14] afforded a mixture of the desired 15β-hydroxy-16α-acetoxy product **7** and its C-16 epimer (*ca.* 40%). A slightly better



1a: R_1, R_2=H
1b: R_1, R_2=OH
1c: R_1=H, R_2=OH

2

Fig. 1. Structures of Xestobergsterols (**1a—c**) and 3-Epi-6,7-dideoxyxestobergsterol A (**2**)

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Dedicated to the memory of Dr. Kyosuke Tsuda.

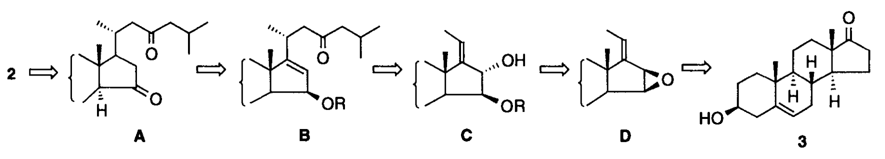
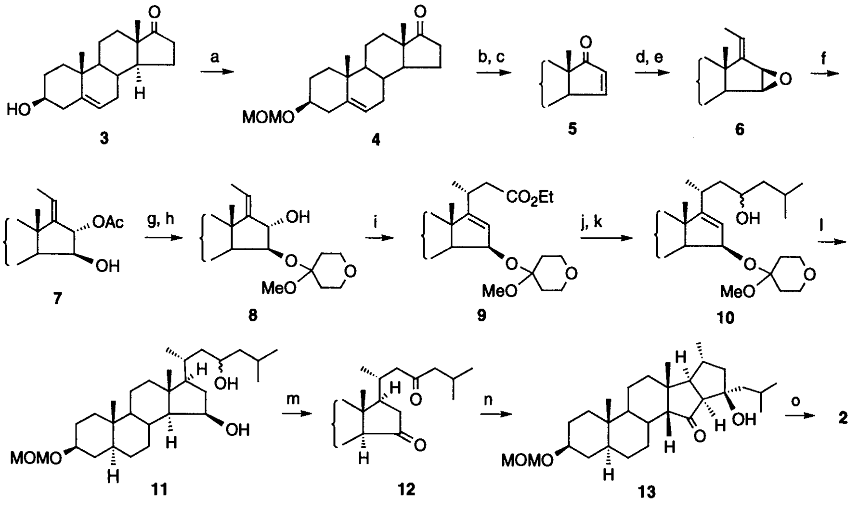


Chart 1. Retrosynthetic Analysis of 3-Epi-6,7-dideoxyxestobergsterol (**2**)



Reagents and conditions: a) MOMCl, $i\mathrm{Pr_2NEt}$, 99%, b) LDA, TMSCl, c) $\mathrm{Pd(OAc)_2}$, 92% from **4**, d) TBHP, Triton-B, 87%, e) $\mathrm{Ph_3PEtI}$, KHMDS, 95%, f) AcOH, $\mathrm{Ti(O}i\mathrm{Pr)_4}$, 56%, g) MDHP, PPTS, h) $\mathrm{LiAlH_4}$, 82% from **7**, i) $\mathrm{(EtO)_3CMe}$, $\mathrm{EtCO_2H}$, 98%, j) DIBAL, k) iBuMgBr, 56% from **9**, l) $\mathrm{H_2}$, Pd/C, 83%, m) PCC, 94%, n) NaOH, EtOH, 88%, o) 6N HCl, 78%.

Chart 2. Synthesis of 3-Epi-6,7-dideoxyxestobergsterol (**2**)

result was obtained by the use of ammonium acetate ($\mathrm{NH_4OAc}$) and titanium tetraisopropoxide [$\mathrm{Ti(O}i\mathrm{Pr)_4}$][15]) to give compound **7** (42%) and 15β-hydroxy-16α-isopropoxy by-product. Further optimization of conditions allowed us to find the use of acetic acid (3 eq) and $\mathrm{Ti(O}i\mathrm{Pr)_4}$ (1.5 eq) in tetrahydrofuran (THF) to reproducibly afford the desired **7** in 55—60% yield from **6**. The α-orientation of the 16-acetoxy group in **7** was determined on the basis of nuclear Overhauser effect (NOE) studies in which irradiation of 18-$\mathrm{H_3}$ (δ 1.21) caused enhancement of the 16β-H (δ 5.23) signal intensity while the β-orientation of the 15-OH group was assigned from a mechanistic consideration of an epoxide ring-opening reaction. Protection of the 15-hydroxyl group of **7** as 4-methoxytetrahydropyran-4-yl (MTHP) ether followed by removal of the 16-acetyl group gave the key intermediate **8** desired for the side chain introduction. The 15β-hydroxyl group appeared to be substantially sterically hindered since an attempted conversion to *tert*-butyldimethylsilyl (TBS) ether under standard conditions (TBSCl/imidazole or *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBSOTf)/lutidine) failed and gave only recovered starting material.

Encouraged by a known example of a 3,3-sigmatropic reaction of a steroidal 16α-hydroxy-$\Delta^{17(20)E}$ system,[11]) the allylic alcohol **8** was subjected to the conditions of orthoester Claisen rearrangement (triethyl orthoacetate, propionic acid, heating in xylene), which gave rise to the rearranged ester **9** as a single isomer in quantitative yield. Carroll rearrangement of **8** using 5-isovaleryl Meldrum's acid[11]) did not succeed, although the 16-(β-keto) ester was obtained in good yield. The side chain of **9** was extended *via* the aldehyde to yield 23-ol **10** as a *ca.* 1 : 1 epimeric mixture at the C-23 position. Hydrogenation of **10** proceeded stereoselectively to yield the saturated product **11** with β-orientation of the side-chain. It should be noted that the MTHP protecting group was cleaved under the hydrogenation conditions ($\mathrm{H_2}$/10% Pd–C). Similar cleavage of the MTHP group under the same hydrogenation condition was also observed for compound **9**. PCC oxidation of the diol **11** furnished the diketone **12**, whose structure including 14α-H, 17α-H and 20*R* stereochemistry was established from comparison of the NMR data with those reported for the corresponding 3-TBS ether.[5c]) As expected from earlier work by Jung's[6,7]) and Krafft's[5c]) groups, treatment of the diketone **12** with NaOH/ethanol (EtOH) afforded the pentacyclic compound **13** in good yield without formation of any other stereoisomers. The ^{1}H- and ^{13}C-NMR data of **13** were essentially identical with those reported for the corresponding 3-TBS ether[5c]) except for the signals around the C-3 positions. Final deprotection of **13** under acidic conditions completed the synthesis of the target compound **2**. The ^{1}H- and ^{13}C-NMR data for **2**, in comparison with those reported for 7-deoxyxestobergsterol A[6]) and xestobergsterol A,[2]) confirmed the synthetic analogue to have the natural C-14,C-16 C-17 and C-23 configurations.

In conclusion, we have developed a relatively simple synthetic route leading to the construction of the E-ring found in xestobergsterols, which is characterized by an appropriate use of compound **8** readily available from the corresponding epoxide **6**. We previously developed a method of preparation of 3α,6α,7β-trihydroxyandrostan-17-one.[8]) The synthesis of xestobergsterol A by combining the latter method with the present approach is in progress in our laboratory.

Experimental

General Methods Melting points were determined on a Yazawa hot-stage melting point apparatus and were uncorrected. NMR spectra were obtained on a JEOL JNM-LA300 (300 MHz for ^1H and 75 MHz for ^{13}C) or JEOL JNM-LA400 (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer in $CDCl_3$ solutions, unless otherwise noted. ^1H chemical shifts are given in ppm relative to tetramethylsilane δ 0.00) and ^{13}C chemical shifts are shown relative to the solvent (δ 77.00). Merck Kieselgel 60 was used for column chromatography.

3β-(Methoxymethoxy)androst-5-en-17-one (4) *i*-Pr_2NEt (10.6 ml, 60.8 mmol) and chloromethyl methyl ether (3.95 ml, 52.0 mmol) were added to a solution of **3** (5.00 g, 17.3 mmol) in dry CH_2Cl_2 (40 ml) at 0 °C under N_2, and the mixture was stirred at the same temperature for 30 min and then at room temperature for 3 h. CH_2Cl_2 and 2 N HCl were added to the mixture, and the separated CH_2Cl_2 layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated to dryness. The residue was chromatographed on silica gel with hexane–AcOEt (7 : 1) to give **4** (5.70 g, 99%) as white needles, mp 131—132 °C (from hexane–AcOEt). ^1H-NMR δ: 0.89 (s, 18-H_3), 1.04 (s, 19-H_3), 3.38 (s, OCH_3), 3.43 (m, 3α-H), 4.69 (s, OCH_2O), 5.39 (m, 6-H). ^{13}C-NMR δ: 13.48, 19.33, 20.29, 21.82, 28.81, 30.75, 31.40, 31.45, 35.77, 36.81, 37.13, 39.48, 47.46, 50.24, 51.72, 55.11, 76.71, 94.66, 120.89, 140.95, 220.90. *Anal.* Calcd for $C_{21}H_{32}O_3$: C, 75.86; H, 9.70. Found: C, 76.03; H, 9.90.

3β-(Methoxymethoxy)androsta-5,15-dien-17-one (5) A solution of **4** (5.70 g, 17.1 mmol) in dry THF (35 ml) was added at −78 °C under N_2 to a solution of LDA [prepared from diisopropylamine (3.84 ml, 27.4 mmol) in dry THF (20 ml) and *n*-BuLi (1.50 N solution in hexane, 17.1 ml, 25.7 mol)]. The mixture was stirred at the same temperature for 1 h, then chlorotrimethylsilane (4.35 ml, 34.3 mmol) was added. The mixture was warmed up to room temperature over a period of 20 min with stirring. Ether and saturated aqueous NH_4Cl were added. The separated organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated to give the crude enol TMS ether (7.4 g) as a yellow solid.

Palladium (II) acetate (3.85 g, 17.1 mmol) was added to a solution of the enol ether in CH_3CN (60 ml) and the mixture was stirred for 14 h. The mixture was filtered through a pad of Celite and the filtrate was concentrated to give a crude product which was chromatographed on silica gel with hexane–AcOEt (7 : 1) to afford **5** (5.20 g, 92%) as white needles, mp 130—132 °C (from hexane–AcOEt). ^1H-NMR δ: 1.09 (s, 18-H_3, 19-H_3), 3.38 (s, OCH_3), 3.44 (m, 3α-H), 4.70 (s, OCH_2O), 5.41 (m, 6-H), 6.05 (dd, *J*=5.9, 3.2 Hz, 16-H), 7.50 (dd, *J*=5.2, 1.2 Hz, 15-H). ^{13}C-NMR δ: 19.30, 19.93, 20.08, 28.76 (×2), 28.99, 30.58, 36.92, 37.00, 39.48, 50.66, 51.24, 55.16, 57.23, 76.58, 94.66, 120.37, 131.83, 141.44, 158.53, 213.08. *Anal.* Calcd for $C_{21}H_{30}O_3$: C, 76.33; H, 9.15. Found: C, 76.39; H, 9.32.

15β,16β-Epoxy-3β-(methoxymethoxy)androst-5,17(20)*E*-diene (6) Triton B (40% aqueous solution, 8.36 ml, 21.2 mmol) and TBHP (90% aqueous solution, 4.66 ml, 41.9 mmol) were added to a solution of **5** (4.84 g, 14.7 mmol) in THF (44 ml) at −20 °C, and the mixture was stirred for 15 min at the same temperature. Ether and aq. $Na_2S_2O_3$ were added, and the separated ether layer was washed with saturated aqueous NH_4Cl, saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated. The residue was chromatographed on silica gel with hexane–AcOEt (7 : 1) to give the epoxide (4.41 g, 87%) as white crystals, mp 131—133 °C (from hexane–AcOEt). ^1H-NMR δ: 1.07 (s, 18-H_3), 1.17 (s, 19-H_3), 3.31 (d, *J*=2.9 Hz, 15α-H), 3.38 (s, OCH_3), 3.44 (m, 3α-H), 3.82 (d, *J*=2.9 Hz, 16α-H), 4.70 (s, OCH_2O), 5.42 (m, 6-H). ^{13}C-NMR δ: 18.91, 19.17, 19.90, 28.51, 28.75, 30.15, 32.74, 36.94, 36.99, 39.45, 41.88, 51.07, 52.93, 53.39, 55.20, 55.66, 76.58, 94.68, 120.25, 141.48, 213.23. *Anal.* Calcd for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.85; H, 9.03.

To a suspension of ethyltriphenylphosphonium iodide (11.4 g, 27.3 mmol) in THF (10 ml) was added potassium hexamethyldisilylazide (0.52 M toluene solution, 45.6 ml, 15.4 mmol) at 0 °C under N_2, and the mixture was stirred at the same temperature for 30 min. The mixture was cooled to −20 °C, and a solution of the epoxide **12** (3.81 g, 11.0 mmol) in THF (3.0 ml) was added and stirring was continued for 15 min. Ether and brine were added and the ether layer was dried over Na_2SO_4 and concentrated to give a yellow solid. This was suspended in hexane, and the insoluble portion was removed and concentrated to give **6** (3.74 g, 95%) as a white solid, mp 149—152 °C. ^1H-NMR δ: 1.06 (s, 18-H_3), 1.15 (s, 19-H_3), 1.75 (d, *J*=7.3 Hz, 21-H_3), 3.38 (s, OCH_3), 3.43 (m, 3α-H), 3.48 (d, *J*=3.2 Hz, 15α-H), 3.61 (d, *J*=3.2 Hz, 16α-H), 4.70 (s, OCH_2O), 5.41 (m, 6-H), 5.71 (q, *J*=7.1 Hz, 20-H). ^{13}C-NMR δ: 13.45, 19.11, 20.86, 22.38, 28.16, 28.83, 31.26, 36.96, 37.05, 37.98, 39.39, 39.51, 51.12, 55.13, 57.10, 57.75, 58.94, 76.79, 94.67, 120.87, 121.80, 141.24, 146.24. *Anal.* Calcd for $C_{23}H_{34}O_3$: C, 77.05; H, 9.56. Found: C, 77.11; H, 9.85.

16α-Acetoxy-15β-Hydroxy-3β-(methoxymethoxy)androst-17(20)*E*-ene (7) Ti(O*i*Pr)$_4$ (4.86 ml, 16.5 mmol) and acetic acid (1.88 ml, 32.8 mmol) were added to a solution of **6** (3.60 g, 10.0 mmol) and the mixture was stirred for 2 h. Ether and saturated aqueous $NaHCO_3$ were added, and the organic layer was washed with brine, dried over Na_2SO_4 and concentrated to dryness. The residue was chromatographed on silica gel with hexane–AcOEt (7 : 1) to give **7** (2.34 g, 56%) as white plates, mp 148—150 °C (from hexane–AcOEt). ^1H-NMR δ: 1.05 (s, 19-H_3), 1.21 (s, 18-H_3), 1.77 (d, *J*=7.3 Hz, 21-H_3), 2.11 (s, OAc), 3.37 (s, CH_3O), 3.45 (m, 3α-H), 3.97 (d, *J*=5.6 Hz, 15β-H), 4.69 (s, OCH_2O), 5.23 (s, 16α-H), 5.37 (m, 6-H), 5.49 (dq, *J*=7.1 Hz, 20-H). ^{13}C-NMR δ: 13.14, 19.09, 20.29, 20.89, 21.23, 27.27, 28.86, 30.69, 36.78, 37.15, 38.22, 39.46, 44.03, 50.35, 55.12, 57.38, 76.28, 76.84, 85.09, 94.64, 119.47, 121.20, 140.65, 147.31, 171.80. *Anal.* Calcd for $C_{25}H_{38}O_5$: C, 71.74; H, 9.15. Found: C, 71.59; H, 9.24.

16α-Hydroxy-15β-(4-methoxytetrahydropyran-4-yloxy)-3β-(methoxymethoxy)-androsta-5,17(20)*E*-diene (8) 5,6-Dihydro-4-methoxy-2*H*-pyran (855 μl, 7.65 mmol) and pyridinium *p*-toluenesulfonate (48 mg, 0.19 mmol) were added to a solution of **7** (1.60 g, 3.82 mmol) in dry CH_2Cl_2 (16 ml) and the mixture was stirred for 13 h. AcOEt and saturated aqueous $NaHCO_3$ were added, and the organic layer was washed with brine, dried over Na_2SO_4 and concentrated to dryness. The residue was chromatographed on silica gel with hexane–AcOEt (5 : 1) to give the ether (1.9 g, 93%) as an oil. ^1H-NMR δ: 1.07 (s, 19-H_3), 1.25 (s, 18-H_3), 1.75 (dd, *J*=9.4 Hz, 21-H_3), 2.04 (s, OAc), 3.21(s, CH_3O), 3.38 (s, CH_3O), 3.44 (m, 3α-H), 3.35—3.82 (m, 3α-H, 2,4-H_4 of MTHP), 4.00 (d, *J*=5.5 Hz, 15α-H), 4.70 (s, OCH_2O), 5.38 (m, 6-H), 5.61 (m, 16β-H, 20-H). ^{13}C-NMR δ: 13.73, 19.16, 20.31, 20.92, 21.44, 27.15, 28.84, 31.24, 34.35, 34.46, 36.99, 37.18, 38.62, 39.46, 43.37, 48.47, 50.48, 55.16, 57.38, 64.98, 65.12, 74.40, 76.83, 81.19, 94.65, 98.77, 121.14, 123.65, 140.98, 149.23, 170.34. *Anal.* Calcd for $C_{31}H_{48}O_7$: C, 69.89; H, 9.08. Found: C, 69.76; H, 8.95.

$LiAlH_4$ (203 mg, 5.35 mmol) was added to a solution of the ether (1.90 g, 3.57 mmol) in ether (19 ml) at 0 °C under N_2 and the mixture was stirred for 10 min. Ether and 2 N HCl were added, the organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated to dryness. The residue was chromatographed on silica gel with hexane–AcOEt (2 : 1) to give **8** (1.54 g, 88%) as a colorless amorphous solid. ^1H-NMR δ: 1.06 (s, 19-H_3), 1.20 (s, 18-H_3), 1.77 (d, *J*=7.2 Hz, 21-H_3), 3.18 (s, CH_3O), 3.38 (s, CH_3O), 3.35—3.82 (m, 3α-H, 2,4-H_4 of MTHP), 4.02 (d, *J*=5.6 Hz, 15α-H), 4.45 (brs, 16β-H), 4.69 (s, OCH_2O), 5.37 (m, 6-H), 5.60 (q, *J*=7.2 Hz, 20-H). ^{13}C-NMR δ: 13.45, 19.15, 20.18, 20.87, 27.19, 28.82, 31.13, 34.57 (×2), 36.91, 37.13, 38.67, 39.45, 43.71, 48.33, 50.49, 55.13, 56.57, 64.90, 65.07, 76.80, 76.86, 79.92, 94.61, 98.63, 120.12, 121.33, 140.77, 153.96. *Anal.* Calcd for $C_{29}H_{46}O_6$: C, 70.99; H, 9.45. Found: C, 70.86; H, 9.68.

Ethyl 15β-(4-Methoxytetrahydropyran-4-yloxy)-3β-(methoxymethoxy)-24-norchola-5,16-dien-23-oate (9) Ethyl orthoacetate (2.86 ml, 15.6 mmol) and propionic acid (140 μl, 1.88 mmol) were added to a solution of **8** (1.53 g, 3.12 mmol) in xylene (15 ml). After the mixture was stirred at 130 °C for 30 min, it was concentrated under reduced pressure. Purification of the crude product on silica gel with hexane–AcOEt (7 : 1) afforded **9** (1.71 g, 98%) as an oil. ^1H-NMR δ: 1.04 (d, *J*=6.8 Hz, 21-H_3), 1.09 (s, 19-H_3), 1.15 (s, 18-H_3), 1.26 (t, *J*=7.1, 7.2 Hz, 21-H_3), 2.76 (m, 20-H), 3.11 (s, CH_3O), 3.38 (s, CH_3O), 3.35—3.82 (m, 3α-H, 2,4-H_4 of MTHP), 4.10 (q, *J*=7.2 Hz, OEt), 4.11 (q, *J*=7.2 Hz, OEt), 4.40 (dd, *J*=5.4, 2.9 Hz, 15α-H), 4.70 (s, OCH_2O), 5.38 (m, 6-H), 5.57 (d, *J*=1.7 Hz, 16-H). ^{13}C-NMR δ: 14.20, 19.18, 20.53, 21.11, 21.47, 27.61, 28.68, 28.85, 30.96, 34.55, 34.76, 35.05, 37.02, 37.15, 39.50, 41.56, 47.34, 48.35, 51.05, 55.15, 59.80, 60.23, 64.90, 65.16, 71.52, 76.78, 94.63, 97.89, 121.49, 124.24, 140.97, 163.45, 172.49. *Anal.* Calcd for $C_{33}H_{52}O_7$: C, C, 70.68; H, 9.35. Found: C, 70.88; H, 9.10.

(23*RS*)-15β-(4-Methoxytetrahydropyran-4-yloxy)-3β-(methoxymethoxy)cholesta-5,16-dien-23-ol (10) DIBAL (1.01 N solution in toluene, 2.39 ml, 2.41 mmol) was added to a solution of **9** (1.50 g, 2.67 mmol) in dry toluene (15 ml) at −78 °C under N_2 and the mixture was stirred for 30 min at the same temperature. Ether and saturated aqueous NH_4Cl were added, and the organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated to give a crude aldehyde (1.28 g) as an oil. ^1H-NMR δ: 1.08 (d, *J*=6.8 Hz, 21-H_3), 1.09 (s, 19-H_3), 1.16 (s, 18-H_3), 2.51 (ddd, *J*=16.2, 7.7, 1.9 Hz, 22-Ha), 2.67 (ddd, *J*=16.2, 7.0, 1.9 Hz, 22-Hb), 2.82 (m, 20-H), 3.09 (s, CH_3O), 3.38 (s, CH_3O), 3.36—3.82 (m, 3α-H, 2,4-H_4 of MTHP), 4.41 (dd, *J*=5.2, 2.8 Hz, 15α-H), 4.70 (s, OCH_2O), 5.38 (m, 6-H), 5.58 (d, *J*=2.0 Hz, 16-H), 9.73 (t, *J*=1.9 Hz, CHO). ^{13}C-NMR δ: 19.17, 20.55, 21.33, 21.76, 26.63, 27.60, 28.86, 30.90, 34.47, 34.77, 35.09, 37.01, 37.16, 39.48, 47.36, 48.43, 50.20, 51.02, 55.14, 59.81, 64.87, 65.15, 71.47, 76.76, 94.62, 97.97, 121.44, 124.96, 140.96, 163.04, 202.09.

Isobutylmagnesium bromide solution, prepared from Mg (130 mg, 5.35 mmol) and isobutyl bromide (582 μl, 5.35 mmol) in THF (8 ml), was added to a solution of the aldehyde (1.28 g) in THF (10 ml) under N_2 and the mixture was stirred for 40 min. Ether and 2 N HCl were added, and the aqueous layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated to dryness. The residue was chromatographed on silica gel with hexane–AcOEt (3 : 1) to give **10** (861 mg, 56% from **9**) as an oil. ^{1}H-NMR δ: 0.86—0.94 (m, 26-H_3, 27-H_3), 1.01 (d, J=6.8 Hz, 21-H_3), 1.09 (s, 19-H_3), 1.149, 1.154 (s, 18-H_3), 3.13, 3.14 (s each, CH_3O), 3.38 (s, CH_3O), 3.38—3.82 (m, 3α-H, 2,4-H_4 of MTHP, 23-H), 4.42 (br s, 15α-H), 4.70 (s, OCH_2O), 5.39 (m, 6-H), 5.53, 5.58 (d each, J=1.7 Hz, 16-H). ^{13}C-NMR δ: 51.06, 51.12, 55.12, 64.88, 64.90, 65.13, 65.16, 71.64, 71.68, 76.78, 94.59, 97.88, 120.51, 123.83, 123.96, 140.93, 164.76, 165.20. *Anal.* Calcd for $C_{35}H_{58}O_6$: C, 73.13; H, 10.17. Found: C, 73.00; H, 10.32.

(23*RS*)-3β-(Methoxymethoxy)cholestane-15β,23-diol (11) A solution of **10** (690 mg, 1.20 mmol) in AcOEt (7 ml) containing 10% Pd/C (Kojima Chemicals Co. Ltd., 200 mg) was stirred under an atmospheric pressure of H_2 for 40 h. The mixture was filtered through a pad of Celite and the filtrate was concentrated to dryness. The residue was chromatographed on silica gel with hexane–AcOEt (3 : 1) to give **11** (462 mg, 83%) as amorphous solid. ^{1}H-NMR δ: 0.84 (s, 19-H_3), 0.91 (d each, J=6.7 Hz, 26, 27-H_3), 0.95 (s, 18-H_3), 0.96 (d, J=6.7 Hz, 21-H_3), 2.41 (m, 20-H), 3.37 (s, CH_3O), 3.50 (m, 3α-H), 3.76 (m, 23-H), 4.17 (m, 15α-H), 4.68 (s, OCH_2O). ^{13}C-NMR δ: 12.15, 14.63, 14.76, 18.58, 19.20, 21.00, 21.54, 22.24, 23.20, 23.96, 24.32, 24.58, 28.54, 28.59, 31.26, 31.30, 31.34, 31.37, 32.08, 33.46, 35.09, 35.65, 36.90 41.12, 41.17, 41.33, 41.36, 42.23, 42.32, 44.32, 44.87, 44.89, 44.98, 46.26, 47.91, 54.66, 54.69, 55.04, 57.07, 60.86, 60.93, 66.73, 68.29, 70.11, 70.12, 76.18, 94.37. *Anal.* Calcd for $C_{29}H_{52}O_4$: C, 74.95; H, 11.28. Found: C, 74.75; H, 11.43.

3β-(Methoxymethoxy)cholestane-15,23-dione (12) PCC (820 mg, 3.81 mmol) and Celite (1.0 g) were added to a solution of **11** (442 mg, 0.951 mmol) in dry CH_2Cl_2 (11 ml) and the mixture was stirred for 2 h. Dry ether (55 ml) was added and the suspension was filtered through a pad of Celite. Concentration of the filtrate gave a crude product which was chromatographed on silica gel with hexane–AcOEt (7 : 1) to give **12** (411 mg, 94%) as white crystals, mp 134—136 °C (from hexane–AcOEt). ^{1}H-NMR δ: 0.78 (s, 18-H_3), 0.81 (s, 19-H_3), 0.90 (d, J=6.6 Hz, 26-H_3), 0.91 (d, J=6.6 Hz, 27-H_3), 0.99 (d, J=6.6 Hz, 21-H_3), 2.65 (m, 7β-H), 3.36 (s, CH_3O), 3.49 (m, 3-H), 4.67 (s, OCH_2O). ^{13}C-NMR δ: 12.06, 12.96, 20.10, 20.64, 22.44, 22.55, 24.46, 28.20, 28.55, 30.53, 31.64, 31.80, 35.00, 35.62, 36.87, 39.65, 41.65, 42.34, 44.72, 49.76, 51.04, 52.52, 53.80, 55.04, 65.81, 76.01, 94.42, 210.06, 214.97. *Anal.* Calcd for $C_{29}H_{48}O_4$: C, 75.61; H, 10.50. Found: C, 75.38; H, 10.73.

3β-(Methoxymethoxy)-3-Epi-6,7-dideoxyxestobergsterol (13) 2 N NaOH (0.38 ml) was added to a solution of **12** (200 mg, 0.434 mmol) in EtOH (6.6 ml) and THF (2.8 ml) and the mixture was stirred for 37 h. Ether and saturated aqueous NH_4Cl were added. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated to dryness. The residue was chromatographed on silica gel with hexane–AcOEt (10 : 1) to give **13** (175 mg, 88%) as white needles, mp 116—117 °C (from MeOH) ^{1}H-NMR δ: 0.76 (s, 19-H_3), 0.95 (d, J=6.8 Hz, 26-H_3), 0.97 (d, J=6.8 Hz, 27-H_3), 1.10 (d, J=6.4 Hz, 21-H_3), 1.13 (s, 18-H_3), 2.11 (t, J=3.1 Hz, 14β-H), 2.28 (m, 20-H), 2.49 (td, J=9.1, 4.9 Hz, 7α-H), 2.61 (d, J=9.8 Hz, 16α-H), 3.36 (s, CH_3O), 3.49 (m, 3-H), 4.68 (s, OCH_2O). ^{13}C-NMR δ: 12.04, 19.79, 20.41 (C-21), 21.09 (C-11), 24.44 (C-26), 24.56 (C-25), 24.65 (C-27), 28.42, 28.86, 28.90, 32.72, 34.35, 35.25 (C-20), 33.54 (C-10), 36.38, 38.05, 38.78 (C-13), 43.90, 47.38 (C-9), 50.67 (C-22), 51.89 (C-24), 55.04, 56.79 (C-14), 57.38 (C-17), 62.94 (C-16), 76.26 (C-3), 81.91 (C-23), 94.51, 220.16 (C-15). *Anal.* Calcd for $C_{29}H_{48}O_4$: C, 75.61; H, 10.50. Found: C, 75.46; H, 10.74.

3-Epi-6,7-dideoxyxestobergsterol (2) 6 N-HCl (0.2 ml) was added to a solution of **13** (124 mg, 0.269 mmol) in THF (2.0 ml) and the mixture was stirred for 48 h. Ether and saturated aqueous $NaHCO_3$ were added, and the organic layer was washed with brine, dried over Na_2SO_4 and concentrated to dryness. The residue was chromatographed on silica gel with hexane–AcOEt (3 : 1) to give **2** (78 mg, 78%) as white needles, mp 97—98 °C (from MeOH). ^{1}H-NMR (pyridine-d_5, chemical shifts are given relative to H-2 of the residual solvent, δ=7.19) δ: 0.74 (s, 19-H_3), 0.95 (d, J=6.5 Hz, 27-H_3), 1.00 (d, J=6.5 Hz, 26-H_3), 1.12 (d, J=6.5 Hz, 21-H_3), 1.14 (s, 18-H_3), 2.49 (brs, H-14), 2.61 (m, 20-H), 2.67 (d, J=10.0 Hz, 16α-H), 2.78 (m, 7α-H), 3.77 (m, 3α-H). ^{13}C-NMR (pyridine-d_5, chemical shifts are given relative to C-3 of the solvent, δ=123.50) δ: 12.22 (C-19), 19.81 (C-21), 20.87 (C-18), 21.62 (C-11), 24.66 (C-26), 24.82 (C-25), 25.12 (C-27), 29.45 (C-6), 29.45 (C-7), 32.20 (C-2), 32.91 (C-12), 34.98 (C-20), 35.78 (C-10), 37.35 (C-1), 38.63 (C-4), 38.67 (C-8), 39.33 (C-13), 44.42 (C-5), 47.45 (C-9), 51.63 (C-22), 51.92 (C-24), 56.64 (C-14), 57.63 (C-17), 62.90 (C-16), 70.47 (C-3), 82.01 (C-23), 217.20 (C-15). *Anal.* Calcd for $C_{27}H_{44}O_3$: C, 77.84; H, 10.64. Found: C, 77.61; H, 10.90.

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Synthesis of (10*Z*)- and (10*E*)-19-Fluoro-1α,25-dihydroxyvitamin D_3: Compounds to Probe Vitamin D Conformation in Receptor Complex by ^{19}F-NMR

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To study the interaction of vitamin D with its receptor by ^{19}F-NMR, (5*Z*,10*Z*)- and (5*Z*,10*E*)-19-fluoro-1α,25-dihydroxyvitamin D_3 were synthesized starting from vitamin D_2 *via* electrophilic fluorination of vitamin D–SO_2 adducts as the key step. Regio- and stereoselective electrophilic fluorination at C(19) of vitamin D–SO_2 adducts was achieved under the conditions using $(PhSO_2)_2NF$ and bulky bases. The stereochemistry of the addition and elimination of SO_2 of various vitamin D derivatives was studied in detail. SO_2 causes *Z–E* isomerization of the 5,6-double bond of vitamin D and adds to the resulting (5*E*)-isomer from the sterically less hindered side opposite to the substituent at C(1). Elimination of SO_2 from 19-substituted vitamin D–SO_2 adducts proceeded exclusively in a suprafacial manner with respect to the diene part under either thermal or reductive conditions. Dye-sensitized photochemical isomerization of 19-fluorovitamin D derivatives was studied in detail. The rapid isomerization at the 5,6-double bond was followed by the slow isomerization at the 10,19-double bond to yield the (5*E*,10*Z*)-isomer (by nomenclature of the 1-OH derivatives) as the major product. (10*Z*)- and (10*E*)-19-Fluorovitamin Ds were also interconverted thermally probably *via* the corresponding previtamin D by 1,7-sigmatropic isomerization.

Key words fluorovitamin D; vitamin D–sulfur dioxide adduct; electrophilic fluorination; synthesis; sigmatropic reaction; cheletropic reaction; photochemical isomerization

1α,25-Dihydroxyvitamin D [1,25-$(OH)_2D_3$] **1a** exhibits various activities including classical actions in calcium metabolism and basic functions of regulating the proliferation and differentiation of cells and the immune response by regulating the transcription of vitamin D target genes.[1] The vitamin D receptor (VDR) is a member of the nuclear receptor (NR) superfamily which includes steroid and thyroid hormone receptors, retinoic acid receptors and numerous orphan receptors for which currently no natural ligand has been defined.[2] Thus, natural and synthetic ligands of NRs have been known as biologically as well as clinically important compounds, and still many efforts have been devoted to finding better and novel drug candidates from NR potential ligands.

Binding of small lipophilic ligands of NRs can induce crucial conformational changes in the cognate receptors whose molecular weights are nearly 100 times those of the ligands.[3] The crystal structures of a number of NR members have been solved in the past five years, and this provides us with indispensable information on the transactivation function of NR-ligand binding domains on structural basis.[4—8] We learned that, upon ligand binding, drastic conformational changes in the C-terminal region called transactivation function 2 (AF-2) are induced to generate the transcriptionally active form to which coactivators can be recruited.

In the course of continuing research on the conformation and activity relationship of vitamin D,[9—12] we turned our attention to the conformation of the A-ring plus the conjugated triene part of vitamin D in the VDR complex. To specifically observe the ligand counterpart in the ligand–VDR complex, we have planned to use the signal of ^{19}F incorporated in the vitamin D molecule by ^{19}F-NMR spectroscopy.[13] Because of the wide chemical-shift range of organic fluorine atoms, we expected to be able to discriminate the fluorine in different ligand conformations. For this purpose, we have synthesized 4,4-difluoro-1,25-dihydroxyvitamin D_3,[14,15] and now we report another probe in this study, the geometrical isomers of 19-fluoro-1,25-dihydroxyvitamin D_3 (**2** and **3**). Whether the reduced electron density on the triene of vitamin D affects the biological property is also interesting.

The synthesis of 6-fluorovitamin D_3 **4** has been reported by Dauben *et al.*, and this compound was shown to have some antagonistic activity *in vivo.*[16,17] An attempted synthesis of 19,19-difluorovitamin D_3 by Mazur's group *via* a general photochemical approach through the corresponding provitamin D was unsuccessful,[18] because the thermal [1,7]-sigmatropic rearrangement of the difluoroprevitamin D to the corresponding vitamin D was prohibited by the strong electronic effect of the two fluorine atoms on C(19) (Chart 2). Therefore, we adopted a novel way of synthesizing 19-fluorovitamin D analogs *via* regioselective electrophilic fluorination of vitamin D–SO_2 adducts **6** or **7** as a key step (Chart 3). In this paper, we describe the first synthesis of (5*Z*,10*Z*)- and (5*Z*,10*E*)-19-fluoro-1α,25-dihydroxyvitamin D_3 [19-F-1α,25-$(OH)_2D_3$] **2a** and **3a** and their related compounds **2b**, **2c**, **3b**, and **3c** (Chart 1).[19] We also describe the stereochemistry of the cheletropic reaction of vitamin D with SO_2 and the photochemical isomerization of 19-fluorovitamin D geometrical isomers.

Results and Discussions

Synthetic Strategy Both geometrical isomers of 19-F-1α,25-$(OH)_2D_3$ **2a** and **3a** were synthesized from vitamin D_2 *via* the route outlined in Chart 3. Vitamin D compounds **1** and **5** possessing the necessary hydroxyl groups at C(1) and C(25) were prepared by known methods.[10,20,21] Briefly, the 1α-hydroxyl was introduced *via* (5*E*)-vitamin D by allylic oxidation using SeO_2.[22] The side chain with the 25-hydroxyl group was readily constructed by exchanging C(23) to C(27)

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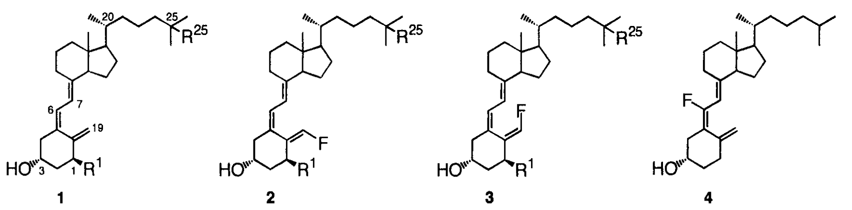
Dedicated to the memory of Dr. Kyosuke Tsuda.



(**a**: $R^1=R^{25}=OH$; **b**: $R^1=OH$, $R^{25}=H$; **c**: $R^1=R^{25}=H$)

Chart 1

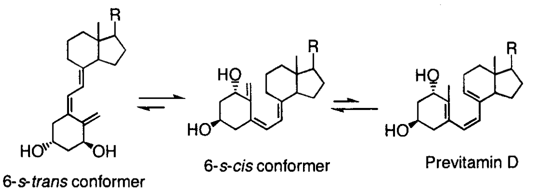


Chart 2. The Dynamic Changes Occurring within the Seco-B, Conjugated Triene Part of Vitamin D Molecule

part *via* the C(22)-aldehyde, which is obtained by the ozonolysis of the vitamin D_2–SO_2 adduct. Treatment of **1** and **5** with liquid SO_2 would afford the SO_2 adduct as a mixture of the two epimers **6** and **7** at C(6).[23,24] The 6 and 19-positions activated by the adjacent SO_2 group can be readily substituted with electrophilic reagents under basic conditions.[25—30] By choosing the proper combination of a base and an electrophilic fluorination reagent, regioselective 19-fluorination to afford **8**—**11** can be achieved in moderate yields.[19] Cheletropic desulfonylation followed by dye-sensitized photoisomerization would afford the desired 19-fluorovitamin D **2** and **3**.

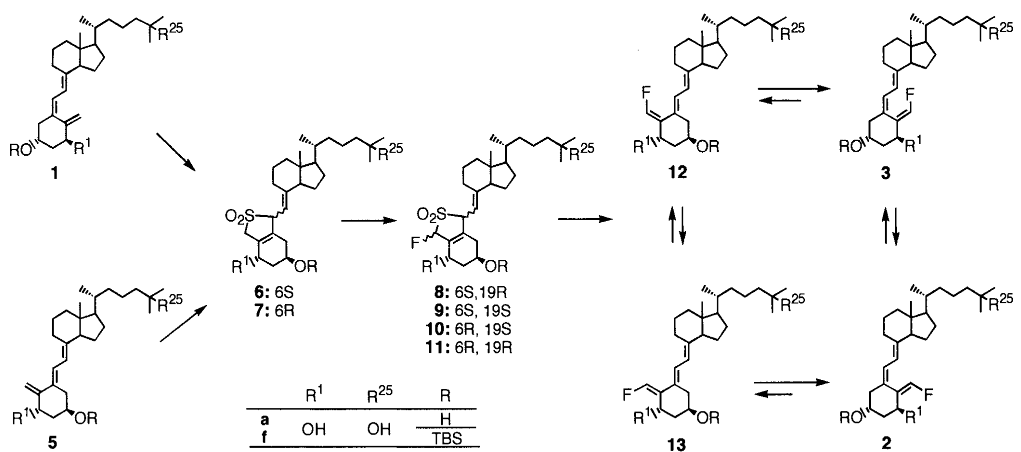
Stereochemistry of the Reaction of (5*Z*)- and (5*E*)-Vitamin D with Liquid SO_2 The s-*cis*-diene part of vitamin D (**1** and **5**) reacts quantitatively with SO_2 to afford the sulfolenes **6** and **7** (Chart 4). The reversible addition–elimination reaction of SO_2 on dienes is a typical cheletropic reaction. The stereochemistry of the extrusion of SO_2 from sulfolenes has been extensively studied and shown to proceed in suprafacial manner with respect to the diene part.[31,32] However, the stereochemistry of the SO_2 addition reactions has not been clearly understood. In the syntheses of vitamin D derivatives, SO_2 adducts (**6** and **7**) have been used for three major purposes:[23,24] (i) to protect the conjugated triene structure under acidic and oxidative conditions;[25] (ii) to introduce electrophiles to the 6- and 19-positions; and (iii) to selectively transform vitamin D (**1**) to (5*E*)-vitamin D (**5**).[22,29,30] Nevertheless, not much attention has been paid to the stereochemistry of the addition of SO_2.

We investigated the effects of the substituents at C(1) and the solvent on the face selectivity of the SO_2 addition, and the results are summarized in Table 1. In the reaction of (5*Z*)-vitamin D **1h** without the 1-OH group, the isomeric adducts (6*S*)-**6h** and (6*R*)-**7h** were produced in about a 1 : 1 ratio regardless of the conditions used (entries 1—3). The face selectivity of vitamin D with a 1α-OH group (**1g**) and its 5*E*-isomer (**5g**) depends on the conditions (entries 4—8). The proportion of the upper-face adduct (6*S*-**6g**) was increased with increasing polarity of the solvents. In the reactions of vitamin D **1i** and **5i** with the bulkier 1α-OMOM group, SO_2 is added preferentially from the upper-face regardless of the conditions (entries 9—13). However, it should be noted that the reactivity of the 5*Z*-isomer (**1i**) was considerably decreased, and the addition did not even proceed with SO_2 in CH_2Cl_2 solution (entry 9). The 5*E*-vitamin D with 1β-OH **14** yielded predominantly the lower-face adduct (6*R*)-**16** (entries 14—16).

Thus, in the case of 5*E*-vitamin D, it is likely that SO_2 adds to the s-*cis*-diene part from the side opposite to the C(1) substituent. The polar solvent MeOH may form a hydrogen bond with the 1α-OH group thereby increasing the steric bulkiness of the C(1) substituent and, in turn, causing the addition of SO_2 from the face opposite to the C(1) substituent. We have observed that vitamin D undergoes reversible *Z*–*E* isomerization at the 5,6-double bond in the presence of SO_2, the 5*E*-isomer being far more predominant. It was also found that the 5*E*-isomer is about 8 times more reactive than the 5*Z*-isomer in the SO_2-adduct formation (unpublished results). Therefore, it is likely that 5*Z*-vitamin D (**1**) reacts with SO_2 after being isomerized to the 5*E*-isomer (**5**). The vitamin D **1i** with a 1α-OMOM group reacts very sluggishly with SO_2 probably because it hardly undergoes *Z* to *E* isomerization (unpublished results). Details of the mechanism of the reaction of SO_2 with the diene in vitamin D will be reported elsewhere.

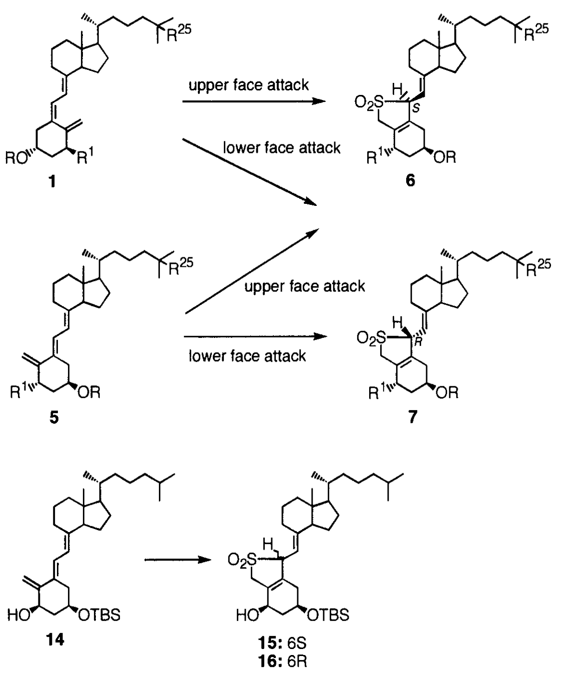
Electrophilic Fluorination of Vitamin D-SO_2 Adducts In the electrophilic substitution of the 6- and 19-positions of vitamin D–SO_2 adducts, the regioselectivity of the reaction can be controlled by selecting the base: for example, the selective electrophilic substitution at C(6) occurs under the conditions using a smaller and weaker base such as NaH, while the selective substitution at C(19) proceeds under conditions using a stronger and bulkier base such as LiHMDS.[25] By forming the SO_2 adducts, vitamin D is activated for the electrophilic substitution at the 6- and 19-positions under basic conditions. On the other hand, vitamin D–SO_2 adducts are unstable under the basic conditions: irreversible double bond isomerization and sulfolene ring opening with SO_2^- as a leaving group occur readily as side reactions under these conditions.[29,33] Therefore, the fluorination reactions described in this study were terminated, while a large amount of the starting material remained.

We investigated the electrophilic fluorination at C(19) of vitamin D–SO_2 adducts (Chart 5) under various conditions, and the results are summarized in Table 2. Upon treatment with $(PhSO_2)_2NF$ in the presence of the bulky base LiHMDS, the (6*S*)-isomer (**6h**) without 1α-OH group afforded the epimeric (*R*)- and (*S*)-19-fluoro-compounds **8h** and **9h** (3 : 1 ratio) in 51% total yield based on the recovered starting material (entry 1). The (6*R*)-isomer **7h** gave the (19*S*)-**10h** and



	R¹	R²⁵	R
a	OH	OH	H
f			TBS
b	OH	H	H
g			TBS
c	H	H	H
h			TBS
d	OMOM	H	H
i			TBS
e	OMOM	OMOM	H
j			TBS

Chart 3



	R¹	R²⁵	R
a	OH	OH	H
f			TBS
b	OH	H	H
g			TBS
c	H	H	H
h			TBS
d	OMOM	H	H
i			TBS
e	OMOM	OMOM	H
j			TBS
k	OTMS	H	TBS

Chart 4. Reaction of Vitamin D with SO_2

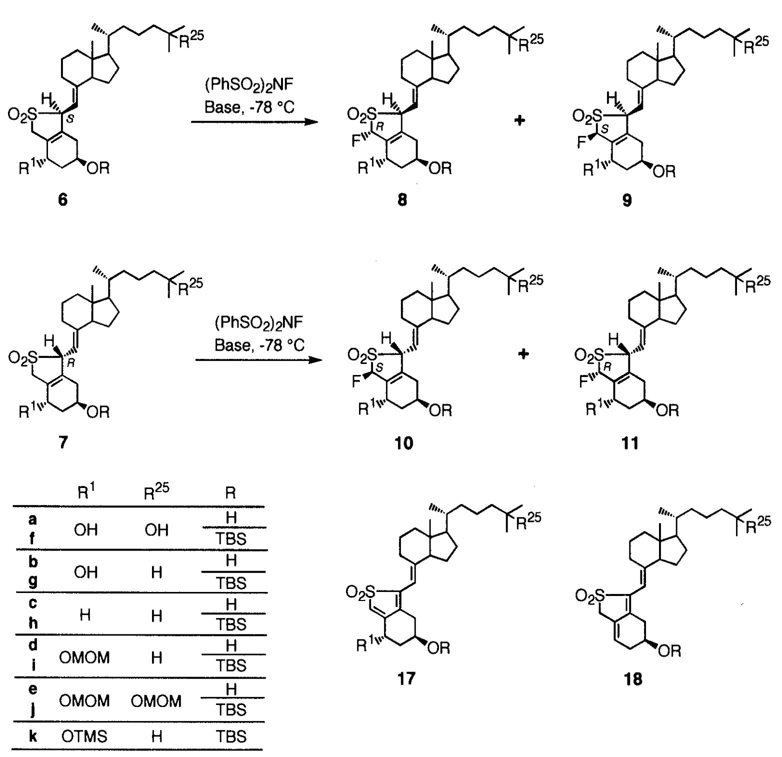
Table 1. Faceselectivity of the Reaction of SO_2 with Vitamin D[a]

Entry	Compound	Substituents C(1)	Substituents C(3)	Conditions	Adducts 6*S* : 6*R*
1	**1h**	—	β-OTBS	SO_2/CH_2Cl_2 (1 : 2)	1 : 1
2	**1h**	—	β-OTBS	SO_2	1 : 1
3	**1h**	—	β-OTBS	SO_2/MeOH (1 : 2)	1 : 1
4	**1g**	α-OH	β-OTBS	SO_2/CH_2Cl_2 (1 : 2)	2 : 3
5	**1g**	α-OH	β-OTBS	SO_2	3 : 2
6	**5g**	α-OH	β-OTBS	SO_2/CH_2Cl_2 (1 : 2)	2 : 3
7	**5g**	α-OH	β-OTBS	SO_2	3 : 2
8	**5g**	α-OH	β-OTBS	SO_2/MeOH (1 : 2)	4 : 1
9	**1i**	α-OMOM	β-OTBS	SO_2/CH_2Cl_2 (1 : 2)	NR[b]
10	**1i**	α-OMOM	β-OTBS	SO_2[c]	3.7 : 1
11	**5i**	α-OMOM	β-OTBS	SO_2/CH_2Cl_2 (1 : 2)	4 : 1
12	**5i**	α-OMOM	β-OTBS	SO_2	4 : 1
13	**5i**	α-OMOM	β-OTBS	SO_2/MeOH (1 : 2)	4 : 1
14	**14**	β-OH	β-OTBS	SO_2/CH_2Cl_2 (1 : 2)	1 : 5
15	**14**	β-OH	β-OTBS	SO_2	1 : 6
16	**14**	β-OH	β-OTBS	SO_2/MeOH (1 : 2)	1 : 4

a) The reaction was conducted at the refluxing temperature of SO_2 (−10 °C) for 30 min. *b*) NR: no SO_2-adduct was produced within 4 h. *c*) The reaction was continued for 4 h.

(19*R*)-**11h** compounds (3 : 1 ratio) in 51% yield (entry 2). In both cases, the fluorination occurred predominantly at the site *trans* to the substituent at C(6), and no epimerization at C(6) was observed in either the fluorinated products or the starting materials. Under the same conditions, no 19-fluorination product was obtained from the (6*S*)-SO_2-adducts (**6g**, **6i** and **6k**) bearing 1α-OH or its protected ones. Instead, a conjugated triene compound **17** or **18** was produced (entries 3, 9 and 10). The by-products (**17i**) are probably formed by the elimination of hydrogen fluoride from the 19-fluorinated adducts (**8i** and/or **9i**). Therefore, the yield of the by-product **17** is higher from sterically congested 1α-substituted adducts. The MOM ether **6i** gave 19-fluoro compounds (**8i** and **9i**) in low yield when a less bulky base was used (entries 4 and 5). The (6*R*)-isomer **7i** afforded solely (19*R*)-fluorinated compound **10i** (entry 6) when LiHMDS was used. The yield (55%) of the fluorination product **10i** was improved with a less bulky base LDA, but the by-product **17i** was also produced (30%) (entry 7).

In summary, the 19-fluorination of vitamin D–SO_2 adducts occurs preferentially *trans* to the C(6) substituent. Fluorination using other electrophilic fluorinating reagents such as *N*-



	R^1	R^{25}	R
a	OH	OH	H
f			TBS
b	OH	H	H
g			TBS
c	H	H	H
h			TBS
d	OMOM	H	H
i			TBS
e	OMOM	OMOM	H
j			TBS
k	OTMS	H	TBS

Chart 5. Electrophilic Fluorination of Vitamin D–SO_2 Adducts

Table 2. Electrophilic Fluorination of Vitamin D–SO_2 Adducts

Entry	Substr.	Base	Products (%)[a]					Recov. (%)
			8	9	10	11	Others	
1	**6h**	LiHMDS	38	13	—	—	**17** (18)	21
2	**7h**	LiHMDS	—	—	39	12	**17** (24)	30
3	**6i**	LiHMDS	—	—	—	—	**17** (65)	48
4	**6i**	LDA	23	36	—	—	**17** (20)	70
5	**6i**	*n*-BuLi	6	8	—	—	**17** (31)	49
6	**7i**	LiHMDS	—	—	47	—	**17** (43)	24
7	**7i**	LDA	—	—	55	—	**17** (30)	40
8	**7i**	*n*-BuLi	—	—	45	—	—	78
9	**6g**	LiHMDS	—	—	—	—	**18** (50)	50
10	**6k**	LiHMDS	—	—	—	—	**18** (69)	46
11	**7j**	LiHMDS	—	—	42	—	**17** (46)	50

a) Yields based on the recovered starting material.

fluoro-pyridinium triflate was unsuccessful. It should be noted that, under the fluorination conditions described above, no epimerization at the C(6) position occurred.

The stereochemistry of the 19-fluorinated compounds was assigned on the basis of the ^{1}H-NMR: The C(6) proton *cis* to the 19-fluorine appears at about 0.2 ppm lower field than the C(6) proton *trans* to the 19-fluorine. The stereochemistry of the cheletropic desulfonylation of the fluorinated adducts **8**, **9**, **10**, and **11** described below provided supporting evidence for the stereochemistry.

Desulfonylation of 19-Fluorinated SO_2-Adducts On thermal desulfonylation ($NaHCO_3$/refluxing EtOH) (Chart 6), both 6,19-*trans*-adducts **8h** and **10h** gave (5*E*,10*Z*)-**12h** and (5*Z*,10*E*)-**2h** in a 5—8 : 1 ratio in high yields (*ca.* 70%), whereas the two 6,19-*cis*-isomers **9h** and **11h** yielded (5*E*)-**13h** as a sole product (60—80%). These results indicate that the desulfonylation proceeded selectively in a suprafacial manner with respect to the diene part and that no *cis–trans* isomerization on the sulfolene ring occurred during the reaction. These results are in contrast with the precedents: the desulfonylation of *trans*-2,5-disubstituted sulfolenes under similar conditions gave predominantly 1,4-disubstituted (*E*,*E*)-dienes, contrary to the selection rule of the cheletropic reaction.[26,27] This stereoselectivity was explained by the *trans–cis* isomerization of the starting sulfolenes occurring under the basic desulfonylation conditions. In the present cases, 6,19-*trans* adducts (**8h** and **10h**) underwent the suprafacial desulfonylation following the selection rule of the cheletropic reaction. Thus no *trans–cis* isomerization occurred with these *trans*-substituted sulfolenes (**8h** and **10h**) probably because the terminal substituent fluorine is smaller than an alkyl group and the pathways to the (*Z*,*E*,*E*)- and (*E*,*Z*,*E*)-trienes (**12h** and **2h**) might not be sterically unfavorable.

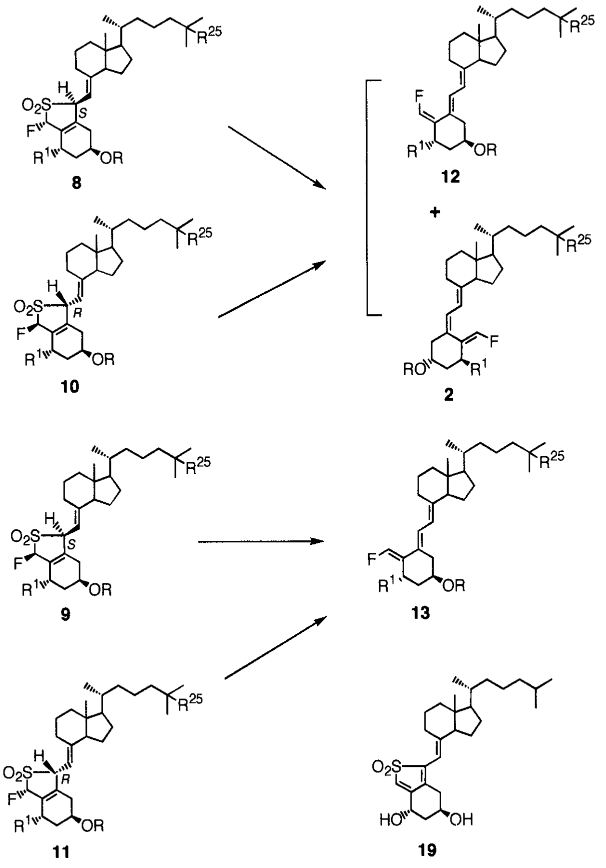
The hydroxyl protecting groups of the fluorinated SO_2-adducts (**8i**, **9i**, **10i** and **8j**, **9j**, **10j**) were deprotected under mild conditions (TMSBr in CH_2Cl_2 at $-20\,°C$, 52—63%), and the resulting sulfolenes (**8a**, **b**, **9a**, **b**, and **10a**, **b**) were subjected to desulfonylation. The hydroxyl protecting groups of **8i**, **9i**, **10i** and **8j**, **9j**, **10j** were removed prior to the desulfonylation reaction, because the vitamin D triene part decomposes under the deprotection conditions. However, thermal desulfonylation of 1α-hydroxylated SO_2-adducts (**8b**, **9b**, and **10b**) in the presence of $NaHCO_3$ yielded no desired 19-fluorovitamin D derivatives but gave only the hydrogen-fluoride elimination product **19**. Reductive desulfonylation ($LiAlH_4$ in ether, room temperature) gave the desired 19-fluorovitamin D as a mixture of (5*Z*,10*Z*)-isomers (**2b**) and (5*E*,10*E*)-(**12b**) in a 2 : 3 ratio (30% yield). The 6,19-*cis*-isomer (**9b**) was desulfonylated under the same conditions, yielding (5*E*,10*Z*)-**13b** (31%) as a single isomer. The above results indicated that the desulfonylation proceeded in the suprafacial manner under both thermal and reductive conditions.

To prepare 19-fluoro-1α-hydroxylated vitamin D derivatives more efficiently, we examined the introduction of the 1α-OH group into (5*E*)-19-fluorinated vitamin D **12c** and **13c**. Allylic oxidation with SeO_2–NMO is a convenient and the most widely used method for introducing a 1α-hydroxyl group into (5*E*)-vitamin D.[22] However, oxidation of **12h** with SeO_2 causes mostly decomposition of the starting material, and the only product detected (less than 5% yield) was a 9-hydroxylated compound. An alternative method using $Hg(OCOCF_3)_2$[34] caused only the geometrical isomerization at C(10) yielding a mixture of isomers **12h** and **13h** in *ca.* 25 : 75 ratio. Thus direct 1α-hydroxylation of the 19-fluorovitamin D was unsuccessful.

Photochemical and Thermal Isomerization of 19-Fluorovitamin D The (5*E*)-19-fluorovitamin D derivatives (**12** and **13**) were converted to the corresponding vitamin D derivatives (**2** and **3**) by photochemical isomerization.[35] (5*E*)-Vitamin D can be readily and selectively converted to the (5*Z*)-vitamin D by dye-sensitized photochemical double bond isomerization. (5*E*)-Vitamin D **12c** and **13c** were irradiated (halogen lamp) in benzene/ethanol in the presence of anthracene as a sensitizer until the starting materials disappeared (10—15 min), both yielding 19-fluorovitamin D_3 **2c** as the sole product (*ca.* 75%). Upon prolonged irradiation, slow isomerization of the 10,19-double bond occurred: thus, **2c** was irradiated until a photostationary state was reached (5 h under the same conditions) to yield a 65 : 35 mixture of **2c** and **3c** without any appreciable decomposition of the substrates. Irradiation of (5*E*,10*E*)-**12b** bearing the 1α-OH group afforded a mixture of three isomers (5*Z*,10*Z*)-**2b**, (5*Z*,10*E*)-isomer **3b** and (5*E*,10*Z*)-**13b** in a 85 : 10 : 5 ratio (70% yield) at a photostationary state (60 min).

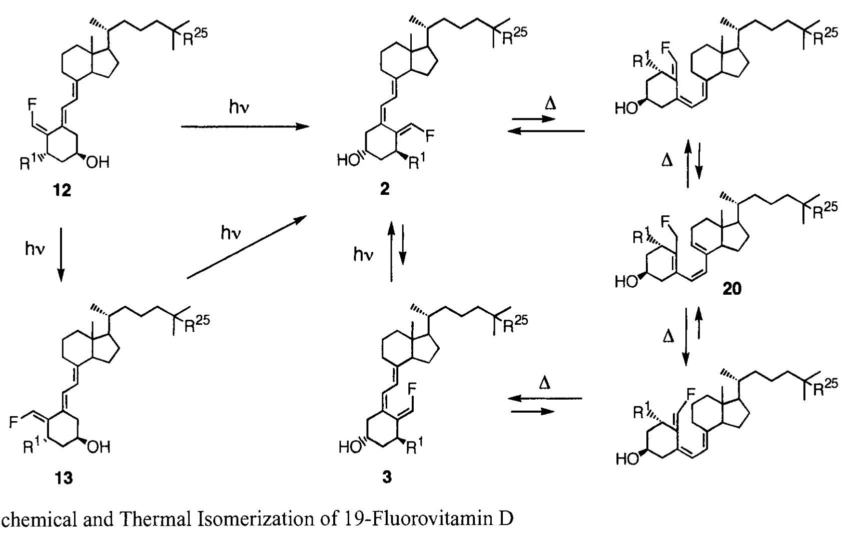
19-Fluorovitamin D **2** also undergoes thermal isomerization to give **3**. When heated in octane at 120 °C in a sealed tube for 4 h, **2c** (or **2b**) was isomerized to afford a 85 : 15 mixture of **2c** and **3c** (or **2b** and **3b**). This isomerization probably occurred *via* the 19-fluoroprevitamin D_3 **20**, though it was not detected in the reaction mixture. This result is in contrast to the results in which 19,19-difluoroprevitamin D did not undergo the thermal 1,7-sigmatropic rearrangement to the corresponding vitamin D (Chart 2).[18] Mono-fluorine substitution would not have much electronic effect on the 1,7-sigmatropic rearrangement.

(10*Z*)- and (10*E*)-19-Fluorovitamins **2b** and **2c** absorb UV at shorter wavelength (262 and 260 nm, respectively) than the parent vitamin D_3 (265 nm) does, while the (10*E*)-isomers **3b**



	R1	R25	R
a	OH	OH	H
f			TBS
b	OH	H	H
g			TBS
c	H	H	H
h			TBS
d	OMOM	H	H
i			TBS
e	OMOM	OMOM	H
j			TBS

Chart 6. Thermal Desulfonylation of 19-Fluorovitamin D–SO_2 Adducts



	R1	R25
a	OH	OH
b	OH	H
c	H	H

Chart 7. Photochemical and Thermal Isomerization of 19-Fluorovitamin D

and **3c** absorb in the normal range of 264 nm.

The stereochemistries of the fluorinated vitamin D_3 analogs **2**, **3**, **12** and **13** at C(5) and C(19) were unambiguously determined by their phase-sensitive 2D NOESY spectra. In **2b** and **2c**, a correlation cross peak was observed between H-7 and H-19, but not between H-1 and H-19. An NOE was observed between H-1 and H-19 in **3b** and **3c**. A cross peak was observed between H-1 and H-19 in **12b** and **12c**, while in **13b** and **13c** a cross peak between H-6 and H-19 was detected.

Synthesis and Biological Activity of (10*Z*)- and (10*E*)-19-Fluoro-1*α*,25-Dihydroxyvitamin D_3 Having examined the chemical and stereochemical details, the syntheses of (10*Z*)- and (10*E*)-19-fluoro-1,25-dihydroxyvitamin D_3, **2a** and **3a**, were achieved as shown in Chart 3. (5*E*)-1,25-Dihydroxyvitamin D_3 3-*tert*-butyldimethylsilyl ether **5f**, which was synthesized from vitamin D_2,[14,20,21)] was converted to the corresponding SO_2 adducts **6f** and **7f** (6*S*:6*R*=2:3, 60%). After protecting hydroxyl groups at C(1) and C(25) as MOM ethers, the major (6*R*)-SO_2 adduct **7j** was fluorinated by $(PhSO_2)_2NF$ to yield only the expected (6*R*,19*S*)-isomer **10j** (41% based on the recovered starting material). Deprotection with TMSBr (55%), followed by reductive desulfonylation, afforded (5*E*,10*Z*)-**12a** (15%) and **2a** (4%). To prepare an isomeric (5*E*,10*Z*)-**13a**, the same sequence of synthetic procedure using the (6*S*)-SO_2 adduct-1,25-MOM ether **6j** was used to give **13a**, together with **12a**. Each (5*E*)-19-fluorovitamin D **12a** and **13a** was converted to **2a** and **3a** by photochemical isomerization.

The binding affinity of **2a** and **2b** for the bovine thymus vitamin D receptor (VDR) was evaluated. The affinity of compounds **2a** and **2b** was about 1/10 as effective as that of the natural ligand **1a**.

In conclusion, we have established a facile method for converting vitamin D to 19-fluorinated vitamin D derivatives *via* regioselective electrophilic fluorination of vitamin D–SO_2 adducts as a key reaction. Using this method, syntheses of (10*Z*)- and (10*E*)-19-fluoro-1*α*,25-dihydroxyvitamin D_3 (**2a** and **3a**) were accomplished starting from vitamin D_2. We are now pursuing a ^{19}F-NMR study of the molecular recognition between VDR and these fluorovitamin D analogs.

Experimental

The NMR spectra were recorded on a Bruker ARX-400 MHz spectrometer, operating at 400 MHz for ^{1}H, 376 MHz for ^{19}F and 101 MHz for ^{13}C. Chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane as an internal standard (δ 0 ppm) for ^{1}H-NMR and trifluorotoluene as an external standard (δ −63 ppm) for ^{19}F-NMR. Low- and high-resolution mass spectra (LR-MS and HR-MS) were obtained with electronic ionization (EI) on a JEOL JMS-AX505HA spectrometer run at 70 eV for EI; *m/z* values are given with relative intensities in parentheses. IR spectra were recorded on a Jasco Janssen Microsampling FT IR spectrometer. UV spectra were obtained on a Hitachi U-3200 spectrophotometer. Column chromatography was carried out on silica gel (Wakogel C-200), unless otherwise indicated. All reactions, unless specifically mentioned, were conducted under a atmosphere of argon gas. Yields are not optimized.

(6*S*) and (6*R*)-SO_2-Adduct of (5*Z*,7*E*)-3-(*tert*-Butyldimethylsilyloxy)-9,10-seco-5,7,10(19)-cholestatriene (6h and 7h) Vitamin D_3–SO_2 adducts **6c** and **7c** were prepared following the reference methods.[23,24)] Vitamin D_3 **1c** (4.10 g, 10.65 mmol) was refluxed in liquid SO_2 (approximately 20 ml) for 30 min and then excess of liquid SO_2 was removed by flashing with the aid of N_2 gas, and the residue was dried *in vacuo* to afford a mixture of (6*S*)- and (6*R*)-SO_2-adducts **6c** and **7c**.

To a stirred solution of crude SO_2-adducts **6c** and **7c** in dry DMF (10 ml) was added imidazole (2.9 g, 42.6 mmol) and TBDMSCl (3.21 g, 21.3 mmol) and the whole mixture was stirred for 2 h at room temperature. The reaction mixture was diluted with H_2O and extracted with hexane, then ether. The combined organic phase was washed with brine, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on silica gel (150 g) using 10% AcOEt–hexane to give **6h** (2.98 g) and **7h** (2.67 g) in a 94.6% total yield.

6h: ^{1}H-NMR ($CDCl_3$) δ: 0.05, 0.06 (each 3H, s, 2×Si–Me), 0.65 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.92 (3H, d, J=6.5 Hz, 21-H), 2.60 (1H, m), 3.65 (2H, m, 19-H), 4.01 (1H, m, 3-H), 4.52 (1H, d, J=9.5 Hz, 7-H), 4.71 (1H, d, J=9.5 Hz, 6-H).

7h: ^{1}H-NMR ($CDCl_3$) δ: 0.05, 0.06 (each 3H, s, 2×Si–Me), 0.57 (3H, s, 18-H), 0.86, 0.87 (each 3H, d, J=6.3 Hz, 26, 27-H), 0.88 (9H, s, Si–*tert*-Bu), 0.93 (3H, d, J=6.1 Hz, 21-H), 2.57 (1H, m), 3.64, 3.68 (each 1H, d, J=16.0 Hz, 19-H), 3.97 (1H, m, 3-H), 4.63 (1H, d, J=10.2 Hz, 7-H), 4.78 (1H, d, J=10.2 Hz, 6-H).

(6*S*)- and (6*R*)-SO_2-Adduct of (5*Z*,7*E*)-3-(*tert*-Butyldimethylsilyloxy)-9,10-seco-5,7,10(19)-cholestatriene-1-ol (6g and 7g) (5*E*)-1*α*-Hydroxyvitamin D_3 3-*tert*-butyldimethylsilyl ether **5g** was synthesized by the published procedure.[20,21)] To a stirred, cold (−10 °C) solution of **5g** (90.1 mg, 0.18 mmol) in dry CH_2Cl_2 or CCl_4 (3 ml) was added liquid SO_2 (*ca.* 1.5 ml) and the mixture was refluxed for 30 min. Excess of liquid SO_2 and the solvent were evaporated *in vacuo*. The crude product was purified by chromatography on silica gel (7 g) with 20% AcOEt–hexane to afford **6g** and **7g** (79.0 mg, 6*S*:6*R*=2:3, 78.0%).

6g: ^{1}H-NMR ($CDCl_3$) δ: 0.061 (6H, s, Si–Me), 0.65 (3H, s, 18-H), 0.87 (9H, s, Si–*tert*-Bu), 0.86—0.88 (6H, 26, 27-H, overlapped with Si–*tert*-Bu), 0.92 (3H, d, J=6.3 Hz, 21-H), 2.60 (1H, m), 3.73 (1H, m, 19-H), 4.02 (1H, m, 19-H), 4.19 (1H, m, 3-H), 4.34 (1H, m, 1-H), 4.62 (1H, d, J=9.5 Hz, 7-H), 4.70 (1H, d, J=9.5 Hz, 6-H).

7g: ^{1}H-NMR ($CDCl_3$) δ: 0.057 (6H, s, Si–Me), 0.56 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.86—0.88 (6H, 26, 27-H, overlapped with Si–*tert*-Bu), 0.93 (3H, d, J=6.0 Hz, 21-H), 2.56 (2H, m), 3.73 (1H, m, 19-H), 4.02 (1H, m, 19-H), 4.16 (1H, m, 3-H), 4.34 (1H, m, 1-H), 4.65 (1H, d, J=10.2 Hz, 7-H), 4.80 (1H, d, J=10.2 Hz, 6-H).

6g and **7g** Mixture: EI-MS *m/z* (%): 514 (M^+-SO_2, 70), 496 (26), 457 (44), 455 (35), 439 (8), 382 (57), 364 (22), 247 (26), 209 (21), 134 (100). HR-EI-MS *m/z*: 514.4243 (M^+-SO_2; Calcd for $C_{33}H_{58}O_2Si$: 514.4206).

(6*S*)- and (6*R*)-SO_2-Adduct of (5*Z*,7*E*)-1-(Methoxymethyloxy)-3-(*tert*-butyldimethylsilyloxy)-9,10-seco-5,7,10(19)-cholestatriene (6i and 7i) A mixture of **6g** and **7g** (170.9 mg, 0.30 mmol), *N*,*N*-diisopropylethyl amine (0.51 ml, 2.95 mmol), chloromethyl methyl ether (0.11 ml, 1.47 mmol) in dry CH_2Cl_2 (5 ml) was stirred at 0 °C. After 5 h, additional *N*,*N*-diisopropylethyl amine (0.51 ml, 2.95 mmol) and chloromethyl methyl ether (0.11 ml, 1.47 mmol) were added and the mixture was stirried for 20 h. The reaction mixture was diluted with CH_2Cl_2 and the organic layer was washed with 1% HCl, 5% $NaHCO_3$, followed by brine, and dried ($MgSO_4$), and then evaporated *in vacuo*. The residue was purified by chromatography on silica gel (22 g) using 20—40% AcOEt–hexane to yield (6*S*)-**6i** (52.6 mg) and (6*R*)-**7i** (97.7 mg) in a total yield of 81.7%.

6i: ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.07 (each 3H, s, Si–Me), 0.66 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.86—0.88 (6H, 26, 27-H, overlapped with Si–*tert*-Bu), 0.92 (3H, d, J=6.3 Hz, 21-H), 2.19 (1H, m), 2.61 (1H, m), 3.38 (3H, s, OMe), 3.66, 3.98 (each 1H, d, J=15.7 Hz, 19-H), 4.16 (1H, m, 3-H), 4.22 (1H, m, 1-H), 4.59, 4.72 (each 1H, d, J=7.0 Hz, OCH_2O), 4.65 (1H, d, J=9.6 Hz, 7-H), 4.72 (1H, d, J=9.6 Hz, 6-H). EI-MS *m/z* (%): 558 (M^+-SO_2, 25), 528 (47), 513 (17), 498 (100), 496 (22), 441 (26), 439 (17), 426 (15), 381 (40), 364 (12), 178 (74). HR-EI-MS *m/z*: 558.4447 (M^+-SO_2; Calcd for $C_{35}H_{62}O_3Si$: 558.4468.

7i: ^{1}H-NMR ($CDCl_3$) δ: 0.05, 0.07 (each 3H, s, Si–Me), 0.56 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.86—0.88 (6H, 26, 27-H, overlapped with Si–*tert*-Bu), 0.93 (3H, d, J=6.1 Hz, 21-H), 2.35 (1H, m), 2.55 (1H, m), 3.39 (3H, s, OMe), 3.68 (1H, dd, J=16.1, 2.9 Hz, 19-H), 3.96 (1H, d, J=16.1 Hz, 19-H), 4.12 (1H, m, 3-H), 4.22 (1H, m, 1-H), 4.60, 4.73 (each 1H, d, J=7.0 Hz, OCH_2O), 4.62 (1H, d, J=10.2 Hz, 7-H), 4.80 (1H, d, J=10.2 Hz, 6-H). EI-MS *m/z* (%): 558 (M^+-SO_2, 29), 528 (39), 513 (15), 498 (100), 496 (30), 441 (22), 439 (16), 426 (14), 381 (31), 364 (14), 178 (62). HR-EI-MS *m/z*: 558.4483 (M^+-SO_2; Calcd for $C_{35}H_{62}O_3Si$: 558.4468).

(6*S*,19*R*)- and (6*S*,19*S*)-SO_2-Adduct of (5*Z*,7*E*)-3-(*tert*-Butyldimethylsilyloxy)-19-fluoro-9,10-seco-5,7,10(19)-cholestatriene (8h and 9h) (6*S*)-SO_2-adduct **6h** (225 mg, 0.40 mmol), HMPA (139 μl, 0.80 mmol) and *N*-fluorodibenzenesulfonamide (151.4 mg, 0.48 mmol) were dissolved in dry THF (3 ml) and the solution was cooled to −78 °C. To this solution was added LiHMDS (1 M solution in THF, 480 μl, 0.48 mmol) and the whole mixture was stirred for 10 min and then quenched with sat. NH_4Cl. The mixture was taken up in ether and the organic layer was washed with brine, dried ($MgSO_4$), and then evaporated to dryness. The residues was separated by chromatography on silica gel (50 g) using 3% AcOEt–hexane to give **8h** (less polar, 69.7 mg, 30.0%), **9h** (more polar, 23.5 mg, 10.1%), **17h** (32.6 mg, 14.0%) and the unreacted starting material (47.9 mg, recovery 21.3%).

8h: ^{1}H-NMR ($CDCl_3$) δ: 0.05, 0.07 (each 3H, s, 2×Si–Me), 0.65 (3H, s, 18-H), 0.87 (9H, s, Si–*tert*-Bu), 0.93 (3H, d, J=6.4 Hz, 21-H), 2.32 (2H, m), 2.60 (1H, m), 4.04 (1H, m, 3-H), 4.66 (2H, m, 6, 7-H), 5.42 (1H, d, J=56.9 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −165.2 (d, J=56.9 Hz). EI-MS *m/z* (%): 516 (M^+-SO_2, 100), 496 (11), 459 (13), 439 (7), 431 (4), 403 (10), 384 (19), 364 (47), 271 (19), 259 (30), 251 (45), 211 (61), 136 (37), 117 (47). HR-EI-MS *m/z*: 516.4178 (M^+-SO_2; Calcd for $C_{33}H_{57}OFSi$: 516.4163).

9h: ^{1}H-NMR ($CDCl_3$) δ: 0.05, 0.07 (each 3H, s, 2×Si–Me), 0.66 (3H, s, 18-H), 0.87 (9H, s, Si–*tert*-Bu), 0.92 (3H, d, J=7.0 Hz, 21-H), 4.04 (1H, m, 3-H), 4.43, 4.65 (each 1H, d, J=9.6 Hz, 6, 7-H), 5.35 (1H, d, J=56.3 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −161.0 (d, J=56.3 Hz). EI-MS *m/z* (%): 516 (M^+-SO_2, 40), 496 (3), 459 (5), 439 (3), 431 (1), 402 (43), 403 (16), 384 (16), 364 (11), 271 (17), 259 (23), 251 (16), 211 (37), 136 (84), 135 (100), 117 (40). HR-EI-MS *m/z*: 516.4135 (M^+-SO_2; Calcd for $C_{33}H_{57}OFSi$: 516.4163).

17h: ^{1}H-NMR ($CDCl_3$) δ: 0.05, 0.07 (each 3H, s, 2×Si–Me), 0.62 (3H, s, 18-H), 0.87 (9H, s, Si–*tert*-Bu), 0.93 (3H, d, J=6.1 Hz, 21-H), 2.38 (1H, dd, J=17.9, 5.8 Hz), 2.51 (2H, m), 2.85 (1H, m), 4.06 (1H, m, 3-H), 5.41 (1H, s, 7-H), 6.24 (1H, s, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −165.2 (d, J=56.9 Hz). EI-MS *m/z* (%): 560 (M^+, 100), 545 (7), 503 (15), 476 (9), 447 (48), 398 (10), 364 (8), 315 (5), 295 (7), 247 (14), 195 (17), 133 (13). HR-EI-MS *m/z*: 560.3719 (Calcd for $C_{33}H_{56}O_3FSiS$: 560.3719).

(6*R*,19*S*)- and (6*R*,19*R*)-SO_2-Adduct of (5*Z*,7*E*)-3-(*tert*-Butyldimethylsilyloxy)-19-fluoro-9,10-seco-5,7,10(19)-cholestatriene (10h and 11h) (6*R*)-SO_2-adduct **7h** (2.64 g, 4.69 mmol) was fluorinated as described for the preparation of **8h** and **9h** to give **10h** (less polar, 744.3 mg, 27.4%), **11h** (more polar, 233.3 mg, 8.6%), **17h** (451.9 mg, 17.2%) and the recovered starting material (796.7 mg, 30.2%).

10h: ^{1}H-NMR ($CDCl_3$) δ: 0.05, 0.06 (each 3H, s, 2×Si–Me), 0.56 (3H, s, 18-H), 0.87 (9H, s, Si–*tert*-Bu), 0.93 (3H, d, J=5.9 Hz, 21-H), 2.33 (1H, m), 2.48 (1H, m), 2.55 (1H, m), 3.99 (1H, m, 3-H), 4.74 (2H, s, 6, 7-H), 5.37 (1H, d, J=56.7 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −164.7 (d, J=56.7 Hz). EI-MS *m/z* (%): 516 (M^+-SO_2, 100), 496 (9), 459 (8), 439 (5), 431 (4), 403 (11), 384 (11), 364 (13), 271 (17), 259 (33), 251 (16), 211 (39), 136 (31), 117 (37). HR-EI-MS *m/z*: 516.4184 (M^+-SO_2; Calcd for $C_{33}H_{57}OFSi$: 516.4163).

11h: ^{1}H-NMR ($CDCl_3$) δ: 0.05, 0.06 (each 3H, s, 2×Si–Me), 0.55 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.93 (3H, d, J=6.0 Hz, 21-H), 2.35 (2H, m), 2.57 (1H, m), 4.01 (1H, m, 3-H), 4.50, 4.69 (each 1H, d, J=10.1 Hz, 6, 7-H), 5.40 (1H, d, J=54.6 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −161.6 (d, J=54.6 Hz). EI-MS *m/z* (%): 516 (M^+-SO_2, 100), 496 (4), 459 (8), 439 (4), 431 (3), 403 (9), 384 (10), 364 (29), 271 (14), 259 (27), 251 (32), 211 (30), 136 (19), 117 (27). HR-EI-MS *m/z*: 516.4181 (M^+-SO_2; Calcd for $C_{33}H_{57}OFSi$: 516.4163).

(6*S*,19*R*)- and (6*S*,19*S*)-SO_2-Adduct of (5*Z*,7*E*)-1-(Methoxymethyloxy)-3-(*tert*-butyldimethylsilyloxy)-19-fluoro-9,10-seco-5,7,10(19)-cholestatriene (8i and 9i) To a stirred, cold (−78 °C) solution of (6*S*)-**6i** (85 mg, 0.14 mmol), *N*-fluorodibenzenesulfonamide (52 mg, 0.16 mmol), HMPA (60 μl) in THF (3 ml) was added LDA (2 M solution in THF, 82 μl, 0.16 mmol) and the mixture was stirred for 10 min. The reaction mixture was quenched by sat. NH_4Cl and extracted with ether. The extracts were rinsed with brine, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was separated by chromatography on silica gel (10 g) with 20% AcOEt–hexane to give **8i** and **9i** (21 mg) as two isomeric fluoro-adducts, **17i** (5.2 mg, 6.1%) and the unreacted starting material **6i** (60 mg, 70%). The epimeric 19-fluoro-adducts were re-purified by chromatography on silica gel with 35% $CHCl_3$-benzene to yield the less polar **9i** (9.4 mg, 10.8%) and the more polar **8i** (6.0 mg, 6.9%).

8i: ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.08 (each 3H, s, Si–Me), 0.63 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.86—0.88 (6H, 26, 27-H, overlapped with Si–*tert*-Bu), 0.93 (3H, d, J=6.3 Hz, 21-H), 2.25 (1H, m, 4-H), 2.62 (1H, m, 9-H), 3.42 (3H, s, OMe), 4.20 (1H, m, 3-H), 4.46 (1H, m, 1-H), 4.64, 4.77 (each 1H, d, J=7.1 Hz, OCH_2O), 4.72 (1H, d, J=10.1 Hz, 7-H), 4.84 (1H, dd, J=10.1, 4.3 Hz, 6-H), 5.51 (1H, d, J=56.4 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −157.5 (d, J=56.4 Hz). EI-MS *m/z* (%): 576 (M^+-SO_2, 14), 546 (35), 512 (15), 494 (3), 399 (4), 362 (29), 135 (21), 75 (100). HR-EI-MS *m/z*: 576.4354 (M^+-SO_2; Calcd for $C_{35}H_{61}O_3FSi$: 576.4374).

9i: ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.08 (each 3H, s, Si–Me), 0.66 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.86—0.88 (6H, 26, 27-H, overlapped with Si–*tert*-Bu), 0.92 (3H, d, J=6.4 Hz, 21-H), 2.11 (1H, m, 4-H), 2.28 (1H, m, 4-H), 2.58 (1H, m, 9-H), 3.40 (3H, s, OMe), 4.20 (1H, m, 3-H), 4.46 (1H, m, 1-H), 4.54 (1H, dd, J=9.6, 6.7 Hz, 6-H), 4.65 (1H, d, J=9.6 Hz, 7-H), 4.67, 4.75 (each 1H, d, J=6.9 Hz, OCH_2O), 5.70 (1H, d, J=56.1 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −163.3 (d, J=56.1 Hz). EI-MS *m/z* (%): 576 (M^+-SO_2, 4), 546 (100), 531 (4), 494 (5), 489 (5), 457 (4), 381 (7), 362 (9), 241 (19), 135 (37). HR-EI-MS *m/z*: 546.4299 (M^+-SO_2; Calcd for $C_{34}H_{59}O_2FSi$: 546.4268).

17i: ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.09 (each 3H, s, Si–Me), 0.61 (3H, s, 18-H), 0.87 (9H, s, Si–*tert*-Bu), 0.86—0.88 (6H, 26, 27-H, overlapped with Si–*tert*-Bu), 0.93 (3H, d, J=6.1 Hz, 21-H), 2.04 (1H, m), 2.12 (1H, m), 2.20 (1H, m, 2-H), 2.46 (1H, m, 4-H), 2.54 (1H, m, 4-H), 3.41 (3H, s, OMe), 4.31 (1H, m, 3-H), 4.71, 4.75 (each 1H, d, J=6.9 Hz, OCH_2O), 4.80 (1H, br s, 1-H), 5.41 (1H, s, 7-H), 6.54 (1H, d, J=2.0 Hz, 19-H). MS *m/z* (%): 620 (M^+, 5), 558 (4), 445 (3), 309 (36), 252 (44), 235 (19), 135 (19), 64 (100).

(6*R*,19*S*)-SO_2-Adduct of (5*Z*,7*E*)-1-(Methoxymethyloxy)-3-(tert-butyldimethylsilyloxy)-19-fluoro-9,10-seco-5,7,10(19)-cholestatriene (10i) (6*R*)-SO_2-adduct **7i** (97.7 mg, 0.16 mmol) was fluorinated as described for the preparation of **8i** and **9i**, but using LiHMDS as a base to give **10i** (36.0 mg, 35.8%), **17i** (31.6 mg, 32.4%) and the unreacted starting material (23.7 mg, 24.3%).

10i: ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.07 (each 3H, s, Si–Me), 0.57 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.86—0.88 (6H, 26, 27-H, overlapped with Si–*tert*-Bu), 0.93 (3H, d, J=5.9 Hz, 21-H), 2.42 (1H, m), 2.54 (1H, m), 3.40 (3H, s, OMe), 4.16 (1H, m, 3-H), 4.44 (1H, br s, 1-H), 4.67, 4.76 (each 1H, d, J=7.0 Hz, OCH_2O), 4.78 (2H, br s, 6, 7-H), 5.70 (1H, d, J=56.9 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −166.3 (d, J=56.9 Hz). EI-MS *m/z* (%): 576 (M^+-SO_2, 33), 546 (100), 531 (8), 514 (8), 489 (5), 465 (6), 457 (6), 381 (10), 363 (7), 259 (28), 207 (29), 135 (83). HR-EI-MS *m/z*: 576.4412 (M^+-SO_2; Calcd for $C_{35}H_{61}O_3FSi$: 576.4374).

(6*S*,19*S*)-SO_2-Adduct of (5*Z*,7*E*,10*E*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-1,3-diol (9b) and (6*R*,19*S*)-SO_2-Adduct of (5*Z*,7*E*,10*Z*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-1,3-diol (10b) To a stirred, cold (−40 °C) solution of (6*R*)-**10i** (21.9 mg, 0.03 mmol) in dry CH_2Cl_2 (1 ml) was added bromotrimethylsilane (18 μl, 0.14 mmol) and the mixture was stirred for 4 h and then an additional trimethylbromosilane (9 μl) was added. After being stirred for 2 h, the reaction was quenched with 5% $NaHCO_3$ and the mixture was extracted with AcOEt. The organic extract was rinsed with H_2O, brine, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on silica gel (5 g) using 50% AcOEt–hexane to yield **10b** (10.2 mg, 62.6%).

The same procedure and work-up as mentioned above except employing (6*S*)-**9i** (8.7 mg, 0.014 mmol) gave **9b** (3.6 mg, 54.0%).

9b: ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-H), 0.86, 0.87 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.92 (3H, d, 21-H), 2.40 (1H, m), 2.58 (1H, m, 9-H), 4.28 (1H, m, 3-H), 4.58 (1H, dd, J=9.8, 6.5 Hz, 6-H), 4.66 (1H, m, 1-H), 4.69 (1H, d, J=9.8 Hz, 7-H), 5.77 (1H, d, J=56.0 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −163.4 (d, J=56.0 Hz). EI-MS *m/z* (%): 418 (M^+-SO_2, 20), 398 (29), 380 (17), 362 (25), 305 (10), 285 (16), 267 (18), 249 (30), 135 (100). HR-EI-MS *m/z*: 418.3217 (M^+-SO_2; Calcd for $C_{27}H_{43}O_2F$: 418.3247).

10b: ^{1}H-NMR ($CDCl_3$) δ: 0.57 (3H, s, 18-H), 0.867, 0.873 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.93 (3H, d, J=6.0 Hz, 21-H), 2.45—2.60 (2H, m), 4.28 (1H, m, 3-H), 4.66 (1H, m, 1-H), 4.75 (1H, d, J=10.0 Hz, 6-H), 4.85 (1H, d, J=10.0 Hz, 7-H), 5.78 (1H, d, J=56.5 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −165.9 (d, J=56.5 Hz). IR: 3354, 1325 cm^{-1}. EI-MS *m/z* (%): 418 (M^+-SO_2, 33), 398 (100), 380 (15), 362 (10), 305 (14), 285 (48), 267 (31), 249 (24), 135 (77). HR-EI-MS *m/z*: 418.3248 (M^+-SO_2; Calcd for $C_{27}H_{43}O_2F$: 418.3247).

(5*E*,7*E*,10*Z*)- and (5*Z*,7*E*,10*E*)-3-(*tert*-Butyldimethylsilyloxy)-19-fluoro-9,10-seco-5,7,10(19)-cholestatriene (12h and 2h) A solution of (6*S*,19*R*)-**8h** (842.5 mg, 1.45 mmol) and $NaHCO_3$ (609.2 mg, 7.25 mmol) in EtOH (20 ml) was heated at 80 °C for 1.5 h in a sealed tube. The mixture was cooled to room temperature and filtered. The filtrate was partitioned between water and AcOEt and the aqueous layer was extracted with AcOEt. The combined organic phase was washed with H_2O, dried ($MgSO_4$), then evaporated to dryness. The crude product was chromatographed on silica gel (80 g), using 2% AcOEt–hexane to yield (5*E*)-**12h** (447.2 mg, 59.7%), (5*Z*)-**2h** (94.0 mg, 12.5%) and the unreacted starting material (193.0 mg, 22.9%).

(6*R*,19*S*)-Isomer **10h** (103.6 mg, 0.18 mmol) was thermal-desulfonylated as mentioned above to yield **12h** (60.3 mg, 65.5%), **2h** (7.5 mg, 8.2%) and the unreacted starting material (8.9 mg, 8.6%).

12h: ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.07 (each 3H, s, 2×Si–Me), 0.56 (3H, s, 18-H), 0.868, 0.873 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.88 (9H, s, Si–*tert*-Bu), 0.92 (3H, d, J=6.4 Hz, 21-H), 2.26 (2H, m), 2.64 (1H, m), 2.81 (1H, m), 3.84 (1H, m, 3-H), 5.92, 6.57 (each 1H, d, J=11.5 Hz, 7-, 6-H), 6.48 (1H, d, J=85.7 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −135.9 (d, J=85.7 Hz). EI-MS *m/z* (%): 516 (M^+, 100), 496 (5), 459 (7), 439 (4), 431 (4), 403 (9), 384 (8), 365 (37), 364 (4), 271 (15), 259 (29), 251 (10), 211 (28), 136 (22), 117 (29). HR-EI-MS *m/z*: 516.4193 (Calcd for $C_{33}H_{57}OFSi$: 516.4163). UV λ_{max} (hexane): 204, 269 nm.

2h: ^{1}H-NMR ($CDCl_3$) δ: 0.069, 0.073 (each 3H, s, 2×Si–Me), 0.55 (3H, s, 18-H), 0.869, 0.874 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.89 (9H, s, Si–*tert*-Bu), 0.92 (3H, d, J=6.4 Hz, 21-H), 2.20, 2.45 (each 1H, m, 4-H), 2.72 (1H, m, 1-H), 2.80 (1H, m), 3.81 (1H, m, 3-H), 5.92, 6.21 (each 1H, d, J=11.0 Hz, 6, 7-H), 6.49 (1H, d, J=87.6 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −133.0 (d, J=87.6 Hz). EI-MS *m/z* (%): 516 (M^+, 44), 496 (7), 459 (18), 439 (10), 431 (2), 403 (6), 384 (23), 365 (21), 364 (20), 271 (13), 259 (9), 251 (17), 211 (100), 136 (81), 117 (83). HR-EI-MS *m/z*: 516.4141 (Calcd for $C_{33}H_{57}OFSi$: 516.4163). UV λ_{max} (hexane): 262 nm.

(5*E*,7*E*,10*E*)-3-(*tert*-Butyldimethylsilyloxy)-19-fluoro-9,10-seco-5,7,10(19)-cholestatriene (13h) (6*S*,19*S*)-Isomer **9h** (206.7 mg, 0.36 mmol) or (6*R*,19*R*)-isomer **11h** (49.7 mg, 0.09 mmol) was thermal-desulfonylated as described for the preparation of **2h** and **12h** to yield **13h** (111.5 mg, 60.6% from **9h**; 35.2 mg, 79.6% from **11h**).

13h: ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.07 (each 3H, s, 2×Si–Me), 0.55 (3H, s, 18-H), 0.86, 0.87 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.89 (9H, s, Si–*tert*-Bu), 0.92 (3H, d, J=6.3 Hz, 21-H), 2.25 (1H, m), 2.61 (2H, m), 2.81 (1H, m), 3.85 (1H, m, 3-H), 5.81, 6.29 (each 1H, d, J=11.4 Hz, 6, 7-H), 6.66 (1H, d, J=87.1 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −137.2 (d, J=87.1 Hz). EI-MS *m/z* (%): 516 (M^+, 100), 496 (5), 459 (10), 439 (6), 431 (3), 403 (7), 384 (14), 365 (13), 364 (6), 271 (18), 259 (21), 251 (12), 211 (94), 136 (74), 117 (84). HR-EI-MS *m/z*: 516.4133 (Calcd for $C_{33}H_{57}OFSi$: 516.4163).

(5*E*,7*E*,10*Z*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-3-ol (12c) and (5*E*,7*E*,10*E*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-3-ol (13c) To a stirred solution of **12h** (27.6 mg, 0.05 mmol) in dry THF (2 ml) was added *n*-Bu_4NF (1 M solution in THF, 0.16 mmol). The mixture was stirred for 1 h at room temperature and diluted with ether. The organic layer was washed with brine, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was purified by chromatography on silica gel (5 g) with 3% AcOEt–hexane to afford **12c** (20.1 mg, 93.6%).

The same procedure and work-up as described in the preceding experiment, but using **13h** (100.0 mg, 0.19 mmol) gave **13c** (68.2 mg, 87.5%).

12c: ^{1}H-NMR ($CDCl_3$) δ: 0.56 (3H, s, 18-H), 0.867, 0.871 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.92 (3H, d, J=6.4 Hz, 21-H), 2.83 (2H, m), 3.86 (1H, m, 3-H), 5.93, 6.63 (each 1H, d, J=11.5 Hz, 6, 7-H), 6.51 (1H, d, J=86.6 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −134.8 (d, J=86.6 Hz). EI-MS *m/z* (%): 402 (M^+, 100), 384 (7), 382 (4), 364 (4), 317 (20), 289 (42), 271 (18), 269 (7), 259 (47), 194 (12), 176 (16), 154 (20), 136 (39), 135 (67). HR-EI-MS *m/z*: 402.3320 (Calcd for $C_{27}H_{43}OF$: 402.3298). UV λ_{max} (95% EtOH): 270 nm (ε 22000).

13c: ^{1}H-NMR ($CDCl_3$) δ: 0.55 (3H, s, 18-H), 0.868, 0.873 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.92 (3H, d, J=6.7 Hz, 21-H), 2.67 (1H, m), 2.82 (2H, m), 3.88 (1H, m, 3-H), 5.83, 6.35 (each 1H, d, J=11.5 Hz, 6, 7-H), 6.69 (1H, d, J=86.7 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −135.4 (d, J=86.7 Hz). EI-MS *m/z* (%): 402 (M^+, 84), 384 (7), 382 (3), 364 (4), 317 (11), 289 (25), 271 (13), 269 (7), 259 (21), 194 (6), 176 (13), 154 (50), 136 (89), 135 (100). HR-EI-MS *m/z*: 402.3268 (Calcd for $C_{27}H_{43}OF$: 402.3298). UV λ_{max} (95% EtOH): 269 nm.

Thermal Desulfonylation of **10b**: A solution of (6*R*,19*S*)-**10b** (10.0 mg, 0.02 mmol) in 95% EtOH was heated at 80 °C for 1 h in the presence of $NaHCO_3$ (35 mg, 0.42 mmol). The mixture was cooled to room temperature and filtered. The filtrate was partitioned between water and AcOEt and the aqueous layer was extracted with AcOEt. The combined organic phase was washed with brine, dried ($MgSO_4$), and evaporated to dryness. The residue was chromatographed on silica gel (4 g) with 80% AcOEt–hexane to give **19** (8.0 mg, 83.0%).

19: ^{1}H-NMR ($CDCl_3$) δ: 0.63 (3H, s, 18-H), 0.87, 0.88 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.93 (3H, d, J=6.1 Hz, 21-H), 2.12 (1H, m), 2.27 (1H, m, 2-H), 2.48, 2.57 (each 1H, m, 4-H), 4.43 (1H, m, 3-H), 4.94 (1H, m, 1-H), 5.43 (1H, s, 7-H), 6.61 (1H, d, J=2.0 Hz, 19-H).

(5*Z*,7*E*,10*Z*)-, (5*E*,7*E*,10*E*)- and (5*E*,7*E*,10*Z*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatri-ene-1,3-diol (2b, 12b and 13b) To a stirred solution of **10b** (8.6 mg, 0.02 mmol) in dry ether (1 ml) was added $LiAlH_4$ (5.4 mg, 0.14 mmol) and the mixture was stirred at room temperature for 30 min and then quenched with sat. sodium potassium tartarate. The resulting slurry was filtered and washed with ether. The ether layer was dried ($MgSO_4$) and evaporated *in vacuo*. The crude product was purified by chromatography on silica gel (3 g) using 40—50% AcOEt–hexane to afford **2b** (0.9 mg, 12.1%), **12b** (1.3 mg, 17.4%) and the unreacted starting material (0.9 mg, 10.2%).

The same procedure and work-up as described in the preceding experiment, but using **9b** (1.2 mg, 0.0025 mmol) gave **13b** (0.32 mg, 30.6%) and the unreacted starting material (0.8 mg, 66.7%).

2b: ^{1}H-NMR ($CDCl_3$) δ: 0.53 (3H, s, 18-H), 0.863, 0.867 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.91 (3H, d, J=6.3 Hz, 21-H), 2.19 (1H, m), 2.32 (1H, m), 2.68 (1H, m), 2.80 (1H, m, 9-H), 4.16 (1H, m, 3-H), 5.09 (1H, br s, 1-H), 5.90 (1H, d, J=11.1 Hz, 7-H), 6.46 (1H, d, J=11.1 Hz, 6-H), 6.50 (1H, d, J=86.0 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −129.8 (d, J=86.0 Hz). EI-MS *m/z* (%): 418 (M^+, 6), 400 (5), 398 (5), 380 (10), 362 (52), 347 (8), 305 (5), 287 (5), 285 (5), 267 (8), 249 (34), 195 (35), 135 (100). HR-EI-MS *m/z*: 418.3224 (Calcd for $C_{27}H_{43}O_2F$: 418.3247). IR: 3290 cm^{-1}. UV λ_{max} (95% EtOH): 262 nm.

12b: ^{1}H-NMR ($CDCl_3$) δ: 0.57 (3H, s, 18-H), 0.87, 0.88 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.93 (3H, d, J=6.3 Hz, 21-H), 2.19 (1H, m), 2.32 (1H, m), 2.68 (1H, m), 2.80 (1H, m, 9-H), 3.07 (1H, dd, 4-H), 4.19 (1H, m, 3-H), 4.34 (1H, br s, 1-H), 5.97 (1H, d, J=11.6 Hz, 7-H), 6.68 (1H, d, J=11.6 Hz, 6-H), 6.74 (1H, d, J=83.4 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −132.8 (d, J=83.4 Hz). EI-MS *m/z* (%): 418 (M^+, 100), 398 (61), 380 (23), 362 (54), 347 (14), 305 (30), 287 (19), 285 (36), 267 (30), 249 (53), 195 (40), 135 (88). HR-EI-MS *m/z*: 418.3234 (Calcd for $C_{27}H_{43}O_2F$: 418.3247). UV λ_{max} (95% EtOH): 272 nm.

13b: ^{1}H-NMR ($CDCl_3$) δ: 0.55 (3H, s, 18-H), 0.868, 0.872 (each 3H, d, J=6.5 Hz, 26, 27-H), 0.93 (3H, d, J=6.2 Hz, 21-H), 2.31 (1H, m), 2.81 (1H, m, 9-H), 3.15 (1H, m, 4-H), 4.15 (1H, m, 3-H), 5.05 (1H, d, J=3.2 Hz, 1-H), 5.87 (1H, d, J=11.4 Hz, 7-H), 6.38 (1H, d, J=11.4 Hz, 6-H), 6.69 (1H, d, J=85.6 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −133.2 (d, J=85.6 Hz). EI-MS *m/z* (%): 418 (M^+, 12), 398 (26), 380 (31), 362 (51), 347 (13), 305 (7), 287 (7), 285 (15), 267 (26), 249 (44), 195 (41), 135 (100). HR-EI-MS *m/z*: 418.3232 (Calcd for $C_{27}H_{43}O_2F$: 418.3247). UV λ_{max} (95% EtOH): 269 nm.

(5*Z*,7*E*,10*E*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-3-ol (2c) A solution of **12c** (20.0 mg, 0.05 mmol) in benzene–EtOH (5 : 95, v/v, 200 ml) was cooled to 0 °C and with Ar passed through it the mixture was irradiated using halogen lamp (200 W) in the presence of anthracene (44.0 mg, 0.25 mmol). After being stirred for 15 min, solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel (5 g) using 4% AcOEt–hexane to yield **2c** (15.4 mg, 76.8%).

A solution of **13c** (4.1 mg, 0.01 mmol) and anthracene (9 mg, 0.05 mmol) dissolved in benzene–EtOH (5 : 95, 100 ml) was photo-irradiated for 10 min and the same work-up and purification as described above gave **2c** (2.7 mg, 65.9%).

2c: ^{1}H-NMR ($CDCl_3$) δ: 0.53 (3H, s, 18-H), 0.86, 0.87 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.92 (3H, d, J=6.4 Hz, 21-H), 2.56 (2H, m), 2.78 (1H, m), 3.93 (1H, m, 3-H), 5.93, 6.28 (each 1H, d, J=11.1 Hz, 6, 7-H), 6.51 (1H, d, J=87.4 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −132.5 (d, J=87.4 Hz). EI-MS *m/z* (%): 402 (M^+, 36), 384 (21), 382 (8), 364 (16), 317 (5), 289 (13), 271 (19), 269 (6), 259 (11), 154 (45), 136 (70), 135 (100). HR-EI-MS *m/z*: 402.3301 (Calcd for $C_{27}H_{43}OF$: 402.3298). UV λ_{max} (95% EtOH): 260 nm (ε 20900).

(5*Z*,7*E*,10*E*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-1,3-diol (3b) A stirred, cold (0 °C) solution of **12b** (7.5 mg, 0.02 mmol), anthracene (15.9 mg, 0.090 mmol) in benzene–EtOH (5 : 95; v/v; 150 ml) was purged with Ar and irradiated at 0 °C for 60 min (halogen lamp; 200 W). The solvent was evaporated to dryness and the residue was chromatographed on silica gel (3 g). A mixture of **2b** and **13b** (6.1 mg) was eluted with 30% AcOEt–hexane and **3b** (549 mg, 7.3%) was eluted with 70% AcOEt–hexane. The mixture of **2b** and **13b** was further purified by HPLC [YMC pack ODS-AM SH-342-5AM 120A; 150 mm×20 mm; 8% H_2O–MeOH; 8 ml/min.; room temperature] to afford **2b** (4.45 mg, 59.3%) and **13b** (227 mg, 3.0%).

3b: ^{1}H-NMR ($CDCl_3$) δ: 0.54 (3H, s, 18-H), 0.86, 0.87 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.92 (3H, d, J=6.3 Hz, 21-H), 2.14 (1H, m), 2.28 (1H, dd, J=13.0, 8.6 Hz, 4-H), 2.66 (1H, dd, J=13.0, 3.9 Hz, 4-H), 2.82 (1H, m, 9-H), 4.20 (1H, m, 3-H), 4.44 (1H, dd, J=9.2, 4.7 Hz, 1-H), 5.62 (1H, dd, J=11.3, 5.4 Hz, 7-H), 6.52 (1H, d, J=11.3 Hz, 6-H), 6.70 (1H, d, J=84.0 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −127.8 (broad s). EI-MS *m/z* (%): 418 (M^+, 55), 400 (7), 398 (12), 380 (22), 362 (100), 347 (21), 305 (16), 287 (9), 285 (10), 267 (16), 249 (68), 195 (52), 135 (43). HR-EI-MS *m/z*: 418.3221 (Calcd for $C_{27}H_{43}O_2F$: 418.3247). UV λ_{max} (95% EtOH): 264 nm.

Thermal Isomerization of 2c and 2b: (5*Z*,7*E*,10*Z*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-3-ol (3c) A solution of **2c** (10 mg, 0.025

mmol) in deoxygenated, dry octane (10 ml) was heated at 120 °C for 4 h in a sealed tube and evaporated to dryness to give a mixture of **2c** : **3c** (approximately 85 : 15 by HPLC analysis). The residue was purified by HPLC (LiChrosorb Si60, 250 mm×10 mm, 1% 2-PrOH–hexane, 6 ml/min, room temperature) to give **2c** (6.98 mg) and its isomer **3c** (1.4 mg).

A solution of **2b** (1.23 mg, 0.003 mmol) in dry octane (2 ml) was heated at 120 °C for 2.5 h in a sealed tube and evaporated *in vacuo*. The residue (**2b** : **3b**=approximately 85 : 15 by HPLC analysis) was purified by HPLC (the same conditions as described above except using 10% 2-PrOH–hexane as a mobile phase) to afford **2b** (0.902 mg) and its isomer **3b** (0.145 mg).

3c: ^{1}H-NMR ($CDCl_3$) δ: 0.55 (3H, s, 18-H), 0.86, 0.87 (each 3H, d, J=6.6 Hz, 26, 27-H), 0.92 (3H, d, J=6.2 Hz, 21-H), 2.60 (1H, dd, J=13.2, 3.5 Hz, 4-H), 2.81 (1H, m, 9-H), 3.95 (1H, tt, J=7.3, 3.6 Hz, 3-H), 5.63 (1H, d, J=11.3, 5.3 Hz, 7-H), 6.37 (1H, d, J=11.3 Hz, 6-H), 6.47 (1H, d, J=85.5 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −127.5 (d, J=85.5 Hz). UV λ_{max} (95% EtOH): 264 nm.

(6*S*)- and (6*R*)-SO_2-Adduct of (5*Z*,7*E*)-1,25-Bis-(methoxymethyloxy)-3-(*tert*-butyldimethylsilyloxy)-9,10-seco-5,7,10(19)-cholestatriene (6j and 7j) (5*E*)-1α,25-Dihydroxyvitamin D_3 3-*tert*-butyldimethylsilyl ether **5f** (190 mg, 0.003 mmol) was synthesized by the published procedure[14,20,21] and was converted to the corresponding (6*S*)- and (6*R*)-SO_2-adducts **6f** and **7f** (127 mg, 60.2%, 6*S* : 6*R*=2 : 3) as described for the preparation of **6g** and **7g**.

A mixture of **6f** and **7f** (320 mg, 0.54 mmol), *N*,*N*-diisopropylethylamine (1.39 g, 10.8 mmol) and chloromethyl methyl ether (434 mg, 5.4 mmol) in dry CH_2Cl_2 (10 ml) was stirred for 2 h at 0 °C and for 7 h at room temperature. The mixture was acidified with 1% HCl and extracted with CH_2Cl_2. The organic phase was washed with 5% $NaHCO_3$, brine and dried ($MgSO_4$). After evaporation of the solvent, the residue was chromatographed on silica gel (30 g) with 10% AcOEt–hexane to give (6*S*)-**6j** (105 mg) and (6*R*)-**7j** (145 mg) (total 68%, *ca.* 6*S* : 6*R*=2 : 3).

6f: ^{1}H-NMR ($CDCl_3$) δ: 0.057, 0.07 (each 3H, s, Si–Me), 0.65 (3H, s, 18-H), 0.87 (9H, s, Si–*tert*-Bu), 0.94 (3H, d, J=6.4 Hz, 21-H), 1.22 (6H, s, 26, 27-H), 2.23 (1H, m), 2.60 (1H, m, 9-H), 3.70, 4.01 (each 1H, br d, J=15.9 Hz, 19-H), 4.18 (1H, m, 3-H), 4.38 (1H, m, 1-H), 4.61 (1H, d, J=9.5 Hz, 6-H), 4.70 (1H, d, J=9.5 Hz, 7-H).

7f: ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.07 (each 3H, s, Si–Me), 0.56 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.95 (3H, d, J=6.3 Hz, 21-H), 1.22 (6H, s, 26, 27-H), 2.34 (1H, m), 2.57 (1H, m, 9-H), 3.70, 4.01 (each 1H, br d, J=15.9 Hz, 19-H), 4.18 (1H, m, 3-H), 4.38 (1H, m, 1-H), 4.65 (1H, d, J=10.1 Hz, 6-H), 4.80 (1H, d, J=10.1 Hz, 7-H).

6j: ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.07 (each 3H, s, Si–Me), 0.65 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.94 (3H, d, J=6.4 Hz, 21-H), 1.21 (6H, s, 26, 27-H), 2.19 (1H, m), 2.62 (1H, m, 9-H), 3.37, 3.38 (each 3H, s, OMe), 3.66 (1H, br d, J=15.8 Hz, 19-H), 3.98 (1H, dm, J=15.8 Hz, 19-H), 4.16 (1H, m, 3-H), 4.21 (1H, m, 1-H), 4.59 (1H, d, J=7.0 Hz, OCH_2O), 4.65 (1H, d, J=9.5 Hz, 6-H), 4.71 (2H, s, OCH_2O), 4.72 (1H, d, J=9.5 Hz, 7-H). EI-MS *m*/*z* (%): 618 (M^+-SO_2, 13), 588 (16), 558 (44), 496 (63), 455 (12), 437 (17), 424 (14), 379 (24), 362 (28), 265 (20), 251 (36), 178 (98), 133 (77), 75 (100). HR-EI-MS *m*/*z*: 618.4698 (M^+-SO_2; Calcd for $C_{37}H_{66}0_5Si$: 618.4680).

7j: ^{1}H-NMR ($CDCl_3$) δ: 0.05, 0.07 (each 3H, s, Si–Me), 0.56 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.94 (3H, d, J=6.2 Hz, 21-H), 1.22 (6H, s, 26, 27-H), 2.35 (1H, m), 2.56 (1H, m, 9-H), 3.37, 3.69 (each 3H, s, OMe), 3.67 (1H, br d, J=16.0 Hz, 19-H), 3.96 (1H, br d, J=16.0 Hz, 19-H), 4.13 (1H, m, 3-H), 4.22 (1H, m, 1-H), 4.60, 4.73 (each 1H, d, J=7.0 Hz, OCH_2O), 4.62 (1H, d, J=10.0 Hz, 6-H), 4.71 (2H, s, OCH_2O), 4.80 (1H, d, J=10.0 Hz, 7-H). EI-MS *m*/*z* (%): 618 (M^+-SO_2, 22), 588 (26), 558 (72), 496 (62), 455 (10), 437 (15), 424 (16), 379 (23), 362 (21), 265 (20), 251 (29), 178 (100), 133 (69), 75 (75). HR-EI-MS *m*/*z*: 618.4655 (M^+-SO_2; Calcd for $C_{37}H_{66}O_5Si$: 618.4680).

(6*R*,19*S*)-SO_2-Adduct of (5*Z*,7*E*,10*Z*)-1,25-Bis-(methoxymethyloxy)-3-(*tert*-butyldimethylsilyloxy)-19-fluoro-9,10-seco-5,7,10(19)-cholestatriene (10j) To a stirred, cold (−78 °C) solution of (6*R*)-**7j** (35.0 mg, 0.05 mmol), *N*-fluorobenzenesulfonimide (19.4 mg, 0.06 mmol), HMPA (22.3 ml, 0.06 mmol) and dry THF (2 ml) was added LiHMDS (1 M solution in THF, 62 μl, 0.06 mmol). The mixture was stirred for 10 min, quenched with sat. NH_4Cl and extracted with ether. The organic layer was washed with brine, dried ($MgSO_4$) and evaporated to dryness. The residue was chromatographed on silica gel (5 g) using 0.5% AcOEt–hexane to afford **10j** (7.7 mg, 21%), **17j** (9.7 mg, 23.0%) and the unreacted starting material (17.7 mg, 50%).

10j: ^{1}H-NMR ($CDCl_3$) δ: 0.05, 0.07 (each 3H, s, Si–Me), 0.56 (3H, s, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.94 (3H, d, J=6.0 Hz, 21-H), 1.22 (6H, s, 26, 27-H), 2.54 (1H, m, 9-H), 3.37, 3.40 (each 3H, s, OMe), 4.31 (1H, m, 3-H), 4.44 (1H, m, 1-H), 4.65—4.80 (6H, m, OCH_2O, 6, 7-H), 5.70 (1H, d, J=56.8 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −166.4 (d, J=56.8 Hz). EI-MS *m*/*z* (%): 636 (M^+-SO_2, 40), 606 (73), 574 (53), 544 (98), 512 (23), 135 (73), 73 (100). HR-EI-MS *m*/*z*: 636.4615 (M^+-SO_2; Calcd for $C_{37}H_{65}O_5FSi$: 636.4585).

17j: ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.09 (each 3H, s, Si–Me), 0.61 (3H, s, 18-H), 0.87 (9H, s, Si–*tert*-Bu), 0.95 (3H, d, J=6.0 Hz, 21-H), 1.22 (6H, s, 26, 27-H), 3.37, 3.40 (each 3H, s, OMe), 4.32 (1H, m, 3-H), 4.44 (1H, m, 1-H), 4.60—4.80 (7H, m, 2×OCH_2O, 1, 6, 7-H), 5.41 (1H, s, 7-H), 6.54 (1H, d, J=2.1 Hz, 19-H).

(6*R*,19*S*)-SO_2-Adduct of (5*Z*,7*E*,10*Z*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (10a) A mixture of **10j** (34.7 mg, 0.05 mmol), bromotrimethylsilane (31.5 μl, 0.29 mmol) and dry CH_2Cl_2 (1.5 ml) was stirred at −40 °C for 6 h. The mixture was poured into cold 5% $NaHCO_3$ and extracted with AcOEt. The AcOEt layer was washed with brine and dried ($MgSO_4$). After evaporation of the solvent, the residue was chromatographed on silica gel (1 g) with 50% $CHCl_3$–benzene to give **10a** (13.3 mg, 55%).

10a: ^{1}H-NMR ($CDCl_3$) δ: 0.57 (3H, s, 18-H), 0.95 (3H, d, J=6.0 Hz, 21-H), 1.22 (6H, s, 26, 27-H), 4.27 (1H, m, 3-H), 4.65 (1H, m, 1-H), 4.75 (1H, d, J=10.3 Hz, 7-H), 4.85 (1H, br d, J=10.3 Hz, 6-H), 5.77 (1H, d, J=56.5 Hz). ^{19}F-NMR ($CDCl_3$) δ: −165.6 (d, J=56.5 Hz). EI-MS *m*/*z* (%): 434 (M^+-SO_2, 40), 416 (24), 396 (52), 378 (45), 360 (66), 305 (17), 287 (18), 285 (25), 267 (31), 265 (36), 249 (57), 133 (100). HR-EI-MS *m*/*z*: 434.3224 (M^+-SO_2; Calcd for $C_{27}H_{43}O_3F$: 434.3196).

(5*Z*,7*E*,10*Z*)- and (5*E*,7*E*,10*E*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (2a and 12a) To a stirred suspension of $LiAlH_4$ (8.1 mg, 0.21 mmol) in dry Et_2O (0.25 ml) was added a solution of **10a** (13.3 mg, 0.03 mmol) in dry Et_2O (0.25 ml) and the slurry was stirred for 30 min at room temperature. Excess of $LiAlH_4$ was decomposed with sat. sodium potassium tartarate. The resulting gray salt was filtered and washed with excess of Et_2O. The filtrate was dried ($MgSO_4$) and evaporated to dryness. The residue was chromatographed on silica gel (1 g) with 10% AcOEt–hexane to give **12a** (1.8 mg, 15%) and **2a** (0.45 mg, 4%).

12a: ^{1}H-NMR ($CDCl_3$) δ: 0.58 (3H, s, 18-H), 0.95 (3H, d, J=6.4 Hz, 21-H), 1.22 (6H, s, 26, 27-H), 2.25 (1H, m), 2.68 (1H, m), 2.81 (1H, m, 9-H), 3.07 (1H, dd, J=13.7, 3.7 Hz, 4-H), 4.19 (1H, tt, J=10.0, 4.4 Hz, 3-H), 4.34 (1H, t, J=3.6 Hz, 1-H), 5.97 (1H, d, J=11.5 Hz, 7-H), 6.68 (1H, d, J=11.5 Hz, 6-H), 6.75 (1H, d, J=83.4 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −132.6 (d, J=83.4 Hz). ^{13}C-NMR (CD_2Cl_2–CD_3OD=2 : 1) δ: 12.3, 19.1, 21.5, 22.9, 24.2, 28.2, 29.0, 29.1, 29.6, 36.8, 37.1, 37.2, 41.2, 42.5, 44.9, 46.5, 57.2, 57.3, 65.6, 67.7 (d, J=9.0 Hz), 71.3, 116.5, 116.5, 125.7 (d, J=6.1 Hz), 127.7 (d, J=5.7 Hz), 145.6, 145.8 (d, J=264.4 Hz). EI-MS *m*/*z* (%): 434 (M^+ 59), 416 (35), 398 (19), 396 (33), 378 (21), 360 (52), 305 (31), 287 (32), 285 (31), 269 (30), 267 (36), 249 (66), 135 (100). HR-EI-MS *m*/*z*: 434.3169 (Calcd for $C_{27}H_{43}O_3F$: 434.3196). UV λ_{max} (95% EtOH): 269 nm.

2a: ^{1}H-NMR ($CDCl_3$) δ: 0.53 (3H, s, 18-H), 0.93 (3H, d, J=6.4 Hz, 21-H), 1.21 (6H, s, 26, 27-H), 2.18 (1H, t, J=11.6 Hz, 4-H), 2.31 (1H, m, 2-H), 2.67 (1H, dm, J=11.6 Hz, 4-H), 2.80 (1H, m, 9-H), 4.17 (1H, tt, J=10.9, 4.4 Hz, 3-H), 5.08 (1H, t, J=3.1 Hz, 1-H), 5.90 (1H, d, J=11.1 Hz, 7-H), 6.46 (1H, d, J=11.1 Hz, 6-H), 6.50 (1H, d, J=86.1 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −129.6 (d, J=86.1 Hz). ^{13}C-NMR ($CDCl_3$) δ: 12.3, 19.0, 21.0, 22.5, 23.6, 27.9, 29.4, 29.6, 29.9, 36.3, 36.6, 40.7, 42.0, 44.6, 46.2, 46.3, 56.5, 56.7, 63.1 (d, J=6.2 Hz), 66.5, 71.4, 116.6, 121.4 (d, J=4.1 Hz), 126.1, 126.7 (d, J=6.1 Hz), 144.5, 146.5 (d, J=267.9 Hz). EI-MS *m*/*z* (%): 434 (M^+, 13), 416 (17), 398 (13), 396 (18), 378 (25), 360 (52), 305 (8), 287 (16), 285 (11), 269 (9), 267 (13), 249 (32), 135 (100). HR-EI-MS *m*/*z*: 434.3204 (Calcd for $C_{27}H_{43}O_3F$: 434.3196). UV λ_{max} (95% EtOH): 261 nm.

(5*E*,7*E*,10*E*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (12a) (6*S*)-SO_2-adduct **6j** (34.4 mg, 0.05 mmol) was fluorinated as described for the preparation of **10j** to give **8j** and **9j** (an approximately 1 : 1 ratio, 3.1 mg, 9%).

The epimeric compounds **8j** and **9j** (28.1 mg, 0.04 mmol) were treated with bromotrimethylsilane as described for the preparation of **10a** affording **8a** and **9a** (10.4 mg, 52%).

The triols **8a** and **9a** (7.2 mg, 0.014 mmol) were reduced with $LiAlH_4$ as described above to give **12a** (0.23 mg, 4%) and **13a** (0.25 mg, 4%).

8j and **9j**: ^{1}H-NMR ($CDCl_3$) δ: 0.07, 0.08 (each 3H, s, Si–Me), 0.63, 0.66 (3H, s, *ca.* 1 : 1, 18-H), 0.88 (9H, s, Si–*tert*-Bu), 0.95 (3H, d, J=6.0 Hz, 21-H), 1.21 (6H, s, 26, 27-H), 2.54 (1H, m, 9-H), 3.37, 3.40 (3H, s, *ca.* 1 : 1, OMe), 3.38, 3.42 (3H, s, *ca.* 1 : 1, OMe), 4.21 (1H, m, 3-H), 4.46 (1H, m, 1-H), 4.6—4.8 (6H, m, OCH_2O, 6, 7-H), 5.50, 5.70 (1H, d, J=56.0 Hz, *ca.*

1 : 1, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −163.1, −157.2 (each d, J=56.0 Hz, *ca.* 1 : 1). **8a** and **9a**: ^{1}H-NMR ($CDCl_3$) δ: 0.65, 0.66 (3H, s, *ca.* 1 : 1, 18-H), 0.94 (3H, d, J=6.0 Hz, 21-H), 1.22 (6H, s, 26, 27-H), 4.27 (1H, m, 3-H), 4.55—4.90 (3H, m, 1, 6, 7-H), 5.60, 5.76 (1H, d, J=56.0 Hz, *ca.* 1 : 1, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −163.5, −156.9 (each d, J=56.0 Hz, *ca.* 1 : 1).

13a: ^{1}H-NMR ($CDCl_3$) δ: 0.56 (3H, s, 18-H), 0.95 (3H, d, J=6.4 Hz, 21-H), 1.22 (6H, s, 26, 27-H), 2.31 (1H, m), 2.81 (1H, m, 9-H), 3.15 (1H, dm, J=13.2 Hz, 4-H), 4.16 (1H, tt, J=11.0, 4.3 Hz, 3-H), 5.05 (1H, brs, 1-H), 5.87 (1H, d, J=11.5 Hz, 7-H), 6.38 (1H, d, J=11.5 Hz, 6-H), 6.69 (1H, d, J=86.5 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −133.2 (d, J=86.5 Hz). ^{13}C-NMR (CD_2Cl_2–CD_3OD=2 : 1) δ: 12.3, 19.1, 21.5, 22.8, 24.1, 28.2, 29.0, 29.1, 29.6, 36.8, 37.1, 37.9, 41.1, 42.0, 44.8, 46.5, 57.1, 57.3, 62.5 (d, J=5.9 Hz), 65.4, 71.3, 116.3, 122.8, 127.6 (d, J=6.2 Hz), 128.4 (d, J=6.2 Hz), 144.1 (d, J=266.1 Hz), 145.6. EI-MS *m/z* (%): 434 (M^+, 25), 416 (18), 398 (10), 396 (19), 378 (15), 360 (61), 305 (13), 287 (18), 285 (21), 269 (15), 267 (21), 249 (53), 135 (100). HR-EI-MS *m/z*: 434.3198 (Calcd for $C_{27}H_{43}O_3F$: 434.3196). UV λ_{max} (95% EtOH): 269 nm.

(5*Z*,7*E*,10*E*)-19-Fluoro-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (3a) A stirred, cold (0 °C) solution of **12a** (0.93 mg, 0.002 mmol), anthracene (8 mg, 0.02 mmol) in benzene–EtOH (1 : 9; 100 ml) was purged with Ar and irradiated at 0 °C for 20 min (halogen lamp, 200 W). The solvent was evaporated to dryness. The residue was purified by HPLC [LiChrosorb Si 60 (250 mm×10 mm); 10% iso-PrOH–hexane; 7 ml/min; room temperature] to give **2a** (0.96 mg, 52%) and **3a** (0.16 mg, 9%).

3a: ^{1}H-NMR ($CDCl_3$) δ: 0.54 (3H, s, 18-H), 0.93 (3H, d, J=6.4 Hz, 21-H), 1.21 (6H, s, 26, 27-H), 2.13 (1H, m, 2-H), 2.27 (1H, dd, J=13.0, 8.5 Hz, 4-H), 2.66 (1H, dd, J=13.0, 3.8 Hz, 4-H), 2.82 (1H, m, 9-H), 4.20 (1H, tt, J=8.5, 3.8 Hz, 3-H), 4.43 (1H, dd, J=5.5, 3.8 Hz, 1-H), 5.63 (1H, d, J=11.3, 5.4 Hz, 7-H), 6.51 (1H, d, J=11.3 Hz, 6-H), 6.70 (1H, d, J=83.8 Hz, 19-H). ^{19}F-NMR ($CDCl_3$) δ: −128.0 (br signal). ^{13}C-NMR ($CDCl_3$) δ: 12.2, 19.0, 21.0, 22.4, 23.8, 27.6, 29.36, 29.39, 29.5, 36.3, 36.6, 40.7, 43.2, 44.6, 45.1, 46.1, 56.5, 56.8, 66.8, 67.7 (d, J=9.5 Hz), 71.4, 117.9 (d, J=3.0 Hz), 122.0 (d, J=4.8 Hz), 124.9, 126.7, 143.4 (d, J=265.2 Hz), 144.4. EI-MS *m/z* (%): 434 (M^+, 28), 416 (31), 398 (13), 396 (11), 378 (20), 360 (100), 305 (16), 287 (14), 285 (10), 269 (14), 267 (15), 249 (69), 135 (42). HR-EI-MS *m/z*: 434.3180 (Calcd for $C_{27}H_{43}O_3F$: 434.3196). UV λ_{max} (95% EtOH): 264 nm.

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α-Glucosidase Inhibitors with a Phthalimide Skeleton: Structure–Activity Relationship Study

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α-Glucosidase inhibitors with a phthalimide skeleton were prepared. Structure–activity relationship studies indicated a critical role for the hydrophobicity of the substituent at the nitrogen atom of the phthalimide skeleton. Introduction of electron-withdrawing groups, including a nitro group and chlorine, influenced the activity. Optimization studies led us to design 4,5,6,7-tetrachloro-*N*-phenylphthalimide (CP0P) and its *N*-phenylalkyl derivatives. CP0P and 4,5,6,7-tetrachloro-*N*-(4-phenylbutyl)phthalimide (CP4P) proved to be more potent α-glucosidase inhibitors than the known inhibitor 1-deoxynojirimycin.

Key words α-glucosidase; enzyme inhibitor; phthalimide; tetrachlorophthalimide; structure–activity relationship

Inhibitors of α-glucosidase (EC 3.2.1.20) have considerable potential for the treatment of various diseases.[1] Because the enzyme catalyzes the final step in the digestive processing of carbohydrates, inhibition of the enzyme may retard the uptake of dietary carbohydrates and suppress postprandial hyperglycemia. Hence, α-glucosidase inhibitors have potential in the management of carbohydrate imbalance diseases, such as diabetes and certain forms of hyperlipoproteinemia, as well as in the treatment of obesity.[2—4] α-Glucosidase inhibitors also have potential as anti-viral agents controlling viral infectivity through interference with the normal biosynthesis of *N*-linked oligosaccharides by glycosidation of viral coat/envelope glycoproteins,[5—7] and are being investigated for the treatment of both cancer and acquired immunodeficiency syndrome (AIDS).[8—10] To date, a number of low-molecular-weight α-glucosidase inhibitors have been identified as a result of screening of natural products. Among them, 1-deoxynojirimycin (dNM, **1**) is a well established inhibitor,[11] and various mechanistic and structural development studies have been reported.[12—15]

The pathophysiological role of tumor necrosis factor-α (TNF-α) in the diseases mentioned above (*i.e.*, diabetes, obesity, cancer, AIDS) has been well documented.[16—19] TNF-α is a member of the TNF family of ligands and receptors, which is a large family of cell surface and secreted molecules, mediating host defence and immune regulation as pleiotropic cytokines.[17,20] TNF-α is produced mainly by macrophages and T cells (and also is smaller amounts by other cell types, including adipocytes and fibroblasts) in response to various stimuli, and causes insulin-independency in type II diabetes/obesity and enhancement of gene expression of human immunodeficiency virus (HIV) in AIDS.[17,19,21] Therefore, agents which suppress TNF-α production are also thought to have potential for the treatment of the above diseases. We have conducted various structural developmental studies of TNF-α production-regulators derived from thalidomide [*N*(α)-phthalimidoglutarimide], and have designed and synthesized a number of potent regulators with a phthalimide skeleton.[22—28] In the course of these studies, we noted the general utility of the phthalimide and homophthalimide skeletons as proto-pharmacophores, and extended the scope of their biological activities to include androgen antagonists,[22,29] proteinase inhibitors,[24,30—32] anti-angiogenic agents,[33] and inhibitors of tumor cell invasion.[24,34] Some of our phthalimide derivatives showed hypoglycemic activity as expected, but the activity did not correlate with their TNF-α production-regulating activity. The results suggested that the hypoglycemic activity of our phthalimide derivatives is elicited *via* some mechanism other than TNF-α production-regulation.

We hypothesized that α-glucosidase might be a target molecule of our phthalimide derivatives, and screening of α-glucosidase-inhibiting activity was performed. Recently, we reported phthalimide derivatives with potent α-glucosidase-inhibiting activity in a communication.[35] In this article, we describe the structure–activity relationship of α-glucosidase inhibitors with a phthalimide skeleton.

Assay System The α-glucosidase-inhibitory activity of phthalimide derivatives (**2—70**, Tables 1—4) was assayed as described in the Experimental (*vide infra*).[36—38] As a positive control, dNM (**1**) was adopted, and it was assayed together with phthalimide derivatives in each set of experiments. All of the active compounds showed dose-dependent inhibitory activity, and the IC_{50} values (μM) are presented in Tables 1—4. Though the IC_{50} values of dNM (**1**) and the test compounds (**2—70**) showed some variation from experiment to experiment [for example, the IC_{50} values (μM) of dNM (**1**) are 34.6 in Table 1, 32.0 in Table 2, 38.1 in Table 3, and 30.7 in Table 4], the order of efficacy of the test compounds was reproducible. Therefore, a typical set of data (repeated at least twice) obtained at the same time is presented in each table. In each set of experiments, the assay was performed in triplicate (the mean value was taken) and at least two times. IC_{50} values in excess of 400 μM could not be assessed because of the insolubility of the compound in the assay buffer,

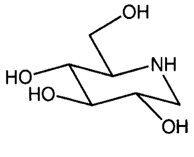

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Fig. 1. Structure of dNM (**1**)

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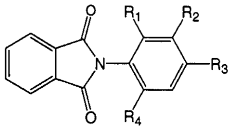
Dedicated to the memory of Dr. Kyosuke Tsuda.

Table 1. α-Glucosidase-Inhibitory Activity of Alkylphenyphthalimide Derivatives



Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)
1 (dNM)	—	—	—	—	34.6
2 (PP00)	H	H	H	H	>400
3 (PP10)	CH_3	H	H	H	>400
4 (PP20)	C_2H_5	H	H	H	>400
5	n-C_3H_7	H	H	H	189.4
6 (PP30)	Iso-C_3H_7	H	H	H	106.4
7 (PP03)	H	Iso-C_3H_7	H	H	324.5
8 (PP003)	H	H	Iso-C_3H_7	H	>400
9 (PP40)	*tert*-C_4H_9	H	H	H	91.4
10	n-C_5H_{11}	H	H	H	26.1
11	n-C_6H_{13}	H	H	H	50.5
12	Adamantyl	H	H	H	32.1
13 (PP11)	CH_3	H	H	CH_3	>400
14 (PP21)	C_2H_5	H	H	CH_3	147.4
15 (PP22)	C_2H_5	H	H	C_2H_5	119.0
16 (PP31)	Iso-C_3H_7	H	H	CH_3	120.0
17 (PP33)	Iso-C_3H_7	H	H	Iso-C_3H_7	45.0
18 (PP404)	*tert*-C_4H_9	H	*tert*-C_4H_9	H	73.4
19	Phenethyl	H	H	H	16.2
20	CH_2-cyclohexyl	H	H	H	41.8
21	F	H	H	F	>400
22	Cl	H	H	Cl	119.2
23	Br	H	H	Br	87.9

and compounds with IC_{50} value of >400 μM were designated as inactive in this paper.

Effects of Alkylation at the *N*-Phenyl Moiety All of the compounds described in this article were derived from *N*-phenylphthalimide (PP00, **2**, Table 1). Though PP00 (**2**) and its methylated and ethylated analogs [PP10 (**3**) and PP20 (**4**), respectively] are inactive (IC_{50}=>400 μM) in α-glucosidase inhibition assay, introduction of a normal- or isopropyl group at the *ortho*-position [**5** and PP30 (**6**), respectively] caused the appearance of moderate activity (Table 1). Comparison of the activities of the regio-isomers of PP30 (**6**—**8**) suggested that the *ortho*-position is the best substitution position for high activity, *i.e.*, the activity decreased in the order of: PP30 (*ortho*-, **6**)>PP03 (*meta*-, **7**)>PP003 (*para*-, **8**).

Introduction of a hydrophobic group more bulky than an isopropyl group, such as *tert*-butyl (PP40, **9**), *n*-pentyl (**10**), *n*-hexyl (**11**), adamantyl (**12**), phenethyl (**19**), and cyclohexylmethyl (**20**), at the *ortho*-position (R_1) enhanced the activity. Among these compounds, the *n*-pentyl (**10**) and phenethyl (**19**) derivatives showed higher activity than dNM (**1**). Comparison of the *ortho*-*n*-alkylated analogs (**3**—**5**, **10**, **11**) suggested that the *n*-pentyl group (**10**) is optimal for the activity (**10** is more potent than **5** and **11**).

Introduction of one more alkyl group at the other *ortho*-position (R_4) seemed to enhance the activity of the corresponding mono-alkylated analogs. Though the mono-ethyl analog (PP20, **4**) is inactive, its methylated analog (PP21, **14**) showed moderate activity. At least one of the alkyl groups should be ethyl or larger for the activity, because the dimethyl analog (PP11, **13**) is inactive. Similarly, the activity of *o*-ethyl-*o*′-alkyl analogs increased in the order of: PP20 (**4**)<PP21 (**14**)<PP22 (**15**).

The two ethyl groups at the *ortho*-positions could be substituted with halogen atoms such as chlorine (**22**) and bromine (**23**), though substitution with fluorine (**21**) caused disappearance of the activity.

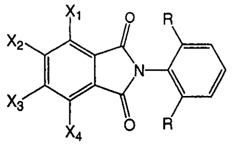
In the case of the *o*-isopropyl analog (PP30, **6**), introduction of a methyl group at the other *ortho*-position (PP31, **16**) did not enhance the activity, while introduction of one more isopropyl group (PP33, **17**) markedly enhanced the activity. However, the introduction of one more *tert*-butyl group at the *para*-position (R_3, PP404, **18**) to the mono-*tert*-butyl analog (PP40, **9**) only slightly enhanced the activity, its efficacy was low.

These results suggest that a bulky hydrophobic group(s) of appropriate size extending perpendicular to the phthalimide ring is critical for potent activity.

Effects of Substitution at the Phenylphthalimide Ring Next, we investigated the effects of substitution at the phthalimide ring moiety (X_1—X_4, Table 2). The compounds listed in Table 2 (**24**—**51**) were derived from inactive PP00 (**2**), PP11 (**13**) and moderately active PP33 (**17**).

Concerning the effect of *o*,*o*′-substituents (R's), the activity of the active compounds decreased in the order of: isopropyl ("33"-series of compounds)>methyl ("11"-series)>hydrogen ("00"-series), in all substituted series (X=methyl, nitro, amino, hydroxy, fluorine), in accordance with the results shown in Table 1, except for the tetrachlorophthalimide derivatives (**46**—**48**, *vide infra*), *i.e.*, the orders of potency are: **26**>**25**>**24** (5-methyl derivatives), **30**>**29**>**28** (4-nitro derivatives: 4NPP's), **33**>**32**>**31** (5-nitro derivatives: 5NPP's), **39**>**38**>**37** (5-amino derivatives: 5APP's), **45**>**44**=**43** (5-hydroxy derivatives: 5HPP's), and **51**>**50**=**49** (tetrafluoro derivatives).

Table 2. α-Glucosidase-Inhibitory Activity of Substituted Phenylphthalimide Derivatives



Compound	X_1	X_2	X_3	X_4	R	IC_{50} (μM)
1 (dNM)	—	—	—	—	—	32.0
17 (PP33)	H	H	H	H	Iso-C_3H_7	43.6
24	H	CH_3	H	H	H	>400
25	H	CH_3	H	H	CH_3	119.6
26	H	CH_3	H	H	Iso-C_3H_7	67.6
27	H	*tert*-C_4H_9	H	H	Iso-C_3H_7	60.1
28 (4NPP00)	NO_2	H	H	H	H	259.3
29 (4NPP11)	NO_2	H	H	H	CH_3	111.8
30 (4NPP33)	NO_2	H	H	H	Iso-C_3H_7	56.5
31 (5NPP00)	H	NO_2	H	H	H	236.9
32 (5NPP11)	H	NO_2	H	H	CH_3	98.3
33 (5NPP33)	H	NO_2	H	H	Iso-C_3H_7	37.5
34 (4APP00)	NH_2	H	H	H	H	>400
35 (4APP11)	NH_2	H	H	H	CH_3	>400
36 (4APP33)	NH_2	H	H	H	Iso-C_3H_7	>400
37 (5APP00)	H	NH_2	H	H	H	>400
38 (5APP11)	H	NH_2	H	H	CH_3	146.6
39 (5APP33)	H	NH_2	H	H	Iso-C_3H_7	60.6
40 (4HPP00)	OH	H	H	H	H	>400
41 (4HPP11)	OH	H	H	H	CH_3	>400
42 (4HPP33)	OH	H	H	H	Iso-C_3H_7	>400
43 (5HPP00)	H	OH	H	H	H	>400
44 (5HPP11)	H	OH	H	H	CH_3	>400
45 (5HPP33)	H	OH	H	H	Iso-C_3H_7	288.5
46 (CPOP)	Cl	Cl	Cl	Cl	H	3.2
47	Cl	Cl	Cl	Cl	CH_3	5.9
48	Cl	Cl	Cl	Cl	Iso-C_3H_7	9.5
49	F	F	F	F	H	>400
50	F	F	F	F	CH_3	>400
51	F	F	F	F	Iso-C_3H_7	26.2

Introduction of a methyl group at the 5-position (X_2) into the inactive PP11 (**13**), *i.e.*, compound **25**, resulted in the appearance of moderate activity. However, the same substitution of active PP33 (**17**), *i.e.*, compound **26**, did not enhance the activity of PP33 (**17**), though **26** was more potent than **25**. Introduction of a *tert*-butyl group at the same position (**27**) did not affect the activity.

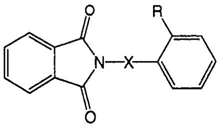
Introduction of an electron-withdrawing nitro group at position 4 (X_1=NO_2, 4NPP's, **28**—**30**) caused the appearance and enhancement of the activity. Though PP00 (**2**) and the corresponding 5-methyl analog (**24**) are inactive, the corresponding 4-nitro (4NPP00: **28**) and 5-nitro (5NPP00: **31**) derivatives showed moderate activity. Position 5 (X_2) seems to be preferable for the appearance and enhancement of the activity, because in all sets (R=H, methyl, isopropyl) of compounds, the 5-nitro derivative is more potent than the corresponding 4-nitro derivative [*i.e.*, the orders of potency are: 5NPP00 (**31**)>4NPP00 (**28**), 5NPP11 (**32**)>4NPP11 (**29**), and 5NPP33 (**33**)>4NPP33 (**30**)].

The effects of substitution with an electron-donating group, such as an amino (**34**—**39**) or a hydroxyl (**40**—**45**) group, are contradictory. Introduction of these electron-donating groups at the 4-position [4APP's (**34**—**36**) and 4HPP's (**40**—**42**)] had no effect on inactive PP00 (**2**) and PP11 (**13**), and resulted in disappearance of the activity of active PP33 (**17**). Introduction of a 5-hydroxyl group [5HPP's (**43**—**45**)] similarly decreased the activity [5HPP33 (**45**) was less potent than PP33 (**17**), and 5HPP00/5HPP11 (**43**/**44**) were inactive]. On the other hand, introduction of an amino group at the 5-position (5APP's: **37**—**39**) had somewhat different effects. The substitution of inactive PP11 (**13**), *i.e.*, 5APP11 (**38**), caused the appearance of the activity, while the same substitution of active PP33 (**17**), *i.e.*, 5APP33 (**39**), decreased the activity (**17**>**39**). Although we cannot clearly interpret these phenomena, we can conclude that i) introduction of a nitro group enhances the activity, ii) substitution at the 5-position is superior to that at the 4-position, and iii) introduction of an amino or a hydroxyl group tends to decrease the activity.

The effects of an electron-withdrawing nitro group mentioned above led us to try introducing electron-withdrawing halogen atoms into the phthalimide skeleton (**46**—**51**). As expected, the tetrafluoro derivative of PP33 (**17**), *i.e.*, compound **51**, showed more potent activity than PP33 (**17**).

Surprisingly, tetrachlorination (**46**—**48**) had dramatic effects on the activity. The tetrachlorinated analog of inactive PP00 (**2**), *i.e.*, CPOP (**46**), showed very potent α-glucosidase-inhibiting activity with an IC_{50} value of 3.2 μM, which is one order of magnitude more potent than dNM (**1**). Though its *o,o'*-alkylated derivatives (**47**, **48**) also showed comparable activity, being more potent than dNM (**1**), the structure–activity relationship was opposite to that of the other derivatives, *i.e.*, the potency decreased in the order of: non-substi-

Table 3. α-Glucosidase-Inhibitory Activity of Phthalimide Derivatives



Compound	X	R	IC$_{50}$ (μM)
1 (dNM)	—	—	38.1
19	None	Phenethyl	16.2
20	None	CH$_2$-cyclohexyl	41.8
52 (P1P)	CH$_2$	H	>400.0
53 (P1P1)	CH$_2$	CH$_3$	94.6
54 (P2P)	(CH$_2$)$_2$	H	>400
55 (P2P1)	(CH$_2$)$_2$	CH$_3$	66.8
56	CH(CH$_3$)CH$_2$	H	100.0
57 (P3P)	(CH$_2$)$_3$	H	>400

tuted (**46**)>*o*,*o*′-dimethyl (**47**)>*o*,*o*′-diisopropyl (**48**). The reason for this is unknown.

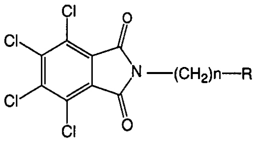
Effect of Distance between the Phthalimide Ring and Phenyl Moiety The potent α-glucosidase-inhibiting activity elicited by *N*-(*o*-phenethylphenyl)phthalimide (**19**) and *N*-(*o*-methylenecyclohexylphenyl)phthalimide (**20**) (Table 1) led us investigate the effect of the distance between the phthalimide ring and the phenyl moiety (Table 3). The compounds listed in Table 3 have methylene spacers of different lengths inserted between the phthalimide moiety and the phenyl group of inactive PP00 (**2**) or PP10 (**3**), or are related analogs.

Insertion of methylene spacers into PP00 (**2**), *i.e.*, P1P (**52**), P2P (**54**), and P3P (**57**), did not cause the appearance of activity. However, the corresponding derivatization of inactive PP10 (**3**), *i.e.*, P1P1 (**53**) and P2P1 (**55**), resulted in the appearance of the activity. P2P1 (**55**) was more potent than P1P1 (**53**), and the introduction of a methyl group on the methylene spacer (**56**) decreased the activity. Though we could not find any potent α-glucosidase inhibitor among the compounds listed in Table 3, it seems that the distance between the phthalimide ring and the phenyl moiety affects the activity (*vide infra*).

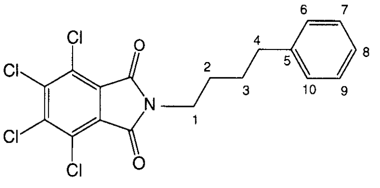
Tetrachlorophthalimide Derivatives The very potent α-glucosidase-inhibiting activity of CP0P (**46**, Table 2) and the results shown in Table 3, as well as the unique features of the tetrachlorinated derivatives (**46**—**48**, Table 2) as regards structure–activity relationship, led us to attempt the structural development of CP0P (**46**). The compounds listed in Table 4 are *N*-phenylalkyl-(**58**—**64**) and *N*-alkyl-(**65**—**70**) tetrachlorophthalimide derivatives.

All of the prepared compounds listed in Table 4 showed much more potent activity than dNM (**1**). As expected, the length of the methylene spacer was critical for the activity. Among *N*-phenylalkyl derivatives (**58**—**64**, $n=1$—10), the potency of the α-glucosidase-inhibitory activity increased as the length of the methylene spacer increased to $n=4$ [CP1P (**58**, $n=1$)<CP2P (**59**, $n=2$)<CP3P (**60**, $n=3$)<CP4P (**61**, $n=4$)], while for methylene spacers longer than $n=4$, the activity conversely decreased [CP4P (**61**, $n=4$)>CP5P (**62**, $n=5$)>CP6P (**63**, $n=6$)≅CPXP (**64**, $n=10$)]. The results suggest that the structure of active tetrachlorophthalimide derivatives is critically recognized, and the length of four methylene units (CP4P: **61**, $n=4$, IC$_{50}$=2.0 μM) is the optimum for the fitting/binding of the compound to α-glucosidase. As already reported, enzyme kinetic studies indicated that CP4P (**61**) inhibits α-glucosidase in a competitive manner.[35] Therefore, CP4P (**61**) is considered to bind the catalytic/substrate-binding site of α-glucosidase. If this is the case, the tetrachlorophthalimide structure might be regarded as a novel pharmacophore with the character of a sugar mimic. In this sense, the potent activity elicited by CP0P (**46**, $n=0$, IC$_{50}$=2.6 μM), comparable to that of CP4P (**61**), is exceptional, and therefore the mechanism/binding site of CP0P (**46**) may be different from that of CP4P.[35]

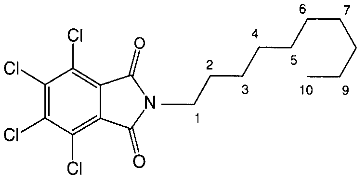
Table 4. α-Glucosidase-Inhibitory Activity of Tetrachlorophthalimide Derivatives



Compound	*n*	R	IC$_{50}$ (μM)
1 (dNM)	—	—	30.7
46 (CP0P)	0	Phenyl	2.6
58 (CP1P)	1	Phenyl	10.9
59 (CP2P)	2	Phenyl	6.0
60 (CP3P)	3	Phenyl	4.5
61 (CP4P)	4	Phenyl	2.0
62 (CP5P)	5	Phenyl	3.5
63 (CP6P)	6	Phenyl	7.0
64 (CPXP)	10	Phenyl	6.8
65 (CP3M)	3	CH$_3$	7.4
66 (CP5M)	5	CH$_3$	2.5
67 (CP7M)	7	CH$_3$	1.1
68 (CP9M)	9	CH$_3$	0.7
69 (CPXIM)	11	CH$_3$	0.8
70	0	Adamantyl	2.9



CP4P (**61**)



CP9M (**68**)

Chart 1

N-Alkyl-tetrachlorophthalimide derivatives (**65**—**70**) also showed very potent activity. The activity again showed a dependency on the length of the methylene spacer; *i.e.*, the potency increased in the order of: CP3M (**65**, $n=3$)<CP5M (**66**, $n=5$)<CP7M (**67**, $n=7$)<CP9M (**68**, $n=9$), and the activity seems to reach optimum at the length of methylene spacer of $n=9$, because the activity of CPXIM (**69**, $n=11$, IC$_{50}$=0.8 μM) was almost the same as that of CP9M (**68**,

$IC_{50}=0.7\,\mu$M). Therefore, in the case of *N*-alkylated derivatives, CP9M (**68**) seems to be the best fitting/binding compound for α-glucosidase inhibition. This result can be regarded as consistent with the findings for *N*-phenylalkyl derivatives (**58**—**64**), because the numbers of carbon atoms introduced at the nitrogen atom of the tetrachlorophthalimide are the same (ten carbon atoms) in CP4P (**61**) and CP9M (**68**). If folding of the *n*-alkyl group of CP9M (**68**) occurs in the binding site of α-glucosidase, the three-dimensional structure might be similar to that of CP4P (**61**), though the flexible CP9M (**68**) is more potent than CP4P (**61**).

Conclusion

Various phthalimide derivatives were screened for α-glucosidase inhibitory activity, and the structure–activity relationships were analyzed. As a result, we identified potent α-glucosidase inhibitors, such as CP0P (**46**), CP4P (**61**) and CP9M (**68**), which possess activity one to two orders of magnitude more potent than that of dNM (**1**), with IC_{50} values of 0.7—2.6 μM in our assay system.

Our studies might imply the usefulness of the tetrachlorophthalimide skeleton as a non-sugar-type sugar mimic and as a versatile pharmacophore. Because some of our α-glucosidase inhibitors also possess TNF-α production-regulating activity,[22—28] and because both α-glucosidase and TNF-α play pathophysiological roles, the active compounds presented in this paper could be superior lead compounds for medicaments treating various diseases, including diabetes, obesity, AIDS and cancer. Application of these α-glucosidase inhibitors as hypoglycemic agents and anti-viral/anti-infectious agents, as well as mechanistic studies of α-glucosidase inhibition are scheduled.

Experimental

Assay of α-Glucosidase Inhibition For assessment of the α-glucosidase-inhibitory activity of the compounds (**1**—**70**), we adopted the reported method.[36—38] α-Glucosidase (*Saccharomyces*. sp., Wako Fine Chemicals Co. Ltd., final concentration: 25 mU/ml) was incubated with *para*-nitrophenylglucopyranoside (*p*-NPG, final concentration: 80 μM) in the presence or absence of various concentrations of test compound in 10 mM phosphate buffer (pH 7.28) containing 1% v/v dimethylsulfoxide (DMSO) at 37 °C for exactly 10 min. After the incubation, an equal volume of stop solution (0.5 M Na_2CO_3) was added, and the amount of released *para*-nitrophenol was measured in terms of the absorbance at 400 nm. The assay was performed in triplicate with five different concentrations around the IC_{50} values which were roughly estimated in the first round experiments, and the mean values were taken. The IC_{50} values calculated with dose–response curves thus obtained in these experiments are presented in the text.

Chemicals Most of the compounds listed in Tables 1—4 were prepared by condensation of phthalic anhydride (or its derivatives) with appropriate amines by usual organic synthetic methods.[25—31] Each product was separated by silica gel column chromatography and recrystallized from an appropriate solvent(s). Physicochemical properties of compounds **2**—**6**, **9**—**23**, **28**—**45**, and **63** were described in our previous reports.[27,28,35,39]

N-(*m*-Isopropylphenyl)phthalimide (PP03: **7**): mp 80 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.29 (6H, d, J=6.96 Hz), 2.98 (1H, quin, J=6.96 Hz), 7.23—7.25 (1H, m), 7.26—7.30 (2H, m), 7.43 (1H, t, J=7.98 Hz), 7.77—7.81 (2H, m), 7.94—7.98 (2H, m). *Anal.* Calcd for $C_{17}H_{15}NO_2$: C, 76.96; H, 5.70; N, 5.28. Found: C, 77.18; H, 5.70; N, 5.24.

N-(*p*-Isopropylphenyl)phthalimide (PP003: **8**): mp 210 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.29 (6H, d, J=6.84 Hz), 2.97 (1H, quin, J=6.84 Hz), 7.32—7.38 (4H, m), 7.76—7.81 (2H, m), 7.93—7.98 (2H, m). *Anal.* Calcd for $C_{17}H_{15}NO_2$: C, 76.96; H, 5.70; N, 5.28. Found: C, 76.90; H, 5.58; N, 5.03.

5-Methyl-*N*-phenylphthalimide (**24**): mp 198—199 °C. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.56 (3H, s), 7.34—7.94 (8H, m). *Anal.* Calcd for $C_{15}H_{11}NO_2$: C, 75.94; H, 4.67; N, 5.90. Found: C, 76.04; H, 4.79; N, 6.08.

5-Methyl-*N*-(*o*,*o*′-dimethylphenyl)phthalimide (**25**): mp 142—144 °C. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.18 (6H, s), 2.53 (3H, s), 7.20 (3H, m), 7.44—7.94 (3H, m)). *Anal.* Calcd for $C_{17}H_{15}NO_2$: C, 76.96; H, 5.70; N, 5.28. Found: C, 76.88; H, 5.68; N, 5.30.

5-Methyl-*N*-(*o*,*o*′-diisopropylphenyl)phthalimide (**26**): mp 163 °C. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.20 (12H, d, J=6.4 Hz), 2.57 (3H, s), 2.73 (2H, hept, J=6.4 Hz), 7.14—7.98 (6H, m)). *Anal.* Calcd for $C_{21}H_{23}NO_2$: C, 78.47; H, 7.21; N, 4.36. Found: C, 78.44; H, 7.24; N, 4.35.

5-*tert*-Butyl-*N*-(*o*,*o*′-diisopropylphenyl)phthalimide (**27**): mp 234—235 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.16 (6H, d, J=6.87 Hz), 1.17 (6H, d, J=6.87 Hz), 1.43 (9H, s), 2.73 (2H, hept, J=6.87 Hz), 7.30 (2H, d, J=7.83 Hz), 7.46 (1H, t, J=7.83 Hz), 7.84 (1H, dd, J=7.79, 1.83 Hz), 7.90 (1H, d, J=7.79 Hz), 8.01 (1H, d, J=1.83 Hz). *Anal.* Calcd for $C_{24}H_{29}NO_2$: C, 79.30; H, 8.04; N, 3.85. Found: C, 79.29; H, 8.26; N, 3.81.

4,5,6,7-Tetrachloro-*N*-phenylphthalimide (CP0P: **46**): mp 258—260 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.50—7.55 (2H, m), 7.42—7.47 (1H, m), 7.50—7.55 (2H, m). *Anal.* Calcd for $C_{14}H_5Cl_4NO_2$: C, 46.58; H, 1.40; N, 3.88; Cl, 39.28. Found: C, 46.56; H, 1.39; N, 3.96; Cl, 39.56.

4,5,6,7-Tetrachloro-*N*-benzylphthalimide (CP1P: **58**): mp 202—205 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 4.85 (2H, s), 7.29—7.35 (3H, m), 7.44 (2H, td, J=8.2 Hz). *Anal.* Calcd for $C_{15}H_7Cl_4NO_2$: C, 48.04; H, 1.88; N, 3.73. Found: C, 47.89; H, 1.93; N, 3.53.

4,5,6,7-Tetrachloro-*N*-phenethylphthalimide (CP2P: **59**): mp 177—178 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.97 (2H, t, J=7.0 Hz), 3.92 (2H, t, J=7.0 Hz), 7.22 (3H, m), 7.28 (2H, m). *Anal.* Calcd for $C_{16}H_9Cl_4NO_2$: C, 49.38; H, 2.31; N, 3.60; Cl, 36.47. Found: C, 49.16; H, 2.17; N, 3.40; Cl, 36.62.

4,5,6,7-Tetrachloro-*N*-(3-phenylpropyl)phthalimide (CP3P: **60**): mp 163 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.07 (2H, tt, J=8, 8 Hz), 2.70 (2H, t, J=8 Hz), 3.76 (2H, t, J=8 Hz), 7.05—7.09 (1H, tt, J=8, 2 Hz), 7.15—7.23 (4H, m). *Anal.* Calcd for $C_{17}H_{11}Cl_4NO_2$: C, 50.66; H, 2.75; N, 3.47. Found: C, 50.48; H, 2.69; N, 3.34.

4,5,6,7-Tetrachloro-*N*-(4-phenylbutyl)phthalimide (CP4P: **61**): mp 118—119 °C. ^{1}H-NMR (500 MHz, $CHCl_3$) δ: 1.67 (2H, tt, J=8, 8 Hz), 1.72 (2H, tt, J=8, 8 Hz), 2.65 (2H, t, J=8 Hz), 3.72 (2H, t, J=8 Hz), 7.14—7.18 (2H, m), 7.24—7.27 (3H, m). *Anal.* Calcd for $C_{18}H_{13}Cl_4NO_2$: C, 51.83; H, 3.14; N, 3.36. Found: C, 51.71; H, 3.21; N, 3.09.

4,5,6,7-Tetrachloro-*N*-(5-phenylpentyl)phthalimide (CP5P: **62**): mp 125 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.37 (2H, tt, J=8, 8 Hz), 1.66 (2H, tt, J=8, 8 Hz), 1.71 (2H, tt, J=8, 8 Hz), 2.60 (2H, t, J=8 Hz), 3.69 (2H, t, J=8 Hz), 7.15 (1H, dd, J=8, 1 Hz), 7.15 (2H, dt, J=8, 1 Hz), 7.26 (2H, ddd, J=8, 1, 1 Hz). *Anal.* Calcd for $C_{19}H_{15}Cl_4NO_2$: C, 52.93; H, 3.51; N, 3.04. Found: C, 52.75; H, 3.57; N, 3.04.

4,5,6,7-Tetrachloro-*N*-(10-phenyldecyl)phthalimide (CPXP: **64**): mp 110—111 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.24 (12H, br), 1.60 (2H, tt, J=8, 8 Hz), 1.66 (2H, tt, J=8, 8 Hz), 2.59 (2H, t, J=8 Hz), 3.68 (2H, t, J=8 Hz), 7.16 (1H, dd, J=8, 2 Hz), 7.17 (2H, dt, J=8, 2 Hz), 7.26 (2H, ddd, J=8, 8, 2 Hz). *Anal.* Calcd for $C_{24}H_{25}Cl_4NO_2$: C, 57.51; H, 5.03; N, 2.79. Found: C, 57.22; H, 5.07; N, 2.69.

4,5,6,7-Tetrachloro-*N*-butylphthalimide (CP3M: **65**): mp 153—155 °C. FAB-MS *m*/*z*: 340 $(M+1)^+$. *Anal.* Calcd for $C_{12}H_9Cl_4NO_2$: C, 42.48; H, 2.65; N, 4.13. Found: C, 42.18; H, 2.84; N, 4.23.

4,5,6,7-Tetrachloro-*N*-hexylphthalimide (CP5M: **66**): mp 153—154 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.88 (3H, t, J=7 Hz), 1.25—1.35 (6H, br), 1.67 (2H, tt, J=7, 8 Hz), 3.69 (2H, t, J=7 Hz). FAB-MS *m*/*z*: 368 $(M+1)^+$. *Anal.* Calcd for $C_{14}H_{13}Cl_4NO_2$: C, 45.78; H, 3.54; N, 3.81. Found: C, 45.51; H, 3.54; N, 3.91.

4,5,6,7-Tetrachloro-*N*-octylphthalimide (CP7M: **67**): mp 142—144 °C. FAB-MS *m*/*z*: 396 $(M+1)^+$. *Anal.* Calcd for $C_{16}H_{17}Cl_4NO_2$: C, 48.61; H, 4.30; N, 3.53. Found: C, 48.43; H, 4.21; N, 3.58.

4,5,6,7-Tetrachloro-*N*-decylphthalimide (CP9M: **68**): mp 139—140 °C. FAB-MS *m*/*z*: 424 $(M+1)^+$. *Anal.* Calcd for $C_{18}H_{21}Cl_4NO_2$: C, 51.06; H, 4.96; N, 3.31. Found: C, 50.98; H, 4.88; N, 3.29.

4,5,6,7-Tetrachloro-*N*-dodecylphthalimide (CPXIM: **69**): mp 137—138 °C. FAB-MS *m*/*z*: 452 $(M+1)^+$. *Anal.* Calcd for $C_{20}H_{25}Cl_4NO_2$: C, 53.22; H, 5.54; N, 3.10. Found: C, 53.05; H, 5.38; N, 3.15.

4,5,6,7-Tetrachloro-*N*-adamantylphthalimide (**70**): mp 206—210 °C. FAB-MS *m*/*z*: 418 $(M+1)^+$. *Anal.* Calcd for $C_{18}H_{15}Cl_4NO_2$: C, 51.58; H, 3.61; N, 3.34. Found: C, 51.57; H, 3.87; N, 3.07.

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Synthesis and Properties of Novel Bifunctional Nitrosamines with ω-Chloroalkyl Groups

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Novel *N*-nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines were synthesized and their chemical and biological properties were evaluated. The nitrosamines were expected to decompose through ω-chloroalkyldiazohydroxides in aqueous solution, and then to alkylate various cellular macromolecules. *N*-Nitroso-*N*-(acetoxymethyl)-2-chloroethylamine rapidly decomposed in aqueous solution, and the reaction rate was apparently independent of the pH of the solution. On the other hand, the rate of decomposition of chloropropyl and chlorobutyl homologs was pH-dependent, and increased in alkaline solution. When mutagenicity was assayed in *Salmonella typhimurium* TA1535 and TA92 for preliminary evaluation, all three compounds were directly mutagenic. The mutagenicity in *Salmonella typhimurium* TA1535, which can detect base-pair change mutation, clearly showed that these compounds induced DNA alkylation *in vivo*. The increase of alkyl chain length in chloroalkyl compounds increased the mutagenic activity, and the activities were stronger than those of the corresponding simple α-acetoxy nitrosamines lacking a chloro group, *N*-nitroso-*N*-(acetoxymethyl)alkylamines. Furthermore, the positive result in TA92 suggested that chlorinated nitrosamines cross-linked DNA like antitumor chloroethylnitrosoureas and that they are expected to be new lead compounds for antitumor agents.

Key words ω-chloroalkyl nitrosamine; antitumor agent; DNA cross-linking; DNA alkylation; mutagenicity

There are various carcinogens in the environment, such as polycyclic aromatic hydrocarbons, aromatic amines and *N*-nitrosamines.[1—3] Among many carcinogenic chemicals, *N*-nitroso compounds are important not only because they are present in the environment, but also because they can be formed under the acidic conditions in the stomach,[3] or under neutral *in vivo* conditions by the action of activated macrophages on amines.[4]

N-Nitrosodialkylamines are a group of environmental carcinogens and are metabolized *in vivo* by cytochrome P450. The metabolites, α-hydroxy nitrosamines, decompose spontaneously to alkanediazohydroxides and then to alkyldiazonium ions.[5] Alkanediazohydroxides and alkyldiazonium ions can react with biological nucleophiles in nucleic acids and proteins. The resulting DNA alkylation is thought to be an important reason why *N*-nitroso compounds show carcinogenic and/or mutagenic activities.[5] Since α-hydroxy nitrosamines are unstable in aqueous solution, α-acetoxy nitrosamines[6] and α-hydroperoxy nitrosamines[7,8] have been used as precursors for α-hydroxy nitrosamines in research on the chemical and biological activities of *N*-nitrosamines. The α-hydroxy nitrosamines were isolated by deoxygenation of α-hydroperoxy nitrosamines,[9,10] and their chemistry and mutagenicity have been elucidated.[10] Furthermore, the mutagenicity and alkylating activity of simple alkanediazohydroxides, which are the ultimate alkylating agents produced from *N*-nitroso compounds, were examined using their potassium salts as the precursors.[11,12]

While many *N*-nitroso compounds are considered as carcinogens, some *N*-nitrosoureas have antitumor activities.[13] Chloroethyl *N*-nitrosoureas (CENUs) such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and chlorozotocin have a chloroethyl moiety as a common bifunctional group, and decompose under physiological conditions to generate chloroethyldiazohydroxides similarly to the decomposition of carcinogenic *N*-nitroso compounds. Of the two geometrical isomers, (*E*)-chloroethyldiazohydroxide can react with DNA to form alkylated base,[14] and the second alkylation reaction due to the presence of the chloro leaving group can lead to DNA cross-links.[13] DNA interstrand cross-links prevents separation of DNA double strands in replication process, which eventually inhibits DNA synthesis, resulting in a cytotoxic effect.

We synthesized *N*-nitrosamines having a chloroethyl group to evaluate their chemical and biological properties. In the present study, in order to investigate the properties of activated *N*-nitrosodialkylamines having a chloroalkyl group with a longer alkyl chain, we synthesized *N*-nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines (**1**—**3**, Fig. 1). The nitrosamines were expected to hydrolyze and decompose to chloroalkyldiazohydroxides in an aqueous solution, then to alkylate various cellular macromolecules (Fig. 2). To confirm the reaction of the nitrosamines with DNA, the stability in an

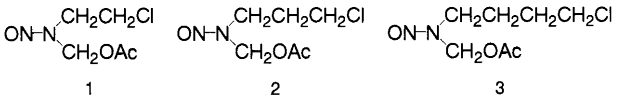

Fig. 1. Structures of *N*-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines Synthesized in This Study

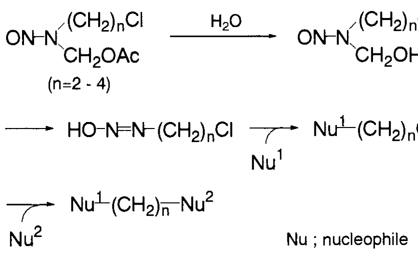

Fig. 2. Possible Reaction of *N*-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines with Nucleophiles

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Dedicated to the memory of Dr. Kyosuke Tsuda.

aqueous solution and mutagenicity towards two bacterial strains were assayed and the results were compared with those of simple nitrosamines without a chloro moiety to elucidate the reaction mechanism of chloroalkyl nitrosamines under physiological conditions.

Experimental

^{1}H-NMR spectra were measured on JEOL JNM-A500 (500 MHz) and JNM-GX270 (270 MHz) spectrometers using tetramethylsilane as an internal reference. The abbreviations for signal patterns are as follows: s, singet; t, triplet; q, quartet; se, sextet; br, broad; m, multiplet. UV spectra were recorded on a Hitachi U-3210 spectrometer. MS spectra were determined on a JEOL JMS-DX303 spectrometer. HPLC analysis was performed on a system consisting of a Shimadzu LC-10AD pump, a Shimadzu SPD-10A UV detector, and a Shimadzu C-R6A chromatopac. Most reagents used were purchased from Wako Pure Chemical Ind. (Osaka, Japan) as the purest grade available. 2-Chloroethylamine hydrochloride, 3-chloropropylamine hydrochloride and 4-amino-1-butanol were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Kieselgel 60 (230—400 mesh) and sodium ammonium hydrogenphosphate were purchased from Merck & Co. (Rahway, NJ, U.S.A.). Dimethyl sulfoxide (DMSO) was freshly distilled from calcium hydride before use (bp$_7$ 58 °C). Bacto agar and nutrient broth were purchased from Difco Laboratories (Detroit, MI, U.S.A.).

***N*-Nitroso-*N*-(acetoxymethyl)-2-chloroethylamine (1)**[15] 2-Chloroethylamine hydrochloride 5.5 g (54 mmol) was dissolved in 150 ml of acetic acid, and 5 ml of formalin (HCHO 62 mmol) was added. The solution was stirred in an ice bath and aqueous sodium nitrite (7.5 g/20 ml, $NaNO_2$ 109 mmol) was added. Reaction temperature was kept below 10 °C for 6 h, and 50 ml of water was added to the resulting solution and it was extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The yellow oil obtained was purified by silica gel column chromatography to give 1.2 g of yellowish oil. Yield: 12.2%. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.06 (3H, S, Z-C$\underline{H}_3$), 2.15 (3H, S, *E*-C$\underline{H}_3$), 3.57 (2H, t, *J*=6.2 Hz, *E*-C$\underline{H}_2$Cl), 3.89 (2H, t, *J*=6.0 Hz, *Z*-C$\underline{H}_2$Cl), 3.92 (2H, t, *J*=6.3 Hz, *E*-C$\underline{H}_2$N), 4.63 (2H, t, *J*=6.3 Hz, *Z*-C$\underline{H}_2$N), 5.40 (2H, s, *Z*-C$\underline{H}_2$O), 6.24 (2H, s, *E*-C$\underline{H}_2$O). $E:Z=89.6:10.4$.

***N*-Nitroso-*N*-(acetoxymethyl)-3-chloropropylamine (2)** 3-Chloropropylamine hydrochloride 5.8 g (50 mmol) was dissolved in 150 ml of acetic acid, and 5 ml of formalin (HCHO 62 mmol) was then added. The solution was stirred in an ice bath and was treated with aqueous sodium nitrite solution (6.9 g/20 ml, $NaNO_2$ 100 mmol). Reaction temperature was kept below 10 °C for 6 h, and 50 ml of water was added to the resulting solution and it was extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The yellow oil obtained was purified by silica gel column chromatography to give 2.02 g of yellowish oil. Yield: 20.7%. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.97 (2H, q, *J*=6.7 Hz, *E*–C–CH_2–C), 2.07 (3H, s, *Z*-C$\underline{H}_3$), 2.15 (3H, s, *E*-C$\underline{H}_3$), 2.30 (2H, m, *Z*–C–C$\underline{H}_2$–C), 3.48 (2H, t, *J*=6.3 Hz, *E*-C$\underline{H}_2$Cl), 3.62 (2H, t, *J*=6.3 Hz, *Z*-C$\underline{H}_2$Cl), 3.73 (2H, t, *J*=7.0 Hz, *E*-C$\underline{H}_2$N), 4.43 (2H, t, *J*=6.8 Hz, *Z*-C$\underline{H}_2$N), 5.37 (1H, s, *Z*-C$\underline{H}_2$O), 6.19 (1H, s, *E*-C$\underline{H}_2$O). $E:Z=90.1:9.9$. UV λ_{max} (CH_3CN) nm (ε): 231 (6900), 369 (110). MS *m/z*: 194 (M^+), 196 ($[M+2]^+$). *Anal.* Calcd for $C_6H_{11}ClN_2O_3$: C, 37.03; N, 14.40; H, 5.70. Found: C, 36.73; N, 14.22; H, 5.53.

***N*-Nitroso-*N*-(acetoxymethyl)-4-chlorobutylamine (3)** 4-Amino-1-butanol 4.7 ml (50 mmol) was added dropwise to 4 ml of thionyl chloride (55 mmol) in an ice bath. When the dark brownish solution was allowed to warm to 50 °C, gas evolution was observed then the solution was evaporated to dryness. The resulting residue was dissolved in 15 ml of ethanol. After refluxing for 30 min, the solution was filtered through activated carbon. Evaporation of the resulting solution gave 3.5 g of yellow gel. ^{1}H-NMR (270 MHz, DMSO-d_6) δ: 1.74 (4H, m, C-C$\underline{H}_2$C$\underline{H}_2$-C), 2.80 (2H, se, *J*=6.4 Hz, C$\underline{H}_2$-NH_3^+), 3.67 (2H, t, *J*=6.4 Hz, C$\underline{H}_2$–Cl), 8.11 (3H, br, N$\underline{H}_3^+$). Yield: 44.7%. Crude 4-chlorobutylamine hydrochloride 3.5 g (*ca.* 25 mmol) was dissolved in 80 ml of acetic acid, and 2.5 ml of formalin (HCHO 31 mmol) was added. The solution was stirred in an ice bath and aqueous sodium nitrite (3.5 g/10 ml, $NaNO_2$ 50 mmol) was added. Reaction temperature was kept below 10 °C for 15 h, and 50 ml of water was added to the resulting solution and it was extracted with dichloromethane.The organic layer was dried over anhydrous sodium sulfate, then evaporated under reduced pressure. The pale yellow oil obtained was purified by silica gel column chromatography to give 0.66 g of yellowish oil. Yield: 12.7%. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.67 (4H, m, *E*-C-C$\underline{H}_2$-C$\underline{H}_2$-C), 1.85 (2H, m, *Z*-C$\underline{H}_2$-C-Cl), 1.98 (2H, m, *Z*-C$\underline{H}_2$-C-N), 2.06 (3H, s, *Z*-C$\underline{H}_3$), 2.14 (3H, s, *E*-C$\underline{H}_3$), 3.52 (2H, t, *J*=6.1 Hz, *E*-C$\underline{H}_2$Cl), 3.60 (2H, t, *J*=7.3 Hz, *Z*-C$\underline{H}_2$Cl), 3.61 (2H, t, *J*=7.4 Hz, *E*-C$\underline{H}_2$N), 4.30 (2H, t, *J*=7.0 Hz, *Z*-C$\underline{H}_2$N), 5.36 (1H, s, *Z*-C$\underline{H}_2$O), 6.16 (1H, s, *E*-C$\underline{H}_2$O). $E:Z=90.1:9.9$. MS *m/z*: 208 (M^+), 210 ($[M+2]^+$). *Anal.* Calcd for $C_7H_{13}ClN_2O_3$: C, 40.29; N, 13.43; H, 6.28. Found: C, 39.99; N, 13.19; H, 6.21.

Decomposition of Nitrosamines Ten μl of an acetonitrile (CH_3CN) solution of *N*-nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines was added to 990 μl of 0.1 M phosphate buffer (pH 5.0, 7.4 and 9.0), and the solution was incubated at 37 °C (final concentration; 1 mM). At 15 min intervals, 5 μl of the solution was injected into the HPLC system, and the amount of nitrosamine remaining was analyzed on a LiChrosorb RP-18 column (10 μm), eluted with mixtures of CH_3CN and water.

Bacterial Strains A culture of *Salmonella typhimurium* TA1535 was kindly provided by Dr. B. N. Ames, University of California, Berkeley, U.S.A., and TA92 was kindly provided by Dr. T. Nohmi, National Institute of Health Sciences, Tokyo, Japan.

Mutation Assay The medium used for overnight culture of bacterial strains was a nutrient broth medium (NB) containing 6 g of nutrient broth and 5 g of NaCl per liter. The minimal media used for mutation assays were the same as reported by Maron and Ames.[16] The agar plates contained 30 ml of the minimal medium with 1.5% Bacto agar.

The bacteria were grown in 5 ml of NB for 15.5 h at 37 °C. Test compounds were diluted in DMSO. To a tube containing 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) and 0.1 ml of a culture of bacterial tester strain, 0.1 ml of sample solution was added, then the mixture was shaken immediately. Two milliliters of top agar was added to the mixture (0.6% agar in 0.6% NaCl) at 45 °C, and it was poured onto a minimal agar plate. After incubation at 37 °C for 2 d, the colonies on the plate were counted manually, and the number of spontaneous revertant colonies was subtracted from that of the induced revertants. The concentration of the test compounds was expressed as μmol/plate. All data reported are representatives of at least three experiments using duplicate plates for each dose level.

Results and Discussion

DNA alkylation and DNA cross-linking are classical mechanisms for activity of cancer chemotherapeutic agents such as mitomycin C and carmustine (BCNU), currently used in clinical treatment. These agents alkylate DNA by reaction with one electrophilic center, allowing a neighboring nucleophile in the alkylated DNA to react with a second electrophilic center to eventually form a DNA cross-link.

The chloroethyl moiety is a common bifunctional group antitumor cross-linking agents. CENUs are one group of effective chemotherapeutic agents with a chloroethyl group, and many analogs have been developed in an effort to improve DNA-directing reactivity and reduce side effects.[13] *N*-Nitrosamides including CENUs show their biological activities by alkylation and carbamoylation, and protein carbamoylation is thought to be the cause of their side effects. In contrast, *N*-nitrosamines degrade *in vivo* by releasing aldehydes and alkylate cellular nucleophiles, with no possibility of carbamoylation. *N*-Nitrosamines are thought to be environmental carcinogens, and little has been reported about their antitumor activity. We are interested in the behavior of nitrosamines having a chloroethyl, or chloroalkyl group with a longer carbon chain because of their potential as lead compounds for novel antitumor agents.

The synthesis of compound **1** and its effect on increasing the life span of tumor-bearing mice has been already reported,[15] but little is known about its precise biological activity. We have additionally synthesized nitrosamines with chloropropyl and chlorobutyl groups, compounds **2** and **3** respectively, and the properties for **2** and **3** were compared with **1**, and the effect of alkyl-chain length and the chloro leaving group was examined.

As one of the key chemical properties, the rate of decom-

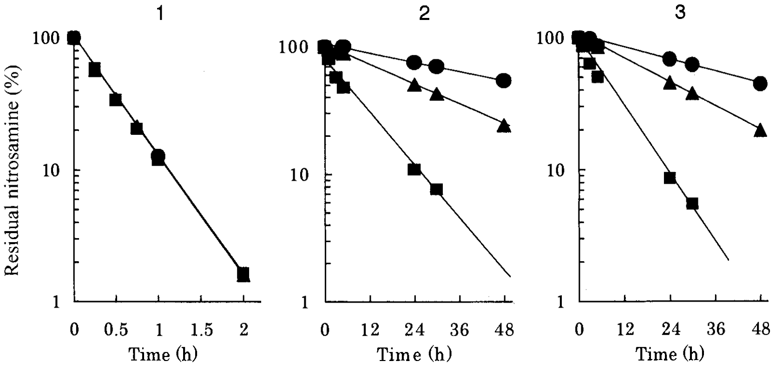


Fig. 3. Decomposition of *N*-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines in Aqueous Solutions
●, pH 5.5; ▲, pH 7.4; ■, pH 9.0.

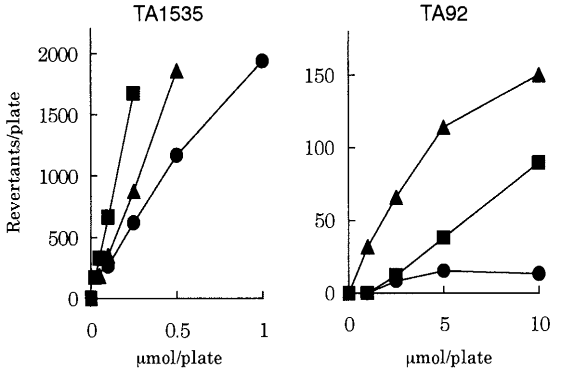
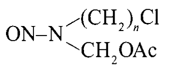
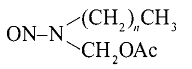


Fig. 4. Mutagenicity of *N*-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines in *Salmonella typhimurium* TA1535 and TA92
●,1; ▲,2 ; ■,3.

position of these nitrosamines in aqueous solution was determined. The concentration of the nitrosamines decreased exponentially in phosphate buffer, which indicates that the nitrosamines time-dependently decomposed, as shown in Fig. 3. Compound **1** rapidly decomposed in aqueous solution, and the rate was apparently independent of the pH of the solution. On the other hand, compounds **2** and **3** were comparably stable and decomposed pH-dependently, and the rate of the reaction increased under alkaline conditions. The half-lives of **1**, **2** and **3** under physiological conditions (pH 7.4) were calculated as 0.3, 25 and 21 h, respectively. The stability of **2** and **3** in aqueous solution and the effect of pH on their decomposition were clearly different from those of **1**, which suggested that the mechanism of decomposition of **2** and **3** was different from that of **1**. That is, **2** and **3** decomposed more rapidly at higher pH, because the acetyl group was hydrolyzed through base catalysis and this step determined the overall rate of decomposition. Compound **1** decomposed pH-independently because of the chloroethyl group, since there is a neighboring group effect as observed in nitrogen mustards, which accelerates decomposition under aqueous conditions.

The mutagenicity of compounds **1**—**3** was assayed in *Salmonella typhimurium* TA1535 and TA92 as a preliminary evaluation of their biological activity. All compounds were directly mutagenic, and the mutagenic potency was linearly related to the concentration of the chemicals (Fig. 4). A specific mutagenicity per μmol of chemical was defined by the slope of the linear part of the dose–mutagenicity relationship, and was calculated by the least squares method. Table 1 shows the specific mutagenicity for three series of chemicals in *Salmonella typhimurium* TA1535 strains in comparison to that for *N*-nitroso-*N*-(acetoxymethyl)alkylamine lacking a chloro group.[6] In the TA1535 strain, which can detect base-pair change mutations, an increase of alkyl chain length potentiated the mutagenic activity. The activities were stronger than those of the corresponding simple α-acetoxy nitrosamines without a chloro group. These results suggested that the nitrosamines reacted with bacterial DNA, and induced DNA alkylation *in vivo* more frequently than simple α-acetoxy nitrosamines did. One of the reasons why the mutagenicity of **1** was weaker than those of **2** and **3** was that the replacement by chlorine enhanced the reactivity due to a neighboring group effect. Thus, **1** rapidly decomposed in aqueous conditions, and the chance to react with DNA decreased *in vivo*, which weakened its mutagenicity in bacteria. Compounds **2** and **3** seem to have sufficient stability to react with bacterial DNA, and their alkylating activity may be reflected in their mutagenic potency.

Table 1. Relative Mutagenicity of *N*-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines in *Salmonella typhimurium* TA1535

Compound		Relative mutagenicity (revertants/μmol)
 ON-N(CH$_2$)$_n$Cl / CH$_2$OAc	**1** (n=2)[a]	2320
	2 (n=3)[a]	3700
	3 (n=4)[a]	6650
 ON-N(CH$_2$)$_n$CH$_3$ / CH$_2$OAc	n=1[b]	530
	n=2[b]	450
	n=3[b]	620

a) This paper. *b*) Ref. 6.

Although chlorinated nitrosamines were reported as a product of nitrosation of spermidine in the presence of chloride ion[17] and as a product from reaction of hydroxylated nitrosamine and chloride ion,[18] there are few reports about chlorinated *N*-nitroso compounds except CENUs. Haloethylnitrosoureas form haloethyldiazotates as ultimate electrophiles, and the *E*-isomers of diazotates are reported to form DNA cross-links in contrast to the *Z*-isomer. Furthermore, *E*-2-chloroethyldiazotate exhibited activity against P388 leukemia *in vivo*.[19] The chloroalkylnitrosamines synthesized in this study may also form chloroalkyldiazotates, and they are expected to act as cross-linking agents. *Salmonella typhimurium* TA92 retains excision repair capabilities and is commonly used for detection of cross-linking agents, so we chose this assay at first for evaluating whether the compounds have cross-linking activity that is essential for antitumor activity. Compound **1** showed weaker mutagenicity in TA92 than the corresponding α-acetoxy nitrosamine lacking a chloro group (data not shown), which was attributed to its instability. On the other hand, compound **2** showed higher mutagenicity than the corresponding α-acetoxy nitrosamine lacking a chloro group, but compound **3** showed the same mutagenic potency as the corresponding α-acetoxy nitrosamine lacking a chloro group (data not shown). Compound **2** showed a weaker mutagenicity than compound **3** towards the TA1535 strain, but a higher mutagenicity towards the TA92 strain. These results suggested that not only a chloroethyl group but also a chloropropyl group could behave as an antitumor bifunctional group, and suggested the possibility that chlorinated nitrosamine derivatives could be applicable as lead compounds for antitumor agents. Further investigation is in progress to study in detail whether ω-chloroalkyl α-acetoxymethyl nitrosamines form DNA cross-links similar to antitumor chloroethylnitrosoureas.

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Retinoidal Pyrimidinecarboxylic Acids. Unexpected Diaza-Substituent Effects in Retinobenzoic Acids

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Several pyridine- and pyrimidine-carboxylic acids were synthesized as ligand candidates for retinoid nuclear receptors, retinoic acid receptors (RARs) and retinoic X receptors (RXRs). Although the pyridine derivatives, 6-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]pyridine-3-carboxylic acid (2b) and 6-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]pyridine-3-carboxylic acid (5b) are more potent than the corresponding benzoic acid-type retinoids, Am80 (2a) and Am580 (5a), the replacement of the benzene ring of Am580 (5a), Am555 (6a), or Am55 (7a) with a pyrimidine ring caused loss of the retinoidal activity both in HL-60 cell differentiation assay and in RAR transactivation assay using COS-1 cells. On the other hand, pyrimidine analogs (PA series, 10 and 11) of potent RXR agonists (retinoid synergists) with a diphenylamine skeleton (DA series, 8 and 9) exhibited potent retinoid synergistic activity in HL-60 cell differentiation assay and activated RXRs. Among the synthesized compounds, 2-[*N*-*n*-propyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)amino]pyrimidine-5-carboxylic acid (PA013, 10e) is most active retinoid synergist in HL-60 assay.

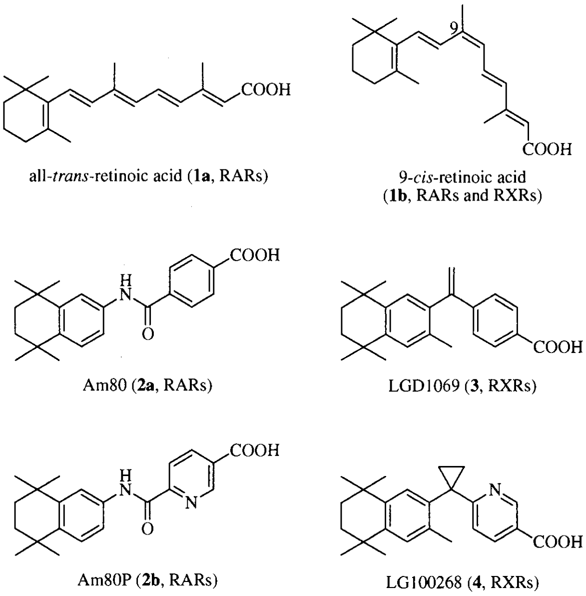
Key words retinoid; retinoic acid receptor; retinoid X receptor; pyrimidine

Retinoids, which are natural and synthetic analogs of retinoic acid (all-*trans*, **1a**, Fig. 1), regulate important biological events, such as cell growth, differentiation, and apoptosis.[1)] Retinoids have significant therapeutic potential in the fields of dermatology and oncology.[2)] For example, retinoic acid (**1a**) induces a high rate of complete remission of acute promyelocytic leukemia (APL),[3)] and some synthetic retinoids have remarkable effects on skin diseases, including acne and psoriasis.[4)] However, the use of retinoid therapy is still limited, owing to undesirable side effects.

The biological activities of retinoids are mediated by binding to and activating two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both having three subtypes (α, β, γ).[5)] These receptors belong to the nuclear receptor superfamily, and are ligand-inducible transcription factors. All-*trans*-retinoic acid (**1a**) binds to RARs, and its 9-*cis* isomer (**1b**) binds to both RARs and RXRs. Recently, several retinoids with receptor-selectivity have been reported. Am80 (**2a**, Fig. 1) is a retinoid (RAR agonist) with RARα (and β) selectivity,[6)] and LGD1069 (**3**) is an RXR-pan-agonist.[7)] Using these receptor-selective compounds, retinoidal activities have been proven to result mainly from the activation of RXR–RAR heterodimers by binding to an RAR ligand. An RXR ligand alone cannot activate RXR–RAR heterodimers, but it can increase the potency of an RAR ligand.[8,9)]

Structurally, most RAR and RXR ligands possess a benzoic acid moiety. Several successful replacements of the benzene ring with a heterocyclic ring have been reported.[10—13)] Thus, Am80P (**2b**, EC_{50} 2.2×10^{-10} M), a pyridinecarboxylic acid analog of Am80 (**2a**), is more potent than Am80 (**2a**, EC_{50} 7.9×10^{-10} M) in HL-60 cell differentiation assay, while another pyridinecarboxylic acid, Am80P2 (**2c**), exhibited weaker activity (EC_{50} 4.0×10^{-8} M).[10)] Similarly, a pyridinecarboxylic acid derivative, LG100268 (**4**), has higher affinities for RXRs than LGD1069 (**3**).[12)] During our investigations on structure–activity relationships of retinoids, we found unique effects caused by replacement of a benzoic acid moiety with a pyrimidinecarboxylic acid, and showed that these effects differed between RAR and RXR ligands. Here, we describe the synthesis and retinoidal activities of several heterocyclic derivatives.

Chemistry Considering the activities of Am80P (**2b**) and Am80P2 (**2c**), we synthesized several pyridine and pyrimidine derivatives, as shown in Fig. 2 and Tables 1 and 2. Compounds **2**, **5**—**7** are analogs of RAR agonists, Am80 (**2a**) and Am580 (**5a**), and compounds **10** and **11** are pyrimidine analogs of potent retinoid synergists (RXR agonists), **8**



all-*trans*-retinoic acid (**1a**, RARs)

9-*cis*-retinoic acid (**1b**, RARs and RXRs)

Am80 (**2a**, RARs)

LGD1069 (**3**, RXRs)

Am80P (**2b**, RARs)

LG100268 (**4**, RXRs)

Fig 1. Structures of Retinoic Acids and RAR- and RXR-Selective Retinoids

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Dedicated to the memory of Dr. Kyosuke Tsuda.

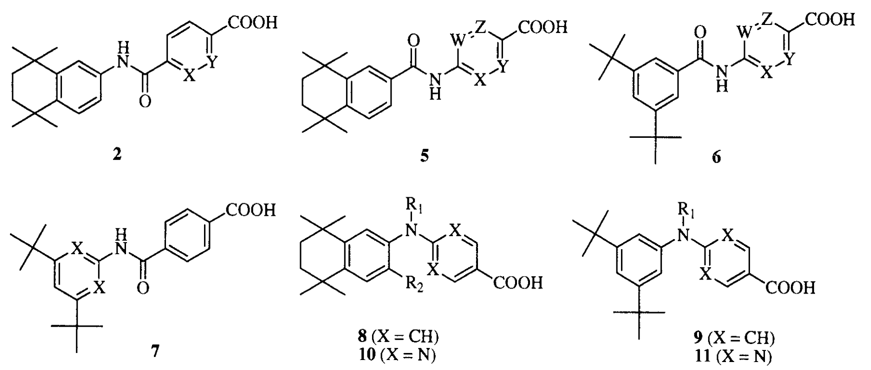


Fig. 2. Structures of Pyridine- and Pyrimidine-Carboxylic Acids

The substituents are shown in Tables 1 and 2.

Table 1. HL-60 Cell Differentiation Activity of Aromatic Amides with A Heterocyclic Ring

Compound	X	Y	Z	W	EC$_{50}$,$^{a)}$ M
Am80 (**2a**)	CH	CH	—	—	7.9×10^{-10}
Am80P (**2b**)	N	CH	—	—	2.2×10^{-10}
Am80P2 (**2c**)	CH	N	—	—	4.0×10^{-8}
Am80P3 (**2d**)	N(–O)	CH	—	—	Inactive
Am80P4 (**2e**)	CH	N(–O)	—	—	$>10^{-6}$
Am580 (**5a**)	CH	CH	CH	CH	3.4×10^{-10}
Am580P (**5b**)	CH	N	CH	CH	4.2×10^{-11}
Am580K (**5c**)	N	N	CH	CH	Inactive
Am580K2 (**5d**)	CH	CH	N	N	Inactive
Am555 (**6a**)	CH	CH	CH	CH	4.8×10^{-8}
Am555P (**6b**)	CH	N	CH	CH	1.0×10^{-8}
Am555K (**6c**)	N	N	CH	CH	Inactive
Am555K2 (**6d**)	CH	CH	N	N	Inactive
Am55 (**7a**)	CH	—	—	—	3.6×10^{-8}
Am55K (**7b**)	N	—	—	—	Inactive

a) 'Inactive' means that there was no effect below 1×10^{-6} M test compound, and '$>10^{-6}$' means that there was a little activty at 1×10^{-6} M.

and **9**,14) respectively.

Two isomeric pyridine derivatives of Am80 (**2a**) were prepared by condensation of an amine **12** with the corresponding monomethyl esters of pyridine-2,5-carboxylic acid, followed by ester hydrolysis (Fig. 3). Oxidation of the pyridine rings of **13** and **14** was performed using *m*-chloroperoxybenzoic acid (*m*CPBA) in chloroform and the following ester hydrolysis afforded Am80P3 (**2d**) and Am80P4 (**2e**). Am580P (**5b**), a pyridine analog of Am580 (**5a**), was prepared by the condensation of an acid chloride **16** and methyl 6-aminonicotinate. Under suitable conditions, the reaction afforded diacyl compounds, which can be selectively hydrolyzed to monoacyl derivatives by refluxing in acidic methanol.

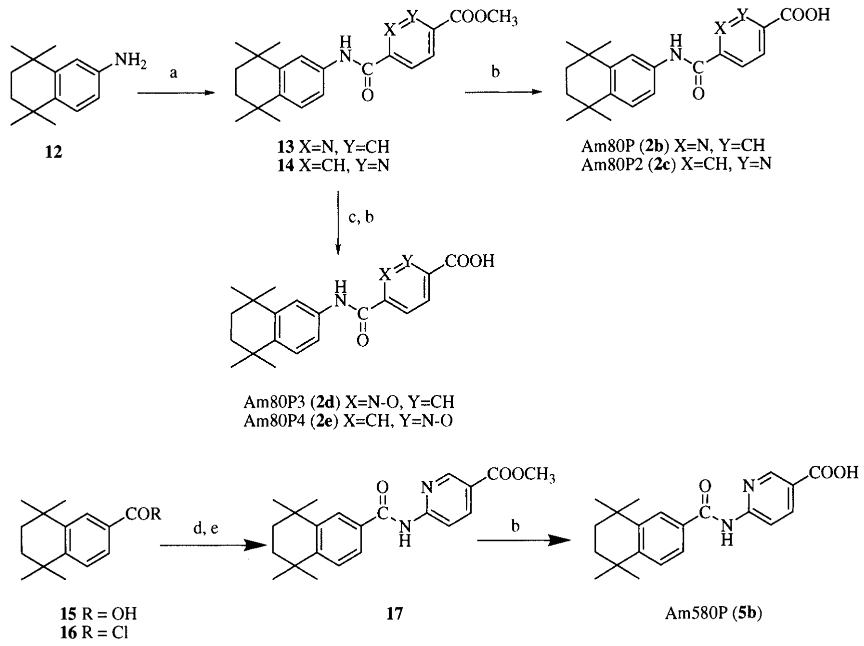
Am580K (**5c**), a pyrimidinecarboxylic acid analog of Am580 (**5a**), was synthesized as shown in Fig 4. Claisen condensation of ethyl 3-ethoxypropionate (**18**) with ethyl formate afforded ethyl 3-ethoxy-2-methoxymethylenepropionate (**19**, 58%),15) which was cyclized with urea in acidic ethanol to give **20** in 66% yield.16) After dehydrogenation of **20** using bromine (99%), **21** was converted to ethyl 2-aminopyrimidine-5-carboxylate (**23**), which was reacted with 5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthoyl chloride (**16**), followed by ester hydrolysis, to afford Am580K (**5c**). For the synthesis of another pyrimidine derivative, Am580K2 (**5d**), the trimethinium salt (**26**), prepared from glycine (**25**), was reacted with *S*-methylthiourea to afford 2-methylthio-5-(dimethylaminomethyleneamino)pyrimidine (**27**, 80%).17) After oxidation of the methylthio group of **27** (87%), **28** was transformed to a cyano group to give **29** (quant). Methyl 5-aminopyrimidine-2-carboxylate (**30**) was obtained by the two-step hydrolyses of **29**, and was converted to Am580K2 (**5d**) by a similar method to that used in the synthesis of Am580K (**5c**). Pyridine (**6b**) and pyrimidine derivatives, **6c** and **6d**, of Am555 (**6a**) were similarly prepared.

A compound (**7b**), having a pyrimidine ring at the hydrophobic region of Am80 (**2a**), was also prepared as shown in Fig. 6. Cyanoguanidine (**32**) was converted to guanidinecarboxamide (**33** 56%), which was cyclized with 2,2,6,6-tetramethylpentane-3,5-dione to give 2-amino-4,6-di-*tert*-butylpyrimidine (**34**) in 62%.18) Compound **34** was reacted with terephthalic acid monomethyl ester chloride (83%), followed by ester hydrolysis (87%) to give **7b**.

RXR ligand candidates with a diarylamine skeleton were prepared as shown in Fig. 7. 5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthylamine (**12**) or 5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthylamine (**36**) was reacted with ethyl *p*-iodobenzoate catalyzed by tris(dibenzylideneacetone)dipalladium(0) (Pd(dba)$_3$) in the presence of (*R*)-2,2′-bis(diphenylphosphino)-1,1′-binaphtyl ((*R*)-BINAP)19) with ethyl 2-chloropyrimidine-5-carboxylate (**22**) to give the diphenylamine **37** (48%) or **38** (60%), respectively. Treatment of **12** or **36** with ethyl 2-chloropyrimidine-5-carboxylate (**22**) in the presence of potassium carbonate afforded 2-(*N*-arylamino)pyrimidine-5-carboxylates, **39** (91%) and **40** (61%), respectively. DA compounds **8** and PA compounds **10** shown in Table 2 were obtained by *N*-alkylation of these diarylamines using NaH and alkyl halide, followed by ester hydrolysis. Compounds **9** and **11** having a di-*tert*-butylphenyl moiety as the hydrophobic region were synthesized similarly.

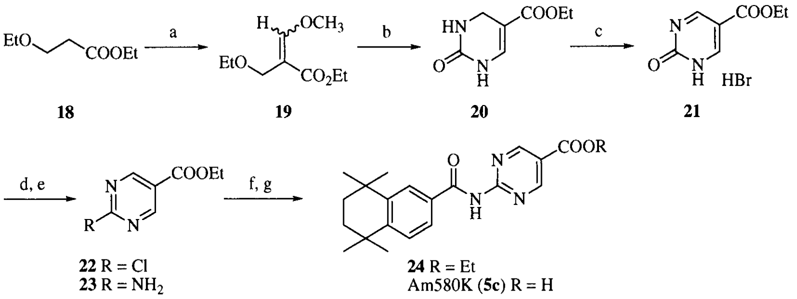
Biological Activity The retinoidal activities of various pyridine and pyrimidine derivatives were evaluated in terms of the ability to induce differentiation of human promyelocytic leukemia cells HL-60.20,21) The morphological changes were examined by microscopy after Wright–Giemsa staining, and the differentiation state was determined by means of nitro blue tetrazolium (NBT) reduction assay as a functional marker of differentiation.22) These two indexes of differentiation correlate well with each other.6a,23)

The differentiation-inducing activities of the amide compounds are shown in Table 1. As described above, replacement of a carbon atom with a nitrogen atom in the benzoic acid moiety of Am80 (**2a**) resulted in different effects, de-



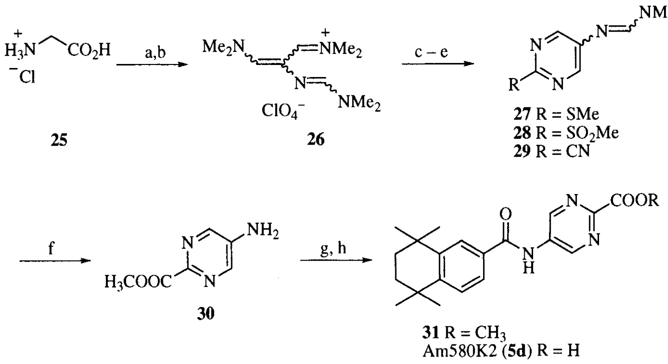
(a) 5-methoxycarbonylpicolinic acid or 2-methoxycarbonylnicotinic acid / pyridine / benzene; (b) NaOH / EtOH; (c) *m*CPBA / $CHCl_3$; (d) $SOCl_2$ / benzene; (e) methyl 6-aminonicotinate, benzene

Fig. 3



(a) Na / EtOH / HCO_2Et / benzene / $(CH_3)_2SO_4$; (b) H_2NCONH_2 / HCl / EtOH; (c) Br_2 / AcOH; (d) N,N-dimethylaniline / $POCl_3$; (e) NH_3 / EtOH; (f) **16** / DMAP / pyridine; (g) NaOH / EtOH

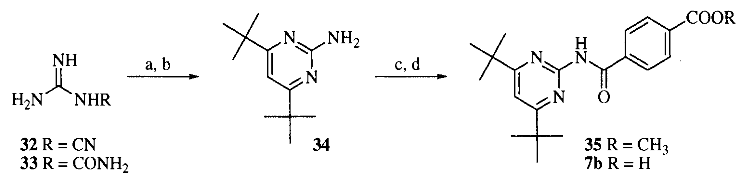
Fig. 4



(a) $POCl_3$ / DMF ; 70% $HClO_4$; (b) Et_3N / EtOH; Activated carbon; (c) *S*-methylthiourenium sulfate / EtOH; NaOMe / MeOH; (d) aq. $NaClO_4$ / $NaHCO_3$; (e) KCN / DMF; (f) 2N NaOH / H_2SO_4 / MeOH; (g) **16** / DMAP / pyridine; (h) NaOH / EtOH

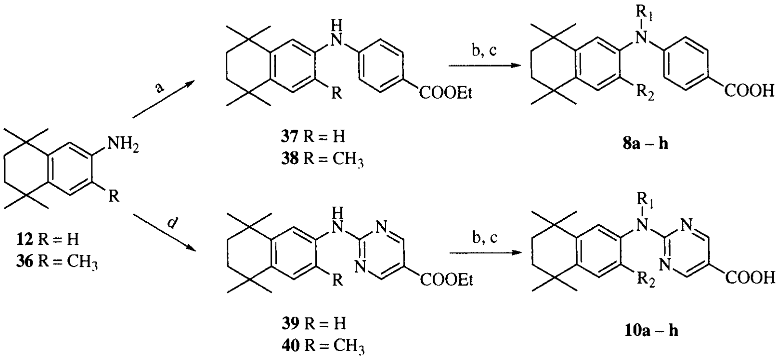
Fig. 5

pending on the position. Am80P (**2b**), having a nitrogen atom *ortho* to the amide bond, is more potent than Am80 (**2a**), while the activity of Am80P2 (**2c**), having a nitrogen atom *ortho* to the carboxyl group is 1/50 of that of Am80 (**2a**) in terms of EC_{50} value. Oxidation of the pyridine nitrogen atom of Am80P (**2b**) and Am80P2 (**2c**) decreased the ac-



(a) HCl / MeOH ; ion exchange resin; (b) 2,2,6,6-tetramethylpentane-3,5-dione / 80 %EtOH; (c) terephthalic acid monomethyl ester chloride / DMAP / pyridine; (d) LiCl / DMF

Fig. 6



(a) ethyl *p*-iodobenzoate / Pd(dba)$_3$ / BINAP / NaO*tert*Bu / toluene; (b) NaH / alkyl halide / DMF; (c) KOH / EtOH; (d) **22** / K_2CO_3

Fig. 7

Table 2. HL-60 Cell Differentiation-Inducing Activity of Pyrimidinecarboxylic Acids (**10** and **11**)

Pyrimidinecarboxylic acid derivatives[a]					Benzoic acid derivatives[a]		
Compound	R_1	R_2	EC_{50}, M[b]	SEC_{50}, M[c]	Compound	EC_{50}, M[b]	SEC_{50}, M[c]
PA010 (**10a**)	H	H	9.8×10^{-7}	2.1×10^{-7}	DA010 (**8a**)	6.8×10^{-7}	8.6×10^{-9}
PA110 (**10b**)	H	CH_3	Inactive	8.4×10^{-9}			
PA011 (**10c**)	CH_3	H	1.7×10^{-7}	8.6×10^{-10}	DA011 (**8c**)	3.6×10^{-7}	7.6×10^{-9}
PA111 (**10d**)	CH_3	CH_3	Inactive	1.5×10^{-9}	DA111 (**8d**)	Inactive	1.3×10^{-10}
PA013 (**10e**)	n-C_3H_7	H	Inactive	6.8×10^{-11}	DA013 (**8e**)	Inactive	2.0×10^{-9}
PA113 (**10f**)	n-C_3H_7	CH_3	Inactive	1.0×10^{-10}	DA113 (**8f**)	Inactive	3.6×10^{-10}
PA024 (**10g**)	$CH_2(c$-$C_3H_5)$	H	Inactive	1.1×10^{-10}	DA024 (**8g**)	Inactive	2.5×10^{-9}
PA124 (**10h**)	$CH_2(c$-$C_3H_5)$	CH_3	Inactive	3.0×10^{-10}	DA124 (**8h**)	Inactive	6.2×10^{-10}
PA211 (**11a**)	CH_3	—	Inactive	3.8×10^{-7}			
PA212 (**11b**)	C_2H_5	—	Inactive	1.4×10^{-8}	DA212 (**9b**)	Inactive	3.8×10^{-8}
PA213 (**11c**)	n-C_3H_7	—	Inactive	1.5×10^{-9}	DA213 (**9c**)	Inactive	2.6×10^{-9}
PA224 (**11d**)	$CH_2(c$-$C_3H_5)$	—	Inactive	2.0×10^{-9}			
LG100268 (**4**)	—	—	Inactive	1.0×10^{-10}			

a) Structures (positions of R_1 and R_2) are shown in Figs. 1 and 2. *b*) EC_{50} was determined as the concentration of a test compound (alone) which induces HL-60 cell differentiation to the extent of 50%. 'Inactive' means there was no activity below 1.0×10^{-6} M test compound. *c*) SEC_{50} was determined as the concentration of a test compound which induces HL-60 cell differentiation to the extent of 50% in the presence of 3×10^{-10} M Am80 (**2a**). This concentration of Am80 (**2a**) alone induced less than 10% differentiation.

tivity by two or more orders of magnitude. In the case of Am580 (**5a**), introduction of a nitrogen atom into the *ortho* position to the amide bond increased the retinoidal activity, and Am580P (**5b**) is more potent than Am580 (**5a**) by one order of magnitude. However, remarkable diminution of the activity was observed in the pyrimidine derivatives of Am580 (**5a**), irrespective of the position of the nitrogen atoms. Thus, introduction of two nitrogen atoms into the benzoic acid moiety of Am580 (**5a**) afforded the pyrimidine derivatives, Am580K (**5c**) and Am580K2 (**5d**), which are completely inactive below 10^{-6} M. Similar results were obtained with pyridine (**6b**) and pyrimidine analogs (**6c**, **d**) of Am555 (**6a**). Further, replacement of the benzene ring bearing alkyl groups in Am55 (**7a**) with a pyrimidine ring also resulted in loss of activity.

The activities of several pyrimidine derivatives (PA compounds, **10** and **11**) of RXR agonists (DA compounds, **8** and **9**)[12] are shown in Table 2. In the case of the DA series, compounds having a methyl group (DA011) or no substituent (DA010) are weak retinoids (RARs agonistic activity) with EC_{50} values of 10^{-6}—10^{-7} M, and increased the potency of a low concentration of Am80 (**2a**) (RXRs agonistic activity). Introduction of a bulky *N*-alkyl group or a methyl group on the aromatic ring (R_2 of **8** in Fig. 2) caused loss of the retinoidal activity, but increased the retinoid synergistic activity. Similarly, pyrimidinecarboxylic acids, PA010 (**10a**) with an *N*-methyl group and PA011 (**10c**) without an *N*-alkyl group, exhibited differentiation-inducing activity alone at high concentrations, and their EC$_{50}$ values are similar to those of **8a** and **8c**, respectively. The other pyrimidine derivatives (**10b**, **10d**—**h**, **11**) are all inactive alone.

The synergistic activities of DA and PA compounds with Am80 (**2a**) were examined, and the potency was evaluated as the synergistic effective concentration (SEC$_{50}$) value, defined as the concentration of a test compound which induces HL-60 cell differentiation to the extent of 50% in the presence of 3×10^{-10} M Am80 (**2a**). This concentration of Am80 (**2a**) alone induced less than 10% differentiation. In contrast to the inactivity of the pyrimidine analogs of retinoidal aromatic amides, replacement of the benzoic acid moiety of DA compounds with a pyrimidinecarboxylic acid moiety resulted in retention or increase of the retinoid synergistic activity. Except in the cases of PA010 (**10a**) and PA111 (**10d**), the activity of pyrimidine derivatives is more potent than that of the corresponding benzoic acid derivatives. In particular, the pyrimidine derivatives, PA011 (**10c**), PA013 (**10e**), and PA024 (**10g**), without a methyl group on the aromatic ring (R$_2$) are more potent than DA011 (**8c**), DA013 (**8e**), and DA024 (**8g**), respectively, by one order or two orders of magnitude. Among the PA compounds (**10**, **11**), the synergistic activity increases as the *N*-substituent become bulkier, as observed in DA compounds (**8**, **9**), and the SEC$_{50}$ values of **10e**—**g** are almost equal to that of the well-known RXR ligand LG100268 (**4**). Interestingly, the effects of methyl group substitution on the aromatic ring differ between DA and PA compounds. Introduction of a methyl group at the R$_2$ position increases the synergistic activities in DA compounds (**8c**—**h**), while the same modification in PA compounds resulted in a decrease of the synergistic activity, except in the case of PA compounds without an *N*-alkyl substituent (**10a** *vs.* **10b**).

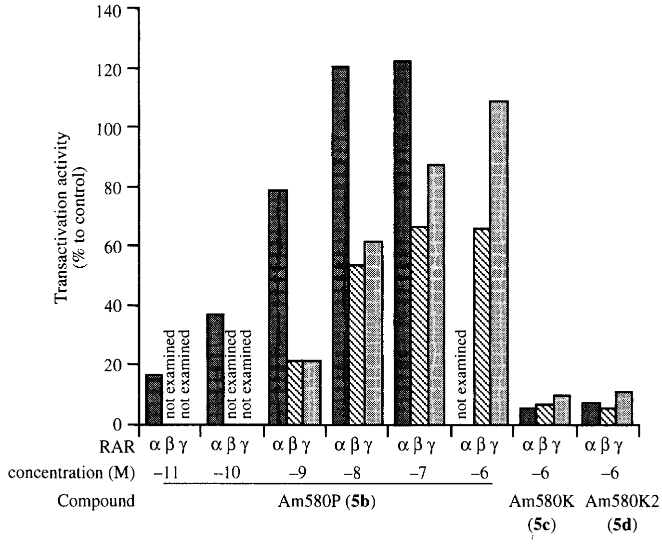


Fig. 8. Transactivation Assays in COS-1 Cells Transiently Transfected with pSG5-hRAR and TREpal×3-TKLUC

The vertical scale is the transactivation relative to that with 1×10^{-7} M Am80, 1×10^{-6} M Am80, and 1×10^{-6} M retinoic acid for RARα, β, and γ, respectively.

In order to elucidate whether the potency of the active and inactive pyrimidine derivatives in HL-60 cell assay can be ascribed to their ability to activate retinoid receptors, transient transactivation assay using RARs and RXRs was conducted for selected compounds.[24] Among three heterocyclic analogs of Am580 (**5a**) examined, only Am580P (**5b**) activated all of the RAR subtypes, and pyrimidine derivatives, Am580K (**5c**) and Am580K2 (**5d**), did not affect the activation of RARs at 10^{-6} M (Fig. 8). None of them activated RXRs (data not shown). Like Am80 (**2a**) and Am580 (**5a**), Am580P (**5b**) showed RARα-selectivity. On the other hand, three retinoid synergists, LG100268 (**4**), DA124 (**8h**) and PA024 (**10g**), activated all three RXR subtypes (Fig. 9), but not RARs (data not shown). Although a diphenylamine derivative, DA124 (**8h**) is nearly 1/6 as active as LG100268 (**4**) in HL-60 cell assay, it exhibited weaker RARs transactivation assay, especially for RXRα and β, by two orders of magnitude. A pyrimidine analog PA024 (**10g**) exhibited more potent RXRs transactivation activities than DA124 (**8h**), and was as potent as LG100268 (**4**) in HL-60 cell assay.

Discussion

Several attempts to introduce nitrogen atoms into hydrophobic skeletons of retinoid structures have afforded highly potent and/or therapeutically superior compounds, including aromatic amides, Am80 (**2a**) and Am580 (**5a**), as RAR agonists with subtype selectivity, and diphenylamines, **8** and **9**, as RXR agonists. The present studies showed that further aza-substitution has differential effects on the activities of RAR and RXR ligands.

Introduction of one nitrogen atom *ortho* to the amide bond in the benzoic acid moiety of an RAR agonist, Am580 (**5a**), increased the retinoidal activity. However, aza-substitution at the second *ortho* position yielding a pyrimidine derivative,

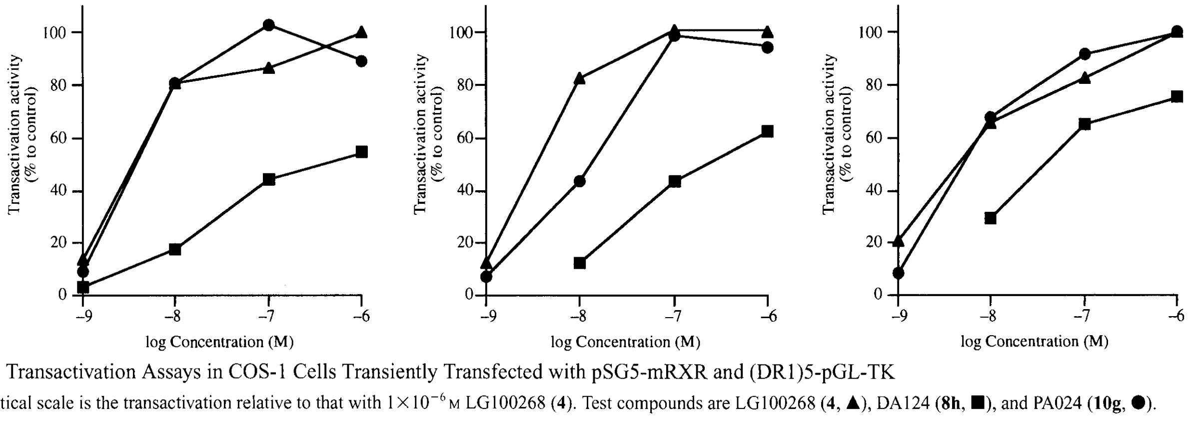


Fig. 9. Transactivation Assays in COS-1 Cells Transiently Transfected with pSG5-mRXR and (DR1)5-pGL-TK

The vertical scale is the transactivation relative to that with 1×10^{-6} M LG100268 (**4**). Test compounds are LG100268 (**4**, ▲), DA124 (**8h**, ■), and PA024 (**10g**, ●).

Am580K (**5c**), with dramatically decreased activity, by more than 5 orders of magnitude. Similar results were observed with other retinoidal amides. Am580K (**5c**) did not activate any RARs in COS-1 cells (Fig. 8), and also did not antagonize retinoids, such as Am80 (**2a**), in HL-60 cell assay (data not shown), which suggests the inability of Am580K (**5c**) to bind to RARs. We confirmed that Am580K (**5c**) did not compete with the binding of [^{3}H]Am80 to RARα (data not shown). Crystal structures of various nuclear receptor ligand-binding domains have recently been reported, making it possible to understand the ligand–receptor interactions at the molecular level. However, we could not find any reason why Am580K (**5c**) would not bind the RARα ligand-binding domain in preliminary computational investigations to compare the binding features of highly active Am580 (**5a**) and inactive Am580K (**5c**). One possible explanation is a difference in the stable conformations of the two molecules, as shown in the case of inactive *N*-methylated analogs of Am80 (**2a**) and Am580 (**5a**).[25] Simple *N*-(pyrimidin-2-yl)benzamide was reported to exist in similar conformations to benzanilide in the crystal,[26] while some secondary *N*-pyrimidin-2-yl amides have *cis*-amide structures in the crystals.[27] ^{1}H-NMR studies indicated that there is no large conformational difference between Am580 (**5a**) and Am580K (**5c**), such as *trans vs. cis* amide structure. Further investigations on the conformation (or structure) of Am580K (**5c**) and its interactions with RAR ligand-binding domains are needed.

The case of RXR ligands was completely different from that of RAR ligands. Replacement of the benzoic acid moiety of potent RXR ligands, **8** and **9** (DA series), with a pyrimidine-5-carboxylic acid afforded potent retinoid synergists, **10** and **11** (PA series). The effects of an *N*-alkyl group are similar in the DA and PA series. Compounds having no or a smaller *N*-alkyl group exhibited weak retinoid synergistic activity and some of them exhibited differentiation-inducing activity alone. A bulkier *N*-alkyl group is more effective, and PA013 (**10e**) and PA024 (**10g**), having an *n*-propyl or a cyclopropylmethyl group, respectively, showed strongest synergistic activity among the synthesized compounds. Interestingly, the effect of a methyl group (R_2 in Fig. 2) on the aromatic ring is different in the PA series (**10**) and DA series (**8**). Thus, in the case of the DA series (**8**), the introduction of a methyl group increased the synergistic activity by one order of magnitude, while the same modification decreased the activity of PA series (**10**), except for PA010 (**10a**), which has no *N*-alkyl group. Since the increased activity of the DA series (**8**) should be ascribed to the twisted conformations of the diphenylamine skeletons induced by the methyl substitution (and also by bulky *N*-alkyl substitution), conformational analyses of the DA and PA compounds may clarify the different substituent effects observed in the PA series (**10**).

In conclusion, several heterocyclic compounds have been synthesized as RAR and RXR ligand candidates. Among them, pyridine derivatives, Am80P (**2b**) and Am580P (**5b**), are potent retinoids, and pyrimidine derivatives, such as PA013 (**10e**) and PA024 (**10g**), are potent retinoid synergists. Although some of the effects of aza substitution on retinoidal benzoic acids remain unclear, these heterocyclic retinoids and retinoid synergists with possibly different physicochemical or pharmacological properties may be candidates for use in retinoid therapy.

Experimental

General Melting points were determined by using a Yanagimoto hot-stage melting point apparatus and are uncorrected. Elemental analyses were carried out in the Microanalytical Laboratory, Faculty of Pharmaceutical Sciences, University of Tokyo, and were within ±0.3% of the theoretical values. NMR spectra were recorded on a JEOL JNM-GX400 (400 MHz) spectrometer. Chemical shifts are expressed in ppm relative to tetramethylsilane. Mass spectra were recorded on a JEOL JMS-DX303 spectrometer.

6-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]-nicotinic Acid (Am80P, 2b) A mixture of 5-methoxycarbonylpicolinic acid (270 mg) and thionyl chloride (1.5 ml) in dry benzene (10 ml) was heated at reflux for 3 h. Solvent and excess thionyl chloride were removed under vacuum, and the residue was dissolved in 5 ml of dry benzene. A mixture of 5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylamine (303 mg) and pyridine (0.5 ml) in dry benzene (5 ml) was added to the solution of the acid chloride, and the whole was stirred for 1.5 h. The reaction mixture was poured into 2 N HCl, extracted with CH_2Cl_2, and dried over Na_2SO_4. After evaporation, the residue was purifed by silica gel flash column chromatography to give methyl 6-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)carbamoyl]nicotinate (**13**, 438 mg, 80%), which was hydrolyzed under basic conditions (NaOH/EtOH) to give Am80P (**2b**).

Am80P (**2b**): Pale yellow needles (AcOEt/*n*-hexane); mp 205.5—207 °C; ^{1}H-NMR ($CDCl_3$) 9.93 (s, 1H), 9.28 (d, J=2 Hz, 1H), 8.57 (dd, J=2, 8 Hz, 1H), 8.43 (d, J=8 Hz, 1H), 7.70 (d, J=2 Hz, 1H), 7.58 (dd, J=2, 8.5 Hz, 1H), 7.34 (d, J=8.5 Hz, 1H), 1.71 (s, 4H), 1.33 (s, 6H), 1.29 (s, 6H); *Anal.* Calcd for $C_{21}H_{24}N_2O_3$: C, 68.09; H, 7.07; N, 7.56. Found: C, 68.07; H, 7.12; N, 7.47.

5-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]picolinic acid (Am80P2, **2c**) was similarly prepared from **12** and 2-methoxycarbonylnicotinic acid.

Am80P2 (**2c**): Pale yellow needles (AcOEt/*n*-hexane); mp 200—201 °C; ^{1}H-NMR ($CDCl_3$) 9.12 (s, 1H), 8.42 (d, J=7.5 Hz, 1H), 8.36 (d, J=8 Hz, 1H), 7.75 (s, 1H), 7.51 (s, 1H), 7.44 (d, J=8.5 Hz, 1H), 7.35 (d, J=8.5 Hz, 1H), 1.70 (s, 4H), 1.30 (s, 12H). *Anal.* Calcd for $C_{21}H_{24}N_2O_3$: C, 68.09; H, 7.07; N, 7.56. Found: C, 67.80; H, 7.12; N, 7.53.

6-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]-nicotinic Acid *N*-Oxide (Am80P3, 2d) *m*CPBA (175 mg) was added to a solution of **13** (65 mg) in 2 ml of $CHCl_3$, and the mixture was stirred for 3 d. The reaction mixture was poured into 1 N $NaHCO_3$, extracted with CH_2Cl_2, and dried over Na_2SO_4. After evaporation, the residue was purified by silica gel flash column chromatography (hexane : AcOEt 5 : 1) to afford methyl 6-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]pyridine-3-carboxylate *N*-oxide (39 mg, 58%), which was hydrolyzed under basic conditions (NaOH/MeOH) to give Am80P3 (**2d**).

Am80P3 (**2d**): Yellow cotton (AcOEt); mp 205.5—207 °C; ^{1}H-NMR ($CDCl_3$) 8.98 (d, J=1.1 Hz, 1H), 8.66 (d, J=8.4 Hz, 1H), 8.13 (dd, J=1.5, 8.4 Hz, 1H), 7.65 (d, J=2.2 Hz, 1H), 7.56 (dd, J=2.2, 8.4 Hz, 1H), 7.33 (d, J=8.6 Hz, 1H), 1.70 (s, 4H), 1.32 (s, 6H), 1.29 (s, 6H). *Anal.* Calcd for $C_{21}H_{24}N_2O_4$: C, 68.46; H, 6.57; N, 7.61. Found: C, 68.45; H, 6.45; N, 7.36.

5-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]picolinic acid *N*-oxide (Am80P4, **2e**) was similarly prepared from **12** and 2-methoxycarbonylnicotinic acid.

Am80P4 (**2e**): White powder (AcOEt); mp 200—201 °C; ^{1}H-NMR ($CDCl_3$) 9.10 (d, J=1.7 Hz, 1H), 8.50 (d, J=8.1 Hz, 1H), 8.33 (dd, J=1.7, 8.2 Hz, 1H), 7.70 (d, J=2.2 Hz, 1H), 7.52 (dd, J=2.4, 8.6 Hz, 1H), 7.38 (d, J=8.6 Hz, 1H), 1.77 (s, 4H), 1.35 (s, 6H), 1.33 (s, 6H). *Anal.* Calcd for $C_{21}H_{24}N_2O_4$: C, 68.46; H, 6.57; N, 7.61. Found: C, 68.31; H, 6.48; N, 7.41.

6-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]nicotinic Acid (Am580P, 5b) A mixture of **15** (464 mg), thionyl chloride (2 ml), and benzene (6 ml) was heated at reflux for 4 h. After removal of the solvent, a mixture of methyl 6-aminonicotinate (304 mg), 4-dimethyl aminopyridine (DMAP) (2 mg), and pyridine (8 ml) was added to the residue, and the whole was stirred overnight. The reaction mixture was poured into 2 N hydrochloric acid, and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and evaporated. The residue was purified by silica gel flash column chromatography (CH_2Cl_2) to give **17** (400 mg, 55%). ^{1}H-NMR (CD_3OD) 8.94 (d, J=2.2 Hz, 1H), 8.71 (s, 1H), 8.48 (d, J=8.8 Hz, 1H), 8.35 (dd, J=2.2, 8.8 Hz, 1H), 7.90 (d, J=2.0 Hz, 1H), 7.64 (dd, J=2.0, 8.2 Hz, 1H), 7.43 (d, J=8.2 Hz, 1H), 3.95 (s, 3H), 1.72 (s, 4H), 1.34 (s, 6H), 1.32 (s, 6H).

A 5% aqueous solution of NaOH (1 ml) was added to a solution of **17** (67 mg) in methanol (5 ml), and the mixture was stirred for 16 h. The reaction mixture was poured into 2 N hydrochloric acid, and extracted with AcOEt. The organic layer was dried over Na_2SO_4, and evaporated. The residue was recrystallized from AcOEt–*n*-hexane to give Am580P (**5b**).

Am580P (**5b**): White powder (AcOEt–*n*-hexane); mp 286 °C; ^{1}H-NMR (DMSO-d_6) 11.08 (s, 1H), 8.89 (s, 1H), 8.33 (d, $J=7.9$ Hz, 1H), 8.05 (d, $J=1.8$ Hz, 1H), 7.78 (d, $J=6.2$ Hz, 1H), 7.46 (d, $J=8.3$ Hz, 1H), 1.68 (s, 4H), 1.32 (s, 6H), 1.28 (s, 6H).

2-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]pyrimidine-5-carboxylic Acid (Am580K, 5c) Sodium (13.8 g) was added to a mixture of benzene (200 ml) and ethanol (27 g) at room temperature. A mixture of ethyl formate (45 g) and ethyl 3-ethoxypropionate (44 g) was added slowly at 0 °C, and the whole was stirred for 2 h. Dimethyl sulfate (76 g) was added to the mixture, and the whole was heated at 50 °C for 3 h. The mixture was filtered, and the filtrate was washed with water three times. Triethylammonium chloride (40 g) and sodium hydroxide (7 g) was added to the organic layer, and stirred for 4 h. The mixture was filtered, and the organic layer was washed with water, and dried over Na$_2$SO$_4$. After evaporation, the residue was distilled under vacuum (105—107 °C at 4 mmHg) to give **19** (a mixture of isomers, 33.1 g, 58%).

A mixture of **19** (21.4 g), urea (5.7 g), and hydrochloric acid (5 ml) in ethanol (300 ml) was heated at reflux overnight. After evaporation, the residue was recrystallized from ethanol to give **20** (7.92 g, 66%) as colorless prisms. ^{1}H-NMR (CDCl$_3$) 7.23 (t, $J=1.1$ Hz, 1H), 4.21 (q, $J=7.3$ Hz, 2H), 4.13 (d, $J=1.1$ Hz, 2H), 1.31 (t, $J=7.3$ Hz, 3H).

A solution of **20** (2.5 g) and bromine (2.4 g) in acetic acid (55 ml) was heated at reflux for 1.5 h. Removal of the solvent afforded crude **21** (3.64 g, 99%). ^{1}H-NMR (DMSO-d_6) 8.74 (s, 2H), 4.27 (q, $J=7.0$ Hz, 2H), 1.29 (t, $J=7.0$ Hz, 3H).

A mixture of **21** (3.64 g), phosphorus oxychloride (25 ml), and *N*,*N*-dimethylaniline (2.5 ml) was heated at reflux for 1.5 h. After removal of the solvent, ice water (10 ml) was added to the residue. The mixture was added to 2 N NaOH (90 ml), and extracted with AcOEt. The organic layer was washed with brine, and dried over Na$_2$SO$_4$. After evaporation, the residue was extracted with *n*-hexane to give crude **22** (1.401 g, 51.5%). ^{1}H-NMR (CDCl$_3$) 9.15 (s, 2H), 4.45 (q, $J=7.3$ Hz, 2H), 1.42 (t, $J=7.3$ Hz, 3H).

22 (1.313 g) was dissolved in NH$_3$-saturated ethanol (10 ml), and the solution was heated at 100 °C in a sealed tube for 1 h. After cooling, the mixture was poured into 2 N aqueous NaOH, and extracted with CH$_2$Cl$_2$. The organic layer was dried over Na$_2$SO$_4$, and evaporated. The residue was purified by silica gel flash column chromatography (CH$_2$Cl$_2$: AcOEt 2 : 1) to give **23** (1.025 g, 87%) as colorless prisms. ^{1}H-NMR (CDCl$_3$) 8.86 (s, 2H), 5.51 (br s, 2H), 4.36 (q, $J=7.0$ Hz, 2H), 1.38 (t, $J=7.0$ Hz, 3H).

A solution of **15** (104 mg) and oxalyl chloride (0.25 ml) in CH$_2$Cl$_2$ (2 ml) was stirred for 1 h. After removal of the solvent, a solution of **23** (75 mg) and DMAP (10 mg) in pyridine (2 ml) was added to the residue, and the mixture was stirred overnight. The reaction mixture was poured into 2 N hydrochloric acid, and extracted with CH$_2$Cl$_2$. The organic layer was dried over Na$_2$SO$_4$, and evaporated. The residue was purified by silica gel flash column chromatography (*n*-hexane : AcOEt 2 : 1) to give **24** (74 mg, 44%). ^{1}H-NMR (CDCl$_3$) 9.20 (s, 2H), 8.83 (br s, 1H), 7.91 (d, $J=1.8$ Hz, 1H), 7.67 (dd, $J=1.8$, 8.1 Hz, 1H), 7.43 (d, $J=8.1$ Hz, 1H), 4.43 (q, $J=7.3$ Hz, 2H), 1.72 (s, 4H), 1.42 (t, $J=7.0$ Hz, 3H), 1.34 (s, 6H), 1.31 (s, 6H).

A 5% aqueous solution of NaOH (0.5 ml) was added to a solution of **24** (74 mg) in ethanol (3 ml), and the mixture was stirred for 2 h. The reaction mixture was poured into 2 N hydrochloric acid, and extracted with CH$_2$Cl$_2$. The organic layer was dried over Na$_2$SO$_4$, and evaporated. The residue was recrystallized from aqueous ethanol to give Am580K (**5c**).

Am580K (**5c**): Colorless prisms (aqueous ethanol); mp 215 °C; ^{1}H-NMR (CD$_3$OD) 9.20 (s, 2H), 8.04 (d, $J=1.8$ Hz, 1H), 7.79 (dd, $J=1.8$, 8.4 Hz, 1H), 7.54 (d, $J=8.4$ Hz, 1H), 1.80 (s, 4H), 1.39 (s, 6H), 1.37 (s, 6H). *Anal.* Calcd for C$_{20}$H$_{23}$N$_3$O$_3$ · 1/2H$_2$O: C, 66.28; H, 6.67; N, 11.59. Found: C, 66.19; H, 6.62; N, 11.55.

5-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]pyrimidine-2-carboxylic Acid (Am580K2, 5d) Phosphorus oxychloride (70 ml) was slowly added to *N*,*N*-dimethylformamide (DMF) (150 ml) at 0 °C, and the mixture was stirred at room temperature for 20 min. Glycine hydrochloride (**25**, 27.88 g) was added to the mixture, and the whole was heated at 80 °C for 4 h, and then at 125 °C for 1 h. After cooling, the reaction mixture was poured into water (225 ml), and cooled at −10 °C. Then 70% perchloric acid (50 ml) was added to the mixture, and the whole was stirred at −10 °C for 2 h. The precipitate was collected, washed with cooled ethanol, and then dissolved in ethanol (300 ml) and triethylamine (20 ml). The mixture was heated at reflux for 1.5 h. Activated carbon was added, and the whole was heated for 30 min. After filtration, the mixture was cooled to give **26** (22.54 g, 75%). ^{1}H-NMR (D$_2$O) 7.36 (s, 1H), 7.01 (s, 2H), 3.21 (s, 12H), 3.05 (s, 3H), 2.95 (s, 3H).

A solution of **26** (19.8 g) and *S*-methylthiourenium sulfate (11.18 g) in ethanol (180 ml) was heated at reflux. A solution of 1 M sodium methoxide in methanol (160 ml) was added dropwise to the mixture, and the whole was heated for 3 h. After removal of solvent, the residue was purified by silica gel flash column chromatography (CH$_2$Cl$_2$) to give **27** (10.44 g, 80%). ^{1}H-NMR (CDCl$_3$) 8.22 (s, 2H), 7.49 (s, 1H), 3.06 (s, 3H), 3.04 (s, 3H), 2.56 (s, 3H).

A mixture of sodium perchlorate (75 ml) and water (75 ml) was added dropwise to a mixture of **27** (10.44 g), and NaHCO$_3$ (1.5 g) in water (15 ml). After 1 h, the reaction mixture was poured into water, and extracted with CH$_2$Cl$_2$. The organic layer was dried over Na$_2$SO$_4$, and evaporated to afford crude **28** (10.53 g, 87%). ^{1}H-NMR (CDCl$_3$) 8.45 (s, 2H), 7.63 (s, 1H), 3.31 (s, 3H), 3.15 (s, 3H), 3.13 (s, 3H).

A solution of **28** (10.53 g) and potassium cyanide (5.56 g) in DMF (140 ml) was heated at reflux for 6 h. After removal of solvent, the residue was dissolved in CH$_2$Cl$_2$, and filtered. The filtrate was washed with saturated NaHCO$_3$, dried over Na$_2$SO$_4$, and evaporated to afford crude **29** (9.0 g, quant). ^{1}H-NMR (CDCl$_3$) 8.40 (s, 2H), 7.65 (s, 1H), 3.16 (s, 3H), 3.12 (s, 3H).

A solution of **29** (3.03 g) in 2 N NaOH was heated at reflux for 6.5 h. Sulfuric acid was added to neutralize the solution, and water was removed under vacuum. The residue was dissolved in methanol (100 ml) and sulfuric acid (2 ml), and heated at reflux overnight. The reaction mixture was poured into saturated NaHCO$_3$, and extracted with CH$_2$Cl$_2$. The organic layer was dried over Na$_2$SO$_4$, and evaporated to give crude **30** (410 mg, 16%). ^{1}H-NMR (DMSO-d_6) 8.24 (s, 2H), 3.98 (s, 3H).

A solution of **15** (98 mg) and oxalyl chloride (0.7 ml) in CH$_2$Cl$_2$ (2 ml) was stirred for 1 h. After removal of the solvent, a solution of **30** (65 mg) and DMAP (60 mg) in pyridine (2 ml) was added to the residue, and the mixture was stirred overnight. The reaction mixture was poured into 2 N hydrochloric acid, and extracted with CH$_2$Cl$_2$. The organic layer was dried over Na$_2$SO$_4$, and evaporated. The residue was purified by silica gel flash column chromatography (*n*-hexane : AcOEt 1 : 1) to give **31** (81 mg, 52%). ^{1}H-NMR (CDCl$_3$) 9.31 (s, 2H), 8.00 (br s, 1H), 7.90 (d, $J=2.2$ Hz, 1H), 7.59 (dd, $J=2.2$, 8.4 Hz, 1H), 7.45 (d, $J=8.1$ Hz, 1H), 4.08 (s, 3H), 1.73 (s, 4H), 1.34 (s, 6H), 1.31 (s, 6H).

A 5% aqueous solution of NaOH (0.5 ml) was added to a solution of **31** (23 mg) in ethanol (3 ml), and the mixture was stirred for 1 h. The reaction mixture was poured into 2 N hydrochloric acid, and extracted with CH$_2$Cl$_2$. The organic layer was dried over Na$_2$SO$_4$, and evaporated. The residue was recrystallized from AcOEt to give Am580K2 (**5d**).

Am580K2 (**5d**): Colorless powder (AcOEt); mp 155 °C; ^{1}H-NMR (CD$_3$OD) 9.42 (s, 2H), 8.05 (d, $J=1.8$ Hz, 1H), 7.80 (dd, $J=1.8$, 8.4 Hz, 1H), 7.55 (d, $J=8.4$ Hz, 1H), 1.80 (s, 4H), 1.40 (s, 6H), 1.37 (s, 6H).

Compounds **6b**—**d** were synthesized by similar methods using 3,5-di-tert-butylbenzoic acid.

Am555P (**6b**): Colorless prisms (MeOH–AcOEt); mp >300 °C; ^{1}H-NMR (DMSO-d_6, 30 °C) 11.27 (s, 1H), 8.89 (d, $J=2.2$ Hz, 1H), 8.34 (d, $J=8.4$ Hz, 1H), 8.31 (dd, $J=2.0$, 8.5 Hz, 1H), 7.87 (d, $J=1.5$ Hz, 1H), 7.63 (br t, 1H), 1.34 (s, 18H). *Anal.* Calcd for C$_{21}$H$_{26}$N$_2$O$_3$: C, 71.16; H, 7.39; N, 7.90. Found: C, 71.19; H, 7.66; N, 7.88.

Am555K (**6c**): Colorless prisms (EtOH); mp 166 °C; ^{1}H-NMR (CD$_3$OD) 9.21 (s, 2H), 7.89 (d, $J=1.8$ Hz, 2H), 7.78 (t, $J=1.8$ Hz, 1H), 1.44 (s, 18H). *Anal.* Calcd for C$_{20}$H$_{25}$N$_3$O$_3$: C, 67.58; H, 7.09; N, 11.83. Found: C, 67.48; H, 7.05; N, 11.71.

Am555K2 (**6d**): Colorless prisms (AcOEt); mp 281 °C; ^{1}H-NMR (CDCl$_3$) 9.43 (s, 2H), 7.92 (d, $J=1.8$ Hz, 2H), 7.78 (t, $J=1.8$ Hz, 1H), 1.44 (s, 18H). *Anal.* Calcd for C$_{20}$H$_{25}$N$_3$O$_3$: C, 67.58; H, 7.09; N, 11.83. Found: C, 67.36; H, 7.00; N, 11.68.

4-[(4,6-Di-*tert*-butylpyrimidin-2-yl)carbamoyl]benzoic Acid (7b) Cyanoguanidine (48 g) was added to methanol (300 ml) saturated with HCl gas at 0 °C. The mixture was stirred at room temperature for 80 min, and cooled in a refrigerator to afford **33** · HCl as a precipitate (70.12 g, 89%). The salt (15 g) was treated with ion exchange resin (250 ml) for 10 min. After removal of solvent, the residue was recrystallized from ethanol to give **33** (7 g, 64%, mp 103—105 °C). ^{1}H-NMR (CD$_3$OD) 6.61 (br s, 4H), 5.47 (br s, 2H), 3.26 (br s, 1H).

A solution of **33** (4.9 g) and 2,2,6,6-tetramethylpentane-3,5-dione (8.71 g) in 80% aqueous ethanol was heated at reflux for 20 h. After cooling, the precipitate was collected, and dissolved in acetone. Filtration and removal of the solvent afforded **34** (6.041 g, 62%). ^{1}H-NMR (DMSO-d_6) 6.58 (s, 1H), 6.13 (s, 2H), 1.22 (s, 18H).

A mixture of **34** (316 mg), terephthalic acid monomethyl ester chloride (360 mg), and DMAP (5 mg) in pyridine (3 ml) was stirred at room temperature for 1 h. The reaction mixture was poured into 2 N hydrochloric acid, and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and evaporated. The residue was purified by silica gel flash column chromatography (*n*-hexane : AcOEt 8 : 1) to give **35** (467 mg, 83%). ^{1}H-NMR (CD_3OD) 8.38 (s, 1H), 8.14 (d, J=8.4 Hz, 2H), 7.94 (d, J=8.4 Hz, 2H), 7.03 (s, 1H), 3.95 (s, 3H), 1.30 (s, 18H).

Lithium chloride (2 g) was added to a solution of **35** (100 mg) in DMF (10 ml), and the mixture was heated at 130 °C for 28 h. The reaction mixture was poured into 2 N hydrochloric acid, and extracted with ether. The organic layer was dried over Na_2SO_4, and evaporated. The residue was recrystallized to give **7b** (84 mg, 87%).

7b: Colorless prisms (AcOEt–CH_3OH–*n*-hexane); mp 227 °C; ^{1}H-NMR (CD_3OD) 10.74 (s, 1H), 8.01 (d, J=8.2 Hz, 2H), 7.93 (d, J=8.4 Hz, 2H), 7.16 (s, 1H), 1.26 (s, 18H).

4-[*N*-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoic Acid (DA010, 8a) Pd(dba)$_3$ (97 mg) and (*R*)-BINAP (158 mg) were added to a mixture of **12** (1.214 g, 5.97 mmol), ethyl 4-iodobenzoate (1.622 g, 5.87 mmol), *tert*-BuONa (600 mg) in dry toluene (60 ml) under an Ar atmosphere, and the mixture was heated at 80 °C for 1 h. After cooling, the reaction mixture was poured into water, and extracted with ether. The organic layer was dried over Na_2SO_4, and evaporated. The residue was purified by silica gel flash column chromatography (AcOEt : *n*-hexane 1 : 19) to afford **37** (1.0 g, 48%). ^{1}H-NMR ($CDCl_3$) 7.91 (d, J=8.8 Hz, 2H), 7.27 (d, J=8.4 Hz, 1H), 7.10 (d, J=2.6 Hz, 1H), 6.96 (dd, J=2.6, 8.8 Hz, 1H), 6.94 (d, J=9.2 Hz, 2H), 4.33 (q, J=7.0 Hz, 2H), 1.69 (s, 4H), 1.37 (t, J=7.3 Hz, 3H), 1.28 (s, 6H), 1.27 (s, 6H).

A 20% aqueous solution of KOH (0.5 ml) was added to a solution of **37** (118 mg) in ethanol (4 ml), and the mixture was stirred for 3 h. The reaction mixture was poured into 1 N hydrochloric acid, and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and evaporated. The residue was recrystallized from AcOEt–*n*-hexane to give DA010 (**8a**, 109 mg, quant).

DA010 (**8a**): Pale colored prisms (AcOEt–*n*-hexane); mp 277 °C; ^{1}H-NMR ($CDCl_3$+DMSO-d_6) 7.89 (dt, J=1.0, 8.8 Hz, 2H), 7.25 (d, J=8.4 Hz, 1H), 7.10 (d, J=2.2 Hz, 1H), 6.98 (dd, J=2.6, 8.4 Hz, 1H), 6.97 (dt, J=1.8, 8.8 Hz, 2H), 6.78 (br s, 1H), 1.69 (s, 4H), 1.28 (s, 6H), 1.27 (s, 6H). *Anal.* Calcd for $C_{21}H_{25}NO_2$: C, 77.98; H, 7.79; N, 4.33. Found: C, 78.02; H, 8.01; N, 4.29.

4-[*N*-Methyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoic Acid (DA011, 8c) A solution of **37** (242 mg) in dry DMF (2 ml) was added to a suspension of NaH (145 mg) in DMF (2 ml), and the mixture was stirred for 20 min. Methyl iodide (1.5 ml) was added to the mixture, and the whole was stirred for 30 min. The reaction mixture was poured into water, and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and evaporated. The residue was purified by silica gel flash column chromatography (AcOEt : *n*-hexane 1 : 10) to afford methyl 4-[*N*-methyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoate (269 mg, quant). Colorless needles (*n*-hexane); mp 131 °C; ^{1}H-NMR ($CDCl_3$) 7.86 (dd, J=2.2, 9.2 Hz, 2H), 7.31 (d, J=8.4 Hz, 1H), 7.13 (d, J=2.6 Hz, 1H), 6.95 (dd, J=2.2, 8.4 Hz, 1H), 6.74 (dd, J=2.2, 9.2 Hz, 2H), 4.32 (q, J=7.0 Hz, 2H), 3.34 (s, 3H), 1.70 (s, 4H), 1.36 (t, J=7.3 Hz, 3H), 1.30 (s, 6H), 1.25 (s, 6H). *Anal.* Calcd for $C_{24}H_{31}NO_2$: C, 78.86; H, 8.55; N, 3.83. Found: C, 78.88; H, 8.68; N, 3.78.

A 20% aqueous solution of KOH (1 ml) was added to a solution of methyl 4-[*N*-methyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]benzoate (367 mg) in ethanol (5 ml), and the mixture was stirred for 5 h. The reaction mixture was poured into 1 N hydrochloric acid, and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and evaporated. The residue was recrystallized from *n*-hexane to give DA011 (**8c**, 335 mg, quant).

DA011 (**8c**): Colorless powders (*n*-hexane); mp 252 °C; ^{1}H-NMR ($CDCl_3$) 7.92 (dd, J=1.8, 9.2 Hz, 2H), 7.35 (d, J=8.4 Hz, 1H), 7.17 (d, J=2.2 Hz, 1H), 6.98 (dd, J=2.6, 8.4 Hz, 1H), 6.76 (dd, J=2.2, 9.2 Hz, 2H), 3.38 (s, 3H), 1.73 (s, 4H), 1.33 (s, 6H), 1.28 (s, 6H). *Anal.* Calcd for $C_{22}H_{27}NO_2$: C, 78.30; H, 8.07; N, 4.15. Found: C, 78.16; H, 8.14; N, 4.16.

Other compounds in the DA series (**8**, **9**) were synthesized similarly.

DA111 (**8d**): Colorless prisms (CH_2Cl_2–*n*-hexane); mp 253—254 °C; ^{1}H-NMR ($CDCl_3$) 7.86 (d, J=9.2 Hz, 2H), 7.19 (s, 1H), 7.04 (s, 1H), 6.48 (d, J=8.4 Hz, 2H), 3.28 (s, 3H), 2.04 (s, 3H), 1.69 (s, 4H), 1.31 (s, 6H), 1.23 (s, 6H). *Anal.* Calcd for $C_{23}H_{29}NO_2$: C, 78.59; H, 8.32; N, 3.99. Found: C, 78.29; H, 8.15; N, 3.89.

DA013 (**8e**): Colorless powder (*n*-hexane); mp 235.5 °C; ^{1}H-NMR ($CDCl_3$) 7.87 (d, J=9.2 Hz, 2H), 7.33 (d, J=8.1 Hz, 1H), 7.11 (d, J=2.2 Hz, 1H), 6.93 (dd, J=2.6, 8.4 Hz, 1H), 6.63 (d, J=9.2 Hz, 2H), 3.63 (t, J=7.7 Hz, 2H), 1.67—1.76 (m, 2H), 1.70 (s, 4H), 1.31 (s, 6H), 1.25 (s, 6H), 0.94 (t, J=7.7 Hz, 3H). *Anal.* Calcd for $C_{24}H_{31}NO_2$: C, 78.86; H, 8.55; N, 3.83. Found: C, 78.64; H, 8.46; N, 3.84.

DA113 (**8f**): Colorless powder (*n*-hexane); mp 245 °C; ^{1}H-NMR ($CDCl_3$) 7.86 (d, J=9.2 Hz, 2H), 7.20 (s, 1H), 7.00 (s, 1H), 6.42 (d, J=8.8 Hz, 2H), 3.52 (br s, 2H), 2.02 (s, 3H), 1.72 (hep, J=7.7 Hz, 2H), 1.69 (s, 4H), 1.31 (s, 6H), 1.23 (s, 6H), 0.95 (t, J=7.7 Hz, 3H). *Anal.* Calcd for $C_{25}H_{33}NO_2$: C, 79.11; H, 8.76; N, 3.69. Found: C, 79.17; H, 8.89; N, 3.64.

DA024 (**8g**): Colorless prisms (*n*-hexane–CH_2Cl_2); mp 245 °C; ^{1}H-NMR ($CDCl_3$) 7.88 (dd, J=1.8, 9.2 Hz, 2H), 7.32 (d, J=8.4 Hz, 1H), 7.16 (d, J=2.6 Hz, 1H), 6.95 (dd, J=2.2, 8.1 Hz, 1H), 6.68 (dd, J=1.8, 9.2 Hz, 2H), 3.56 (d, J=6.6 Hz, 2H), 1.70 (s, 4H), 1.31 (s, 6H), 1.25 (s, 6H), 1.15—1.22 (m, 1H), 0.50 (ddd, J=4.4, 5.9, 8.1 Hz, 2H), 0.14 (q, J=4.8 Hz, 2H). *Anal.* Calcd for $C_{25}H_{31}NO_2$: C, 79.53; H, 8.28; N, 3.71. Found: C, 79.33; H, 8.36; N, 3.82.

DA124 (**8h**): Colorless plates (*n*-hexane–CH_2Cl_2); mp 213 °C; ^{1}H-NMR ($CDCl_3$) 7.88 (d, J=9.2 Hz, 2H), 7.17 (s, 1H), 7.11 (s, 1H), 6.50 (d, J=8.8 Hz, 2H), 3.50 (br s, 2H), 2.03 (s, 3H), 1.69 (s, 4H), 1.30 (s, 6H), 1.24 (s, 6H), 1.22 (m, 1H), 0.51 (ddd, J=4.8, 5.5, 8.1 Hz, 2H), 0.13 (q, J=4.8 Hz, 2H). *Anal.* Calcd for $C_{26}H_{33}NO_2$: C, 79.76; H, 8.49; N, 3.58. Found: C, 79.65; H, 8.53; N, 3.66.

DA212 (**9b**): Colorless prisms (*n*-hexane–CH_2Cl_2); mp 225 °C; ^{1}H-NMR ($CDCl_3$) 7.88 (dd, J=2.2, 9.2 Hz, 2H), 7.33 (t, J=1.8 Hz, 1H), 7.02 (d, J=1.8 Hz, 2H), 6.63 (dd, J=1.8, 8.8 Hz, 2H), 3.80 (q, J=7.0 Hz, 2H), 1.32 (s, 18H), 1.27 (t, J=7.0 Hz, 3H). *Anal.* Calcd for $C_{23}H_{31}NO_2$: C, 78.14; H, 8.84; N, 3.96. Found: C, 78.20; H, 8.91; N, 3.92.

DA213 (**9c**): Colorless prisms (*n*-hexane–CH_2Cl_2); mp 247—248 °C; ^{1}H-NMR ($CDCl_3$) 7.87 (dd, J=2.2, 9.2 Hz, 2H), 7.33 (t, J=1.8 Hz, 1H), 7.03 (d, J=1.8 Hz, 2H), 6.61 (dd, J=1.8, 9.2 Hz, 2H), 3.66 (t, J=7.7 Hz, 2H), 1.74 (hex, J=7.7 Hz, 2H), 1.32 (s, 18H), 0.95 (t, J=7.7 Hz, 3H). *Anal.* Calcd for $C_{24}H_{33}NO_2$: C, 78.43; H, 9.05; N, 3.81. Found: C, 78.55; H, 8.94; N, 3.59.

2-[*N*-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]pyrimidine-5-carboxylic Acid (PA010, 10a) A mixture of **12** (108 mg), **22** (100 mg), and potassium carbonate (400 mg) was heated at 110 °C overnight. The reaction mixture was poured into water, and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and evaporated. The residue was purified by silica gel flash column chromatography to give **39** (91%). Colorless cotton (*n*-hexane–AcOEt); ^{1}H-NMR ($CDCl_3$) 8.95 (s, 2H), 7.56 (br s, 1H), 7.44 (dd, J=2.4, 9.0 Hz, 1H), 7.44 (d, J=2.4 Hz, 1H), 7.31 (d, J=9.2 Hz, 1H), 4.37 (q, J=7.0 Hz, 2H), 1.69 (s, 4H), 1.39 (t, J=7.1 Hz, 3H), 1.30 (s, 6H), 1.28 (s, 6H).

A 20% aqueous solution of KOH (0.5 ml) was added to a solution of **39** (52 mg) in ethanol (3 ml), and the mixture was heated at reflux overnight. The reaction mixture was poured into 1 N hydrochloric acid, and extracted with AcOEt. The organic layer was dried over Na_2SO_4, and evaporated. The residue was recrystallized from *n*-hexane to give PA010 (**10a**, 52 mg, quant).

PA010 (**10a**): Colorless prisms (*n*-hexane–AcOEt); mp >300 °C; ^{1}H-NMR ($CDCl_3$) 9.94 (s, 1H), 8.83 (s, 2H), 7.57 (s, 1H), 7.55 (d, J=4.5 Hz, 1H), 7.26 (d, J=8.4 Hz, 1H), 1.65 (s, 4H), 1.25 (s, 6H), 1.24 (s, 6H). *Anal.* Calcd for $C_{19}H_{23}N_3O_2 \cdot 1/2H_2O$: C, 68.24; H, 7.23; N, 12.57. Found: C, 68.51; H, 7.02; N, 12.59.

2-[*N*-Methyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]pyrimidine-5-carboxylic Acid (PA011, 10c) A solution of **39** (104 mg) in dry DMF (5 ml) was added to a suspension of NaH (40 mg) in DMF (2 ml), and the mixture was stirred for 20 min. Methyl iodide (1.5 ml) was added to the mixture, and the whole was stirred for 3 h. The reaction mixture was poured into water, and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and evaporated. The residue was purified by silica gel flash column chromatography (AcOEt : *n*-hexane 1 : 20) to afford ethyl 2-[*N*-methyl-*N*-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-yl)amino]pyrimidine-5-carboxylate (105 mg, 97%), which was hydrolyzed under basic conditions to afford PA011 (**10c**, quant).

PA011 (**10c**): Colorless needles (EtOH); mp >300 °C; ^{1}H-NMR (DMSO-d_6) 8.75 (s, 2H), 7.34 (d, J=8.4 Hz, 1H), 7.26 (d, J=2.2 Hz, 1H), 7.07 (dd, J=2.2, 8.2 Hz, 1H), 3.49 (s, 3H), 1.67 (s, 4H), 1.27 (s, 6H), 1.24 (s, 6H). *Anal.* Calcd for $C_{20}H_{25}N_3O_2$: C, 70.77; H, 7.42; N, 12.38. Found: C, 70.49; H, 7.43; N, 12.26.

Other compounds in the PA series (**10**, **11**) were synthesized similarly.

PA110 (**10b**): Colorless needles (*n*-hexane–AcOEt); mp 267 °C; ^{1}H-NMR (DMSO-d_6) 9.39 (s, 1H), 8.73 (s, 2H), 7.20 (s, 1H), 7.16 (s, 1H), 2.10 (s, 3H), 1.64 (s, 4H), 1.25 (s, 6H), 1.21 (s, 6H). *Anal.* Calcd for $C_{20}H_{25}N_3O_2$: C, 70.77; H, 7.42; N, 12.38. Found: C, 70.49; H, 7.42; N, 12.27.

PA111 (**10d**): Colorless needles (EtOH); mp >300 °C; ^{1}H-NMR (DMSO-d_6) 8.83 (br s, 1H), 8.68 (br s, 1H), 7.22 (s, 1H), 7.14 (s, 1H), 3.41 (s, 3H), 1.96 (s, 3H), 1.65 (s, 4H), 1.28 (s, 3H), 1.26 (s, 3H), 1.22 (s, 3H), 1.20 (s, 3H). *Anal.* Calcd for $C_{21}H_{27}N_3O_2$: C, 71.36; H, 7.70; N, 11.89. Found: C, 71.26; H, 7.74; N, 11.77.

PA013 (**10e**): Colorless prisms (*n*-hexane–AcOEt); mp 222 °C; ^{1}H-NMR ($CDCl_3$) 8.80 (s, 2H), 7.43 (d, J=8.5 Hz, 1H), 7.26 (d, J=2.3 Hz, 1H), 7.08 (dd, J=2.3, 8.5 Hz, 1H), 3.99 (t, J=7.8 Hz, 2H), 1.74 (s, 4H), 1.66 (hex, J=7.1 Hz, 2H), 1.35 (s, 6H), 1.31 (s, 6H), 0.93 (t, J=7.3 Hz, 3H). *Anal.* Calcd for $C_{22}H_{29}N_3O_2$: C, 71.90; H, 7.95; N, 11.44. Found: C, 71.79; H, 7.99; N, 11.25.

PA113 (**10f**): Colorless needles (*n*-hexane–AcOEt); mp 193 °C; ^{1}H-NMR ($CDCl_3$) 9.01 (s, 1H), 8.84 (s, 1H), 7.21 (s, 1H), 7.0 (s, 1H), 4.09 (m, 1H), 3.64 (m, 1H), 2.07 (s, 3H), 1.71 (hex, J=7.3 Hz, 2H), 1.69 (s, 4H), 1.33 (s, 3H), 1.28 (s, 3H), 1.26 (s, 6H), 0.95 (t, J=7.3 Hz, 3H).

PA024 (**10g**): Colorless needles (*n*-hexane–AcOEt); mp 232 °C; ^{1}H-NMR (DMSO-d_6) 8.73 (s, 2H), 7.36 (d, J=8.4 Hz, 1H), 7.22 (d, J=1.2 Hz, 1H), 7.02 (dd, J=1.2, 8.4 Hz, 1H), 3.84 (d, J=6.8 Hz, 2H), 1.67 (s, 4H), 1.28 (s, 6H), 1.24 (s, 6H), 1.18 (br m, 1H), 0.42 (dd, J=5.1, 13 Hz, 2H), 0.14 (dd, J=5.1, 10 Hz, 2H). *Anal.* Calcd for $C_{23}H_{28}N_3O_2 \cdot 1/4H_2O$: C, 72.13; H, 7.50; N, 10.97. Found: C, 72.37; H, 7.74; N, 10.89.

PA124 (**10h**): Pale yellow prisms (CH_3OH); mp 123 °C; ^{1}H-NMR ($CDCl_3$) 8.96 (s, 1H), 8.83 (s, 1H), 7.18 (s, 1H), 7.11 (s, 1H), 4.10 (dd, J=6.6, 14.1 Hz, 1H), 3.47 (d, J=7.5 Hz, 1H), 2.08 (s, 3H), 1.69 (br s, 2H), 1.68 (br s, 2H), 1.33 (s, 3H), 1.27 (s, 3H), 1.26 (s, 6H), 1.19 (br m, 1H), 0.47 (br m, 2H), 0.22 (br m, 2H). *Anal.* Calcd for $C_{24}H_{31}N_3O_2 \cdot 1/4H_2O$: C, 72.42; H, 7.98; N, 10.56. Found: C, 72.45; H, 7.94; N, 10.35.

PA211 (**11a**): Colorless needles (*n*-hexane–AcOEt); mp 261—263 °C; ^{1}H-NMR (DMSO-d_6) 8.74 (s, 2H), 7.31 (br s, 1H), 7.14 (br s, 2H), 3.51 (s, 3H), 1.29 (s, 18H). *Anal.* Calcd for $C_{20}H_{27}N_3O_2 \cdot 1/3H_2O$: C, 69.13; H, 8.03; N, 12.10. Found: C, 69.19; H, 7.78; N, 11.87.

PA212 (**11b**): Colorless cotton (*n*-hexane–AcOEt); mp 277 °C; ^{1}H-NMR (DMSO-d_6) 8.73 (s, 2H), 7.34 (br s, 1H), 7.06 (br s, 2H), 3.99 (q, J=7.0 Hz, 2H), 1.29 (s, 18H), 1.17 (t, J=7.0 Hz, 3H). *Anal.* Calcd for $C_{21}H_{29}N_3O_2 \cdot 1/4H_2O$: C, 70.07; H, 8.26; N, 11.67. Found: C, 69.92; H, 8.16; N, 11.61.

PA213 (**11c**): Colorless prisms (*n*-hexane–CH_2Cl_2); mp 219 °C; ^{1}H-NMR ($CDCl_3$) 8.87 (s, 2H), 7.39 (t, J=1.8 Hz, 1H), 7.06 (d, J=1.7 Hz, 2H), 3.96 (m, 2H), 1.71 (hex, J=7.5 Hz, 2H), 1.34 (s, 18H), 0.94 (t, J=7.4 Hz, 3H). *Anal.* Calcd for $C_{22}H_{31}N_3O_2 \cdot 1/4H_2O$: C, 70.65; H, 8.49; N, 11.23. Found: C, 70.91; H, 8.42; N, 11.17.

PA224 (**11d**): Colorless powder (*n*-hexane–CH_2Cl_2); mp 194 °C; ^{1}H-NMR ($CDCl_3$) 8.89 (s, 2H), 7.40 (t, J=1.8 Hz, 1H), 7.11 (d, J=1.8 Hz, 2H), 3.89 (d, J=7.0 Hz, 2H), 1.34 (s, 18H), 1.18 (br m, 1H), 0.48 (dd, J=4.6, 13 Hz, 2H), 0.20 (dd, J=5.0, 10.1 Hz, 2H). *Anal.* Calcd for $C_{23}H_{31}N_3O_2 \cdot 1/4H_2O$: C, 71.56; H, 8.22; N, 10.89. Found: C, 71.42; H, 8.18; N, 10.66.

Differentiation-Inducing Assay The human promyelocytic leukemia cell line HL-60[20,21] was provided by Prof. F. Takaku (Faculty of Medicine, University of Tokyo) in 1980, and has been maintained in continuous suspension culture. The cells are cultured in plastic flasks in RPMI1640 medium, supplemented with 5% fetal bovine serum (FBS, not delipidized) and antibiotics (penicillin G and streptomycin), in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Test compounds were dissolved in ethanol at 2 mM and added to the cells, which were seeded at about 8×10^4 cells/ml; the final ethanol concentration was kept below 0.5%. Control cells were given only the same volume of ethanol. Am80 (**2a**), as a positive control, was always assayed at the same time. The cells were incubated for 4 d and stained with Wright–Giemsa in order to check for morphological change. The percentages of differentiated cells were determined by NBT reduction assay as described.[22] Cells were incubated for 20 min at 37 °C in RPMI1640 medium (5% FBS) and an equal volume of phosphate-buffered saline (PBS) containing NBT (0.2%) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 200 ng/ml). The percentage of cells containing blue-black formazan was determined on a minimum of 200 cells. The evaluation of the differentiation from NBT reduction assay was always consistent with the morphological result.

Synergistic activity with Am80 (**2a**) was examined in the presence of a suitable concentration of the test compound according to the method described above. In this experiment, the independent effects of Am80 (**2a**) and the test compound were always assayed, and the percentages of differentiated cells were determined by NBT reduction assay. The assays of test compounds were performed at least twice. The values in figures are the average of the two experiments. IC_{50} values of active compounds were calculated from the NBT reduction assay data by means of the van der Waerden method.[28]

Transactivation Assay Transient transactivation assays were carried out using COS-1 cells transfected with hRAR (α, β or γ) and (TREpal)3-TKLUC or mRXR (α, β or γ) and (DR1)5-pGL-TK, using Am80 (**2a**), retinoic acid (**1a**) or HX630[29] as the activating ligand. The COS-1 cells were obtained from the Japanese Cancer Research Resources Bank (JCRB) and were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO), supplemented with 10% FBS. The reporter plasmid, (TREpal)3-TKLUC contains three copies of the thyroid hormone-responsive palindromic element AGGTCA-TGACCT.[30] The reporter plasmid, (DR1)5-pGL-TK, was constructed by introducing five copies of RXRE, GGTTCA-GAGTTCA, and the herpes simplex virus thymidine kinase promoter into the NheI–Hind III sites of the pGL3-Basic luciferase reporter vector (Promega). For reporter gene assay, COS-1 cells were seeded in 24-well tissue culture plates at 7×10^4 cells per well with assay medium (5% FBS/DMEM). The cells were cultured at 37 °C in 5% CO_2 overnight and allowed to attach to the plates. Then, the medium were removed and transfection was performed by the standard calcium phosphate method. For each well, cells were transfected with 60 ng of receptor-expression plasmid, 240 ng of (TREpal)3-TKLUC or (DR1)5-pGL-TK, 60 ng of the reference plasmid pCMVβ (Clontech), and carrier plasmid pUC18, to adjust the total DNA amount to 600 ng. After 6 h, cells were washed and fresh assay medium was added. Each ligand was added as an EtOH solution (final 0.5% EtOH). After an additional 16 h of incubation, the cells were harvested, and luciferase assay was performed with the Luciferase Assay System (Toyo Ink Mfg Co., Ltd). The luciferase activities were normalized to β-galactosidase activities. Assay was done in triplicate under each condition.

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Superoxide Dismutase Activity of Iron(II)TPEN Complex and Its Derivatives

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Superoxide is involved in the pathogenesis of various diseases, such as inflammation, ischemia-reperfusion injury and carcinogenesis. Superoxide dismutases (SODs) catalyze the disproportionation reaction of superoxide to produce oxygen and hydrogen peroxide, and can protect living cells against the toxicity of free radicals derived from oxygen. Thus, SODs and their functional mimics have potential value as pharmaceuticals. We have previously reported that Fe(II)tetrakis-*N,N,N′,N′*-(2-pyridylmethyl)ethylenediamine (Fe(II)TPEN) has an excellent SOD activity ($IC_{50}=0.5\,\mu M$) among many iron complexes examined (*J. Biol. Chem.*, 264, 9243—9249 (1989)). Fe(II)TPEN can act like native SOD in living cells, and protect *Escherichia coli* cells from free radical toxicity caused by paraquat.

In order to develop more effective SOD functional mimics, we synthesized Fe(II)TPEN derivatives with electron-donating or electron-withdrawing groups at the 4-position of all pyridines of TPEN, and measured the SOD activities and the redox potentials of these complexes. Fe(II) tetrakis-*N,N,N′,N′*-(4-methoxy-2-pyridylmethyl)ethylenediamine (Fe(II)(4MeO)$_4$TPEN) had the highest SOD activity ($IC_{50}=0.1\,\mu M$) among these iron-based SOD mimics. In addition, a good correlation was found between the redox potential and the SOD activity of 15 Fe(II) complexes, including iron-based SOD mimics reported in the previous paper (*J. Organometal. Chem.*, in press). Iron-based SOD mimics may be clinically applicable, because these complexes are generally tissue-permeable and show low toxicity. Therefore our findings should be significant for the development of clinically useful SOD mimics.

Key words superoxide; Fe-TPEN; Fe complex; superoxide dismutase; SOD mimic; reactive species.

Superoxide (O_2^-) is involved in the pathology of many diseases such as inflammation, ischemic injury and carcinogenesis, and is a mediator of reperfusion diseases, such as those following acute myocardial infarct or stroke. It is also associated with the development and progression of inflammatory processes involved in diseases such as arthritis, and plays a major role in the initiation of neurological disorders such as Parkinson's disease. Superoxide dismutases (SODs), which can destroy superoxide very rapidly, protect the organism from this radical burden. Indeed, native SODs have been shown in many studies to have a protective effect in animal models of inflammatory diseases.[1,2] However, there are problems intrinsic to their protein nature (cost, stability, tissue permeability, oral bioavailability, specific tissue targeting, immunogenicity, *etc.*), which make it difficult to use SODs as pharmaceuticals. Since synthetic SOD mimics may overcome these problems, many low-molecular metal complexes, mainly copper and manganese complexes, have been synthesized[3—19] and their SOD activity has been examined *in vitro* and *in vivo*. Several complexes have been reported to have SOD activity *in vivo*.[15,17,20—26] In particular, Fe(II) tetrakis-*N,N,N′,N′*-(2-pyridylmethyl)ethylenediamine (Fe(II)TPEN) can act like native SOD in living cells, and protect *Escherichia coli* cells from free radical toxicity caused by paraquat.[27,28]

Recently, we showed that Fe(II) *N*-benzyl-*N′*-(6-methyl-2-pyridylmethyl)-*N,N′*-bis(2-pyridylmethyl)ethylenediamine (Fe(II)(*N′*6MeBPEN)), Fe(II)*N*-(6-methyl-2-pyridylmethyl)-*N,N′,N′*-tris(2-pyridylmethyl)trimethylenediamine (Fe(II)-(6MeTPTN)) and Fe(II)*N*-methyl-*N,N′,N′*-tris(-2-pyridylmethyl)trimethylenediamine (Fe(II)(*N*MeTPTN)) have lower SOD activity than Fe(II)TPEN and Fe(II) *N*-benzyl-*N,N′,N′*-tris(-2-pyridylmethyl)ethylenediamine (Fe(II)-BPEN).[29] This may be because the redox potentials of the former three complexes are more positive than those of the latter two. This result suggests that the redox potential influences SOD activity.

We synthesized Fe(II)TPEN analogues bearing electron-donating or electron-withdrawing groups at the 4-position of all pyridines of TPEN in order to examine the correlation between their redox potentials and SOD activity.

Materials and Methods

Materials Catalase, cytochrome c (type III), and xanthine sodium salt were purchased from Sigma. Xanthine oxidase was purchased from Boehringer Mannheim GmbH. The other chemicals were all reagent-grade products from Aldrich, Tokyo Kasei Kogyo Ltd., and Wako Ltd.

Assay of SOD Activity SOD activity was measured according to the method of McCord and Fridovich,[30] except for omission of EDTA and inclusion of catalase. The reaction mixture was prepared with 50 mM potassium phosphate buffer (pH 7.4) containing 10 μM cytochrome *c*, 50 μM xanthine and catalase (1200 U/ml). The reaction was started by adding xanthine oxidase at 25 °C, and reduction of cytochrome *c* was monitored at 550 nm. The amount of xanthine oxidase added was adjusted so as to give a rate of $\Delta A_{550\,nm}=0.025$/min in the absence of SOD mimic. The SOD activity (IC_{50}) was determined as the concentration which halves the rate of reduction of cytochrome c.

Cyclic Voltammetry The redox potentials were measured in CH_3CN containing 0.1 M tetraethylammonium perchlorate as the supporting electrolyte under argon. The half wave potentials ($E_{1/2}$) *versus* a saturated calomel electrode (S.C.E.) were determined by cyclic voltammetry employing a platinum electrode.

Synthesis 4-Nitro-2-picoline *N*-Oxide **2**: To 2-picoline *N*-oxide **1** in c. H_2SO_4 28 ml, fum. HNO_3 21 ml was added dropwise at 0 °C. The reaction mixture was heated at 110—115 °C for 2 h, and poured into ice-water after having cooled to room temperature. The solution was basified with K_2CO_3, and the product was extracted with $CHCl_3$. The $CHCl_3$ phase was dried over K_2CO_3 and evaporated to afford **2** (83.9%). ^{1}H-NMR ($CDCl_3$/TMS) 2.58 (s, 3H), 8.01 (dd, 1H, J=7.1, 2.9 Hz), 8.15 (d, 1H, J=2.9 Hz), 8.33 (d, 1H, J=7.1 Hz).

4-Chloro-2-picoline *N*-Oxide **3**: **2** was heated in c. HCl in a sealed tube at

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Dedicated to the memory of Dr. Kyosuke Tsuda.

160 °C for 10 h. The HCl was evaporated, and the residue was neutralized with Na_2CO_3 aqueous solution. The product was extracted with $CHCl_3$. The $CHCl_3$ phase was dried over K_2CO_3 and evaporated. The residue was chromatographed on alumina (CH_2Cl_2) to afford **3** (77.6%). ^{1}H-NMR ($CDCl_3$/TMS) 2.51 (s, 3H), 7.15 (dd, 1H, J=6.9, 2.9 Hz), 7.28 (d, 1H, J=2.9 Hz), 8.18 (d, 1H, J=6.9 Hz).

4-Methoxy-2-picoline *N*-Oxide **4**: NaOMe 1.164 g (29.1 mmol) in MeOH was added dropwise to **2** (4.07 g, 26.5 mmol) in MeOH at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h. The MeOH was evaporated, and the residue was chromatographed on alumina to afford **4** (79.1%). ^{1}H-NMR ($CDCl_3$/TMS) 2.53 (s, 3H), 3.85 (s, 3H), 6.71 (dd, 1H, J=7.1, 3.3 Hz), 6.79 (d, 1H, J=3.3 Hz), 8.19 (d, 1H, J=7.1 Hz).

(4-Chloro-2-pyridyl)methyl Chloride **8**: Compound **3** (1.253 g, 8.73 mmol) was added to acetic anhydride 10 ml preheated at 110—120 °C and stirring was continued at the same temperature for 30 min. EtOH 10 ml was added and refluxed. The solvent was evaporated, and the residue was neutralized with 2 N Na_2CO_3 aqueous solution. The product was extracted with CH_2Cl_2, then the CH_2Cl_2 phase was dried over K_2CO_3 and evaporated. The residue was chromatographed on silica gel (*n*-hexane : CH_2Cl_2=1 : 1) to afford 2-acetoxymethyl-4-chloropyridine **5** (68.4%). Compound **5** (1.108 g, 5.97 mmol) was hydrolyzed in MeOH 15 ml and 1 N NaOH aqueous solution 8 ml at room temperature for 45 min. The MeOH was evaporated, the residue was diluted with H_2O, and the product was extracted with CH_2Cl_2. The CH_2Cl_2 phase was dried over K_2CO_3 and evaporated to afford 4-chloro-2-pyridinemethanol (69.2%). To 4-chloro-2-pyridinemethanol 1.110 g (7.74 mmol) in CH_2Cl_2, thionyl chloride 1.64 g (13.8 mmol) was added dropwise under reflux. The solvent was evaporated, and the residue was basified with 2 N Na_2CO_3 aqueous solution. The product was extracted with CH_2Cl_2. The CH_2Cl_2 phase was dried over K_2CO_3 and evaporated. The residue was chromatographed on silica gel (CH_2Cl_2) to afford **8** (88.8%). ^{1}H-NMR ($CDCl_3$/TMS) 4.65 (s, 2H), 7.25—7.28 (m, 1H), 7.52 (d, 1H, J=1.7 Hz), 8.48 (d, 1H, J=5.3 Hz).

(4-Nitro-2-pyridyl)methyl Chloride **9**: (4-Nitro-2-pyridyl)methyl chloride **9** was prepared analogously to **8**. ^{1}H-NMR ($CDCl_3$/TMS) 4.77 (s, 2H), 7.96 (dd, 1H, J=5.0, 2.0 Hz), 8.24 (d, 1H, J=2.0 Hz), 8.90 (d, 1H, J=5.0 Hz).

(4-Methoxy-2-pyridyl)methyl Chloride **10**: (4-Methoxy-2-pyridyl)methyl chloride **10** was prepared analogously to **8**. ^{1}H-NMR ($CDCl_3$/TMS) 3.88 (s, 3H), 4.63 (s, 2H), 6.76 (dd, 1H, J=5.7, 2.4 Hz), 7.01 (d, 1H, J=2.4 Hz), 8.39 (d, 1H, J=5.7 Hz).

2,4-Lutidine *N*-Oxide **12**: To 2,4-lutidine **11** (3.21 g, 30.0 mml) in $CHCl_3$, *m*CPBA 5.4 g in $CHCl_3$ was added dropwise at room temperature. The reaction mixture was stirred at room temperature for 3 h and Na_2SO_3 2.5 g was added. The reaction mixture was washed with 2 N Na_2CO_3 aqueous solution, dried over K_2CO_3 and evaporated to afford **12** (quant.). ^{1}H-NMR ($CDCl_3$/TMS) 2.32 (s, 3H), 2,50 (s, 3H), 6.95 (dd, 1H, J=6.6, 1.8 Hz), 7.07 (d, 1H, J=1.8 Hz), 8.15 (d, 1H, J=6.6 Hz).

(4-Methyl-2-pyridyl)methyl Chloride **14**: (4-Methyl-2-pyridyl)methyl chloride **14** was prepared analogously to **8**. ^{1}H-NMR ($CDCl_3$/TMS) 2.38 (s, 3H), 4.64 (s, 2H), 7.06 (d, 1H, J=5.0 Hz), 7.29 (d, 1H), 8.43 (d, 1H, J=5.0 Hz).

N,*N*,*N'*,*N'*-Tetrakis(4-methoxy-2-pyridylmethyl)ethylenediamine ($(4MeO)_4$-TPEN): $(4MeO)_4$TPEN was prepared according to Sato *et al.*[31] from **10**. ^{1}H-NMR ($CDCl_3$/TMS) 2.80 (s, 4H), 3.75 (s, 8H), 3.76 (s, 12H), 6.63—6.66 (m, 4H), 7.04 (d, 4H, J=2.4 Hz), 8.29 (d, 4H, J=6.0 Hz); MS 544 (M^+).

N,*N*,*N'*,*N'*-Tetrakis(4-methyl-2-pyridylmethyl)ethylenediamine ($(4Me)_4$-TPEN): $(4Me)_4$TPEN was prepared analogously to $(4MeO)_4$TPEN from **14**. ^{1}H-NMR ($CDCl_3$/TMS) 2.26 (s, 12H), 2.77 (s, 4H), 3.73 (s, 8H), 6.92 (d, 4H, J=5.2 Hz), 7.26 (s, 4H), 8.34 (d, 4H, J=5.2 Hz); MS 480 (M^+).

N,*N*,*N'*,*N'*-Tetrakis(4-chloro-2-pyridylmethyl)ethylenediamine ($(4Cl)_4$-TPEN): $(4Cl)_4$TPEN was prepared analogously to $(4MeO)_4$TPEN from **8**. ^{1}H-NMR ($CDCl_3$/TMS) 2.78 (s, 4H), 3.77 (s, 8H), 7.14—7.17 (m, 4H), 7.47 (d, 4H, J=1.6 Hz), 8.40 (d, 4H, J=5.2 Hz); MS 560 (M^+): 562 (M^++2)=3 : 4, 434 (M^+−ClPyCH_2): 436 (M^+−ClPyCH_2+2): 438 (M^+−ClPyCH_2+4): 440 (M^+−ClPyCH_2+6)=27 : 27 : 9 : 1.

N,*N*,*N'*,*N'*-Tetrakis(4-nitro-2-pyridylmethyl)ethylenediamine ($(4NO_2)_4$TPEN): $(4NO_2)_4$TPEN was prepared analogously to $(4MeO)_4$TPEN from **9**. ^{1}H-NMR ($CDCl_3$/TMS) 2.93 (s, 4H), 4.02 (s, 8H), 7.87—7.90 (m, 4H), 8.18 (d, 4H, J=2.0 Hz), 8.82 (d, 4H, J=5.6 Hz); MS 604 (M^+).

Formation of Ferrous Complexes Ferrous complexes were prepared by reacting equimolar $FeSO_4 \cdot 7H_2O$ (or $Fe(ClO_4)_2 \cdot 6H_2O$) and the chelators in MeOH at room temperature for several minutes, followed by evaporation of the solvent and drying *in vacuo*.

Results and Discussion

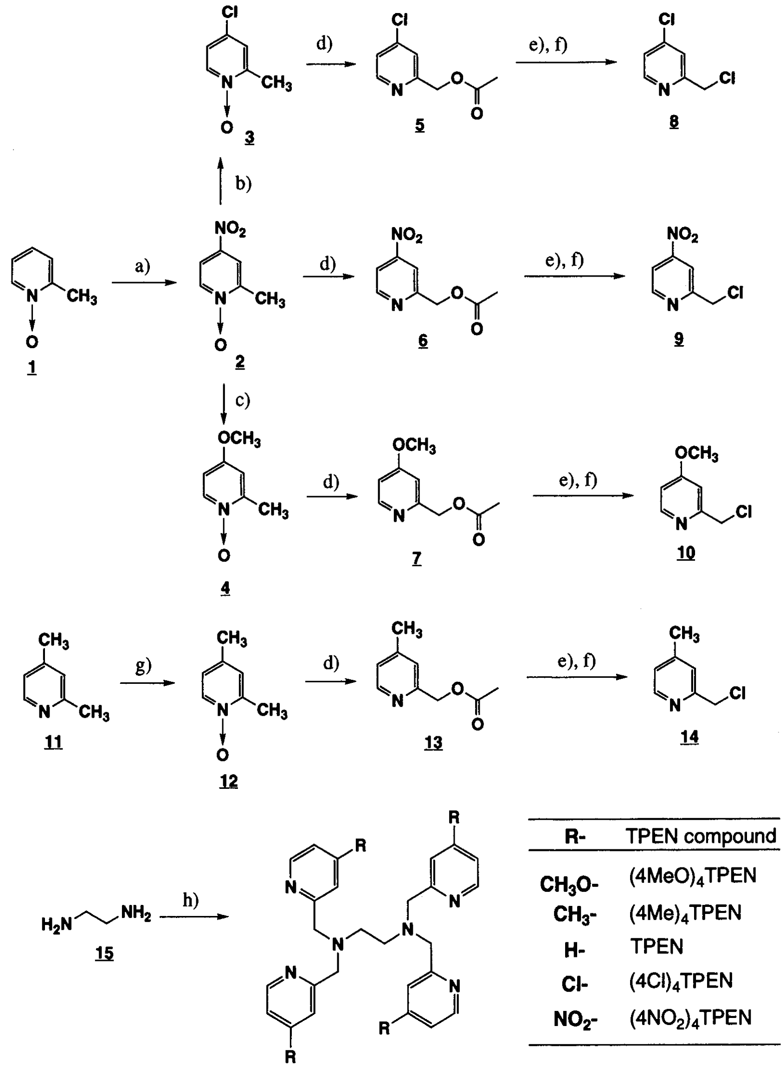
Synthesis of 4-Substituted TPEN We synthesized four 4-substituted TPEN derivatives bearing electron-donating (MeO, Me) or electron-withdrawing (Cl, NO_2) groups. 4-Substituted-2-pyridylmethyl chloride was prepared according to Chart 1. 4-NO_2-2-picoline *N*-oxide **2** was prepared by the nitration of 2-picoline *N*-oxide **1** with fum. HNO_3 and c. H_2SO_4. 4-Cl-2-picoline *N*-oxide **3** was prepared by reacting **2** with c. HCl and 4-MeO-2-picoline *N*-oxide **4** was prepared by reacting **2** with NaOMe. 4-Me-2-picoline *N*-oxide **12** was prepared by oxidizing 2,4-lutidine **11** with *m*CPBA. 4-Substituted-2-pyridylmethyl chloride was prepared according to Ashimori *et al.*[32] by deoxygenation and 2-acetoxymethylation with acetic anhydride, followed by hydrolysis and chlorination of the alcohol. Finally, 4-substituted TPEN was synthesized by reacting ethylenediamine and 4-substituted-2-pyridylmethyl chloride in alkaline aqueous solution.

Fe(II)(4-substituted TPEN) was prepared by reacting equimolar $FeSO_4 \cdot 7H_2O$ (or $Fe(ClO_4)_2 \cdot 6H_2O$) and 4-substituted TPEN in MeOH at room temperature for several minutes, followed by evaporation of the solvent and drying *in vacuo*. These complexes have strong absorption in the visible region and their wavelengths of maximal absorption and molar extinction coefficients are listed in Table 1. We could not measure the spectrum of Fe(II)$(4NO_2)_4$TPEN in potassium phosphate buffer because it did not dissolve.

SOD Activity and Redox Potentials of Fe(II)(4-Substituted TPEN) SOD activities of Fe(II)(4-substituted TPEN) were determined by the xanthine–xanthine oxidase-cytochrome *c* method. Table 2 shows the IC_{50} values of SOD activity. The IC_{50} means the concentration of the complex which inhibits by 50% the reduction rate of cytochrome *c* and exerts SOD activity equivalent to 1 unit of native SOD. Interestingly, all Fe(II)(4-substituted TPEN) except Fe(II)$(4NO_2)_4$TPEN had higher SOD activity than Fe(II)TPEN. The IC_{50} of Fe(II)$(4MeO)_4$TPEN was 0.1 μM, and this complex is the most active Fe-based SOD mimic so far synthesized.

The introduction of electron-donating groups at the 4-position of pyridine was expected to make the redox potentials of Fe(II) complexes less positive. We measured the redox potentials of Fe(II)(4-substituted TPEN) (Table 2). The nature of the substituent groups was reflected in the redox potential: elecron-donating groups made the redox potentials less positive and electron-withdrawing groups made them more positive, as expected. The most active SOD mimic, Fe(II)$(4MeO)_4$TPEN, had the most least-positive redox potential among these complexes.

Correlation between SOD Activity and Redox Potentials To examine the correlation between the redox potential and SOD activity, we plotted the SOD activity of 15 Fe(II) complexes, including the iron-based SOD mimics reported in the previous paper,[29] against the redox potential (Table 3, Fig. 1 and Chart 2). The redox potentials of Fe(II)(4-substituted BPEN) and Fe(II)(NMeTPTN) were used instead of those of Fe(II)(4-substituted TPEN) and Fe(II)(6MeTPTN) because the intermediate of Fe(II)(4-substituted TPEN) and Fe(II)(6MeTPTN) during the reaction with O_2^- are considered to be the structures (Form B in Chart 3) in which one pyridine or 6-methylpyridine is replaced and so are the same as Fe(II)(4-substituted BPEN) and



R-	TPEN compound
CH_3O-	$(4MeO)_4TPEN$
CH_3-	$(4Me)_4TPEN$
H-	TPEN
Cl-	$(4Cl)_4TPEN$
NO_2-	$(4NO_2)_4TPEN$

Chart 1. Synthetic Scheme of 4-Substituted TPEN

a) c. H_2SO_4, fum. HNO_3, 110—120 °C, b) c. HCl, 160 °C, c) NaOMe, r.t., d) Ac_2O, 110—120 °C, e) NaOH, r.t., f) $SOCl_2$, reflux, g) *m*CPBA, h) 8), 9), 10) or 14), NaOH, r.t.

Table 1. Maximal Absoption and Molar Extinction Coefficients of Fe(II) Complexes

Fe(II) complex	λ_{max} (nm)	ε (M^{-1} cm^{-1})
$Fe(II)((4MeO)_4TPEN)$	375	6000
$Fe(II)((4Me)_4TPEN)$	410	9000
Fe(II)TPEN	416	10000
$Fe(II)((4Cl)_4TPEN)$	420	8000
$Fe(II)((4NO_2)_4TPEN)$[a]	—	—

Fe(II) complexes were measured in 50 mM potassium phosphate buffer (pH 7.4). *a*) The spectra of $Fe(II)((4NO_2)_4TPEN)$ was not measured because $Fe(II)((4NO_2)_4TPEN)$ could not be dissolved in 50 mM potassium phosphate buffer (pH 7.4).

Table 2. IC_{50} Values and Redox Potentials of Fe(II) Complexes

Fe(II) complex	IC_{50} (μM)	$E_{1/2}$ (mV) (*vs*. S.C.E.)
$Fe(II)((4MeO)_4TPEN)$	0.1	+580
$Fe(II)((4Me)_4TPEN)$	0.2	+680
Fe(II)TPEN	0.5	+780
$Fe(II)((4Cl)_4TPEN)$	0.25	+910
$Fe(II)((4NO_2)_4TPEN)$	—[a]	+1180

IC_{50} value means the concentration of Fe(II) complex required to inhibit 50% of the reduction rate of cytochrome *c* by superoxide. *a*) SOD activity of $Fe(II)((4NO_2)_4TPEN)$ was not measured because $Fe(II)((4NO_2)_4TPEN)$ could not be dissolved.

Table 3. Apparent Rate Constants and Redox Potentials of 15 Fe(II) Complexes

No.	Fe(II) complex	k_{cat} (M^{-1} s^{-1})	$E_{1/2}$ (mV) (*vs*. S.C.E.)[a]
1	$Fe(II)((4MeO)_4TPEN)$	7.0×10^7	+425[a]
2	$Fe(II)((4Me)_4TPEN)$	3.5×10^7	+510[a]
3	$Fe(II)(N,N'(3Me)_2TPEN)$	8.8×10^6	+520
4	$Fe(II)(N,N'(5Me)_2TPEN)$	1.8×10^7	+540
5	Fe(II)PeTPEN	1.4×10^7	+570
6	Fe(II)BPEN	1.8×10^7	+590
7	Fe(II)TPEN	1.4×10^7	+590[a]
8	Fe(II)(6MeTPEN)	1.8×10^7	+595
9	Fe(II)(*N*MeTPTN)	4.7×10^6	+640
10	Fe(II)(6MeTPTN)	3.5×10^6	+640[a]
11	$Fe(II)(N,N(6Me)_2TPEN)$	2.3×10^6	+640
12	Fe(II)(*N*'6MeBPEN)	2.0×10^6	+640
13	Fe(II)(*N*6MeBPEN)	2.3×10^6	+645
14	$Fe(II)(N,N'(6Me)_2TPEN)$	2.3×10^6	+655
15	$Fe(II)((4Cl)_4TPEN)$	2.8×10^7	+685[a]

a) The redox potentials mean those of the proposed intermediates (Form C in Chart 3). The redox potentials of Fe(II)(4-substituted BPEN) and Fe(II)(NMeTPTN) are used instead of those of corresponding Fe(II)(4-substituted TPEN) and Fe(II)(6MeTPTN) respectively.

Fe(II)(NMeTPTN) (Chart 3). In other words, the first step in the reaction with O_2^- is the conversion of Form A to Form B, as reported in the previous paper.[29]

The SOD activities, IC_{50}s, were converted to the apparent rate constant (k_{cat}) by the use of Eq. (1);

$$k_{cat} = k_{cyt.c} \times \frac{[\text{cyt.}c^{III}]}{2 \times IC_{50}} \qquad (1)$$

$k_{cat} = 1.4 \times 10^6$ $(M^{-1} s^{-1})$[33]

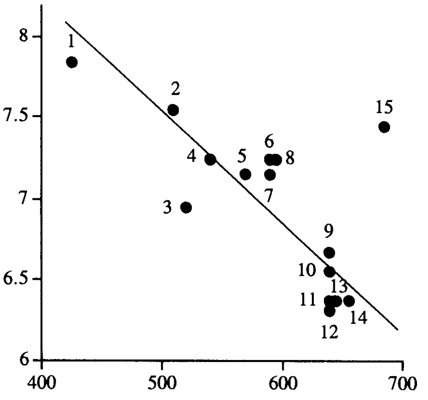


Fig. 1. Correlation between Redox Potential and SOD Activity

The structures of the chelators in the Fe complexes are shown in Chart 2. 1. Fe(II)($(4MeO)_4$TPEN). 2. Fe(II)($(4Me)_4$TPEN). 3. Fe(II)(*N,N*'$(3Me)_2$TPEN). 4. Fe(II)(*N,N*'$(5Me)_2$TPEN). 5. Fe(II)PeTPEN. 6. Fe(II)BPEN. 7. Fe(II)TPEN. 8. Fe(II)(6MeTPEN). 9. Fe(II)(*N*MeTPTN). 10. Fe(II)(6MeTPTN). 11. Fe(II)(*N,N*$(6Me)_2$TPEN). 12. Fe(II)(*N*'6MeBPEN). 13. Fe(II)(*N*6MeBPEN). 14. Fe(II)(*N,N*'$(6Me)_2$TPEN). 15. Fe(II)($(4Cl)_4$TPEN).

Figure 1 shows that SOD activity is correlated with redox potential for Fe(II) complexes. It is suggested that SOD catalyses O_2^- through the redox cycle of the metal center as represented by the Eqs. (2) and (3).[34] These Fe(II) complexes inhibited the reduction cytochrome *c* by O_2^- at the less concentration than O_2^- produced in this assay and therefore it is considered that they quenched O_2^- catalytically through the redox cycle of Fe(II)/Fe(III) like SOD. To take this mechanism into consideration, these data suggests that the oxidation process of Fe(II) by O_2^- (Eq. (2)) is the rate-determing step.

$$\text{Fe(II)} + O_2^- + 2H^+ \rightarrow \text{Fe(III)} + H_2O_2 \qquad (2)$$

$$\text{Fe(III)} + O_2^- \rightarrow \text{Fe(II)} + O_2 \qquad (3)$$

The catalase activity of Mn-complexes has been reported to correlate with redox potential,[35] though there has been no report yet about the correlation between SOD activity and redox potential. Our findings should be helpful in developing more active SOD mimics.

In summary, we have established the relationship between redox potential and SOD activity, and developed the most active iron-based SOD mimic, Fe(II)$(4MeO)_4$TPEN, currently known. Iron-based SOD mimics may have potential for clinical application, because these complexes are generally tissue-permeable and have low toxicity.[27] Further studies on the biological application of these SOD mimics are in progress.

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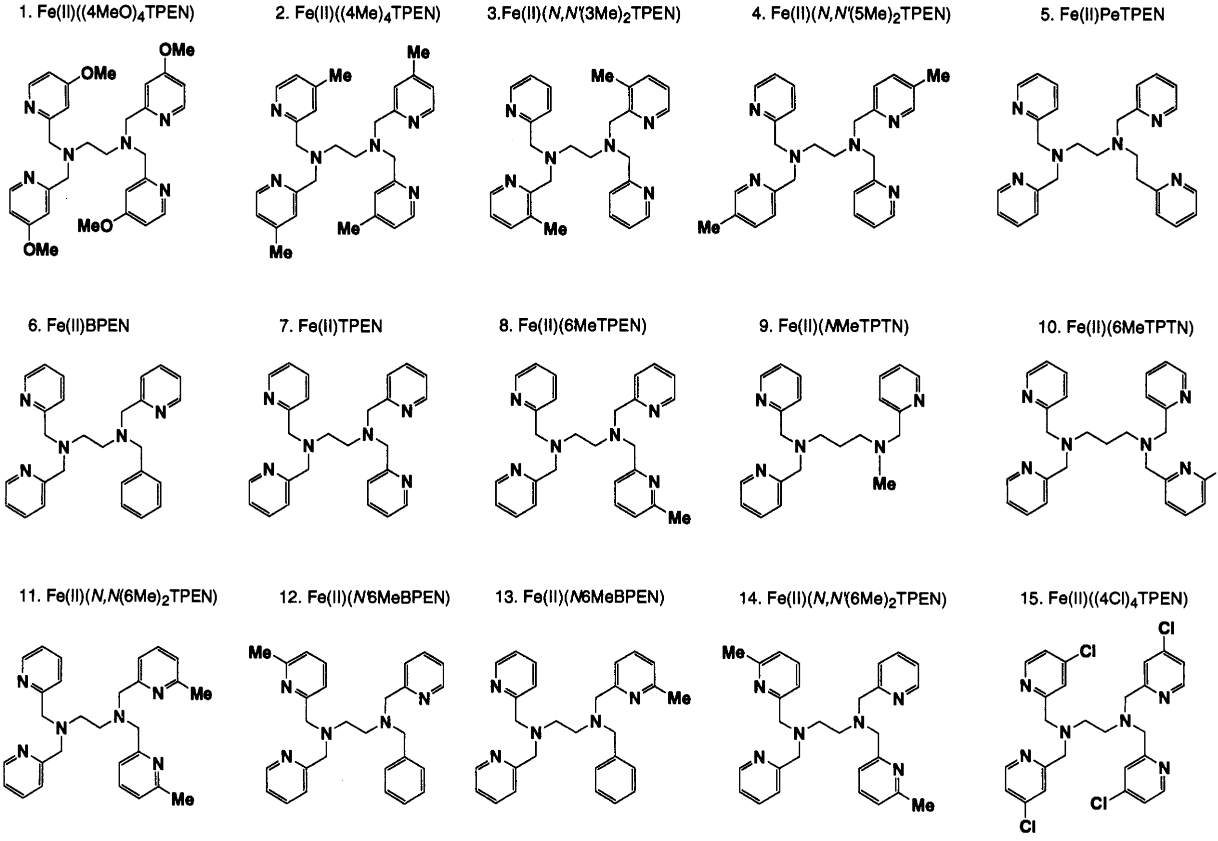
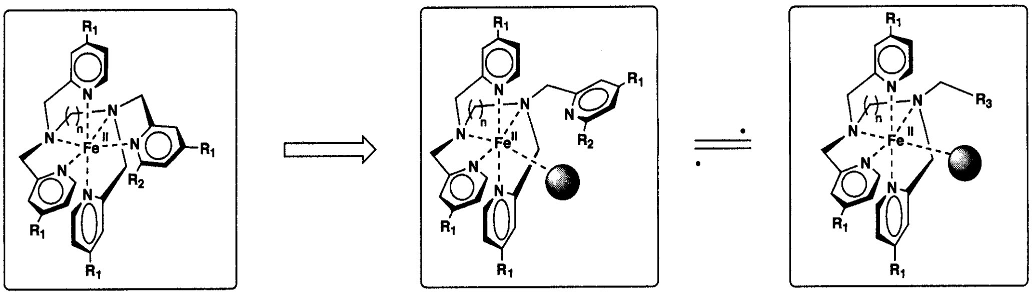


Chart 2. Chelators of Fe Complexes with SOD Activity



Form A **Form B** **Form C**

Chart 3. First Step in the Reaction of Fe Complex with Superoxide

Fe complex (Form A) reacts with superoxide by ligand substitution to produce the intermediate (Form B), which is similar to the structure of Fe complex having five coordinated ligands (Form C).

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Lipase-Catalyzed Asymmetric Desymmetrization of Prochiral 2,2-Disubstituted 1,3-Propanediols Using 1-Ethoxyvinyl Benzoate

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The lipase-catalyzed asymmetric desymmetrization of the prochiral 2,2-disubstituted 1,3-propanediols was studied using various types of 1-ethoxyvinyl esters (1a—i). Although 1a—e with aliphatic acyl groups were not sufficient, use of the benzoate (1f) in combination with *Candida rugosa* lipases converted acyclic diols (2, 6) and cyclic diols (11—14) to the optically active compounds (3f, 7f, 15f—18f), bearing a quaternary carbon center, with moderate-to-high optical yields. These products were fairly stable against racemization under acidic conditions.

Key words lipase; desymmetrization; 1-ethoxyvinyl benzoate; prochiral 2,2-disubstituted 1,3-propanediol

The development of highly enantioselective, catalytic methodology for the construction of optically active quaternary carbon centers has been a challenging subject during this decade.[1] In additon to an increasing number of novel chemical methods, methodologies using biocatalysts have also become significant. The latter include the enzyme-catalyzed asymmetric desymmetrization reactions of molecules having a prochiral quaternary carbon center such as the esterification of 2,2-disubstituted 1,3-propanediols (**I**) (Chart 1),[2] the hydrolysis of 2,2-disubstituted malonates,[1a,3] the hydrolysis of the esters of 2,2-disubstituted 1,3-propanediols,[4] and the reduction of 2,2-disubstituted 1,3-diones.[1a] Among them, the process of Chart 1 is the most attractive in terms of easy operation in organic solvents and simple work up.

However, this approach has rarely been reported due to the low reactivity of sterically congested substrates (**I**) and the easy racemization of products (**II**) through acyl group migration.[2] Although Fadel *et al.* first reported practical enzymatic desymmetrization of **I** using vinyl and isopropenyl esters as an acyl donor, the reaction took several days or more. More seriously, a decrease in the optical purity of **II** (R=Me) was observed in some cases under acidic conditions. During the course of our research on the enzyme-catalyzed transesterification of alcohols with novel acyl donors, 1-ethoxyvinyl esters (**1**),[5] we have briefly presented a solution of these problems using 1-ethoxyvinyl benzoate (**1f**).[6] In this paper, we fully describe the details of our results.

Results and Discussion

In order to overcome the previously discussed problems, namely, low reactivity of the substrate (**I**) and acyl migration of the product (**II**), the following two points were thought to be of significance: 1) improvement of the reactivity of the acyl donors and 2) the choice of a suitable acyl group for both the efficient enzymatic desymmetrization[7] and the inertness to the acyl migration of **II**. When we started this project, we envisioned that use of the 1-ethoxyvinyl esters (**1**) would provide an efficient solution for the following two reasons: First, we had already found that **1** were in some cases more reactive than the vinyl esters and isopropenyl esters.[5a,c] Second, **1** with various acyl groups (COR^1) can be easily prepared by the addition of carboxylic acids (R^{1}COOH) to ethoxyacetylene.[8] We believed that by screening various **1**, we would be able to find such an effective acyl group.

First, we examined the applicability of 1-ethoxyvinyl acetate (**1a**) for the desymmetrization of the diol (**2**) in the presence of various types of lipases in hexane or wet iso-Pr$_2$O (see, General in the Experimental) at 30 °C.[9] The maximum ee of the desired monoacetate (**3a**) was 37% which was obtained using lipase AY (from *Candida rugosa*) in wet iso-Pr$_2$O (run 1 in Table 1). Next, several other ethoxyvinyl esters (**1b**—**e**) possessing different alkyl chain lengths were investigated using lipase AY. Although the reaction was completed in a short period when ethoxyvinyl butyrate (**1c**) was used, the optical purity of the product (**3c**) was not so high (63% ee) (run 3). Other aliphatic acyl donors (**1b, d, e**) were less effective (runs 2, 4, 5).

We then focused our attention on the use of an aroyl reagent, ethoxyvinyl benzoate (**1f**). Although aroyl esters, *e.g.*, vinyl benzoate (**5**), were not familiar acyl donors in enzymatic acylation reactions due to their low reactivity,[10] we were encouraged at the preliminary results, in which **1f** showed several times higher reactivity for lipase AY-catalyzed esterification of 1-octanol than **5** (Fig. 1). In addition, judging from the general tendency for the benzoic acid derivatives to be less reactive for chemical hydrolysis (or alcoholysis) under both acidic and basic conditions than the acetic acid derivatives,[11] the benzoate (**II**, R=Ph) was expected to be less sensitive to the acyl group migration than the acetate (**II**, R=Me).

The reaction of **2** with the benzoate (**1f**), as well as with a few related benzoyl donors (**1g**—**i**), was investigated in the presence of lipase AY (Table 2). Several aspects are noteworthy. 1) The benzoate (**1f**) was the most effective in terms of reactivity and selectivity among all the esters (**1a**—**i**) examined. Substituents on the phenyl ring improved neither reactivity nor enantiotopic selectivity (runs 3—5). 2) A prolonged reaction caused kinetic resolution of the product (**3f**)

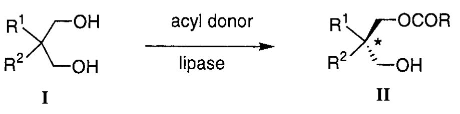

Chart 1

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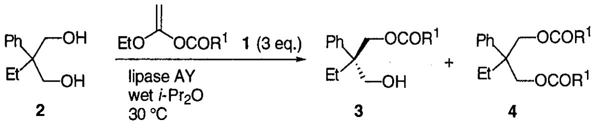
Dedicated to the memory of Dr. Kyosuke Tsuda.

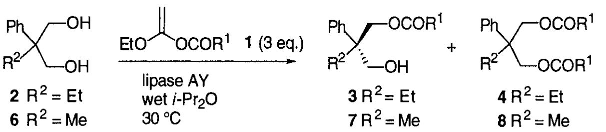
Table 1. Lipase-Catalyzed Desymmetrization of the 1,3-Diol (**2**) Using Aliphatic Esters (**1a**—**e**)



Run	1, R^1	Reaction time	Monoester (3)			Diester (4)	
				Isolated yield (%)[a]	Ee (%)[b]		Isolated yield (%)[a]
1	**1a** Me	4 d	**3a**	66	37	**4a**	—
2	**1b** CH_2Cl	7 d	**3b**	36	1[c]	**4b**	64
3	**1c** *n*-Pr	5 h	**3c**	86	63	**4c**	14
4	**1d** *n*-C_7H_{15}	4 d	**3d**	85	36	**4d**	12
5	**1e** *n*-$C_{11}H_{23}$	21 d	**3e**	78	0[c]	**4e**	—

a) Purified by SiO_2-column chromatography. *b*) Determined by HPLC using a Daicel CHIRALCEL OD column (hexane–iso-PrOH). *c*) Determined after transformation into **9** by a method similar to that shown in Chart 2.

Table 2. Lipase-Catalyzed Desymmetrization of the 1,3-Diols (**2**, **6**) Using Aromatic Esters (**1f**—**i**)



Run	Diol	1, R^1	Reaction time	Monoester (3, 7)			Diester (4, 8)	
					Isolated yield (%)[a]	Ee (%)[b]		Isolated yield (%)[a]
1	**2**	**1f** Ph	6 h	**3f**	90	81	**4f**	—
2	**2**	**1f** Ph	18 h	**3f**	39	91	**4f**	61
3	**2**	**1g** 4-MeO–C_6H_4	8.5 d	**3g**	50	69	**4g**	33
4	**2**	**1h** 4-NO_2–C_6H_4	13 d	**3h**	64	53	**4h**	[c]
5	**2**	**1i** 2,6-diMe–C_6H_3	30 d	**3i**	<5	—	**4i**	—
6	**6**	**1a** Me	8 d	**7a**	46	1	**8a**	*ca.* 10
7	**6**	**1f** Ph	3 d	**7f**	71	84	**8f**	26

a) Purified by SiO_2-column chromatography. *b*) Determined by HPLC using a Daicel CHIRALCEL OD column (hexane–iso-PrOH). *c*) Not determined.

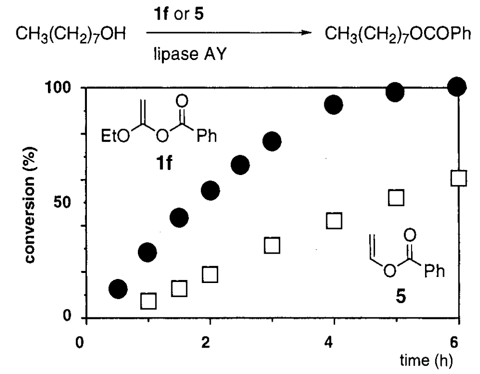


Fig. 1. Time-Course of the Transesterification of 1-Octanol (0.77 mmol) with the Acyl Donor (**1f** or **5**, 2.3 mmol) Catalyzed by Lipase AY (300 mg) in Wet Iso-Pr_2O (20 ml) at 30 °C

The conversion was determined by GLC using a G-100 column.

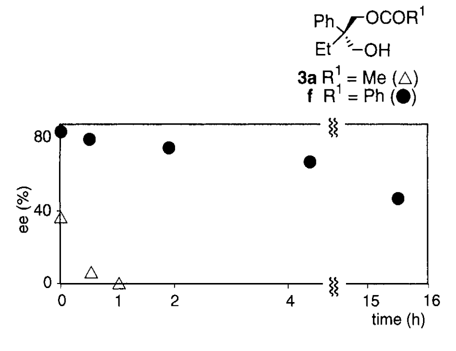


Fig. 2. Racemization of Optically Active **3a, f** in the Presence of CSA (0.1 eq) at Room Temperature

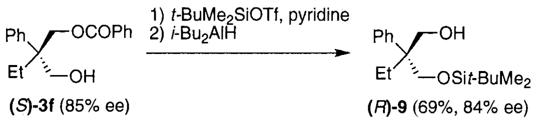


Chart 2

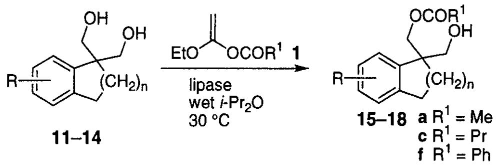
to give a slightly better ee of **3f**, although its yield decreased (run 2). 3) The product (**3f**) was found to be fairly stable against racemization. A comparison of the stability of **3a** and **3f** under acidic conditions [0.1 eq of camphorsulfonic acid (CSA), 4×10^{-4} M in CH_2Cl_2, room temperature] is shown in Fig. 2.[12]

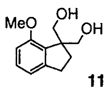
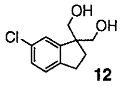
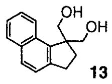
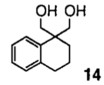
Similarly, the desymmetrization of **6** using **1f** and lipase AY gave the optically active product (**7f**) in 71% yield with 84% ee, while a similar reaction using **1a** was not satisfactory (runs 6, 7).

The transformation of the functional group of **3f** into the silyl ether (**9**) was attained without loss of its optical purity (Chart 2).

The absolute stereochemistry of **3f** was determined to be *S* by its derivation to the known hydroxyacid (**10**) and by com-

Table 3. Lipase-Catalyzed Desymmetrization of the 1,3-Diols (**11**—**14**)



Run	Diol	Lipase	**1**, R^1	Reaction time	Product		
						Isolated yield (%)[a]	Ee (%)[b]
1	 **11**	AY	**1a** Me	25 d	**15a**	41	10
2		AY	**1c** *n*-Pr	25 d	**15c**	37	10
3		AY	**1f** Ph	4 d	**15f**	50	72
4		MY	**1f** Ph	4 d	**15f**	82	71
5		OF	**1f** Ph	4 d	**15f**	67	58
6	 **12**	MY	**1f** Ph	4 d	**16f**	74	74
7	 **13**	MY	**1f** Ph	7 d	**17f**	53	73
8	 **14**	MY	**1f** Ph	7 d	**18f**	74	46

a) Purified by SiO_2-column chromatography. *b*) Determined by HPLC using a Daicel CHIRALCEL OD column (hexane–iso-PrOH).

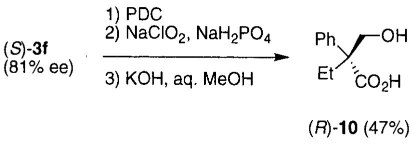


Chart 3

parison of its specific rotation with that of the reported compound (Chart 3).[3a)] On the basis of the similarity of the specific rotation of **7f** to that of **3f**, the absolute stereochemistry of **7f** could be considered to be *S*.

The reagent (**1f**) was also applicable to the more hindered diol (**11**) to give 72% ee of **15f** in 50% isolated yield (Table 3, run 3), while the use of **1a** or **1c** was again insufficient (runs 1, 2). A better result (82% yield, 71% ee) was obtained using a similar lipase MY (from *Candida rugosa*) (run 4). Similarly, the desymmetrization of **12**, **13** and **14** was also attained using **1f** and lipase MY to give the products (**16f**—**18f**) with 74% ee, 73% ee and 46% ee, respectively (runs 6—8).

The product (**15f**) (71% ee) was converted to the carboxylic acid **19** (81% yield). An enantiomerically pure **19** was obtained through the formation of a crystalline salt (**20**) with (*S*)-1-phenylethylamine followed by recrystallization from ethyl acetate (Chart 4). In contrast to the cases of **3f** and **7f**, the absolute stereochemistry of the chiral center of **15f** was disclosed to be *R*, because the X-ray crystallographic analysis of **20** unambiguously showed that the chiral center of **19** was *S* (Fig. 3).

Conclusions

In this study, the use of 1-ethoxyvinyl benzoate (**1f**) provided promising access to efficient enantiotopic differentiation of the prochiral 2,2-disubstituted 1,3-propanediols. The advantages of this method are good-to-high optical yields of the products and their reduced racemization under acidic conditions. These are outstanding results because the successful applications of vinyl benzoate for the enzymatic reactions have been reported in limited cases.[10)]

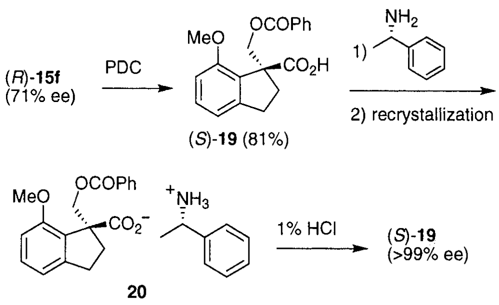


Chart 4

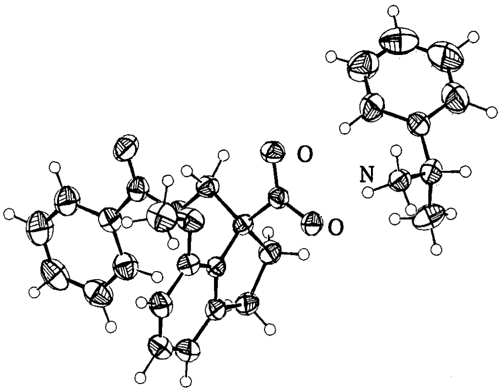


Fig. 3. The X-Ray Crystallographic Structure of the Salt (**20**)

Experimental

General All melting points (mp) and boiling points (bp) are uncorrected. Gas-liquid chromatography (GLC) analyses were carried out using a G-100 column (40 m×1.2 mm, Chemical Inspection and Testing Institute, Japan). ^{1}H-NMR spectra were measured at 200—300 MHz with tetramethylsilane as an internal standard. IR spectra were recorded by diffuse reflectance measurement of samples dispersed in KBr powder or as a $CHCl_3$ solution. Chiral HPLC analyses of the products (**3a—i**, **7a**, **7f**, **9**, **15a**, **15c**, **15f—18f**) were carried out using a Daicel CHIRALCEL OD column (250 mm×4.6 mm, eluent: hexane–iso-PrOH). Column chromatographic purification was performed using Silica gel 60 (70—230 mesh, Merck Co., Ltd.) or Silica gel BW-300 (200—400 mesh, Fuji Silysia Chemical Co., Ltd., Japan). Precoated Silica gel 60 F_{254} plates (E. Merck) were used for preparative TLC. Lipase AY (from *Candida rugosa*) was a gift from Amano Pharmaceutical Co., Ltd., Japan. Lipase MY (from *Candida rugosa*) and OF (from *Candida rugosa*) were gifts from Meito Sangyo Co., Ltd., Japan. Enzymes were dried (1 mmHg, room temperature, overnight) prior to use. Wet iso-Pr_2O was prepared by vigorously stirring a 1000 : 1 mixture of distilled iso-Pr_2O and water for 20—30 min using a magnetic stirrer and decanting the ether layer after settling down. Yields refer to isolated materials of ≥95% purity as determined by ^{1}H-NMR analysis.

Known ethoxyvinyl esters (**1a**, **1b**, **1d**, **1f—i**) were prepared by the reported method.[8] Unknown esters (**1c**, **e**) were similarly prepared.

1-Ethoxyvinyl Butyrate (1c) 81% yield. A colorless oil, bp 80—82 °C (25 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, t, *J*=7.0 Hz), 1.33 (3H, t, *J*=7.0 Hz), 1.71 (2H, sext., *J*=7.5 Hz), 2.41 (2H, t, *J*=7.5 Hz), 3.76 (1H, d, *J*=3.0 Hz), 3.81 (1H, d, *J*=3.0 Hz), 3.87 (2H, q, *J*=7.0 Hz). IR ($CHCl_3$) cm^{-1}: 1765, 1675. *Anal.* Calcd for $C_8H_{14}O_3$: C, 60.74; H, 8.92. Found: C, 60.96; H, 8.84.

1-Ethoxyvinyl Dodecanoate (1e) 77% yield. A pale yellow oil, bp 85—90 °C (0.2 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, *J*=6.5 Hz), 1.20—1.45 (19H, m), 1.66 (2H, quint., *J*=7.0 Hz), 2.41 (2H, t, *J*=7.0 Hz), 3.76 (1H, d, *J*=3.5 Hz), 3.81 (1H, d, *J*=3.5 Hz), 3.87 (2H, q, *J*=7.0 Hz). IR ($CHCl_3$) cm^{-1}: 1765, 1675. *Anal.* Calcd for $C_{16}H_{30}O_3$: C, 71.07; H, 11.18. Found: C, 71.28; H, 11.08.

2,2-Disubstituted 1,3-propanediols (**2**, **6**, **11—14**) were prepared by a standard method using $LiAlH_4$ from the corresponding malonates.[13]

2-Ethyl-2-phenyl-1,3-propanediol (2) Colorless crystals, mp 79—80 °C (ethyl acetate–hexane). ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, t, *J*=7.5 Hz), 1.68 (2H, q, *J*=7.5 Hz), 2.17 (2H, t, *J*=5.5 Hz), 3.94 (2H, dd, *J*=10.5, 5.5 Hz), 4.13 (2H, dd, *J*=10.5, 5.5 Hz), 7.22—7.45 (5H, m). IR (KBr) cm^{-1}: 3460. *Anal.* Calcd for $C_{11}H_{16}O_2$: C, 73.30; H, 8.95. Found: C, 73.18; H, 8.85.

2-Methyl-2-phenyl-1,3-propanediol (6) Colorless crystals, mp 88—89 °C (ethyl acetate–hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.31 (3H, s), 2.00 (2H, t, *J*=6.0 Hz), 3.86 (2H, dd, *J*=10.5, 6.0 Hz), 3.99 (2H, dd, *J*=10.5, 6.0 Hz), 7.24—7.46 (5H, m). IR (KBr) cm^{-1}: 3300. *Anal.* Calcd for $C_{10}H_{14}O_2$: C, 72.26; H, 8.49. Found: C, 72.37; H, 8.40.

1,1-Bis(hydroxymethyl)-7-methoxyindan (11) Colorless crystals, mp 129—130 °C (ethyl acetate–hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.96—2.02 (2H, m), 2.86 (2H, dd, *J*=8.0, 2.0 Hz), 2.90 (2H, t, *J*=8.0 Hz), 3.69 (2H, dd, *J*=11.0, 9.0 Hz), 3.88 (3H, s), 3.93 (2H, dd, *J*=11.0, 4.5 Hz), 6.75 (1H, d, *J*=8.0 Hz), 6.88 (1H, dd, *J*=8.0, 1.0 Hz), 7.22 (1H, t, *J*=8.0 Hz). IR (KBr) cm^{-1}: 3300. *Anal.* Calcd for $C_{12}H_{16}O_3$: C, 69.21; H, 7.74. Found: C, 69.29; H, 7.64.

1,1-Bis(hydroxymethyl)-6-chloroindan (12) Colorless crystals, mp 116—117 °C (ethyl acetate–hexane). ^{1}H-NMR ($CDCl_3$) δ: 2.04 (2H, t, *J*=5.5 Hz), 2.09 (2H, t, *J*=7.5 Hz), 2.91 (2H, t, *J*=7.5 Hz), 3.78 (2H, dd, *J*=11.0, 5.5 Hz), 3.86 (2H, dd, *J*=11.0, 5.5 Hz), 7.16 (1H, d, *J*=8.0 Hz), 7.18 (1H, d, *J*=8.0 Hz), 7.29 (2H, d, *J*=2.0 Hz). IR (KBr) cm^{-1}: 3260. *Anal.* Calcd for $C_{11}H_{13}ClO_2$: C, 62.12; H, 6.16. Found: C, 62.19; H, 6.03.

1,1-Bis(hydroxymethyl)benz[*e*]indan (13) Colorless crystals, mp 104—105 °C (ethyl acetate–hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.89 (2H, t, *J*=6.0 Hz), 2.39 (2H, t, *J*=7.5 Hz), 3.09 (1H, t, *J*=7.5 Hz), 4.07 (2H, dd, *J*=11.0, 6.0 Hz), 4.28 (2H, dd *J*=11.0, 6.0 Hz), 7.37—7.52 (3H, m), 7.74 (1H, d, *J*=8.0 Hz), 7.87 (1H, d, *J*=8.5 Hz), 8.20 (1H, d, *J*=8.0 Hz). IR (KBr) cm^{-1}: 3250. *Anal.* Calcd for $C_{15}H_{16}O_2$: C, 78.92; H, 7.06. Found: C, 78.98; H, 7.10.

1,1-Bis(hydroxymethyl)-1,2,3,4-tetrahydronaphthalene (14) Colorless crystals, mp 114—115 °C (ethyl acetate–hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.57—2.06 (6H, m), 2.79 (2H, t, *J*=6.0 Hz), 3.78 (2H, dd, *J*=11.0, 5.5 Hz), 3.95 (2H, dd, *J*=11.0, 6.0 Hz), 7.11—7.19 (3H, m), 7.34—7.37 (1H, m). IR (KBr) cm^{-1}: 3545. *Anal.* Calcd for $C_{12}H_{16}O_2$: C, 74.97; H, 8.39. Found: C, 75.12; H, 8.28.

Enzymatic Desymmetrization of the 1,3-Diols (2, 6, 11—14): A Typical Procedure To a suspension of **2** (0.55 mmol) and lipase (300 mg) in wet iso-Pr_2O (3.5 ml) was added a solution of **1** (1.65 mmol) in wet iso-Pr_2O (2.0 ml). The reaction mixture was stirred at 30 °C for the time shown in Table 1 and filtered through a Celite pad. The filtrate was concentrated *in vacuo*, and the residue was purified by column chromatography (hexane–ethyl acetate) to give the mono ester (**3**) and the diester (**4**). The isolated yields of the products (**3**, **4**, **7**, **8**, **15—18**) are listed in Tables 1—3.

(*S*)-2-Hydroxymethyl-2-phenyl-1-butyl Benzoate (**3f**): A colorless oil, 91% ee, $[\alpha]_D^{22}$ −28.7° (*c*=1.5, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.76 (3H, t, *J*=8.0 Hz), 1.88 (2H, q, *J*=8.0 Hz), 1.96—2.15 (1H, m), 3.90 (2H, d, *J*=5.0 Hz), 4.73 (2H, br s), 7.25—7.70 (8H, m), 7.99 (2H, d, *J*=7.5 Hz). IR ($CHCl_3$) cm^{-1}: 3490, 1715. *Anal.* Calcd for $C_{18}H_{20}O_3$: C, 76.03; H, 7.09. Found: C, 75.60; H, 7.10.

2-Hydroxymethyl-2-phenyl-1-propyl Benzoate (**7f**): A colorless oil, 84% ee, $[\alpha]_D^{22}$ −9.9° (*c*=1.3, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.46 (3H, s), 2.20 (1H, br s), 3.84 (2H, s), 4.61 (2H, s), 7.30—7.60 (8H, m), 7.99 (2H, d, *J*=7.5 Hz). IR (KBr) cm^{-1}: 3500, 1713. *Anal.* Calcd for $C_{17}H_{18}O_3$: C, 75.53; H, 6.71. Found: C, 75.07; H, 6.82.

(1-Hydroxymethyl-7-methoxyindan-1-yl)methyl Benzoate (**15f**): A colorless gum, 71% ee, $[\alpha]_D^{24}$ −39.9° (*c*=0.4, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 2.00—2.07 (1H, m), 2.18—2.26 (1H, m), 2.80 (1H, dd, *J*=8.0, 5.0 Hz), 2.94—3.01 (2H, m), 3.83 (3H, s), 3.90 (1H, dd, *J*=11.0, 4.5 Hz), 4.02 (1H, dd, *J*=11.0, 8.0 Hz), 4.49 (1H, d, *J*=11.0 Hz), 4.69 (1H, d, *J*=11.0 Hz), 6.74 (1H, d, *J*=8.5 Hz), 6.90 (1H, d, *J*=7.0 Hz), 7.22 (1H, t, *J*=7.5 Hz), 7.42 (2H, t, *J*=7.5 Hz), 7.55 (1H, t, *J*=7.5 Hz), 7.97 (2H, d, *J*=7.5 Hz). IR (KBr) cm^{-1}: 3350, 1720. *Anal.* Calcd for $C_{19}H_{20}O_4$: C, 73.06; H, 6.45. Found: C, 72.79; H, 6.60.

[6-Chloro-1-(hydroxymethyl)indan-1-yl]methyl Benzoate (**16f**): A colorless gum, 74% ee, $[\alpha]_D^{21}$ +11.8° (*c*=1.1, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 2.06—2.23 (2H, m), 2.96 (2H, t, *J*=7.5 Hz), 3.72 (2H, br s), 4.49 (2H, br s), 7.18 (1H, d, *J*=7.5 Hz), 7.22 (1H, dd, *J*=7.5, 1.5 Hz), 7.37 (1H, d, *J*=1.5 Hz), 7.47 (2H, t, *J*=7.5 Hz), 7.60 (1H, tt, *J*=7.5, 1.5 Hz), 8.05 (2H, br d, *J*=7.0 Hz). IR (KBr) cm^{-1}: 3540, 1720. *Anal.* Calcd for $C_{18}H_{17}ClO_3$: C, 68.25; H, 5.41. Found: C, 67.95; H, 5.61.

[1-(Hydroxymethyl)benz[*e*]indan-1-yl]methyl Benzoate (**17f**): A colorless gum, 73% ee, $[\alpha]_D^{21}$ −32.5° (*c*=1.4, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 2.30—2.70 (2H, m), 3.13 (2H, t, *J*=7.5 Hz), 4.11 (1H, d, *J*=12.0 Hz), 4.32 (1H, d, *J*=12.0 Hz), 4.68 (1H, d *J*=11.5 Hz), 4.95 (1H, d, *J*=11.5 Hz), 7.30—7.70 (5H, m), 7.76 (1H, d, *J*=8.0 Hz), 7.87 (1H, d, *J*=8.0 Hz), 7.92 (2H, d, *J*=8.0 Hz), 8.11 (1H, d, *J*=8.0 Hz), 8.18 (1H, d, *J*=8.0 Hz). IR (KBr) cm^{-1}: 3560, 1720. *Anal.* Calcd for $C_{22}H_{20}O_3$: C, 79.50; H, 6.06. Found: C, 79.12; H, 6.30.

(1-Hydroxymethyl-1,2,3,4-tetrahydronaphthalen-1-yl)methyl Benzoate (**18f**): A colorless gum, 46% ee, $[\alpha]_D^{21}$ +11.2° (*c*=1.2, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.78—2.08 (4H, m), 2.83 (2H, t, *J*=6.5 Hz), 3.81 (1H, d, *J*=11.5 Hz), 3.88 (1H, d, *J*=11.5 Hz), 4.49 (1H, d, *J*=11.0 Hz), 4.53 (1H, d, *J*=11.0 Hz), 7.13—7.30 (3H, m), 7.38—7.62 (4H, m), 8.03 (2H, d, *J*=8.5 Hz). IR (KBr) cm^{-1}: 3490, 1720. *Anal.* Calcd for $C_{19}H_{20}O_3$: C, 77.00; H, 6.80. Found: C, 76.64; H, 7.02.

(*R*)-2-(*tert*-Butyldimethylsilyloxy)methyl-2-phenyl-1-butanol (9) Under a nitrogen atmosphere, *tert*-butyldimethylsilyl trifluoromethanesulfonate (*tert*-$BuMe_2SiOTf$) (0.050 ml, 0.22 mmol) was added to an ice-cooled solution of (*S*)-**3f** (85% ee) (25 mg, 0.088 mmol) and pyridine (0.10 ml) in dry *N*,*N*-dimethylformamide (DMF) (0.5 ml). The reaction mixture was stirred at room temperature for 25 min, and diethyl ether and water were added to it. The organic layer was separated, and the aqueous layer was extracted with diethyl ether. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo* to give the crude silyl ether of **3f**. A solution of this crude product in dry CH_2Cl_2 (1 ml) was cooled to −50 °C, to which was added (iso-Bu)$_2$AlH (0.95 M solution in hexane, 0.28 ml, 0.27 mmol) over a period of 2 min. The reaction mixture was stirred at −50 °C for 30 min, and sat. aqueous NH_4Cl was added. The whole mixture was stirred at room temperature for 10 min and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by preparative TLC (hexane–ethyl acetate 6 : 1) to give (*R*)-**9** (19 mg, 69%, 84% ee) as a pale yellow syrup. $[\alpha]_D^{19}$ −3.7° (*c*=1.0, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.05 (3H, s), 0.08 (3H, s), 0.68 (3H, t, *J*=7.5 Hz), 0.85 (9H, s), 1.70—1.90 (2H, m), 2.76—2.81 (1H, m), 3.84—3.93 (2H, m), 4.02—4.07 (2H, m), 7.20—7.36 (5H, m). IR (KBr) cm^{-1}: 3480. *Anal.* Calcd for $C_{17}H_{30}O_2Si$: C, 69.33; H, 10.27. Found: C, 69.88; H, 10.30. HR-FAB-MS *m/z*: 295.2094 $[M+H]^+$ (Calcd for $C_{17}H_{31}O_2Si$: 295.2093).

(*R*)-2-Hydroxymethyl-2-phenylbutanoic Acid (10) A mixture of (*S*)-**3f** (81% ee) (47 mg, 0.17 mmol) and pyridinium dichromate (PDC) (0.19 g, 0.51 mmol) in DMF (1 ml) was stirred at room temperature for 6 h, and diethyl ether and water were added to it. The organic layer was separated, washed with brine, dried with $MgSO_4$, and concentrated *in vacuo* to give the crude aldehyde (47 mg), which was dissolved in *tert*-BuOH (2.0 ml) and water (0.4 ml). NaH_2PO_4 (50 mg, 0.42 mmol), 2-methyl-2-butene (0.10 ml, 0.94 mmol), and $NaClO_2$ (20 mg, 0.17 mmol) were successively added in turn. The reaction mixture was stirred at room temperature for 3 h and concentrated *in vacuo* to about one-fifth of its original volume. The residue was extracted with CH_2Cl_2, and the organic layer was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo* to give the crude carboxylic acid (46 mg). This product was dissolved in MeOH (1 ml), and a solution of KOH (48 mg) in water (1 ml) was added. The reaction mixture was stirred at room temperature for 3 d, and CH_2Cl_2 and 1% aqueous NaOH were added to it. The aqueous layer was separated, and the organic layer was extracted with 1% aqueous NaOH. After the addition of CH_2Cl_2, the combined aqueous layer was made acidic (pH 2—3) by the addition of 10% HCl with vigorous stirring. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by preparative TLC (ethyl acetate) to give (*R*)-**10** (16 mg, 47%) as colorless crystals, mp 75—77 °C (Et_2O–hexane), $[\alpha]_D^{24}$ +12.1° (c=1.1, $CHCl_3$) {lit.[3a] mp 75.3 °C, $[\alpha]_D^{20}$ −16.5° (c=1.0, $CHCl_3$) for 97% ee of (*S*)-form}. ^1H-NMR ($CDCl_3$) δ: 0.94 (3H, t, J=7.5 Hz), 2.17 (2H, q, J=7.5 Hz), 4.03 (1H, d, J=11.5 Hz), 4.13 (1H, d, J=11.5 Hz), 7.20—7.45 (5H, m). IR (KBr) cm^{-1}: 3650—2250, 1705.

(*S*)-1-Benzoyloxymethyl-7-methoxyindan-1-carboxylic Acid (19) (a) By Oxidation of (*R*)-**15f**: To a solution of (*R*)-**15f** (71% ee) (0.92 g, 2.9 mmol) in dry DMF (10 ml) was added PDC (3.9 g, 10 mmol). The mixture was stirred at room temperature for 3 d. Water was added to the reaction mixture, and the product was extracted with diethyl ether. The organic layer was concentrated *in vacuo*. The residue was dissolved in 1% aqueous NaOH and washed with CH_2Cl_2. After the addition of ethyl acetate, the aqueous layer was made acidic (pH 2—3) by the addition of 1% HCl with vigorous stirring. The organic layer was separated, dried over Na_2SO_4, and concentrated *in vacuo* to give (*S*)-**19** (0.78 g, 81%) as a colorless solid. This product was subjected to the formation of a salt with (*S*)-phenylethylamine (*vide infra*).

(b) From the Optically Pure Salt (**20**): To a stirred suspension of optically pure **20** (70 mg, 0.16 mmol) in ethyl acetate (10 ml) was added 1% HCl (5 ml) at 0 °C, and the mixture was stirred at room temperature for 10 min. The organic layer was separated and worked up as usual to give optically pure (*S*)-**19** (51 mg, quant.) as a colorless solid, mp 122—125 °C (ethyl acetate–hexane), $[\alpha]_D^{20}$ −93.6° (c=0.5, MeOH). ^1H-NMR (dimethylsulfoxide-d_6) δ: 2.25—2.65 (2H, m), 2.95—3.10 (2H, m), 3.73 (3H, s), 4.57 (1H, d, J=11.0 Hz), 4.76 (1H, d, J=11.0 Hz), 6.79 (1H, d, J=8.5 Hz), 6.87 (1H, d, J=8.0 Hz), 7.21 (1H, t, J=8.0 Hz), 7.47 (2H, t, J=7.5 Hz), 7.62 (1H, t, J=7.0 Hz), 7.76 (2H, d, J=7.5 Hz), 12.53 (1H, br s). IR (KBr) cm^{-1}: 3500—2500, 1735, 1715, 1700, 1595. *Anal.* Calcd for $C_{19}H_{18}O_5$: C, 69.93; H, 5.56. Found: C, 69.78; H, 5.63.

(*S*)-1-Phenylethylammonium (*S*)-1-Benzoyloxymethyl-7-methoxyindan-1-carboxylate (20) To a solution of (*S*)-**19** (1.8 g, 5.8 mmol) in dry CH_2Cl_2 (18 ml) was added (*S*)-1-phenylethylamine (99% ee) (0.75 ml, 5.8 mmol). After being stirred for 20 min, the reaction mixture was concentrated *in vacuo*. The residual solid was recrystallized from ethyl acetate twice to give the optically pure **20** (1.0 g, 40%) as colorless crystals, mp 165—170 °C (ethyl acetate), $[\alpha]_D^{20}$ −83.0° (c=0.5, MeOH). ^1H-NMR ($CDCl_3$) δ: 1.41 (3H, d, J=7.0 Hz), 2.35—2.45 (1H, m), 2.60—2.75 (1H, m), 3.08 (2H, t, J=7.5 Hz), 3.77 (3H, br s), 4.11 (1H, q, J=7.0 Hz), 4.69 (1H, d, J=11.0 Hz), 4.95 (1H, d, J=11.0 Hz), 6.67 (1H, d, J=7.5 Hz), 6.87 (1H, d, J=7.5 Hz), 7.18—7.39 (9H, m), 7.51 (1H, tt, J=7.5, 1.0 Hz), 7.85 (1H, dd, J=8.5, 1.0 Hz). IR (KBr) cm^{-1}: 3500—2500, 1710, 1560. *Anal.* Calcd for $C_{27}H_{29}NO_5$: C, 72.46; H, 6.53; N, 3.13. Found: C, 72.32; H, 6.55; N, 3.08.

Crystallography of 20 A single crystal (0.30×0.20×0.10 mm) of **20** was obtained by recrystallization from EtOH. Crystal data of **20**: $C_{27}H_{29}NO_5$, M.W.=447, monoclinic, space group $P2_1$, a=14.574(2) Å, b=7.014(2) Å, c=11.641(2) Å, β=102.17(1)°, V=1163.3(4) Å, Z=2, D_{calc}=1.28 g/cm^3, λ(Cu-$K\alpha$)=1.54178 Å, μ=6.28 cm^{-1}, R=0.029 and R_w=0.032 for 2079 reflections. All measurements were carried out with a Mac Science MXC 18 four-circle automated diffractometer with graphite monochromated Cu-$K\alpha$ radiation and an 18 kW rotating anode generator. Cell constants and an orientation matrix for data collection were obtained from a least-squares refinement. The data were collected at 288 K using the ω–2θ scan technique to an above maximum 2θ value of 130°. All intensities were corrected for Lorentz and polarization effects. The structure was solved by direct methods using SHELXS86.[14] The non-hydrogen atoms were refined anisotropically, while only the coordinates of the hydrogen atoms were refined. All calculations were performed using CRYSTAN-G crystallographic software from Mac Science. Tables of fractional atomic coordinates, bond lengths, bond angles, and thermal parameters have been deposited at the Cambridge Crystallographic Data Centre (CCDC-137241).

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Cleavage of S–S Bond by Nitric Oxide (NO) in the Presence of Oxygen: A Disproportionation Reaction of Two Disulfides

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Disulfide bond was cleaved by a catalytic amount of nitric oxide in the presence of oxygen, which was confirmed by experiments employing two symmetrical disulfides. The reaction resulted in the formation of unsymmetrical disulfides in nearly 50% yields. The steric hindrance of alkyl disulfide slowed the reaction rate, and an electron-donating group on the aryl disulfide promoted the reaction. The substituent and *S*-nitrosothiol effects suggested that the reaction was initialized with an oxidative process by NO^+.

Key words disulfide; nitric oxide; disproportionation; nitrogen oxide; oxidation; *S*-nitrosothiol

Nitric oxide (NO)[1] is an important biological messenger that plays a role in physiological and pathological conditions such as endothelium-dependent vasorelaxation and septic shock.[2,3] In the reaction with organic molecules, NO has been recognized to react with various amines[4] and thiols[5] in the presence of oxygen, and the interaction of NO with sulfhydryl group in biomolecules and enzymes has recently gained considerable importance.[6] In addition, one of the NO donors employed frequently is *S*-nitrosothiol, and there are a lot of papers that treat the decomposition of this compound.[7] In the course of the cleavage of *S*-nitrosothiol to release NO, the remaining thiyl group was converted to disulfide,[8] and a recent paper showed that *S*-nitrosothiols reacted with thiol groups in protein molecules to afford mixed disulfide compounds.[9] Thus, disulfide and NO have a close relationship in the biological system, but their interaction has never been reported thus far. We have recently been investigating the chemical reactivity of NO,[10] and found that NO and O_2 reacted with disulfide to cleave the disulfide bond to form an unsymmetrical disulfide when two symmetric disulfides were mixed. The results suggest that the reaction has potential to participate in the biological systems. This paper describes detailed results of the reaction.[11]

Results and Discussion

Two symmetrical disulfides brought about the disproportionation reaction to form an unsymmetrical disulfide in yields up to 50% in the presence of a catalytic amount of NO and the results are summarized in Chart 1 and Table 1. It was found that the reaction must be performed in the dark; without a shield, the reaction rate became faster, and unreproducible. These facts indicated participation of photosensitive intermediate(s) in the reaction.

In most cases, the reaction converged on the product ratio of 1 : 2 : 1 as shown in Chart 1. When di(*tert*-butyl) disulfide was used, the exchange reaction was not observed (entries 4, 9), and diphenyl disulfide reacted fairly slowly. These results suggested that the reaction was considerably influenced by steric hindrance. The presence of a hydroxyl group in the substrate slowed the reaction (entry 13), but a carboxyl group did not affect the rate (entries 14, 15). Nitrogen oxides are known to react with hydroxyl groups to give stable esters, thus the active species was assumed to be trapped in the case of entry 13.

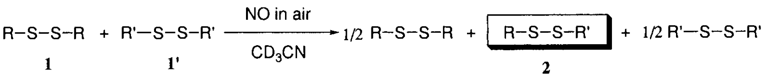
Next, the reaction was carried out in the presence of various amounts of NO and O_2 (or NO_2) using diethyl and dibenzyl disulfides (Chart 2, Table 2).

The reaction proceeded by catalytic amounts of NO in air (Table 2, entries 2—4), but not in the absence of NO (entry 1) and/or O_2 (entry 5). Thus, both gases were found to be necessary for the reaction. Since it is well-known that NO and O_2 react rapidly to form active nitrogen oxides such as N_2O_3 and NO_2 (N_2O_4),[12] the above results indicated the participation of these species in the process. Among nitrogen oxides, dinitrogen trioxide N_2O_3 is formed by the reaction of NO and NO_2. Thus a catalytic amount of NO_2 (0.0005 eq to

Table 1. Formation of Unsymmetrical Disulfide by the Reaction of Two Symmetrical Disulfides in the Presence of a Catalytic Amount of Nitric Oxide in Air

Entry	R	R′	Reaction time (h)	Yield of **2** (%)[a]
1	Me	Et	1	49
2	Me	Bu	1	40
3	Me	*sec*-Bu	1	47
4	Me	*tert*-Bu	5	0
5	Me	Bn	1	49
6	Me	Ph	1 (3)	2 (50)
7	Me	*p*-MePh	1 (3)	18 (50)
8	Me	*p*-MeOPh	1	48
9	Et	*tert*-Bu	5	0
10	Et	Bn	1 (5)	12 (47)
11	Et	Ph	1 (5)	0 (8)
12	Bu	Bn	1 (5)	2 (17)
13	Me	$-CH_2CH_2OH$	5	35
14	Me	$-CH_2CO_2H$	1	50
15	Me	$-CH_2CH(NHCbz)CO_2H$	1	49

a) The yield was estimated based on the stoichiometry shown in Chart 1.



$$\underset{\mathbf{1}}{R\text{–}S\text{–}S\text{–}R} + \underset{\mathbf{1'}}{R'\text{–}S\text{–}S\text{–}R'} \xrightarrow[CD_3CN]{\text{NO in air}} 1/2\ R\text{–}S\text{–}S\text{–}R + \underset{\mathbf{2}}{\boxed{R\text{–}S\text{–}S\text{–}R'}} + 1/2\ R'\text{–}S\text{–}S\text{–}R'$$

Chart 1

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Dedicated to the memory of Dr. Kyosuke Tsuda.

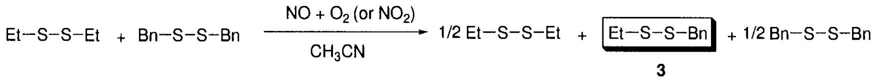


Chart 2

Table 2. The Effect of NO and O_2 (or NO_2) on the Reaction of Diethyl and Dibenzyl Disulfides

Entry	Amount of NO (eq)	Amount of O_2 (or NO_2) (eq)	Yield of **3** (%)[a] Reaction time (h) 0.5	1	3	5
1	0	In air (*ca.*0.18)	0	0	0	0
2	0.0025	In air (*ca.*0.18)	3	5	10	18
3	0.005	In air (*ca.*0.18)	2	13	44	47
4	0.01	In air (*ca.*0.18)	37	47	47	47
5	0.005	0	0	0	0	0
6	0.005	0.005	1	2	21	36
7	0.005	0.05	2	12	40	50
8	0.005	NO_2 0.0005	0	2	28	43
9	0.05	NO_2 0.0005	0	3	27	41
10	0	NO_2 0.0005	0	1	4	5
11	0	NO_2 0.005	17	30	47	49

a) The yield was estimated based on the stoichiometry shown in Chart 2.

Table 3. The Solvent Effect on the Reaction of Diethyl and Dibenzyl Disulfides

Entry	Solvent	NO (eq)	Yield of **3** (%)[a] Reaction time (h) 1	3	5	24
1	CH_3CN	0.005	12	42	48	48
2	CH_3CH_2CN	0.005	0	0	0	0
3	$CH_3CH_2CH_2CN$	0.005	0	0	0	0
4	$(CH_3)_3CCN$	0.005	2	4	8	30
5	$ClCH_2CH_2Cl$	0.005	50	50	49	—
6	$BrCH_2CH_2Br$	0.005	5	15	20	—
7	CH_2Cl_2	0.05	2	13	38	—
8	CH_2Br_2	0.05	50	50	48	—
9	CH_3CN+H_2O (0.05 eq)	0.005	8	41	49	48
10	CH_3CN+H_2O (0.5eq)	0.005	2	16	31	46
11	CH_3CN+H_2O (5 eq)	0.005	0	0	0	39[b]

a) The yield was estimated based on the stoichiometry shown in Chart 2. *b*) 72 h.

the total amount of disulfides) was added to the reaction mixture of two disulfides (1.0 eq of each) and NO (0.005 eq), and the disproportionation reaction proceeded in spite of the absence of O_2 (entry 8). In the presence of the same amount of NO_2 (0.0005 eq), the increase of NO by 10 times (from 0.005 to 0.05) did not affect the reaction yields (entries 8, 9). These facts suggest that N_2O_3 is the active species, and NO_2 is completely transformed to N_2O_3 under these conditions. As another possible active species, NO_2 was applied to the reaction (entries 10, 11), and it was found that 0.005 eq of NO_2 afforded a result between those of entries 3 and 4. Thus, NO_2 (probably in the form of N_2O_4) was also reactive toward disulfides.

In order to obtain information about the reaction mechanism, the solvent effect was investigated, and the results are summarized in Table 3.

Acetonitrile and 1,2-dichloroethane were found to be the best solvents for the reaction (Table 3, entries 1, 5).[13] Among the nitriles, only acetonitrile and pivalonitrile yielded **3** (entries 1—4). The α-hydrogens of the nitrile group in propionitrile or butyronitrle were assumed to be abstracted more readily than those of acetonitrile, thus we speculate that the active species in the reaction medium might be quenched by the reaction with the α-hydrogens in propionitrile or butyronitrle. Addition of water to the reaction fairly slowed the reaction (entries 9—11), because the amount of the active species was catalytic, and N_2O_3 and NO_2 are rapidly hydrolyzed by water. In other solvents such as methanol, dimethylformamide (DMF) ether, and tetrahydrofuran (THF) the reaction did not proceed within 5 h.

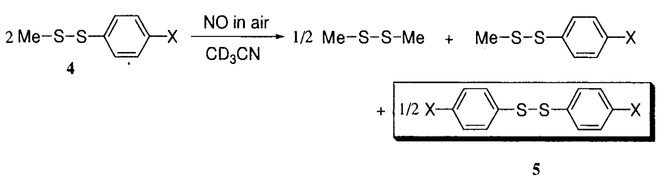


Chart 3

Table 4. Substituent Effects on the Reaction of Aryl Methyl Disulfides with a Catalytic Amount of Nitric Oxide in Air.

Entry	X	Yield of **5** (%)[a] Reaction time (min) 10	30	60
1	H	0	39	47
2	Me	26	50	—
3	Cl	0	8	35

a) The yield was estimated based on the stoichiometry shown in Chart 3.

Next, unsymmetrical aryl methyl disulfides were employed for the reaction (Chart 3, Table 4).

The data shown in Table 4 suggest that electron rich substrates reacted faster than those having an electron-withdrawing group. Thus, the reaction was supposed to proceed *via* an oxidative step. It was shown that superoxide,[14] which is also a ubiquitous biological radical, reacted with disulfide in a reductive manner,[15] thus nitrogen oxides are suggested to have the opposite reactivity toward disulfides.

There have been a few reports concerning the reaction of disulfides with nitrogen oxides, and Oae *et al.* reported the stoichiometrical reactions of N_2O_4 with disulfides.[16] It was suggested that the reaction was initialized by *S*-oxygenation to form thiosulfinate **6**, which was nitrosated by N_2O_4 to give *S*-nitrosothiol **7**. And *S*-nitrosothiol thus obtained was further decomposed by N_2O_4 to disulfides, thiosulfonates, or sulfonates depending upon the reaction conditions (Chart 4).[17]

In our reaction system, it should be possible that an intermediary radical species formed in the above scheme is responsible for the radical chain reaction to obtain the mixture of three disulfides.

When diethyl disulfide was allowed to react with NO (0.1 eq) in air, the ^{1}H-NMR of the mixture showed two major products (product **A**, **B**) other than the starting material. After 3 d, the measurement of the same sample showed that the peaks of the product **A** disappeared and those of **B** and diethyl disulfide increased instead. There have been several reports that thiosulfinates were unstable and readily decomposed to thiosulfonate and disulfide,[18] thus we assumed that the spectral change was derived from the disproportionation of two thiosulfinates into a thiosulfonate and a disulfide (Chart 5). During the disproportionation, there might be radical species that catalyze the cleavage of disulfides.

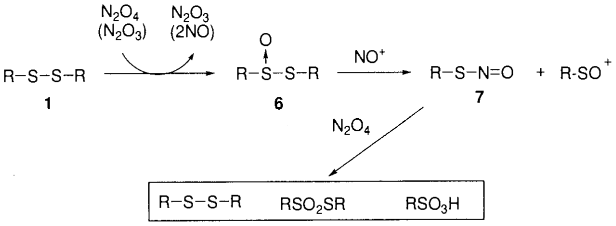


Chart 4

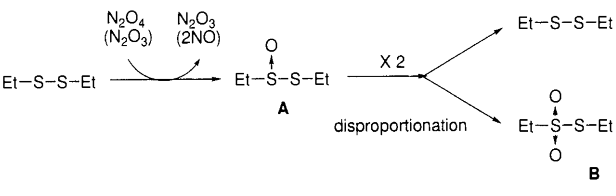


Chart 5

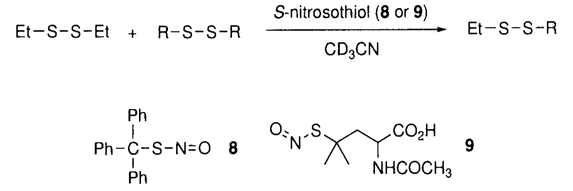


Chart 6

Table 5. Formation of Unsymmetrical Disulfide by the Reaction of Two Symmetrical Disulfides in the Presence of a Catalytic Amount of *S*-Nitrosothiol

Entry	*S*-Nitrosothiol (eq to the substrate)	Reaction time (h)	Conditions	Yield of Et–S–S–R (%)
1	**8** (0.005)	24	In air	47[a]
2	**8** (0.0005)	24	In air	47[a]
3	**8** (0.0005)	24	Under Ar	36[a]
4	**9** (0.0005)	24	In air	10[a]
5	**9** (0.0005)	24	Under Ar	10[a]
6	**9** (0.005)	5	In air	48[a]
		24		42[a]
7	**9** (0.005)	5	Under Ar	5[a]
		24		47[a]
8	**9** (0.005)	16	Under Ar	46[a]
9	**9** (0.005)	16	Under Ar	16[b]

a) The yield of benzyl ethyl disulfide. *b*) The yield of ethyl phenyl disulfide.

The participation of *S*-nitrosothiol in the reaciton was examined by the use of triphenylmethylthionitrite (**8**) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) (**9**) as reagents, whose results are summarized in Chart 6 and Table 5. The reaction proceeded in the presence of a catalytic amount of *S*-nitrosothiol, and the presence of air accelerated the reaction progress (entries 2, 3, entries 6, 7). The results suggested that *S*-nitrosothiol enhanced the reaction progress by both pathways which did involve N_2O_3 (or N_2O_4) and did not. Accordingly, it was supposed that *S*-nitrosothiol acted as both NO donor and thiyl radical donor to catalyze the reaction.

In addition, when a catalytic amount of nitrosonium tetrafluoroborate (0.00025 eq to the total amount of disulfides) was used under Ar, the reaction proceeded in 42% yield after 5 h, and in the case of 0.005 eq, the reaction was complete within 1 h (Chart 7). These results suggest that the step of *S*-oxygenation is not absolutely necessary for the disproportionation reaction.

The UV–vis spectrum of *S*-butyl thionitrite was observed when dibutyl disulfide, NO (2 eq), and O_2 (0.2 eq) were allowed to react in acetonitrile.[19] Consequently, there is a possible pathway that the S–S bond was cleaved by the direct attack of NO⁺ without *S*-oxidation to afford *S*-nitrosothiol **7** and sulfenium cation **10** (Chart 8). *S*-Nitrosothiol thus formed might be decomposed to NO and a thiyl radical, which would attack another disulfide molecule to give unsymmetrical disulfide and a new thiyl radical, which reiterated the reaction to the statistically most probable 1 : 2 : 1 mixture of three disulfides. Moreover, there is a recent paper that claimed a disulfide bond was cleaved by the attack of sulfenium cation to bring about the disproportionation reaction.[20] Consequently, if the reaction proceeds *via* the pathway shown in Chart 8, both of the two intermediates will take part in the disproportionation reaction.

In this paper, we described a new disproportionation reaction of disulfide by nitric oxide in the presence of oxygen. The reaction proceeded in a catalytic fashion, and this process might be of importance from the physiological point of view, since disulfide and NO are closely related to each other in biological system. In addition, it was suggested that the S–S bond was cleaved by the direct attack of nitrosation reagents to form *S*-nitrtosothiol. Although the reaction be-

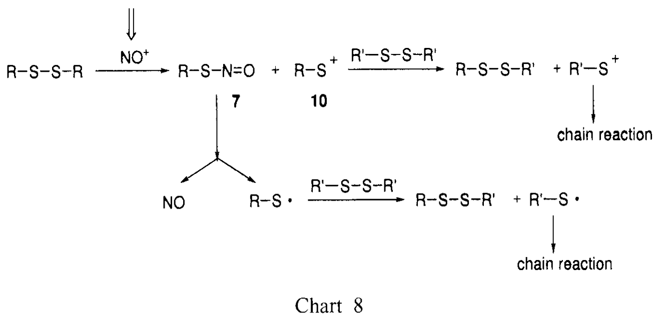
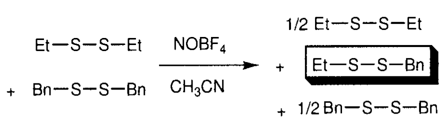


Chart 8



$NOBF_4$	Reaction Time	Yield (%)
0.00025 equiv	1 h	31
0.00025 equiv	5 h	42
0.005 equiv	1 h	48

Chart 7

came fairly slow in the presence of water, it might progress in lipophilic sites or under the situation of continuous generation of NO.

Experimental

Melting points were measured with Büchi535 micromelting point apparatus and are uncorrected. The NMR spectra were measured with JEOL GX400 and LA500 spectrometers using tetramethylsilane as an internal standard. The abbreviations used are as follows: s, singlet; d, doublet; dd, double doublet; ddd, double double doublet; t, triplet; q, quartet; sext, sextet; m, multiplet. High performance liquid chromatography (HPLC) analysis was carried out using JASCO UVIDEC-100-V TRIROTAR-V system.

Materials Nitric oxide gas (99.9%) was purchased from Takachiho Chemical Company Ltd, and was passed through 10 M NaOH aqueous solution and a column of 4—8 mesh soda lime to remove NOx impurities.

Oily disulfides (dimethyl disulfide, diethyl disulfide, dibutyl disulfide, di-*sec*-butyl disulfide, di-*tert*-butyl disulfide) were purchased from Wako Chemicals Co. and used as received. 2,2′-Dithiodiethanol and dithioacetic acid were purchased form Tokyo Kasei Kogyo Co. and used as received. Diphenyl disulfide, di(*p*-tolyl) disulfide and dibenzyl disulfide were recrystallized until analytically pure.

Synthesis of Bis(*p*-methoxyphenyl) Disulfide and Bis(*p*-chlorophenyl) Disulfide The corresponding thiol was oxidized by I_2 and a catalytic amount of KI in acetonitrile at room temperature until the thiol was entirely consumed. Bis(*p*-methoxyphenyl) disulfide was purified by recrystallization from hexane after the evaporation of the reaction solvent (yellow plates; mp 39—39.5°C (lit.[21]) mp 42—44 °C)). Bis(*p*-chlorophenyl) disulfide was purified by silica gel column chromatography (hexane) and recrystallization from hexane (pale yellow plates; mp 70.5 °C (lit.[22]) mp 70.7—71.9 °C)).

Synthesis of Unsymmetrical Disulfides Diaryl disulfide and excess amount of dimethyl disulfide were dissolved in acetonitrile or 1,2-dichloroethane, and the vessel was sealed with a septum. NO gas (up to 0.1 eq to diaryl disulfide) was added and the mixture was allowed to react for several hours at room temperature. In the case of bis(*p*-chlorophenyl) disulfide, KO_2 was used for the reaction according to the reported procedure.[15]) Methyl *p*-tolyl disulfide[23]) and *p*-chlorophenyl methyl disulfide were purified by distillation *in vacuo*. Methyl phenyl disulfide[23]) was purified by silica gel column chromatography (hexane).

Other chemicals were of analytical grade and were used as received.

Reaction of Two Disulfides in the Presence of Catalytic Amounts of NO in Air Though the reaction also proceeded on a preparative scale, NMR was used for precise quantitative analyses of volatile disulfides. In the typical procedure, two symmetrical disulfides (0.1 mmol of each) were dissolved in CD_3CN (1 ml), and the reaction vessel was sealed with a septum cap. Then 22 μl (0.01 eq to each disulfide) of NO was added to the mixture using a gas tight syringe, and the reaction mixture was allowed to react at room temperature in the dark. The reaction mixture was subjected to NMR measurement to estimate the product ratio. Each NMR showed only the signals of unsymmetrical disulfides other than those of two starting materials, and each signal was separated sufficiently to estimate the reaction yields. The spectra of symmetrical disulfides are as follows: dimethyl disulfide (2.41 (s)); diethyl disulfide (1.28 (6H, t, J=7.3 Hz), 2.71 (4H, q, J=7.3 Hz)); dibutyl disulfide (0.92 (6H, t, J=7.3 Hz), 1.40 (4H, sext, J=7.3 Hz), 1.64 (4H, tt, J=7.5, 7.3 Hz), 2.71 (4H, t, J=7.5 Hz)); di(*sec*-butyl) disulfide (0.96 (6H, t, J=7.3 Hz), 1.27 (6H, d, J=6.8 Hz), 1.46—1.56 (2H, m), 1.62—1.73 (2H, m), 2.73—2.81 (2H, m)); di(*tert*-butyl) disulfide (1.30 (s)); dibenzyl disulfide (3.69 (4H, s), 7.26—7.36 (10H, m)); diphenyl disulfide (7.26—7.30 (2H, m), 7.33—7.38 (4H, m), 7.51—7.55 (4H, m)); di(*p*-tolyl) disulfide (2.30 (6H, s), 7.16 (4H, d, J=8.0 Hz), 7.40 (4H, d, J=8.0 Hz)); bis(*p*-methoxyphenyl) disulfide (3.78 (6H, s), 6.90 (4H, d, J=8.9 Hz), 7.41 (4H, d, J=8.9 Hz)); bis(*p*-chlorophenyl) disulfide (7.36 (4H, d, J=8.8 Hz), 7.49 (4H, d, J=8.8 Hz); 2,2′-dithiodiethanol (2.82 (4H, t, J=6.0 Hz), 2.95 (2H, t, J=6.0 Hz), 3.73 (4H, q, J=6.0 Hz)); dithiodiacetic acid (3.60 (S)); *N,N*′-bis(benzyloxycarbonyl)-L-cystine (3.02 (2H, dd, J=14.0, 8.5 Hz), 3.22 (2H, dd, J=14.0, 4.6 Hz), 4.47 (2H, ddd, J=8.5, 7.6, 4.6 Hz), 5.08 (4H, s), 6.15 (2H, br d, J=7.6 Hz), 7.32—7.37 (10H, m)). The spectra of unsymmetrical disulfides are as follows: ethyl methyl disulfide (1.30 (3H, t, J=7.3 Hz), 2.40 (3H, s), 2.74 (2H, q, J=7.3 Hz)); butyl methyl disulfide (0.92 (3H, t, J=7.3 Hz), 1.36—1.46 (2H, m), 1.61—1.70 (2H, m), 2.39 (3H, s), 2.74 (2H, t, J=7.3 Hz)); *sec*-butyl methyl disulfide (0.97 (3H, t, J=7.3 Hz), 1.29 (3H, d, J=6.8 Hz), 1.48—1.59 (1H, m), 1.62—1.74 (1H, m), 2.39 (3H, m), 2.83 (1H, sext, J=6.8 Hz)); benzyl methyl disulfide (2.18 (3H, s), 3.94 (2H, s), 7.26—7.38 (5H, m)); methyl phenyl disulfide (2.46 (3H, s), 7.26—7.30 (1H, m), 7.36—7.40 (2H, m), 7.54—7.56 (2H, m)); methyl (*p*-tolyl) disulfide (2.33 (3H, s), 2.44 (3H, s), 7.20 (2H, d, J=8.1 Hz), 7.44 (2H, d, J=8.1 Hz)); (*p*-methoxyphenyl) methyl disulfide (2.44 (3H, s), 3.80 (3H, s), 6.94 (2H, d, J=8.9 Hz), 7.50 (2H, d, J=8.9 Hz)); (*p*-chlorophenyl) methyl disulfide (2.45 (3H, s), 7.38 (2H, d, J=8.8 Hz), 7.53 (2H, d, J=8.8 Hz)); benzyl ethyl disulfide (1.21 (3H, t, J=7.3 Hz), 2.51 (2H, q, J=7.3 Hz), 3.92 (2H, s), 7.26—7.37 (5H, m)); ethyl phenyl disulfide (1.28 (3H, t, J=7.3 Hz), 2.78 (2H, q, J=7.3 Hz), 7.24—7.30 (1H, m), 7.33—7.39 (2H, m), 7.52—7.59 (2H, m)); benzyl butyl disulfide (0.87 (3H, t, J=7.3 Hz), 1.27—1.35 (2H, m), 1.51—1.58 (2H, m), 2.48 (2H, t, J=7.3 Hz), 3.91 (2H, s), 7.26—7.36 (5H, m)); 2-hydroxyethyl methyl disulfide (2.41 (3H, s), 2.83 (2H, t, J=6.1 Hz), 2.93 (1H, t, J=6.1 Hz), 3.74 (2H, q, J=6.1 Hz)); 2-carboxymethyl methyl disulfide (2.46 (3H, s), 3.50 (3H, s)); *N*-benzyloxycarbonyl-L-cysteinyl methyl disulfide (2. 39 (3H, s), 3.00 (1H, dd, J=14.0, 8.8 Hz), 3.23 (1H, dd, J=14.0, 4.3 Hz), 4.49 (1H, ddd, J=8.8, 8.2, 4.3 Hz), 5.09 (2H, s), 6.17 (1H, d, J=8.2 Hz), 7.30—7.39 (5H, m)).

The Effect of NO and O_2 (or NO_2) on the Reaction of Diethyl and Dibenzyl Disulfides Dibenzyl disulfide (1 mmol) was dissolved in acetonitrile (10 ml), and argon was bubbled into the solvent for 15 min except for the reactions in air. Then the reaction vessel was sealed with a septum rubber, and diethyl disulfide was introduced by a syringe. NO and O_2 gas were added by the use of a Hamilton gas tight syringe. The mixture was allowed to react in the dark at room temperature. The reaction was monitored using HPLC analysis (column: Merck Lichrospher Si60 (5 μm), eluent; hexane: dichloromethane=2 : 1).

Reaction of Unsymmetrical Disulfides with NO Aryl methyl disulfide (0.2 mmol) was dissolved in CD_3CN (1 ml), and the reaction vessel was sealed with a septum cap. Then 22 μl of NO was added to the mixture using a gas tight syringe, and the reaction mixture was allowed to react at room temperature in the dark. The reaction mixture was subjected to NMR measurement to estimate the product ratio.

Reaction of Diethyl Disulfide with NO in Air Diethyl disulfide (1 mmol) was dissolved in CH_3CN (10 ml), and the reaction vessel was sealed with a septum cap. Then 2.2 ml of NO was added to the mixture using a gas tight syringe, and the reaction mixture was allowed to react at room temperature in the dark for 3 h. Then the solvent was evaporated off, and the residue was subjected to NMR measurement. The sample was stored in the dark for 3 d at ambient temperature, then the NMR was re-measured. The reaction mixture after 3 h showed the spectra (in $CDCl_3$) mentioned below: diethyl disulfide; 1.32 (t), 2.71 (q). Product **A** (diethyl thiosulfinate); 1.42 (t), 1.48 (t), 3.07—3.25 (m). After 3 d, the spectra derived from product **A** had entirely disappeared, and product **B** was the only compound formed other than diethyl disulfide. Product **B** (diethyl thiosulfonate); 1.44 (t), 1.48 (t), 3.16 (q), 3.33 (q).

Reaction of Two Disulfides with *S*-Nitrosothiol Triphenylmethylthionitrite (**8**) was synthesized according to the reported method.[24]) A certain amount of *S*-nitrosothiol was dissolved in CD_3CN (1 ml), and argon was bubbled into the solvent for 15 min except for the reactions in air. Then the reaction vessel was sealed with a septum rubber, and the CD_3CN solution of two disulfides was introduced by a syringe. The mixture was allowed to react at room temperature in the dark. The reaction progress was monitored by NMR analysis.

Reaction with Nitrosonium Tetrafluoroborate Dibenzyl disulfide (1 mmol) was dissolved in acetonitrile (10 ml), and argon was bubbled into the solvent for 15 min except for the reactions in air. Then the reaction vessel was sealed with a septum rubber, and diethyl disulfide (1 mmol) was introduced by a syringe. Then the acetonitrile solution (100 μl) of nitrosonium tetrafluoroborate (0.5 or 10 μmol) was added to the mixture, which was allowed to stir at room temperature. Product analysis was carried out using HPLC.

Reaction of Dibutyl Disulfide with NO and O_2: Detection of *S*-Nitrosothiol NO (45 ml) and O_2 (4.5 ml) was added to a reaction vessel degassed with Ar bubbling containing acetonitrile (10 ml). Then dibutyl disulfide (1 mmol) was added and the mixture was allowed to stand for 10 min, and the solution was subjected to UV–vis spectrum measurement (551.5 nm). A similar spectrum was observed by the reaction with diethyl disulfide. By comparison to the reported data, the yield of *S*-nitrosothiol was estimated at about 2%.

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Rate Enhancement with High Ratio of the Monoalkylated Product to the Dialkylated Product in the Alkylation of the Lithium Enolate of 1-Tetralone with Reactive Alkyl Halides

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The reactions of the lithium enolate of 1-tetralone with reactive alkyl halides were examined in the absence and in the presence of 3 eq of various ligands for the lithium. It is shown that the rates of the reactions are enhanced greatly in the presence a tetradentate amine (1,1,4,7,10,10-hexamethyltriethylenetetramine), and the ratio of the monoalkylated product to the dialkylated product is increased under shorter reaction times.

Key words alkylation; bidentate amine; tridentate amine; tetradentate amine; pentadentate amine; rate enhancement

Lithium enolates play a central role in synthetic organic chemistry, because they can be prepared easily from the corresponding carbonyl compounds by deprotonation using lithium amides such as lithium diisopropylamide (LDA), and they react with various electrophiles to undergo a variety of important reactions, such as alkylation, acylation, aldolization, protonation, *etc.*[2)] In the alkylation reaction, the rate of the reaction is usually slow, and the formation of polyalkylated products is frequently a serious problem. To enhance the rate of the alkylation reaction, hexamethylphosphoric triamide (HMPA) was found to be a useful reagent which coordinates to the lithium of the lithium enolate to deaggregate it.[3)] However, due to the possible carcinogenicity of HMPA, cyclic urea derivatives such as *N*,*N*′-dimethyl-*N*,*N*′-propyleneurea (DMPU) and *N*,*N*′-dimethyl-*N*,*N*′-ethyleneurea (DMEU) were developed as safe substitutes for HMPA having similar properties.[4)]

It is known that the lithium enolate prepared from a carbonyl compound and a lithium amide forms a complex in solution with the amine coming from the lithium amide employed.[5)] It is, therefore, reasonable to assume that the achiral lithium enolate prepared by using a chiral lithium amide forms a complex with the chiral amine coming from the chiral lithium amide employed, where the symmetrical π-system of the enolate is expected to exist in chiral environment, and to react with electrophiles enantioselectively. Based on this strategy, we have previously examined enantioselective alkylations of achiral lithium enolates prepared by deprotonation of cyclohexanone and 1-tetralone using various chiral lithium amides (for example, **1**—**4**), followed by treatment of the resulting solution with reactive alkyl halides in toluene.[6)] It is shown that the chemical yields of the products depend heavily on the lithium amide employed. For example, in benzylation reaction of cyclohexanone under the same conditions (deprotonation using **1**—**4** in toluene, and then −20 °C, 18 h after addition of benzyl bromide), chemical yields of 2-benzylcyclohexanone were 1%, 7%, 15%, and 62% by using 1 eq of **1**, **2**, **3**, and **4**, respectively.[6d)] These data suggest that the reactivity of the lithium enolate of cyclohexanone is enhanced by forming a complex with a tetradentate amine coming from **4** as a ligand to the lithium.

In order to examine the effect of the number of ligation sites of the ligands on the rate of the reactions of the lithium enolates with reactive alkyl halides, 1-tetralone (**5**) was treated with LDA to give the corresponding lithium enolate, and the resulting solution was treated with 5 eq of reactive alkyl halides in the absence and in the presence of 3 eq of various ligands [HMPA, DMPU, [2.1.1]-cryptand, bidentate (**8**), tridentate (**9**), tetradentate (**10**), and pentadentate (**11**) amines], which are expected to interact with the lithium of the lithium enolate. Chemical yields of the products [monoalkylated (**6**) and dialkylated (**7**) products] and the recovered yield of **5** were determined by HPLC using 2-acetonaphthone as an internal standard. The results are summarized in Table 1.[7)]

Benzylation reaction of **5** to give **6a** and **7a** was first examined in dimethoxyethane (DME) at −23 °C. In the absence of any ligand, the reaction was slow, and needed longer reaction time to get the products in certain yields (runs 1, 2). In the presence of HMPA, the rate of the reaction was enhanced with reasonably high selectivity for **6a**/**7a** after 40 min (run 3), while DMPU had little effect (runs 4, 5 *vs.* runs 1, 2). In the presence of [2.1.1]-cryptand, the reaction was actually enhanced, but the ratio of **6a**/**7a** decreased greatly (run 6). This means that further deprotonation of **6a** is also enhanced greatly during the reaction in the presence of [2.1.1]-cryptand. Among multidentate amines (**8**—**11**), bidentate amine (**8**) showed little effect (runs 7, 8 *vs.* runs 1, 2), while the reaction was enhanced in the presence of tridentate (**9**), tetradentate (**10**), and pentadentate (**11**) amines to give the products in higher yields (runs 9, 10, 13 *vs.* run 1). It is shown that **10** is the most effective, giving **6a** in 72% yield and **7a** in 1% yield after 40 min (run 10), while **6a** in 83% yield and **7a** in 7% yield after 3 h (run 12). Thus, longer reaction time decreases the ratio of **6a**/**7a**. The effect of the tetradentate amine (**10**) on the rate of the reaction is superior to that of HMPA (run 10 *vs.* run 3).

For the enantioselective reactions of the lithium enolate of **5** with benzyl bromide mediated by chiral tetradentate amines, the presence of lithium bromide is necessary to get the corresponding **6a** in higher ee's.[6)] It is shown that the presence of lithium bromide does not affect the rate of the alkylation reaction (run 11 *vs.* run 10).

From our evaluation of **10** as the most effective ligand to enhance the rate of benzylation reaction with high ratio of **6a**/**7a**, benzylation reaction of **5** was examined in tetrahydro-

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furan (THF), ether, and toluene in the absence and in the presence of **10** (runs 14—19). It is shown that chemical yields of the products are low in the absence of **10** (runs 14, 16, 18), but they increase in the presence of **10** (runs 15, 17, 19). Judging from the chemical yield of **6a** and the ratio of **6a**/**7a**, it is concluded that DME is the solvent of choice for this reaction.

Using DME as a solvent, the reactions with other reactive alkyl halides were examined in the absence and in the presence of **10** (runs 20—23). It is again shown that the corresponding monoalkylated products (**6b**, **6c**) were obtained in reasonably high chemical yields and in high ratio of **6b**/**7b** and **6c**/**7c** in the presence of **10** (runs 21, 23). Since the reaction with butyl iodide was extremely slow at −23 °C, the reactions were examined at 25 °C (runs 24, 25). Rate enhancement in the presence of **10** was also observed.

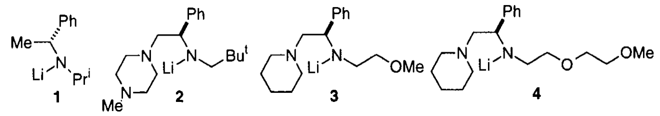

Chart 1

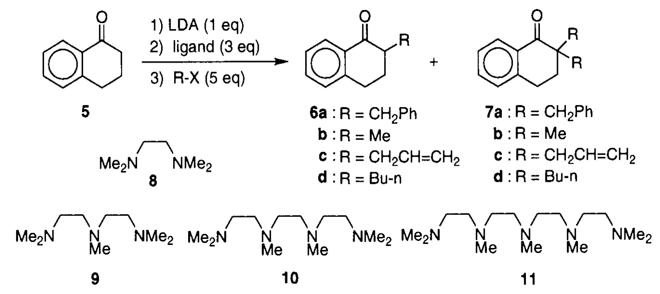

Chart 2

It is known that lithium enolates are aggregated in solution, and the reactivity of the lithium enolate can be dramatically modified by formation of mixed aggregates with amines.[4—6] In the present case, it is conceivable that the rates of the alkylation reactions are enhanced due to the formation of the complex(es) between the lithium enolate of **5** and **10** in solution. Studies on the structure(s) of the complex(es) are a matter of future investigation.

In conclusion, it is shown that the rates of the reactions of the lithium enolate of **5** with alkyl halides are enhanced in the presence of a tetradentate amine (**10**). It is also shown that the ratio of the monoalkylated product to the dialkylated product (**6**/**7**) is increased under shorter reaction time. Since **10** is commercially available, the results shown here should be widely useful in the reactions of lithium enolates.

Experimental

General All melting and boiling points are uncorrected. IR spectra were recorded on a JASCO IR Report-100 spectrometer. ^{1}H-NMR spectra were recorded in $\mathrm{CDCl_3}$ on a JEOL EX-270 spectrometer. Chemical shifts are given in δ (ppm) using tetramethylsilane as an internal standard. High resolution mass spectra (HRMS) were recorded on a JEOL DX-300 mass spectrometer under electron impact (EI) conditions. For anhydrous solvents, DME, THF, toluene, and ether were distilled from sodium/benzophenone ketyl. HMPA and amines (**8**—**11**) were distilled from $\mathrm{CaH_2}$. 2,2,-Dimethyl-1-tetralone (**7b**),[8] 2-allyl-1-tetralone (**6c**),[9] 2,2-diallyl-1-tetralone (**7c**),[9] and 2-butyl-1-tetralone (**6d**)[10] are known.

A Typical Procedure (Run 10) Under argon atmosphere, a solution of **5** (155 mg, 1.06 mmol) in DME (2 ml) was added to a solution of LDA [prepared from butyllithium in hexane (1.52 N, 0.77 ml, 1.17 mmol) and diisopropylamine (0.18 ml, 1.28 mmol) as usual] in DME (6 ml) at −45 °C. A solution of **10** (730 mg, 3.17 mmol) in DME (1 ml) was added, and the whole was stirred for 50 min. A solution of benzyl bromide (0.63 ml, 5.30 mmol) in

Table 1. Alkylation Reaction of the Lithium Enolate of 5[a)]

Run	RX (5 eq)	Ligand (3 eq)	Solvent	Temp. (°C)	Reaction time	Yield (%)		Recovered 5 (%)
						6	7	
1	$\mathrm{PhCH_2Br}$	None	DME	−23	40 min	6	*ca.* 0	92
2	$\mathrm{PhCH_2Br}$	None	DME	−23	18 h	54	13	25
3	$\mathrm{PhCH_2Br}$	HMPA	DME	−23	40 min	34	2	58
4	$\mathrm{PhCH_2Br}$	DMPU	DME	−23	40 min	3	*ca.* 0	93
5	$\mathrm{PhCH_2Br}$	DMPU	DME	−23	18 h	30	6	61
6	$\mathrm{PhCH_2Br}$	[2.1.1]-Cryptand	DME	−23	40 min	24	31	36
7	$\mathrm{PhCH_2Br}$	**8**	DME	−23	40 min	6	*ca.* 0	93
8	$\mathrm{PhCH_2Br}$	**8**	DME	−23	18 h	42	4	46
9	$\mathrm{PhCH_2Br}$	**9**	DME	−23	40 min	50	1	51
10	$\mathrm{PhCH_2Br}$	**10**	DME	−23	40 min	72	1	24
11[b)]	$\mathrm{PhCH_2Br}$	**10**	DME	−23	40 min	70	1	27
12	$\mathrm{PhCH_2Br}$	**10**	DME	−23	3 h	83	7	6
13	$\mathrm{PhCH_2Br}$	**11**	DME	−23	40 min	33	*ca.* 0	64
14	$\mathrm{PhCH_2Br}$	None	THF	−23	40 min	3	*ca.* 0	94
15	$\mathrm{PhCH_2Br}$	**10**	THF	−23	40 min	12	*ca.* 0	85
16	$\mathrm{PhCH_2Br}$	None	Ether	−23	40 min	1	*ca.* 0	98
17	$\mathrm{PhCH_2Br}$	**10**	Ether	−23	40 min	45	2	51
18	$\mathrm{PhCH_2Br}$	None	Toluene	−23	40 min	1	*ca.* 0	88
19	$\mathrm{PhCH_2Br}$	**10**	Toluene	−23	40 min	75	6	15
20	MeI	None	DME	−23	40 min	27	*ca.* 0	65
21	MeI	**10**	DME	−23	40 min	80	2	7
22	$\mathrm{CH_2{=}CH\text{-}CH_2Br}$	None	DME	−23	40 min	2	*ca.* 0	93
23	$\mathrm{CH_2{=}CH\text{-}CH_2Br}$	**10**	DME	−23	40 min	60	1	34
24	*n*-BuI	None	DME	25	2 h	12	1	77
25	*n*-BuI	**10**	DME	25	2 h	39	6[c)]	45

a) A typical procedure is described in Experimental. *b*) The reaction was carried out in the presence of LiBr (1 eq). *c*) *C*,*O*-Dibutylated product (1-butoxy-2-butyl-3,4-dihydronaphthalene) (1%) was observed.

DME (1.5 ml) was added at −78 °C, and the whole was stirred at −23 °C for 40 min. After addition of 40% aqueous citric acid (10 ml), the whole was allowed to warm to room temperature under stirring, and was extracted with AcOEt (40 ml×3). The organic extracts were combined, washed with saturated aqueous sodium bicarbonate (30 ml) and brine (30 ml), dried over $MgSO_4$ and filtered. The filtrate was mixed with 2-acetonaphthone (59.3 mg) as an internal standard, and was subjected to HPLC analysis [μ PORASIL, hexane–AcOEt (100 : 1)]. It is shown that the filtrate solution contains **6a** (180 mg, 72%), **7a** (3.5 mg, 1%), and **5** (36.2 mg, 24%).

2,2-Dibenzyl-1-tetralone (7a) Under argon atmosphere, a solution of **6a**[11] (204.6 mg, 0.866 mmol) in THF (2 ml) was added to a solution of diisopropylamine (0.14 ml, 1.02 mmol), BuLi in hexane (1.57 N, 0.62 ml, 0.97 mmol), and HMPA (0.44 ml, 2.54 mmol) in THF (6 ml), and the whole was stirred at −78 °C for 20 min. A solution of benzyl bromide (0.51 ml, 4.23 mmol) in THF (2 ml) was added dropwise, and the whole was stirred at −23 °C for 2 h. After addition of 40% aqueous citric acid (4 ml), the whole was allowed to warm to room temperature. Water (6 ml) was added, and the whole was extracted with hexane (50 ml×3). The combined organic extracts were washed with water (25 ml×2), brine (30 ml), dried over $MgSO_4$, and evaporated *in vacuo* . The residual oil was recrystallized from hexane to give **7b** (120 mg, 42%) as colorless plates of mp 98—98.5 °C. IR (KBr) cm^{-1}: 1680. ^{1}H-NMR: 1.9 (2H, m, $Ar–CH_2C\underline{H}_2–$), 2.65 (2H, d, J=13 Hz, $Ph–CH\underline{H}–$), 3.0 (2H, m, $Ar–C\underline{H}_2–$), 3.31 (2H, d, J=13 Hz, Ph–CHH–), 7.1—8.0 (14H, m, aromatic protons). *Anal.* Calcd for $C_{24}H_{22}O$: C, 88.31; H, 6.79. Found: C, 88.30; H, 6.78.

2,2-Dibutyl-1-tetralone (7d) Under argon atmosphere, a solution of **6d**[11] (1.8 g, 8.9 mmol) in THF (5 ml) was added to a solution of diisopropylamine (1.50 ml, 10.7 mmol), BuLi in hexane (1.62 N, 6.00 ml, 9.72 mmol), and HMPA (4.7 ml, 26.8 mmol) in THF (55 ml), and the whole was stirred at −78 °C for 20 min. A solution of BuI (5.10 ml, 44.8 mmol) in THF (10 ml) was added dropwise, and the whole was stirred at −23 °C for 2 h, and at room temperature for 2 h. After addition of 40% aqueous citric acid (20 ml), the whole was extracted with hexane (50 ml×3). The combined organic extracts were washed with water (50 ml), saturated aqueous $NaHCO_3$ (50 ml), brine (50 ml), dried over $MgSO_4$, and evaporated *in vacuo* to dryness. The residual pale yellow oil was subjected to column chromatography [silica gel, hexane–ether (30 : 1)] to give **7d** (0.80 g, 33% yield) as a colorless oil. IR (neat) cm^{-1}: 1675. ^{1}H-NMR: 0.88 (6H, t, J=7 Hz, $C\underline{H}_3–CH_2–$), 1.1—1.4 (8H, m, $CH_3–C\underline{H}_2–C\underline{H}_2–CH_2–$), 1.4-1.75 (4H, m, $CH_3–CH_2–CH_2–C\underline{H}_2–$), 2.04 (2H, t, J=6 Hz, $Ar–CH_2–CH_2–$), 2.96 (2H, t, J=6 Hz, $Ar–CH_2–CH_2–$), 7.1—8.1 (4H, m, aromatic protons). HRMS *m/z*: Calcd for $C_{18}H_{26}O$: 258.1984. Found: 258.1974.

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Hydroxylation of Nitrated Naphthalenes with KO_2/Crown Ether

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Superoxide radical anion ($O_2^{\cdot -}$), generated by KO_2/crown ether, is effective for hydroxylation of nitronaphthalenes. When mono- and di-nitronaphthalenes are treated with KO_2/crown ehter, hydroxylation results at the electron-deficient site caused by the electron withdrawing effect of the substituted nitro group. Kinetic experiments suggest that the hydroxylation proceeds by two different mechanisms dependent on the first one-electron reduction potential of nitronaphthalenes.

Key words superoxide; nitroarene; hydroxylation; nitronaphthalene

Studying the introduction of a hydroxyl group to aromatic compounds is important not only for the development of the methodology for organic synthesis,[1] but also for the clarification of the oxidative metabolisms in both endogenous and exogenous aromatic hydrocarbons by monooxygenases, such as cytochrome p450 enzymes.[2] The microsomal cytochrome p450-dependent hydroxylation of benzene is mediated by hydroxyl radicals formed in a Haber–Weiss reaction with hydrogen peroxide and superoxide radical anion ($O_2^{\cdot -}$).[3] Hydroxyl radical-generating systems, which mimic the activity of monooxygenase, have been developed as synthetic tools for hydroxylation.[4] However, hydroxylation is limited to substituted benzene and a few other arenes. The reactivity of the hydroxyl radical is very low when an aromatic ring is substituted by an electron-withdrawing group, such as the nitro group.[5] Among the active oxygen species formed in biological systems, $O_2^{\cdot -}$ is of interest because of its unique reactivity toward electron-deficient sites in organic compounds.[6] We recently reported that $O_2^{\cdot -}$ generated by KO_2/18-crown-6 was effective for the oxidation of 1-nitropyrene and 3-nitrofluorantene, and that direct hydroxylation without the accompanying nitro replacement reaction was characteristic of $O_2^{\cdot -}$ oxidation.[7] The objective of the present study is to investigate the possibility of KO_2/crown ether as a synthetic tool for hydroxylation of mono- and dinitronaphthalenes and the mechanistic aspect of aromatic hydroxylation with $O_2^{\cdot -}$.

Experimental

General ^{1}H-NMR and ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY) spectra were recorded on a Varian Gemini 300 and VXR-400S in deuterated chloroform with tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ, ppm) are expressed relative to TMS, and coupling constants (J value) in hertz (Hz). Chemical shift assignments of all synthetic chemicals were made by the combination of one-dimensional nuclear overhauser effect (1D NOE) and COSY spectra. Preparative high-performance liquid chromatography (HPLC) was conducted on a Shimadzu LC8A HPLC instrument with a silica gel column (5 μm, 20 mm i.d.×300 mm length) obtained from Soken Chemical & Engineering Co. (Tokyo, Japan). Cyclic voltammetric measurements were made with a Bioanalytical Systems BAS-100B (IN, U.S.A.) electrochemical analyzer using platinum working and auxiliary electrodes and a saturated calomel electrode as the reference. Ultraviolet (UV) spectra were run on a Shimadzu UV-240 instrument.

Chemicals KO_2 and 1,3-dinitronaphthalene were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Dicyclohexano-18-crown-6, dimethylformamide (DMF), dichloromethane, and benzene were from Wako Pure Chemical Industries (Osaka, Japan) and 1- and 2-nitronaphthalenes and 1,5-dinitronaphthalene were from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan). The 1,6- and 1,7-dinitronaphthalenes were synthesized according to the procedure of Ward and Hawkins.[8]

Reaction of Nitronaphthalene with KO_2/18-Crown-6 The general procedure for hydroxylation consisted of adding nitronaphthalene (3 mmol) in dry benzene (50 ml) to a solution of KO_2 (8 molar excess) and dicyclohexano-18-crown-6 (2 or 4 molar excess) in dry benzene (50 ml) in the dark. The consumption of the starting nitronaphthalenes and formation of hydroxylated products were monitored by HPLC. After suitable reaction times (0.5—4 h), the reaction mixture was quenched by acidification. The identity of the products was confirmed by NMR and mass spectral data after acetylation and purification by silica gel column chromatography.

Reaction of 1-nitronaphthalene with KO_2/18-Crown-6 is as follows. To a mixture of KO_2 (1.70 g, 24 mmol) and dicyclohexano-18-crown-6 (3.17 g, 12 mmol) in the solution (50 ml) of benzene and DMF (1 : 2) was added a solution (50 ml) of 1-nitronaphthalene (0.52 g, 3 mmol) in benzene and DMF (1 : 2). The mixture was stirred for 1.5 h at 25 °C in the dark and then quenched with water, acidified with concentrated HCl to pH 5, and extracted with dichloromethane. The extract was washed, dried over Na_2SO_4, evaporated, and treated with acetic anhydride (5 ml) and pyridine (2.5 ml) for 10 h. After the mixture was concentrated *in vacuo*, the residue was column chromatographed on silica gel with 30% CH_2Cl_2–*n*-hexane – 10% EtOAc–30% CH_2Cl_2–*n*-hexane to obtain a mixture of products. The crude products were redissolved in dichloromethane and purified by HPLC eluted with *n*-hexane, dichloromethane, and ethyl acetate (8 : 1 : 1) to obtain 4-acetoxy-1-nitronaphthalene (70.9%) and 3-acetoxy-1-nitronaphthalene (11.6%) in that order.

2-Acetoxynaphthalene: ^{1}H-NMR δ (ppm): 2.35 (s, OAc), 7.23 (d, H_3, J=8.9), 7.46 (t, H_7), 7.49 (s, H_6), 7.56 (s, H_1), 7.80 (d, H_5, J=9.1), 7.84 (d, H_8, J=9.5), 7.85 (d, H_4, J=8.9). Electron impact mass spectrum (EI-MS) *m/z*: 186 $[M]^+$, 144 (base), 115. High resolution mass spectrum (HR-MS) Calcd for $C_{12}H_{10}O_2$: 186.0681. Found: 186.0689.

1-Acetoxy-2-nitronaphthalene: ^{1}H-NMR δ (ppm): 2.58 (s, OAc), 7.68 (t, H_7), 7.72 (t, H_6), 7.85 (d, H_4, J=10.2), 7.95 (d, H_5, J=7.9), 8.10 (d, H_8, J=6.7), 8.13 (d, H_3, J=10.2). EI-MS *m/z*: 231 $[M]^+$, 189 (base), 172, 114. HR-MS Calcd for $C_{12}H_9NO_4$: 231.0532. Found: 231.0541.

1-Acetoxy-7-nitronaphthalene: ^{1}H-NMR δ (ppm): 2.54 (s, OAc), 7.45 (d, H_2, J=7.6), 7.70 (t, H_3), 7.85 (d, H_4, J=8.3), 8.02 (d, H_5, J=9.1), 8.29 (d, H_6, J=9.1), 8.87 (s, H_8). EI-MS *m/z*: 231 $[M]^+$, 189 (base), 131, 115. HR-MS Calcd for $C_{12}H_9NO_4$: 231.0532. Found: 231.0550.

3-Acetoxy-1-nitronaphthalene: ^{1}H-NMR δ (ppm): 2.40 (s, OAc), 7.65 (t, H_6), 7.71 (s, H_4), 7.72 (t, H_7), 7.92 (d, H_5, J=8.7), 8.07 (s, H_2), 8.58 (d, H_8, J=7.9). EI-MS *m/z*: 231 $[M]^+$, 189 (base), 131, 115. HR-MS Calcd for $C_{12}H_9NO_4$: 231.0532. Found: 231.0546.

4-Acetoxy-1-nitronaphthalene: ^{1}H-NMR δ (ppm): 2.52 (s, OAc), 7.39 (d, H_3, J=8.5), 7.68 (t, H_6), 7.78 (t, H_7), 8.06 (d, H_5, J=8.4), 8.31 (d, H_2, J=8.5), 8.66 (d, H_8, J=8.9). EI-MS *m/z*: 231 $[M]^+$, 189 (base), 159, 115. HR-MS Calcd for $C_{12}H_9NO_4$: 231.0532. Found: 231.0541.

5-Acetoxy-1-nitronaphthalene: ^{1}H-NMR δ (ppm): 2.50 (s, OAc), 7.43 (d, H_6, J=8.6), 7.60 (t, H_3), 7.72 (t, H_7), 8.23 (d, H_4, J=7.7), 8.26 (d, H_2, J=8.9), 8.45 (d, H_8, J=8.9). EI-MS *m/z*: 231 $[M]^+$, 189 (base), 115. HR-MS Calcd for $C_{12}H_9NO_4$: 231.0532. Found: 231.0550.

7-Acetoxy-1-nitronaphthalene: ^{1}H-NMR δ (ppm): 2.54 (s, OAc), 7.43 (d, H_6, J=8.9), 7.56 (t, H_3), 8.04 (d, H_5, J=8.9), 8.15 (d, H_4, J=8.3), 8.35 (d, H_2, J=7.7), 8.40 (s, H_8). EI-MS *m/z*: 231 $[M]^+$, 189 (base), 115. HR-MS Calcd for $C_{12}H_9NO_4$: 231.0532. Found: 231.0538.

2-Acetoxy-1,5-dinitronaphthalene: ^{1}H-NMR δ (ppm): 2.58 (s, OAc), 7.79 (t, H_7), 8.33 (d, H_3, J=7.9), 8.43 (d, H_6, J=6.6), 8.45 (d, H_8, J=7.6), 8.61 (d, H_4, J=8.8). EI-MS *m/z*: 276 $[M]^+$, 234 (base), 113. HR-MS Calcd for

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Dedicated to the memory of Dr. Kyosuke Tsuda.

$C_{12}H_8N_2O_6$: 276.0382. Found: 276.0386.

4-Acetoxy-1,3-dinitronaphthalene: ^{1}H-NMR δ (ppm): 2.61 (s, OAc), 7.85 (t, H_6), 7.98 (t, H_7), 8.29 (d, H_5, J=8.6), 8.70 (d, H_8, J=7.9), 8.97 (s, H_2). EI-MS m/z: 276 $[M]^+$, 234 (base), 217, 113. HR-MS Calcd for $C_{12}H_8N_2O_6$: 276.0382. Found: 276.0399.

4-Acetoxy-1,5-dinitronaphthalene: ^{1}H-NMR δ (ppm): 2.37 (s, OAc), 7.58 (d, H_3, J=8.6), 7.76 (d, H_6, J=7.5), 7.81 (t, H_7), 8.39 (d, H_2, J=8.6), 8.78 (d, H_8, J=9.9). EI-MS m/z: 276 $[M]^+$, 234 (base), 218, 204. HR-MS Calcd for $C_{12}H_8N_2O_6$: 276.0382. Found: 276.0391.

4-Acetoxy-1,6-dinitronaphthalene: ^{1}H-NMR δ (ppm): 2.52 (s, OAc), 7.64 (d, H_3, J=8.5), 8.52 (d, H_7, J=9.6), 8.54 (d, H_2, J=8.5), 8.90 (d, H_8, J=9.6), 9.02 (s, H_5). EI-MS m/z: 276 $[M]^+$, 234 (base), 204. HR-MS Calcd for $C_{12}H_8N_2O_6$: 276.0382. Found: 276.0393.

4-Acetoxy-1,7-dinitronaphthalene: ^{1}H-NMR δ (ppm): 2.54 (s, OAc), 7.66 (d, H_3, J=8.5), 8.27 (d, H_5, J=9.2), 8.43 (d, H_6, J=9.2), 8.52 (d, H_2, J=8.5), 9.69 (s, H_8). EI-MS m/z: 276 $[M]^+$, 234 (base), 218, 204. HR-MS Calcd for $C_{12}H_8N_2O_6$: 276.0382. Found: 276.0399.

5-Acetoxy-1,6-dinitronaphthalene: ^{1}H-NMR δ (ppm): 2.52 (s, OAc), 7.79 (t, H_3), 8.33 (d, H_7, J=9.3), 8.44 (d, H_2, H_4), 8.62 (d, H_8, J=9.3). EI-MS m/z: 276 $[M]^+$, 234 (base). HR-MS Calcd for $C_{12}H_8N_2O_6$: 276.0382. Found: 276.0393.

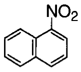
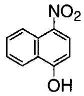
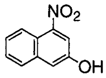
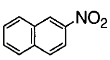
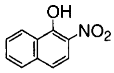
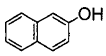
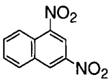
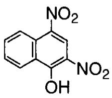
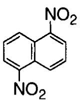
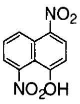
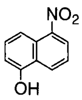
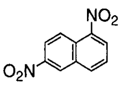
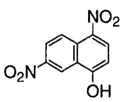
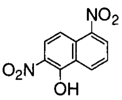
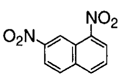
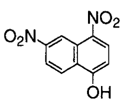
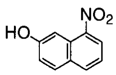
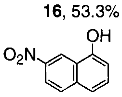
Electrochemical Reduction by Cyclic Voltammetry In DMF, tetramethylammonium perchlorate was used as the supporting electrolyte at a concentration of 0.1 M. After transfer of the solution containing the test chemical to the cell, it was purged of oxygen by bubbling with Ar for 15 min. The cyclic voltammograms were recorded at a scan rate of 100 mV/s while maintaining the test solution under a steady stream of Ar.

Kinetic Studies of the Hydroxylation of 1,3- and 1,6-Dinitronaphthalenes A solution of KO_2 (56.8 mg, 0.8 mmol) and dicyclohexano-18-crown-6 (53 mg, 0.2 mmol) in dry benzene (5 ml) was prepared and stirred for 20 min in the dark. Immediately after mixing with a supernatant (0.5 ml) of the suspension and 1,3-dinitronaphthalene (0.087 mg, 0.4 μmol) in 2.5 ml of dry benzene, the UV spectrum was monitored 16 times over a period of 30 min at regular intervals (2 min). For 1,6-dinitronaphthalene, the UV spectrum was monitored using the same procedure as for 1,3-dinitronaphthalene.

Results and Discussion

Nitronaphthalenes (**1**—**6**) were oxidized by KO_2/crown ether to afford several hydroxylated compounds. In case of the mononitronaphthalenes (**1**, **2**), the reaction was carried out in a mixture of benzene and DMF (1 : 2), because the reaction rate of mononitronaphthalene was very slow in benzene. In contrast, dinitronaphthalenes (**3**, **4**, **5**, **6**) were readily oxidized in benzene. The results are shown in Table 1. Throughout all reactions, it was clearly observed that KO_2/crown ether effectively reacted with the mono- and dinitronaphthalenes, and hydroxylation preferentially proceeded at the p-position to the nitro substituents. No such oxidation was observed when the reaction was carried out in the absence of crown ether (data not shown), suggesting that $O_2^{\cdot -}$ generated from KO_2/crown ether is responsible for the oxidation of nitrated naphthalenes. In the case of 2-nitronaphthalene **2**, which lacks the p-position, the reaction was slow and nucleophilic displacement of the nitro group by $O_2^{\cdot -}$ occurred. Replacement of the nitro group during the reaction of 1,5-dinitronapthathalene **4** in high yield is presumably the result of decreased hydroxylation at the 4-position due to the steric effect (*peri* strain) of the nitro group at the 5-position. Although both 1,6- and 1,7-dinitronaphthalenes have an α- and β-nitro group in each benzene ring, 1,6-dinitronaphthalene **5** has strong electron-deficient sites at the 2-, 4-, 5- and 7-positions where both the 1- and 6-nitro group reinforce their electron withdrawal. No such electron-deficient positions are present in 1,7-dinitronaphthalene **6** because the electron withdrawing effect of the two nitro groups do not coincide. Thus this electrochemical property of **5** facilitated the reaction with $O_2^{\cdot -}$ to form hydroxylated products at the 4- and 5-positions. In contrast, the reaction of **6** was slow, and, in addition to the hydroxylation at the 4-position, a nitro replacement reaction at the 1- and 7-positions by $O_2^{\cdot -}$ also occurred.

Table 1. Results from the Treatment of Mono- and Dinitronaphthalenes with KO_2/Crown Ether

Entry	Substrate Epc1 *vs.* S.C.E[a]	Reaction conditions	Products[b]
		KO_2/crown (eq) solvent, time	
1	 **1**,-1082 mV	8/4 Benzene/DMF[c] 1.5 h	 **7**, 70.9%  **8**, 11.6%
2	 **2**, -1096mV	8/4 Benzene/DMF[c] 4 h	 **9**, 9.4%  **10**, 39.8%
3	 **3**, -760mV	8/2 Benzene 1.5 h	 **11**, 90.4%
4	 **4**, -856mV	8/2 Benzene 2 h	 **12**, 24.4%  **13**, 59.7%
5	 **5**, -910mV	8/2 Benzene 0.5 h	 **14**, 64.3%  **15**, 13.6%
6	 **6**, -813mV	8/2 Benzene 2 h	 **16**, 53.3%  **17**, 35.4%  **18**, 0.3%

a) Epc1 of nitronaphthalenes were determined by cyclic voltammetry. All measurements were recorded in DMF solution in 0.1 mol dm^{-3} Et_4NClO_4 using platinum working and auxiliary electrodes and a saturated calomel electrode as a reference. *b*) Yields for the reaction by KO_2/18-crown-6 were obtained as acetylated derivatives. *c*) The ratio of benzene and DMF was 1 : 2.

Since trace amounts of hydroxy or nitro replacement products were obtained when KOH was used instead of KO_2 in these reactions, as shown in Table 2, it is expected that $O_2^{\cdot -}$ was the substantial reactant for hydroxylation in this system. Two possible mechanisms for the first step of the hydroxylation by $O_2^{\cdot -}$ are considered. One is oxygen dependent: a one-electron reduction by $O_2^{\cdot -}$ to form the aromatic radical anion which is followed by the addition of molecular oxygen. The other is oxygen independent: the direct addition of $O_2^{\cdot -}$ to the aromatic ring. The reaction was run under Ar to investigate the participation of molecular oxygen. As shown in Table 2, the reaction by 1,3-dinitronaphthalene **3** gave a much lower yield than the reaction in the atmosphere, while the reaction yield was increased in the case of **5**. Taking into account the one-electron reduction potentials (Epc1) of the mono- and dinitronaphthalenes and half-wave reduction potentials ($E_{1/2}$) of O_2, it is concluded that the first step for hydroxylation of **3** and **5** proceeds by a different mechanism, as shown in Chart 1. That is, **3** with an Epc1 (−760 mV) more positive than the $E_{1/2}$ of O_2 (−877 mV) was easily reduced by $O_2^{\cdot-}$, and thus formed the radical anion combined with O_2 to give the hydroxylated product. In contrast, the reduction of **5**, which has a more negative Epc1 (−910 mV) than the E1(1/2) of O_2, was unfavorable under this condition; hydroxylation of **5** proceeded *via* the direct addition of $O_2^{\cdot-}$. The difference in the mechanism was also observed by UV–vis spectroscopy. As shown in Fig. 1a, absorbance at 510 nm, which clearly indicated the formation of the radical anion of **3**, appeared just after adding the solution of KO_2/18-crown-6 and, along with the decreasing absorbance at 510 nm, new absorbance as the result of the addition of O_2 appeared at 380 and 450 nm. Alternatively, as demonstrated in Fig. 1b, no absorbance associated with the radical anion of **5** due to the reduction of $O_2^{\cdot-}$ was observed, and increasing absorbance at 490 nm originating from the addition of $O_2^{\cdot-}$ was observed under pseudo-first-order conditions ($k_{obs}=2.57\times10^{-3}\,s^{-1}$).

Table 2. Effects of Reaction Conditions on Hydroxylation of **3** and **5** with KO_2/Crown Ether

Entry	Substrate	Reaction conditions Ratio,[a] time (min)	Products	Yield (%)	Recovery (%)
1	**3**	1/8/4, 45	**11**	81.5	14.8
2	**3**	1/8/4, 45[b]	**11**	38.6	58.0
3	**3**	1/8[c]/4, 45	**11**	0.2	99.4
4	**5**	1/8/2, 15	**14, 15**	40.3, 25.7	25.6
5	**5**	1/8/2, 15[b]	**14, 15**	55.6, 29.9	5.4
6	**5**	1/8[c]/2, 30	**14**	3.6	94.2

a) The ratio of substrates: KO_2 : 18-crown-6 was 1 : 8 : 4 for **3** and 1 : 8 : 2 for **5**. *b*) Reaction was run under Ar. *c*) KOH was used instead of KO_2.

The same mechanism as that for the hydroxylation of **3** has been proposed for the formation of 2,4-dinitrophenol by the reaction of 1-bromo-2,4-dinitrobenzene with $O_2^{\cdot-}$ by Frimer and Rosenthal.[9] On the other hand, the mechanism for nucleophilic addition by $O_2^{\cdot-}$ to **5** is similar to that of hydroxylation of 1-nitropyrene by $O_2^{\cdot-}$, as previously reported.[7] The same result was also shown by the reaction of 1-nitronaphthalene **1** with KO_2/crown ether (data not shown); the reaction was oxygen independent and the same spectral change as that in **5** was observed. The nucleophilic addition of $O_2^{\cdot-}$ to **1** was also evidenced by the greater facility of the reaction in DMF solution as a solvent compared with that in benzene

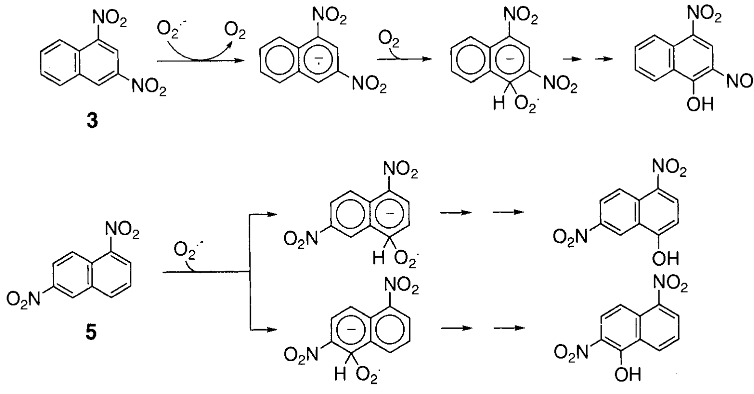


Chart 1. Possible Mechanism for Hydroxylation of **3** and **5** with KO_2/Crown Ether

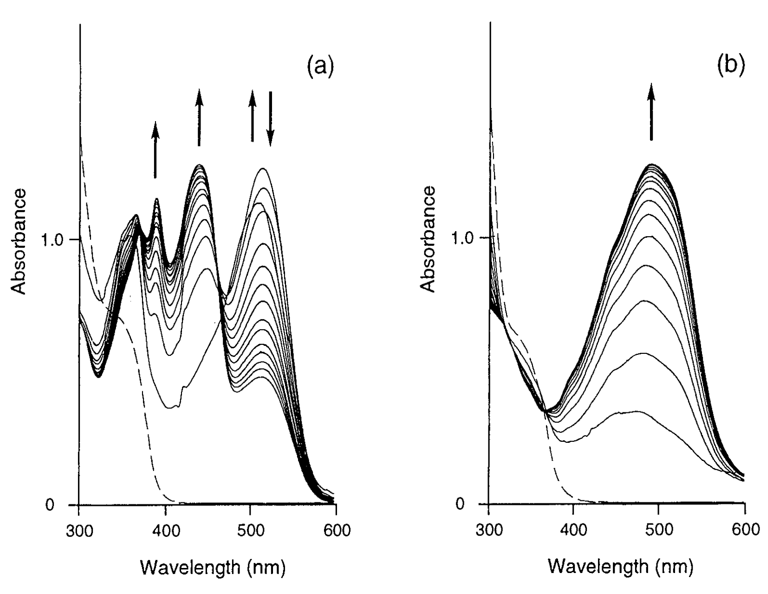


Fig. 1. Absorption Spectra of **3** (a) and **5** (b) after Treatment with KO_2/Crown Ether

Spectra were recorded at 2 min intervals over 0—30 min. The spectra represented by the dashed line indicate the absorption of the original **3** and **5** in benzene solution before adding the KO_2/18-crown-6.

solution (data not shown). Therefore it was concluded that the relative Epc1 of nitrated aromatic compounds toward the E1(1/2) of O_2 was an important factor for determining the mechanism of hydroxylation by $O_2^{\cdot -}$.

In conclusion, $O_2^{\cdot -}$ is useful for introducing the hydroxy group to the nitrated aromatic ring. The electric and steric effects of the nitro groups are important for determining which positions are hydroxylated. Two possible mechanisms that depend on the first reduction potential of nitronaphthalenes are postulated for the first steps in hydroxylation processes by $O_2^{\cdot -}$. Further studies to clarify the subsequent steps are now in progress.

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Insight into Acid-Mediated Asymmetric Spirocyclization in the Presence of a Chiral Diol

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Asymmetric spirocyclization based on intramolecular conjugate addition using a combination of a Lewis acid and an optically active cyclohexane-1,2-diol has been studied in connection with 1) the effect of substituents on the cyclohexane-1,2-diol and 2) the effect of substituents on the substrate. This reaction was found to be both thermodynamically and kinetically controlled under restricted conditions.

Key words asymmetric spirocyclization; Lewis acid; 1,2-diol; kinetic control; thermodynamic control

We previously reported asymmetric spirocyclization of 3-(4-oxobutyl)-2-cyclohexenone **1** into spiro[5.5]undecane-2,8-dione **2** using the combination of a Lewis acid and an optically active cyclohexane-1,2-diol, in which the best result in terms of enantiomeric excess (ee) of **2** was up to 85% ee as shown in Chart 1.[1] Our working hypothesis in the study of this reaction was an enol ether intermediate **A** as mentioned in the earlier paper. To further study the aspects of this reaction, we planned the following two approaches: 1) determination of the effects of substituents on the cyclohexane-1,2-diol, and 2) of the effects of geminal dimethyl substituents at the C5-position of the substrate.

Effects of Substituents on Cyclohexane-1,2-diol Preparation of Cyclohexane-1,2-diols: First, we planned to study this reaction of using several kinds of cyclohexane-1,2-diols, **4**, (*dl*)-**5**, (*dl*)-**6** and optically active **3**, **7**—**10**. Racemic *trans*-1,2-diols **5**, **6** were prepared by conventional methods from 1-methycyclohexene and dimedone, respectively, according to reported procedures.[2]

Optically active diols **7**—**10** were prepared from *l*-menthol *via* **11**, which was obtained by *trans*-elimination of the corresponding mesylate in 95% yield.[3] Diastereoselective *trans*-dihydroxylation of **11** into **7** was performed by epoxidation with *m*-chloroperbenzoic acid (*m*CPBA) and subsequent epoxide ring cleavage under acidic conditions. Preparation of the minor *trans*-product **8** was established by another method as described below. The stereochemistry of **7** and **8** was determined based on ^{1}H-NMR spectra. In the case of **7**, signals at δ 3.98 and δ 3.68, attributable to C1-H and C2-H, respectively, were observed with a value of W_H=7.9 Hz, which suggests that the hydroxy functions at the C1- and C2-positions are in an axial orientation. In the ^{1}H-NMR spectrum of **8**, the corresponding signals were observed at δ 3.20 (J=10.6, 8.6 Hz) and δ 2.97 (J=9.9, 8.6 Hz), which suggests all the substituents are in equatorial orientation.

Osmium-catalyzed *cis*-dihydroxylation of **11** gave an inseparable diastereomeric mixture of **9** and **10** in a ratio of 2 to 7 in 98% yield, which were separated after conversion into monobenzoates **12** and **13**. In this process, it was noted that a hydroxy group in equatorial orientation reacted regioselectively to afford the above products despite the use of 2 eq of benzoyl chloride (BzCl). The structure of **12** and **13** was determined by ^{1}H–^{1}H correlation spectroscopy (COSY) NMR

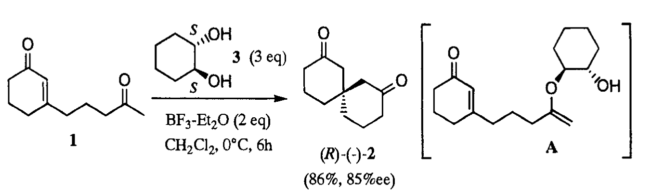

Chart 1. Asymmetric Spirocyclization Using **1**

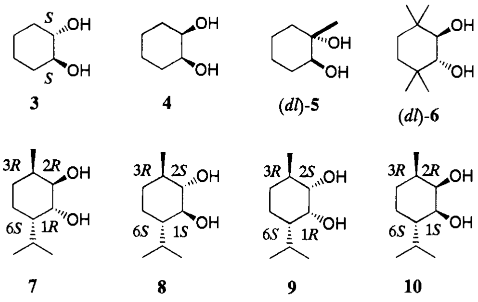

Fig. 1. Cyclohexane-1,2-diols

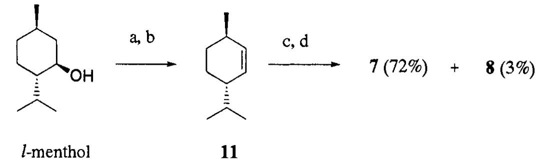

Chart 2. Preparation of **7** and **8**

(a) methanesulfonyl chloride (MsCl), pyridine, CH_2Cl_2, r.t. (quant.); (b) *tert*-BuOK, dimethyl sulfoxide (DMSO), 70 °C (95%); (c) *m*-CPBA, CH_2Cl_2, 0 °C; (d) 3% H_2SO_4 aq., tetrahydrofuran (THF), 0 °C.

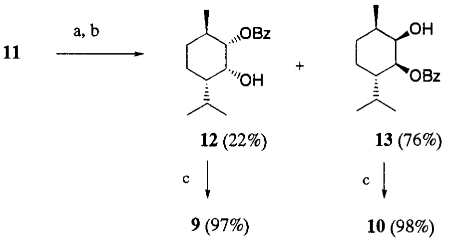

Chart 3. Preparation of **9** and **10**

(a) OsO_4, 4-methylmorpholine *N*-oxide (NMO), acetone, H_2O, r.t.; (b) BzCl, pyridine, CH_2Cl_2, r.t. and separation; (c) K_2CO_3, MeOH.

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Dedicated to the memory of Dr. Kyosuke Tsuda.

spectra. These compounds were easily converted into the corresponding diols **9**, **10** in quantitative yields.

Next, *trans*-diol **8** was prepared starting from compound **13**. For inversion of stereochemistry at the C2-position, mesylation of **13** in refluxing $CHCl_3$ gave a mesylate **14** in 67% yield. After solvolysis of **14** into **15**, reaction with CsOAc at 100 °C in *N*,*N*-dimethylformamide (DMF) according to Ikegami's procedure[4] gave the desired products, a 1 to 1 mixture of **16** and **17** in 55% yield.[5] The acyl migration from **16** was believed to afford **17**. Further solvolysis of a mixture of **16** and **17** afforded **8** in 82% yield.

Results and Discussion of Spirocyclization of **1**: Reaction of **1** under acidic conditions in the presence of a diol was studied. The reaction conditions were based on our previous study,[1b)] that is to say, BF_3–Et_2O (2 eq) as a Lewis acid, 3 eq of a diol and CH_2Cl_2 as a solvent were used. The results are summarized in Table 1.

In entries 1 and 2, our previous results are shown. In entry 1, reaction without a diol proceeded slowly to give **2** (31%) and an intramolecular aldol-dehydration product **18** (30%) accompanied by recovery of **1** (28%). In entry 2, spirocyclization was highly accelerated by addition of **3** to afford (*R*)-(−)-**2** (86%) of 85% ee.

As a preliminary study, the effects of substituents at the C1- and C3,6-positions were studied using (*dl*)-**5**, **6**. In the case of entry 3 using (*dl*)-**5**, the reaction was not at all affected by addition of a diol. Furthermore, in entry 4 using (*dl*)-**6**, the obtained result was similar to that of entry 1. This result of entry 4 prompted us to study what kind of substituents inhibited the roll of a diol such as **3** in entry 2.

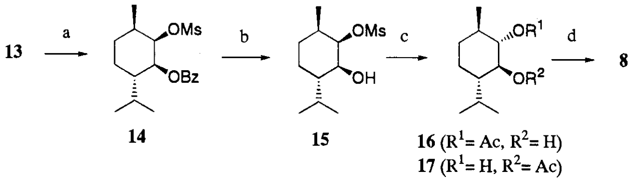


Chart 4. Conversion from **13** to **8**

(a) MsCl, pyridine, 4-dimethylaminopyridine (DMAP), $CHCl_3$, reflux (67%); (b) K_2CO_3, MeOH, r.t. (99%); (c) CsOAc, DMF, 100 °C (55%, **16**:**17**=1:1); (d) K_2CO_3, MeOH, r.t. (82%).

When *trans*-diols **7**, **8** were used, the respective results were very different. As regards the effect on reactivity when using diol **7** in entry 5, the yield of **2** (86%) was evaluated as high as when **3** was used, but the effect of diol **8** was similar to that of **6**. These results suggest that alkyl substituents at the C3,6-positions which locate in vicinal *trans* orientation to the C1- or C2-hydroxy group might inhibit the acceleration of spirocyclization. In connection with asymmetric induction, the absolute stereochemistry of **2** was dependent on that of the C1,2-position of the *trans*-diols used (entries 2, 5, 6). However, ees of **2** in entries 5 and 6 were not as high as that in entry 2. Taking the configurational factor of **3** and **7** into consideration, both hydroxy groups of **3** were predominantly in equatorial orientation. On the other hand, those of **7** were in axial orientation as aforementioned. The relationship between this conformational aspect and ee of product **2** is still undetermined.

Next, spirocyclization of **1** using *cis*-cyclohexane-1,2-diol **4** was examined in entry 7, which afforded **2** in 53% yield and **18** in 16% yield. These results, which showed some acceleration of the reaction rate compared to entry 1, prompted us to study the reaction using optically active *cis*-diols **9** and **10**. As shown in entries 8—11 in Table 1, both diols gave similar results. That is to say, reactions at 0 °C for 24 h gave the corresponding acetals **19** (60%) and **20** (65%), respectively (entries 8, 10).[6] In addition, reactions in refluxing CH_2Cl_2 for 48 h gave (*S*)-(+)-**2** of 13% ee (44% yield) and 16% ee (49% yield), respectively (entries 9, 11).

Throughout these studies, a more efficient diol than **3** to produce **2** of high ee was not found, but the effects of alkyl substituents at C3,6-positions were observed in connection with the reactivity of **1**.

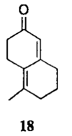
Spirocyclization of 5,5-dimethyl-3-(4-oxobutyl)-2-cyclo-

Table 1. Reaction of **1** with Cyclohexane-1,2-diols

$$\mathbf{1} \xrightarrow[\text{diol (3 eq), CH}_2\text{Cl}_2]{\text{BF}_3\text{–Et}_2\text{O (2 eq)}} \mathbf{2}$$

Entry	Diol	Temp.	Time (h)	Product 2			Recovery (%)	Other products (%)
				Yield (%)	Abs. config.	% ee		
1[a)]	—	r.t.	72	31	—	—	28	**18** (30)
2[a)]	**3**	0 °C	6	86	*R*	85	—	—
3	(*dl*)-**5**	r.t.	96	27	—	—	68	—
4	(*dl*)-**6**	r.t.	24	25	—	—	38	**18** (15)
5	**7**	0 °C	12	86	*S*	28	—	—
6	**8**	r.t.	24	31	*R*	26	44	**18** (13)
7	**4**	r.t.	24	53	—	—	—	**18** (16)
8	**9**	0 °C	24	—	—	—	39	**19** (60)
9	**9**	Reflux	48	44	*S*	13	—	—
10	**10**	0 °C	24	—	—	—	33	**20** (65)
11	**10**	Reflux	48	49	*S*	16	—	—

a) Previous result.[1]



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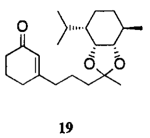

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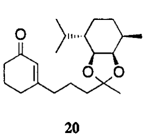

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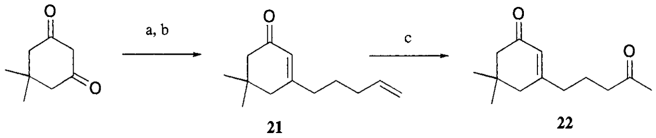
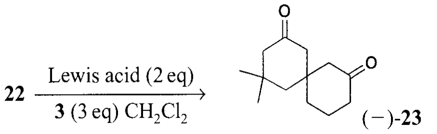


Chart 5. Preparation of Substrate **22**

(a) iso-BuOH, *p*-toluenesulfonic acid (*p*-TsOH), benzene, reflux (quant.); (b) (i) BrMg(CH$_2$)$_3$CH=CH$_2$, THF, r.t., (ii) 10% HCl, THF, r.t. (78% 2 steps); (c) PdCl$_2$, CuCl, O$_2$, DMF, H$_2$O, r.t. (73%).

Table 2. Spirocyclization of **22**



Entry	Lewis acid	Temp.	Time	Yield (%)	% ee	Recovery (%)
1[a]	BF$_3$·Et$_2$O	r.t.	3 d	0	—	98
2	BF$_3$·Et$_2$O	r.t.	3 d	0	—	90
3[a]	TMSOTf	r.t.	3 d	20	—	36
4	TMSOTf	r.t.	3 h	56	33	9
5	TMSOTf	0 °C	5 h	41	64	15
6	TMSOTf	−20 °C	1 d	30	86	50
7	TMSOTf	−20 °C	7 d	36	91	48
8	TMSOTf	−30 °C	10 d	0	—	98

a) Reaction was performed without diol.

hexenone Next, compound **22** was prepared as a substrate by a similar sequence of reactions as for preparation of **1** as shown in Chart 5. The objective in setting up **22** was to investigate the effect of C-5 geminal dimethyl functions on asymmetric spirocyclization under the previously optimized conditions, using BF$_3$–Et$_2$O (2 eq) or trimethylsilyl trifluoromethanesulfonate (TMSOTf) (2 eq) as the Lewis acid, and **3** (3 eq) in CH$_2$Cl$_2$. The results are summarized in Table 2. In reactions using BF$_3$–Et$_2$O as the Lewis acid, the spirocyclized product **23** was not obtained at all, and most of the substrate was recovered, regardless of the absence or presence of **3** (entries 1, 2). The desired product **23** was obtained using TMSOTf (entries 3—7). This result was interesting, because when **1** was used as a substrate, BF$_3$–Et$_2$O was the first choice as Lewis acid.

Acceleration of the reaction by addition of **3** can be clearly observed by comparing entries 3 and 4. That is to say, reaction for 3 d at room temperature without a diol afforded (*dl*)-**23** in 20% yield (entry 3), while the reaction for 3 h at the same temperature in the presence of **3** gave (−)-**23** in 56% yield (33% ee) (entry 4). The ee of (−)-**23** was strongly influenced by the reaction temperature. Reaction at −20 °C for 1 d gave (−)-**23** of 86% ee and that at the same temperature for 7 d gave (−)-**23** of 91% ee, although chemical yields were unsatisfactory (30—36% yields) (entries 6, 7). This value of 91% ee was the best among our studies of asymmetric spirocyclization.[7]

The ee of (−)-**23** was determined by ^{1}H-NMR spectroscopy after conversion into bisacetal of (*R*,*R*)-butane-2,3-diol **24** under azeotropic conditions (*p*-TsOH, benzene). This was the same procedure used for the determination of ee of **1**. In both cases, no decrease of ee was confirmed by repeating acetalization and deacetalization (5—10% aqueous HCl), which led us to believe that the asymmetric spirocyclization proceeded in an irreversible manner (Chart 6). However, surprisingly, this assumption had to be corrected based on the following results.

Compound (−)-**23** (81% ee, 91% ee) was treated under the same reaction conditions of spirocyclization (TMSOTf, **3** in CH$_2$Cl$_2$) at room temperature and −20 °C, respectively. In the former case, the ee of recovered (−)-**23** (58% yield) was decreased from 81% ee to 28% ee and the formation of **22** (25%) was observed. In the latter case, the ee of (−)-**23** (84%) was slightly decreased from 91% ee to 86% ee and a small amount of **22** (5%) was obtained (Chart 6). Each ee was similar to that of the product at the corresponding reaction temperature in Table 2 (entries 4, 6). In addition, it is noteworthy that this reverse reaction from (−)-**23** to **22** proceeded in a regioselective manner, that is to say, formation of the other possible product B was not detected at all. These results suggest that this asymmetric spirocyclization might be both kinetically and thermodynamically controlled under restricted conditions.

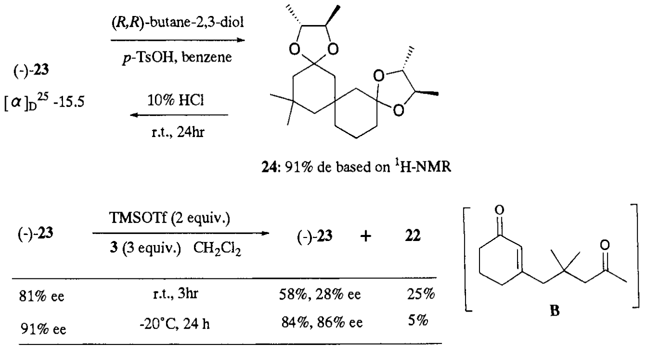


Chart 6

Experimental

Melting points were measured on Yanaco micro melting point apparatus without correction. ^{1}H-NMR spectra were taken on a JEOL GX-270 (270 MHz) spectrometer. ^{13}C-NMR were recorded on a JEOL GX-270 (67.8 MHz) spectrometer. IR spectra were measured on a JASCO IR A-100 IR spectrophotometer. Mass spectra (electron ionization (EI), FAB) were measured on a JEOL JMS-D300 or JEOL JMS-SX102 spectrometer, and specific rotations were measured on a JASCO DIP-360 digital polarimeter. The elemental analyses were performed on a Yanaco MT2 CHN recorder. Column chromatography were carried out on silica gel 70—230 mesh (Merck, Kieselgel 60). Solvents were distilled and dried before use. All the extracts were dried with Na$_2$SO$_4$.

(1*R*,2*R*,3*R*,6*S*)-3-Methyl-6-(methylethyl)cyclohexane-1,2-diol (7) and (1*S*,2*S*,3*R*,6*S*)-3-Methyl-6-(methylethyl)cyclohexane-1,2-diol (8) A solution of *m*-CPBA (11.6 g, 47.1 mmol) in CH$_2$Cl$_2$ (30 ml) was added dropwise to a solution of **11** (5.00 g, 36.2 mmol) in CH$_2$Cl$_2$ (200 ml) at 0 °C. The whole was stirred at the same temperature for 8 h. The reaction mixture was successively washed with 5% of aqueous sodium bisulfite, 5% of aqueous NaHCO$_3$ and brine, then dried. After removal of the solvent *in vacuo*, the residue was diluted with THF (20 ml), which was added to 3% of aqueous H$_2$SO$_4$ (150 ml) at 0 °C. The whole was stirred at 0 °C for 7 h. After neutralization by addition of NaHCO$_3$, the reaction mixture was extracted with AcOEt (50 ml×3). The combined extracts were washed with brine, and dried. The solvent was removed *in vacuo*, and the residue was purified by column chromatography (hexane : AcOEt=3 : 1) to afford **7** (4.49 g, 72%) and **8** (160 mg, 3%).

7: Colorless oil. $[\alpha]_D^{26}$ 38.6° (c=1.01, CHCl$_3$). IR (neat) cm^{-1}: 3400, 2940, 2900, 2850, 1120, 1010, 970. ^{1}H-NMR (CDCl$_3$): δ 3.98 (br, 1H), 3.68 (br d, J=2.6 Hz, 1H), 1.88 (m, 1H), 1.65—1.18 (m, 8H), 0.97 (d, J=6.9 Hz, 3H), 0.96 (d, J=6.9 Hz, 3H), 0.95 (d, J=6.6 Hz, 3H). ^{13}C-NMR (CDCl$_3$): δ 74.4 (d), 71.1 (d), 41.7 (d), 30.1 (d), 28.7 (d), 27.6 (t), 23.6 (t), 21.1 (q), 20.6 (q), 17.5 (q). MS *m/z* : 172 (M$^+$). FAB-MS *m/z*: 172.1455 (Calcd for C$_{10}$H$_{20}$O$_2$ (M$^+$): 172.1463).

8: A colorless solid. $[\alpha]_D^{28}$ −41.1° (c=0.99, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3400, 2950, 2920, 2860, 1450, 1200, 1050. ^{1}H-NMR ($CDCl_3$): δ 3.20 (m, 1H), 2.97 (m, 1H), 2.49 (br s, 1H), 2.44 (br s, 1H), 2.14 (m, 1H), 1.69 (m, 1H), 1.57 (m, 1H), 1.46—1.26 (m, 2H), 1.17—0.92 (m, 2H), 1.03 (d, J=6.3 Hz, 3H), 0.93 (d, J=6.9 Hz, 3H), 0.82 (d, J=6.9 Hz, 3H). ^{13}C-NMR ($CDCl_3$): δ 81.5 (d), 76.4 (d), 47.7 (d), 37.6 (d), 32.4 (t), 25.9 (d), 22.5 (t), 20.9 (q), 18.3 (q), 15.9 (q). MS m/z: 154 (M^+-H_2O), 137, 136. FAB-MS m/z: 172.1469 (Calcd for $C_{10}H_{20}O_2$ (M^+): 172.1463).

(1*S*,2*R*,3*S*,6*R*)-2-Hydroxy-6-methyl-3-(methylethyl)cyclohexyl Benzoate (12) and (1*S*,2*R*,3*R*,6*S*)-2-Hydroxy-3-methyl-6-(methylethyl)cyclohexyl Benzoate (13) Aqueous 2% OsO_4 (5 ml, 0.394 mmol) was added to a mixed solution of aqueous 50% NMO (31 ml, 130 mmol) and **11** (6.00 g, 43.5 mmol) in acetone (200 ml) and H_2O (100 ml) at 0 °C. The whole was stirred at room temperature for 16 h. After addition of 5% of aqueous sodium bisulfite (16 ml), the whole was stirred for 30 min, then concentrated *in vacuo* to remove acetone, and the residue was extracted with AcOEt (50 ml×3). The combined extracts were washed with brine and dried. After removal of the solvent *in vacuo*, the residue was purified by column chromatography (hexane : AcOEt=1 : 1) to afford a mixture of **9** and **10** (7.13 g; 95%; **9** : **10**=2 : 7). Benzoyl chloride (9.59 ml, 82.8 mmol) was added dropwise to a mixed solution of a mixture of **9** and **10** (7.13 g, 41.4 mmol) and pyridine (13.4 ml, 165 mmol) in CH_2Cl_2 (200 ml) at 0 °C. The whole was stirred at room temperature for 6 h. Reaction was quenched by addition of 5% of aqueous $NaHCO_3$, then the organic layer was washed with 5% of aqueous HCl and brine, and dried. After removal of the solvent *in vacuo*, the residue was purified by column chromatography (hexane : AcOEt=20 : 1) to give **12** (2.51 g, 22%) and **13** (8.79 g, 76%).

12: A colorless solid. mp 95—98 °C (hexane). $[\alpha]_D^{27}$ −50.1° (c=1.02, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3600, 2950, 2850, 1700, 1450, 1310, 1270, 1100. ^{1}H-NMR ($CDCl_3$): δ 8.16—8.04 (m, 2H), 7.65—7.43 (m, 3H), 4.72 (dd, J=11.2, 2.6 Hz, 1H), 4.19 (br d, J=2.3 Hz, 1H), 2.14 (m, 1H), 1.84 (m, 1H), 1.76—1.59 (m, 2H), 1.44 (m, 1H), 1.19—1.06 (m, 2H), 0.95 (d, J=6.6 Hz, 3H), 0.94 (d, J=6.9 Hz, 3H), 0.94 (d, J=6.9 Hz, 3H). ^{13}C-NMR ($CDCl_3$): δ 166.0 (s), 133.0 (d), 130.3 (s), 129.6 (d), 128.4 (d), 81.9 (d), 69.1 (d), 47.7 (d), 32.9 (t), 30.3 (d), 28.9 (d), 23.2 (t), 21.1 (q), 20.6 (q), 18.1 (q). MS m/z: 277 (M^++H), 137, 105, 77.

13: A colorless solid. mp 86—88 °C (hexane). $[\alpha]_D^{27}$ −57.2° (c=1.04, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3600, 2950, 2920, 2850, 1700, 1440, 1260, 1100 1090. ^{1}H-NMR ($CDCl_3$): δ 8.11—8.04 (m, 2H), 7.61—7.43 (m, 3H), 4.99 (dd, J=11.9, 2.6 Hz, 1H), 4.00 (br t, J=2.3 Hz, 1H), 2.06 (m, 1H), 1.89 (dqq, J=6.9, 6.9, 3.0 Hz, 1H), 1.76—1.60 (m, 2H), 1.49—1.41 (m, 2H), 1.12 (m, 1H), 1.03 (d, J=6.6 Hz, 3H), 0.93 (d, J=6.9 Hz, 3H), 0.79 (d, J=6.9 Hz, 3H). ^{13}C-NMR ($CDCl_3$): δ 165.8 (s), 133.0 (d), 130.3 (s), 129.6 (d), 128.4 (d), 77.6 (d), 72.4 (d), 39.5 (d), 35.6 (d), 26.9 (t), 26.2 (d), 23.3 (t), 20.6 (q), 17.7 (q), 16.3 (q). MS m/z: 277 (M^++H), 137, 105, 77.

(1*R*,2*S*,3*R*,6*S*)-3-Methyl-6-(methylethyl)cyclohexane-1,2-diol (9) K_2CO_3 (4.08 g, 29.6 mmol) was added to a solution of **12** (2.72 g, 9.86 mmol) in MeOH (40 ml) at room temperature, and the whole was stirred for 7 h at the same temperature. After neutralization by addition of 10% aqueous HCl, MeOH was removed *in vacuo*. The aqueous residue was extracted with AcOEt (40 ml×4). The combined extracts were washed with brine and dried. After removal of the solvent, the residue was purified by column chromatography (hexane : AcOEt=2 : 1) to give **9** (1.64 g, 97%) as a colorless solid. mp 78—80 °C. $[\alpha]_D^{26}$ −26.3° (c=1.02, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3420, 3025, 2930, 2860, 1450, 1380, 1200, 940. ^{1}H-NMR ($CDCl_3$): δ 4.01 (br s, 1H), 3.06 (br d, J=9.9 Hz, 1H), 2.05 (br s, 1H), 1.90 (br s, 1H), 1.75—1.54 (m, 4H), 1.27 (m, 1H), 1.07—0.89 (m, 2H), 1.00 (d, J=6.3 Hz, 3H), 0.97 (d, J=6.9 Hz, 3H), 0.93 (d, J=6.6 Hz, 3H). ^{13}C-NMR ($CDCl_3$): δ 78.7 (d), 71.1 (d), 47.9 (d), 32.8 (t), 32.8 (d), 29.0 (d), 23.3 (t), 21.2 (q), 20.7 (q), 18.3 (q). MS m/z: 172 (M^+), 154, 137, 136. FAB-MS m/z: 172.1466 (Calcd for $C_{10}H_{20}O_2$ (M^+): 172.1463).

(1*S*,2*R*,3*R*,6*S*)-3-Methyl-6-(methylethyl)cyclohexane-1,2-diol (10) Solvolysis of **13** (1.55 g, 5.60 mmol) in a similar manner to that described for preparation of **9** gave **10** (944 mg; 98%) as a colorless solid. mp 69—70 °C. $[\alpha]_D^{25}$ −32.1° (c=1.03, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3400, 2950, 2860, 1450, 1380, 1360, 1090, 960. ^{1}H-NMR ($CDCl_3$): δ 3.79 (br s, 1H), 3.37 (br d, J=10.2 Hz, 1H), 2.15—2.01 (m, 3H), 1.62—1.21 (m, 5H), 1.09 (m, 1H), 1.02 (d, J=6.9 Hz, 3H), 0.94 (d, J=6.9 Hz, 3H), 0.81 (d, J=6.9 Hz, 3H). ^{13}C-NMR ($CDCl_3$): δ 74.9 (d), 73.7 (d), 42.3 (d), 35.7 (d), 27.2 (t), 25.8 (d), 22.8 (t), 20.9 (q), 17.9 (q), 16.0 (q). MS m/z: 172 (M^+), 154, 136. FAB-MS m/z: 172.1478 (Calcd for $C_{10}H_{20}O_2$ (M^+): 172.1463).

(1*S*,2*R*,3*R*,6*S*)-3-Methyl-6-(methylethyl)-2-(methylsulfonyloxy)cyclohexyl Benzoate (14) MsCl (3.05 ml, 26.3 mmol) was added to a mixed solution of **13** (3.63 g, 13.2 mmol), pyridine (8.51 ml, 105 mmol) and small amount of DMAP in CH_2Cl_2 (130 ml) at 0 °C, and the whole was heated under reflux for 2 d. The whole was successively washed with 5% of aqueous $NaHCO_3$, 5% of aqueous HCl, and brine, and dried. After concentration *in vacuo*, the residue was purified by column chromatography (hexane : AcOEt=6 : 1) to afford **14** (3.12 g, 67%) as colorless needles. mp 133—135 °C (hexane). $[\alpha]_D^{30}$ −42.6° (c=1.08, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 2950, 1720, 1340, 1280, 1180, 1100. ^{1}H-NMR ($CDCl_3$): δ 8.11—8.06 (m, 2H), 7.61—7.43 (m, 3H), 5.10 (br s, 1H), 4.99 (dd, J=11.7, 2.5 Hz, 1H), 2.95 (s, 3H), 2.12—1.73 (m, 4H), 1.58 (m, 1H), 1.41 (m, 1H), 1.20 (m, 1H), 1.07 (d, J=6.9 Hz, 3H), 0.95 (d, J=6.9 Hz, 3H), 0.77 (d, J=6.9 Hz, 3H). MS m/z: 355 (M^++H), 259, 137, 105, 77.

(1*R*,2*S*,3*S*,6*R*)-2-Hydroxy-6-methyl-3-(methylethyl)cyclohexyl Methylsulfonate (15) Solvolysis of **14** (103 mg, 0.291 mmol) in a similar manner to that described for preparation of **9** gave **15** (72 mg; 99%) as colorless needles. mp 83—84 °C (hexane). $[\alpha]_D^{30}$ −18.1° (c=1.03, $CHCl_3$). IR (KBr) cm^{-1}: 3530, 2940, 1320, 1150, 1030. ^{1}H-NMR ($CDCl_3$): δ 4.94 (br s, 1H), 3.51 (ddd, J=10.9, 6.3, 2.3 Hz, 1H), 3.15 (s, 3H), 2.20—2.09 (m, 2H), 1.75—1.45 (m, 5H), 1.29 (m, 1H), 1.05 (d, J=6.9 Hz, 3H), 0.94 (d, J=6.9 Hz, 3H), 0.81 (d, J=7.3 Hz, 3H). MS m/z: 251 (M^++H), 155, 137.

8 from 15 A stirred solution of **15** (2.52 g, 10.1 mmol) and CsOAc (7.16 g, 37.3 mmol) in DMF (100 ml) was heated at 100 °C for 5 h. The reaction mixture was poured into water, and the whole was extracted with AcOEt. The combined extracts were washed with brine, then dried. After removal of the solvent *in vacuo*, the residue was purified by column chromatography (hexane : AcOEt=4 : 1) to afford a mixture of **16** and **17**) (1.19 g, 55%, **16** : **17**=1 : 1). The mixture (1.10 g) submitted to solvolysis by a similar manner to that described for preparation of **9** gave **8** (728 mg, 82%).

General Procedure for Spirocyclization Reaction without Diol: To a solution of substrate (2 mmol) in CH_2Cl_2 (10 ml) was added dropwise Lewis acid, and the mixture was stirred. The resulting mixture was washed with saturated aqueous $NaHCO_3$ and extracted with AcOEt. The combined organic layers were washed with brine, and dried. After removal of the solvent *in vacuo*, the residue was purified by column chromatography (hexane/AcOEt=4 : 1 to 2 : 1).

Reaction with Diol: To a solution of substrate and diol in CH_2Cl_2 was added dropwise Lewis acid and the mixture was stirred. The reaction mixture was treated in a similar manner as above.

5,5-Dimethyl-3-(4-pentenyl)cyclohex-2-en-1-one (21) A solution of 5,5-dimethyl-3-(2-methylpropoxy)cyclohex-2-en-1-one (2.0 g, 10.2 mmol) in THF (7 ml) was added at 0 °C to a solution of 4-pentenylmagnesium bromide prepared from magnesium (270 mg, 11.2 mmol) and 5-bromo-1-pentene (1.2 ml, 10.2 mmol) in THF (25 ml), and the mixture was stirred for 30 min at room temperature. The reaction was quenched with saturated aqueous NH_4Cl, and extracted with ethyl acetate. The combined organic layers were washed with brine, and dried. After removal of the solvent *in vacuo*, the residue was purified by column chromatography (hexane/AcOEt=10 : 1) to give **21** (1.53 g, 78%) as a colorless oil. IR (neat) cm^{-1}: 2975, 2945, 1660, 1360, 1300, 1280. ^{1}H-NMR ($CDCl_3$): δ 5.89 (m, 1H), 5.80 (m, 1H), 5.07—4.94 (m, 2H), 2.28—2.05 (m, 6H), 1.65—1.07 (m, 4H), 1.04 (s, 6H). MS m/z: 193 (M^++1).

5,5-Dimethyl-3-(4-oxopentyl)cyclohex-2-en-1-one (22) A solution of palladium(II) chloride (0.36 g, 2.04 mmol) and copper(I) chloride (0.5 g, 5.14 mmol) in DMF (21 ml) and water (7 ml) was stirred for 2 h at room temperature under an O_2 atmosphere. To the solution was added a solution of **21** (1.0 g, 5.14 mmol) in DMF (6 ml) and water (2 ml), and the mixture was stirred for 3 h at room temperature. The resulting mixture was washed with saturated NH_4Cl, and extracted with ether. The organic layer was washed with brine, and dried. After removal of solvent *in vacuo*, the residue was purified by column chromatography (hexane/AcOEt=2 : 1) to give **22** (0.78 g, 73%) as a colorless oil. IR (neat) cm^{-1}: 2950, 1700, 1680, 1620, 1400, 1360, 1300. ^{1}H-NMR ($CDCl_3$): δ 5.87 (t, J=1.32 Hz, 1H), 2.53 (t, J=7.3 Hz, 2H), 2.22—2.16 (m, 6H), 2.15 (s, 3H), 1.78 (tt, J=7.3, 6.9 Hz, 2H), 1.03 (s, 3H). MS m/z: 209 (M^++1).

4,4-Dimethylspiro[5.5]undecane-2,8-dione (23) A colorless oil. IR (neat) cm^{-1}: 2950, 1700, 1440, 1380, 1340, 1320. ^{1}H-NMR ($CDCl_3$): δ 2.36—2.17 (m, 8H), 1.95—1.85 (m, 2H), 1.80—1.54 (m, 4H), 1.07 (s, 3H), 1.06 (s, 3H). ^{13}C-NMR ($CDCl_3$): δ 210.0 (s), 209.9 (s), 55.1 (t), 54.0 (t), 51.7 (t), 47.8 (t), 43.7 (s), 40.6 (t), 37.2 (t), 35.8 (s), 31.9 (q), 30.7 (q), 21.7 (t). MS m/z: 209 (M^++1). FAB-MS m/z: 209.1553 (Calcd for $C_{13}H_{21}O_2$ $(M+H)^+$: 209.1541). $[\alpha]_D^{25}$ −15.5° (c=1.0, $CHCl_3$) (91% ee) (Table 2, entry 7).

Determination of the Enantiomeric Excess of 23 p-TsOH–H_2O (10 mg) was added to a solution of compound (−)-**23** (52.7 mg, 0.25 mmol) and (*R*,*R*)-2,3-butanediol (68 mg, 0.76 mmol) in benzene (15 ml), and the whole was refluxed with azeotropic removal of water for 2 h. The resulting mixture was washed with saturated aqueous $NaHCO_3$ and extracted with AcOEt. The combined organic layers were washed with brine, and dried. After removal of solvent *in vacuo*, the residue was purified by column chromatography (hexane/AcOEt=9 : 1) to give the corresponding bisacetal (**24**) (87 mg, 98% yield) as a colorless oil. IR (neat) cm^{-1}: 2970, 2940, 2860, 1450, 1370, 1320, 1300, 1220, 1200. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.63—3.49 (m, 4H), 2.08—2.00 (m, 2H), 1.66—1.23 (m, 12H), 1.18—1.20 (m, 3H×4), 1.03 [0.97] (s, 3H), 0.93 [0.99] (s, 3H). ^{13}C-NMR (125.7 MHz, $CDCl_3$): δ 109.5 (s), 109.4 (s), 77.81 [77.85] (d), 77.71 [77.56] (d), 77.48 [77.19] (d), 76.52 [76.68] (d), 51.90 [49.68] (t), 48.6 [48.2] (t), 46.1 [45.8] (t), 43.5 [43.9] (t), 40.2 [38.3] (t), 37.1 [37.0] (t), 36.9 (s), 33.4 [32.4] (q), 32.2 (s), 31.43 [30.40] (q), 19.3 [19.7] (t), 17.22 [17.21] (q), 17.14 [17.08] (q), 17.04 [16.96] (q), 16.9 [16.8] (q). Chemical shifts are those of the major diastereomer, bisacetal of (−)-**23**, and those in parentheses are based on the minor diastereomer, bisacetal of (+)-**23**. The ee of **23** was determined based on the average ratio of these peak intensities.

References and Notes

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4) Torisawa Y., Okabe H., Ikegami S., *Chem. Lett.*, **1984**, 1555.
5) Reaction of **14** with CsOAc at 150 °C in DMF gave elimination product (1*S*,6*S*)-3-methyl-6-(methylethyl)cyclohex-2-enyl benzoate in 43% yield.
6) For compounds **19** and **20**, absolute stereochemistry of acetal carbons was not determined.
7) The absolute stereochemistry of (−)-**23** was not determined.

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Sterically Constrained 'Roofed' 2-Thiazolidinones as Excellent Chiral Auxiliaries

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The "roofed" chiral 2-thiazolidinones, which are sterically congested and conformational rigid, and which are prepared by the [4+2] cycloaddition of 2-thiazolone to the cyclic dienes, dimethylanthracene and hexamethylcyclopentadiene, followed by optical resolution with (1S,2R)-2-methoxy-1-apocamphanecarbonic acid (MAC acid) are of considerable promise for use as chiral auxiliaries for the alkylation of enolates.

Key words 2-thiazolone; [4+2] cycloadduct; 2-thiazolidinone; chiral auxiliary; optical resolution

Methods involving the use of heterocyclic chiral auxiliaries for a wide range of asymmetric transformations have been highly successful, in terms of the stereoselective construction of a number of natural products and biologically important compounds. Of the heterocyclic auxiliaries which have been explored thus far, the chiral 2-oxazolidinones[1] and 2-imidazolidinones[2] hold considerable promise, because of their high versatility and high degrees of asymmetric induction.

We previously reported on the preparation of sterically congested and conformationally fixed 2-oxazolidinones and 2-imidazolidinones with "roofed" structures such as **1** (**a**: DMAOx[3], **b**: DMAIm[4]) and **2** (**a**: HMCOx[5], **b**: HMCIm[6]). These substances serve as excellent chiral auxiliaries in enantiocontrolled typical transformations ranging from alkylations to Michael type additions as well as Diels–Alder reactions.

This paper describes the facile synthesis of the "roofed" type of chiral 2-thiazolidinones **8**—**11** with the same steric demands as those of the tricyclic compounds **1** and **2**, in which conformational rigidity and steric shielding, largely the result of the bridgehead methyl groups, would allow these compounds to serve as highly promising chiral auxiliaries, as previously pointed out.[3—6]

By simply heating the 3-acetyl-2-thiazolone[7] **3**, which is readily derived from commercially available 2-bromothiazole, with a slight excess of 9,10-dimethylanthracene **4** and 1,1,2,3,4,5-hexamethylcyclopentadiene[8] **5** at 140 °C, the uncatalyzed cycloaddition reaction proceeded smoothly to give the [4+2] cycloadducts, **6a** and **7a**, respectively. The straightforward optical resolution of the resulting cycloadducts was performed in a manner analogous to that used in the preparation of the same types of 2-oxazolidinones (**1a**, **2a**)[3,5] and 2-imidazolidinones (**1b**, **2b**).[4,6] The cycloadducts **6c** and **7c**, after deacetylation with cesium carbonate in methanol, were converted with the aid of (1*S*,2*R*)-2-methoxy-1-apocamphanecarbonyl chloride (MAC chloride)[9] to the readily separable *N*-acylated diastereomers **8b**, **9b** and **10b**, **11b**, respectively, in which the MAC auxiliary was smoothly removed with the $LiBH_4$–MeOH agents,[10] to give nearly quantitative yields of both enantiomers. These simple and high yield processes are of practical use for a relatively large scale preparation of the cycloadduct-based chiral, crystalline 2-thiazolidinones **8c**—**11c**.

The stereochemistry of the sterically constrained 2-thiazo-

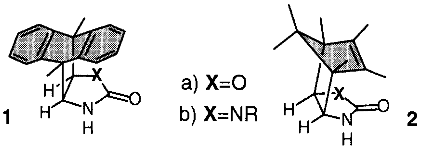

Fig. 1

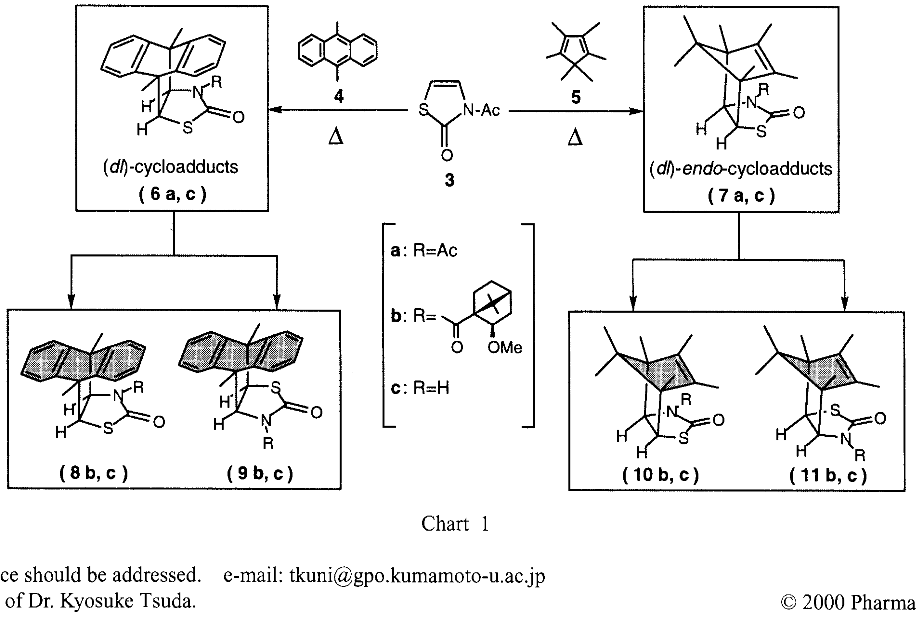

Chart 1

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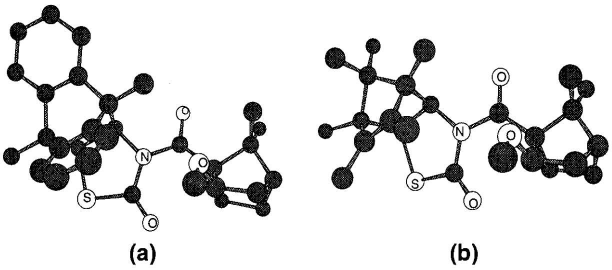
Dedicated to the memory of Dr. Kyosuke Tsuda.

lidinone chiral auxiliaries **8c** and **10c** thus obtained was unequivocally confirmed by X-ray crystal analysis[11] of the *N*-MAC isomers **8b** and **10b**, as shown in Fig. 2.



(a) **(b)**

Fig. 2. Perspective View of **8b** (**a**) and **10b** (**b**) by X-Ray Analysis

The high potential of this type 2-thiazolidinones as chiral auxiliaries was demonstrated by diastereocontrolled alkylations of the *N*-acyl-2-thiazolidinones (**12**—**15**) *via* the enolates. As can be seen in Table 1, the reactions proceeded with high to excellent diastereoselectivity, as expected, and both types of compounds **8c** (**9c**) and **10c** (**11c**) were equally effective for a high level of asymmetric induction, although the latter gave a somewhat higher selectivity on treatment with Lithium bis(trimethyl silyl) amide (LHMDS) as a base.[12] Even in the case of the methylation of *N*-butyryl-2-thiazolidinone enolates with methyl iodide, a reaction which is generally difficult to control with high degrees of diastereoselection,[10a,13] an excellent diastereomer ratio of 118 : 1 was obtained using auxiliary **10c**.

In conclusion, newly synthesized 2-thiazolidinones **8c**—**11c**, which are conformationally fixed and sterically congested, all serve equally well as useful chiral heterocyclic auxiliaries, which belong to the same category of excellent chiral 2-oxazolidinone and 2-imidazolidinone auxiliaries (**1**, **2**) which have been previously developed.[3—6]

Experimental

General Methods Melting points were determined with a Yanaco micro melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP 370 polarimeter. ^{1}H-NMR spectra were recorded in $CDCl_3$ with tetramethylsilane as the internal standard on a JEOL ALPHA-500 (500 MHz) and JEOL JNM-GX400 (400 MHz) spectrometers. MS and HRMS were obtained with a JEOL JMS-DX303HF mass spectrometer. Column chromatography was performed using Silica gel 60 (70—230 mesh, Merck). HPLC analysis was performed on a COSMOSIL® column (Nacalai Tesque, Inc.). Capillary gas chromatography was performed on a Shimadzu GC-14A equipped with a flame ionization detector using a TC-17 column. X-ray crystallographic data was measured on a Rigaku AFC7R diffractometer with graphite monochromated Cu*K*α radiation and a rotating anode generator. All solvents were distilled prior to use; THF over Na/benzophenone, Et_2O over $LiAlH_4$, CH_2Cl_2 over CaH_2, MeOH over NaOMe and benzene over CaH_2.

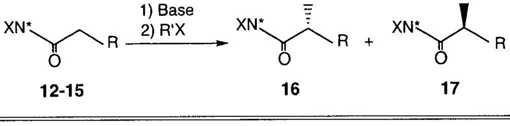
3-Acetyl-2-thiazolone (3) The reaction mixture of 2-bromothiazole (5.0 g) and NaOH (3.6 g) in *tert*-butyl alcohol (30 ml) was refluxed with stirring for 2 d, after which time and acetic anhydride (30 ml) was added. The reaction mixture was then heated at 100 °C for an additional 2 h. Removal of the precipitates followed by evaporation *in vacuo* and chromatographic purification on silica gel (hexane–AcOEt (20 : 1)) gave 3-acetyl-2-thiazolone (**3**) (2.2 g, 51%) as colorless crystals, mp 37—38 °C (from CH_2Cl_2–hexane). ^{1}H-NMR δ: 2.62 (3H, s), 6.25 (1H, d, J=6.1 Hz), 7.36 (1H, d, J=6.1 Hz). *Anal.* Calcd for $C_5H_5NO_2S$: C, 41.95; H, 3.52; N, 9.78. Found: C, 41.71; H, 3.37; N, 9.55.

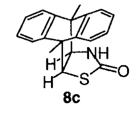
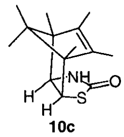
(*dl*)-5-Acetyl-1,7-dimethyl-3-thio-5-aza-dibenzo[h,k]tricyclo[5.2.2.0^{2,6}]-undeca-8,10-dien-4-one (6a) The solution of 3-acetyl-2-thiazolone (**3**) (1.0 g, 7.0 mmol) and 9,10-dimethylanthracene (**4**) (1.6 g, 7.7 mmol) in *m*-xylene (7.0 ml) was heated at 150—160 °C in a sealed tube for 8 d. Removal of the solvent, followed by chromatography on silica gel (CH_2Cl_2) gave the *N*-acetyl-cycloadducts **6a** (2.2 g, 89%) as colorless crystals, mp 238—239 °C (from CH_2Cl_2–hexane). ^{1}H-NMR δ: 1.86 (3H, s), 1.95 (3H, s), 2.43 (3H, s), 3.89 (1H, d, J=9.7 Hz), 4.97 (1H, d, J=9.7 Hz), 7.22—7.40 (8H, m). *Anal.* Calcd for $C_{21}H_{19}NO_2S$: C, 72.18; H, 5.48; N, 4.01. Found: C, 72.18; H, 5.24; N, 3.99.

(*dl*)-1,7-Dimethyl-3-thio-5-aza-dibenzo[h,k]tricyclo[5.2.2.0^{2,6}]undeca-8,10-dien-4-one (6c) A solution of the *N*-acetyl-cycloadducts **6a** (2.1 g, 6.0 mmol) in MeOH (6.0 ml) was treated with Cs_2CO_3 (0.2 g, 0.6 mmol) at room temperature for 2 h. The mixture was acidified with 3 M aqueous solution of citric acid and extracted with AcOEt. The usual work-up followed by chromatographic purification on silica gel (CH_2Cl_2–AcOEt (9 : 1)) gave the crystalline *N*-deacetylated cycloadducts **6c** (1.5 g, 81%), mp >300 °C (from EtOH). ^{1}H-NMR δ: 1.92 (3H, s), 1.93 (3H, s), 3.89 (1H, d, J=9.7 Hz), 4.03 (1H, d, J=9.7 Hz), 6.08 (1H, br), 7.20—7.34 (8H, m). *Anal.* Calcd for $C_{19}H_{17}NOS$: C, 74.23; H, 5.57; N, 4.56. Found: C, 74.23; H, 5.36; N, 4.35.

5-[(1*S*,2*R*)-2-Methoxy-7,7-dimethylbicyclo[2.2.1]heptane-1-carbonyl]-1,7-dimethyl-3-thio-5-aza-dibenzo[h,k]tricyclo[5.2.2.0^{2,6}]undeca-8,10-dien-4-ones. Diastereoisomers, 8b and 9b To a solution of *N*-deacetylated cycloadducts **6c** (2.3 g, 7.3 mmol) and BuLi (1.5 M hexane solution; 5.4 ml, 8.0 mmol) in THF (70 ml), the MAC chloride prepared from (1*S*,2*R*)-7,7-dimethyl-2-methoxybicyclo[2.2.1]heptane-1-carboxylic acid (MAC acid) (1.6 g, 8.0 mmol) was added along with an excess $SOCl_2$ and the entire reaction mixture was heated at 50 °C for 5 h. The mixture was treated with a saturated aqueous solution of NH_4Cl and extracted with AcOEt. The usual work-up gave a diastereomeric mixture of the *N*-MAC derivatives which were cleanly separated by chromatography on silica gel (CH_2Cl_2–hexane

Table 1. Diastereoselective Alkylation of Chiral *N*-Propionyl and *N*-Butyryl-2-thiazolidinones (**12**—**15**)



HXN*	R	Base (1.05 eq)	R'X	Temp (°C)	Yield (%)[a]	**16**:**17**
 **8c**	Me (**12**)	NaHMDS	$PhCH_2Br$	−78	68	53 : 1[b]
	Me (**12**)	NaHMDS	CH_2=$CHCH_2Br$	−78	56	39 : 1[b]
	Et (**13**)	NaHMDS	CH_3I	−78	93	32 : 1[b]
 **10c**	Me (**14**)	LHMDS	$PhCH_2Br$	−30	87	110 : 1[c]
	Me (**14**)	LHMDS	CH_2=$CHCH_2Br$	−30	71	>300 : 1[c]
	Et (**15**)	LHMDS	CH_3I	−30	90	66 : 1[c]
	Et (**15**)	LHMDS	CH_3I	−50	91	118 : 1[c]

a) Isolated yields. *b*) Determined by HPLC analysis. *c*) Determined by capillary GC.

(3 : 7)) to give the *N*-MAC diastereomers **8b** (1.6 g, 44%) and **9b** (1.6 g, 44%).

Isomer **8b**: mp 215—216 °C (from CH_2Cl_2–hexane). $[\alpha]_D^{27}$ −235.4° (c=1.00, $CHCl_3$). ^{1}H-NMR δ: 1.04—1.08 (1H, m), 1.14 (3H, s), 1.34 (3H, s), 1.61—1.71 (4H, m), 1.86—1.98 (2H, m), 1.92 (3H, s), 1.93 (3H, s), 3.17 (3H, s), 3.95 (1H, d, J=10.3 Hz), 4.25—4.28 (1H, m), 5.19 (1H, d, J=10.3 Hz), 7.18—7.40 (8H, m). *Anal.* Calcd for $C_{30}H_{33}NO_3S$: C, 73.89; H, 6.82; N, 2.87. Found: C, 73.76; H, 6.94; N, 2.71.

X-Ray Crystal Data for **8b**: $C_{30}H_{33}NO_3S$, monoclinic, $P2_1$, a=13.246(2) Å, b=8.326(2) Å, c=11.720(1) Å, β=98.478(9)°, V=1278.4(4) Å^3, Z=2, $\mu(\mathrm{Cu}K\alpha)$=13.37 cm^{-1}, R=0.060.

Isomer **9b**: mp 182—183 °C (from CH_2Cl_2–hexane). $[\alpha]_D^{29}$ +232.4° (c=1.00, $CHCl_3$). ^{1}H-NMR δ: 1.09—1.10 (1H, m), 1.17 (3H, s), 1.32 (3H, s), 1.57—1.63 (2H, m), 1.78—1.80 (2H, m), 1.84 (3H, s), 1.89—1.91 (1H, m), 1.93 (3H, s), 2.05—2.09 (1H, m), 3.07 (3H, s), 3.92 (1H, d, J=9.7 Hz), 4.13—4.15 (1H, m), 5.17 (1H, d, J=9.7 Hz), 7.20—7.37 (8H, m). *Anal.* Calcd for $C_{30}H_{33}NO_3S$: C, 73.89; H, 6.82; N, 2.87. Found: C, 73.97; H, 6.86; N, 2.92.

(−)-1,7-Dimethyl-3-thio-5-aza-dibenzo[h,k]tricyclo[5.2.2.0^{2,6}]undeca-5H-8,10-dien-4-one [(−)-DMATh] (8c) A solution of diastereomer **8b** (0.4 g, 0.8 mmol) in THF (8.3 ml) was treated with $LiBH_4$ (3.4 mmol)–MeOH (0.3 ml) at 50 °C for 10 h. Purification of the deacylated product by chromatography on silica gel (hexane–AcOEt (9 : 1)) gave the (−)-enantiomer **8c** as colorless crystals (0.25 g, 99%), mp 125—126 °C (from EtOH). $[\alpha]_D^{27}$ −122.5° (c=1.00, $CHCl_3$). *Anal.* Calcd for $C_{19}H_{17}NOS$: C, 74.23; H, 5.57; N, 4.56. Found: C, 74.39; H, 5.70; N, 4.55.

(+)-1,7-Dimethyl-3-thio-5-aza-dibenzo[h,k]tricyclo[5.2.2.0^{2,6}]undeca-5H-8,10-dien-4-one [(+)-DMATh] (9c) A similar treatment of the *N*-MAC isomer **9b** (0.6 g, 1.2 mmol) as above gave the (+)-enantiomer **9c** (0.22 g, 58%) as colorless crystals, mp 121—122 °C (from EtOH). $[\alpha]_D^{25}$ +123.6° (c=1.00, $CHCl_3$). *Anal.* Calcd for $C_{19}H_{17}NOS$: C, 74.23; H, 5.57; N, 4.56. Found: C, 74.36; H, 5.69; N, 4.52.

(*dl*)-5-Acetyl-1,7,8,9,10,10-Hexamethyl-3-thio-5-azatricyclo[5.2.1.0^{2,6}]-dec-8-en-4-one (7a) A solution of 3-acetyl-2-thiazolone (**3**) (2.0 g, 13.9 mmol) and hexamethylcyclopentadiene$^{8)}$ (**5**) (3.14 g, 20.9 mmol) in *m*-xylene (7.0 ml) was heated at 150—160 °C in a sealed tube for 3 d. Removal of the solvent, followed by chromatography on silica gel (hexane–AcOEt (9 : 1)) gave the cycloadducts **7a** (2.0 g, 49%) as a yellow oil. ^{1}H-NMR δ: 0.67 (3H, s), 0.79 (3H, s), 1.01 (3H, s), 1.10 (3H, s), 1.50 (3H, d, J=1.2 Hz), 1.67 (3H, d, J=1.2 Hz), 2.42 (3H, s), 3.80 (1H, d, J=9.1 Hz), 4.96 (1H, d, J=9.1 Hz). MS (FAB) *m/z*: 294 (MH$^+$), 252, 150.

(*dl*)-1,7,8,9,10,10-Hexamethyl-3-thio-5-azatricyclo[5.2.1.0^{2,6}]dec-8-en-4-one (7c) Analogous to the procedure for **6c**, deacetylation of cycloadducts **7a** (1.3 g, 4.4 mmol) with Cs_2CO_3 (0.1 g, 0.4 mmol) gave crystalline **7c** (0.9 g, 86%), mp 185 °C (from hexane). ^{1}H-NMR δ: 0.68 (3H, s), 0.70 (3H, s), 0.99 (3H, s), 1.04 (3H, s), 1.58 (3H, d, J=1.2 Hz), 1.68 (3H, d, J=1.2 Hz), 3.97 (1H, d, J=9.1 Hz), 4.02 (1H, d, J=9.1 Hz), 6.50 (1H, br). *Anal.* Calcd for $C_{14}H_{21}NOS$: C, 66.89; H, 8.42; N, 5.57. Found: C, 66.68; H,8.43; N, 5.53.

5-[(1*S*,2*R*)-7,7-Dimethyl-2-methoxybicyclo[2.2.1]heptane-1-carbonyl]-1,7,8,9,10,10-hexa-methyl-3-thio-5-azatricyclo[5.2.1.0^{2,6}]dec-8-en-4-ones. Diastereoisomers 10b and 11b By analogy with the cycloadducts **6c**, **7c** (0.5 g, 1.9 mmol) was acylated with the MAC chloride to give the diastereomeric *N*-MAC derivatives which were cleanly separated by chromatography on silica gel (CH_2Cl_2–hexane (3 : 7)) to the diastereomers **10b** (0.4 g, 47%) and **11b** (0.4 g, 47%).

Isomer **10b**: mp 119—120 °C (from CH_2Cl_2–hexane). $[\alpha]_D^{30}$ −293.6° (c=1.00, $CHCl_3$). ^{1}H-NMR δ: 0.68 (3H, s), 0.78 (3H, s), 1.00 (3H, s), 1.05 (3H, s), 1.08—1.11 (1H, m), 1.14 (3H, s), 1.35 (3H, s), 1.57 (3H, d, J=1.2 Hz), 1.53—1.69 (4H, m), 1.65 (3H, d, J=1.2 Hz), 1.90—1.93 (2H, m), 3.14 (3H, s), 3.76 (1H, d, J=8.5 Hz), 4.36—4.38 (1H, m), 4.94 (1H, d, J=8.5 Hz). *Anal.* Calcd for $C_{25}H_{37}NO_3S$: C, 69.57; H, 8.64; N, 3.25. Found: C,69.41; H, 8.58; N, 3.18.

X-Ray Crystal Data for **10b**: $C_{25}H_{37}NO_3S$, orthorhombic, $P2_12_12_1$, a=11.459(1) Å, b=25.324(1) Å, c=8.303(1) Å, V=2409.4(4) Å^3, Z=4, $\mu(\mathrm{Cu}K\alpha)$=13.83 cm^{-1}, R=0.043.

Isomer **11b**: mp 114—115 °C (from CH_2Cl_2–hexane). $[\alpha]_D^{31}$ +250.6° (c=0.98, $CHCl_3$). ^{1}H-NMR δ: 0.67 (3H, s), 0.77 (3H, s), 0.99 (3H, s), 1.05 (3H, s), 1.09—1.12 (1H, m), 1.14 (3H, s), 1.28 (3H, s), 1.57 (3H, d, J=1.2 Hz), 1.65 (3H, d, J=1.2 Hz), 1.60—1.63 (2H, m), 1.75—1.88 (3H, m), 2.23—2.27 (1H, m), 3.13 (3H, s), 3.75 (1H, d, J=8.5 Hz), 4.12—4.15 (1H, m), 5.15 (1H, d, J=8.5 Hz). *Anal.* Calcd for $C_{25}H_{37}NO_3S$: C, 69.57; H, 8.64; N, 3.25. Found: C,69.58; H, 8.73; N, 3.14.

(−)-1,7,8,9,10,10-Hexamethyl-3-thio-5-azatricyclo[5.2.1.0^{2,6}]dec-8-en-4-one (10c) Treatment of the diastereomer **10b** (1.0 g, 2.3 mmol) with $LiBH_4$ (9.2 mmol)–MeOH (0.7 ml) at 50 °C for 10 h gave the (−)-enantiomer **10c** as colorless crystals (0.5 g, 82%), mp 219—220 °C (from EtOH). $[\alpha]_D^{31}$ −163.2° (c=1.00, $CHCl_3$). *Anal.* Calcd for $C_{14}H_{21}NOS$: C, 66.89; H, 8.42; N, 5.57. Found: C, 66.71; H,8.55; N, 5.33.

(+)-1,7,8,9,10,10-Hexamethyl-3-thio-5-azatricyclo[5.2.1.0^{2,6}]dec-8-en-4-one (11c) Similar treatment of the *N*-MAC isomer **11b** (1.0 g, 2.3 mmol), as described above, gave the (+)-enantiomer **11c** (0.3 g, 60%) as colorless crystals, mp 218—219 °C (from EtOH). $[\alpha]_D^{31}$ +162.0° (c=1.00, $CHCl_3$). *Anal.* Calcd for $C_{14}H_{21}NOS$: C, 66.89; H, 8.42; N, 5.57. Found: C, 66.71; H,8.55; N, 5.33.

(−)-5-Propionyl-1,7-dimetyl-3-thio-5-aza-dibenzo[h,k]tricyclo-[5.2.2.0^{2,6}]undeca-8,10-dien-4-one (12) The lithium salts derived from **8c** (1,2 g, 3.9 mmol) and BuLi (4.3 mmol) in THF (39 ml), were treated with propionyl chloride (4.3 mmol) at −78 °C for 1 h and then at 0 °C for 2 h. The usual work-up followed by column chromatography on silica gel (CH_2Cl_2–hexane (3 : 7)) gave the *N*-butyryl derivative **12** (1.36 g, 95%) as colorless crystals, mp 170—171 °C (from hexane). $[\alpha]_D^{30}$ −361.2° (c=1.00, $CHCl_3$). ^{1}H-NMR δ: 1.15 (3H, t, J=7.3 Hz), 1.84 (3H, s), 1.95 (3H, s), 2.73—2.86 (2H, m), 3.97 (1H, d, J=9.7 Hz), 5.00 (1H, d, J=9.7 Hz), 7.22—7.39 (8H, m). *Anal.* Calcd for $C_{22}H_{21}NO_2S$: C, 72.70; H, 5.82; N, 3.85. Found: C, 73.00; H, 5.88; N, 3.86.

(−)-5-Butyryl-1,7-dimethyl-3-thio-5-aza-dibenzo[h,k]tricyclo-[5.2.2.0^{2,6}]undeca-8,10-dien-4-one (13) Analogous to the above procedure, this compound was prepared from **8c** (0.8 g, 2.6 mmol) in 98% yield (0.99 g) as colorless crystals, mp 104—105 °C (from hexane). $[\alpha]_D^{31}$ −356.6° (c=1.00, $CHCl_3$). ^{1}H-NMR δ: 0.97 (3H, t, J=7.3 Hz), 1.65—1.69 (2H, m), 1.84 (3H, s), 1.95 (3H, s), 2.67—2.84 (2H, m), 3.97 (1H, d, J=9.7 Hz), 5.01 (1H, d, J=9.7 Hz), 7.21—7.39 (8H, m). *Anal.* Calcd for $C_{23}H_{23}NO_2S$: C, 73.18; H, 6.14; N, 3.71. Found: C, 73.36; H, 5.99; N, 3.64.

(−)-5-Propionyl-1,7,8,9,10,10-hexamethyl-3-thio-5-azatricyclo-[5.2.1.0^{2,6}]dec-8- en-4-one (14) A solution of (−)-enantiomer **10c** (0.35 g, 1.3 mmol) and propionyl chloride (1.5 mmol) in CH_2Cl_2 (13 ml) was treated in the presence of triethylamine (4.1 mmol) at 0 °C for 1 h and then at 50 °C for another 1 h to give the *N*-butyryl derivative **14** (0.36 g, 85%) as colorless crystals, mp 55—56 °C (from hexane). $[\alpha]_D^{28}$ −297.6° (c=1.00, $CHCl_3$). ^{1}H-NMR δ: 0.67 (3H, s), 0.79 (3H, s), 1.01 (3H, s), 1.09 (3H, s), 1.13 (3H, t, J=7.3 Hz), 1.50 (3H, d, J=1.2 Hz), 1.66 (3H, d, J=1.2 Hz), 2.77—2.82 (2H, m), 3.80 (1H, d, J=9.1 Hz), 4.99 (1H, d, J=9.1 Hz). *Anal.* Calcd for $C_{17}H_{25}NO_2S$: C, 66.41; H, 8.20; N, 4.56. Found: C, 66.38; H, 8.23; N, 4.44.

(−)-5-Butyryl-1,7,8,9,10,10-hexamethyl-3-thio-5-azatricyclo-[5.2.1.0^{2,6}]dec-8- en-4-one (15) Analogous to the above procedure, this compound was prepared from **10c** (0.39 g, 1.5 mmol) in 86% yield (0.43 g) as colorless crystals, mp 78—79 °C (from hexane). $[\alpha]_D^{28}$ −290.2° (c=0.50, $CHCl_3$). ^{1}H-NMR δ: 0.67 (3H, s), 0.79 (3H, s), 0.96 (3H, t, J=7.3 Hz), 1.01 (3H, s), 1.09 (3H, s), 1.50 (3H, d, J=1.2 Hz), 1.66 (3H, d, J=1.2 Hz), 1.64—1.67 (2H, m), 2.74—2.78 (2H, m), 3.80 (1H, d, J=9.1 Hz), 5.00 (1H, d, J=9.1 Hz). *Anal.* Calcd for $C_{18}H_{27}NO_2S$: C, 67.25; H, 8.47; N, 4.36. Found: C, 67.09; H, 8.59; N, 4.11.

General Procedure for Diastereoselective Alkylation To a solution of the *N*-propionyl or *N*-butyryl derivatives **12**—**15** (0.2 mmol) in THF (0.3 ml) was slowly added the base (1.05 eq), prepared *in situ* from hexamethyldisilazane (HMDS) and NaH or BuLi, under an atmosphere of Ar at −30 °C and, after standing for 1 h, alkyl halide was added. The mixture was treated with a saturated solution of NH_4Cl, and then extracted with AcOEt. The crude mixture of alkylated diastereomers thus obtained was directly analyzed by HPLC or capillary gas chromatography.

Absolute configulation of the alkylated adducts were determined as follows:

i) The benzylated products (R′=$PhCH_2$): the optical rotations ($[\alpha]_D$ −10.3° (C_6H_6) from **8c** and $[\alpha]_D$ −10.5° (C_6H_6) from **10c**) of 2-methyl-3-phenyl-1-propanol derived from reductive cleavage with $LiBH_4$–MeOH (1 : 2) were compared with the data previously reported ([lit., for (*S*)-form, $[\alpha]_D$ −11.1° (C_6H_6)]).$^{14)}$

ii) The allylated products (R′=allyl): the authentic species were prepared from the 2-thiazolidinone auxiliaries and (2*R*)-2-methyl-4-propenoic acid.$^{14)}$

16 (HXN*=**8c**, R=Me, R′=allyl): ^{1}H-NMR δ: 1.05 (3H, d, J=6.7 Hz), 1.82 (3H, s), 1.95 (3H, s), 2.04—2.15 (1H, m), 2.53—2.60 (1H, m), 3.56—3.58 (1H, m), 3.97 (1H, d, J=9.7 Hz), 5.00 (1H, d, J=9.7 Hz), 5.09—5.14 (2H, m), 5.78—5.83 (1H, m), 7.22—7.39 (8H, m).

16 (HXN*=**10c**, R=Me, R′=allyl): ^{1}H-NMR δ: 0.67 (3H, s), 0.79 (3H, s), 1.01 (3H, s), 1.06 (3H, s), 1.07 (3H, d, J=6.7 Hz), 1.51 (3H, d, J=1.2 Hz), 1.66 (3H, d, J=1.2 Hz), 2.08—2.14 (1H, m), 2.50—2.56 (1H, m), 3.60—3.64 (1H, m), 3.79 (1H, d, J=9.1 Hz), 5.01 (1H, d, J=9.1 Hz), 5.03—

5.11 (2H, m), 5.72—5.79 (1H, m).

iii) The methylated products (R′=Me): the authentic samples were prepared from the 2-thiazolidinone auxiliaries and commercially available (2*S*)-2-methylbutyric acid.

16 (HXN*=**8c**, R=Et, R′=Me): ^{1}H-NMR δ: 0.85 (3H, t, J=7.9 Hz), 1.22 (3H, d, J=7.3 Hz), 1.34—1.37 (1H, m), 1.65—1.72 (1H, m), 1.83 (3H, s), 1.95 (3H, s), 3.38—3.40 (1H, m), 3.95 (1H, d, J=9.7 Hz), 5.06 (1H, d, J=9.7 Hz), 7.21—7.39 (8H, m).

16 (HXN*=**10c**, R=Et, R′=Me): ^{1}H-NMR δ: 0.67 (3H, s), 0.79 (3H, s), 0.86 (3H, t, J=7.3 Hz), 1.01 (3H, s), 1.08 (3H, s), 1.18 (3H, d, J=6.7 Hz), 1.33—1.39 (1H, m), 1.52 (3H, d, J=1.2 Hz), 1.67 (3H, d, J=1.2 Hz), 1.67—1.69 (1H, m), 3.41—3.45 (1H, m), 3.79 (1H, d, J=9.1 Hz), 5.01 (1H, d, J=9.1 Hz).

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Construction of an Enantiomerically Pure 6-Substituted 3,5-*syn*-Dihydroxyhexanoic Acid System by an Enantioselective Deprotonation Strategy: Formal Synthesis of an Antiobesity Agent, (−)-Tetrahydrolipstatin

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Preparation of 6-substituted 4-hydroxytetrahydro-2*H*-pyran-2-one (12), a key intermediate for the synthesis of (−)-tetrahydrolipstatin, was achieved in an optically pure form by employing an enantioselective deprotonation reaction of the prochiral bicyclic derivative (5) as a key step.

Key words enantioselective deprotonation; 3,5-*O*-benzylidenecyclohexanone; chiral base; (−)-tetrahydrolipstatin; antiobesity agent

(−)-Tetrahydrolipstatin (**1**),[1] a member of the lipstatin family of β-lactone microbial agents, acts as a potent inhibitor of pancreatic lipases,[2] and is used clinically as an antiobesity drug (the trade name Xenical® marketed by Roche) to block the digestion of dietary fat in overweight patients.[3] Due to its attractive pharmacological properties and structural novelty, this lipase inhibitor has been the subject of extensive synthetic efforts which have culminated in several total syntheses in recent years.[4]

Recently we established the asymmetric synthesis of (+)-(4*R*,6*R*)-4-hydroxy-6-(2-phenylethyl)-tetrahydro-2*H*-pyran-2-one, the lactone moiety of HMG-CoA reductase inhibitor, by employing an enantioselective deprotonation[5] of a prochiral 3,5-dibenzyloxycyclohexanone with lithium (*S*,*S*′)-α,α'-dimethyldibenzylamide[6]; however, the enantiomeric excess (ee) of the product in this reaction at −100 °C was determined to be 74%. When this reaction was carried out at −78 °C, the ee was decreased to 70%.[7] We thought that the observed low enantioselectivity arose from the conformational change of the starting material, where, in part, the π–π stacking interaction of the two aromatic rings resulted in the two benzyloxy-substituents moving to the axial orientation. As an extension of our work on the synthesis of biologically active natural products utilizing an enantioselective deprotonation strategy,[8] we are interested in the synthesis of (−)-tetrahydrolipstatin, in which we chose a conformationally rigid starting material based on the consideration of the above result.

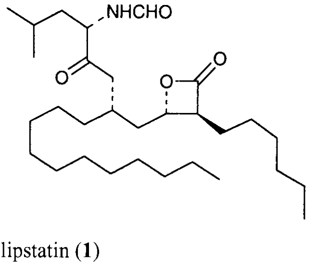

Fig. 1. Tetrahydrolipstatin (**1**)

In analyzing the structure of **1** for retrosynthetic disconnection, we focused our attention on the construction of a 1,3-*syn*-dihydroxy system, in an optically pure form, which could easily be transformed to 6-substituted 4-hydroxytetrahydro-2*H*-pyran-2-one, the key intermediate for the synthesis of (−)-tetrahydrolipstatin, and decided to adopt an enantioselective deprotonation of a prochiral 3,5-disubstituted cyclohexanone derivative as a key reaction.[9]

The requisite starting material for an enantioselective deprotonation reaction was prepared as follows.

Treatment of *cis*,*cis*-1,3,5-cyclohexanetriol (**2**) with trimethyl orthobenzoate in the presence of boron trifluoride etherate gave the *ortho*-ester (**3**) in quantitative yield, which was also prepared from the much cheaper 1,3,5-cyclohexanetriol (*cis*- and *trans*-mixture) by using essentially the same procedure as above, in 50% yield. Partial reduction of the *ortho*-ester (**3**) with borane in tetrahydrofuran (THF) in the presence of hexamethylphosphoric triamide (HMPA) afforded the alcohol (**4**) in 70% yield, which was then subjected to oxidation with Dess–Martin periodinane to provide

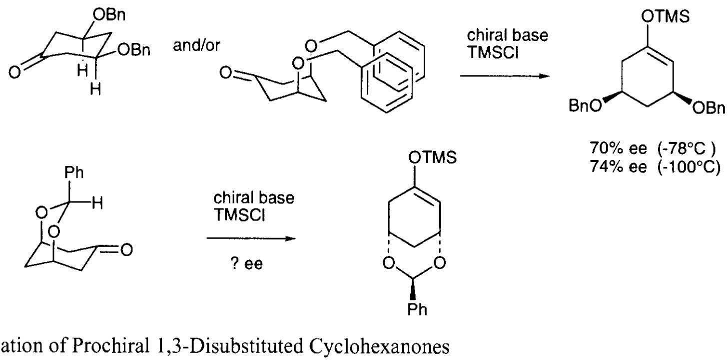

Fig. 2. Enantioselective Deprotonation of Prochiral 1,3-Disubstituted Cyclohexanones

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Dedicated to the memory of Dr. Kyosuke Tsuda.

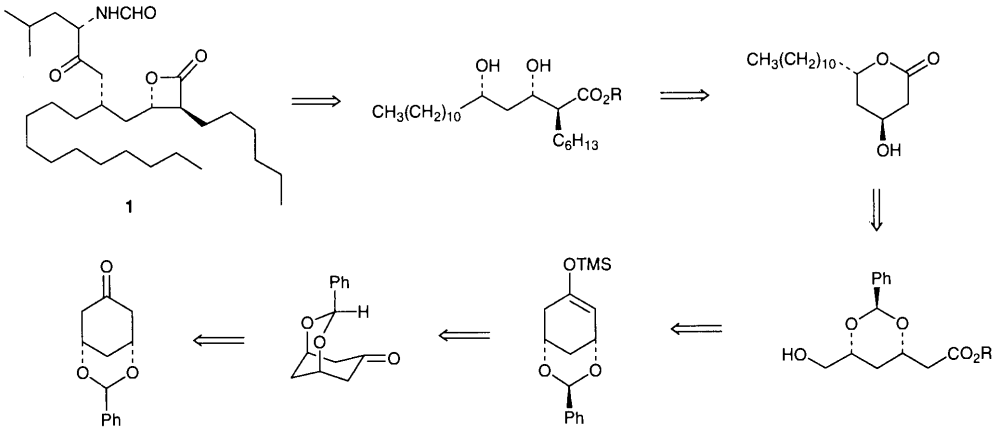
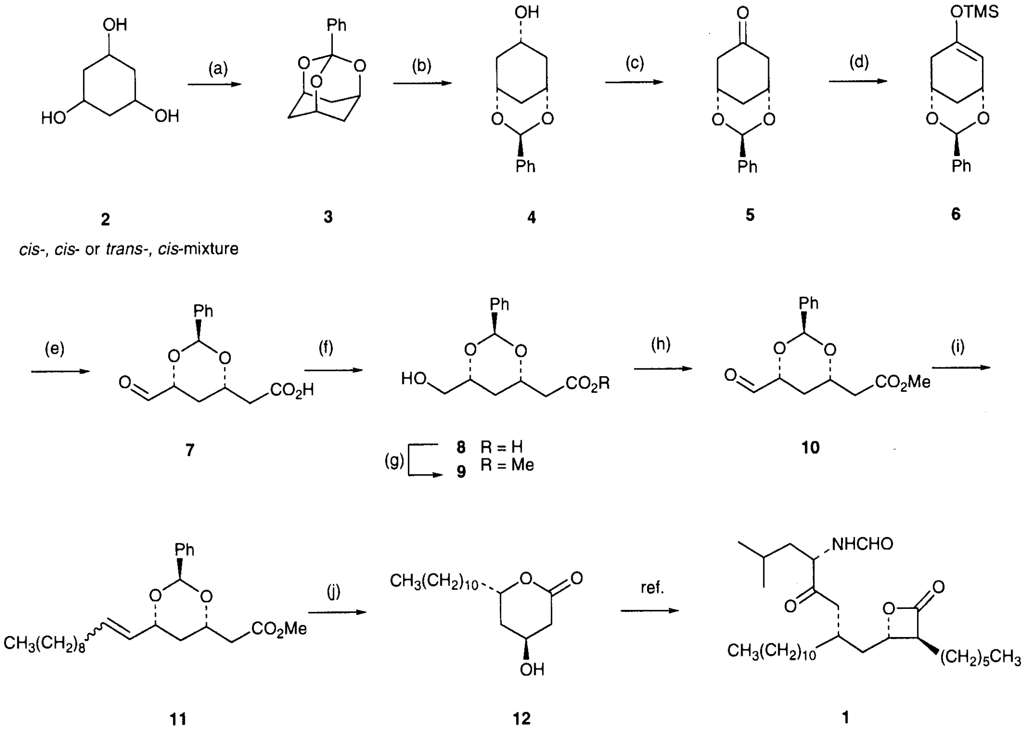


Fig. 3. Retrosynthetic Analysis of Tetrahydrolipstatin (1)



(a) $Ph(OCH_3)_3$, $BF_3 \cdot Et_2O$, CH_2Cl_2, room temp.; (b) $BH_3 \cdot THF$, HMPA–THF, −15 °C; (c) Dess–Martin oxidation; (d) lithium (*S*,*S*′)-α,α'-dimethyldibenzylamide, TMSCl, −100 °C; (e) O_3, MeOH–CH_2Cl_2, −78 °C; then PPh_3; (f) $NaBH_4$, MeOH, room temp.; (g) MeI, K_2CO_3, DMF, room temp.; (h) $(COCl)_2$, DMSO, −78 to −30 °C; then Et_3N, CH_2Cl_2, 0 °C; (i) $C_{10}H_{21}PPh_3Br$, *n*-BuLi, THF, −78 to 0 °C; (j) H_2, $Pd(OH)_2$ on C, AcOH, room temp.

Chart 1

the desired ketone (**5**) in quantitative yield. Enantioselective deprotonation of the ketone (**5**) with lithium (*S*,*S*′)-α,α'-dimethyldibenzylamide in THF in the presence of trimethylsilyl chloride (TMSCl) at −100 °C gave the silyl ether (**6**) in 75% yield. Since the enantiomeric excess of the silyl ether could not be determined at this stage, unfortunately, we decided to use the silyl ether in the next step. Ozonolysis of the silyl ether (**6**) in CH_2Cl_2 at −78 °C, followed by reduction of the ozonide with triphenylphosphine, gave the aldehyde (**7**) in 85% yield. Since we noted in advance[7] that the direct esterification of the aldehyde (**7**) caused partial epimerization of the hydroxy group at the 5-position, the aldehyde (**7**) was converted into the alcohol (**8**) by reduction with sodium borohydride in MeOH. The resulting alcohol (**8**) was further esterified with iodomethane in the presence of potassium carbonate in dimethylformamide (DMF) to give the ester (**9**) in 93% yield from **7**. The enantiomeric excess of the ester (**9**) was determined at this stage to be >99.9% by HPLC analysis with the chiral column CHIRALCEL OJ (Daicel Chemical Industries, Ltd.). It is noteworthy that the enantioselective deprotonation of the bicyclic compound (**5**) afforded the ester (**9**) with >95% ee when this reaction was carried out, even at −78 °C. Thus, the stereocontrolled construction of a 1,3-*syn*-dihydroxy system was achieved, in optically pure form, by adopting an enantioselective deprotonation strategy for the conformationally rigid bicyclic compound. Although the stereostructure of the hydroxy groups could be assumed based on the consideration of previous results,[7] the determination of its absolute configuration still remained obscure.

As a means of determining the absolute configuration of **9** and also by application of this methodology to the natural product synthesis, the alcohol (**9**) was converted into the known lactone (**12**) as follows. Swern oxidation of the alcohol (**9**) gave the aldehyde (**10**) in quantitative yield, which on treatment with Wittig reagent provided the olefin (**11**) as an inseparable mixture of (*E*)- and (*Z*)-isomers in 56% yield. Hydrogenation of the olefin and deprotection of the benzylidene acetal in **11** over palladium hydroxide in acetic acid at room temperature simultaneously brought about a lactone formation to furnish the δ-lactone (**12**), $[\alpha]_D$ −29.9° (c=0.3, $CHCl_3$) in 69% yield.

Since the sign of rotation of our synthetic product (**12**) corresponds to those of the antipodal forms of the structurally related δ-lactonic compounds,[7,9)] the absolute stereochemistry of **12** can now be unambiguously assigned as depicted in Chart 1. This lactone was already converted into (−)-tetrahydrolipstatin by Ghosh; therefore, this synthesis also constitutes its formal synthesis.[10)]

Experimental

IR spectra were recorded for thin films on a JASCO FT/IR-200 Fourier transform IR spectrophotometer. ^{1}H-NMR spectra were obtained for solution in $CDCl_3$ on a JEOL PMX-270 instrument, and chemical shifts are reported on the δ-scale from internal tetramethylsilane (TMS). MS spectra were measured with a JEOL JMS D-300 spectrometer.

3-Phenyl-2,4,10-trioxaadamantane (3) A solution of *cis,cis*-1,3,5-cyclohexanetriol (**2**) (0.5 g, 3.78 mmol), trimethyl orthobenzoate (0.97 ml, 5.67 mmol) and boron trifluoride etherate (0.05 ml, 0.38 mmol) in CH_2Cl_2 (10 ml) was stirred until the solution became clear at room temperature. After the addition of saturated sodium hydrogen carbonate solution, the mixture was extracted with CH_2Cl_2, then the organic layer was washed with water and dried over Na_2SO_4. Evaporation of the solvent gave a residue which was subjected to column chromatography on silica gel. Elution with hexane : EtOAc : CH_2Cl_2 (16 : 4 : 1, v/v) gave **3** as colorless prisms (830 mg, 100%); mp 158—160 °C (from hexane–CH_2Cl_2). IR cm^{-1}: 1124. ^{1}H-NMR ($CDCl_3$) δ: 1.82 (3H, d, J=12.4 Hz, 6-, 8-, 9-H_{ax}), 2.70—2.82 (3H, m, 6-, 8-, 9-H_{eq}), 4.53—4.62 (3H, m, 1-, 5-, 7-H), 7.32—7.41 (3H, m, aromatic protons), 7.64—7.71 (2H, m, aromatic protons). ^{13}C-NMR ($CDCl_3$) δ: 32.7, 68.7, 108.7, 124.8, 127.8, 128.9, 139.4. High-resolution mass spectrometry (HRMS) m/z: Calcd for $C_{13}H_{14}O_3$ (M^+) 218.0943; Found: 218.0960. *Anal.* Calcd for $C_{13}H_{14}O_3$: C, 71.54; H, 6.47. Found: C, 71.38; H, 6.50.

***cis*-3,5-*O*-Benzylidenecyclohexanol (4)** To a stirred solution of the ortho-ester (**3**) (1.0 g, 4.6 mmol) in dry THF (10 ml) containing HMPA (0.88 ml) was added portionwise BH_3–THF (1 M THF solution, 22.9 ml, 22.9 mmol) at −15 °C under argon, and the resulting solution was stirred for a further 60 h at the same temperature. To this solution were added water (50 ml) and ether (50 ml) at −20 °C, and the mixture was stirred for 30 min at ambient temperature. The separated organic layer was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave a residue which was subjected to column chromatography on silica gel. Elution with hexane : EtOAc (2 : 1, v/v) gave the alcohol **4** as colorless needles (708 mg, 70%); mp 185—187 °C (from hexane–CH_2Cl_2). IR (KBr) cm^{-1}: 3560, 1123. ^{1}H-NMR ($CDCl_3$) δ: 1.57 (1H, d, J=14.5 Hz, 4-H), 1.78 (2H, dd, J=4.9, 15.0 Hz, 2-, 6-H), 2.52 (2H, d, J=15.5 Hz, 2-, 6-H), 2.96—3.09 (1H, m, 4-H), 3.80 (1H, d, J=11.0 Hz, OH), 4.03—4.15 (1H, m, 1-H), 4.72 (2H, br s, 3-, 5-H), 6.20 (1H, s, PhCH), 7.32—7.42 (3H, m, aromatic protons), 7.47—7.54 (2H, m, aromatic protons). ^{13}C-NMR ($CDCl_3$) δ: 26.9, 39.2, 65.9, 69.2, 91.8, 126.0, 128.3, 128.8, 138.2. HRMS m/z: Calcd for $C_{13}H_{16}O_3$ (M^+) 220.1100; Found: 220.1100. *Anal.* Calcd for $C_{13}H_{16}O_3 \cdot 5/6H_2O$: C, 66.36; H, 7.57. Found: C, 66.34; H, 7.74.

3,5-*O*-Benzylidenecyclohexanone (5) To a stirred solution of the alcohol (**4**) (1.3 g, 5.91 mmol) in CH_2Cl_2 (10 ml) was added periodinane (7.52 g, 17.7 mmol) at 0 °C under argon. The resulting mixture was stirred for a further 15 min at the same temperature. After treatment with saturated sodium hydrogen carbonate solution, the mixture was extracted with ether and the ethereal layer was washed with brine, dried over Na_2SO_4 and concentrated to leave a residue which was subjected to column chromatography on silica gel. Elution with hexane : EtOAc (2 : 1, v/v) gave the ketone **5** as colorless needles (708 mg, 70%); mp 117—118 °C (from hexane–CH_2Cl_2). IR cm^{-1}: 1713, 1119. ^{1}H-NMR ($CDCl_3$) δ: 1.97 (1H, d, J=14.8 Hz, 4-H), 2.49 (2H, d, J=15.8 Hz, 2-, 6-H), 2.94 (2H, d, J=17.0 Hz, 2-, 6-H), 3.04—3.18 (1H, m, 4-H), 4.82—4.90 (2H, m, 3-, 5-H), 6.00 (1H, s, PhCH), 7.30—7.44 (5H, m, aromatic protons). ^{13}C-NMR ($CDCl_3$) δ: 26.3, 49.7, 69.7, 91.9, 126.6, 128.3, 129.3, 138.0, 207.2. HRMS m/z: Calcd for $C_{13}H_{14}O_3$ (M^+) 218.0941; Found: 218.0939. *Anal.* Calcd for $C_{13}H_{14}O_3 \cdot 1/6H_2O$: C, 70.57; H, 6.53. Found: C, 70.50; H, 6.63.

(3*R*,5*S*)-3,5-*O*-Benzylidene-1-trimethylsiloxycyclohex-1-ene (6) To a stirred solution of (*S,S*′)-α,α'-dimethyldibenzylamine (341 mg, 1.51 mmol) in dry THF (10 ml) was added *n*-BuLi (1.66 M hexane solution, 0.9 ml, 1.49 mmol) at −78 °C under argon, and the resulting solution was stirred for a further 5 min at the same temperature. TMSCl (0.32 ml, 2.52 mmol) was added to the solution and the mixture was cooled to −100 °C. To this solution was added dropwise a solution of the ketone (**5**) (110 mg, 0.51 mmol) in THF (2 ml), and the resulting mixture was stirred for a further 10 min at the same temperature. The mixture was treated with saturated NaCl solution at the same temperature, and the organic solvent was evaporated *in vacuo* to leave an aqueous layer, which was then extracted with ethyl acetate. The extract was washed with brine, dried over Na_2SO_4 and concentrated to leave a residue, which was subjected to column chromatography on silica gel. Elution with hexane : EtOAc (50 : 1, v/v) gave the silyl ether (**6**) as a colorless oil (110 mg, 75%). IR cm^{-1}: 1665, 1111. ^{1}H-NMR ($CDCl_3$) $\delta\delta$: 0.28 (9H, s, 3×Me), 1.60—1.73 (1H, m, 4-H), 2.23—2.45 (2H, m, 6-H_2), 2.78—2.91 (1H, m, 4-H), 4.62—4.74 (2H, m, 3-, 5-H), 5.37 (1H, d, J=5.9 Hz, 2-H), 5.99 (1H, s, PhCH), 7.31—7.41 (3H, m, aromatic protons), 7.41—7.53 (2H, m, aromatic protons). ^{13}C-NMR ($CDCl_3$) δ: 0.44, 25.6, 39.4, 65.7, 67.9, 91.3, 107.3, 126.9, 128.3, 128.9, 139.5, 152.2. HRMS m/z: Calcd for $C_{16}H_{22}O_3Si$ (M^+) 290.1336; Found: 290.1335. *Anal.* Calcd for $C_{16}H_{22}O_3Si$: C, 66.17; H, 7.64. Found: C, 66.46; H, 7.66.

(3*R*,5*S*)-3,5-*O*-Benzylidene-6-oxohexanoic Acid (7) A stream of ozone was bubbled through a stirred solution of the silyl ether (**6**) (150 mg, 0.52 mmol) in CH_2Cl_2 (10 ml) at −78 °C until the disappearance of the starting material on TLC. The reaction mixture was flushed with argon and treated with triphenylphosphine (680 mg, 2.59 mmol) at the same temperature. The mixture was allowed to warm to room temperature over 1 h, then stirred for another 90 min. After evaporation of the solvent, ethyl acetate was added to the residue, and the organic layer was extracted with saturated sodium hydrogen carbonate solution. The aqueous layer was acidified with saturated potassium hydrogen sulfate solution and extracted with ethyl acetate. The extract was washed with brine, dried over Na_2SO_4 and concentrated to leave a residue, which was subjected to column chromatography on silica gel. Elution with EtOAc : MeOH (10 : 1, v/v) gave the acid (**7**) as a colorless solid (110 mg, 85%). IR cm^{-1}: 3420, 1720, 1712. ^{1}H-NMR ($CDCl_3$) δ: 1.56—1.74 (1H, m, 4-H), 1.99—2.10 (1H, m, 4-H), 2.64 (1H, dd, J=5.8, 16.3 Hz, 2-H), 2.82 (1H, dd, J=7.1, 16.1 Hz, 2-H), 4.32—4.46 (2H, m, 3-, 5-H), 5.68 (1H, s, PhCH), 7.33—7.45 (3H, m, aromatic protons), 7.45—7.59 (2H, m, aromatic protons), 9.74 (1H, s, 6-H). ^{13}C-NMR ($CDCl_3$) δ: 30.8, 40.1, 72.7, 79.9, 100.9, 126.2, 128.4, 129.3, 137.2, 173.7. HRMS m/z: Calcd for $C_{13}H_{14}O_5$ (M^+) 250.0840; Found: 250.0855.

(3*R*,5*S*)-3,5-*O*-Benzylidene-6-hydroxyhexanoic Acid (8) To a stirred solution of the aldehyde (**7**) (50 mg, 0.20 mmol) in MeOH (2 ml) was added portionwise sodium borohydride (76 mg, 2.0 mmol) at room temperature. The resulting solution was stirred for a further 3 h. After evaporation of the solvent, the residue was treated with saturated potassium hydrogensulfate solution, and the resulting mixture was extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated to leave a residue, which was subjected to column chromatography on silica gel. Elution with EtOAc : MeOH (1 : 1, v/v) gave the acid (**8**) as a colorless solid (47 mg, 93%); mp 30.5—31.0 °C. IR cm^{-1}: 3426, 1716. ^{1}H-NMR (CD_3OD) δ: 1.45 (1H, dt, J=11.4, 12.9 Hz, 4-H), 1.73 (1H, dt, J=2.5, 12.9 Hz, 4-H), 2.54, 2.62 (each 1H, each dd, J=5.9, 15.7 Hz, 2-H_2), 3.53—3.68 (2H, m, 6-H_2), 3.92—4.05 (1H, m, 3-H), 4.26—4.39 (1H, m, 5-H), 5.59 (1H, s, PhCH), 7.24—7.38 (3H, m, aromatic protons), 7.40—7.54 (2H, m, aromatic protons). ^{13}C-NMR (CD_3OD) δ: 33.6, 42.1, 66.0, 74.7, 78.7, 102.1, 127.5, 128.9, 129.7, 140.1, 174.8. HRMS m/z: Calcd for $C_{13}H_{16}O_5$ (M^+) 252.0996; Found: 252.0996. *Anal.* Calcd for $C_{13}H_{16}O_5 \cdot 1/2H_2O$: C, 59.76; H, 6.79. Found: C, 59.92; H, 6.63.

(3*R*,5*S*)-Methyl 3,5-*O*-Benzylidene-6-hydroxyhexanoate (9) A mixture of the acid (**8**) (356 mg, 1.41 mmol), potassium carbonate (215 mg, 1.55 mmol), iodomethane (0.10 ml, 1.55 mmol) and DMF (3 ml) was stirred for 10 min at room temperature. The mixture was poured into water and extracted with ethyl acetate. The extract was washed with brine, dried over Na_2SO_4 and concentrated to leave a residue which was subjected to column chromatography on silica gel. Elution with hexane : EtOAc (2 : 1, v/v) gave the ester (**9**) as a colorless oil (380 mg, 100%). $[\alpha]_D$ +5.2° (c=1.2, $CHCl_3$).

IR cm^{-1}: 3450, 1736. ^{1}H-NMR (CDCl$_3$) δ: 1.50—1.73 (2H, m, 4-H$_2$), 2.04 (1H, t, J=6.5 Hz, OH), 2.55 (1H, dd, J=6.1, 15.8 Hz, 2-H), 2.77 (1H, dd, J=6.9, 15.8 Hz, 2-H), 3.59—3.79 (2H, m, 6-H$_2$), 3.70 (3H, s, Me), 3.98—4.10 (1H, m, 3-H), 4.29—4.42 (1H, m, 5-H), 5.61 (1H, s, PhCH), 7.29—7.43 (3H, m, aromatic protons), 7.43—7.54 (2H, m, aromatic protons). ^{13}C-NMR (CDCl$_3$) δ: 32.0, 40.7, 51.9, 65.5, 72.8, 77.0, 100.8, 126.2, 128.3, 129.0, 138.0, 171.0. HRMS m/z: Calcd for $C_{14}H_{18}O_5$ (M$^+$) 266.1153; Found: 266.1153. *Anal.* Calcd for $C_{14}H_{18}O_5$: C, 63.15; H, 6.81. Found: C, 62.93; H, 6.96.

(3*R*,5*S*)-Methyl 3,5-*O*-Benzylidene-6-oxohexanoate (10) To a stirred solution of oxalyl chloride (33 μl, 0.38 mmol) in CH$_2$Cl$_2$ (2 ml) was added dropwise dimethyl sulfoxide (36 μl, 0.51 mmol) at −78 °C under argon, and the resulting solution was stirred for a further 10 min. To this solution was slowly added a solution of the alcohol (**9**) (50 mg, 0.19 mmol) in CH$_2$Cl$_2$ (2 ml), and the mixture was further stirred for 30 min at the same temperature and for 30 min at −45 °C. This mixture was allowed to warm to 0 °C, then triethylamine (0.19 ml, 1.37 mmol) was added to the mixture, and the whole was stirred for a further 10 min at the same temperature. After treatment with saturated ammonium chloride solution, the organic solvent was removed *in vacuo* and the residue was extracted with ethyl acetate. The extract was washed with brine, dried over Na$_2$SO$_4$ and concentrated to leave a residue, which was subjected to column chromatography on silica gel. Elution with hexane : EtOAc (1 : 1, v/v) gave the aldehyde (**10**) as a colorless oil (50 mg, 100%). IR cm^{-1}: 1740, 1734. ^{1}H-NMR (CDCl$_3$) δ: 1.53—1.71 (1H, m, 4-H), 1.65 (1H, dt, J=2.6, 13.2 Hz, 4-H), 2.58 (1H, dd, J=6.1, 15.8 Hz, 2-H), 2.78 (1H, dd, J=6.9, 15.8 Hz, 2-H), 3.71 (3H, s, Me), 4.33—4.47 (2H, m, 3, 5-H), 5.67 (1H, s, PhCH), 7.32—7.45 (3H, m, aromatic protons), 7.48—7.57 (2H, m, aromatic protons), 9.74 (1H, s, 6-H). ^{13}C-NMR (CDCl$_3$) δ: 30.9, 40.5, 51.9, 72.9, 80.0, 100.8, 126.2, 128.4, 129.3, 137.3, 170.7. HRMS m/z: Calcd for $C_{14}H_{15}O_5$ (M$^+$−1) 263.0918; Found: 263.0917.

(3*R*,5*S*)-Methyl 3,5-*O*-Benzylidene-6-hexadecenoate (11) To a stirred solution of decyltriphenylphosphonium bromide (155 mg, 0.32 mmol) in THF (2 ml) was added *n*-BuLi (1.59 M hexane solution, 0.19 ml, 0.19 mmol) at −78 °C under argon, and the solution was stirred for 20 min at the same temperature. To this solution was added a solution of the aldehyde (**10**) (65 mg, 0.31 mmol) in THF (1 ml) at −78 °C. The resulting mixture was stirred for another 30 min at the same temperature and then for 2 h at room temperature. The mixture was treated with saturated ammonium chloride solution, and the organic solvent was evaporated *in vacuo* to leave an aqueous layer, which was extracted with ethyl acetate. The extract was washed with brine, dried over Na$_2$SO$_4$ and concentrated to leave a residue which was subjected to column chromatography on silica gel. Elution with hexane : EtOAc (20 : 1, v/v) gave the olefin (**11**) as an inseparable mixture of (*E*)- and (*Z*)-isomers as a colorless oil (53 mg, 56%). $[\alpha]_D$ −34.3° (c=0.3, CHCl$_3$). IR cm^{-1}: 1742. ^{1}H-NMR (CDCl$_3$) δ: 0.88 (3H, t, J=6.5 Hz, Me), 1.11—1.46 (14H, m, 9-, 10-, 11-, 12-, 13-, 14-, 15-H$_2$), 1.44—1.54 (2H, m, 4-H$_2$), 1.97—2.23 (2H, m, 8-H$_2$), 2.53 (1H, dd, J=6.1, 15.8 Hz, 2-H), 2.75 (1H, dd, J=7.1, 15.8 Hz, 2-H), 3.71 (3H, s, OMe), 4.26—4.44 (1H, m, 3-H), 4.60—4.75 (1H, m, 5-H), 5.39—5.61 (2H, m, 6-, 7-H), 5.63 (1H, s, PhCH), 7.29—7.40 (3H, m, aromatic protons), 7.40—7.53 (2H, m, aromatic protons). ^{13}C-NMR (CDCl$_3$) δ: 14.1, 22.7, 28.0, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 36.6, 40.7, 51.8, 73.0, 73.1, 100.7, 126.2, 128.2, 128.7, 129.0, 133.4, 138.4, 171.2. HRMS m/z: Calcd for $C_{24}H_{36}O_4$ (M$^+$) 388.2613; Found: 388.2605.

(4*S*,6*S*)-4-Hydroxy-6-undecyltetrahydro-2*H*-pyran-2-one (12) A solution of the benzylidene acetal (**11**) (87 mg, 0.22 mmol) in acetic acid (3 ml) was hydrogenated over 10% palladium hydroxide on carbon (87 mg) for 24 h under an atmosphere of hydrogen. The catalyst was filtered off and the filtrate was concentrated to leave a residue, which was subjected to column chromatography on silica gel. Elution with hexane : EtOAc (2 : 1, v/v) gave the lactone (**12**) as colorless needles (42 mg, 69%); mp 57.5—58.0 °C (from hexane–CH$_2$Cl$_2$). $[\alpha]_D$ −29.9° (c=0.3, CHCl$_3$). IR cm^{-1}: 3420, 1728. ^{1}H-NMR (CDCl$_3$) δ: 0.88 (3H, t, J=6.6 Hz, Me), 1.26 (18H, br s, 2′-, 3′-, 4′-, 5′-, 6′-, 7′-, 8′-, 9′-, 10′-H$_2$), 1.53—1.62 (1H, m, 1′-H), 1.69—1.86 (2H, m, 5-, 1′-H), 1.90—2.01 (1H, m, 5-H), 2.61 (1H, ddd, J=1.6, 3.8, 17.6 Hz, 3-H), 2.75 (1H, dd, J=4.9, 17.6 Hz, 2-H), 4.34—4.45 (1H, m, 4-H), 4.62—4.76 (1H, m, 6-H). ^{13}C-NMR (CDCl$_3$) δ: 14.1, 22.7, 24.9, 29.35, 29.4, 29.5, 29.56, 29.62, 29.65, 31.9, 35.5, 36.1, 38.7, 62.9, 75.7, 170.2. HRMS m/z: Calcd for $C_{16}H_{28}O_2$ (M$^+$−18) 252.2087; Found: 252.2087. *Anal.* Calcd for $C_{16}H_{30}O_3$: C, 71.07; H, 11.18. Found: C, 70.54; H, 11.22.

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A Practical Synthesis of the ABC Ring Model of Ecteinascidins

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A practical synthesis of 1,2,3,4,5,6-hexahydro-1,5-imino-10-hydroxy-9-methoxy-3,8,11-trimethyl-3-benzazocin-4-one (3) as an ABC ring model compound of ecteinascidin 743 and safracins from 3-hydroxy-4-methoxy-5-methylbenzaldehyde (7) is described. The overall yield in 15 steps is 27%.

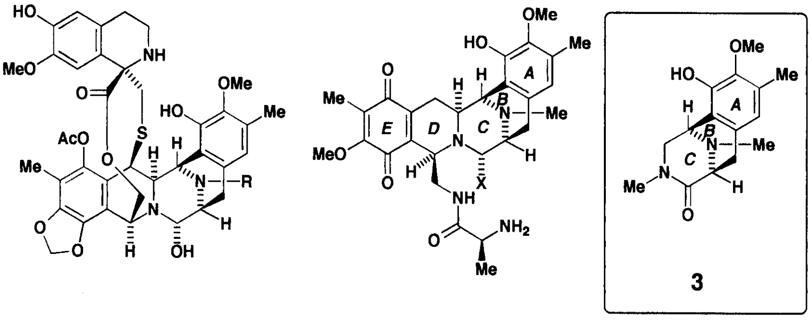
Key words ecteinascidin; safracin; synthesis; Baeyer–Villiger oxidation; regioselectivity

Ecteinascidin 743 (**1a**) is a novel natural marine product discovered from the Caribbean tunicate *Ecteinascidia turbinata* along with ecteinascidin 729 (**1b**) and related compounds.[1] Among this group, **1a** has exceedingly potent antitumor activity and is now undergoing phase II clinical trials.[2] It is a tetrahydroisoquinoline derivative that is structurally related to microbial safracins.[3] Because of their novel and diverse structure, general approaches to their construction have been explored.[4]

We are very interested in the preparation of the ABC ring model compound (**3**), which has a structure common to **1a** and **2**. We previously reported the preparation of **3** with the idea of introducing the hydroxy group at a later stage[5]; however, the overall yield was low (1.4—1.7%). Recently, we completed an improved synthesis of **3** from **4** in 11 steps *via* the useful intermediates **5** and **6**, which introduces a phenolic hydroxy group, at an early stage.[6] In this paper, we report the full account of our synthesis of **3** along with the practical synthesis from a benzaldehyde derivative **7**, which was easily prepared in seven steps from 2,3-dihydroxytoluene and is used to protect the phenolic hydroxy functionality as an isopropyl group.[7]

A mixture of **7** and 1,4-diacetyl-2,5-piperazinedione (**8**) was treated with potassium *tert*-butoxide in dimethylformamide (DMF) to give 3-arylidene-2,5-piperazinedione (**9**) in 74% yield according to the procedure of Gallina and Liberatori (Chart 1).[8] The geometric structure of **9** was confirmed by the chemical shift of the olefinic proton at δ 7.18. Alkylation of **9** with 4-methoxybenzyl chloride in the presence of sodium hydride in DMF furnished **10** in quantitative yield, and successive treatment with hydrazine hydrate afforded **11** in 94% yield. Methylation of **11** with methyl iodide (1.1 eq) in the presence of sodium hydride in DMF afforded **12** in 79% yield.[9] Deprotection of **12** with trifluoroacetic acid (TFA) and concentrated H_2SO_4 produced the phenol **13** in 85% yield. Reduction of the double bond of **13** with hydrogen over 20% palladium hydroxide on carbon in ethanol occurred cleanly accompanied by debromination to afford **5** in 88% yield.

On the other hand, we studied the conversion of **4**[5] into **5**

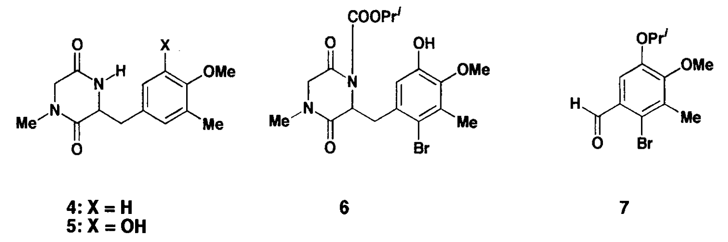


ecteinascidins
743 (1a): R = Me
729 (1b): R = H

safracins
A (2a): X = H
B (2b): X = OH

3

Fig. 1



4: X = H
5: X = OH

6

7

Fig. 2

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Dedicated to the memory of Dr. Kyosuke Tsuda.

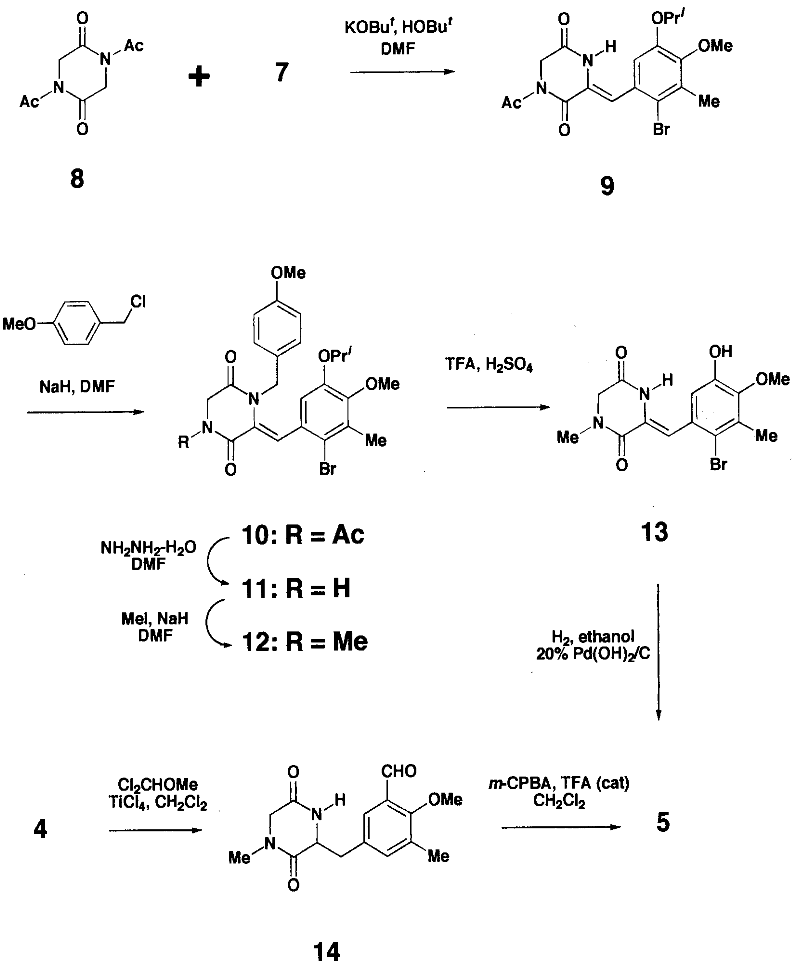

Chart 1

Table 1. Baeyer–Villiger Oxidation of **14** with *m*-CPBA in CH_2Cl_2[a]

Entry	Catalyst[b]	Eq	Time (h)	Temp (°C)	Yield (%)[c]
1	None		18	40	21[d]
2	None		24	25	38[d]
3	TsOH-H_2O	0.2	8	25	30
4	TfOH	0.2	8	25	38
5	TFA	0.2	8	25	75
6	TFA	0.1	8	25	51
7	CSA	0.2	8	25	25

[a] The reactions were carried out on a 0.4 mol scale. [b] TsOH=*p*-toluenesulfonic acid; TfOH=trifluoromethanesulfonic acid; CSA=10-camphorsulfonic acid. [c] Isolated yield. [d] Basic workup with 5% $NaHCO_3$.

via the benzaldehyde derivative **14**. Titanium tetrachloride and α,α-dichloromethyl methyl ether promoted formylation to afford **14** in 77% yield. Baeyer–Villiger oxidation of **14** with *m*-chloroperbenzoic acid (*m*-CPBA) was performed in the absence of a protic acid to give **5** with a maximum yield of only 38%. Knölker and Fröhner recently demonstrated that some acetophenone derivatives are able to be transformed into the corresponding acetates by acid-catalyzed Baeyer–Villiger oxidation.[10] Numerous efforts for the preparation of **5** employing *m*-CPBA with a variety of protic acids are summarized in Table 1. We found the reaction conditions in entry 5 were best in terms of product yield on a small scale. Unfortunately, the product yield was dramatically decreased (<25—30%) on a large scale. From a practical point of view, the process from **7** to **5** as described above is better in terms of overall yield and reproducibility in a large scale preparation.

We then studied the conversion of **5** to a cyclized compound (Chart 2). Protection of the phenol (**5**) with benzyl bromide and K_2CO_3 in DMF afforded the *O*-benzylated compound (**15**), which was then transformed by introduction of an isopropyloxycarbonyl group using isopropyl chloroformate, triethylamine, and 4-dimethylaminopyridine (DMAP) to give **16** in 89% overall yield. Deprotection of **16** by hydrogenolysis afforded **17** in 93% yield. The chemoselective reduction of **17** with an excess of lithium tri-*tert*-butoxyaluminium hydride in tetrahydrofuran (THF) gave a diastereomeric mixture of the alcohol (**18**), which was treated with TFA at 25 °C for 1 h to generate **19a** and **19b** in 22% and 66% yields, respectively. The structure of the cyclized compounds were fully supported by the molecular weight determined by mass spectrometry along with elemental analysis. The regiochemistry of both products, however, was undetermined at this stage because the signals in the ^{1}H-NMR spectra of **19a** and **19b** were not split, which indicated that they were a mixture of two rotational isomers. Conversion of **19a** to the final product (**3**) was accomplished using deprotection with TFA and H_2SO_4 at room temperature followed by reductive methylation in 74% overall yield. Similarly, **19b** was converted to **21** *via* **20b** in 61% overall yield. In the ^{1}H-NMR spectra of **3**, when H-7 (δ 6.48) was irradiated, nuclear Overhauser enhancement (NOE) (10%) of the methyl protons (δ 2.25) was observed, whereas NOE was negligible in the

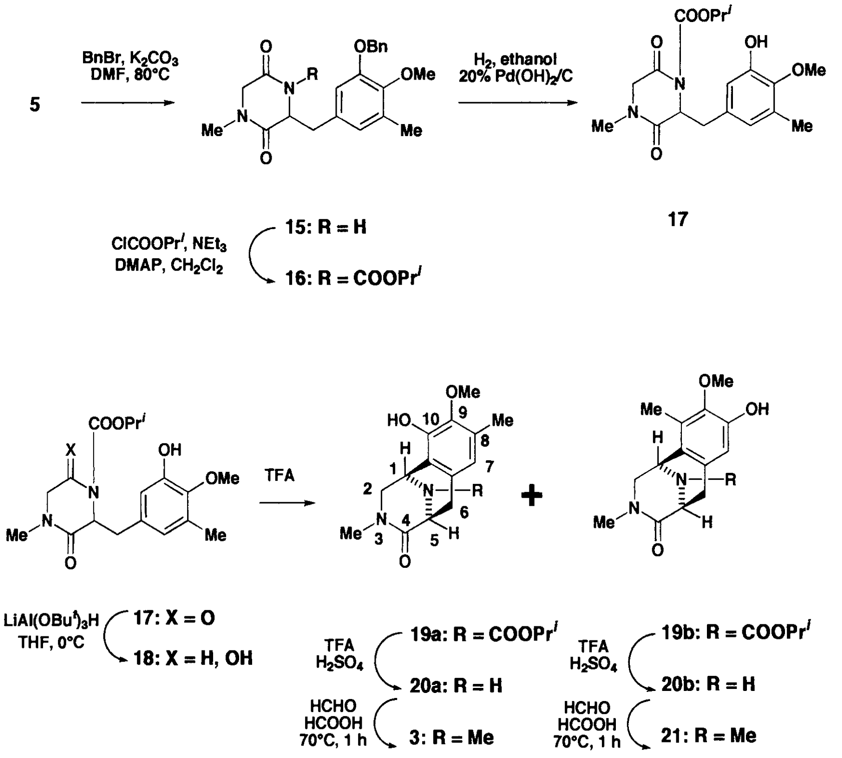

Chart 2

spectra of **21**.

The yield of this process was low because of the formation of an unwanted *para*-cyclized product (**19b**). Thus, we attempted to block the *para* position by introducing a bromine (Chart 3). Selective bromination of **5** with bromine in dichloromethane–THF (2 : 1) at 0 °C for 2 h gave the desired *para*-brominated compound **22** in 91% yield along with the regioisomer (**23a**) and the bisbrominated compound (**23b**) in 2.0% and 1.8% yields, respectively. The structure of **22** was supported by the ^{1}H-NMR spectrum, and the NOE was negligible between the aromatic proton at δ 6.76 and the methyl protons at δ 2.37, whereas the NOE (8.3%) was observed in the ^{1}H-NMR spectrum of **23a**. These results indicated that **22** was a *para*-brominated compound and **23a** was an *ortho*-brominated compound. Direct conversion of **22** to **6** was not possible, and produced the *O*-acylated product **24** in 67% yield. Accordingly, the sequence of reactions was studied. Protection of the phenol **22** with benzyl bromide gave **25** in 90% yield. This compound was converted into the imide (**26**) in 95% yield. The subsequent removal of the benzyl group of **26** proved troublesome. The acid-catalyzed removal of the benzyl unit in **26** with TFA at room temperature for 18 h afforded the desired compound (**6**) in only 51% yield because of the migration of the *O*-substituent to the free α-position to give **27a** in 31% yield.11) The structure of **27a** was supported by the spectral data, and this compound was transformed to **28** in 73% yield. To trap the benzyl carbocation that was produced, treatment of **26** in TFA with an excess of 1,3-dimethoxybenzaldehyde gave **6** in 72% yield, but the migration product **27a** (21%) was also produced. When the transformation was performed with trimethylsilyl iodide (TMSI) in chloroform at 50 °C for 24 h to give **6** in 18% yield, the major product was the catecohol (**27b**) in 28% yield.12) On the other hand, the direct bromination of the phenol (**17**) with bromine in dichloromethane at 0 °C for 1 h afforded **6** in 89% yield.

With the precursors for construction of the ABC ring model in hand, we then attempted to convert **26** or **6** into the final goal **3** (Chart 4). Attempts under a variety of conditions to cyclize **29** directed from **26** were fruitless; the yield was disappointingly low because of deprotection and migration of the benzyl group. When this dehydration/cyclization reaction was performed with methanesulfonic anhydride (Ms_2O) in dichloromethane, the cyclized compound could not be isolated; instead, the enamine (**30**) was formed in quantitative yield.13) Numerous efforts to convert **30** to the cyclized compound were unsuccessful, indicating that relatively slow cyclization of **29** or **30** was the reason for the steric hindrance of the benzyl protecting group which was unstable under acidic conditions. Indeed, treatment of **29** under strong acid conditions (TFA, H_2SO_4) generated **31** in 65% overall yield. In contrast, treatment of **32** (which was prepared from **6**) with TFA gave the desired cyclized compound (**33**) in 96% overall yield. Deprotection of **33** with TFA and H_2SO_4 at room temperature for 19 h gave the amine (**31**) in 94% yield. Methylation of **31** with formaldehyde and formic acid at 70 °C for 1 h gave the *N*-methylamine (**34**) in 94% yield. Finally, debromination of **34** with hydrogen over 20% palladium hydroxide on carbon in ethanol achieved our final goal (**3**) in 95% yield.

In summary, we succeeded in a practical synthesis of the ABC ring model of ecteinascidins from **7** in 15 steps. The overall yield of **3** from **7** was 27%. Application of this strategy to the total synthesis of ecteinascidin natural products are under intensive investigation in our laboratories.

Experimental

All melting points were determined with a Yanagimoto micromelting

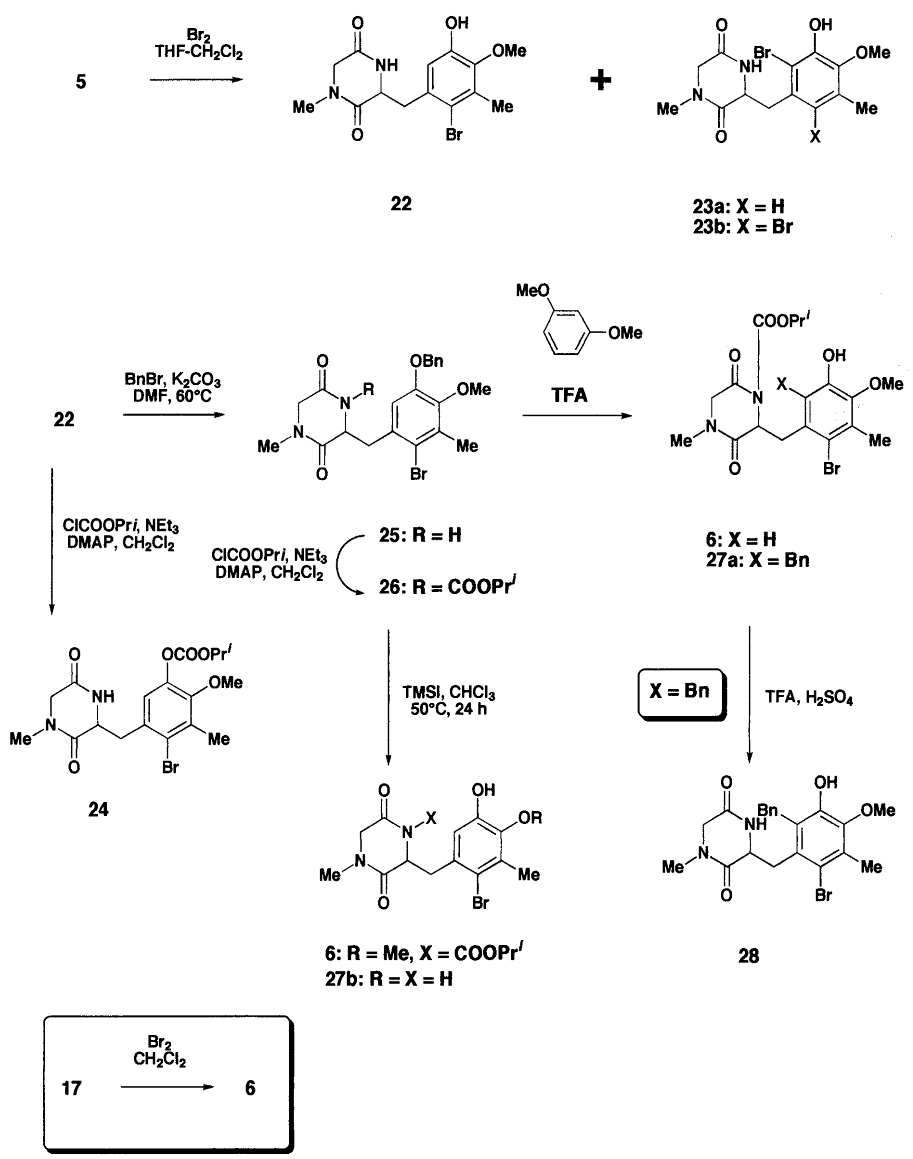

Chart 3

points apparatus and are uncorrected. IR spectra were obtained with a Hitachi 260-10 IR Fourier-transform spectrometer. ^{1}H-NMR were recorded at 270 MHz with a JEOL JNM-EX 270 spectrometer and at 300 MHz with a JEOL JNM-AL300 spectrometer. Peak multiplicities are denoted by s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), sept (septet), m (multiplet) or by a combination of these *e.g.* dd (double doublet), with coupling constants (J) given in Hz. ^{13}C-NMR spectra were recorded at 67.5 MHz (multiplicity determined from off-resonance decoupled or distortionless enhancement by polarization transfer (DEPT) spectra). NMR spectra were measured in $CDCl_3$, and chemical shifts were recorded in δ_H values relative to internal $(CH_3)_4Si$ as a standard. Mass spectra were recorded on a JMS-DX 302 instrument with a direct inlet system operating at 70 eV. Elemental analyses were obtained on a Perkin-Elmer Model 240B elemental analyzer. All reactions were conducted under an argon atmosphere. Dry solvents and reagents were obtained using standard procedure. Anhydrous sodium sulfate was used for drying organic solvent extracts. Removal of the solvent was done with a rotary evaporator and, finally, under high vacuum. Column chromatography was performed with E. Merck silica gel (70—230 mesh).

(*Z*)-1-Acetyl-3-(2-bromo-5-isopropoxy-4-methoxy-3-methylphenylmethylene)-2,5-piperazinedione (9) A solution of potassium *tert*-butoxide (5.633 g, 50.2 mmol) in *tert*-butyl alcohol (100.4 ml) was added to a stirred solution of 2-bromo-5-isopropoxy-4-methoxy-3-methylbenzaldehyde **7** (14.427 g, 50.2 mmol) and 1,4-diacetyl-2,5-piperazinedione **8** (9.957 g, 50.2 mmol) in dry DMF (100.4 ml). After being stirred at room temperature for 1 h, the reaction mixture was poured into brine (600 ml) and extracted with ethyl acetate (600 ml×3). The combined extracts were washed with water (600 ml), dried, and concentrated *in vacuo* to give the residue as a pale yellow solid, recrystallization of which from ethyl acetate gave **9** (15.876 g, 74.3%) as pale yellow prisms, mp 145—147 °C. ^{1}H-NMR δ: 1.36 (6H, d, J=6.1 Hz, $CH(C\underline{H}_3)_2$), 2.38 (3H, s, $ArCH_3$), 2.68 (3H, s, $COCH_3$), 3.84 (3H, s, OCH_3), 4.50 (1H, sept, J=6.1 Hz, OCH), 4.51 (2H, s, 6-H_2), 6.70 (1H, s, ArH), 7.18 (1H, s, C=CH), 7.64 (1H, br s, NH). IR (KBr) cm^{-1}: 3230, 1710, 1685, 1640. MS *m/z* (%): 426 (M^++2, 10), 424 (M^+, 10), 346 (13), 345 (54), 304 (14), 303 (69), 262 (16), 261 (100), 246 (19), 43 (13). *Anal.* Calcd for $C_{18}H_{21}BrN_2O_5$: C, 50.84; H, 4.98; N, 6.59. Found: C, 50.83, H, 4.98; N, 6.56.

(*Z*)-3-(2-Bromo-5-isopropoxy-4-methoxy-3-methylphenylmethylene)-4-(4-methoxyphenylmethyl)-2,5-piperazinedione (11) Sodium hydride (60% oil dispersion, washed with dry hexane three times, 300 mg, 12.5 mmol) was added to a stirred solution of **9** (4.241 g, 10 mmol) in dry DMF (80 ml) under ice-cooling, and stirring was continued at 0 °C for 30 min. 4-Methoxybenzyl chloride (1.96 g, 12.5 mmol) in dry DMF (20 ml) was added during 10 min, and the reaction mixture was stirred at room temperature for 42 h. The reaction mixture was concentrated *in vacuo*, and the residue was diluted with water (100 ml) and extracted with benzene (100 ml×3). The combined extracts were washed with brine (100 ml), dried, and concentrated *in vacuo* to furnish **10** (5.441 g, 100%) as a pale yellow amorphous powder, which was used for the next step without further purification. ^{1}H-NMR δ: 1.36 (6H, d, J=6.1 Hz, $CH(C\underline{H}_3)_2$), 2.40 (3H, s, $ArCH_3$), 2.55 (3H, s, $COCH_3$), 3.75, 3.84 (each 3H, s, OCH_3), 4.47 (1H, sept, J=6.1 Hz, OCH), 4.51 (2H, s, 6-H_2), 4.54 (2H, s, NCH_2), 6.72 (1H, s, ArH), 6.74, 6.87 (each 2H, d, J=8.6 Hz, 2×ArH), 7.35 (1H, s, C=CH). IR ($CHCl_3$) cm^{-1}: 1708, 1630. MS *m/z* (%): 546 (M^++ 2, 5), 544 (M^+, 5), 466 (22), 465 (71), 424 (12), 423 (42), 122 (10), 121 (100). High-resolution MS

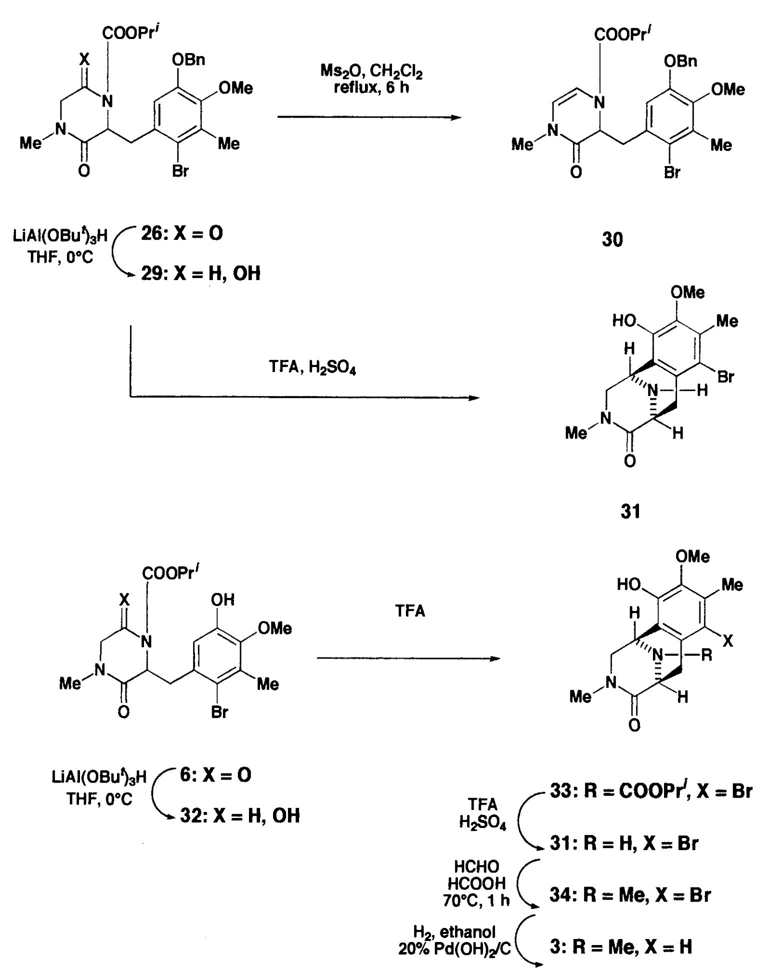

Chart 4

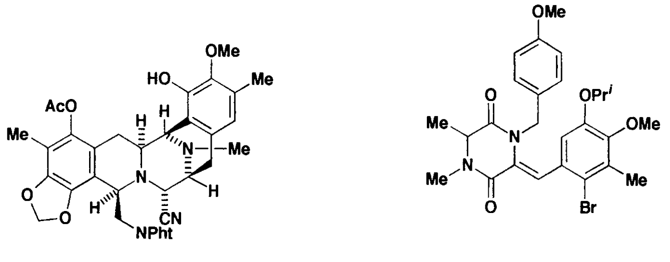

Fig. 3

Calcd for $C_{26}H_{29}BrN_2O_6$: 544.1209. Found: 544.1207.

Hydrazine monohydrate (2.43 ml) was added to a stirred solution of the crude **10** (5.441 g) in dry DMF (100 ml), and the resulting solution was stirred at room temperature for 1 h. The reaction mixture was poured into water (100 ml), and extracted with ethyl acetate (100 ml×3). The combined extracts were washed with brine (100 ml), dried, and concentrated *in vacuo* to give a residue. Chromatography on a silica gel (120 g) column with dichloromethane–methanol (80 : 1) as the eluent gave **11** (4.720 g, 93.8%) as pale yellow amorphous powder. ^{1}H-NMR δ: 1.35 (6H, d, J=6.1 Hz, CH(C$\underline{H}_3$)$_2$), 2.39 (3H, s, ArCH$_3$), 3.74, 3.85 (each 3H, s, OCH$_3$), 4.12 (2H, d, J=2.2 Hz, 6-H$_2$), 4.44 (1H, sept, J=6.1 Hz, OCH), 4.54 (2H, s, NCH$_2$), 6.67 (1H, s, ArH), 6.72, 6.87 (each 2H, d, J=8.8 Hz, 2×ArH), 7.18 (1H, s, C=CH), 7.48 (1H, br s, NH). IR (CHCl$_3$) cm^{-1}: 3420, 1692, 1632. MS *m/z* (%): 504 (M$^+$+2, 2), 502 (M$^+$, 2), 424 (30), 423 (100), 381 (6), 121 (66). High-resolution MS Calcd for $C_{24}H_{27}BrN_2O_5$: 502.1103. Found: 502.1101.

(*Z*)-3-(2-Bromo-5-isopropoxy-4-methoxy-3-methylphenylmethylene)-4-(4-methoxyphenylmethyl)-1-methyl-2,5-piperazinedione (12). Method A Sodium hydride (60% oil dispersion, washed with dry hexane three times, 247.2 mg, 10.3 mmol) was added to a stirred solution of **11** (4.720 g, 9.38 mmol) in dry DMF (94 ml) under ice-cooling, and stirring was continued at 0 °C for 30 min. Methyl iodide (0.642 ml, 10.31 mmol) was added during 30 min, and the reaction mixture was stirred at room temperature for 3 h. The reaction mixture was concentrated *in vacuo*, and the residue was diluted with water (200 ml) and extracted with ether (200 ml×3). The combined extracts were washed with brine (200 ml), dried, and concentrated *in vacuo* to give the residue. Chromatography on a silica gel (120 g) column with hexane–ethyl acetate (1 : 1) as the eluent gave **12** (3.184 g, 78.6%) as a pale yellow amorphous powder. ^{1}H-NMR δ: 1.35 (6H, d, J=6.1 Hz, CH(C$\underline{H}_3$)$_2$), 2.38 (3H, s, ArCH$_3$), 3.05 (3H, s, NCH$_3$), 3.74, 3.84 (each 3H, s, OCH$_3$), 4.10 (2H, s, 6-H$_2$), 4.43 (1H, sept, J=6.1 Hz, OCH), 4.52 (2H, s, NCH$_2$), 6.65 (1H, s, ArH), 6.72, 6.87 (each 2H, d, J=8.6 Hz, 2×ArH), 7.22 (1H, s, C=CH). IR (CHCl$_3$) cm^{-1}: 1686, 1630. MS *m/z* (%): 518 (M$^+$+2, 1), 516 (M$^+$, 1), 438 (31), 437 (100), 121 (42). High-resolution MS Calcd for $C_{25}H_{29}BrN_2O_5$: 516.1260. Found: 516.1253.

Method B The same procedure as described above, but using **11** (617.8 mg, 1.227 mmol) with sodium hydride (44.2 mg, 1.842 mmol) and methyl iodide (0.115 mmol, 1.841 mmol) in dry DMF (15 ml) at room temperature for 20 h, gave the residue (894 mg). Chromatography on a silica gel (30 g) column with hexane–ethyl acetate (3 : 2) as the eluent gave **35** (282.1 mg, 43.4%) as a colorless amorphous powder and further elution with hexane–ethyl acetate (1 : 1) gave **12** (266.8 mg, 42.0%).

(*Z*)-3-(2-Bromo-5-isopropoxy-4-methoxy-3-methylphenylmethylene)-4-(4-methoxyphenylmethyl)-1,6-dimethyl-2,5-piperazinedione (**35**): ^{1}H-NMR δ: 1.34, 1.36 (each 3H, d, J=6.1 Hz, CH(CH$_3$)$_2$), 1.56 (3H, d, J=7.0 Hz, 6-CH$_3$), 2.38 (3H, s, ArCH$_3$), 3.03 (3H, s, NCH$_3$), 3.74, 3.84 (each 3H, s, OCH$_3$), 3.94 (1H, d, J=14.9 Hz, NCH), 4.04 (1H, q, J=7.0 Hz, 6-H), 4.39 (1H, sept, J=6.1 Hz, OCH), 5.07 (1H, d, J=14.9 Hz, NCH), 6.61 (1H, s, ArH), 6.71, 6.86 (each 2H, d, J=8.6 Hz, 2×ArH), 7.19 (1H, s, C=CH). IR (CHCl$_3$) cm^{-1}: 1682, 1628. MS *m/z* (%): 532 (M$^+$+2, 1), 530 (M$^+$, 1), 452 (31), 451 (100), 121 (33). High-resolution MS Calcd for $C_{26}H_{31}BrN_2O_5$: 530.1416. Found: 530.1423.

(*Z*)-3-(2-Bromo-5-hydroxy-4-methoxy-3-methylphenylmethylene)-1-methyl-2,5-piperazinedione (13) Concentrated H$_2$SO$_4$ (1.0 ml) was added to a stirred solution of **12** (3.814 g, 7.37 mmol) in TFA (20 ml), and stirring was continued at room temperature for 1 h. The reaction mixture was poured into water (100 ml) and extracted with chloroform–methanol (9 : 1, 100 ml×3). The combined extracts were washed with brine (100 ml), dried, and concentrated *in vacuo* to give a solid. Recrystallization of which from methanol gave **13** (2.231 g, 85.2%) as pale yellow prisms, mp 248—250 °C. ^{1}H-NMR δ: 2.37 (3H, s, ArCH$_3$), 2.45 (1H, br s, OH), 3.10 (3H, s, NCH$_3$), 3.81 (3H, s, OCH$_3$), 4.17 (2H, s, 6-H2), 6.76 (1H, s, ArH), 7.02 (1H, s, C=CH), 8.16 (1H, br s, NH). IR (KBr) cm^{-1}: 3264, 1688, 1638. MS *m/z* (%): 356 (M$^+$+2, 6), 354 (M$^+$, 6), 276 (18), 275 (100), 260 (60). *Anal.* Calcd for $C_{14}H_{15}BrN_2O_4 \cdot 1/8H_2O$: C, 47.04; H, 4.30; N, 7.30. Found: C, 46.91; H, 4.20; N, 7.75.

3-(3-Hydroxy-4-methoxy-5-methylphenylmethyl)-1-methyl-2,5-piperazinedione (5) A solution of **13** (1.471 g, 4.14 mmol) in ethanol (140 ml) was hydrogenated over 20% Pd(OH)$_2$/C (700 mg) at 1 atm for 5 h. The catalyst was removed by filtration and washed with ethanol (100 ml). The combined filtrates were evaporated to give a soild, recrystallization of which from acetone gave **5** (1.020 g, 88.4%) as colorless needles, mp 201—203 °C. ^{1}H-NMR δ: 2.24 (3H, s, ArCH$_3$), 2.88 (3H, s, NCH$_3$), 2.96 (1H, dd, *J*=13.8, 6.5 Hz, ArCH), 3.00 (1H, br s, OH), 3.04 (1H, dd, *J*=13.8, 4.1 Hz, ArCH), 3.19, 3.64 (each 1H, d, *J*=17.6 Hz, 6-H), 3.78 (3H, s, OCH$_3$), 4.23 (1H, m, 3-H), 6.50, 6.63 (each 1H, d, *J*=2.0 Hz, ArH), 6.96 (1H, br s, NH). ^{13}C-NMR δ: 15.8 (q), 33.7 (q), 40.2 (t), 51.2 (t), 56.3 (d), 60.6 (q), 114.7 (d), 123.5 (d), 131.3 (s), 131.5 (s), 145.1 (s), 149.2 (s), 165.5 (s), 165.7 (s). IR (KBr) cm^{-1}: 3184, 3120, 1690, 1646. MS *m/z* (%): 278 (M$^+$, 17), 152 (12), 151 (100). *Anal.* Calcd for $C_{14}H_{18}N_2O_4$: C, 60.42; H, 6.52; N, 10.07. Found: C, 60.22; H, 6.50; N, 9.93.

3-(3-Formyl-4-methoxy-5-methylphenylmethyl)-1-methyl-2,5-piperazinedione (14) A stirred solution of **4** (471.6 mg, 1.8 mmol) and α,α-dichloromethyl methyl ether (1.06 ml, 11.7 mmol) in dry dichloromethane (9 ml) was cooled in ice-water, and TiCl$_4$ (0.594 ml, 5.4 mmol) was added dropwise to it over 1 h. The resulting solution was stirred at 0 °C for 2 h. The reaction mixture was diluted with water (30 ml) and the phane were separated. The aqueous layer was extracted with dichloromethane (30 ml×2). The combined extracts were washed with water (50 ml), dried, and concentrated *in vacuo* to give a soild, recrystallization of which from ethyl acetate gave **14** (400.4 mg, 76.7%) as pale yellow prisms, mp 148—150 °C. ^{1}H-NMR δ: 2.31 (3H, s, ArCH$_3$), 2.90 (3H, s, NCH$_3$), 3.15 (2H, d, *J*=5.3 Hz, ArCH$_2$), 3.26, 3.70 (each 1H, d, *J*=17.5 Hz, 6-H), 3.87 (3H, s, OCH$_3$), 4.31 (1H, t like, 3-H), 7.30 (1H, d, *J*=2.0 Hz, ArH), 7.34 (1H, br s, NH), 7.52 (1H, d, *J*=2.0 Hz, ArH), 10.31 (1H, s, CHO). ^{13}C-NMR δ: 15.4 (q), 33.6 (q), 39.5 (t), 51.1 (t), 56.1 (d), 63.2 (q), 127.6 (d), 129.1 (s), 131.5 (s), 132.8 (s), 138.9 (d), 161.0 (s), 165.2 (s), 165.7 (s), 189.8 (s). IR (KBr) cm^{-1}: 3280, 1680, 1665, 1655, 1640, 1605. MS *m/z* (%): 290 (M$^+$, 25), 163 (100). *Anal.* Calcd for $C_{15}H_{18}N_2O_4 \cdot 1/10H_2O$: C, 61.68; H, 6.28; N, 9.59. Found: C, 61.58; H, 6.29; N, 9.65.

Baeyer–Villiger Oxidation of 14 A solution of **14** (116.0 mg, 0.4 mmol) and TFA (0.0062 ml, 0.08 mmol) in dry dichloromethane (10 ml) was cooled with ice-water, and *m*-CPBA acid (80%, 259 mg, 1.2 mmol) was added over 10 min, the reaction mixture was stirred at room temperature for 8 h. The reaction mixture was diluted with 5% NaHCO$_3$ solution (30 ml), and extracted with chloroform (30 ml×3). The combined extracts were washed with water (30 ml), dried, and concentrated *in vacuo* to give a residue (225.6 mg). Chromatography on a silica gel (10 g) column with chloroform–methanol (50 : 1) afforded **5** (83.3 mg, 75.0%) as colorless needles, mp 199—201 °C, which were identical in all respects with an authentic sample described above.

3-(3-Benzyloxy-4-methoxy-5-methylphenylmethyl)-1-methyl-2,5-piperazinedione (15) A suspended solution of **5** (1.113 g, 4.0 mmol) and anhydrous K$_2$CO$_3$ (1.106 g, 8.0 mmol) in dry DMF (80 ml) was cooled with ice-water, and benzyl bromide (0.952 ml, 8.0 mmol) was added over 5 min. The reaction mixture was heated at 80 °C for 24 h. The reaction mixture was diluted with water (100 ml), and extracted with chloroform (100 ml×3). The combined extracts were washed with brine (100 ml), dried, and concentrated *in vacuo* to give a residue (2.509 g). Chromatography on a silica gel (10 g) column with chloroform–methanol (100 : 1) afforded a solid, recrystallization of which from ethyl acetate–ether gave **15** (1.334 g, 91.2%) as colorless prisms, mp 140—141 °C. ^{1}H-NMR δ: 2.23 (3H, s, ArCH$_3$), 2.83 (3H, s, NCH$_3$), 2.97 (1H, dd, *J*=13.8, 6.4 Hz, ArCH), 3.05 (1H, dd, *J*=13.8, 4.4 Hz, ArCH), 3.16, 3.61 (each 1H, d, *J*=17.5 Hz, 6-H), 3.82 (3H, s, OCH$_3$), 4.20 (1H, m, 3-H), 5.04 (2H, s, OCH$_2$), 6.61, 6.64 (each 1H, d, *J*=2.0 Hz, ArH), 6.76 (1H, br s, NH), 7.27—7.45 (5H, m, 5×ArH). ^{13}C-NMR δ: 16.1 (q), 33.8 (q), 40.8 (t), 51.4 (t), 56.7 (d), 60.5 (q), 71.0 (t), 113.5 (d), 124.8 (d), 127.5 (d×2), 128.2 (d), 128.8 (d×2), 130.5 (s), 132.7 (s), 137.1 (s), 147.5 (s), 152.2 (s), 165.7 (s), 166.0 (s). IR (KBr) cm^{-1}: 3590, 3450, 1680, 1670, 1660. MS *m/z* (%): 368 (M$^+$, 51), 242 (18), 241 (100), 228 (12), 181 (18), 151 (20), 91 (79). High-resolution MS Calcd for $C_{21}H_{24}N_2O_4$: 368.1736. Found: 368.1731. *Anal.* Calcd for $C_{21}H_{24}N_2O_4 \cdot 1/2H_2O$: C, 66.83; H, 6.68; N, 7.42. Found: C, 67.07; H, 6.49; N, 7.41.

3-(3-Benzyloxy-4-methoxy-5-methylphenylmethyl)-4-isopropyloxycarbonyl-1-methyl-2,5-piperazinedione (16) A solution of **15** (921.1 mg, 2.5 mmol), triethylamine (0.697 ml, 5.0 mmol), and (DMAP) (610.9 mg, 5.0 mmol) in dichloromethane (50 ml) was cooled with ice-water, and isopropyl chloroformate (1.14 ml, 10.0 mmol) was added dropwise over 10 min. The solution was stirred at room temperature for 43 h. The organic layer was washed with 1 N HCl (50 ml), and then water (50 ml), dried, and concentrated *in vacuo* to give a residue (1.695 g). Chromatography on a silica gel (40 g) column with dichloromethane–methanol (100 : 1) as the eluent gave **16** (1.114 g, 98.0%) as a colorless amophous powder. ^{1}H-NMR δ: 1.35, 1.38 (each 3H, d, *J*=6.3 Hz, CH(C$\underline{H}_3$)$_2$), 2.22 (3H, s, ArCH$_3$), 2.46 (1H, d, *J*=18.2 Hz, 6-H), 2.72 (3H, s, NCH$_3$), 3.11 (1H, dd, *J*=13.9, 4.3 Hz, ArCH), 3.23 (1H, dd, *J*=13.9, 4.0 Hz, ArCH), 3.45 (1H, d, *J*=18.2 Hz, 6-H), 3.82 (3H, s, OCH$_3$), 5.01 (3H, m, 3-H, OCH$_2$), 5.14 (1H, sept, *J*=6.3 Hz, OCH), 6.54, 6.55 (each 1H, br s, ArH), 7.34—7.47 (5H, m, 5×ArH). IR (CHCl$_3$) cm^{-1}: 1780, 1730, 1670. MS *m/z* (%): 454 (M$^+$, 55), 277 (13), 242 (22), 241 (100), 209 (13), 181 (18), 151 (14), 91 (51). High-resolution MS Calcd for $C_{25}H_{30}N_2O_6$: 454.2104. Found: 454.2102.

3-(3-Hydroxy-4-methoxy-5-methylphenylmethyl)-4-isopropyloxy-carbonyl-1-methyl-2,5-piperazinedione (17) A solution of **16** (1.114 g, 2.45 mmol) in ethanol (40 ml) was hydrogenated over 20% Pd(OH)$_2$/C (500 mg) at 1 atm for 1 h. The catalyst was removed by filtration and washed with ethanol (100 ml). The combined filtrates were evaporated to give a solid, recrystallization of which from acetone gave **17** (833.3 mg, 93.3%) as colorless prisms, mp 123—124 °C. ^{1}H-NMR δ: 1.35, 1.37 (each 3H, d, *J*=6.2 Hz, CH(C$\underline{H}_3$)$_2$), 2.23 (3H, s, ArCH$_3$), 2.56 (1H, d, *J*=18.2 Hz, 6-H), 2.80 (3H, s, NCH$_3$), 3.08 (1H, dd, *J*=14.0, 4.6 Hz, ArCH), 3.21 (1H, dd, *J*=14.0, 4.0 Hz, ArCH), 3.49 (1H, d, *J*=18.2 Hz, 6-H), 3.78 (3H, s, OCH$_3$), 4.98 (1H, dd, *J*=4.6, 4.0 Hz, 3-H), 5.13 (1H, sept, *J*=6.2 Hz, OCH), 6.17 (1H, br s, OH), 6.43, 6.55 (each 1H, d, *J*=2.0 Hz, ArH). ^{13}C-NMR δ: 15.7 (q), 21.6 (q), 21.7 (q), 32.9 (q), 38.8 (t), 52.2 (t), 60.0 (d), 60.8 (q), 72.3 (d), 114.9 (d), 124.3 (d), 130.9 (s), 131.4 (s), 145.4 (s), 149.2 (s), 151.1 (s), 164.4 (s), 166.0 (s). IR (KBr) cm^{-1}: 3352, 1730, 1664. MS *m/z* (%): 364 (M$^+$, 20), 278 (12), 153 (13), 152 (100). *Anal.* Calcd for $C_{18}H_{24}N_2O_6$: C, 59.33; H, 6.64; N, 7.69. Found: C, 59.31; H, 6.64; N, 7.56.

Attempted Cyclization of 17 *via* 18 A stirred solution of **17** (36.4 mg, 0.1 mmol) in dry THF (4 ml) was cooled with ice-water, and lithium tri-*tert*-butoxyaluminium hydride (178.0 mg, 0.7 mmol) was added to it over 5 min. After continued stirring at the same temperature for 1 h, the reaction mixture was quenched by the addition of water (1 ml) and then filtered through a Celite pad, and the filtrate was concentrated *in vacuo*. The unstable diastereomeric mixture of the alcohols **18** (37 mg) obtained was used for the next step without further purification. A solution of **18** in TFA (1 ml) was stirred at room temperature for 1 h. The reaction mixture was diluted with water (10 ml), and extracted with chloroform (20 ml×3). The combined extracts were washed with 5% NaHCO$_3$ (20 ml), dried, and concentrated *in vacuo* to give the residue (36.7 mg). Column chromatography on a silica gel (12 g) gave **19a** (7.8 mg, 22.4%) as a solid and **19b** (23.0 mg, 66.1%) as a solid.

Isopropyl 1,2,3,4,5,6-Hexahydro-1,5-imino-10-hydroxy-9-methoxy-3,8-dimethyl-4-oxo-3-benzazocine-11-carboxylic Acid (**19a**): mp 237—239 °C (from acetone). ^{1}H-NMR δ (at 50 °C): 1.25 (6H, d, *J*=6.3 Hz, CH(C$\underline{H}_3$)$_2$), 2.24 (3H, s, ArCH$_3$), 2.84 (3H, s, NCH$_3$), 2.99 (1H, dd, *J*=16.5, 1.0 Hz, 6-Hβ), 3.10 (1H, dd, *J*=16.5, 5.9 Hz, 6-Hα), 3.29 (1H, dd, *J*=11.9, 1.0 Hz, 2-Hβ), 3.77 (3H, s, OCH$_3$), 3.84 (1H, dd, *J*=11.9, 4.6 Hz, 2-Hα), 4.92 (1H, br s, 5-H), 4.96 (1H, sept, *J*=6.3 Hz, OCH), 5.61 (1H, br s, 1-H), 5.97 (1H, br s, OH), 6.48 (1H, s, ArH). ^{13}C-NMR δ (at 55 °C): 15.7 (q), 22.1 (q), 22.1 (q), 31.4 (t), 34.3 (q), 44.9 (d), 53.0 (d), 54.2 (t), 60.8 (q), 69.4 (d), 119.3 (s), 122.3 (d), 129.4 (s), 129.8 (s), 143.7 (s), 144.9 (s), 153.5 (s), 168.4 (s). IR (KBr) cm^{-1}: 3220, 1715, 1640. MS *m/z* (%): 348 (M$^+$, 88), 277 (21), 276 (40), 262 (48), 261 (43), 235 (38), 234 (62), 191 (18), 190 (100), 175 (21), 158 (11), 43 (20). *Anal.* Calcd for $C_{18}H_{24}N_2O_5$: C, 62.05; H, 6.94; N, 8.04. Found: C, 61.98; H, 6.99; N, 7.93.

Isopropyl 1,2,3,4,5,6-Hexahydro-1,5-imino-8-hydroxy-9-methoxy-3,10-dimethyl-4-oxo-3-benzazocine-11-carboxylic Acid (**19b**): mp 206—207 °C (from ethyl acetate). ^{1}H-NMR δ (at 50 °C): 1.25 (6H, d, *J*=6.3 Hz, CH(C$\underline{H}_3$)$_2$), 2.25 (3H, s, ArCH$_3$), 2.86 (3H, s, NCH$_3$), 3.04 (2H, AB d, *J*=16.2, 1.0 Hz, 6-H$_2$), 3.14 (1H, d, *J*=11.5 Hz, 2-Hβ), 3.76 (3H, s, OCH$_3$), 3.89 (1H, dd, *J*=11.5, 4.6 Hz, 2-Hα), 4.90 (1H, br s, 5-H), 4.94 (1H, sept, *J*=6.3 Hz, OCH), 5.44 (1H, br s, 1-H), 5.82 (1H, br s, OH), 6.55 (1H, s, ArH). ^{13}C-NMR δ (at 55 °C): 11.6 (q), 22.1 (q), 22.1 (q), 32.2 (t), 34.3 (q), 46.2 (d), 52.6 (d), 54.7 (t), 60.8 (q), 69.6 (d), 113.7 (d), 125.7 (s), 127.3 (s), 129.6 (s), 144.7 (s), 148.5 (s), 153.6 (s), 168.5 (s). IR (KBr) cm^{-1}: 3280, 1710, 1685, 1670, 1645. MS *m/z* (%): 348 (M$^+$, 64), 277 (27), 276 (73), 235 (43), 234 (100), 191 (12), 190 (76), 175 (21), 43 (22). *Anal.* Calcd for $C_{18}H_{24}N_2O_5$: C, 62.05; H, 6.94; N, 8.04. Found: C, 61.84; H, 7.00; N, 7.90.

1,2,3,4,5,6-Hexahydro-1,5-imino-10-hydroxy-9-methoxy-3,8,11-trimethyl-3-benzazocin-4-one (3) from 19a *via* 20a Concentrated H_2SO_4 (0.05 ml) was added to a stirred solution of **19a** (5.9 mg, 0.024 mmol) in TFA (1 ml), and stirring was continued at room temperature for 15 h. The reaction mixture was poured into water (10 ml), made alkaline with concentrated NH_4OH, and extracted with chloroform–methanol (9 : 1). The combined extracts were washed with brine (10 ml), dried, and concentrated *in vacuo* to give **20a** (4.8 mg, 100%) as a solid, which was used for the next step without further purification. ^{1}H-NMR δ: 2.25 (3H, s, ArCH$_3$), 2.86 (3H, s, NCH$_3$), 2.90—3.10 (2H, br, NH, OH), 2.99 (2H, d, J=5.9 Hz, 6-H$_2$), 3.30 (1H, dd, J=11.9, 1.0 Hz, 2-Hβ), 3.78 (3H, s, OCH$_3$), 3.82 (1H, dd, J=11.9, 4.6 Hz, 2-Hα), 3.95 (1H, d, J=5.9 Hz, 5-H), 4.52 (1H, d, J=4.9 Hz, 1-H), 6.49 (1H, s, ArH). ^{13}C-NMR δ (CDCl$_3$+CD$_3$OD): 15.6 (q), 31.9 (t), 34.2 (q), 44.7 (d), 52.8 (d), 55.1 (t), 60.5 (q), 120.5 (s), 121.8 (d), 128.4 (s), 129.8 (s), 143.4 (s), 145.0 (s), 170.5 (s). IR (KBr) cm^{-1}: 3280, 1650. MS *m/z* (%): 262 (M$^+$, 26), 191 (19), 190 (100), 175 (16). High-resolution MS Calcd for $C_{14}H_{18}N_2O_3$: 262.1317. Found: 262.1320.

Formaldehyde (37% solution in water, 0.29 ml, 0.365 mmol) was added to a stirred solution of **20a** (4.8 mg, 0.0183 mmol) in formic acid (0.34 ml) at 60 °C. After having been stirred at 70 °C for 2 h, the reaction mixture was poured into water (10 ml), and extracted with chloroform (10 ml×3). The combined extracts were washed with 5% NaHCO$_3$, dried, and concentrated *in vacuo* to give a residue (5.6 mg). Chromatography on a silica gel (8 g) column with dichloromethane–methanol (25 : 1) gave **3** (3.9 mg, 74.0%) as colorless prisms, mp 216—217.5 °C, which were identical in all respects with an authentic sample described earlier.[5]

1,2,3,4,5,6-Hexahydro-1,5-imino-8-hydroxy-9-methoxy-3,10,11-trimethyl-3-benzazocin-4-one (21) from 19b *via* 20b The same procedure as described above but using **19b** (27.8 mg, 0.08 mmol) in concentrated H_2SO_4 (0.05 ml) and TFA (1 ml), gave **20b** (21.0 mg, 100%) as a solid, which was used for the next step without further purification. ^{1}H-NMR δ: 2.21 (3H, s, ArCH$_3$), 2.20—2.40 (2H, br, NH, OH), 2.88 (3H, s, NCH$_3$), 2.97 (1H, d, J=16.5 Hz, 6-Hβ), 3.08 (1H, dd, J=16.5, 6.6 Hz, 6-Hα), 3.14 (1H, dd, J=11.5, 0.5 Hz, 2-Hβ), 3.76 (3H, s, OCH$_3$), 3.87 (1H, dd, J=11.5, 4.9 Hz, 2-Hα), 3.94 (1H, d, J=5.0 Hz, 5-H), 4.34 (1H, d, J=4.9 Hz, 1-H), 6.52 (1H, s, ArH). ^{13}C-NMR δ (CDCl$_3$+CD$_3$OD): 11.2 (q), 32.5 (t), 34.3 (q), 46.5 (d), 52.4 (d), 55.1 (t), 60.5 (q), 113.8 (d), 125.1 (s), 127.5 (s), 128.9 (s), 144.6 (s), 148.6 (s), 169.8 (s). IR (CHCl$_3$) cm^{-1}: 3290, 1645, 1605. MS *m/z* (%): 262 (M$^+$, 17), 191 (16), 190 (100), 175 (13). High-resolution MS Calcd for $C_{14}H_{18}N_2O_3$: 262.1317. Found: 262.1313.

Formaldehyde (37% solution in water, 0.29 ml, 0.365 mmol) was added to a stirred solution of **20b** (21.0 mg, 0.08 mmol) in formic acid (1.16 ml) at 60 °C. After having been stirred at 70 °C for 2 h, the reaction mixture was poured into water (10 ml), and extracted with chloroform (10 ml×3). The combined extracts were washed with 5% NaHCO$_3$, dried, and concentrated *in vacuo* to give a residue (35.0 mg). Chromatography on a silica gel (10 g) column with dichloromethane–methanol (25 : 1) gave **21** (13.4 mg, 60.7%) as colorless prisms, mp 223—225 °C (from ethyl acetate). ^{1}H-NMR δ: 2.20 (3H, s, ArCH$_3$), 2.49 (3H, s, NCH$_3$), 2.81 (1H, d, J=17.1 Hz, 6-Hβ), 2.86 (3H, s, NCH$_3$), 3.02 (1H, dd, J=14.0, 3.0 Hz, 2-Hβ), 3.17 (1H, dd, J=17.1, 7.0 Hz, 6-Hα), 3.60 (1H, d, J=7.0 Hz, 5-H), 3.77 (3H, s, OCH$_3$), 3.95 (1H, dd, J=14.0, 4.9 Hz, 2-Hα), 3.96 (1H, br s, 1-H), 6.55 (1H, s, ArH). ^{13}C-NMR δ: 11.4 (q), 28.1 (t), 34.1 (q), 40.3 (q), 52.9 (t), 52.9 (d), 59.0 (d), 60.7 (q), 113.3 (d), 124.9 (s), 127.7 (s), 129.0 (s), 144.6 (s), 148.2 (s), 170.5 (s). IR (KBr) cm^{-1}: 3400—3050, 1650, 1620. MS *m/z* (%): 276 (M$^+$, 15), 205 (17), 204 (100), 189 (12). High-resolution MS Calcd for $C_{15}H_{20}N_2O_3$: 276.1474. Found: 276.1471. *Anal.* Calcd for $C_{15}H_{20}N_2O_3 \cdot 1/5H_2O$: C, 64.36; H, 7.35; N, 10.01. Found: C, 64.34; H, 7.33; N, 9.76.

3-(2-Bromo-5-hydroxy-4-methoxy-3-methylphenylmethyl)-1-methyl-2,5-piperazinedione (22) A carbon tetrachlorode solution of bromine (1.0 M, 1.3 ml, 1.3 mmol) was added to a stirred solution of **5** (278.0 mg, 1.0 mmol) in dry THF–dichloromethane (2 : 1, 10 ml) at 0 °C for 10 min, and the mixture was stirred at the same temperature for 2 h. The reaction mixture was diluted with water (100 ml), and extracted with chloroform (50 ml×3). The combined extracts were washed with water (50 ml), dried, and concentrated *in vacuo* to give a solid (363 mg). Chromatography on a silica gel (50 g) column with dichloromethane–methanol (100 : 3) gave a soild (352 mg), recrystallization of which from methanol gave **22** (324 mg, 91%) as colorless needles, mp 232—234 °C. ^{1}H-NMR δ: 2.37 (3H, s, ArCH$_3$), 3.00 (3H, s, NCH$_3$), 3.01 (1H, dd, J=14.0, 9.1 Hz, ArCH), 3.61 (1H, dd, J=14.0, 4.0 Hz, ArCH), 3.75 (1H, d, J=17.7 Hz, 6-H), 3.78 (3H, s, OCH$_3$), 3.88 (1H, d, J=17.7 Hz, ArCH), 4.34 (1H, dd, J=9.1, 4.0 Hz, 3-H), 5.87 (2H, br s, NH, OH), 6.76 (1H, s, ArH). ^{13}C-NMR δ (CDCl$_3$+CD$_3$OD): 16.8 (q), 33.8 (q), 40.2 (t), 51.0 (t), 55.5 (d), 60.4 (q), 116.0 (d), 117.3 (s), 130.5 (s), 133.0 (s), 146.0 (s), 148.9 (s), 165.5 (s), 166.0 (s). IR (KBr) cm^{-1}: 3280, 1685, 1655. MS *m/z* (%): no M$^+$, 277 (100), 231 (52), 229 (54). *Anal.* Calcd for $C_{14}H_{17}BrN_2O_4$: C, 47.07; H, 4.80; N, 7.84. Found: C, 46.83; H, 4.81; N, 7.79.

Further elution with dichloromethane–methanol (50 : 3) gave the residue (20 mg), which showed two major spots on TLC (*Rf* 0.35 and 0.45, 4 : 5 acetone–chloroform). This material was subjected to chromatography on preparative layer silica gel plates (Merck 5715, solvent, 1 : 2 acetone–chloroform) to afford **23a** (7.0 mg, 2.0%) and **23b** (8.0 mg, 1.8%).

3-(2-Bromo-3-hydroxy-4-methoxy-5-methylphenylmethyl)-1-methyl-2,5-piperazinedione (**23a**): Recrystallization from methanol gave colorless prisms, mp 113—115.5 °C. ^{1}H-NMR δ: 2.25 (3H, s, ArCH$_3$), 2.98 (1H, dd, J=14.0, 9.2 Hz, ArCH), 2.99 (3H, s, NCH$_3$), 3.54 (1H, dd, J=14.0, 3.6 Hz, ArCH), 3.70 (1H, d, J=17.7 Hz, 6-H), 3.81 (3H, s, OCH$_3$), 3.85 (1H, d, J=17.7 Hz, ArCH), 4.32 (1H, dd, J=9.2, 3.6 Hz, 3-H), 6.16 (1H, br s, OH), 6.34 (1H, br s, NH), 6.63 (1H, s, ArH). ^{13}C-NMR δ: 15.7 (q), 33.9 (q), 39.7 (t), 51.6 (t), 55.2 (d), 60.6 (q), 109.7 (s), 124.2 (d), 130.6 (s), 130.8 (s), 145.4 (s), 147.0 (s), 165.1 (s), 165.4 (s). IR (KBr) cm^{-1}: 3600—2800, 1705, 1660. MS *m/z* (%): 358 (M$^+$+2, 4), 356 (M$^+$, 4), 278 (15), 277 (85), 231 (98), 229 (100). *Anal.* Calcd for $C_{14}H_{17}BrN_2O_4 \cdot 1/2H_2O$: C, 45.92; H, 4.95; N, 7.65. Found: C, 46.12; H, 4.82; N, 7.54.

3-(2,6-Dibromo-3-hydroxy-4-methoxy-5-methylphenylmethyl)-1-methyl-2,5-piperazinedione (**23b**): Recrystallization from methanol gave colorless prisms, mp 222—224 °C. ^{1}H-NMR δ: 2.32 (3H, s, ArCH$_3$), 3.04 (3H, s, NCH$_3$), 3.50 (1H, dd, J=13.9, 10.2 Hz, ArCH), 3.72 (1H, dd, J=13.9, 5.0 Hz, ArCH), 3.77 (3H, s, OCH$_3$), 3.94, 4.14 (each 1H, d, J=17.5 Hz, ArCH), 4.46 (1H, ddd, J=10.2, 5.0, 3.0 Hz, 3-H), 6.18 (1H, br s, NH), 6.80 (1H, br s, OH). ^{13}C-NMR δ: 17.3 (q), 34.1 (q), 41.2 (t), 51.6 (t), 53.9 (d), 61.0 (q), 110.7 (s), 118.5 (s), 130.5 (s), 132.1 (s), 145.4 (s), 146.5 (s), 165.2 (s), 165.7 (s). IR (KBr) cm^{-1}: 3280, 1685, 1655. MS *m/z* (%): no M$^+$, 358 (17), 357 (98), 356 (20), 355 (100), 311 (49), 309 (97), 307 (52). *Anal.* Calcd for $C_{14}H_{16}Br_2N_2O_4$: C, 38.56; H, 3.70; N, 6.42. Found: C, 38.52; H, 3.78; N, 6.37.

Attempted Direct Conversion of 22 to 6 A solution of **22** (35.8 mg, 0.1 mmol), triethylamine (0.028 ml, 0.2 mmol), and DMAP (24.5 mg, 0.2 mmol) in dry dichloromethane (2 ml) was cooled with ice-water, and isopropyl chloroformate (0.0455 ml, 0.4 mmol) was added dropwise over 10 min. The reaction mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with dichloromethane (20 ml), the organic layer was washed with 1 N HCl (10 ml), and then water (20 ml), dried, and concentrated *in vacuo* to give the residue. Chromatography on a silica gel (5 g) column with dichloromethane–methanol (80 : 1) as the eluent gave **24** (29.4 mg, 66.7%) as a solid, recrystallization of which from acetone afforded colorless needles, mp 146—147.5 °C. Further elution with dichloromethane–methanol (30 : 1) as the eluent gave the starting material (8.1 mg, 22.6% recovery). This reaction was done using NaH as base in DMF to give **24** and **22** in 74.1% and 5.6% yields, respectively.

Compound **24**: ^{1}H-NMR δ: 1.38 (6H, d, J=6.3 Hz, CH(C$\underline{H}_3$)$_2$), 2.39 (3H, s, ArCH$_3$), 2.95 (3H, s, NCH$_3$), 3.15 (1H, dd, J=13.9, 6.9 Hz, ArCH), 3.56 (1H, d, J=17.5 Hz, 6-H), 3.60 (1H, dd, J=13.9, 4.6 Hz, ArCH), 3.73 (1H, d, J=17.7 Hz, 6-H), 3.78 (3H, s, OCH$_3$), 4.34 (1H, m, 3-H), 4.96 (1H, sept, J=6.3 Hz, OCH), 6.36 (1H, br s, NH), 6.93 (1H, s, ArH). IR (KBr) cm^{-1}: 3400—3050, 1785, 1715, 1695. MS *m/z* (%): 444 (M$^+$+2, 2), 442 (M$^+$, 2), 364 (11), 363 (50), 319 (10), 278 (18), 277 (100), 231 (49), 229 (50), 43 (19). *Anal.* Calcd for $C_{18}H_{23}BrN_2O_6$: C, 48.77; H, 5.23; N, 6.32. Found: C, 48.72; H, 5.24; N, 6.32.

3-(5-Benzyloxy-2-bromo-4-methoxy-3-methylphenylmethyl)-1-methyl-2,5-piperazinedione (25) A suspension of **22** (1.074 g, 3.0 mmol) and anhydrous K_2CO_3 (0.622 g, 4.5 mmol) in dry DMF (60 ml) was cooled with ice-water, and benzyl bromide (0.428 ml, 3.6 mmol) was added over 5 min. This mixture was heated at 60 °C for 3 h. The reaction mixture was diluted with water (100 ml), and extracted with chloroform (100 ml×3). The combined extracts were washed with brine (100 ml), dried, and concentrated *in vacuo* to give a residue, recrystallization of which from ethyl acetate–ether afforded **25** (1.207 g, 89.8%) as colorless prisms, mp 150.5—151 °C. ^{1}H-NMR δ: 2.38 (3H, s, ArCH$_3$), 2.96 (3H, s, NCH$_3$), 3.06 (1H, dd, J=14.2, 8.6 Hz, ArCH), 3.57 (1H, dd, J=14.2, 4.0 Hz, ArCH), 3.68 (1H, d, J=17.8 Hz, 6-H), 3.82 (3H, s, OCH$_3$), 3.84 (1H, d, J=17.8 Hz, ArCH), 4.30

(1H, m, 3-H), 5.04, 5.09 (each 1H, d, J=11.9 Hz, OCHAr), 5.87 (1H, br s, NH), 6.73 (1H, s, ArH), 7.31—7.45 (5H, m, 5×ArH). IR (KBr) cm^{-1}: 3350—2850, 1680. MS m/z (%): 448 (M^{+}+2, 7), 446 (M^{+}, 7), 368 (28), 367 (97), 321 (39), 319 (28), 277 (11), 276 (13), 275 (8), 91 (100). *Anal.* Calcd for $C_{21}H_{23}BrN_2O_4$: C, 56.39; H, 5.18; N, 6.26. Found: C, 56.19; H, 5.23; N, 6.14.

3-(5-Benzyloxy-2-bromo-4-methoxy-3-methylphenylmethyl)-4-isopropyloxycarbonyl-1-methyl-2,5-piperazinedione (26) A solution of **25** (626.3 mg, 1.40 mmol), triethylamine (0.488 ml, 3.50 mmol), and DMAP (428.0 mg, 3.50 mmol) in dry dichloromethane (30 ml) was cooled with ice-water, and isopropyl chloroformate (0.795 ml, 7.00 mmol) was added dropwise over 10 min. The reaction mixture was stirred at room temperature for 4 h. The reaction mixture was diluted with dichloromethane (30 ml), the organic layer was washed with 1 N HCl (30 ml), and then water (30 ml), dried, and concentrated *in vacuo* to give a solid, recrystallization of which from acetone afforded **26** (706.3 mg, 94.6%) as colorless prisms, mp 146—147.5 °C. ^{1}H-NMR δ: 1.27, 1.32 (each 3H, d, J=6.3 Hz, $OCH(C\underline{H}_3)_2$), 2.35 (3H, s, $ArCH_3$), 2.84 (3H, s, NCH_3), 3.13 (1H, d, J=18.2 Hz, 6-H), 3.28, 3.60 (each 1H, dd, J=14.2, 5.3 Hz, ArCH), 3.65 (1H, d, J=18.2 Hz, 6-H), 3.81 (3H, s, OCH_3), 5.03 (2H, s, OCH_2Ar), 5.05 (1H, sept, J=6.3 Hz, OCH), 5.10 (1H, t, J=5.3 Hz, 3-H), 6.68 (1H, s, ArH), 7.33—7.46 (5H, m, 5×ArH). ^{13}C-NMR δ: 17.7 (q), 22.2 (q), 22.4 (q), 33.9 (q), 39.4 (t), 53.2 (t), 60.3 (d), 61.4 (q), 71.6 (t), 72.8 (d), 114.9 (d), 120.5 (s), 128.0 (d×2), 128.8 (d), 129.2 (s), 129.3 (d×2), 131.0 (s), 134.8 (s), 137.1 (s), 148.6 (s), 151.7 (s), 164.6 (s), 166.5 (s). IR (KBr) cm^{-1}: 1790, 1690, 1665. MS m/z (%): 534 (M^{+}+2, 26), 532 (M^{+}, 26), 454 (32), 453 (100), 367 (31), 321 (37), 319 (38), 276 (11), 275 (17), 240 (17), 91 (89), 43 (14). *Anal.* Calcd for $C_{25}H_{29}BrN_2O_6$: C, 56.29; H, 5.48; N, 5.25. Found: C, 56.14; H, 5.49; N, 5.20.

Debenzylation of 26. Method A A solution of **26** (32.0 mg, 0.06 mmol) in TFA (1 ml) was stirred at room temperature for 18 h. The reaction mixture was diluted with water (10 ml) and extracted with chloroform (10 ml×3). The combined extracts were washed with water (10 ml), dried, and concentrated *in vacuo*. The residue (38.4 mg), which showed two major spots on TLC (*Rf* 0.3 and 0.1, 1 : 1 hexane–ethyl acetate), was subjected to chromatography on preparative layer silica gel plate (Merck 5715, solvent 1 : 1 hexane–ethyl acetate) afforded **6** (13.4 mg, 51.6%) and **27a** (9.8 mg, 30.6%).

Method B A solution of **26** (53.3 mg, 0.1 mmol) and 1,3-dimethoxybenzene (0.262 ml, 2.0 mmol) in TFA (2 ml) was stirred at room temperature for 18 h. After usual work up described as above gave **6** (31.9 mg, 72.0%) and **27a** (10.9 mg, 20.5%).

Method C A solution of **26** (53.3 mg, 0.1 mmol) in chloroform (2 ml) was cooled with ice-water, and TMSI (0.021 ml, 0.15 mmol) was added dropwise over 10 min. The reaction mixture was heated at 50 °C for 24 h. The reaction mixture was diluted with methanol (5 ml) and brine (10 ml), and extracted with chloroform (10 ml×3). The combined extracts were washed with water (10 ml), dried, and concentrated *in vacuo* to give the residue (40.3 mg). Chromatography on a silica gel (8 g) column with dichloromethane–methanol (50 : 1) gave **6** (7.8 mg, 17.6%). Further elution with dichloromethane–methanol (25 : 1) gave **27b** (9.9 mg, 27.7%).

3-(2-Bromo-5-hydroxy-4-methoxy-3-methylphenylmethyl)-4-iso-propyloxycarbonyl-1-methyl-2,5-piperazinedione (**6**): Recrystallization from ethyl acetate–ether gave colorless prisms, mp 156—158 °C. ^{1}H-NMR δ: 1.29, 1.33 (each 3H, d, J=6.2 Hz, $OCH(C\underline{H}_3)_2$), 2.34 (3H, s, $ArCH_3$), 2.90 (3H, s, NCH_3), 3.12 (1H, d, J=18.2 Hz, 6-H), 3.22 (1H, dd, J=14.0, 5.3 Hz, ArH), 3.63 (1H, dd, J=14.2, 5.5 Hz, ArCH), 3.67 (1H, d, J=18.2 Hz, 6-H), 3.78 (3H, s, OCH_3), 5.04 (1H, sept, J=6.2 Hz, OCH), 5.10 (1H, dd, J=5.5, 5.3 Hz, 3-H), 6.54 (1H, br s, OH), 6.74 (1H, s, ArH). ^{13}C-NMR δ: 17.2 (q), 21.6 (q), 21.7 (q), 33.3 (q), 38.5 (t), 52.5 (t), 59.7 (d), 61.1 (q), 72.2 (d), 115.6 (d), 118.6 (s), 131.3 (s), 132.5 (s), 145.8 (s), 148.3 (s), 150.9 (s), 163.8 (s), 166.0 (s). IR (KBr) cm^{-1}: 3600—3050, 1734, 1720, 1662. MS m/z (%): 444 (M^{+}+2, 3), 442 (M^{+}, 3), 364 (21), 363 (100), 278 (10), 277 (55), 231 (60), 229 (63), 43 (12). *Anal.* Calcd for $C_{18}H_{23}BrN_2O_6$: C, 48.77; H, 5.23; N, 6.32. Found: C, 48.74; H, 5.16; N, 6.25.

3-(2-Bromo-5-hydroxy-4-methoxy-3-methyl-6-phenylmethyl-phenylmethyl)-4-isopropyloxycarbonyl-1-methyl-2,5-piperazinedione (**27a**): Recrystallization from methanol gave colorless prisms, mp 255.5—257 °C. ^{1}H-NMR δ ($CDCl_3$+CD_3OD, 55 °C): 1.17, 1.24 (each 3H, d, J=6.3 Hz, $OCH(C\underline{H}_3)_2$), 2.36 (3H, s, $ArCH_3$), 2.93 (3H, s, NCH_3), 3.34 (2H, d, J=7.6 Hz, $ArCH_2$), 3.76 (3H, s, OCH_3), 3.86, 4.13 (each 1H, d, J=17.8 Hz, 6-H), 4.21 (2H, s, OCH_2), 4.94 (1H, sept, J=6.3 Hz, OCH), 5.11 (1H, t, J=7.6 Hz, 3-H), 7.10—7.42 (5H, m, 5×ArH). ^{13}C-NMR δ ($CDCl_3$+CD_3OD, 55 °C): 17.3 (q), 21.3 (q), 21.5 (q), 32.0 (t), 33.5 (q), 35.9 (t), 58.2 (t), 59.0 (d), 61.0 (q), 72.2 (d), 119.5 (s), 126.0 (d), 128.2 (d×2), 128.4 (d×2), 129.9 (s), 130.4 (s), 134.2 (s), 139.8 (s), 145.5 (s), 147.0 (s), 151.2 (s), 164.7 (s), 165.7 (s). IR (KBr) cm^{-1}: 3160, 1780, 1700, 1670. MS m/z (%): 534 (M^{+}+2, 3), 532 (M^{+}, 3), 454 (31), 453 (100), 368 (11), 367 (42), 240 (42), 209 (11), 208 (14), 91 (14). *Anal.* Calcd for $C_{25}H_{29}BrN_2O_6$: C, 56.29; H, 5.48; N, 5.25. Found: C, 56.24; H, 5.53; N, 5.15.

3-(2-Bromo-4,5-dihydroxy-3-methylphenylmethyl)-1-methyl-2,5-piperazinedione (**27b**): Recrystallization from methanol gave colorless prisms, mp 206—208 °C. ^{1}H-NMR δ ($CDCl_3$+CD_3OD): 2.31 (3H, s, $ArCH_3$), 2.92 (3H, s, NCH_3), 3.06 (1H, dd, J=13.9, 6.9 Hz, ArCH), 3.37 (1H, d, J=17.2 Hz, ArCH), 3.64—3.78 (2H, m, 6-H, ArCH), 4.25 (1H, t like, 3-H), 6.52 (1H, s, ArH). IR (KBr) cm^{-1}: 3340, 1700, 1655. MS m/z (%): 344 (M^{+}+2, 1), 342 (M^{+}, 1), 264 (8), 263 (100), 217 (35), 215 (35), 128 (85). *Anal.* Calcd for $C_{13}H_{15}BrN_2O_4$: C, 45.50; H, 4.41; N, 8.16. Found: C, 45.29; H, 4.39; N, 8.06.

3-(2-Bromo-5-hydroxy-4-methoxy-3-methyl-6-phenylmethyl-phenylmethyl)-1-methyl-2,5-piperazinedione (28) Concentrated H_2SO_4 (0.025 ml) was added to a stirred solution of **27a** (9.8 mg, 0.0184 mmol) in TFA (0.5 ml). The resulting solution was stirred at room temperature for 60 h. The reaction mixture was poured into water (10 ml), made alkaline with 5% $NaHCO_3$, and extracted with chloroform (10 ml×3). The combined extracts were washed with brine (10 ml), dried, and concentrated *in vacuo* to give a residue, recrystallization of which from ethyl acetate–ether gave **28** (6.0 mg, 73.0%) as colorless prisms, mp 193—195 °C. ^{1}H-NMR δ: 2.38 (3H, s, $ArCH_3$), 2.99 (3H, s, NCH_3), 3.23 (1H, dd, J=14.2, 10.9 Hz, ArCH), 3.56 (1H, dd, J=14.2, 4.6 Hz, ArCH), 3.79 (3H, s, OCH_3), 3.86, 3.98 (each 1H, d, J=17.8 Hz, 6-H), 4.07, 4.22 (each 1H, d, J=15.8 Hz, ArCHAr′), 4.22 (1H, m, 3-H), 5.72, 6.05 (each 1 H, br s, D_2O exchangeable, NH and OH), 7.08—7.24 (5H, m, 5×ArH). ^{13}C-NMR δ: 17.4 (q), 32.4 (t), 33.9 (q), 37.1 (t), 51.5 (t), 54.4 (d), 61.2 (q), 119.2 (s), 125.7 (s), 126.2 (d), 127.9 (d×2), 128.6 (d×2), 130.6 (s), 130.8 (s), 139.5 (s), 145.2 (s), 147.1 (s), 165.2 (s), 165.4 (s). IR (KBr) cm^{-1}: 3450—3150, 1700, 1670. MS m/z (%): 448 (M^{+}+ 2, 3), 446 (M^{+}, 3), 368 (27), 367 (100), 240 (29), 225 (12), 209 (12), 208 (14), 91 (11). *Anal.* Calcd for $C_{21}H_{23}BrN_2O_4$: C, 56.39; H, 5.18; N, 6.26. Found: C, 56.52; H, 5.37; N, 6.06.

3-(2-Bromo-5-hydroxy-4-methoxy-3-methylphenylmethyl)-4-isopropyloxycarbonyl-1-methyl-2,5-piperazinedione (6) from 17 A carbon tetrachloride solution of bromine (1.0 M, 2.2 ml, 2.2 mmol) was added to a stirred solution of **17** (728.8 mg, 2.0 mmol) in dry dichloromethane (20 ml) at 0 °C for 10 min, and the mixture was stirred at the same temparature for 1 h. The reaction mixture was diluted with water (40 ml), and extracted with chloroform (40 ml×3). The combined extracts were washed with water (40 ml), dried, and concentrated *in vacuo* to give a solid, recrystallization of which from ethyl acetate–ether afforded **6** (790.7 mg, 89.2%) as colorless prisms, mp 156—158 °C, which were identical in all respects with an authentic sample described above.

Conversion of 26 to 30 *via* 29 A stirred solution of **26** (53.3 mg, 0.1 mmol) in dry THF (5 ml) was cooled with ice-water, and lithium tri-*tert*-butoxyaluminium hydride (152.6 mg, 0.6 mmol) was added to it over 5 min. After continued stirring at the same temperature for 1 h, the reaction mixture was quenched by the addition of water (1 ml) and then filtered through a Celite pad, and the filtrate was concentrated *in vacuo*. The unstable diastereomeric mixture of the alcohols **29** (71 mg) obtained was used for the next step without further purification. Methanesulfonic anhydride (20.9 mg, 0.12 mmol) was added to a stirred solution of the crude **29** in dry dichloromethane (4 ml) at 0 °C dropwise for 10 min, the reaction mixture was heated reflux for 6 h. The reaction mixture was diluted with water (10 ml) and extracted with chloroform (20 ml×3). The combined extracts were washed with 5% $NaHCO_3$ (20 ml), dried, and concentrated *in vacuo* to give a solid, recrystallization of which from ether gave **30** (51.7 mg, 100%) as colorless needles, mp 125—126.5 °C. ^{1}H-NMR δ (71 : 29 a pair of rotamers was existed): major rotamer 0.79, 1.10 (each 3H, d, J=6.3 Hz, $OCH(C\underline{H}_3)_2$), 2.36 (3H, s, $ArCH_3$), 2.84 (1H, dd, J=13.9, 10.2 Hz, ArCH), 3.11 (3H, s, NCH_3), 3.18 (1H, dd, J=13.9, 4.0 Hz, ArCH), 3.79 (3H, s, OCH_3), 4.63 (1H, sept, J=6.2 Hz, OCH), 5.05 (2H, s, OCH_2Ar), 5.10 (1H, ddd, J=10.2, 4.0, 1.7 Hz, 3-H), 5.63 (1H, d, J=5.9 Hz, 6-H), 6.28 (1H, dd, J=5.9, 1.7 Hz, 5-H), 6.56 (1H, s, ArH), 7.31—7.47 (5H, m, 5×ArH). Minor rotamer 1.13, 1.22 (each 3H, d, J=6.3 Hz, $OCH(C\underline{H}_3)_2$), 2.35 (3H, s, $ArCH_3$), 2.97 (1H, dd, J=13.5, 8.3 Hz, ArCH), 3.06 (3H, s, NCH_3), 3.28 (1H, dd, J=13.5, 5.3 Hz, ArCH), 3.78 (3H, s, OCH_3), 4.83 (1H, sept, J=6.2 Hz, OCH), 5.05 (2H, s, OCH_2Ar), 5.11 (1H, m, 3-H), 5.37 (1H, d, J=5.9 Hz, 6-H), 6.12 (1H, dd, J=5.9, 1.3 Hz, 5-H), 6.71 (1H, s, ArH), 7.31—7.47 (5H, m, 5×ArH). IR (KBr) cm^{-1}: 1705, 1670. MS m/z (%): 516 (M^{+}+2, 2), 514 (M^{+}, 2), 321 (11), 319 (11), 197 (67), 155 (10), 153 (11), 111 (100), 91 (25), 43 (28). *Anal.* Calcd for $C_{25}H_{27}BrN_2O_5 \cdot 1/5H_2O$: C, 57.86; H, 5.32; N, 5.40. Found: C, 57.82; H, 5.61; N, 5.36.

Attempted Cyclization of 26 to 31 *via* 29 The same procedure for the reduction of **26** (53.3 mg, 0.1 mmol) as descrived above afforded **29** (87.3 mg). Concentrated H_2SO_4 (0.1 ml) was added to a solution of **29** in TFA (2 ml), and the resulting solution was stirred at room temperature for 21 h. The reaction mixture was diluted with water (10 ml) and extracted with chloroform (20 ml×3). The combined extracts were washed with 5% $NaHCO_3$ (20 ml), dried, and concentrated *in vacuo* to give a soild (58.9 mg). Recrystallization of which from acetone–methanol afforded **31** (22.0 mg, 64.5%) as colorless prisms, mp 268—270 °C. ^{1}H-NMR δ ($CDCl_3$+CD_3OD): 2.35 (3H, s, ArCH_3), 2.87 (3H, s, NCH_3), 2.88 (1H, dd, J=17.8, 6.9 Hz, 6-Hα), 3.06 (1H, dd, J=17.8, 1.7 Hz, 6-Hβ), 3.34 (1H, dd, J=12.2, 1.0 Hz, 2-Hβ), 3.74 (3H, s, OCH_3), 3.83 (1H, dd, J=12.2, 5.0 Hz, 2-Hα), 3.99 (1H, dd, J=6.9, 1.7 Hz, 5-H), 4.55 (1H, dd, J=5.0, 1.0 Hz, 1-H). ^{13}C-NMR δ ($CDCl_3$+CD_3OD): 16.4 (q), 34.1 (q), 34.3 (t), 44.8 (d), 53.1 (d), 55.1 (t), 60.9 (q), 117.4 (s), 122.9 (s), 129.3 (s), 130.5 (s), 144.1 (s), 144.5 (s), 170.5 (s). IR (KBr) cm^{-1}: 3330, 3250, 1640, 1615. MS *m/z* (%): 342 (M$^+$+2, 22), 340 (M$^+$, 24), 271 (19), 270 (97), 269 (25), 268 (100), 255 (12), 253 (11). *Anal.* Calcd for $C_{14}H_{17}BrN_2O_3$: C, 49.28; H, 5.02; N, 8.21. Found: C, 49.44; H, 5.10; N, 8.12.

Isopropyl 1,2,3,4,5,6-Hexahydro-1,5-imino-7-bromo-10-hydroxy-9-methoxy-3,8-dimethyl-4-oxo-3-benzazocine-11-carboxylic Acid (33) A stirred solution of **6** (541.6 mg, 1.25 mmol) in dry THF (50 ml) was cooled with ice-water, and lithium tri-*tert*-butoxyaluminium hydride (2.225 g, 8.75 mmol) was added to it over 5 min. After continued stirring at the same temperature for 1 h, the reaction mixture was quenched by the addition of water (1 ml) and filtered through a Celite pad, and the filtrate was concentrated *in vacuo*. The unstable diastereomeric mixture of the alcohols **32** (1.249 g) obtained was used for the next step without further purification. A stirred solution of the crude **32** in TFA (10 ml) was stirred at room temperature for 13 h. The reaction mixture was diluted with water (60 ml) and extracted with chloroform (80 ml×3). The combined extracts were washed with 5% $NaHCO_3$ (80 ml), dried, and concentrated *in vacuo* to give a solid, recrystallization of which from acetone gave **33** (520.0 mg, 97.4%) as colorless prisms, mp 265—266 °C. ^{1}H-NMR δ (at 50 °C): 1.25 (6H, d, J=6.3 Hz, CH(C$\underline{H}_3$)$_2$), 2.35 (3H, s, ArCH_3), 2.85 (3H, s, NCH_3), 2.96 (1H, dd, J=17.5, 5.9 Hz, 6-Hα), 3.17 (1H, dd, J=17.5, 1.7 Hz, 6-Hβ), 3.31 (1H, d, J=11.9 Hz, 2-Hβ), 3.77 (3H, s, OCH_3), 3.85 (1H, dd, J=11.9, 4.6 Hz, 2-Hα), 4.90—4.98 (2H, m, OCH and 5-H), 5.62 (1H, br s, 1-H), 5.83 (1H, br s, OH). ^{13}C-NMR δ (at 55 °C): 16.7 (q), 22.1 (q), 22.1 (q), 33.8 (t), 34.2 (q), 44.8 (d), 53.0 (d), 54.0 (t), 61.2 (q), 69.7 (d), 121.2 (s), 129.8 (s), 130.8 (s), 138.8 (s), 144.2 (s), 144.3 (s), 153.4 (s), 168.1 (s). IR (KBr) cm^{-1}: 3500—3050, 1720, 1650, 1610. MS *m/z* (%): 428 (M$^+$+2, 78), 426 (M$^+$, 79), 386 (11), 385 (12), 384 (12), 357 (19), 356 (40), 355 (20), 354 (37), 342 (39), 341 (36), 340 (41), 339 (30), 315 (36), 314 (45), 313 (38), 312 (45), 271 (19), 270 (98), 269 (24), 268 (100). *Anal.* Calcd for $C_{18}H_{23}BrN_2O_5$: C, 50.60; H, 5.43; N, 6.56. Found: C, 50.52; H, 5.42; N, 6.44.

1,2,3,4,5,6-Hexahydro-1,5-imino-7-bromo-10-hydroxy-9-methoxy-3,8-dimethyl-3-benzazocin-4-one (31) Concentrated H_2SO_4 (1.5 ml) was added to a stirred solution of **33** (427.0 mg, 0.1 mmol) in TFA (30 ml), and the resulting solution was stirred at room temperature for 19 h. The reaction mixture was diluted with water (100 ml) and extracted with chloroform (100 ml×3). The combined extracts were washed with 5% $NaHCO_3$ (100 ml), dried, and concentrated *in vacuo* to give a solid (58.9 mg), recrystallization of which from acetone–methanol afforded **31** (319.0 mg, 93.5%) as colorless prisms, mp 269—270 °C, which were identical in all respects with an authentic sample described above.

1,2,3,4,5,6-Hexahydro-1,5-imino-7-bromo-10-hydroxy-9-methoxy-3,8,11-trimethyl-3-benzazocin-4-one (34) Formaldehyde (37% solution in water, 10.0 ml, 125.0 mmol) was added to a stirred solution of **31** (214.0 mg, 0.627 mmol) in formic acid (11.6 ml, 308.0 mmol) at 60 °C. After having been stirred at 70 °C for 1 h, the reaction mixture was poured into water (100 ml), and extracted with chloroform (100 ml×3). The combined extracts were washed with 5% $NaHCO_3$ (100 ml), dried, and concentrated *in vacuo* to give a solid, recrystallization of which from methanol gave **34** (209.0 mg, 94.0%) as colorless prisms, mp 265.5—267 °C.^{1}H-NMR δ: 2.36 (3H, s, ArCH_3), 2.48, 2.86 (each 3H, s, NCH_3), 2.92 (1H, d, J=18.1 Hz, 6-Hβ), 3.01 (1H, dd, J=18.1, 5.6 Hz, 6-Hα), 3.21 (1H, d, J=11.9 Hz, 2-Hβ), 3.73 (1H, d, J=5.6 Hz, 5-H), 3.76 (3H, s, OCH_3), 3.94 (1H, dd, J=11.9, 4.6 Hz, 2-Hα), 4.24 (1H, d, J=4.6 Hz, 1-H), 6.06 (1H, br s, OH). ^{13}C-NMR δ: 16.7 (q), 29.5 (t), 33.9 (q), 39.7 (q), 51.0 (d), 52.5 (t), 59.4 (d), 61.3 (q), 117.8 (s), 120.6 (s), 129.2 (s), 130.4 (s), 144.0 (s), 144.8 (s), 169.6 (s). IR (KBr) cm^{-1}: 3500—3050, 1655, 1620. MS *m/z* (%): 356 (M$^+$+2, 23), 354 (M$^+$, 23), 285 (18), 284 (98), 283 (21), 282 (100), 269 (13), 267 (12). *Anal.* Calcd for $C_{15}H_{19}BrN_2O_3$: C, 50.72; H, 5.39; N, 7.89. Found: C, 50.65; H, 5.47; N,7.77.

1,2,3,4,5,6-Hexahydro-1,5-imino-10-hydroxy-9-methoxy-3,8,11-trimethyl-3-benzazocin-4-one (3) A solution of **34** (355.0 mg, 1 mmol) in ethanol (20 ml) was hydrogenated over 20% Pd(OH)$_2$/C (200 mg) at 1 atm for 6 h. The catalyst was removed by filtration and washed with ethanol (100 ml). The combined filtrates were evaporated to give a solid, recrystallization of which from ethyl acetate afforded **3** (262.2 mg, 95.0%) as colorless prisms, mp 216—217.5 °C. ^{1}H-NMR δ: 2.25 (3H, s, ArCH_3), 2.48 (3H, s, NCH_3), 2.80 (1H, d, J=17.3 Hz, 6-Hβ), 2.87 (3H, s, NCH_3), 3.13 (1H, dd, J=17.3, 6.6 Hz, 6-Hα), 3.18 (1H, d, J=11.9 Hz, 2-Hβ), 3.62 (1H, d, J=6.6 Hz, 5-H), 3.78 (3H, s, OCH_3), 3.90 (1H, dd, J=11.9, 4.9 Hz, 2-Hα), 4.18 (1H, d, J=4.6 Hz, 1-H), 5.96 (1H, br s, OH), 6.48 (1H, s, ArH). ^{13}C-NMR δ: 15.8 (q), 27.3 (t), 34.0 (q), 39.9 (q), 50.9 (d), 52.6 (t), 59.2 (d), 60.8 (q), 118.8 (s), 122.2 (d), 128.7 (s), 129.5 (s), 143.4 (s), 145.5 (s), 169.8 (s). IR (KBr) cm^{-1}: 3500—3050, 1730, 1625, 1605. MS *m/z* (%): 276 (M$^+$, 24), 205 (19), 204 (100), 189 (15). *Anal.* Calcd for $C_{15}H_{20}N_2O_3$: C, 65.19; H, 7.30; N, 10.14. Found: C, 65.03; H, 7.28; N, 9.96.

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Preparation of Benzene, Furan, and Thiophene Analogs of Duocarmycin SA Employing a Newly-Devised Phenol-Forming Reaction

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Five A-ring analogs of duocarmycin SA 9a—e were synthesized in racemic form modifying our second synthetic route toward duocarmycin SA. The problem encountered at the crucial phenol forming step to secure 17a, b from 16a, b under the conventionally used Kuwajima conditions was overcome by devising a more convenient method: simple heating of 16a—c in benzene in the presence of bis(triphenylphosphine)palladium(II) chloride (10 mol%), cesium carbonate (3 eq), and triphenylphosphine (0.3 eq) gave 17a—c in high yields of 86—91%. The intermediates 17a—e were readily led to the A-ring analogs (±)-9a—e almost according to the reported route.

Key words duocarmycin SA; analog synthesis; ketone α-arylation; antitumor antibiotic; palladium-catalyzed reaction; phenol-forming reaction

Duocarmycin SA (DSA, **1**) is an exceptionally potent antitumor antibiotic isolated from a culture broth of *Streptomyces* species in 1990 (Chart 1).1) Among the several structurally related antibiotics, CC-1065,2) duocarmycin A,3,4) duocarmycins B$_1$,4,5) B$_2$,4,5) C$_1$4,6) (=pyrindamycin B7)), C$_2$4,6) (=pyrindamycin A7)), and D,4) **1** is the newest and most promising member since it has proved to be the most potent and most stable.8) We have already reported three independent synthetic routes toward **1**.9—11) Two more total syntheses of **1** have been reported by Boger and his colleagues12) and by Fukuda and Terashima.13)

In our first route, (±)-**1** was synthesized starting from methyl 5-acetyl-4-bromo-1*H*-pyrrole-2-carboxylate (**2**) by way of pyrrolo[3,2-*f*]quinoline derivative **3** in 15 total steps, in 10% overall yield.9) The second route also commenced from **2** involving two palladium-catalyzed carbon–carbon bond formation reactions to construct the tricyclic heteroaromatic intermediate **4**. (±)-**1** was prepared in 13 steps, in 22% overall yield from **2** by the second route.10) In both routes, optical resolution was readily executed by the HPLC separation of (*R*)-*O*-methylmandelate of the pyrrolo[3,2-*f*]quinolinol intermediate **5**.14) The separated unnatural (*R*)-**5** was converted to natural (*S*)-**5** by the inversion of the hydroxy group under the Mitsunobu reaction conditions. Thus, two enantioselective syntheses of **1** were established in an enantio-convergent manner. By the third route, optically pure **1** was synthesized starting from L-malic acid by way of the intermediate **6** in 19 steps, in 2% overall yield.11) The absolute configuration of **1** was unequivocally shown to be 7b*R* for the first time by the third route.

The structure of the A-ring of **1** and its congeners has been proved to greatly influence the cytotoxic activity as well as the chemical stability, and some A-ring analogs have been selected as clinical candidates.15) Furthermore, the simpler structure of DSA compared to those of taxol, mitomycin C, adriamycin *etc.* would permit a perpetual supply of the analogs by total synthesis. With this background in mind, we turned our attention to the preparation of A-ring analogs of **1** and earlier reported the synthesis of furan and thiophene analogs of DSA **7** and **8** applying our first synthetic route (Chart 2).16) Among the above three routes, however, the second one10) is the most suitable for analog synthesis, because it is the most practical from the viewpoints of number of steps, overall yield, and simple operations. Herein we describe the full details of our studies on the preparation of five A-ring analogs of DSA **9a**—**e**, modifying our second route.17) The crucial palladium-catalyzed phenol-forming step (**10**→**11**) was revised and was much improved by the development of direct intramolecular α-arylation reaction of ketone instead of the Kuwajima conditions18) which we had conventionally employed. Thus, as shown in Chart 4, Table 1 (*vide infra*), the direct cyclization of **16a**—**c** to **17a**—**c** (isolated after protection of phenol as methyl carbonate) was attained in high yields under novel palladium-catalyzed conditions: bis(triphenylphosphine)palladium(II) chloride $[PdCl_2(Ph_3P)_2]$–cesium carbonate (Cs_2CO_3)–triphenylphosphine (Ph_3P) in boiling benzene. The benzene [(±)-**9a**], furan [(±)-**9b**], thiophene [(±)-**9c**], and benzothiophene [(±)-**9d**] analogs of DSA lack methoxycarbonyl group at the 2-position which contributes to the chemical stability of the cyclopropanoindolinone pharmacophore. A preparation of (±)-**9e** bearing a bulky 3,4,5-trimethoxybenzyl group at the 2-position is also described, aiming for enhancement of the cytotoxic activity.

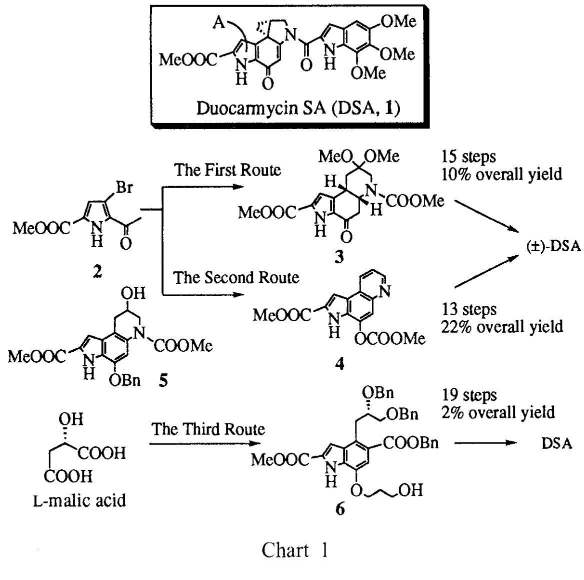

Chart 1

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Dedicated to the memory of Dr. Kyosuke Tsuda.

The transformation of **16a**—**c** to **17a**—**c** (Chart 4) is substantially regarded as an intramolecular version of the α-arylation reaction of aliphatic ketone. We have already reported an extension of the above palladium-catalyzed reaction conditions to the intramolecular α-arylation reaction of ketone,[19] aldehyde,[20] and nitro[20] groups as communications.

Preparation of Tricyclic Heteroaromatic Intermediates 17a—e At the outset, tricyclic aromatic intermediates **17a**—**e** corresponding to compound **4** were prepared according to our second route. The compounds **17a**—**d** were synthesized from the corresponding **12a**—**d** as shown in Chart 3. Compound **17e** was prepared later by substitution at 2-position of **17c** as shown in Chart 6.

i) Preparation of the Precursors **16a**—**d** for Cyclization: Among the starting materials **12a**—**d**, *o*-bromoacetophenone (**12a**) is commercially available (Chart 3). The yields of the known compounds **12b**,[21] **c**[22] were improved as follows. Treatment of 3-bromofuran in dichloromethane (CH_2Cl_2) with acetic anhydride (Ac_2O) and boron trifluoride etherate ($BF_3 \cdot OEt_2$) afforded **12b** in 76% yield accompanied by an isomer, 1-(4-bromo-2-furanyl)ethanone in 10% yield. The starting material, 1-(3-bromo-2-thienyl)ethanone (**12c**) prepared from 3-bromothiophene in 79% yield by treatment with Ac_2O and aluminum chloride ($AlCl_3$) in CH_2Cl_2 was found to contain an inseparable minor isomer, 1-(4-bromo-2-thienyl)ethanone in a ratio of 16 : 1 estimated by the ^{1}H-NMR integral values of the methyl signal observed at δ 2.67 (major) and δ 2.53 (minor). The same acylation procedure on 3-bromobenzo[*b*]thiophene with $AlCl_3$ readily gave **12d** in 78% yield.

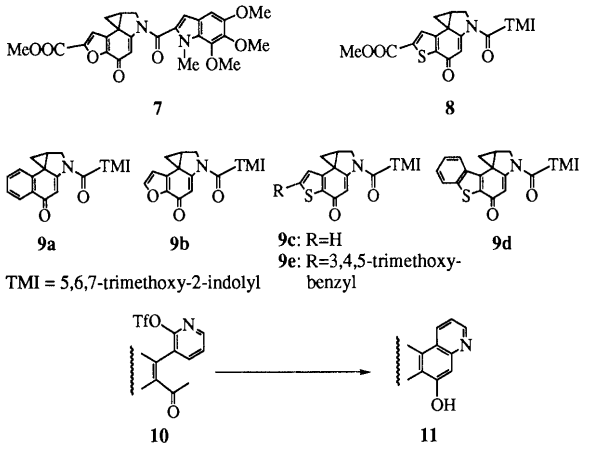


Chart 2

The Stille coupling reaction[23] of thus prepared **12a**—**d** with 2-fluoro-3-(trimethylstannyl)pyridine (**13**)[10] catalyzed by $PdCl_2(Ph_3P)_2$ proceeded as before without trouble affording **14a**—**d** in good to high yields (Chart 3). The yield of **14c** was calculated from used **13** because of the impurity of **12c**. Subsequent hydrolysis in 10% aqueous hydrochloric acid (HCl)-1,2-dimethoxyethane (DME) at 60 °C readily afforded pyridones **15a**—**d**, which were then transformed to the required precursors **16a**—**d** with trifluoromethanesulfonic anhydride (Tf_2O) and pyridine (Py) in CH_2Cl_2 at 0 °C to ambient temperature in high yields.

ii) Cyclization of **16a**—**d** to **17a**—**d** by the Conventional Method: The obtained triflates **16a**—**d** were subjected to the palladium-catalyzed cyclization under the Kuwajima conditions[18] following the conventional method of our second route (Chart 3).[10] First, the acetyl group was converted to enol silyl ether with *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMSOTf) and triethylamine (Et_3N). Then the crude product was heated in boiling xylene with 5 mol% of $PdCl_2(Ph_3P)_2$, 1.2 eq of tributyltin fluoride (Bu_3SnF), and 3 eq of lithium chloride (LiCl) to give a crude phenol derivative. At this stage, the xylene solution of the crude product from furan derivative **16b** contained some *tert*-butyldimethylsilyl ether judging from silica gel thin layer chromatography, and so it was treated with 10% aqueous HCl and methanol (MeOH) to liberate the phenol. Isolation of the products was executed after protection of the phenol group as methyl carbonate **17a**—**d** with methyl chloroformate (ClCOOMe) and sodium hydride (NaH) in tetrahydrofuran (THF)-*N*,*N*-dimethylformamide (DMF) (3 : 1). The respective yields for **17a** and **17b** were, however, only 24% and 27% accompanied by recovered **16a** and **16b** in 7% and 55%, respectively. In contrast, **17c**, **d** were obtained in good respective yields of 75% and 80%. It is unclear at present why the yields of **17a**, **b** were so low compared to those of **4** and **17c**, **d**. Since slight modification [increase of the palladium catalyst to 10 mol%, exchange of solvent to toluene, or exchange of TBDMSOTf to triisopropylsilyl trifluoromethanesulfonate] of the reaction conditions for **a** and **b** brought about no improvement, we decided to reinvestigate thoroughly the palladium-catalyzed cyclization conditions for **16a**, **b**, and **c**.

iii) Improved Palladium-Catalyzed Cyclization Conditions: At first, cyclization of the furan derivative **16b** was tried with various combinations of bases and solvents (at reflux) in the presence of a fixed palladium catalyst $PdCl_2(Ph_3P)_2$, and the resulting products were isolated as methyl carbonates as before (Chart 4) (Table 1). As can be

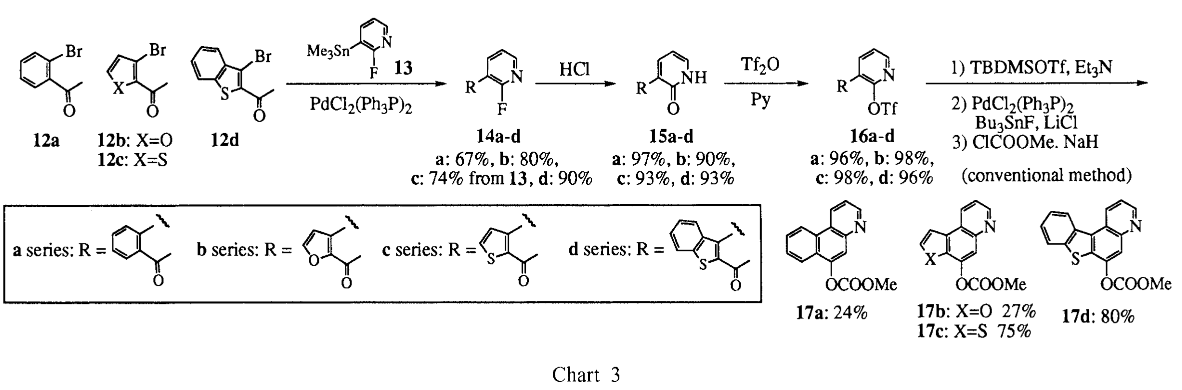


Chart 3

seen from Table 1, NaH and Cs_2CO_3 are preferable as base (run 2, 3, 7—10). Potassium *tert*-butoxide (*t*-BuOK) or potassium carbonate (K_2CO_3) gave a trace of or no desired **17b**, but afforded by-products **19**, **20** and recovery of starting material **16b** (run 4—6). Less polar solvent such as toluene was more suitable than polar THF; this is probably because a polar solvent tends to accelerate an intermolecular aldol reaction (run 1 *vs*. 2). The yield of **17b** was dramatically improved by the addition of a catalytic amount (0.3 eq) of Ph_3P (run 7 *vs*. 8). Eventually, the carbonate **17b** was obtained in 90% yield by stirring of **16b** with 10 mol% of $PdCl_2(Ph_3P)_2$, 3 eq of Cs_2CO_3, and 0.3 eq of Ph_3P in boiling benzene for 6 h (run 10). These reaction conditions of run 10 were also applicable to **16a** and **16c**, and the desired products **17a** and **17c** were readily isolated in 91 and 86% yields, respectively (run 11, 13). The addition of Ph_3P was pivotal, and without this the yield of **17a** dropped to only 6% accompanied by by-products **18** and the pyridone **15a** (run 12). It must be noted that the separation of the product is much easier than that in the conventionally employed Kuwajima conditions, because Bu_3SnF which causes considerable trouble at the separation step is not used under these novel conditions. Furthermore, the novel method requires no activation of the acetyl group as silyl enol ether prior to the palladium-catalyzed cyclization.

iv) On the Reaction Mechanism of the Improved Cyclization Conditions: The most probable mechanisms for the above novel palladium-catalyzed intramolecular cyclization reaction are shown in Chart 5, giving the run 7 in Table 1 as an example. After the oxidative addition of Pd(0) to the substrate **16b**, the acetyl group is partially deprotonated with Cs_2CO_3 to form an enolate **21** even in a less polar solvent, benzene, toluene, or xylene. Two pathways are possible from the intermediate **21**. Nucleophilic attack of the enolate on Pd(II) leads **21** to a palladacycle **22**, and the Heck type of insertion of the enolic olefin group to the aryl-Pd(II) bond affords a tricyclic hemiacetal-type compound **23**. Pd(0) is reductively eliminated from **22** to give a phenol **24**, which is converted to the desired product **17b** by the subsequent treatment with ClCOOMe–NaH. Coexisting starting material **16b** can behave as a trifluoromethanesulfonating agent, and by-product **19** is partially formed from **24** in the presence of Cs_2CO_3. On the other hand, a release of cesium hydroxide from **23** affords aromatic compound **25**, which is in turn transformed to the by-product **19**. Furthermore, the positional exchange of the palladium takes place from **23** to give **27** by way of **26**. Elimination of **28** from **27** furnishes deoxygenated product **20**. Usual reductive elimination from **23**, of course, gives cesium salt of **24**. The Ph_3P would play a role not only to reduce Pd(II) to Pd(0) but to increase the spatial congestion around the palladium of **21**, preventing the cyclization course toward **23** which is more congested than **22**.

v) Preparation of **17e** from **17c**: For the preparation of **17e**, the phenol protecting group of **17c** was first changed from methyl carbonate to benzyl ether **29**. Stirring of **17c** in MeOH–Et_3N (10 : 1) at room temperature (25 °C) readily afforded deprotected phenol, and after evaporation of volatile materials, introduction of benzyl group was effected under conditions A, B or C (Chart 6). The Mitsunobu reaction con-

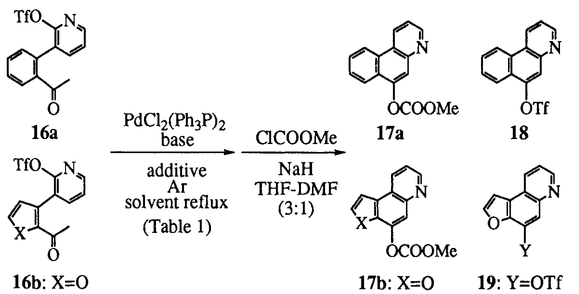


Chart 4

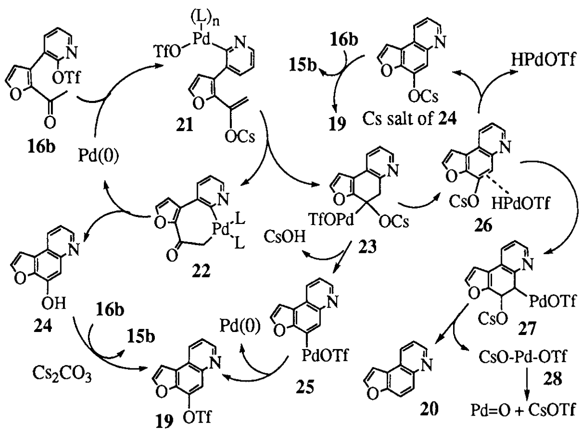


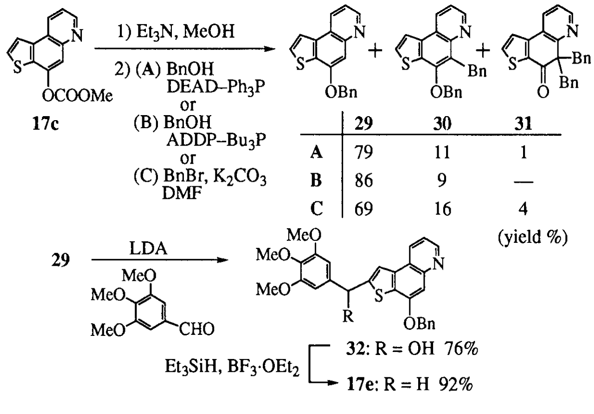
Chart 5

Table 1. Improved Palladium-Catalyzed Cyclization of **16a**, **b**, and **c** to Form **17a**, **b**, and **c**

Run	Starting material	Pd cat. (mol %)	Conditions				Product yield (%)		
			Base (eq)	Additive (eq)	Solvent	Time (h)	**17**	By-product	Recovery of **16**
1	**16b**	5	NaH (1.5)	—	THF	4	—	—	—
2	**16b**	5	NaH (3)	—	Xylene	16.5	**b**: 9	**19**: 11, **20**: 6, **15b**: 13	19
3	**16b**	20	NaH (3)	—	Xylene	5.5	**b**: 50	—	—
4	**16b**	5	*t*-BuOK (3)	—	Toluene	16	**b**: 1	**19**: 3	5
5	**16b**	5	K_2CO_3 (3)	—	THF	18.5	—	**19**: 6, **20**: 3	63
6	**16b**	5	K_2CO_3 (3)	—	Xylene	18	—	**19**: 18, **20**: 3, **15b**: 16	25
7	**16b**	5	CS_2CO_3 (3)	—	Xylene	4.5	**b**: 26	**19**: 6, **20**: 2	6
8	**16b**	5	CS_2CO_3 (3)	Ph_3P (0.3)	Xylene	5	**b**: 57	—	—
9	**16b**	10	CS_2CO_3 (3)	Ph_3P (0.3)	Xylene	2	**b**: 88	—	—
⑩	**16b**	10	CS_2CO_3 (3)	Ph_3P (0.3)	Benzene	6	**b**: 90	—	—
⑪	**16a**	10	CS_2CO_3 (3)	Ph_3P (0.3)	Benzene	1.5	**a**: 91	—	—
12	**16a**	10	CS_2CO_3 (3)	—	Benzene	5	**a**: 6	**18**: 16, **15a**: 38	—
⑬	**16c**	10	CS_2CO_3 (3)	Ph_3P (0.3)	Benzene	3	**c**: 86	—	—

ditions[24] A [benzyl alcohol (BnOH), diethyl azodicarboxylate (DEAD), Ph_3P in THF] gave desired **29** in 79% along with 5-benzyl by-product **30** and 5,5-dibenzyl-4-oxo by-product **31** in 11% and 1% respective yields. The yield of **29** was slightly improved to 86% employing conditions B [BnOH, 1,1′-(azodicarbonyl)dipiperidine (ADDP), tributylphosphine (Bu_3P)[25] in THF)]. These by-products were, however, increased under the basic conditions C [benzyl bromide (BnBr), K_2CO_3 in DMF]. After treatment of **29** with lithium diisopropylamide (LDA) in THF at -80—-75 °C, resulting lithium salt was trapped with 3,4,5-trimethoxybenzaldehyde to yield alcohol **32** in 76%. The benzylic alcohol **32** was easily reduced to the desired **17e** with triethylsilane (Et_3SiH) and $BF_3 \cdot OEt_2$ in CH_2Cl_2 at 0 °C in 92% yield.

Synthesis of DSA A-Ring Analogs 9a—e from 17a—e With the requisite quinolinol derivatives **17a**—**e** in hand, we then carried out further transformation to DSA A-ring analogs **9a**—**e** according to the reported procedure (Chart 7). Thus the heteroaromatic intermediates **17a**—**e** were subjected to reduction with sodium borohydride ($NaBH_4$) in the presence of ClCOOMe in THF–2-propanol (1 : 2) at room temperature to give a mixture of dihydroderivatives **33a**—**e** and **34a**—**e**. Without further purification due to their slight instability, these were oxidized with a catalytic amount of osmium tetroxide and trimethylamine *N*-oxide in acetone–H_2O (9 : 1) at room temperature. The resulting mixture of vicinal diol derivatives was then treated with Et_3SiH and $BF_3 \cdot OEt_2$ in CH_2Cl_2 to execute reductive cleavage of the hydroxy groups located at the benzylic and the α-carbamate positions. Desired **35a**—**e** were obtained in modest to good overall yields calculated from **17a**—**e** along with a slight amount of tetrahydro derivatives **36a**—**e**. The mechanism for the formation of the by-products **36a**—**e** through hydroboration and subsequent air oxidation reaction of **33a**—**e** or **34a**—**e** was reported previously.[10b] Methanesulfonates **37a**—**e** were obtained in high yields from **35a**—**e** in the usual way with methanesulfonyl chloride (MsCl) and Et_3N in CH_2Cl_2 at 0 °C. The compounds **37a**—**d** were readily cyclized to the cyclopropanoindolinone derivatives **38a**—**d** in high yields with K_2CO_3 in MeOH at room temperature. Among them, the compound **38a** was reported earlier by Boger *et al.*[26] For **37e**, hydrogenolysis of the benzyl protecting group on palladium hydroxide in MeOH–DME (5 : 1) was executed prior to the cyclization. Unexpectedly, the benzyl group strongly resisted the hydrogenolysis, and **38e** was obtained in 64% yield accompanied by recovered **37e** in 32% even after hydrogenolysis for 52 h under the atmospheric pressure of hydrogen. The final step, coupling of **38a**—**e** with imidazolide **39**[9] was carried out with K_2CO_3 in DMF at ambient temperature to complete the synthesis of DSA A-ring analogs (±)-**9a**—**e**. The low yield of (±)-**9b** is attributable to instability of the product. The above K_2CO_3 method[10] was found to be more conveniently applicable in these cases in better yields than the NaH in DMF method[9] employed in our first route toward DSA.

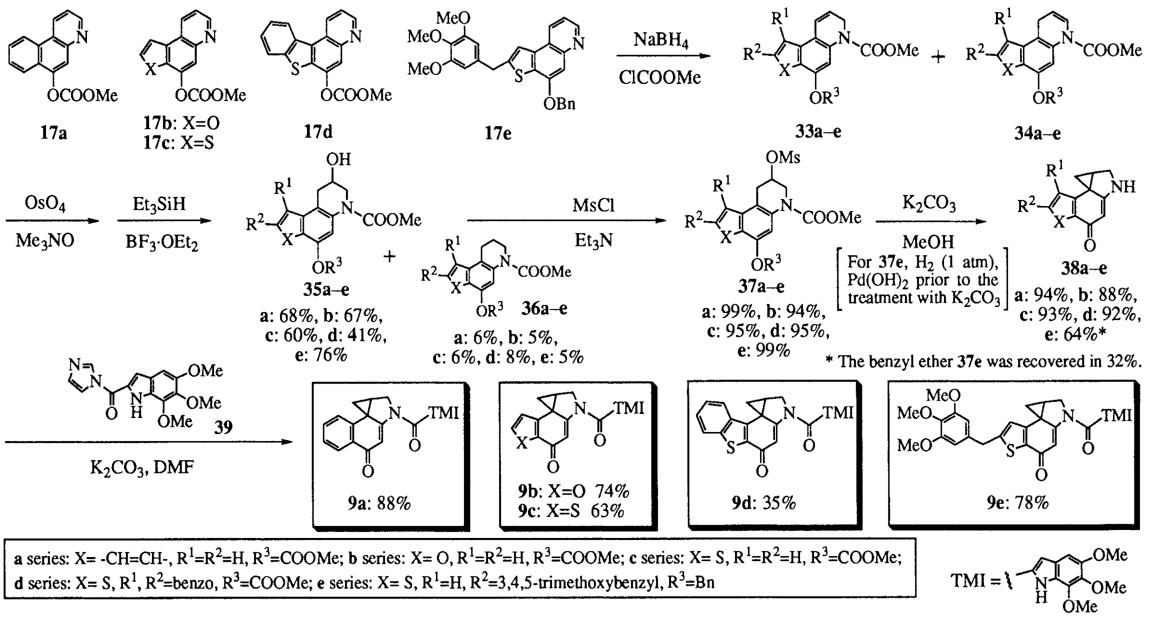


	29	30	31
A	79	11	1
B	86	9	—
C	69	16	4

(yield %)

Chart 6

Summary Five DSA A-ring analogs (±)-**9a**—**e** were synthesized modifying our second synthetic route of DSA. We encountered a problem at the phenol forming step of securing **17a,b** from **16a,b** with the conventional palladium-catalyzed arylation reaction of enol silyl ether in the presence of Bu_3SnF and LiCl (the Kuwajima conditions). The arylation method lacks the generality affording **17a**, **17b** in low



* The benzyl ether **37e** was recovered in 32%.

a series: X= -CH=CH-, R^1=R^2=H, R^3=COOMe; b series: X= O, R^1=R^2=H, R^3=COOMe; c series: X= S, R^1=R^2=H, R^3=COOMe; d series: X= S, R^1, R^2=benzo, R^3=COOMe; e series: X= S, R^1=H, R^2=3,4,5-trimethoxybenzyl, R^3=Bn

Chart 7

yields of 24% and 27%, respectively. This difficulty was, however, overcome by devising a more convenient method: simple heating of **16a**—**c** in benzene in the presence of $PdCl_2(Ph_3P)_2$ (10 mol%), Cs_2CO_3 (3 eq), and Ph_3P (0.3 eq) gave **17a**—**c** in 86—91% yields. The analogs (±)-**9a**—**e** were readily synthesized from the intermediates **17a**—**e** almost according to the reported route.

Experimental

Melting points were measured on a Yanagimoto micro-melting point apparatus and are not corrected. MS and high-resolution MS (HRMS) were recorded on a Hitachi M-80B spectrometer, and figures in parentheses indicate the relative intensities. IR spectra were determined on a Hitachi 215 spectrophotometer. ^1H-NMR spectra were obtained on a Varian EM 390 (90 MHz) spectrometer in $CDCl_3$ unless otherwise specified with tetramethylsilane (TMS) as an internal reference. Column chromatography was carried out on silica gel, Fuji Davison BW 200 and preparative TLC (PTLC) was conducted on glass plates (20×20 cm) coated with Merck Silica gel 60 PF_{254} (1 mm thick). Usual workup refers to washing of the organic layer with water or brine, drying over anhydrous Na_2SO_4, and evaporating off the solvents under reduced pressure.

1-(3-Bromo-2-furanyl)ethanone (12b) A CH_2Cl_2 (20 ml) solution of 3-bromofuran (2.449 g, 16.7 mmol) was cooled to 0 °C, and Ac_2O (4.71 ml, 50.0 mmol) and $BF_3 \cdot OEt_2$ (2.25 ml, 18.3 mmol) were added to this. The mixture was stirred at 0 °C for 15 min, and at 21 °C for 4 h. The reaction was quenched by addition of H_2O and the whole was extracted with CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$–H_2O and then treated as usual to give a residue (4.54 g). Purification by silica gel column chromatography [hexane–EtOAc (19 : 1)] afforded 1-(4-bromo-2-furanyl)ethanone (0.320 g, 10%) as a less polar isomer and **12b** (2.406 g, 76%) as a more polar isomer. **12b**: Slightly yellow oil. GC-HRMS Calcd for $C_6H_5BrO_2$: 189.9453 and 187.9473. Found: 189.9459 and 187.9485. GC-MS *m/z*: 190, 188 (M^+, 50, 52); 175, 173 (100, 100); 148, 146 (4, 4); 147, 145 (4, 4); 119, 117 (14, 14); 84 (12). IR ($CHCl_3$): 1676 cm^{-1}. ^1H-NMR δ: 2.50 (3H, s), 6.63 (1H, d, J=2 Hz), 7.52 (1H, d, J=2 Hz). 1-(4-Bromo-2-furanyl)ethanone: Colorless scales, mp 65—66 °C (hexane). *Anal.* Calcd for $C_6H_5BrO_2$: C, 38.13; H, 2.67; Br, 42.28. Found: C, 37.87; H, 2.83; Br, 42.04. GC-HRMS Calcd for $C_6H_5BrO_2$: 189.9453 and 187.9473. Found: 189.9460 and 187.9498. GC-MS *m/z*: 190, 188 (M^+, 40, 42); 175, 173 (92, 100); 147, 145 (6, 4); 119, 117 (19, 22); 84 (31). IR (KBr): 1660 cm^{-1}. ^1H-NMR δ: 2.46 (3H, s), 7.17 (1H, s), 7.58 (1H, s).

Crude 1-(3-Bromo-2-thienyl)ethanone (12c) $AlCl_3$ (43.9 g, 0.330 mol) was added over 20 min to a cooled (−20 °C) solution of 3-bromothiophene (17.93 g, 0.110 mol) and Ac_2O (27.4 ml, 0.220 mol) in CH_2Cl_2 (200 ml). The mixture was vigorously stirred at this temperature for 30 min and at 0 °C for 2 h. The resulting mixture was poured into ice-water and the whole was extracted with CH_2Cl_2. After washing with saturated $NaHCO_3$–H_2O and usual workup, the residue was distilled to give recovered 3-bromothiophene [1.43 g, 8%, bp 43—46 °C (10 mmHg)] and crude **12c** [17.82 g, 79%, bp 121—124 °C (10 mmHg)]. The latter consists of **12c** and 1-(4-bromo-2-thienyl)ethanone in a ratio of 16 : 1. Crude **12c**: Colorless oil. GC-HRMS Calcd for C_6H_5BrOS: 205.9225 and 203.9245. Found: 205.9237 and 203.9265. GC-MS *m/z*: 206, 204 (M^+, 38, 36); 191, 189 (100, 96); 163, 161 (5, 5); 82 (35); 45 (29); 43 (46). IR ($CHCl_3$): 1658 cm^{-1}. ^1H-NMR δ: 2.67 (3H, s), 7.07 (1H, d, J=5.5 Hz), 7.50 (1H, d, J=5.5 Hz).

1-(3-Bromo-2-benzo[*b*]thienyl)ethanone (12d) In a similar way to the preparation of **12c**, 3-bromobenzo[*b*]thiophene (226 mg, 1.06 mmol) in CH_2Cl_2 (8 ml) was stirred with Ac_2O (0.30 ml, 3.18 mmol) and $AlCl_3$ (848 mg, 6.38 mmol) at 0—27 °C for 6 h. After the same workup as for **12c**, the residue was purified by PTLC [hexane–CH_2Cl_2 (3 : 1)] to give **12d** (210 mg, 78%) as colorless needles, mp 99.5—100 °C (CH_2Cl_2–hexane). *Anal.* Calcd for $C_{10}H_7BrOS$: C, 47.08; H, 2.77; Br, 31.32; S, 12.57. Found: C, 46.67; H, 2.95; Br, 31.55; S, 12.45. GC-HRMS Calcd for $C_{10}H_7BrOS$: 255.9381 and 253.9401. Found: 255.9406 and 253.9395. GC-MS *m/z*: 256, 254 (M^+, 57, 53); 241, 239 (100, 93); 213, 211 (23, 20); 132 (72); 93 (28); 43 (63). IR (KBr): 1635 cm^{-1}. ^1H-NMR δ: 2.76 (3H, s), 7.25—7.57 (2H, m), 7.62—7.97 (2H, m).

Stille Coupling Reaction of 12a—d with 13 to Form 14a—d Preparation of 1-[3-(2-fluoro-3-pyridinyl)-2-furanyl]ethanone (**14b**) is described as a typical example. A toluene solution of **12b** (144 mg, 0.762 mmol), **13** (239 mg, 0.916 mmol), and $PdCl_2(Ph_3P)_2$ (16 mg, 0.023 mmol) was stirred under reflux for 14 h. After the mixture had cooled, saturated $NaHCO_3$–H_2O was added and the whole was extracted with CH_2Cl_2. Usual workup and separation by PTLC [hexane–EtOAc (6 : 1)] afforded **14b** (125 mg, 80%) as colorless scales, mp 75.5—76.5 °C (CH_2Cl_2–hexane). *Anal.* Calcd for $C_{11}H_8FNO_2$: C, 64.39; H, 3.93; N, 6.83. Found: C, 64.58; H, 4.00; N, 6.75. GC-HRMS Calcd for $C_{11}H_8FNO_2$: 205.0539. Found: 205.0522. GC-MS *m/z*: 205 (M^+, 48), 190 (100), 134 (27), 107 (15). IR (KBr): 1668 cm^{-1}. ^1H-NMR δ: 2.51 (3H, s), 6.73 (1H, dd, J=2, 1.5 Hz), 7.22 (1H, ddd, J=7.5, 5, 1.5 Hz), 7.58 (1H, d, J=1.5 Hz), 8.06 (1H, ddd, J=9.5, 7.5, 2 Hz), 8.21 (1H, ddd, J=5, 2, 1.5 Hz).

Similarly, on treatment of **12a** (317 mg, 1.59 mmol) with **13** (456 mg, 1.75 mmol) and $PdCl_2(Ph_3P)_2$ (34 mg, 0.048 mmol) in boiling toluene (10 ml) for 8 h, 1-[2-(2-fluoro-3-pyridinyl)phenyl]ethanone (**14a**, 228 mg, 67%) was obtained as a colorless syrup. HRMS Calcd for $C_{13}H_{10}FNO$: 215.0746. Found: 215.0748. MS *m/z*: 215 (M^+, 35), 200 (100), 172 (40), 145 (17), 43 (41). IR (neat): 1692 cm^{-1}. ^1H-NMR δ: 2.46 (3H, s), 7.14—7.90 (6H, m), 8.13—8.34 (1H, m).

Similarly, on treatment of the crude **12c** (171 mg, 0.834 mmol) with **13** (189 mg, 0.724 mmol) and $PdCl_2(Ph_3P)_2$ (15 mg, 0.021 mmol) in toluene (5 ml) at 125—130 °C (sealed tube) for 5 h, 1-[3-(2-fluoro-3-pyridinyl)-2-thienyl]ethanone (**14c**, 118 mg, 74% from **13**) was obtained as colorless prisms, mp 83—84.5 °C (CH_2Cl_2–hexane). *Anal.* Calcd for $C_{11}H_8FNOS$: C, 59.71; H, 3.64; N, 6.33. Found: C, 59.61; H, 3.77; N, 6.35. GC-HRMS Calcd for $C_{11}H_8FNOS$: 221.0310. Found: 221.0309. GC-MS *m/z*: 221 (M^+, 39), 206 (100), 202 (7), 134 (17), 107 (9), 43 (24). IR (KBr): 1652 cm^{-1}. ^1H-NMR δ: 2.36 (3H, s), 7.08 (1H, d, J=5.5 Hz), 7.23 (1H, ddd, J=7.5, 5, 2 Hz), 7.58 (1H, d, J=5.5 Hz), 7.76 (1H, ddd, J=9, 7.5, 2 Hz), 8.23 (1H, ddd, J=5, 2, 1 Hz).

Similarly, on treatment of **12d** (65 mg, 0.255 mmol) with **13** (73 mg, 0.280 mmol) and $PdCl_2(Ph_3P)_2$ (9 mg, 0.013 mmol) in boiling toluene (5 ml) for 16 h, 1-[3-(2-fluoro-3-pyridinyl)-2-benzo[*b*]thienyl]ethanone (**14d**, 62 mg, 90%) was obtained as colorless prisms, mp 125—126 °C (CH_2Cl_2–hexane). *Anal.* Calcd for $C_{15}H_{10}FNOS$: C, 66.40; H, 3.72; N, 5.16. Found: C, 66.25; H, 3.86; N, 5.19. HRMS Calcd for $C_{15}H_{10}FNOS$: 271.0467. Found: 271.0452. MS *m/z*: 271 (M^+, 76), 256 (100), 228 (25), 209 (15), 184 (27), 43 (46). IR (KBr): 1664 cm^{-1}. ^1H-NMR δ: 2.37 (3H, s), 7.14—7.63 (4H, m), 7.81 (1H, ddd, J=9.5, 7, 2 Hz), *ca.* 7.81—8.02 (1H, m), 8.37 (1H, ddd, J=5, 2, 1 Hz).

Hydrolysis of 14a—d with HCl to Form 15a—d Preparation of 3-(2-acetylphenyl)-2(1*H*)-pyridinone (**15a**) from **14a** is described as representative. A solution of **14a** (169 mg, 0.786 mmol) in DME (2 ml) and 10% HCl–H_2O (2 ml) was stirred at 55—60 °C for 2 h. After the mixture had cooled, saturated $NaHCO_3$–H_2O and NaCl powder were added and the whole was extracted with 10% MeOH–CH_2Cl_2. Usual workup afforded a crystalline residue, which was recrystallized from MeOH–CH_2Cl_2 to give **15a** (162 mg, 97%) as colorless prisms, mp 195—195.5 °C. *Anal.* Calcd for $C_{13}H_{11}NO_2$: C, 73.22; H, 5.20; N, 6.57. Found: C, 73.28; H, 5.25; N, 6.67. HRMS Calcd for $C_{13}H_{11}NO_2$: 213.0789. Found: 213.0782. MS *m/z*: 213 (M^+, 14), 198 (86), 195 (23), 170 (100), 115 (32), 43 (23). IR (KBr): 1679, 1640 cm^{-1}. ^1H-NMR δ: 2.48 (3H, s), 6.31 (1H, dd, J=7, 7 Hz), 7.11—7.75 (6H, m), 12.78 (1H, br s, NH).

In the same way, 3-(2-acetyl-3-furanyl)-2(1*H*)-pyridinone (**15b**, 86 mg, 90%) was obtained from **14b** (96 mg, 0.468 mmol) as colorless needles, mp 236—237 °C (MeOH–CH_2Cl_2). *Anal.* Calcd for $C_{11}H_9NO_3$: C, 65.02; H, 4.46; N, 6.89. Found: C, 64.90; H, 4.43; N, 6.86. HRMS Calcd for $C_{11}H_9NO_3$: 203.0582. Found: 203.0584. MS *m/z*: 203 (M^+, 64), 188 (100), 160 (28), 132 (41), 104 (35), 44 (65). IR (KBr): 1675, 1653, 1617 cm^{-1}. ^1H-NMR (10% CD_3OD–$CDCl_3$) δ: 2.50 (3H, s), 6.37 (1H, dd, J=7, 6.5 Hz), 6.90 (1H, d, J=1.5 Hz), 7.37 (1H, dd, J=6.5, 2 Hz), 7.58 (1H, d, J=1.5 Hz), 7.87 (1H, dd, J=7, 2 Hz).

In the same way, 3-(2-acetyl-3-thienyl)-2(1*H*)-pyridinone (**15c**, 77.5 mg, 93%) was obtained from **14c** (84 mg, 0.380 mmol) as colorless prisms, mp 208—209.5 °C (MeOH–CH_2Cl_2). *Anal.* Calcd for $C_{11}H_9NO_2S$: C, 60.25; H, 4.14; N, 6.39. Found: C, 60.21; H, 4.25; N, 6.42. HRMS Calcd for $C_{11}H_9NO_2S$: 219.0353. Found: 219.0344. MS *m/z*: 219 (M^+, 27), 204 (100), 201 (38), 176 (26), 121 (14), 104 (14), 45 (20), 43 (35). IR (KBr): 1659, 1639, 1617 cm^{-1}. ^1H-NMR (10% CD_3OD–$CDCl_3$) δ: 2.41 (3H, s), 6.36 (1H, dd, J=6.5, 6.5 Hz), 7.09 (1H, d, J=5 Hz), 7.37 (1H, dd, J=6.5, 1.5 Hz), 7.51 (1H, dd, J=6.5, 1.5 Hz), 7.56 (1H, d, J=5 Hz).

In the same way, 3-(2-acetyl-3-benzo[*b*]thienyl)-2(1*H*)-pyridinone (**15d**, 125 mg, 93%) was obtained from **14d** (135 mg, 0.498 mmol) as colorless needles, mp 280—283 °C (dec., MeOH–CH_2Cl_2). *Anal.* Calcd for $C_{15}H_{11}NO_2S$: C, 66.89; H, 4.12; N, 5.20. Found: C, 66.34; H, 4.30; N, 5.15. HRMS Calcd for $C_{15}H_{11}NO_2S$: 269.0510. Found: 269.0507. MS *m/z*: 269 (M^+, 67), 254 (93), 251 (26), 226 (100), 198 (20), 171 (31), 154 (25), 43

(75). IR (KBr): 1656, 1637, 1611 cm^{-1}. ^{1}H-NMR (10% CD$_3$OD–CDCl$_3$) δ: 2.46 (3H, s), 6.45 (1H, dd, J=7, 7 Hz), 7.21—7.63 (5H, m), 7.78—7.94 (1H, m).

Preparation of the Triflates 16a—d from 15a—d Formation of 3-(2-acetylphenyl)-2-pyridinyl trifluoromethanesulfonate (**16a**) is described as representative. Tf$_2$O (97 μl, 0.577 mmol) was added to a solution of **15a** (49 mg, 0.230 mmol) and pyridine (0.30 ml, 3.71 mmol) in CH$_2$Cl$_2$ (2.7 ml) at 0 °C. After stirring was continued at that temperature for 5 min and at 19 °C for 2 h, the reaction was quenched by addition of saturated NaHCO$_3$–H$_2$O. Extraction with CH$_2$Cl$_2$ followed by usual workup and separation by PTLC [hexane–EtOAc (3 : 1)] afforded **16a** (76 mg, 96%) as a colorless syrup. MS *m/z*: 196 (M$^+$–OTf, 100), 69 (24), 43 (25). IR (neat): 1693 cm^{-1}. ^{1}H-NMR δ: 2.50 (3H, s), 8.33 (1H, dd, J=5, 2 Hz), 7.23—7.99 (6H, m).

In the same way, **15b** (34 mg, 0.167 mmol) was allowed to react with Tf$_2$O (71 μl, 0.422 mmol) in CH$_2$Cl$_2$–pyridine (9 : 1, 3 ml) to afford 3-(2-acetyl-3-furanyl)-2-pyridinyl trifluoromethanesulfonate (**16b**, 55 mg, 98%) as colorless prisms, mp 60.5—61 °C (CH$_2$Cl$_2$–hexane). *Anal.* Calcd for C$_{12}$H$_8$F$_3$NO$_5$S: C, 42.99; H, 2.41; N, 4.18. Found: C, 43.00; H, 2.46; N, 4.24. GC-MS *m/z*: 186 (M$^+$–OTf, 100), 160 (11), 69 (24), 44 (55). IR (KBr): 1675 cm^{-1}. ^{1}H-NMR δ: 2.53 (3H, s), 6.74 (1H, d, J=2 Hz), 7.48 (1H, dd, J=8, 5 Hz), 7.70 (1H, d, J=2 Hz), 8.08 (1H, dd, J=8, 2 Hz), 8.46 (1H, dd, J=5, 2 Hz).

In the same way, **15c** (69 mg, 0.315 mmol) was allowed to react with Tf$_2$O (132 μl, 0.785 mmol) in CH$_2$Cl$_2$–pyridine (9 : 1, 3 ml) to afford 3-(2-acetyl-3-thienyl)-2-pyridinyl trifluoromethanesulfonate (**16c**, 108 mg, 98%) as colorless prisms, mp 64—65 °C (CH$_2$Cl$_2$–hexane). *Anal.* Calcd for C$_{12}$H$_8$F$_3$NO$_4$S$_2$: C, 41.02; H, 2.30; N, 3.99. Found: C, 41.02; H, 2.43; N, 4.21. GC-MS *m/z*: 202 (M$^+$–OTf, 100), 176 (7), 69 (21), 43 (36). IR (KBr): 1662 cm^{-1}. ^{1}H-NMR δ: 2.38 (3H, s), 7.06 (1H, d, J=5 Hz), 7.39 (1H, dd, J=7.5, 5 Hz), 7.60 (1H, d, J=5 Hz), 7.83 (1H, dd, J=7.5, 2 Hz), 8.34 (1H, dd, J=5, 2 Hz).

In the same way, **15d** (63 mg, 0.234 mmol) was allowed to react with Tf$_2$O (99 μl, 0.589 mmol) in CH$_2$Cl$_2$–pyridine (9 : 1, 3 ml) to afford 3-(2-acetyl-3-benzo[*b*]thienyl)-2-pyridinyl trifluoromethanesulfonate (**16d**, 90 mg, 96%) as a colorless syrup. HRMS Calcd for C$_{16}$H$_{10}$F$_3$NO$_4$S$_2$: 401.0003. Found: 400.9996. MS *m/z*: 401 (M$^+$, 2), 252 (100), 226 (11), 69 (20), 43 (38). IR (CHCl$_3$): 1676 cm^{-1}. ^{1}H-NMR δ: 2.44 (3H, s), 7.28—7.64 (3H, m), 7.50 (1H, dd, J=7.5, 4.5 Hz), *ca.* 7.80—8.01 (1H, m), 7.87 (1H, dd, J=7.5, 2 Hz), 8.49 (1H, dd, J=4.5, 2 Hz).

Palladium-Catalyzed Cyclization of 16a—d to Form 17a—d by the Conventional Method (Kuwajima Conditions) Preparation of Furo[3,2-*f*]quinoline-4-yl methyl carbonate (**17b**) is described as a typical example. A CH$_2$Cl$_2$ (3 ml) solution of **16b** (51 mg, 0.512 mmol) and Et$_3$N (85 μl, 0.611 mmol) was stirred with TBDMSOTf (87 μl, 0.379 mmol) under an Ar atmosphere at 0 °C for 1 h. Saturated NaHCO$_3$–H$_2$O was added and the mixture was extracted with CH$_2$Cl$_2$. Usual workup left a residue (78 mg) which was dissolved in xylene (4 ml). PdCl$_2$(Ph$_3$P)$_2$ (5.5 mg, 7.83 μmol), Bu$_3$SnF (57 mg, 0.184 mmol), and LiCl (20 mg, 0.471 mmol) were added to this, and the resulting mixture was refluxed under an Ar atmosphere for 1 h. After the mixture had cooled in an ice bath, MeOH (4.5 ml) and 10% HCl–H$_2$O (0.5 ml) were added, and then the whole was stirred at 0 °C for 5 min and at 21 °C for 30 min. Saturated NaHCO$_3$–H$_2$O was added and the aqueous layer was saturated with NaCl. The whole was extracted with 10% MeOH–CH$_2$Cl$_2$ and then the organic layer was treated as usual. The residue was roughly purified by PTLC [benzene–EtOAc (5 : 1)] to give recovered **16b** (28 mg, 55%) and a crude phenolic material (39 mg). The crude phenol was dissolved in THF (3 ml) and DME (1 ml) and the solution was cooled at −18 °C. NaH (60% dispersion in mineral oil, 40 mg, 1.00 mmol) was added to this under an Ar atmosphere and the mixture was stirred for 5 min. A solution of ClCOOMe (77 μl, 1.00 mmol) in THF (1 ml) was added and the resulting mixture was stirred at −18—0 °C for 1.5 h. Saturated NH$_4$Cl–H$_2$O was added and the whole was extracted with CH$_2$Cl$_2$. Usual workup and separation by PTLC [benzene–EtOAc (2 : 1)] afforded **17b** (10 mg, 27%). ^{1}H-NMR of the crude silyl enol ether δ: −0.04 (6H, s), 0.72 (9H, s), 4.45 (1H, d, J=1.5 Hz), 4.84 (1H, d, J=1.5 Hz), 6.45 (1H, d, J=1.5 Hz), 7.34 (1H, dd, J=7.5, 4.5 Hz), 7.41 (1H, d, J=1.5 Hz), 7.85 (1H, dd, J=7.5, 21 Hz), 8.28 (1H, dd, J=4.5, 2 Hz). **17b**: Slightly yellow prisms, mp 69—70 °C (CH$_2$Cl$_2$–hexane). *Anal.* Calcd for C$_{13}$H$_9$NO$_4$: C, 64.20; H, 3.73; N, 5.76. Found: C, 64.05; H, 3.83; N, 5.90. HRMS Calcd for C$_{13}$H$_9$NO$_4$: 243.0531. Found: 243.0513. MS *m/z*: 243 (M$^+$, 50), 199 (42), 169 (25), 156 (100), 101 (22), 59 (24). IR (CHCl$_3$): 1772 cm^{-1}. ^{1}H-NMR δ: 4.01 (3H, s), 7.28 (1H, d, J=2 Hz), 7.48 (1H, dd, J=8.5, 4.5 Hz), 7.86 (1H, d, J=2 Hz), 7.90 (1H, s), 8.44 (1H, br d, J=8.5 Hz), 8.96 (1H, dd, J=4.5, 1.5 Hz).

In a similar way, benzo[*f*]quinolin-6-yl methyl carbonate (**17a**, 14 mg, 24%), along with recovered **16a** (5 mg, 7%), was obtained from **16a** (73 mg, 0.212 mmol) after separation by PTLC [hexane–CH$_2$Cl$_2$ (1 : 4)]. ^{1}H-NMR of the crude silyl enol ether δ: 0.03 (6H, s), 0.74 (9H, s), 4.29 (1H, d, J=1.5 Hz), 4.38 (1H, d, J=1.5 Hz), 7.15—7.63 (5H, m), 7.86 (1H, dd, J=7, 2 Hz), 8.32 (1H, dd, J=5, 2 Hz). **17a**: Colorless prisms, mp 107—108 °C (Et$_2$O–hexane). *Anal.* Calcd for C$_{15}$H$_{11}$NO$_3$: C, 71.14; H, 4.38; N, 5.53. Found: C, 71.08; H, 4.56; N, 5.49. HRMS Calcd for C$_{15}$H$_{11}$NO$_3$: 253.0738. Found: 253.0736. MS *m/z*: 253 (M$^+$, 44), 209 (38), 166 (100), 139 (36), 59 (30). IR (KBr): 1750 cm^{-1}. ^{1}H-NMR δ: 3.98 (3H, s), 7.46 (1H, dd, J=8.5, 4.5 Hz), 7.61—7.81 (2H, m), 7.91 (1H, s), 7.98—8.21 (1H, m), 8.44—8.69 (1H, m), 8.83 (1H, br d, J=8.5 Hz), 8.92 (1H, dd, J=4.5, 1.5 Hz).

In a similar way, methyl thieno[3,2-*f*]quinolin-4-yl carbonate (**17c**, 342 mg, 75%) was obtained from **16c** (617 mg, 1.76 mmol) after separation by PTLC [benzene–EtOAc (5 : 1)]. ^{1}H-NMR of the crude silyl enol ether δ: 0.10 (6H, s), 0.84 (9H, s), 4.27 (1H, d, J=2 Hz), 4.31 (1H, d, J=2 Hz), 6.93 (1H, d, J=5.5 Hz), 7.24 (1H, d, J=5.5 Hz), 7.34 (1H, dd, J=7.5, 5 Hz), 7.84 (1H, dd, J=7.5, 2 Hz), 8.29 (1H, dd, J=5, 2 Hz). **17c**: Slightly yellow prisms, mp 122—123 °C (CH$_2$Cl$_2$–hexane). *Anal.* Calcd for C$_{13}$H$_9$NO$_3$S: C, 60.22; H, 3.50; N, 5.40. Found: C, 60.00; H, 3.68; N, 5.42. GC-HRMS Calcd for C$_{13}$H$_9$NO$_3$S: 259.0303. Found: 259.0312. GC-MS *m/z*: 259 (M$^+$, 45), 215 (31), 186 (14), 172 (100), 145 (15), 59 (19). IR (KBr): 1759 cm^{-1}. ^{1}H-NMR δ: 3.99 (3H, s), 7.49 (1H, dd, J=8.5, 4.5 Hz), 7.67 (1H, d, J=5.5 Hz), 7.95 (1H, s), 7.96 (1H, d, J=5.5 Hz), 8.58 (1H, dd, J=8.5, 2 Hz), 8.93 (1H, dd, J=4.5, 2 Hz).

In a similar way, [1]benzothieno[3,2-*f*]quinolin-6-yl methyl carbonate (**17d**, 44 mg, 80%) was obtained from **16d** (71 mg, 0.177 mmol) after separation by PTLC [benzene–EtOAc (3 : 1)]. ^{1}H-NMR of the crude silyl enol ether δ: 0.12 (3H, s), 0.16 (3H, s), 0.76 (9H, s), 4.22 (1H, d, J=2.5 Hz), 4.30 (1H, d, J=2.5 Hz), *ca.* 6.98—7.35 (3H, m), 7.30 (1H, dd, J=7.5, 5 Hz), *ca.* 7.50—7.74 (1H, m), 7.74 (1H, dd, J=7.5, 2 Hz), 8.26 (1H, dd, J=5, 2 Hz). **17d**: Slightly yellow needles, mp 180.5—181.5 °C (dec., CH$_2$Cl$_2$–hexane). *Anal.* Calcd for C$_{17}$H$_{11}$NO$_3$S: C, 66.00; H, 3.58; N, 4.53. Found: C, 66.02; H, 3.73; N, 4.62. HRMS Calcd for C$_{17}$H$_{11}$NO$_3$S: 309.0459. Found: 309.0448. MS *m/z*: 309 (M$^+$, 50), 265 (27), 222 (100), 195 (11), 59 (19). IR (KBr): 1759 cm^{-1}. ^{1}H-NMR δ: 4.01 (3H, s), 7.38—7.69 (3H, m), 7.86—8.11 (1H, m), 8.11 (1H, s), 8.56—8.79 (1H, m), 8.96 (1H, dd, J=4.5, 1.5 Hz), 9.21 (1H, br d, J=8.5 Hz).

Improved Palladium-Catalyzed Cyclization of 16a—c to Form 17a—c Among runs 10, 11, and 13 of Table 1 carried out under the optimized conditions, preparation of **17b** (run 10) is described as a typical example. A slurry of **16b** (64 mg, 0.191 mmol), PdCl$_2$(Ph$_3$P)$_2$ (13.5 mg, 0.019 mmol), Ph$_3$P (15 mg, 0.057 mmol), and Cs$_2$CO$_3$ (187 mg, 0.574 mmol) in benzene (4 ml) was refluxed with stirring under an Ar atmosphere for 6 h. After the mixture had cooled in an ice bath, citric acid monohydrate (121 mg, 0.630 mmol) and H$_2$O (5 ml) were added and the whole was stirred for 5 min. The whole was adjusted to pH 6—7 by addition of several drops of saturated NaHCO$_3$–H$_2$O, then thoroughly extracted with 10% MeOH–CH$_2$Cl$_2$. Usual workup gave a crystalline material (72 mg) which was then dissolved in THF (3 ml) and DMF (1 ml). NaH (60% dispersion in mineral oil, 38 mg, 0.950 mmol) was added to the solution at −18 °C under an Ar atmosphere and the mixture was stirred at that temperature for 15 min. A THF (1 ml) solution of ClCOOMe (74 μl, 0.958 mmol) was added and the whole was stirred at −18—0 °C for 1 h. The reaction was quenched by addition of saturated NH$_4$Cl—H$_2$O and the whole was extracted with CH$_2$Cl$_2$. Usual workup and purification by PTLC [benzene–EtOAc (2 : 1)] afforded **17b** (42 mg, 90%) as slightly yellow prisms, mp 69—70 °C (CH$_2$Cl$_2$–hexane), whose spectral data were identical with those of the specimen prepared above by the conventional method.

In the same way, **17a** (40 mg, 91%), colorless prisms, mp 107—108 °C (Et$_2$O–hexane), was obtained from **16a** (60 mg, 0.174 mmol). The spectral data of **17a** were described above.

In the same way, **17c** (43 mg, 86%), slightly yellow prisms, mp 120—122 °C (CH$_2$Cl$_2$–hexane), was obtained from **16c** (68 mg, 0.194 mmol). The spectral data of **17c** were described above.

The following by-products were isolated from the other runs carried out under the conditions shown in Table 1. Benzo[*f*]quinolin-6-yl trifluoromethanesulfonate (**18**, Table 1, run 12): Colorless glass. HRMS Calcd for C$_{14}$H$_8$F$_3$NO$_3$S: 327.0176. Found: 327.0166. MS *m/z*: 327 (M$^+$, 22), 194 (29), 166 (100), 139 (24), 69 (29). ^{1}H-NMR δ: 7.63 (1H, dd, J=8.5, 4.5 Hz), *ca.* 7.71—7.96 (2H, m), 8.04 (1H, s), 8.12—8.37 (1H, m), 8.56—8.82 (1H, m), 8.95 (1H, dd, J=8.5, 1.5 Hz), 9.03 (1H, dd, J=4.5, 1.5 Hz). Furo[3,2-*f*]quinolin-4-yl trifluoromethanesulfonate (**19**, Table 1, runs 2, 4—7): Colorless prisms, mp 59—59.5 °C (hexane). *Anal.* Calcd for C$_{12}$H$_6$F$_3$NO$_4$S: C, 45.43; H, 1.91; N, 4.42. Found: C, 45.34; H, 2.14; N, 4.47. GC-HRMS

Calcd for $C_{12}H_6F_3NO_4S$: 316.9969. Found: 316.9981. GC-MS *m/z*: 317 (M^+, 28), 253 (4), 184 (14), 156 (100), 101 (15), 69 (31). ^{1}H-NMR δ: 7.36 (1H, d, *J*=2.5 Hz), 7.59 (1H, dd, *J*=8.5, 4.5 Hz), 7.96 (1H, d, *J*=2.5 Hz), 8.00 (1H, s), 8.49 (1H, dd, *J*=8.5, 2 Hz), 9.02 (1H, dd, *J*=4.5, 2 Hz). Furo[3,2-*f*]quinoline (**20**, Table 1, runs 2, 5—7): Colorless syrup. GC-HRMS Calcd for $C_{11}H_7NO$: 169.0527. Found: 169.0553. GC-MS *m/z*: 169 (M^+, 100), 140 (23), 114 (22), 87 (11), 63 (18). ^{1}H-NMR δ: 7.18 (1H, d, *J*=2 Hz), 7.38 (1H, dd, *J*=8.5, 4.5 Hz), 7.76 (1H, d, *J*=2 Hz), 7.82 (1H, d, *J*=9 Hz), 8.00 (1H, d, *J*=9 Hz), 8.35 (1H, dd, *J*=8.5, 1.5 Hz), 8.85 (1H, dd, *J*=4.5, 1.5 Hz).

Benzyl Thieno[3,2-*f*]quinolin-4-yl Ether (29) (i) Condition A: Et_3N (0.20 ml, 1.44 mmol) was added to a solution of **17c** (27 mg, 0.104 mmol) in MeOH (2 ml) and the mixture was stirred at 25 °C for 1.5 h. The volatile materials were evaporated off, and benzene (5 ml) was added to the residue. The solvent was again evaporated to dryness to leave a crystalline residue. BnOH (43 μl, 0.416 mmol), Ph_3P (82 mg, 0.313 mmol), and DEAD (49 μl, 0.311 mmol) were added in this order to a cooled (0 °C) slurry of the residue in THF (3 ml) under an Ar atmosphere, and the mixture was stirred at 0 °C for 10 min, then at 21 °C for 1.5 h. Saturated $NaHCO_3$–H_2O was added and the whole was extracted with CH_2Cl_2. Usual workup and purification by PTLC [benzene–EtOAc (14 : 1)] afforded crude **30** (5.5 mg), 5,5-dibenzyl-4,5-dihydrothieno[3,2-*f*]quinolin-4-one (**31**, 0.5 mg, 1%), and crude **29** (69 mg, contaminated with Ph_3PO) in order of increasing polarity. The crude **30** was further separated by PTLC [hexane–CH_2Cl_2 (3 : 2)] to give benzyl 5-benzylthieno[3,2-*f*]quinolin-4-yl ether (**30**, 4.5 mg, 11%). The crude **29** was purified by PTLC [hexane–EtOAc (4 : 1)] to afford **29** (24 mg, 79%) as colorless prisms, mp 116—117 °C (CH_2Cl_2–hexane). *Anal.* Calcd for $C_{18}H_{13}NOS$: C, 74.20; H, 4.50; N, 4.81. Found: C, 73.90; H, 4.47; N, 5.10. HRMS Calcd for $C_{18}H_{13}NOS$: 291.0717. Found: 291.0698. MS *m/z*: 291 (M^+, 24), 262 (4), 186 (5), 172 (8), 91 (100), 65 (11). ^{1}H-NMR δ: 5.39 (2H, s), 7.22—7.63 (7H, m), 7.63 (1H, d, *J*=5.5 Hz), 7.92 (1H, d, *J*=5.5 Hz), 8.49 (1H, br d, *J*=8 Hz), 8.83 (1H, dd, *J*=4.5, 1.5 Hz). **30**: Colorless prisms, mp 154—155 °C (CH_2Cl_2–hexane). *Anal.* Calcd for $C_{25}H_{19}NOS$: C, 78.71; H, 5.02; N, 3.67. Found: C, 78.72; H, 5.09; N, 3.75. HRMS Calcd for $C_{25}H_{19}NOS$: 381.1186. Found: 381.1192. MS *m/z*: 381 (M^+, 5), 290 (100), 262 (9), 260 (9), 91 (83), 65 (12). ^{1}H-NMR δ: 4.79 (2H, s), 5.08 (2H, s), 7.04—7.60 (6H, m), 7.60 (1H, d, *J*=5.5 Hz), 7.97 (1H, d, *J*=5.5 Hz), 8.58 (1H, dd, *J*=8, 1.5 Hz), 8.97 (1H, dd, *J*=4.5, 1.5 Hz). **31**: Colorless needles, mp 159—161 °C (CH_2Cl_2–hexane). HRMS Calcd for $C_{25}H_{19}NOS$: 381.1186. Found: 381.1192. MS *m/z*: 381 (M^+, 2), 290 (100), 260 (8), 91 (67), 65 (16). IR (KBr): 1636 cm^{-1}. ^{1}H-NMR δ: 3.65 (2H, d, *J*=11.5 Hz), 3.81 (2H, d, *J*=11.5 Hz), 6.54—6.93 (10H, m), 7.04 (1H, d, *J*=5 Hz), 7.20 (1H, dd, *J*=8, 4.5 Hz), 7.48 (1H, d, *J*=5 Hz), 7.63 (1H, dd, *J*=8, 2 Hz), 8.80 (1H, dd, *J*=4.5, 2 Hz).

(ii) Condition B: The deprotected phenol prepared from **17c** (30 mg, 0.116 mmol) as above was dissolved in THF (4 ml). BnOH (48 μl, 0.464 mmol), ADDP (88 mg, 0.349 mmol), and Bu_3P (87 μl, 0.350 mmol) were successively added to this at 21 °C under an Ar atmosphere. After stirring for 1 h, the mixture was treated and purified by PTLC as above to yield **29** (29 mg, 86%) and **30** (4 mg, 9%).

(iii) Condition C: The deprotected phenol prepared from **17c** (35 mg, 0.135 mmol) as above was dissolved in DMF (2.5 ml). K_2CO_3 (93 mg, 0.674 mmol) and BnBr (21 μl, 0.177 mmol) were added to this and the mixture was stirred at 22 °C for 14 h. Saturated NH_4Cl–H_2O was added and the whole was extracted with EtOAc. Usual workup and separation by PTLC [hexane–CH_2Cl_2 (2 : 3)] afforded **29** (27 mg, 69%), **30** (8 mg, 16%), and **31** (2 mg, 4%).

4-Benzyloxy-α-(3,4,5-trimethoxyphenyl)thieno[3,2-*f*]quinoline-2-methanol (32) Butyllithium (1.65 M in hexane, 0.33 ml, 0.545 mmol) was added to a THF (2 ml) solution of diisopropylamine (0.12 ml, 0.858 mmol) at −18 °C and the mixture was stirred for 10 min. The mixture was cooled to −81 °C, and a THF (2 ml) solution of **29** (40 mg, 0.137 mmol) was added dropwise to this. The stirring was continued at −81—67 °C for 2 h. The resulting solution was again cooled to −80 °C and 3,4,5-trimethoxybenzaldehyde (67 mg, 0.342 mmol) was added portionwise to this. After the stirring was continued at −80—75 °C for 30 min, saturated NH_4Cl–H_2O was added and the whole was extracted with CH_2Cl_2. Usual workup and purification by PTLC (2% MeOH–CH_2Cl_2) afforded **32** (51 mg, 76%) as colorless fine needles, mp 190—191 °C (MeOH–CH_2Cl_2). *Anal.* Calcd for $C_{28}H_{25}NO_5S$: C, 68.97; H, 5.17; N, 2.87. Found: C, 68.93; H, 5.18; N, 3.00. HRMS Calcd for $C_{28}H_{25}NO_5S$: 487.1452. Found: 487.1447. MS *m/z*: 487 (M^+, 19), 195 (5), 172 (7), 91 (100), 65 (7). IR (KBr): 1600 cm^{-1}. ^{1}H-NMR δ: 3.86 (9H, s), 4.00 (1H, br s, OH), 5.30 (2H, s), 6.13 (1H, s), 6.77 (2H, s), 7.19—7.59 (7H, m), 7.59 (1H, s), 8.33 (1H, br d, *J*=8.5 Hz), 8.76 (1H, dd, *J*=4.5, 1.5 Hz).

Benzyl 2-(3,4,5-Trimethoxybenzyl)thieno[3,2-*f*]quinolin-4-yl Ether (17e) $BF_3 \cdot OEt_2$ (35 μl, 0.284 mmol) was added to a solution of **32** (56 mg, 0.115 mmol) and Et_3SiH (64 μl, 0.402 mmol) in CH_2Cl_2 (5 ml) under an Ar atmosphere at 0 °C and the mixture was stirred at that temperature for 30 min. Saturated $NaHCO_3$–H_2O was added and the whole was extracted with CH_2Cl_2. Usual workup and separation by PTLC [benzene–EtOAc (4 : 1)] provided **17e** (50 mg, 92%) as a colorless foam. HRMS Calcd for $C_{28}H_{25}NO_4S$: 471.1503. Found: 471.1511. MS *m/z*: 471 (M^+ 29), 366 (4), 352 (4), 185 (4), 181 (3), 91 (100), 65 (7). ^{1}H-NMR δ: 3.85 (9H, s), 4.28 (2H, s), 5.40 (2H, s), 6.57 (2H, s), 7.28—7.75 (7H, m), 7.66 (1H, s), 8.49 (1H, d, *J*=8 Hz), 8.88 (1H, br d, *J*=4.5 Hz).

Transformation of 17a—e to 35a—e by Way of 33a—e and 34a—e Preparation of methyl (±)-8,9-dihydro-8-hydroxy-4-[(methoxycarbonyl)oxy]furo[3,2-*f*]quinoline-6(7*H*)-carboxylate (**35b**) from **17b** is described as a typical example. $NaBH_4$ (53 mg, 1.39 mmol) was added to a solution of **17b** (34 mg, 0.140 mmol) and ClCOOMe (108 μl, 1.40 mmol) in 2-PrOH–THF (2 : 1, 4.5 ml) at 0 °C under an Ar atmosphere and the mixture was stirred at 0 °C for 5 min, then at 22 °C for 19 h. Saturated NH_4Cl–H_2O was added and the whole was extracted with CH_2Cl_2. Usual workup left a mixture of **33b** and **34b** (50 mg), which was dissolved in acetone–H_2O (9 : 1, 4 ml). $Me_3NO \cdot 2H_2O$ (23 mg, 0.207 mmol) and OsO_4 (1.5 mg, 0.006 mmol) were added to the solution at 0 °C and the mixture was stirred at 21 °C for 1 h. The solvent was evaporated off at an ambient temperature and the resulting residue was dried over P_2O_5 for 2 h. The residue was dissolved in CH_2Cl_2 (4 ml), and the solution was cooled in an ice bath. Et_3SiH (134 μl, 0.841 mmol) and $BF_3 \cdot OEt_2$ (52 μl, 0.423 mmol) were successively added to this and the mixture was stirred under an Ar atmosphere at 0 °C for 5 min and at 25 °C for 3 h. Saturated $NaHCO_3$–H_2O was added and the whole was extracted with CH_2Cl_2. Usual workup and purification by PTLC [benzene–EtOAc (2 : 1)] gave **35b** (30 mg, 67%) and methyl 8,9-dihydro-4-[(methoxycarbonyl)oxy]furo[3,2-*f*]quinoline-6(7*H*)-carboxylate (**36b**, 2 mg, 5%) in order of decreasing polarity. **35b**: Colorless foam. HRMS Calcd for $C_{15}H_{15}NO_7$: 321.0847. Found: 321.0858. MS *m/z*: 321 (M^+, 100), 303 (6), 302 (6), 263 (16), 228 (18), 202 (29), 200 (30), 186 (26), 89 (20), 59 (91). IR ($CHCl_3$): 1768, 1689 cm^{-1}. ^{1}H-NMR δ: 2.30 (1H, br s, OH), 2.85 (1H, dd, *J*=17, 5 Hz), 3.22 (1H, dd, *J*=17, 5 Hz), *ca.* 3.64—4.04 (2H, m), 3.79 (3H, s), 3.94 (3H, s), 4.17—4.49 (1H, m), 6.72 (1H, d, *J*=2 Hz), 7.54 (1H, s), 7.61 (1H, d, *J*=2 Hz). **36b**: Colorless glass. HRMS Calcd for $C_{15}H_{15}NO_6$: 305.0898. Found: 305.0883. MS *m/z*: 305 (M^+, 100), 260 (14), 246 (16), 202 (33), 186 (21), 174 (22), 158 (23), 130 (23), 59 (73). IR ($CHCl_3$): 1771, 1698 cm^{-1}. ^{1}H-NMR δ: 1.88—2.17 (2H, m), 2.94 (2H, t, *J*=6.5 Hz), 3.80 (3H, s), 3.84 (2H, t, *J*=6.5 Hz), 3.96 (3H, s), 6.73 (1H, d, *J*=2 Hz), 7.56 (1H, s), 7.60 (1H, d, *J*=2 Hz).

In the same way, starting from **17a** (44 mg, 0.174 mmol), methyl (±)-2,3-dihydro-2-hydroxy-6-[(methoxycarbonyl)oxy]benzo[*f*]quinoline-4(1*H*)-carboxylate (**35a**, 39 mg, 68%) and methyl 2,3-dihydro-6-[(methoxycarbonyl)oxy]benzo[*f*]quinoline-4(1*H*)-carboxylate (**36a**, 3.5 mg, 6%) along with recovered **17a** (4 mg, 9%) were obtained after separation by PTLC [benzene–EtOAc (3:1)]. **35a**: Colorless needles, mp 189—190 °C (CH_2Cl_2–hexane). *Anal.* Calcd for $C_{17}H_{17}NO_6$: C, 61.62; H, 5.17; N, 4.23. Found: C, 61.31; H, 5.14; N, 4.17. HRMS Calcd for $C_{17}H_{17}NO_6$: 331.1055. Found: 331.1046. MS *m/z*: 331 (M^+, 100), 212 (22), 196 (16), 184 (17), 167 (18), 127 (25), 59 (88). IR (KBr): 1757, 1672 cm^{-1}. ^{1}H-NMR δ: 1.99 (1H, br s, OH), 3.09 (1H, dd, *J*=17.5, 5 Hz), 3.46 (1H, dd, *J*=17.5, 6 Hz), *ca.* 3.74—4.03 (2H, m), 3.84 (3H, s), 3.97 (3H, s), 4.33—4.57 (1H, m), 7.38—7.69 (2H, m), 7.78 (1H, s), 7.78—8.05 (2H, m). **36a**: Colorless glass. HRMS Calcd for $C_{17}H_{17}NO_5$: 315.1106. Found: 315.1101. MS *m/z*: 315 (M^+, 100), 271 (8), 256 (16), 212 (17), 196 (25), 168 (30), 141 (19), 115 (19), 59 (72). IR ($CHCl_3$): 1764, 1696 cm^{-1}. ^{1}H-NMR δ: 1.95—2.29 (2H, m), 3.14 (2H, t, *J*=7 Hz), *ca.* 3.74—4.00 (2H, m), 3.82 (3H, s), 3.94 (3H, s), 7.37—7.67 (2H, m), 7.79 (1H, s), 7.85—8.06 (2H, m).

In the same way, starting from **17c** (41 mg, 0.158 mmol), methyl (±)-8,9-dihydro-8-hydroxy-4-[(methoxycarbonyl)oxy]thieno[3,2-*f*]quinoline-6(7*H*)-carboxylate (**35c**, 32 mg, 60%) and methyl 8,9-dihydro-4-[(methoxycarbonyl)oxy]thieno[3,2-*f*]quinoline-6(7*H*)-carboxylate (**36c**, 3 mg, 6%) were obtained by way of dihydroderivatives **33c** and **34c** (*ca.* 4.5 : 1) after purification by PTLC [benzene–EtOAc (2 : 1)]. ^{1}H-NMR of the crude **33c** δ: 3.78 (3H, s), 3.92 (3H, s), 4.42 (2H, dd, *J*=4.5, 1.5 Hz), 6.08 (1H, dt, *J*=9.5, 4.5 Hz), 6.89 (1H, br d, *J*=9.5 Hz), 7.37 (1H, d, *J*=5 Hz), 7.45 (1H, d, *J*=5 Hz), 7.59 (1H, s). **35c**: Colorless foam. HRMS Calcd for $C_{15}H_{15}NO_6S$: 337.0619. Found: 337.0624. MS *m/z*: 337 (M^+, 100), 319 (5), 279 (16), 218 (22), 216 (25), 202 (25), 190 (18), 173 (18), 89 (16), 59 (77). IR ($CHCl_3$): 1768, 1699 cm^{-1}. ^{1}H-NMR δ: 2.67 (1H, br s, OH), 2.92 (1H, dd, *J*=17, 5 Hz), 3.28 (1H, dd, *J*=17, 6 Hz), *ca.* 3.66—3.89 (2H, m), 3.78 (3H, s), 3.93 (3H, s), 4.13—4.40 (1H, m), 7.23 (1H, d, *J*=5 Hz), 7.44 (1H, d, *J*=5 Hz), 7.65 (1H, s). **36c**:

Colorless glass. HRMS Calcd for $C_{15}H_{15}NO_5S$: 321.0670. Found: 321.0667. MS *m/z*: 321 (M^+, 100), 262 (15), 218 (24), 202 (24), 174 (38), 147 (25), 59 (72). IR ($CHCl_3$): 1767, 1695 cm^{-1}. ^{1}H-NMR δ: 1.89—2.23 (2H, m), 3.05 (2H, t, *J*=6.5 Hz), 3.79 (3H, s), 3.84 (2H, t, *J*=7 Hz), 3.94 (3H, s), 7.32 (1H, d, *J*=5 Hz), 7.46 (1H, d, *J*=5 Hz), 7.68 (1H, s).

In the same way, starting from **17d** (27 mg, 0.087 mmol), methyl (±)-2,3-dihydro-2-hydroxy-6-[(methoxycarbonyl)oxy][1]benzothieno[3,2-*f*]quinoline-4(1*H*)-carboxylate (**35d**, 14 mg, 41%) and methyl 2,3-dihydro-6-[(methoxycarbonyl)oxy][1]benzothieno[3,2-*f*]quinoline-4(1*H*)-carboxylate (**36d**, 2.5 mg, 8%) along with recovered **17d** (2 mg, 7%) were obtained after purification by PTLC [hexane–CH_2Cl_2 (1 : 3)]. **35d**: Colorless glass. HRMS Calcd for $C_{19}H_{17}NO_6S$: 387.0775. Found: 387.0775. MS *m/z*: 387 (M^+, 100), 328 (5), 266 (12), 223 (14), 184 (12), 139 (12), 59 (53). IR ($CHCl_3$): 1769, 1692 cm^{-1}. ^{1}H-NMR δ: 2.51 (1H, br s, OH), 3.29 (1H, dd, *J*=17, 5 Hz), 3.67 (1H, dd, *J*=17, 6 Hz), *ca.* 3.67—4.09 (2H, m), 3.80 (3H, s), 3.96 (3H, s), 4.24—4.53 (1H, m), 7.24—7.58 (2H, m), 7.66—7.97 (1H, m), 7.73 (1H, s), 8.12—8.40 (1H, m). **36d**: Colorless glass. HRMS Calcd for $C_{19}H_{17}NO_5S$: 371.0826. Found: 371.0835. MS *m/z*: 371 (M^+, 100), 312 (9), 284 (14), 224 (29), 197 (19), 59 (54). IR ($CHCl_3$): 1771, 1698 cm^{-1}. ^{1}H-NMR δ: 1.99—2.35 (2H, m), 3.43 (2H, t, *J*=7 Hz), 3.73—4.03 (2H, m), 3.81 (3H, s), 3.96 (3H, s), 7.31—7.57 (2H, m), 7.71—7.98 (1H, m), 7.77 (1H, s), 8.21—8.46 (1H, m).

In the same way, starting from **17e** (45 mg, 0.096 mmol), methyl (±)-4-(benzyloxy)-8,9-dihydro-8-hydroxy-2-(3,4,5-trimethoxybenzyl)thieno[3,2-*f*]quinoline-6(7*H*)-carboxylate (**35e**, 40 mg, 76%) and methyl 4-(benzyloxy)-8,9-dihydro-2-(3,4,5-trimethoxybenzyl)thieno[3,2-*f*]quinoline-6(7*H*)-carboxylate (**36e**, 2.5 mg, 5%) along with recovered **17e** (2 mg, 4%) were isolated after separation by PTLC [benzene–EtOAc (5 : 2)]. **35e**: Colorless glass. HRMS Calcd for $C_{30}H_{31}NO_7S$: 549.1819. Found: 549.1806. MS *m/z*: 549 (M^+, 38), 458 (4), 430 (5), 181 (20), 91 (100), 65 (8), 59 (11). IR ($CHCl_3$): 1691 cm^{-1}. ^{1}H-NMR δ: 2.09 (1H, br s, OH), 2.91 (1H, dd, *J*=17.5, 5.5 Hz), 3.29 (1H, dd, *J*=17.5, 6 Hz), *ca.* 3.61—4.03 (2H, m), 3.76 (3H, s), 3.87 (9H, s), 4.20 (2H, s), 4.23—4.52 (1H, m), 5.27 (2H, s), 6.56 (2H, s), 7.06 (1H, s), 7.27 (1H, s), 7.27—7.65 (5H, m). **36e**: Colorless glass. HRMS Calcd for $C_{30}H_{31}NO_6S$: 533.1870. Found: 533.1860. MS *m/z*: 533 (M^+, 56), 473 (5), 442 (13), 414 (15), 354 (12), 181 (22), 91 (100). IR ($CHCl_3$): 1687 cm^{-1}. ^{1}H-NMR δ: 1.87—2.20 (2H, m), 2.96 (2H, t, *J*=6.5 Hz), 3.67—3.94 (2H, m), 3.75 (3H, s), 3.86 (9H, s), 4.17 (2H, s), 5.24 (2H, s), 6.55 (2H, s), 7.06 (1H, s), 7.26 (1H, s), 7.26—7.62 (5H, m).

Mesylation of 35a—e to Form 37a—e Preparation of methyl (±)-2,3-dihydro-6-[(methoxycarbonyl)oxy]-2-[(methylsulfonyl)oxy]benzo[*f*]quinoline-4(1*H*)-carboxylate (**37a**) from **35a** is described as representative. MsCl (13 μl, 0.168 mmol) was added to a cooled (0 °C) solution of **35a** (27 mg, 0.082 mmol) and Et_3N (45 μl, 0.323 mmol) in CH_2Cl_2 (2.5 ml) under an Ar atmosphere. After having been stirred at 0 °C for 30 min, the reaction was quenched by addition of saturated $NaHCO_3$–H_2O and the mixture was extracted with CH_2Cl_2. The organic layer was successively washed with saturated $CuSO_4$–H_2O and then with saturated $NaHCO_3$–H_2O. Usual workup followed by purification by PTLC [benzene–EtOAc (3 : 1)] provided **37a** (33 mg, 99%) as a colorless foam. HRMS Calcd for $C_{18}H_{19}NO_8S$: 409.0830. Found: 409.0830. MS *m/z*: 409 (M^+, 35), 313 (15), 312 (19), 254, (21), 238 (51), 210 (57), 167 (38), 59 (100). IR ($CHCl_3$): 1766, 1708 cm^{-1}. ^{1}H-NMR δ: 3.04 (3H, s), 3.33 (1H, dd, *J*=18, 4.5 Hz), 3.57 (1H, dd, *J*=18, 5.5 Hz), 3.81 (1H, dd, *J*=13.5, 2.5 Hz), 3.84 (3H, s), 3.96 (3H, s), 4.36 (1H, dd, *J*=13.5, 5.5 Hz), 5.20—5.47 (1H, m), 7.40—7.68 (2H, m), 7.74 (1H, s), *ca.* 7.74—8.06 (2H, m).

In the same manner, **35b** (29 mg, 0.090 mmol) was mesylated to afford methyl (±)-8,9-dihydro-4-[(methoxycarbonyl)oxy]-8-[(methylsulfonyl)oxy]-furo[3,2-*f*]quinoline-6(7*H*)-carboxylate (**37b**, 34 mg, 94%) as a colorless foam. HRMS Calcd for $C_{16}H_{17}NO_9S$: 399.0623. Found: 399.0614. MS *m/z*: 399 (M^+, 41), 302 (29), 258 (18), 244 (19), 228 (89), 200 (88), 185 (38), 59 (100). IR ($CHCl_3$): 1769, 1706 cm^{-1}. ^{1}H-NMR δ: 3.03 (3H, s), 3.16 (1H, dd, *J*=17.5, 4 Hz), 3.40 (1H, dd, *J*=17.5, 5 Hz), 3.80 (3H, s), 3.80 (1H, dd, *J*=14, 3 Hz), 3.95 (3H, s), 4.31 (1H, dd, *J*=14, 6 Hz), 5.15—5.41 (1H, m), 6.72 (1H, d, *J*=2.5 Hz), 7.54 (1H, s), 7.63 (1H, d, *J*=2.5 Hz).

In the same manner, **35c** (30 mg, 0.089 mmol) was mesylated to afford methyl (±)-8,9-dihydro-4-[(methoxycarbonyl)oxy]-8-[(methylsulfonyl)oxy]-thieno[3,2-*f*]quinoline-6(7*H*)-carboxylate (**37c**, 35 mg, 95%) as a colorless glass. HRMS Calcd for $C_{16}H_{17}NO_8S_2$: 415.0394. Found: 415.0370. MS *m/z*: 415 (M^+, 38), 318 (23), 306 (12), 274 (12), 244 (53), 216 (62), 201 (38), 173 (40), 59 (100). IR ($CHCl_3$): 1767, 1710 cm^{-1}. ^{1}H-NMR δ: 3.02 (3H, s), 3.26 (1H, dd, *J*=17.5, 4 Hz), 3.48 (1H, dd, *J*=17.5, 5.5 Hz), 3.80 (1H, dd, *J*=13.5, 2.5 Hz), 3.81 (3H, s), 3.93 (3H, s), 4.32 (1H, dd, *J*=13.5, 5.5 Hz), 5.29 (1H, dddd, *J*=5.5, 5.5, 4, 2.5 Hz), 7.25 (1H, d, *J*=5.5 Hz), 7.48 (1H, d, *J*=5.5 Hz), 7.65 (1H, s).

In the same manner, **35d** (14 mg, 0.033 mmol) was mesylated to afford methyl (±)-2,3-dihydro-6-[(methoxycarbonyl)oxy]-2-[(methylsulfonyl)oxy]-[1]benzothieno[3,2-*f*]quinoline-4(1*H*)-carboxylate (**37d**, 16 mg, 95%) as a colorless glass. HRMS Calcd for $C_{20}H_{19}NO_8S_2$: 465.0551. Found: 465.0555. MS *m/z*: 465 (M^+, 69), 369 (14), 368 (15), 310 (20), 294 (27), 266 (41), 223 (44), 59 (100). IR ($CHCl_3$): 1768, 1710 cm^{-1}. ^{1}H-NMR δ: 3.08 (3H, s), 3.47—4.07 (3H, m), 3.84 (3H, s), 3.97 (3H, s), 4.34 (1H, dd, *J*=13.5, 6 Hz), 5.24—5.54 (1H, m), 7.33—7.60 (2H, m), 7.75 (1H, s), 7.75—7.97 (1H, m), 8.09—8.32 (1H, m).

In the same manner, **35e** (40 mg, 0.073 mmol) was mesylated to afford methyl (±)-4-(benzyloxy)-8,9-dihydro-8-[(methylsulfonyl)oxy]-2-(3,4,5-trimethoxybenzyl)thieno[3,2-*f*]quinoline-6(7*H*)-carboxylate (**37e**, 45 mg, 99%) as a colorless glass. HRMS Calcd for $C_{31}H_{33}NO_9S_2$: 627.1595. Found: 627.1603. MS *m/z*: 627 (M^+, 13), 531 (9), 518 (6), 471 (9), 440 (13), 181 (21), 91 (100), 79 (5), 65 (7), 59 (9). IR ($CHCl_3$): 1704, 1596 cm^{-1}. ^{1}H-NMR δ: 3.04 (3H, s), 3.18 (1H, dd, *J*=17, 4.5 Hz), 3.43 (1H, dd, *J*=17, 6 Hz), 3.63—3.96 (1H, m), 3.73 (3H, s), 3.82 (9H, s), 4.13 (2H, s), 4.25 (1H, dd, *J*=13.5, 5 Hz), 5.11—5.40 (1H, m), 5.19 (2H, s), 6.47 (2H, s), 6.96 (1H, s), 7.13 (1H, s), 7.23—7.57 (5H, m).

Cyclization of 37a—d to Form the Precursors 38a—d Preparation of (8b*RS*,9a*SR*)-2,4,9,9a-tetrahydro-1*H*-benzo[*e*]cycloprop[*c*]indol-4-one[26] (**38a**) from **37a** is described as a typical example. K_2CO_3 (39 mg, 0.283 mmol) was added to a solution of **37a** (33 mg, 0.081 mmol) in MeOH (3 ml). After having been stirred at 17 °C for 4 h, the mixture was cooled in an ice bath. CH_2Cl_2 (5 ml), citric acid monohydrate (59 mg, 0.281 mmol), and H_2O (5 ml) were successively added to the mixture and the whole was extracted with 10% MeOH–CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$–H_2O and then treated as usual. Purification by PTLC (4% MeOH–CH_2Cl_2) provided **38a** (15 mg, 94%) as a colorless glass. HRMS Calcd for $C_{13}H_{11}NO$: 197.0840. Found: 197.0832. MS *m/z*: 197 (M^+, 100), 180 (32), 168 (42), 154 (18), 139 (32), 115 (20), 83 (21). IR ($CHCl_3$): 1620, 1596 cm^{-1}. ^{1}H-NMR δ: 1.35 (1H, dd, *J*=4, 4 Hz), 1.54 (1H, dd, *J*=7.5, 4 Hz), 2.36—2.95 (1H, m), 3.63 (1H, d, *J*=10.5 Hz), 3.84 (1H, dd, *J*=10.5, 5 Hz), 5.76 (1H, s), 6.34 (1H, br s, NH), 6.73—6.91 (1H, m), 7.22—7.53 (2H, m), 8.11—8.30 (1H, m).

In the same way, (7b*RS*,8a*SR*)-1,2,8,8a-tetrahydro-4*H*-cyclopropa[*c*]furo-[3,2-*e*]indol-4-one (**38b**, 14 mg, 88%) was obtained from **37b** (34 mg, 0.085 mmol) after separation by PTLC (5% MeOH–CH_2Cl_2). **38b**: Colorless foam. HRMS Calcd for $C_{11}H_9NO_2$: 187.0633. Found: 187.0630. MS *m/z*: 187 (M^+, 100), 170 (11), 158 (18), 130 (68), 77 (30), 63 (29), 52 (29). IR ($CHCl_3$): 1624, 1560 cm^{-1}. ^{1}H-NMR δ: 1.30 (1H, dd, *J*=5, 4 Hz), 1.58 (1H, dd, *J*=8, 4 Hz), 2.76 (1H, ddd, *J*=8, 6, 5 Hz), 3.63 (1H, d, *J*=11 Hz), 3.84 (1H, br dd, *J*=11, 6 Hz), 5.56 (1H, s), 6.16 (1H, d, *J*=2 Hz), 6.40 (1H, br s, NH), 7.50 (1H, d, *J*=2 Hz).

In the same way, (7b*RS*,8a*SR*)-1,2,8,8a-tetrahydro-4*H*-cyclopropa[*c*]thieno[3,2-*e*]indol-4-one (**38c**, 15.5 mg, 93%) was obtained from **37c** (34 mg, 0.082 mmol) after separation by PTLC (5% MeOH–CH_2Cl_2). **38c**: Colorless foam. HRMS Calcd for $C_{11}H_9NOS$: 203.0404. Found: 203.0403. MS *m/z*: 203 (M^+, 100), 188 (13), 186 (15), 174 (44), 160 (18), 147 (14), 145 (14), 121 (10), 102 (13), 63 (15), 45 (16). IR ($CHCl_3$): 1607, 1581 cm^{-1}. ^{1}H-NMR δ: 1.32 (1H, dd, *J*=5, 4 Hz), 1.60 (1H, dd, *J*=8, 4 Hz), 2.81 (1H, ddd, *J*=8, 5, 5 Hz), 3.61 (1H, d, *J*=10.5 Hz), 3.83 (1H, dd, *J*=10.5, 5 Hz), 5.61 (1H, s), 6.62 (1H, d, *J*=5 Hz), 6.64 (1H, br s, NH), 7.42 (1H, d, *J*=5 Hz).

In the same way, (9c*RS*,10a*SR*)-1,2,10,10a-tetrahydro-4*H*-cyclopropa[*c*]-[1]benzothieno[3,2-*e*]indol-4-one (**38d**, 7.5 mg, 92%) was obtained from **37d** (15 mg, 0.032 mmol) after purification by PTLC (4% MeOH–CH_2Cl_2). **38d**: Slightly yellow glass. HRMS Calcd for $C_{15}H_{11}NOS$: 253.0561. Found: 253.0553. MS *m/z*: 253 (M^+, 100), 224 (40), 210 (16), 197 (14), 195 (16), 152 (12), 63 (11), 44 (11). IR ($CHCl_3$): 1612, 1581 cm^{-1}. ^{1}H-NMR δ: 1.32 (1H, dd, *J*=4.5, 4.5 Hz), 2.09 (1H, dd, *J*=8, 4.5 Hz), 3.18—3.46 (1H, m), 3.74 (1H, d, *J*=10.5 Hz), 3.93 (1H, dd, *J*=10.5, 5 Hz), 5.74 (1H, s), 6.44 (1H, br s, NH), 7.23—7.47 (3H, m), 7.77—7.96 (1H, m).

(7b*RS*,8a*SR*)-1,2,8,8a-tetrahydro-6-(3,4,5-trimethoxybenzyl)-4*H*-cyclopropa[*c*]thieno[3,2-*e*]indol-4-one (38e) A slurry of **37e** (14 mg, 0.022 mmol) and 20% $Pd(OH)_2$ on carbon (10 mg) in MeOH (5 ml) and DME (1 ml) was hydrogenated under H_2 atmosphere (1 atm) at 19 °C for 52 h. The mixture was filtered and the filtrate was evaporated to give a residue (16 mg). The residue was dissolved in MeOH (3 ml) and K_2CO_3 (11 mg, 0.080 mmol) was added to this with stirring at 21 °C. The stirring was continued at this temperature for 2.5 h, then the mixture was treated as for the preparation of **38a** from **37a**. Purification by PTLC (3% MeOH–CH_2Cl_2) afforded **38e** (5.5 mg, 64%) and recovered **37e** (4.5 mg, 32%). **38e**: Colorless glass. HRMS Calcd for $C_{21}H_{21}NO_4S$: 383.1190. Found: 383.1175. MS *m/z*: 383

(M^+, 15), 381 (14), 368 (6), 366 (6), 352 (4), 57 (51), 56 (55), 42 (100). IR ($CHCl_3$): 1608, 1593 cm^{-1}. ^{1}H-NMR δ: 1.36 (1H, dd, J=5, 4 Hz), 1.61 (1H, dd, J=8, 4 Hz), 2.67—2.92 (1H, m), 3.59 (1H, d, J=10.5 Hz), 3.81 (1H, dd, J=10.5, 5 Hz), 3.83 (9H, s), 4.06 (2H, s), 5.29 (1H, br s, NH), 5.62 (1H, s), 6.39 (1H,s), 6.49 (2H, s).

Duocarmycin SA A-Ring Analogs 9a—e The coupling reaction to prepare (8b*RS*,9a*SR*)-1,2,9,9a-tetrahydro-2-[(5,6,7-trimethoxy-1*H*-indol-2-yl)-carbonyl]-4*H*-benzo[*e*]cycloprop[*c*]indol-4-one (**9a**) is described as a typical example. K_2CO_3 (91 mg, 0.659 mmol) was added to a solution of **38a** (13 mg, 0.066 mmol) and the imidazolide **39** (40 mg, 0.133 mmol) in DMF (2.5 ml) under an Ar atmosphere and the mixture was stirred at 27 °C for 3 h. CH_2Cl_2 (5 ml), citric acid monohydrate (151 mg, 0.719 mmol), and H_2O (10 ml) were successively added and the whole was thoroughly extracted with 10% MeOH–CH_2Cl_2. After washing with saturated $NaHCO_3$–H_2O, the organic layer was worked-up as usual. The resulting residue was separated by PTLC (0.7% MeOH–CH_2Cl_2) to afford crude **9a** (29 mg). The crude **9a** was further purified by PTLC [benzene–EtOAc (5 : 2)] to yield **9a** (25 mg, 88%) as a slightly yellow powder. HRMS Calcd for $C_{25}H_{22}N_2O_5$: 430.1527. Found: 430.1514. MS *m/z*: 430 (M^+, 73), 413 (34), 234 (100), 204 (19), 179 (18). IR ($CHCl_3$): 1648, 1627, 1604 cm^{-1}. ^{1}H-NMR δ: 1.55 (1H, dd, J=5, 4.5 Hz), 1.71 (1H, dd, J=7.5, 4.5 Hz), 2.74—3.00 (1H, m), 3.89 (3H, s), 3.94 (3H, s), 4.07 (3H, s), *ca.* 4.27—4.61 (2H, m), 6.79 (1H, s), 6.79—7.02 (1H, m), 6.96 (1H, d, J=2 Hz, changed to s with D_2O), 7.14 (1H, s), 7.27—7.64 (2H, m), 8.25 (1H, dd, J=7, 2 Hz), 9.37 (1H, br s, NH).

Similarly, **38b** (9 mg, 0.048 mmol) was allowed to react with **39** (29 mg, 0.096 mmol) in the presence of K_2CO_3 (66 mg, 0.478 mmol) in DMF (1.5 ml) to yield (7b*RS*,8a*SR*)-1,2,8,8a-tetrahydro-2-[(5,6,7-trimethoxy-1*H*-indol-2-yl)carbonyl]-4*H*-cyclopropa[*c*]furo[3,2-*e*]indol-4-one (**9b**, 15 mg, 74%) as a colorless powder. HRMS Calcd for $C_{23}H_{20}N_2O_6$: 420.1320. Found: 420.1306. MS *m/z*: 420 (M^+, 34), 234 (100), 186 (37), 185 (43). IR (KBr): 1631, 1592 cm^{-1}. ^{1}H-NMR δ: 1.59 (1H, dd, J=5.5, 4.5 Hz), 1.78 (1H, dd, J=7.5, 4.5 Hz), 2.69—2.94 (1H, m), 3.89 (3H, s), 3.94 (3H, s), 4.07 (3H, s), *ca.* 4.27—4.61 (2H, m), 6.26 (1H, d, J=2.5 Hz), 6.79 (1H, s), 6.93 (1H, d, J=2 Hz, changed to s with D_2O), 6.97 (1H, s), 7.63 (1H, d, J=2.5 Hz), 9.28 (1H, br s, NH).

Similarly, **38c** (7 mg, 0.034 mmol) was allowed to react with **39** (21 mg, 0.070 mmol) in the presence of K_2CO_3 (48 mg, 0.348 mmol) in DMF (1.5 ml) to provide (7b*RS*,8a*SR*)-1,2,8,8a-tetrahydro-2-[(5,6,7-trimethoxy-1*H*-indol-2-yl)carbonyl]-4*H*-cyclopropa[*c*]thieno[3,2-*e*]indol-4-one (**9c**, 9.5 mg, 63%) as a slightly yellow powder. HRMS Calcd for $C_{23}H_{20}N_2O_5S$: 436.1092. Found: 436.1086. MS *m/z*: 436 (M^+, 20), 234 (100), 203 (34), 191 (10), 173 (13), 160 (10), 77 (11). IR ($CHCl_3$): 1645, 1617 cm^{-1}. ^{1}H-NMR δ: 1.61 (1H, dd, J=5, 4.5 Hz), 1.82 (1H, dd, J=7.5, 4.5 Hz), 2.73—2.99 (1H, m), 3.88 (3H, s), 3.94 (3H, s), 4.07 (3H, s), *ca.* 4.27—4.60 (2H, m), 6.74 (1H, d, J=5 Hz), 6.80 (1H, s), 6.96 (1H, d, J=2 Hz, changed to s with D_2O), 7.02 (1H, s), 7.61 (1H, d, J=5 Hz), 9.36 (1H, br s, NH).

Similarly, **38d** (4.5 mg, 0.018 mmol) was allowed to react with **39** (11 mg, 0.037 mmol) in the presence of K_2CO_3 (25 mg, 0.181 mmol) in DMF (1.5 ml) to give (9c*RS*,10a*SR*)-1,2,10,10a-tetrahydro-2-[(5,6,7-trimethoxy-1*H*-indol-2-yl)carbonyl]-4*H*-cyclopropa[*c*][1]benzothieno[3,2-*e*]indol-4-one (**9d**, 3 mg, 35%) as a slightly yellow powder. HRMS Calcd for $C_{27}H_{22}N_2O_5S$: 486.1248. Found: 486.1250. MS *m/z*: 486 (M^+, 34), 253 (49), 251 (65), 234 (100). IR ($CHCl_3$): 1643, 1610 cm^{-1}. ^{1}H-NMR (10% CD_3OD–$CDCl_3$) δ: 1.68 (1H, dd, J=4.5, 4.5 Hz), 2.39 (1H, dd, J=8.5, 4.5 Hz), *ca.* 3.31—3.52 (1H, m), 3.90 (3H, s), 3.94 (3H, s), 4.09 (3H, s), *ca.* 4.39—4.74 (2H, m), 6.84 (1H, s), 7.02 (1H, s), 7.13 (1H, s), 7.33—7.67 (3H, m), 7.87—8.10 (1H, m).

Similarly, **38e** (6 mg, 0.016 mmol) was allowed to react with **39** (10 mg, 0.033 mmol) in the presence of K_2CO_3 (22 mg, 0.159 mmol) in DMF (1.5 ml) to give (7b*RS*,8a*SR*)-1,2,8,8a-tetrahydro-6-(3,4,5-trimethoxybenzyl)-2-[(5,6,7-trimethoxy-1*H*-indol-2-yl)carbonyl]-4*H*-cyclopropa[*c*]thieno[3,2-*e*]indol-4-one (**9e**, 7.5 mg, 78%) as a slightly yellow glass. HRMS Calcd for $C_{33}H_{32}N_2O_8S$: 616.1877. Found: 616.1894. MS *m/z*: 616 (M^+, 30), 452 (27), 383 (100), 234 (88). IR ($CHCl_3$): 1644, 1613 cm^{-1}. ^{1}H-NMR δ: 1.58 (1H, dd, J=5, 5 Hz), 1.76 (1H, dd, J=7.5, 5 Hz), 2.67—2.93 (1H, m), 3.84 (9H, s), 3.87 (3H, s), 3.93 (3H, s), 4.07 (5H, s), *ca.* 4.22—4.58 (2H, m), 6.42 (1H, s), 6.47 (2H, s), 6.79 (1H, s), 6.93 (1H, d, J=2.5 Hz, changed to s with D_2O), 6.97 (1H, s), 9.31 (1H, br s, NH).

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Enantioselective Synthesis of the Key Intermediate of the Acyl-CoA: Cholesterol Acyltransferase (ACAT) Inhibitor (R-106578) Using 2,2′-Bis(diphenylphosphino)-1,1′-binaphthyl (BINAP)–Ru(OAc)$_2$ as a Catalyst

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Acidic segment of an acyl-CoA: cholesterol acyltransferase (ACAT) inhibitor, R-106578 was synthesized by enantioselective hydrogenation of the *Z*-olefine (9-(*Z*)) using (*R*)-2,2′-bis(diphenylphosphino)-1,1′-binaphthyl (BINAP)–Ru(OAc)$_2$ as a catalyst in methanol at 100 °C, 5 kgf/cm^2 of H$_2$ pressure. The requisite *Z*-olefine was prepared regioselectively *via* coumarin derivative (5).

Key words enantioselective hydrogenation; (*R*)-BINAP–Ru(OAc)$_2$; coumarin derivative; ACAT inhibitor

Targeting the inhibition of acyl-CoA: cholesterol acyltransferase (ACAT) in the absorption process of cholesterol in the small intestines is currently being undertaken widely all over the world in the research and development of new medicine with potential hypolipidemic and anti-atherosclerotic activities. Several compounds have been reported as promising ones such as CI-1011,[2] F-1394[3] or YM-750.[4] Along these lines we also devoted ourselves to discover our own compound, R-106578 (**1**).[5] Here, we would like to describe our synthesis of the key intermediate (**2**)[6] in the optically active form using a ruthenium(II) complex of 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl (BINAP) as a catalyst. At the starting point of this work the optical form of the acid intermediate (**2**) was prepared by chromatographic separation of the diastereomeric mixture of 3-[2-(2,4-dimethoxyphenyl)heptanoyl]-4(*S*)-benzyl-2-oxazolinone followed by lithium aluminum hydride (LAH) reduction, cyanation and then alkaline hydrolysis, or the optical resolution of the *dl* form of the acid (**2**) using (*S*)-(−)-α-methylbenzylamine as a chiral base.[5]

Therefore, the aim of the present work is to develop an efficient and practical method for the preparation of the desired chiral acid (**2**). The key features of our approach are the asymmetric hydrogenation with the BINAP–Ru(OAc)$_2$ catalyst and the regiospecific synthesis of *Z*-olefin (**4**) *via* coumarin analogue **5** (Charts 1, 2).

At first, we prepared α,β-unsaturated carboxylic acid derivatives (**8**-(*E*),-(*Z*), **9**-(*E*),-(*Z*)) to investigate the enantioselectivities of the hydrogenation of their olefinic bonds. Friedel–Crafts acylation[7] of 1,3-dimethoxybenzene (**6**) followed by Horner–Emmons olefination gave the α,β-unsaturated carboxylate (**8**). The (*E*)/(*Z*)-selectivity of this olefination was *ca.* 1/3, and each **8**-(*E*) and **8**-(*Z*) was separated by silica gel column chromatography. Alkaline hydrolysis of the isolated **8**-(*E*) and **8**-(*Z*) yielded the corresponding acids **9**-(*E*) and **9**-(*Z*) respectively.

With the requisite olefins (**8**-(*E*) and **8**-(*Z*), and **9**-(*E*) and **9**-(*Z*)) in hand we examined the asymmetric hydrogenation. Although varied conditions are generally applied in pilot plant scale synthesis, we performed the hydrogenation reaction under fixed conditions using (*S*)- or (*R*)-BINAP–Ru(OAc)$_2$ as a catalyst in methanol at 100 °C, 5 kgf/cm^2 of H$_2$ pressure.[8] The results are shown in Table 1, in which the asymmetric hydrogenation of **9**-(*Z*) is satisfactory (>97% ee), but the other three have poor selectivity (**9**-(*E*)) or poor reactivity (**8**-(*E*), **8**-(*Z*)). Furthermore, when this reaction was conducted in methanol, the methyl ester of **9**-(*Z*) was produced in a small quantity. To avoid this side reaction a mixed solvent of 2-propanol and water (9 : 1) was used expecting the steric effect to form fewer amount of the isopropyl ester. The ee of this reaction proved to be over 97%. When this reaction was performed in 2-propanol, the ee was *ca.* 96%. From these experiments, it became clear that the best result was obtainable from **9**-(*Z*) using (*R*)-BINAP–Ru(OAc)$_2$ as a

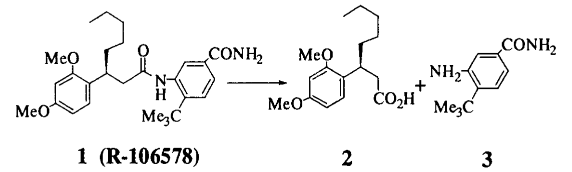

Chart 1

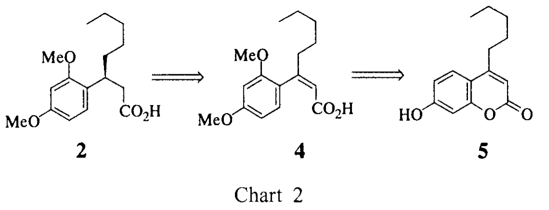

Chart 2

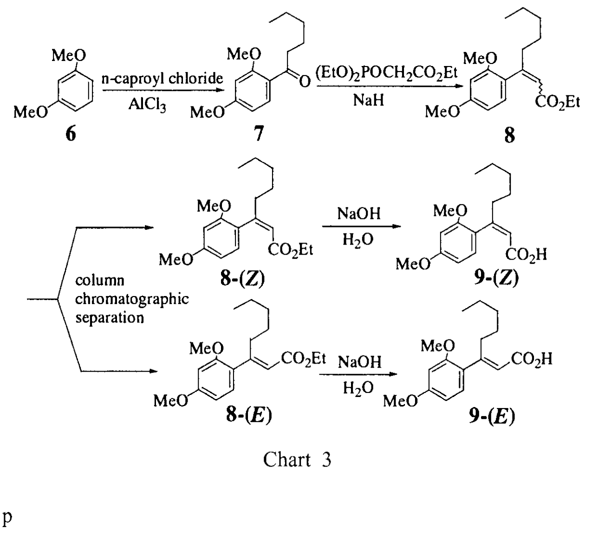

Chart 3

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Dedicated to the memory of Dr. Kyosuke Tsuda.

Table 1. Hydrogenation of the Olefinic Starting Materials

Starting Substrate	BINAP–Ru(OAc)$_2$	ee[a] (%)	Rxn time	Abs. config.[a]	Yield[b]
8-(*E*)	*S*	44.3	7 h	*S*	3%
8-(*Z*)	*S*	92.8	14 h	*R*	64%
9-(*E*)	*S*	3.7	2 h	*S*	Quant.
9-(*Z*)	*S*	97.0	2 h	*R*	Quant.
9-(*Z*)	*R*	97.0	1 h	*S*	Quant.

a) The absolute configuration and enantiomeric excesses (ee) were determined by HPLC analysis (CHIRALCEL OD by Daicel) after the optically active carboxylic acid was converted to the methyl ester by TMSCHN$_2$. Column, Daicel Chiralcel OD (ϕ4.6×250 mm); eluent, *n*-hexane/2-propanol (99 : 1); flow rate, 1.0 ml min^{-1}; oven, 25 °C; detect, UV (280 nm); t_R of (*R*)-methyl ester 8.5 min, t_R of (*S*)-methyl ester 17.1 min, t_R of (*R*)-ethyl ester 6.9 min, t_R of (*S*)-ethyl ester 10.7 min. *b*) Yields were determined by the above HPLC analysis.

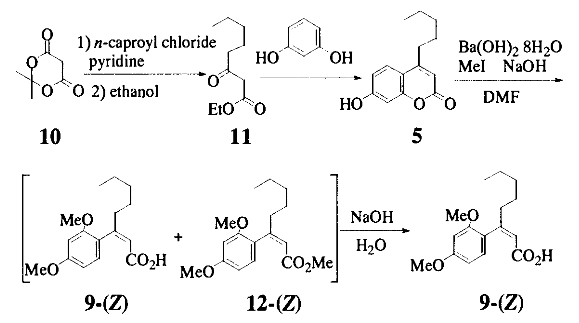

Chart 4

catalyst.

The above described route to the starting acid (**9**-(*Z*)) is multi-step using column chromatography for the separation of the isomers (**8**-(*E*)) and (**8**-(*Z*)), and consequently is unrealistic for large scale production of the desired acid (**2**). Therefore, we developed a new regiospecific synthetic method for the acid **9**-(*Z*) *via* the coumarin analogue (**5**).

According to the documented procedure[9] ethyl *n*-hexanoylacetate (**11**) was easily synthesized from Meldrum's acid (**10**) and *n*-caproyl chloride followed by refluxing in ethanol. Treatment of **11** and resorcinol in the presence of Amberlyst® 15[10] or *p*-toluenesulfonic acid afforded the coumarin derivative (**5**).[11] The lactone ring of this coumarin derivative (**5**) was cleaved with Ba(OH)$_2$ 8H$_2$O, excess NaOH (flake) and excess methyl iodide in *N*,*N*-dimethylformamide (DMF)[12] to yield a mixture of the acid (**9**-(*Z*)) and methyl ester (**12**-(*Z*)). This mixture was further hydrolyzed under basic conditions to the desired (**9**-(*Z*)), mp 51—52 °C.

In conclusion, we succeeded in discovering a very efficient synthetic method for the optically active 3-(*S*)-(2,4-dimethoxyphenyl)octanoic acid by asymmetric hydrogenation (using (*R*)-BINAP–Ru(OAc)$_2$) of 3-(2,4-dimethoxyphenyl)-2-octenoic acid (**9**-(*Z*)), which was prepared regiospecifically *via* the coumarin derivative (**5**).

Experimental

Melting points were determined on a melting point instrument, FP62 (Mettler) and are uncorrected. Infrared (IR) spectra were recorded on an FT-IR spectrometer, Spectrum 2000 (Perkin Elmer). ^{1}H-NMR spectra were recorded on a FT-NMR system, JNM-LA400 (400 MHz) (JEOL). Coupling constants are reported in hertz (Hz) and chemical shift in ppm downfield from internal tetramethylsilane. Mass spectra were recorded on a JEOL JMS-BU20 or JMS-700 spectrometer. Optical rotations were measured with a high sensitive polarimeter, SEPA-3000 (Horiba). Column chromatography was performed on silica gel (Microbeads 4B, 100—200 mesh, Fuji silysia). All chemicals were obtained from commercial sources and were used without further purification.

1-(2,4-Dimethoxyphenyl)hexan-1-one (7) To a slurry of AlCl$_3$ (10.6 g) in CH$_2$Cl$_2$ (50 ml) were added 1,3-dimethoxybenzene (**6**, 10.4 ml) and then *n*-caproyl chloride (11.1 ml) while maintaining the reaction temperature at 0—10 °C. The whole mixture was stirred at 0—5 °C for an additional 2.5 h. Water (50 ml) was added to the reaction mixture and the product was separated. The organic layer was washed with saturated aq. NaHCO$_3$, 20% aq. NaCl, and then the solvent was removed *in vacuo*. *n*-Hexane (50 ml) was added to the residue, and then cooled to 0—5 °C for 0.5 h. The precipitate was filtered and dried *in vacuo* to afford 16.94 g of **7** (90%). mp 36.0 °C. ^{1}H-NMR (CDCl$_3$) δ: 0.90 (3H, t, J=3.9 Hz), 1.20—1.40 (4H, m), 1.66 (2H, t, J=7.6 Hz), 2.92 (2H, t, J=7.3 Hz), 3.85 (3H, s), 3.87 (3H, s), 6.40—6.55 (2H, m), 7.78 (1H, d, J=8.5 Hz). IR (KBr) cm^{-1}: 2938, 1662, 1597. Electron ionization (EI)-MS *m/z*: 236 (M$^+$). *Anal.* Calcd for $C_{14}H_{20}O_3$; C, 71.16; H, 8.53; O, 20.31. Found: C, 71.22; H, 8.30; O, 20.36.

Ethyl (*E*)- and (*Z*)-3-(2,4-Dimethoxyphenyl)-2-octenate (8) In a 500 ml four-necked flask, 60% oil dispersion of NaH (10.2 g) was washed with *n*-hexane (20 ml) and then tetrahydrofuran (THF, 240 ml) was added. Triethyl phosphonoacetate (62.9 g) was added dropwise to the slurry solution at −10 °C and then a solution of ketone (**7**) (30.0 g) in THF (60 ml) was added successively. The whole mixture was refluxed for 7 h. After cooling, saturated aq. NH$_4$Cl (120 ml), water (50 ml) and diethyl ether (150 ml) were added with careful stirring. The organic layer was separated and washed with 20% aq. NaCl three times, dried over anhydrous Na$_2$SO$_4$, and then the solvent was removed *in vacuo*. The residue was chromatographed on silica gel (2 l). Elution with *n*-hexane/ethyl acetate (10/1—8/2) gave the less polar **8**-(*E*) (9.30 g, 24%) as oil and the more polar **8**-(*Z*) (27.82 g, 72%) as oil. **8**-(*E*): ^{1}H-NMR (CDCl$_3$) δ: 0.83 (3H, t, J=7.1 Hz), 1.13—1.45 (9H, m), 3.02 (2H, t, J=8.0 Hz), 3.78 (3H, s), 3.82 (3H, s), 4.20 (2H, q, J=7.8 Hz), 5.79 (1H, s), 6.46 (2H, m), 7.03 (1H, m). **8**-(*Z*): ^{1}H-NMR (CDCl$_3$, 400 MHz, ppm) δ: 0.86 (3H, t, J=7.1 Hz), 1.09 (3H, t, J=7.1 Hz), 1.25—1.40 (6H, m), 2.40 (2H, t, J=7.6 Hz), 3.74 (3H, s), 3.79 (3H, s), 4.00 (2H, q, J=7.1 Hz), 5.90 (1H, s), 6.47 (2H, m), 6.90 (1H, m).

(*E*)-3-(2,4-Dimethoxyphenyl)oct-2-enoic Acid (9-(*E*)) To a solution of **8**-(*E*) (1.0 g) in ethanol (3 ml) was added 5 M aq. NaOH (0.78 ml) and the whole solution was heated at 60 °C for 5 h. After evaporation of the solvent *in vacuo*, 1 M aq. HCl (5 ml) was added to the residue and **9**-(*E*) was extracted with toluene (10 ml). Another toluene (10 ml) was added to the water layer and extracted again. The combined organic layer was washed with water (10 ml), and evaporated. *n*-Hexane (10 ml) was added to the residue, and cooled at 0—5 °C for 0.5 h. The resulting precipitate was filtered and dried *in vacuo* to afford the acid **9**-(*E*) (0.75 g, 82%). mp 95.8 °C. ^{1}H-NMR (CDCl$_3$) δ: 0.83 (3H, t, J=7.3 Hz), 1.20—1.40 (6H, m), 3.05 (2H, t, J=7.8 Hz), 3.80 (3H, s), 3.83 (3H, s), 5.85 (1H, s), 6.46 (2H, m), 7.06 (1H, m). IR (KBr) cm^{-1}: 2935, 1682, 1611, 1500. EI-MS *m/z*: 278 (M$^+$). *Anal.* Calcd for $C_{16}H_{22}O_4$; C, 69.04; H, 7.97; O, 22.99. Found: C, 68.79; H, 7.75; O; 23.01.

(*Z*)-3-(2,4-Dimethoxyphenyl)oct-2-enoic Acid (9-(*Z*)) from 8-(*Z*) To a solution of **8**-(*Z*) (1.0 g) in ethanol (3 ml) was added 5 M aq. NaOH (0.78 ml) and the whole was heated at 60 °C for 6 h. After evaporation the desired compound was isolated by the same procedure as in the case of the isomer (**9**-(*E*)) to give the desired **9**-(*Z*) (0.86 g, 95%). mp 84.5 °C. ^{1}H-NMR (CDCl$_3$) δ: 0.84 (3H, t, J=6.8 Hz), 1.20—1.40 (6H, m), 2.41 (2H, t, J=6.8 Hz), 3.71 (3H, s), 3.81 (3H, s), 5.88 (1H, s), 6.46 (2H, m), 6.90 (1H, m). IR (KBr) cm^{-1}: 2930, 1691, 1626, 1508. EI-MS *m/z*: 278 (M$^+$). *Anal.* Calcd for $C_{16}H_{22}O_4$; C, 69.04; H, 7.97; O, 22.99. Found: C, 68.93; H, 7.88; O; 23.09.

Ethyl 3-Oxooctanoate (11) According to the literature[7] a solution of *n*-caproyl chloride (40.0 g) in CH$_2$Cl$_2$ (100 ml) was added dropwise to a solution of Meldrum's acid (**10**) (39.7 g) in pyridine (50 ml) at 10 °C. The red solution was stirred for 4.5 h at room temperature and then 2 M aq. HCl (300 ml) was added. The organic layer was separated and the aqueous layer was extracted with CH$_2$Cl$_2$ (50 ml). The combined organic layer was washed with water (100 ml), dried (Na$_2$SO$_4$), and evaporated to yield a red oil (67.63 g). A solution of the oil in ethanol (200 ml) was refluxed for 7 h and evaporated. The residue was distilled to give the title compound (**11**) (48.04 g, 94%), bp 84—86 °C (2 mmHg).

4-*n*-Pentyl-7-hydroxycoumarin (5) (Using Amberlyst® 15) To a solution of **11** (15.0 g) in toluene (150 ml) were added resorcinol (17.74 g) and Amberlyst® 15 (7.5 g), and the mixture was refluxed for 5.5 h with azeotropic removal of water and ethanol using Dean–Stark equipment. After filtration in the hot state, water (50 ml) was added carefully to the filtrate. The slurry formed was stirred for 0.5 h at 0—5 °C. The appeared precipitate was filtered, dried *in vacuo* to afford the coumarin derivative (**5**) (14.41 g, 81%). ^{1}H-NMR (CD$_3$OD) δ: 0.91 (3H, t, J=6.6 Hz), 1.30—1.50 (4H, m), 1.60—1.75 (2H, m), 2.76 (2H, t, J=7.8 Hz), 3.30 (3H, s), 6.06 (1H, s), 6.65—6.85 (2H, m), 7.61 (1H, m).

5 (Using *p*-Toluenesulfonic Acid) To a solution of **11** (100 g) in toluene (1 l) were added resorcinol (118.24 g) and *p*-toluenesulfonic acid (10.21 g), and the mixture was refluxed for 1.5 h with azeotropic removal of water and ethanol with Dean–Stark equipment. Water (1 l) was added to the reaction mixture in hot state and cooled to 20 °C during 1 h. The slurry was stirred for 0.5 h at 0—5 °C. The appeared precipitates were filtered, dried *in vacuo* to afford the coumarin derivative **5** (105.91 g, 85%).

9-(*Z*) from 5 To a slurry of NaOH (flake, 86.1 g) and coumarin derivative (**5**) (100 g) in DMF (500 ml) was added Ba(OH)$_2$ 8H$_2$O (67.91 g) and MeI (221.5 ml) successively at 25—30 °C. The whole mixture was stirred at 30—33 °C for 2 h. Then toluene (1 l), water (1.5 l) and 36% aq. HCl (80 ml) were added successively. The organic layer was washed with water (1 l) twice, and evaporated to yield the mixture of **9**-(*Z*) and **12**-(*Z*) as orange-colored oil. Methyl ester (**12**-(*Z*)), ^{1}H-NMR (CDCl$_3$) δ: 0.85 (3H, t, J=7.2 Hz), 1.20—1.50 (6H, m), 2.41 (2H, t, J=8.0 Hz), 3.54 (3H, s), 3.75 (3H, s), 3.83 (3H, s), 5.91 (1H, s), 6.47 (2H, m), 6.91 (1H, m). IR (KBr) cm^{-1}: 2930, 1691, 1626, 1508. To this mixture of **9**-(*Z*) and **12**-(*Z*) in toluene (200 ml), methanol (300 ml) and water (50 ml) was added NaOH (34.5 g), and the whole was refluxed for 2 h. Toluene (300 ml), water (300 ml) and 36% aq. HCl (80 ml) were added successively and the organic layer was washed with water (500 ml) and evaporated. The residue was dissolved in ethylcyclohexane at 70 °C, then cooled to 0—5 °C and the slurry was stirred for 0.5 h. The formed precipitate was filtered and dried *in vacuo* to give the desired (*Z*)-olefine (**9**-(*Z*)) (107.12 g, 89% from **5**).

(*S*)-3-(2,4-Dimethoxy)phenyloctanoic Acid (2) The (*Z*)-olefine (**9**-(*Z*)) (106.0 g), (*R*)-BINAP–Ru(OAc)$_2$ (53 mg), 2-propanol (85 ml) and water (21.3 ml) were bubbled with nitrogen for 2 h and then the whole was poured in the autoclave. After replacing the atmosphere with nitrogen and adjusting the pressure gauge with hydrogen at 5 kgf/cm^2, the yellowish solution was heated at 100 °C for 5 h. The reaction mixture was concentrated *in vacuo*, and the residue was dissolved in methanol (318 ml). Water (114 ml) was added to this and the whole was cooled to 0—5 °C for 1 h. The resulting precipitates were filtered and dried *in vacuo* to yield the desired title (*S*)-acid (**2**) (103.63 g, 97%, content: 97.6%, 97.7% ee) which is identical with the reported data.5) mp 52.0 °C. ^{1}H-NMR (CDCl$_3$) δ: 0.83 (3H, t, J=6.8 Hz), 1.10—1.30 (6H, m), 1.50—1.70 (2H, m), 2.62 (2H, m), 3.39 (1H, quintet, J=7.5 Hz), 3.77 (3H, d, J=3.4 Hz), 3.79 (3H, d, J=3.4 Hz), 4.40—6.50 (2H, m), 7.01 (1H, dd, J=3.4, 9.0 Hz). IR (KBr) cm^{-1}: 2925, 1697, 1611, 1505, 1209, 1155, 830. EI-MS *m/z*: 280 (M$^+$). $[\alpha]_D^{27}$ +6.1 (c=1.07, CHCl$_3$). *Anal.* Calcd for C$_{16}$H$_{24}$O$_4$; C, 68.54; H, 8.63; O, 22.83. Found: C, 68.83; H, 8.41; O; 23.05.

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Total Synthesis of Polyamine Toxin HO-416b and Agel-489 Using a 2-Nitrobenzenesulfonamide Strategy

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Total synthesis of spider toxins HO-416b (1) and Agel-489 (2) was accomplished using the 2-nitrobenzenesulfonamide (Ns) group as both a protecting and activating group. In this strategy, the C–N bonds were constructed by alkylation of sulfonamides with alkyl halides or Mitsunobu reaction with the corresponding alcohol. Beginning with monoprotection of the symmetrical diamine, the construction of the backbone from diamine 3 was efficiently accomplished in 7 steps for 14 and 9 steps for 29. Removal of the Ns group while the substrate was attached to a novel solid support enabled the efficient isolation of this highly polar compound.

Key words 2-nitrobenzenesulfonamide (Ns) group; protecting and activating group; alkylation; monoprotection; solid support

Polyamine toxins derived from spider venom have been shown to be specific glutamate receptor blockers.[1] They are expected to be useful as tools for studying neurophysiology and as lead structures for pharmacological and agrochemical agents. Although many synthetic studies of these compounds have been reported,[2] there are still few versatile syntheses of sequential secondary amines.[3] Recently, we reported an efficient method for the construction of secondary amines using the Ns group as a protecting and activating group.[4] We envisioned that this protocol would provide an efficient synthetic route to polyamine toxins. Described herein is our practical total synthesis of the polyamine spider toxin HO-416b (**1**)[5] and Agel-489 (**2**).[6]

Monoprotected diamines seemed to be ideal starting materials for incorporation into a polyamine chain. Selective protection and purification of diamines is reported to be difficult[7]; however, the Ns group provided good results. Thus, treatment of 1,3-diaminopropane with 2-nitrobenzenesulfonyl chloride, followed by neutralization with NaOEt afforded the monosulfonyl adduct **3** in high yield. This procedure was applied to other diamines to provide **4** and **5**. We used these compounds as the key building blocks in our total synthesis of **1** and **2**.

The synthesis of **1** began from monoprotected diamine **3**. Treatment of **3** with Boc_2O and selective alkylation with 1,3-dibromopropane afforded bromide **7**. Sulfonamide **9**, readily obtained from 3-aminopropanol (**8**), was converted to the right-hand triamine **10** by treatment with **7** and Cs_2CO_3. The left-hand fragment **13** was obtained by condensation of 3-indoleacetic acid (**12**) and **4** under mixed-anhydride conditions. Although the two sides can be coupled under Mitsunobu conditions, we chose the conventional alkylation methods to simplify the purification of alkyl adduct **14**. Conversion of alcohol **10** to the iodide **11** was performed by mesylation and iodide displacement. Upon treatment with **11** and Cs_2CO_3, the sulfonamide **13** underwent smooth alkylation to provide **14**. Subsequent removal of the Boc group under acidic conditions gave the primary amine **15**.

Solid phase supports have proven effective as tools for the isolation of highly polar compounds, making their use in our final deprotection attractive. Initial attempts to load **15** onto a commercially available 2-chlorotrityl chloride resin were inefficient. We thus planned to prepare the novel resin **16**. This resin would be more reactive since a phenol unit separated the bulky polystyrene support from the reactive site and an alkoxy group stabilized the trityl cation. Treatment of Merrifield resin with *p*-hydroxytrityl alcohol[8] and K_2CO_3, followed by reaction with $SOCl_2$, afforded the desired resin **16** (Chart 3). This resin could be recycled by treatment with $SOCl_2 : CH_2Cl_2$ (1 : 9) after cleavage of the substrates.

Linkage of Ns-protected HO-416b **15** to the resin **16** was induced by *i*-Pr_2NEt (Chart 4). Upon treatment of the resin with 2-mercaptoethanol and DBU, the Ns groups were removed.[9] Cleavage from the resin under acidic conditions (1% TFA/CH_2Cl_2) and evaporation of the solvent provided **1** without the need for any chromatographic purification (Chart 4). ^1H- and ^{13}C-NMR spectral data of **1** indicated the presence of highly pure material, and tandem FAB MS-MS spectroscopy also proved **1** was identical with naturally occurring HO-416b.

Next, we turned our attention to the synthesis of Agel-489

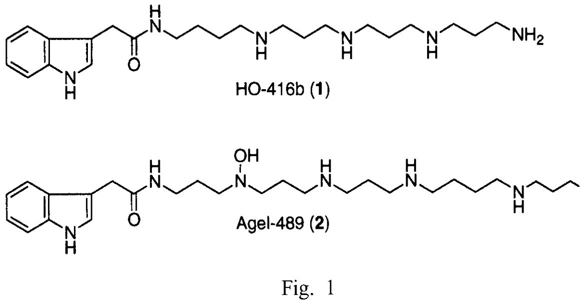

Fig. 1

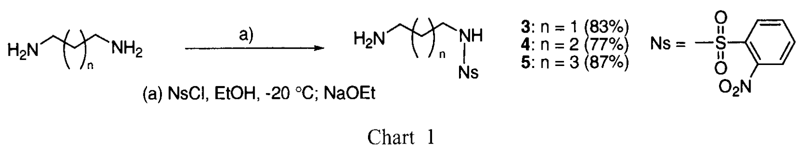

Chart 1

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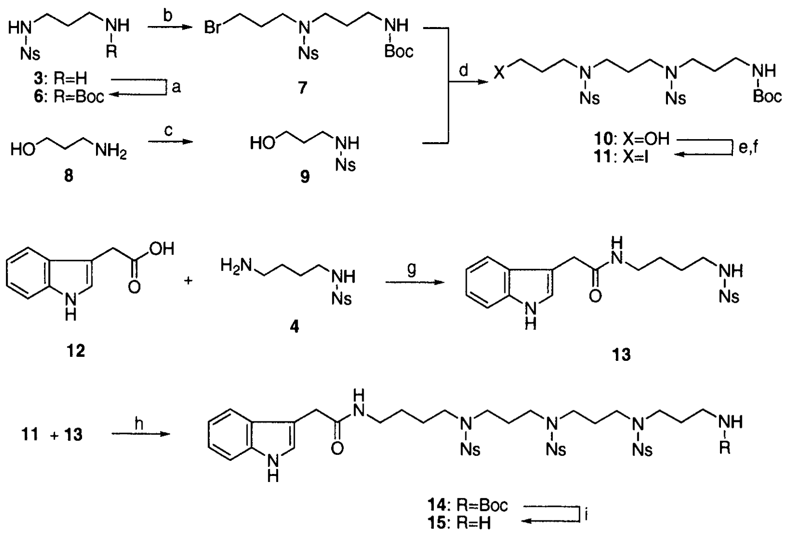
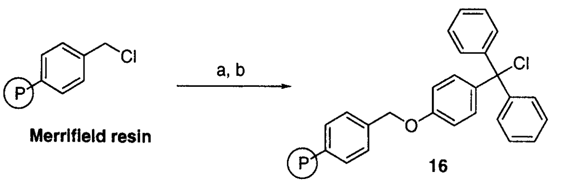
Dedicated to the memory of Dr. Kyosuke Tsuda.



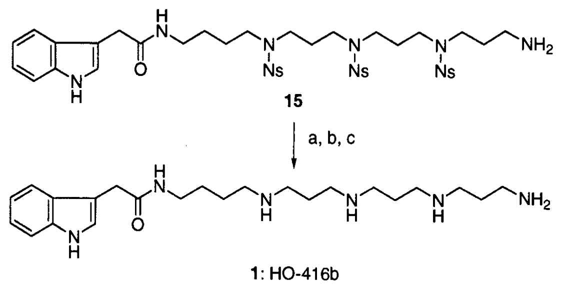
(a) Boc_2O, Et_3N, CH_2Cl_2, rt (99%) (b) 1,3-dibromopropane, K_2CO_3, DMF, 60°C (97%) (c) NsCl, Py, CH_2Cl_2, 0°C (99%) (d) Cs_2CO_3, *n*-Bu_4NI, CH_3CN, 60°C (86%) (e) MsCl, Et_3N, CH_2Cl_2, rt (99%) (f) NaI, 2-butanone, 60°C (99%) (g) PivCl, Et_3N, CH_2Cl_2, rt; **4**, Et_3N, DMAP (97%) (h) Cs_2CO_3, *n*-Bu_4NI, CH_3CN, 60°C (94%) (i) $SOCl_2$, MeOH, rt (96%)

Chart 2



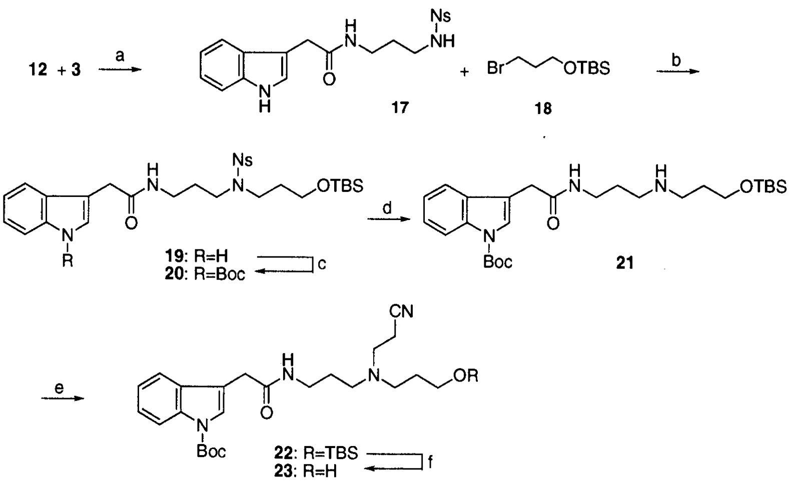
(a) *p*-hydroxytrityl alcohol, K_2CO_3, DMF, 60 °C (b) $SOCl_2$, CH_2Cl_2, rt

Chart 3



(a) resin **16**, *i*-Pr_2NEt, CH_2Cl_2, rt (b) 2-mercaptoethanol, DBU, DMF, rt (c) TFA, CH_2Cl_2 (68% from **15**)

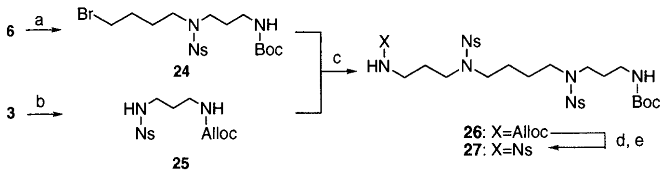
Chart 4



(a) PivCl, Et_3N, CH_2Cl_2, rt; **3**, Et_3N, DMAP (95%) (b) K_2CO_3, *n*-Bu_4NI, CH_3CN, 60°C (80%) (c) Boc_2O, Et_3N, DMAP, CH_2Cl_2, rt (98%) (d) PhSH, Cs_2CO_3, CH_3CN, rt (97%) (e) acrylonitrile, EtOH, 60 °C, (93%) (f) HF-NH_3, CH_3CN, 60 °C, (98%)

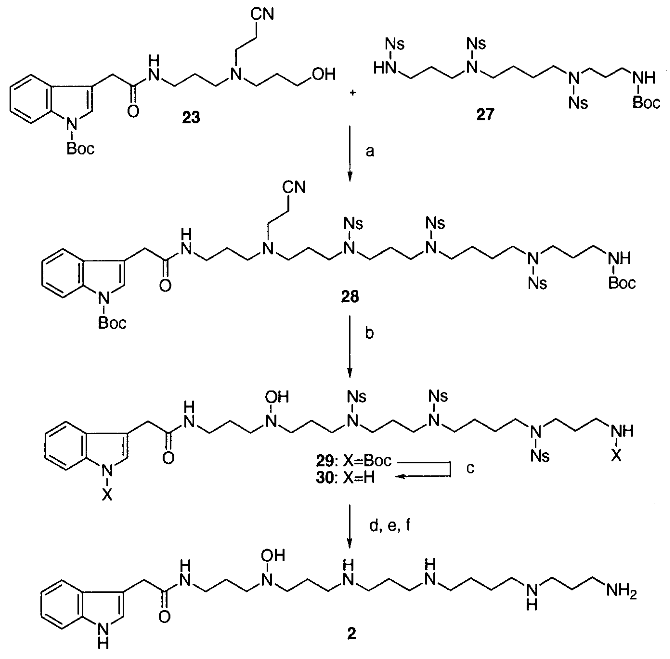
Chart 5

(**2**), isolated from the venomous spider *Agelenopsis apearta*. The key structural feature of **2** is a hydroxylamine-containing polyamine chain. Because of the inherent instability of secondary hydroxylamines, we planned to generate this functionality late in our synthesis. Although several methods have been reported for the transformation,[10)] we planned to construct this group by oxidation of a 2-cyanoethylamine and subsequent elimination of acrylonitrile by *retro*-Michael re-



(a) dibromobutane, K_2CO_3, DMF, 60°C (81%) (b) AllocCl, Et_3N, CH_2Cl_2, 0°C (89%) (c) Cs_2CO_3, *n*-Bu_4NI, CH_3CN, 60°C (97%) (d) $Pd(PPh_3)_4$, PPh_3, pyrrolidine, CH_2Cl_2, rt (99%) (e) NsCl, Et_3N, CH_2Cl_2, rt (94%)

Chart 6



(a) PPh_3, DEAD, benzene : CH_2Cl_2 (4 : 1), rt (90%) (b) *m*-CPBA, CH_2Cl_2, -10 °C (90%) (c) SO_2Cl, MeOH, rt (95%) (d) resin **16**, *i*-Pr_2NEt, CH_2Cl_2, rt (e) 2-mercaptoethanol, DBU, DMF, rt (f) TFA, CH_2Cl_2, rt (68% from **30**)

Chart 7

action.[11] We would assemble the backbone **2** by coupling the cyanoethylamine **23** with the spermine derivative **27**.

The left-hand fragment **23** was obtained by an efficient 6-step synthesis (Chart 5). Condensation of 3-indoleacetic acid (**12**) and diamine **3** under mixed-anhydride conditions provided the sulfonamide **17**. Bromide **18**, readily obtained from 3-bromopropanol, was converted to a precursor of **19** by treatment with **17** and Cs_2CO_3. Protection of the indole **19** with Boc_2O and selective removal of the Ns group with thiophenol[3a] yielded amine **21**. Upon treatment with acrylonitrile, the amine **21** underwent smooth cyanoethylation. Removal of the TBS group gave the primary alcohol **23**.

The protected spermine derivative **27** was also synthesized from the monoprotected diamine **3** (Chart 6). Selective alkylation of **6** with 1,3-dibromopropane afforded bromide **24**. Sulfonamide **25**, readily prepared from diamine **3**, was converted to the tetraamine **26** by treatment with **24** in the presence of Cs_2CO_3. A change of protecting groups from *N*-Alloc to Ns provided sulfonamide **27**.

Condensation of primary alcohol **23** and sulfonamide **27** was accomplished under Mitsunobu conditions. Treatment of **23** and **27** with DEAD and triphenylphosphine provided hexamine **28**. Upon treatment of cyanoethylamine **28** with *m*-CPBA, smooth oxidation and *retro*-Michael reaction gave hydroxy amine **29**. Subsequent removal of the Boc group with TFA afforded the primary amine **30**. A similar treatment to that used for the Ns deprotection of HO-416 provided the natural product **2** (Chart 7). $^1H/^{13}C$ NMR spectra and tandem FAB MS-MS spectroscopy data of synthetic **2** were identical with those of naturally occurring Agel-489.

In conclusion, utilizing the 2-nitrobenzenesulfonamide (Ns) group as both a protecting and activating group (Ns-strategy), the total synthesis of **1** was accomplished in 11 steps and 41% total yield, while the total synthesis of **2** was achieved in 12 steps and 31% yield. In both cases, efficient monoprotection of symmetrical diamines allowed very short syntheses. All C–N bond formations were accomplished in high yield by alkylation of 2-nitrobenzenesulfonamides. Finally, removal of the Ns group while the substrate was attached to a novel solid support enabled the efficient isolation of these highly polar compounds.

Experimental

General Comments IR spectra were recorded on a JASCO FT/IR-410 spectrophotometer. Nuclear magnetic resonance. ^1H- and ^{13}C-NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as the internal standard. Mass spectra (MS) and high resolution mass spectra (HRMS) were measured with a JEOL JMS-GCmate instrument.

***N*-(3-Aminopropan-1-yl)-2-nitrobenzenesulfonamide (3) (Large-Scale Preparation)** To a stirred solution of 55.6 g (75.0 mmol) of 1,3-diaminopropane in 1 l of ethanol was slowly added 55.4 g (25.0 mmol) of 2-nitrobenzenesulfonyl chloride at −20 °C under an argon atmosphere. After 30 min, the reaction mixture was quenched with 1 N sodium ethoxide, filtered with celite, concentrated *in vacuo* and excess 1,3-diaminopropane was removed under reduced pressure (40—50 °C/0.02 mmHg). Purification of the crude product by recrystallization with ethyl acetate in ether yielded sulfonamide **3** (38.0 g, 59%) as a yellow powder. IR ($CHCl_3$) cm^{-1}: 3368, 3310, 3099, 2939, 2877, 1540, 1440, 1368, 1334, 1162, 1127, 1092, 853, 782, 741; ^1H-NMR (DMSO) δ: 1.46 (2H, tt, J=6.7, 6.7 Hz), 2.52 (2H, t, J=6.7 Hz), 2.93 (2H, t, J=6.7 Hz), 3.94 (1H, bs), 7.81—7.87 (2H, m), 7.92—7.99 (2H, m); ^{13}C-NMR (DMSO) δ: 32.3, 41.0, 124.3, 129.4, 132.5, 132.9, 133.8, 147.8; MS: m/z=260 (MH^+); HRMS ($C_9H_{14}N_3O_3S$, MH^+): Calcd for 260.0705. Found: 260.0701.

***N*-(3-Aminopropan-1-yl)-2-nitrobenzenesulfonamide (3)** (Small-Scale Preparation) To a stirred solution of 2.64 g (30 mmol) of 1,3-diaminopropane in 50 ml of ethanol was slowly added 2.2 g (10 mmol) of 2-nitrobenzenesulfonyl chloride at 0 °C under an argon atmosphere. After 30 min, the reaction mixture was quenched with a solution of 1 N sodium ethoxide, filtered with celite, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel, eluted with a gradient of isopropylamine : methanol : dichloromethane (0 : 2 : 98 to 2.5 : 2.5 : 95), furnished sulfonamide **3** (2.15 g, 83%) as a yellow powder.

***N*-(4-Aminobutan-1-yl)-2-nitrobenzenesulfonamide (4)** Similar treatment of 2.64 g (30 mmol) of 1,4-diaminobutane and 2.2 g (10 mmol) of 2-nitrobenzenesulfonyl chloride to the reaction of 1,3-diaminopropane provided sulfonamide **4** (2.10 g, 77%) as a yellow powder. IR ($CHCl_3$): 3365, 3308, 3094, 2936, 2867, 1734, 1592, 1540, 1466, 1440, 1417, 1369, 1335, 1242, 1163, 1127, 1092, 853, 782, 742 cm^{-1}; ^1H-NMR (DMSO) δ: 1.31 (2H, tt, J=6.8, 6.8 Hz), 1.43 (2H, tt, J=6.8, 6.8 Hz), 2.47 (2H, t, J=6.8 Hz), 2.85 (2H, t, J=6.8 Hz), 4.25 (1H, bs), 7.80—7.87 (2H, m), 7.92—7.98 (2H, m); ^{13}C-NMR (DMSO) δ: 27.0, 29.8, 40.8, 42.8, 124.2, 129.4, 132.4, 133.2, 133.7, 147.8; MS: m/z=274 (47, MH^+); HRMS ($C_{10}H_{16}N_3O_4S$, MH^+): Calcd for 274.0862. Found: 274.0868.

***N*-(5-Aminopentan-1-yl)-2-nitrobenzenesulfonamide (5)** Similar treatment of 3.06 g (30 mmol) of 1,5-diaminopentane and 2.2 g (10 mmol) of 2-nitrobenzenesulfonyl chloride to the reaction of 1,3-diaminopropane provided sulfonamide **5** (2.50 g, 87%) as a yellow powder. IR ($CHCl_3$): 3365, 3308, 3094, 2936, 2867, 1734, 1592, 1540, 1466, 1440, 1417, 1369, 1335, 1242, 1163, 1127, 1092, 853, 782, 742 cm^{-1}; ^1H-NMR (DMSO) δ: 1.31 (2H, tt, J=6.8, 6.8 Hz), 1.43 (2H, tt, J=6.8, 6.8 Hz), 2.47 (2H, t, J=6.8 Hz), 2.85 (2H, t, J=6.8 Hz), 4.25 (1H, bs), 7.80—7.87 (2H, m), 7.92—7.98 (2H, m); ^{13}C-NMR (DMSO) δ: 27.0, 29.8, 40.8, 42.8, 124.2, 129.4, 132.4, 133.2, 133.7, 147.8; MS: m/z=274 (47, MH^+); HRMS ($C_{10}H_{16}N_3O_4S$, MH^+): Calcd for 274.0862. Found: 274.0868.

***N*-(3-*tert*-Butoxycarbonylaminopropan-1-yl)-2-nitrobenzenesulfonamide (6)** To a stirred solution of 2.23 g (8.61 mmol) of diamine **3** in 30 ml of dichloromethane were added 1.43 ml (10.3 mmol) of triethylamine and 2.37 ml (10.3 mmol) of di-*tert*-butyl dicarbonate at room temperature under an argon atmosphere. After 1 h, the reaction mixture was poured into a solution of 1 N hydrochloric acid, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (60% ethyl acetate in hexane) yielded diamine **6** (2.43 g, 99%) as a yellow solid. IR ($CHCl_3$): 3344, 2977, 1690, 1593, 1542, 1442, 1414, 1366, 1342, 1301, 1274, 1254, 1166, 1127, 1088, 854, 783, 741; ^1H-NMR ($CDCl_3$) δ: 1.42 (9H, s), 1.69 (2H, tt, $J=6.3$, 6.3 Hz), 3.16 (2H, dt, $J=6.3$, 6.3 Hz), 3.21 (2H, dt, $J=6.3$, 6.3 Hz), 4.67 (1H, bs), 5.87 (1H, bs), 7.72—7.74 (2H, m), 7.84—7.86 (1H, m), 8.12—8.14 (1H, m); ^{13}C-NMR ($CDCl_3$) δ: 28.3, 30.6, 37.1, 40.8, 79.6, 125.2, 130.9, 132.7, 133.4, 148.0, 156.4; MS: 360 (MH^+), 359 (M^+); HRMS: Calcd for ($C_{10}H_{12}N_3O_5S$, $M^+-C_4H_9O$): 287.0576. Found: 287.0576.

7-*tert*-Butoxycarbonylamino-4-(2-nitrobenzenesulfonyl)-4-azaheptan-1-yl Bromide (7) To a stirred solution of 3.57 g (25.8 mmol) of potassium carbonate in 4.3 ml (43 mmol) of 1,3-dibromopropane was added slowly the crude diamine **6** in 10 ml of DMF at 60 °C under an argon atmosphere. After 1 h, the reaction mixture was poured into water, and the aqueous layer was extracted thoroughly with ether (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (20% ethyl acetate in hexane) yielded bromide **7** (3.27 g, 97%) as a yellow oil. IR ($CHCl_3$): 3423, 2976, 1707, 1545, 1458, 1367, 1348, 1251, 1163, 1125, 1060, 1040, 852, 779, 748 cm^{-1}; ^1H-NMR ($CDCl_3$) δ: 1.43 (9H, s), 1.77 (2H, tt, $J=6.7$, 6.7 Hz), 2.11 (2H, tt, $J=6.7$, 6.7 Hz), 3.17 (2H, m), 3.37 (2H, t, $J=6.7$ Hz), 3.38 (2H, t, $J=6.7$ Hz), 3.44 (2H, t, $J=6.7$ Hz), 4.81 (1H, bs), 7.64—7.66 (1H, m), 7.69—7.74 (2H, m), 8.03—8.05 (1H, m); ^{13}C-NMR ($CDCl_3$) δ: 28.5, 28.6, 30.1, 31.4, 37.5, 45.8, 46.3, 80.0, 124.5, 131.2, 131.9, 133.9, 148.2, 156.2; MS: $m/z=$ 408 ($MH^+-C_4H_9O$); HR MS ($C_{13}H_{17}BrN_3O_5S$, $M^+-C_4H_9O$): Calcd for 407.0151. Found: 407.0150.

3-(2-Nitrobenzenesulfonylamino)propan-1-ol (9) To a stirred solution of 1.54 g (20.5 mmol) of 3-aminopropanol in 50 ml of dichloromethane were added 3.48 g (15.7 mmol) of 2-nitrobenzenesulfonyl chloride and 1.48 ml (17.3 mmol) of pyridine at 0 °C under an argon atmosphere. After 10 min, the reaction mixture was poured into a solution of 1 N hydrochloric acid, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give amino alcohol **9** (4.04 g, 99%) as a white solid. IR ($CHCl_3$): 3546, 3336, 3100, 2948, 2886, 1593, 1541, 1441, 1413, 1364, 1338, 1164, 1126, 1068, 1005, 960, 854, 784, 741, 731 cm^{-1}; ^1H-NMR ($CDCl_3$) δ: 1.79 (2H, tt, $J=6.1$, 6.1 Hz), 3.26 (2H, dt, $J=6.1$, 6.1 Hz), 3.77 (2H, t, $J=6.1$ Hz), 5.83 (1H, bs), 7.72—7.78 (2H, m), 7.84—7.90 (1H, m), 8.12—8.17 (1H, m); ^{13}C-NMR ($CDCl_3$) δ: 31.5, 41.5, 60.3, 125.4, 131.1, 132.8, 133.5, 148.0. MS: $m/z=264$ (MH^+), 260 (M^+); HRMS ($C_9H_{12}N_2O_5S$, MH^+): Calcd for 260.0467. Found: 260.0475.

11-*tert*-Butoxycarbonylamino-4,8-bis(2-nitrobenzenesulfonyl)-4,8-diazaundecan-1-ol (10) To a stirred solution of 310 mg (1.19 mmol) of amino alcohol **9**, 800 mg (1.67 mmol) of bromide **7**, and 1.16 g (3.58 mmol) of cesium carbonate in 4 ml of acetonitrile was added a catalytic amount of *n*-tetrabutylammonium iodide (88 mg, 0.24 mmol) at 60 °C under an argon atmosphere. After 90 min, the reaction mixture was poured into brine, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (80% ethyl acetate in hexanes) yielded alcohol **10** (675 mg, 86%) as a yellow oil. IR ($CHCl_3$): 3423, 3094, 2937, 1695, 1544, 1459, 1440, 1370, 1346, 1252, 1161, 1126, 1059, 979, 914, 852, 779, 734 cm^{-1}; ^1H-NMR ($CDCl_3$) δ: 1.44 (9H, s), 1.74 (2H, tt, $J=7.4$, 7.4 Hz), 1.78 (2H, tt, $J=7.0$, 7.0 Hz), 1.89 (2H, tt, $J=7.6$, 7.6 Hz), 3.12 (2H, m), 3.29 (2H, t, $J=7.6$ Hz), 3.30 (2H, t, $J=7.6$ Hz), 3.32 (2H, t, $J=7.4$ Hz), 3.42 (2H, t, $J=7.0$ Hz), 3.66 (2H, bs), 4.85 (1H, bs), 7.61—7.65 (2H, m), 7.69—7.74 (4H, m), 7.98—8.00 (2H, m); ^{13}C-NMR ($CDCl_3$) δ: 27.5, 28.4, 28.8, 31.1, 37.6, 45.0, 45.5, 45.6, 45.7, 59.1, 79.4, 124.2, 130.7, 131.8, 132.8, 133.7, 148.1, 156.1; MS: $m/z=660$ (MH^+); *Anal.* ($C_{26}H_{37}N_5O_{11}S_2$): Calcd for C, 47.33; H, 5.65; N, 10.62. Found: C, 47.54; H, 5.58; N, 10.33.

11-*tert*-Butoxycarbonylamino-4,8-bis(2-nitrobenzenesulfonyl)-4,8-diazaundecan-1-yl Iodide (11) To a stirred solution of 1.98 g (3.00 mmol) of alcohol **10** in 10 ml of dichloromethane were added 0.50 ml (3.61 mmol) of triethylamine, and 0.28 ml (3.61 mmol) of methanesulfonyl chloride at 0 °C under an argon atmosphere. After 10 min at room temperature, the reaction mixture was poured into a solution of 1 N hydrochloric acid, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give crude mesylate which was used in the subsequent step without purification.

To a stirred solution of 2.20 g (2.98 mmol) of mesylate in 20 ml of 2-butanone was added 1.34 g (8.94 mmol) of sodium iodide at 60 °C under an argon atmosphere. After 1 h, the reaction mixture was poured into water, and the aqueous layer was extracted thoroughly with ether (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give the iodide **11** (2.28 g, 99%) as a yellow oil. IR ($CHCl_3$): 3423, 3093, 2931, 1706, 1544, 1458, 1439, 1369, 1347, 1251, 1162, 1125, 1059, 914, 852, 778, 734 cm^{-1}; ^1H-NMR ($CDCl_3$) δ: 1.44 (9H, s), 1.74 (2H, tt, $J=6.9$, 6.9 Hz), 1.87 (2H, tt, $J=7.4$, 7.4 Hz), 2.04 (2H, tt, $J=6.8$, 6.8 Hz), 3.11 (2H, t, $J=6.8$ Hz), 3.14 (2H, m), 3.29 (2H, t, $J=7.4$ Hz), 3.31 (2H, t, $J=7.4$ Hz), 3.34 (2H, t, $J=6.9$ Hz), 3.36 (2H, t, $J=6.8$ Hz), 4.80 (1H, bs), 7.62—7.66 (2H, m), 7.70—7.75 (4H, m), 7.99—8.03 (1H, m), 8.04—8.06, (1H, m); ^{13}C-NMR ($CDCl_3$) δ: 1.65, 27.4, 28.4, 28.6, 31.8, 37.4, 45.2, 45.4, 45.6, 48.2, 124.3, 124.3, 130.1, 131.1, 131.9, 132.0, 132.8, 133.7, 133.9, 148.0; MS: $m/z=770$ (MH^+), 769 (M^+); HRMS ($C_{26}H_{37}IN_5O_{10}S_2$, MH^+): Calcd for 770.1071. Found: 770.1046.

***N*-[4-(2-Nitrobenzenesulfonyl)aminobutan-1-yl]-1*H*-indole-3-acetamide (13)** To a solution of 1.08 g (6.16 mmol) of indole-3-acetic acid in 15 ml of dichloromethane were added 0.94 ml (6.78 mmol) of triethylamine and 0.83 ml (6.78 mmol) of pivaloyl chloride at 0 °C under an argon atmosphere. After 3 min at room temperature, the reaction mixture was cooled at 0 °C. To the above stirred solution was added 2.07 g (8.01 mmol) of diamine **4**, 0.94 ml (6.78 mmol) of triethylamine and 76 mg (0.62 mmol) of 4-dimethylaminopyridine (DMAP). After 10 min at room temperature, the reaction mixture was poured into a solution of 1 N hydrochloric acid, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel, eluted with a gradient of ether in hexane (50 to 70%), furnished sulfonamide **13** (2.43 g, 97%) as a yellow solid. IR ($CHCl_3$): 3402, 2931, 1647, 1540, 1457, 1339, 1164, 1125, 853, 782, 741 cm^{-1}; ^1H-NMR ($CDCl_3$) δ: 1.40 (4H, m), 3.00 (2H, bs), 3.14 (2H, m), 3.72 (2H, s), 5.30 (1H, bs), 5.73 (1H, bs), 7.14 (1H, dd, $J=7.4$, 1.0 Hz), 7.16 (1H, d, $J=7.4$ Hz), 7.24 (1H, dd, $J=7.4$, 1.0 Hz), 7.42 (1H, d, $J=8.0$ Hz), 7.53 (1H, d, $J=7.4$ Hz), 7.69—7.74 (2H, m), 7.80—7.84 (1H, m), 8.06—8.10 (1H, m), 8.38 (1H, bs); ^{13}C-NMR ($CDCl_3$) δ: 26.1, 26.4, 33.1, 38.3, 42.9, 108.7, 111.2, 118.3, 119.8, 122.4, 123.5, 125.0, 126.6, 130.7, 132.5, 133.2, 133.2, 136.1, 171.4; MS: $m/z=431$ (MH^+), 430 (M^+); HRMS ($C_{20}H_{22}N_4O_5S$, M^+): Calcd for 430.1311. Found: 430.1333.

***N*-[16-*tert*-Butoxycarbonylamino-5,9,13-tris(2-nitrobenzenesulfonyl)-5,9,13-triazahexadecan-1-yl]-1*H*-indole-3-acetamide (14)** To a stirred solution of 1.20 g (1.55 mmol) of iodide **11**, and 977 mg (3.00 mmol) of cesium carbonate in 6 ml of acetonitrile was added 430 mg (1.00 mmol) of sulfonamide **13** at 60 °C under an argon atmosphere. After 1 h, the reaction mixture was poured into brine, and the aqueous layer was extracted thoroughly with ethyl acetate (3×). The combined organic extracts were dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (80% ethyl acetate in hexanes) yielded polyamine **14** (1.01 g, 94%) as a yellow powder. IR ($CHCl_3$): 3412, 2936, 1702, 1655, 1544, 1458, 1439, 1370, 1344, 1162, 1125, 1060, 942, 852, 779, 742 cm^{-1}; ^1H-NMR ($CDCl_3$) δ: 1.35 (2H, bs), 1.44 (9H, s), 1.61 (2H, bs), 1.72 (4H, m), 1.82 (2H, m), 3.09—3.28 (14H, m), 3.31 (2H, t, $J=7.20$ Hz), 3.72 (2H, s), 4.88 (1H, bs), 5.85 (1H, m), 7.11 (2H, dt, $J=7.6$, 1.0 Hz), 7.19 (2H, dt, $J=7.6$, 1.0 Hz), 7.20 (1H, bs), 7.41 (1H, d, $J=8.1$ Hz), 7.54 (1H, d, $J=7.6$ Hz), 7.57—7.63 (3H, m), 7.66—7.72 (6H, m), 7.88—7.99 (3H, m), 8.62 (1H, bs); ^{13}C-NMR ($CDCl_3$) δ: 25.1, 26.1, 27.3, 27.3, 28.1, 28.4, 33.1, 37.1, 37.2, 38.3, 47.4, 108.6, 111.3, 118.4, 119.5, 122.1, 123.8, 123.9, 123.9, 124.0, 126.8, 130.2, 130.6, 131.6, 131.7, 131.8, 132.2, 132.5, 133.4, 133.5, 133.6, 136.1, 147.6, 147.7, 155.8, 171.5; HRMS ($C_{46}H_{57}N_9O_{15}S_3Na$, MH^++Na^+): Calcd for 1094.3034. Found: 1094.3057.

***N*-[16-Amino-5,9,13-tris(2-nitrobenzenesulfonyl)-5,9,13-triazahexadecan-1-yl]-1*H*-indole-3-acetamide (15)** To a stirred solution of 1.61 g (1.50 mmol) of polyamine **14** in 3 ml of dichloromethane and 6.0 ml of methanol was added excess thionyl chloride (1.0 ml, 13.7 mmol) at 0 °C under an argon atmosphere. After 1 h, the reaction mixture was evaporated.

Purification of the crude product by column chromatography on silica gel (10% methanol in chloroform) yielded polyamine **15** (1.40 g, 96%) as a yellow powder. IR ($CHCl_3$): 2961, 1638, 1543, 1458, 1439, 1373, 1344, 1262, 1216, 1160, 1124, 1059, 941, 852, 750 cm^{-1}; ^{1}H-NMR (DMSO) δ: 1.28 (2H, m), 1.37 (2H, m), 1.59 (2H, m), 1.68 (2H, m), 1.78 (2H, m), 2.73 (2H, m), 2.98 (2H, m), 3.09—3.23 (6H, m), 3.47 (2H, s), 6.94 (1H, dt, J=7.7, 1.0 Hz), 7.03 (1H, dt, J=7.7, 1.0 Hz), 7.16 (1H, bs), 7.31 (1H, d, J=8.1 Hz), 7.51 (1H, d, J=7.7 Hz), 7.80—7.98 (12H, m), 8.31 (1H, s); ^{13}C-NMR (DMSO) δ: 26.1, 26.2, 26.6, 32.7, 36.4, 38.0, 44.8, 44.8, 47.1, 108.9, 111.3, 118.2, 118.6, 120.9, 123.7, 124.3, 124.4, 124.5, 127.2, 129.6, 129.6, 131.4, 131.6, 132.4, 132.5, 132.6, 134.5, 134.7, 136.1, 147.4, 170.6; HRMS ($C_{41}H_{50}N_9O_{13}S_3$, M^+): Calcd for 972.2690. Found: 972.2682.

4-(Chlorodiphenylmethyl)phenoxymethylated Polystyrene Polymer (16) Merrifield resin 2.14 g (1% cross-linked polystyrene beads with 1.20 mmol of benzyl groups per gram of resin), excess *p*-hydroxytrityl alcohol (6.66 g, 24.1 mmol) and excess potassium carbonate (16.6 g, 120.5 mmol) were suspended in 30 ml of DMF under an argon atmosphere. After 24 h at 60 °C, the resin was washed five times with THF–H_2O (1 : 1), five times with THF, and five times with dichloromethane, and dried under vacuum for 24 h to give 2.72 g of trityl alcohol resin. To a suspension of 305 mg (0.27 mmol) of trityl alcohol resin in 2.3 ml of dichloromethane was added excess thionyl chloride (0.25 ml, 3.4 mmol) at room temperature under an argon atmosphere. After 1 h, the resin was washed five times with dichloromethane and dried under vacuum for 8 h to give trityl chloride resin **16**.

HO 416b (1) To a suspension of the freshly prepared resin **16** and 65.0 mg (0.068 mmol) of amine **15** in 2.5 ml of CH_2Cl_2 was added 0.141 ml (0.828 mmol) of iso-Pr_2NEt at room temperature. After shaking for 48 h, 0.1 ml of MeOH was added to the reaction mixture. The resin was filtered, washed with MeOH : CH_2Cl_2 (1 : 9), H_2O : MeOH : CH_2Cl_2 (1 : 1 : 8), and CH_2Cl_2, and then dried *in vacuo* for 8 h to give the resin. To a suspension of the above resin in 1.5 ml of DMF was added 0.140 ml (2.00 mmol) of 2-mercaptoethanol and 0.30 ml (2.00 mmol) of DBU at room temperature under an argon atmosphere. After shaking for 26 h, the resin was filtered, washed with H_2O : THF (1 : 9), MeOH : CH_2Cl_2 (1 : 9), and CH_2Cl_2 and dried *in vacuo* for 8 h to give the resin. To a mixture of the resulting resin in 2.5 ml of CH_2Cl_2 was added 25 μl (0.324 mmol) of TFA at room temperature. After shaking for 5 min, the resin was filtered and washed with MeOH : CH_2Cl_2 (1 : 9). The combined washings were evaporated and dried *in vacuo* to provide **1** (25.5 mg, 68%) as the TFA salt. ^{1}H-NMR (D_2O) δ: 1.36—1.40 (4H, m), 1.87—1.98 (6H, m), 2.80—3.05 (16H, m), 3.59 (2H, s), 7.02 (1H, dt, J=7.6, 1.0 Hz), 7.12 (1H, dt, J=7.6, 1.0 Hz,), 7.18 (1H, s), 7.38 (1H, d, J=8.0 Hz), 7.46 (1H, d, J=7.6 Hz); ^{13}C-NMR (D_2O) δ: 23.4, 24.5, 26.3, 33.3, 37.2, 39.2, 44.8, 45.3, 45.5, 47.9, 57.2, 108.4, 112.8, 119.1, 120.3, 122.9, 125.9, 127.4, 137.1, 176.2; MS: 417 (MH^+), 416 (M^+); HRMS ($C_{23}H_{41}N_6O$, MH^+): Calcd for 417.3342. Found: 417.3355; MS/MS (FAB); 422 ($M^+-H^++Na^+-NH_2$), 408 ($M^+-H^++Na^+-CH_4N$), 394 ($M^+-H^++Na^+-C_2H_6N$), 380 ($M^+-H^++Na^+-C_3H_8N$), 365 ($M^+-H^++Na^+-C_3H_9N_2$), 351 ($M^+-H^++Na^+-C_4H_{11}N_2$), 337 ($M^+-H+Na-C_5H_{13}N_2$), 323 ($M^+-H+Na-C_6H_{15}N_2$), 308 ($M^+-H+Na-C_6H_{16}N_3$), 294 ($M^+-H^++Na^+-C_7H_{18}N_3$), 280 ($M^+-H^++Na^+-C_8H_{20}N_3$), 266 ($M^+-H^++Na^+-C_9H_{22}N_3$), 252 ($M^+-H^++Na^+-C_9H_{22}N_4$), 238 ($M^+-H^++Na^+-C_{10}H_{24}N_4$), 223 ($M^+-H^++Na^+-C_{11}H_{27}N_4$), 210 ($M^+-H^++Na^+-C_{12}H_{28}N_4H_5$), 130 ($C_9H_8N^+$).

***N*-[3-(2-Nitrobenzenesulfonyl)amino-propan-1-yl]-1*H*-indole-3-acetamide (17)** To a stirred solution of 1.08 g (6.16 mmol) of 3-indoleacetic acid in 15 ml of dichloromethane were added 0.94 ml (6.78 mmol) of triethylamine and 0.83 ml (6.78 mmol) of pivaloyl chloride at 0 °C under an argon atmosphere. After 3 min at room temperature, the reaction mixture was cooled at 0 °C. To the above stirred solution was added 0.94 ml (6.78 mmol) of triethylamine, 2.07 g (8.01 mmol) of diamine **3** and 76 mg (0.62 mmol) of 4-dimethylaminopyridine. After 10 min at room temperature, the reaction mixture was poured into a solution of 1 N hydrochloric acid, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel, eluted with a gradient of ether in hexane (50 to 70%), furnished sulfonamide **17** (2.43 g, 95%) as a yellow solid. IR ($CHCl_3$): 3401, 1645, 1539, 1457, 1339, 1164, 1125, 1094, 911, 853, 740 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.58 (2H, tt, J=6.3, 6.3 Hz), 2.98 (2H, dt, J=6.3, 6.3 Hz), 3.27 (2H, dt, J=6.3, 6.3 Hz), 3.74 (2H, s), 5.87 (1H, m), 6.00 (1H, m), 7.13 (1H, dt, J=7.7, 1.0 Hz), 7.19 (1H, d, J=2.4 Hz), 7.25 (1H, dt, J=7.7, 1.0 Hz), 7.43 (1H, d, J=8.3 Hz), 7.52 (1H, d, J=7.7 Hz), 7.64—7.72 (2H, m), 7.78—7.82 (2H, m), 8.35 (1H, bs); ^{13}C-NMR ($CDCl_3$) δ: 30.0, 33.3, 36.0, 40.7, 108.8, 111.5, 118.6, 120.1, 122.6, 124.1, 125.2, 127.1, 130.7, 132.7, 133.4, 133.9, 136.5, 148.0, 172.4; MS: m/z=417 (MH^+), 416 (M^+); HRMS ($C_{19}H_{21}N_4O_5S$, MH^+): Calcd for 417.1233. Found: 417.1233.

3-*tert*-Butyldimethylsilyloxypropan-1-yl-bromide (18) To a stirred solution of 2.95 g (21.2 mmol) of 3-bromopropan-1-ol in 3 ml of dichloromethane were added 4.16 g (27.6 mmol) of *t*-butyldimethylsilyl chloride and 4.82 ml (27.6 mmol) of *N*,*N*-diisopropylethylamine at 0 °C under an argon atmosphere. After 20 min at room temperature, the reaction mixture was poured into water, and the aqueous layer was extracted thoroughly with hexane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (hexane) yielded silyl ether **18** (4.40 g, 83%) as a colorless oil. IR ($CHCl_3$): 2955, 2929, 2858, 1472, 1387, 1361, 1257, 1211, 1147, 1103, 1062, 1006, 952, 836, 777 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 0.01 (6H, s), 0.83 (9H, s), 1.97 (2H, tt, J=6.1, 6.1 Hz), 3.50 (2H, t, J=6.1 Hz), 3.67 (2H, t, J=6.1 Hz); ^{13}C-NMR ($CDCl_3$) δ: −5.40, 18.3, 25.9, 30.7, 35.5, 60.4; MS: m/z=254 (MH^+); HRMS ($C_9H_{22}BrOSi$, M^+): Calcd for 253.0623. Found: 253.0620.

***N*-(7-*tert*-Butyldimethylsilyloxy-4-(2-nitrobenzenesulfonyl)-4-azaheptan-1-yl)-1*H*-indole-3-acetamide (19)** To a stirred solution of 3.10 g (7.45 mmol) of sulfonamide **17**, 7.28 g (22.3 mmol) of cesium carbonate and 1.37 g of (3.73 mmol) *n*-tetrabutylammonium iodide in 10 ml of acetonitrile was added slowly 1.89 ml (8.20 mmol) of bromide **18** at 60 °C under an argon atmosphere. After 90 min, the reaction mixture was poured into water, and the aqueous layer was extracted thoroughly with ethyl acetate (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (70% ethyl acetate in hexane) yielded indole **19** (3.47 g, 80%) as a yellow oil. IR ($CHCl_3$): 3403, 2929, 2856, 1653, 1545, 1458, 1437, 1373, 1342, 1256, 1163, 1099, 1009, 957, 836, 777, 744 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 0.01 (6H, s), 0.85 (9H, s), 3.24—3.31 (4H, m), 3.12 (2H, t, J=6.5 Hz), 3.24 (2H, t, J=5.6 Hz), 3.29 (2H, t, J=6.5 Hz), 3.51 (2H, t, J=5.6 Hz), 3.75 (2H, s), 6.24 (1H, bs), 7.13 (1H, t, J=7.7 Hz), 7.20 (1H, d, J=7.7 Hz), 7.23 (1H, bs), 7.37 (1H, d, J=7.6 Hz), 7.58 (1H, d, J=7.7 Hz), 7.59—7.68 (4H, m), 8.29 (1H, bs); ^{13}C-NMR ($CDCl_3$) δ: −5.45, 18.2, 25.8, 27.6, 31.4, 33.4, 35.6, 44.8, 45.1, 60.0, 109.0, 111.4, 118.6, 119.8, 122.3, 124.0, 124.2, 127.2, 130.4, 131.6, 133.3, 136.9, 171.9; MS: m/z=588 (M^+); HRMS ($C_{28}H_{40}N_4O_6SSi$, M^+): Calcd for 588.2438. Found: 588.2423.

***N*-[7-*tert*-Butyldimethylsilyloxy-4-(2-nitrobenzenesulfonyl)-4-azaheptan-1-yl]-1-*tert*-butoxycarbonyl-indole-3-acetamide (20)** To a stirred solution of 3.14 g (5.33 mmol) of indole **19** in 25 ml of dichloromethane were added 0.8 ml (5.86 mmol) of triethylamine, 1.3 ml (5.86 mmol) of di-*tert*-butyl dicarbonate, and 65 mg (0.53 mmol) of 4-dimethylaminopyridine (DMAP) at room temperature under an argon atmosphere. After 1 h, the reaction mixture was poured into a solution of 1 N hydrochloric acid, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give indole **20** (3.60 g, 98%) as a yellow oil. IR ($CHCl_3$): 3298, 2930, 1734, 1654, 1546, 1453, 1370, 1308, 1257, 1227, 1159, 1087, 1016, 957, 836, 774, 747 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 0.01 (6H, s), 0.85 (9H, s), 1.64 (9H, s), 1.68—1.71 (4H, m), 3.16 (2H, t, J=6.3 Hz), 3.28 (2H, t, J=5.9 Hz), 3.30 (2H, t, J=6.3 Hz), 3.52 (2H, t, J=5.9 Hz), 3.68 (2H, s), 6.20 (1H, bs), 7.25 (1H, d, J=7.7 Hz), 7.27 (1H, bs), 7.34 (1H, t, J=7.7 Hz), 7.54 (1H, d, J=7.7 Hz), 7.57—7.70 (4H, m), 8.17 (1H, bs); ^{13}C-NMR ($CDCl_3$) δ: −5.36, 3.90, 18.3, 25.9, 27.7, 28.2, 31.4, 33.5, 36.0, 44.9, 45.2, 60.1, 83.8, 113.9, 115.4, 119.0, 122.9, 124.1, 124.8, 125.2, 130.5, 131.7, 133.2, 133.5, 170.6; MS: m/z=588 (MH^+−Boc); HRMS ($C_{33}H_{49}N_4O_8SSi$, MH^+): Calcd 689.3040. Found: 689.3070.

***N*-(7-*tert*-Butyldimethylsilyloxy-4-azaheptan-1-yl)-1-*tert*-butoxycarbonyl-indole-3-acetamide (21)** To a stirred solution of 1.11 g (1.61 mmol) of indole **21** and 2.62 g (8.06 mmol) of cesium carbonate in 5 ml of acetonitrile was added 0.33 ml (3.22 mmol) of thiophenol at room temperature under an argon atmosphere. After 10 h, the reaction mixture was poured into brine, and the aqueous layer was extracted thoroughly with ethyl acetate (3×). The combined organic extracts were dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (10% methanol in chloroform) yielded secondary amine **21** (787 mg, 97%) as a yellow oil. IR ($CHCl_3$): 2958, 2928, 2853, 1734, 1654, 1560, 1455, 1369, 1256, 1159, 1086, 835, 776, 747 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 0.01 (6H, s), 0.84 (9H, s), 1.61 (4H, m), 1.63 (9H, s), 2.59—2.63 (4H, m), 3.28 (2H, m), 3.59 (2H, t, J=7.3 Hz), 3.62 (2H, s), 6.67 (1H, bs), 7.21 (1H, t, J=7.5 Hz), 7.30 (1H, t, J=7.5 Hz), 7.47 (1H, d, J=7.5 Hz), 7.51 (1H, s), 8.10 (1H, m); ^{13}C-NMR ($CDCl_3$) δ: −5.4, 0.0, 18.3, 25.9, 28.0, 28.2, 29.7, 31.1, 33.2, 37.9, 47.0, 61.5, 83.9, 113.8, 115.4, 119.0, 122.9, 124.9, 129.8, 149.5; MS: m/z=504 (MH$^+$), 503 (M$^+$); HRMS ($C_{27}H_{46}N_3O_4SSi$, MH$^+$): Calcd for 504.3258. Found: 504.3254.

***N*-(7-*tert*-Butyldimethylsilyloxy-4-(2-cyanoethyl)-4-azaheptan-1-yl)-1-*tert*-butoxycarbonyl-indole-3-acetamide (22)** To a stirred solution of 2.27 g (4.50 mmol) of secondary amine **21** in 9 ml of ethanol was added excess acrylonitrile (9.0 ml, 37.4 mmol) at 60 °C under an argon atmosphere. After 24 h, the reaction mixture was evaporated. Purification of the crude product by column chromatography on silica gel, eluted with a gradient of ethyl acetate in hexane (30 to 70%), furnished indole **22** (2.22 g, 93%) as a yellow oil. IR ($CHCl_3$): 3297, 2930, 2857, 1732, 1649, 1547, 1453, 1369, 1308, 1256, 1227, 1158, 1086, 1016, 836, 774, 747 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 0.01 (6H, s), 0.85 (9H, s), 1.43 (2H, tt, J=6.6, 6.6 Hz), 1.55 (2H, tt, J=6.3, 6.3 Hz), 1.67 (9H, s), 2.14 (2H, t, J=6.3 Hz), 2.30 (2H, t, J=6.5 Hz), 2.35 (2H, t, J=6.6 Hz), 2.49 (2H, t, J=6.5 Hz), 3.27 (2H, dt, J=6.3, 6.3 Hz), 3.51 (2H, t, J=6.6 Hz), 3.64 (2H, s), 6.13 (1H, bs), 7.25 (1H, t, J=7.0 Hz), 7.34 (1H, t, J=7.0 Hz), 7.51 (1H, d, J=7.0 Hz), 7.54 (1H, s), 8.15 (1H, m); ^{13}C-NMR ($CDCl_3$) δ: −5.85, 15.4, 17.7, 25.4, 26.1, 27.7, 29.1, 37.7, 49.0, 50.9, 60.1, 83.5, 113.5, 114.9, 118.6, 122.4, 124.4, 124.5, 129.4, 149.0, 169.8; MS: m/z=556 (M$^+$); HRMS ($C_{30}H_{49}N_4O_4Si$, MH$^+$): Calcd for 557.3523. Found: 557.3505.

***N*-[4-(2-Cyanoethyl)-7-hydroxy-4-azaheptan-1-yl]-1-*tert*-butoxycarbonyl-indole-3-acetamide (23)** To a stirred solution of 1.90 g (3.41 mmol) of indole **22** in 20 ml of acetonitrile was added 973 mg (17.1 mmol) of ammonium fluoride at 60 °C under an argon atmosphere. After 10 h, the reaction mixture was poured into a solution of saturated sodium bicarbonate, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel, eluted with a gradient of methanol in chloroform (10 to 30%), furnished alcohol **23** (1.47 g, 98%) as a yellow oil. IR ($CHCl_3$): 3298, 2934, 1731, 1654, 1540, 1453, 1369, 1308, 1257, 1227, 1158, 1086, 1017, 942, 856, 768, 748 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.44 (2H, tt, J=6.2, 6.2 Hz), 1.60 (2H, tt, J=6.8, 6.8 Hz), 1.68 (9H, s), 2.29 (2H, t, J=6.8 Hz), 2.32 (2H, t, J=7.1 Hz), 2.46 (2H, t, J=6.2 Hz), 2.61 (2H, t, J=7.1 Hz), 3.26 (2H, dt, J=6.8, 6.8 Hz), 3.62 (2H, t, J=6.2 Hz), 3.66 (2H, s), 6.19 (1H, bs), 7.27 (1H, t, J=7.6 Hz), 7.36 (1H, t, J=7.6 Hz), 7.53 (1H, d, J=7.6 Hz), 7.57 (1H, s), 8.16 (1H, bs); ^{13}C-NMR ($CDCl_3$) δ: 15.7, 26.6, 28.3, 28.5, 33.4, 37.7, 49.3, 50.8, 52.3, 62.3, 84.1, 114.0, 115.5, 118.7, 119.1, 123.0, 125.0, 125.1, 130.0, 135.7, 149.6, 170.5; MS: m/z=442 (M$^+$); HRMS ($C_{24}H_{35}N_4O_4$, MH$^+$): Calcd for 443.2658. Found: 443.2669.

8-*tert*-Butoxycarbonylamino-5-(2-nitrobenzenesulfonyl)-5-azaoctan-1-yl Bromide (24) To a stirred solution of 8.50 g (61.8 mmol) of potassium carbonate in 3.7 ml (30.9 mmol) of 1,4-dibromobutane was added the 1.60 g (6.18 mmol) of diamine **6** in 10 ml of DMF at 60 °C under an argon atmosphere. After 30 min, the reaction mixture was poured into water, and the aqueous layer was extracted thoroughly with ether (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (30% ethyl acetate in hexane) yielded bromide **24** (2.47 g, 81%) as a yellow oil. IR ($CHCl_3$): 2975, 1700, 1545, 1457, 1367, 1252, 1162, 852, 778 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.44 (9H, s), 1.70 (2H, tt, J=7.4, 7.4 Hz), 1.76 (2H, tt, J=6.9, 6.9 Hz), 1.83 (2H, tt, J=6.9, 6.9 Hz), 3.15 (2H, m), 3.33 (2H, t, J=7.4 Hz), 3.35 (2H, t, J=6.9 Hz), 3.38 (2H, t, J=6.9 Hz), 4.78 (1H, bs), 7.62—7.66 (1H, m), 7.67—7.74 (2H, m), 8.00—8.02 (1H, m); ^{13}C-NMR ($CDCl_3$) δ: 26.6, 26.6, 28.4, 29.4, 32.9, 37.4, 45.0, 46.6, 124.3, 130.7, 131.7, 133.3, 133.6, 148.1; MS: m/z=438 (MH$^+$−*tert*-Bu), 394 (MH$^+$−Boc); *Anal.* ($C_{18}H_{28}BrN_3O_6S$): Calcd for C, 43.73; H, 5.71; N, 8.50. Found: C, 44.03; H, 5.52; N, 8.35.

***N*-(3-Allyloxycarbonylaminopropan-1-yl)-2-nitrobenzenesulfonamide (25)** To a stirred solution of 1.99 g (7.68 mmol) of diamine **3** in 30 ml of dichloromethane were added 1.28 ml (9.22 mmol) of triethylamine and 0.97 ml (9.22 mmol) of allyl chloroformate at room temperature under an argon atmosphere. After 30 min, the reaction mixture was poured into a solution of 1 N hydrochloric acid, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (40% ethyl acetate in hexane) yielded diamine **25** (2.33 g, 89%) as a yellow powder. IR ($CHCl_3$): 3340, 3096, 2946, 2883, 1699, 1647, 1593, 1539, 1442, 1418, 1363, 1340, 1302, 1259, 1165, 1126, 1089, 1037, 996, 933, 854, 782, 741, 730 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.73 (2H, tt, J=6.3, 6.3 Hz), 3.17 (2H, dt, J=6.3, 6.3 Hz), 3.30 (2H, dt, J=6.3, 6.3 Hz), 4.55 (2H, d, J=6.0 Hz), 4.96 (1H, bs), 5.21 (1H, d, J=10.8 Hz), 5.30 (1H, d, J=15.6 Hz), 5.78 (1H, bs), 5.91 (1H, ddt, J=15.6, 10.8, 6.0 Hz), 7.72—7.76 (2H, m), 7.85—7.88 (1H, m), 8.12—8.15 (1H, m); ^{13}C-NMR ($CDCl_3$) δ: 30.4, 37.5, 40.7, 65.7, 117.8, 125.4, 130.9, 132.8, 133.5, 133.9, 148.1, 156.7, 167.6, 173.1; MS: m/z=344 (MH$^+$), 343 (M$^+$); HRMS ($C_{13}H_{18}N_3O_6S$, MH$^+$): Calcd for 344.0916. Found: 344.0910.

***N*-(12-Allyloxycarbonylamino-4,9-bis(2-nitrobenzenesulfonyl)-4,9-diazadodecan-1-yl)-*tert*-butoxycarbamide (26)** To a stirred solution of 1.95 g (3.95 mmol) of bromide **24**, 3.49 g (10.7 mmol) of cesium carbonate, and 133 mg (0.36 mmol) of *n*-tetrabutylammonium iodide in 5 ml of acetonitrile was added 1.12 g (3.29 mmol) of diamine **25** at 60 °C under an argon atmosphere. After 1 h, the reaction mixture was poured into brine, and the aqueous layer was extracted thoroughly with ethyl acetate (3×). The combined organic extracts were dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (60% ethyl acetate in hexane) yielded tetramine **26** (2.39 g, 97%) as a yellow solid. IR ($CHCl_3$): 3414, 3092, 2934, 1709, 1544, 1460, 1440, 1372, 1346, 1251, 1162, 1125, 1060, 1032, 914, 852, 779, 733 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.43 (9H, s), 1.51 (4H, bs), 1.73 (4H, m), 3.12 (2H, m), 3.20 (2H, m), 3.25—3.36 (8H, m), 4.55 (2H, d, J=4.8 Hz), 4.81 (1H, bs), 5.13 (1H, bs), 5.21 (1H, d, J=8.8 Hz), 5.30 (1H, d, J=18.8 Hz), 5.92 (1H, ddt, J=18.8, 8.8, 4.8 Hz), 7.61—7.64 (2H, m), 7.70—7.72 (4H, m), 7.98—7.99 (2H, m); ^{13}C-NMR ($CDCl_3$) δ: 25.1, 28.4, 28.7, 37.7, 45.1, 45.2, 47.0, 65.5, 68.2, 117.6, 124.3, 130.6, 130.6, 131.8, 132.9, 133.7, 148.0; MS: m/z=757 (MH$^+$); *Anal.* ($C_{31}H_{44}N_6O_{12}S_2$): Calcd for C, 49.20; H, 5.86; N, 11.10. Found: C, 48.82; H, 5.74; N, 10.60.

***N*-(12-*tert*-Butoxycarbonylamino-4,9-bis(2-nitrobenzenesulfonyl)-4,9-diazadodecan-1-yl)-2-nitrobenzenesulfonamide (27)** To a stirred solution of 2.29 g (3.03 mmol) of tetramine **26** in 20 ml of dichloromethane were added 159 mg (0.61 mmol) of triphenylphosphine, 175 mg (0.15 mmol) of $Pd(PPh_3)_4$ and 0.8 ml (9.09 mmol) of pyrrolidine consecutively at 0 °C under an argon atmosphere. After 90 min at room temperature, the reaction mixture was poured into a solution of 1 N hydrochloric acid, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel, eluted with a gradient of methanol in chloroform (0 to 10%) furnished tetramine (2.13 g, 99%) as a yellow solid. IR ($CHCl_3$): 2962, 1685, 1542, 1461, 1370, 1260, 1160, 1028, 852, 798 cm^{-1}; ^{1}H-NMR (DMSO) δ: 1.29 (9H, s), 1.36 (2H, bs), 1.50 (2H, m), 1.73 (2H, m), 2.67 (2H, m), 2.80 (2H, m), 3.11—3.20 (4H, m), 4.29 (2H, bs), 6.75 (1H, bs), 7.75—7.85 (4H, m), 7.89—7.96 (4H, m); ^{13}C-NMR (DMSO) δ: 18.5, 24.8, 25.0, 26.4, 28.2, 28.5, 36.5, 37.3, 44.7, 45.3, 47.0, 56.0, 77.6, 124.3, 124.3, 129.6, 131.6, 131.7, 132.4, 132.5, 134.5, 134.6, 147.5, 155.6; MS: m/z=672 (M$^+$); HRMS ($C_{27}H_{41}N_6O_{10}S_2$, MH$^+$): Calcd for 673.2325. Found: 673.2324.

To a stirred solution of 2.02 g (3.00 mmol) of the above tetramine and 798 mg (3.60 mmol) of 2-nitrobenzenesulfonyl chloride in 20 ml of dichloromethane was added 0.50 ml (3.60 mmol) of triethylamine at 0 °C under an argon atmosphere. After 30 min at room temperature, the reaction mixture was poured into a solution of 1 N hydrochloric acid, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by column chromatography on silica gel (50% ethyl acetate in hexane) yielded sulfonamide **27** (2.42 g, 94%) as a yellow solid. IR ($CHCl_3$): 3414, 2937, 1706, 1544, 1465, 1373, 1345, 1252, 1160, 1125, 1060, 998, 852, 778, 744 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.43 (9H, s), 1.51 (4H, bs), 1.72 (2H, m), 1.85 (2H, m), 3.13 (2H, m), 3.23—3.38 (10H, m), 4.83 (1H, bs), 7.60—7.63 (3H, m), 7.69—7.72 (6H, m), 7.97—8.01 (3H, m); ^{13}C-NMR ($CDCl_3$) δ: 25.0, 25.0, 27.4, 28.4, 42.4, 44.4, 44.9, 45.1, 46.9, 47.1, 124.2, 130.5, 130.6, 130.8, 130.8, 130.9, 131.8, 131.9, 131.9, 133.0, 133.6, 133.7, 148.0; MS: m/z=758 (MH$^+$−Boc); *Anal.* ($C_{33}H_{43}N_7O_{14}S_3$): Calcd for C, 46.20; H, 5.05; N, 11.43. Found: C, 46.08; H, 5.04; N, 10.77.

***N*-[20-*tert*-Butoxycarbonylamino-4-(2-cyanoethyl)-8,12,17-tris(2-nitrobenzenesulfonyl)-4,8,12,17-tetraazaicosan-1-yl]-1-*tert*-butoxycarbonyl-indole-3-acetamide (28)** To a stirred solution of 225 mg (0.51 mmol) of alcohol **23**, 520 mg (0.61 mmol) of sulfonamide **27** and 200 mg (0.76 mmol) of triphenylphosphine in 2 ml of dichloromethane and 8 ml of benzene was slowly added 345 μl (0.76 mmol) of diethyl azodicarboxylate (40% in toluene) at room temperature under an argon atmosphere. After 10 min, the reaction mixture was evaporated. Purification of the crude product by column chromatography on silica gel, eluted with a gradient of ethyl acetate in hexane (80 to 100%), furnished polyamine **28** (587 mg, 90%) as a yellow powder. IR ($CHCl_3$): 2931, 1715, 1669, 1545, 1455, 1369, 1256, 1160, 1086, 852, 736 cm^{-1}; ^1H-NMR ($CDCl_3$) δ: 1.32 (2H, m), 1.43 (9H, s), 1.49 (2H, m), 1.58 (4H, m), 1.66 (9H, s), 1.68 (2H, m), 1.83 (2H, m), 2.26 (2H, t, J=6.4 Hz), 2.32 (4H, t, J=6.4 Hz), 2.55 (2H, t, J=6.4 Hz), 3.11 (2H, m), 3.20—3.31 (14H, m), 3.64 (2H, s), 4.84 (1H, bs), 6.16 (1H, bs), 7.23 (1H, t, J=7.5 Hz), 7.32 (1H, t, J=7.5 Hz), 7.47—7.65 (5H, m), 7.67—7.71 (6H, m), 7.95—7.98 (3H, m), 8.14 (1H, bs); ^{13}C-NMR ($CDCl_3$) δ: 16.5, 23.9, 25.1, 25.2, 27.9, 28.3, 28.5, 28.8, 30.4, 33.4, 37.9, 38.9, 45.2, 45.5, 46.5, 47.2, 47.4, 49.4, 50.6, 51.1, 114.2, 115.5, 119.2, 123.0, 124.2, 124.3, 124.4, 124.9, 125.1, 128.6, 128.7, 128.9, 128.9, 130.1, 130.6, 130.8, 131.0, 132.0, 132.1, 132.1, 132.2, 132.3, 132.9, 132.9, 133.0, 133.2, 133.8, 148.1, 170.6; HRMS ($C_{57}H_{76}N_{11}O_{17}S_3$, MH^+): Calcd for 1282.4583. Found: 1282.4578.

***N*-[20-*tert*-Butoxycarbonylamino-4-hydroxy-8,12,17-tris(2-nitrobenzenesulfonyl)-4,8,12,17-tetraazaicosan-1-yl]-1-*tert*-butoxycarbonyl-indole-3-acetamide (29)** To a stirred solution of 220 mg (0.172 mmol) of polyamine **28** in 5 ml of dichloromethane was slowly added 30 mg (0.173 mmol) of 3-chloroperoxybenzoic acid (*m*-CPBA) in 3 ml of dichloromethane at −10 °C. After 1 h, excess dimethyl sulfide (0.25 ml, 3.42 mmol) was added to the reaction mixture, and the vessel was allowed to warm to room temperature. The mixture was poured into a solution of saturated sodium bicarbonate, and the aqueous layer was extracted thoroughly with dichloromethane (3×). The combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by preparative thin layer chromatography (10% methanol in chloroform) afforded hydroxylamine **29** (192 mg, 90%) as a yellow powder. IR ($CHCl_3$): 3413, 3093, 2934, 1729, 1654, 1591, 1544, 1453, 1370, 1309, 1256, 1227, 1160, 1125, 1086, 1060, 1017, 912, 852, 773, 733 cm^{-1}; ^1H-NMR ($CDCl_3$) δ: 1.23 (2H, m), 1.42 (9H, s), 1.49 (2H, bs), 1.66 (9H, s), 1.70 (4H, m), 1.84 (2H, m), 2.47 (2H, t, J=6.4 Hz), 2.51 (2H, t, J=6.4 Hz), 3.10 (2H, m), 3.20—3.34 (14H, m), 3.64 (2H, s), 4.86 (1H, bs), 6.21 (1H, bs), 7.24 (1H, t, J=7.5 Hz), 7.33 (1H, t, J=7.5 Hz), 7.51 (1H, d, J=7.5 Hz), 7.56 (1H, bs), 7.59—7.62 (3H, m), 7.67—7.71 (6H, m), 7.95—7.98 (3H, m), 8.14 (1H, bs); ^{13}C-NMR ($CDCl_3$) δ: 13.7, 22.2, 24.5, 24.7, 25.3, 26.2, 27.1, 27.8, 28.0, 28.2, 31.1, 32.8, 37.6, 44.7, 44.8, 45.4, 46.6, 56.7, 57.8, 111.9, 113.7, 115.0, 118.6, 122.5, 123.7, 123.8, 124.4, 129.4, 130.1, 130.3, 131.4, 131.5, 132.4, 132.5, 132.6, 133.2, 133.3, 147.5, 147.5, 155.6, 169.8; HRMS ($C_{54}H_{73}N_{10}O_{18}S_3$, MH^+): Calcd for 1245.4266. Found: 1245.4265.

***N*-(20-Amino-4-hydroxy-8,12,17-tris(2-nitrobenzenesulfonyl)-4,8,12,17-tetraazaicosan-1-yl)-1*H*-indole-3-acetamide (30)** To a stirred solution of 108 mg (0.0867 mmol) of hydroxylamine **29** in 0.4 ml of dichloromethane and 1.2 ml of methanol was added excess thionyl chloride (0.4 ml, 5.48 mmol) at 0 °C under an argon atmosphere. After 2.5 h at room temperature, the reaction mixture was evaporated. Purification of the crude product by column chromatography on silica gel (10% methanol in chloroform) yielded polyamine **30** (89 mg, 95%) as a yellow powder. IR ($CHCl_3$): 3020, 1653, 1541, 1457, 1373, 1340, 1215, 1160, 1125, 1060, 852, 757 cm^{-1}; ^1H-NMR ($CDCl_3$) δ: 1.39 (4H, m), 1.69 (2H, m), 1.79 (4H, m), 1.90 (2H, m), 2.73 (2H, m), 3.09—3.32 (18H, m), 3.49 (2H, s), 6.95 (1H, t, J=7.4 Hz), 7.04 (1H, t, J=7.4 Hz), 7.18 (1H, s), 7.32 (1H, d, J=8.0 Hz), 7.53 (1H, d, J=7.4 Hz), 7.81—7.90 (9H, m), 7.93—8.00 (3H, m), 10.9 (1H, s); ^{13}C-NMR ($CDCl_3$) δ: 24.7, 24.8, 26.2, 32.7, 36.4, 44.9, 47.1, 111.3, 118.3, 118.6, 120.9, 123.8, 124.3, 124.4, 127.2, 129.6, 131.5, 131.6, 132.5, 134.6, 136.1, 147.4, 147.9; HRMS ($C_{44}H_{57}N_{10}O_{14}S_3$, MH^+): Calcd for 1045.3218. Found: 1045.3214.

Agel 489 (2) To a suspension of the freshly prepared resin **16** (0.13 mmol) and 27.0 mg of amine **30** (0.025 mmol) in 1.5 ml of dichloromethane was added excess *N*,*N*-diisopropylethylamine (55 μl, 0.32 mmol) at room temperature. After shaking for 24 h, excess methanol (0.3 ml, 7.5 mmol) was added to the reaction mixture. The resin was filtered, washed with MeOH : CH_2Cl_2 (1 : 9), H_2O : MeOH : CH_2Cl_2 (1 : 1 : 8), and CH_2Cl_2, and then dried *in vacuo* for 8 h to give the resin. To a suspension of the resulting resin in 1.5 ml of DMF was added excess 2-mercaptoethanol (0.053 ml, 0.75 mmol) and excess DBU (0.11 ml, 0.75 mmol) at room temperature under an argon atmosphere. After shaking for 26 h, the resin was filtered, washed with H_2O : THF (1 : 9), MeOH : CH_2Cl_2 (1 : 9), and CH_2Cl_2 and dried *in vacuo* for 8 h to give the resin. To a mixture of the resulting resin in 2.5 ml of CH_2Cl_2 was added trifluoroacetic acid (30 μl, 0.36 mmol) at room temperature. After shaking for 5 min, the resin was filtered and washed with MeOH : CH_2Cl_2 (1 : 1). The combined washings were evaporated and dried *in vacuo* to provide **2** (27.0 mg, 92%) as the TFA salt. ^1H-NMR (D_2O) δ: 1.72 (4H, m), 1.92—2.06 (8H, m), 3.02—3.17 (20H, m), 3.71 (2H, s), 7.13 (1H, dt, J=7.3, 1.0 Hz), 7.22 (1H, dt, J=7.3, 1.0 Hz), 7.29 (1H, s), 7.48 (1H, d, J=8.0 Hz), 7.56 (1H, d, J=7.3 Hz); ^{13}C-NMR (D_2O) δ: 23.5, 24.5, 33.2, 36.8, 37.3, 45.2, 45.3, 47.8, 56.4, 57.4, 108.4, 112.8, 119.1, 120.3, 122.9, 126.0, 127.3, 137.1, 176.8; HRMS ($C_{26}H_{48}N_7O_2$, MH^+): Calcd for 490.3869. Found: 490.3878; MS/MS (FAB) 474 ($MH^+ - NH_2$), 460 ($MH^+ - CH_4N$), 446 ($MH^+ - C_2H_6N$), 432 ($MH^+ - C_3H_8N$), 416 ($M^+ - C_3H_9N_2$), 402 ($M^+ - C_4H_{11}N_2$), 374 ($M^+ - C_6H_{13}N_2$), 360 ($M^+ - C_7H_{17}N_2$), 345 ($M^+ - C_7H_{18}N_3$), 317 ($M^+ - C_9H_{22}N_3$), 275 ($MH^+ - C_{11}H_{27}N_4$), 258 ($M^+ - C_{11}H_{28}N_4O$), 244 ($M^+ - C_{12}H_{30}N_4O$), 215 ($M^+ - C_{13}H_{32}N_5O$), 130 ($C_9H_8N^+$).

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References and Notes

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Controlling Factors in Chiral Bisoxazoline-Catalyzed Asymmetric Lithium Ester Enolate–Imine Condensation Producing a *β*-Lactam

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A catalytic amount of external chiral bisoxazoline ligand 3a bearing an isopropyl group as a stereocontrolling group catalyzed a reaction of a lithium ester enolate 4b, generated from 3-pentyl 2-methylpropionate, with benzaldehyde anisidine-imine 5 to afford corresponding *β*-lactam 6 in higher 70% ee than that obtained by the reaction using a stoichiometric amount of the ligand. A bulkier ligand 3d bearing a phenyl group gave 81% and 6% ees in stoichiometric and catalytic reactions, respectively. Examination of the varying factors suggested the involvement of mixed aggregates as a reactive species. A working model is presented for prediction of the sense of asymmetric induction.

Key words asymmetric reaction; catalysis; enolate; oxazoline

Condensation of a lithium ester enolate with an imine through an addition–elimination sequence is the established synthetic methodology of a *β*-lactam.[1] Diastereoselective asymmetric reactions have been developed using chiral enolates or chiral imines.[2] Although asymmetric reactions of achiral silyl enol ethers with achiral imines have been reported using chiral promoters,[3] an asymmetric reaction of an achiral lithium ester enolate with a prochiral imine by an external chiral ligand methodology[4] has been the subject undeveloped.[5] We have already reported asymmetric condensation of a lithium ester enolate with an imine, which was mediated by binary as well as ternary complex reagents.[6] These reagents are constituted from two components of a lithium enolate and a chiral diether **1**[7] or an aminodiether ligand, and three components[8] of an enolate, a chiral ligand, and an achiral lithium amide, respectively. The external ligand methodology has been shown useful in synthesis of pharmaceutically active compounds.[9] During our efforts directed towards development of more efficient ligands, we found that a chiral bisoxazoline (Box) ligand **3** catalyzed asymmetric condensation of lithium ester enolate **4** with an imine **5** to afford *β*-lactam **6** in high enantiomeric excess (ee).[10] Unexpected behavior of the Box ligand was that the reaction, which uses a catalytic amount of Box, afforded the corresponding *β*-lactam **6** in higher ee than that obtained by the reaction using a stoichiometric amount of the ligand. The structural features of the Box ligand **3** crucially govern such efficiency of the reaction. As a continuing effort to determine critical controlling factors of the reaction, we studied the reaction systematically concerning equivalency, structure of the Box ligand, and involvement of co-products of the reaction.[11] We describe herein the full detail of our study directed towards deeper understanding of chiral Box ligand behavior.[12]

Catalytic Asymmetric Reaction of Lithium Ester Enolate 4 The lithium ester enolate **4b** (R^2=CH(Et)$_2$) was generated *in situ* by lithium diisopropylamide (LDA) treatment of 3-pentyl 2-methylpropionate in toluene. Reaction of 2 eq of **4b** with benzaldehyde anisidine-imine **5** was mediated by 2.6 eq of **1** in toluene at −20 °C for 7 h to afford (+)-**6** in 95% yield (Table 1, entry 1). The ee was determined to be 60% by HPLC analysis using a chiral stationary phase column (DAICEL Chiralcel OD, iso-PrOH/hexane=1/100).[8c] The same reaction at 0 °C for 5 h using 0.1 eq of **1** gave (+)-**6** in 99% yield and 45% ee (entry 2). Although it was surprising to find that the ether ligand **1** catalyzed the reaction and afforded **6** in 45% ee, the efficiency of the catalytic reaction is apparently inferior to that of the stoichiometric reaction. The Box ligand **2** was a poor stoichiometric ligand and afforded (−)-**6** in only 10% ee (entry 3). We were very pleased to find that two stoichiometric Box ligands **3b,c** bearing bulky phenyl and *tert*-butyl groups as R^1 gave (−)-**6** in 81% and 75% ees (entries 4, 6). However, it was again very disappointing to learn that the catalytic reactions using 0.1 eq of **3b,c** proceeded at higher temperature to give **6** in miserable 6% and 26% ees (entries 5, 7). The poor efficiency of **3b,c** in the catalytic asymmetric induction and rate acceleration is attributable to the bulkiness of R^1, which prevents formation of a chelate with lithium enolate **4b** to allow the racemic reaction.

We next examined a Box ligand **3a**[13] bearing a smaller iso-Pr group as R^1. Enantioselectivity was moderate, 56% ee. However, it was very surprising to find that the reactions using 0.2 and 0.1 eq of **3a** proceeded at −20 °C, the same temperature as the stoichiometric reaction, and gave (−)-**6** in 70% and 69% ees apparently higher than that obtained in the stoichiometric reaction (entries 8—10). The size of a base affected the enantioselectivity to afford **6** in 45% ee when lithium cyclohexyl-isopropylamide (LICA) was used as a lithiation reagent (entry 11). A catalytic amount of **3a** gave **6** in higher 55% ee than that of stoichiometric reaction (entry 12).

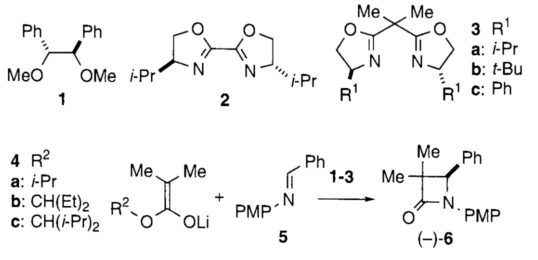

Fig. 1. Lithium Enolate–Imine Condensation Using a Chiral Ligand

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Dedicated to the memory of Dr. Kyosuke Tsuda.

Table 1. Asymmetric Reaction of **4** with **5** Catalyzed by **1**—**3** Giving (−)-**6**

Entry	1—3	R^1	Eq	4	R^2	Base	Temp/°C	Time/h	Yield/%	Ee/%
1	**1**		2.6	**b**	$CH(Et)_2$	LDA	−20	7	95	60[a]
2			0.1			LDA	0	5	99	45[a]
3	**2**		2.6			LDA	0	1	99	10
4	**3c**	Ph	2.6			LDA	−20	5	99	81
5			0.1			LDA	20	4	99	6
6	**3b**	*tert*-Bu	2.6			LDA	0	1	97	75
7			0.1			LDA	20	4	95	26
8	**3a**	iso-Pr	2.6			LDA	−20	2	99	56
9			0.2			LDA	−20	4	96	70
10			0.1			LDA	−20	4	92	69
11			2.6			LICA	−20	2	90	45
12			0.2			LICA	−20	4	96	55
13			2.6	**a**	iso-Pr	LDA	0	1	91	45
14			0.2			LDA	0	2	94	65
15			2.6	**c**	$CH(iso\text{-}Pr)_2$	LDA	−20	2	98	58
16			0.1			LDA	−20	4	95	12

a) The enantiomer (+)-**6** was obtained.

Table 2. Influence of the Equivalency of **3a** on Asymmetric Reaction of **4b** at −20 °C Giving (−)-**6**[a]

Entry	3a/eq	Time/h	Yield/%	Ee/%
1	4.0	2	70	37
2	2.6	2	99	56
3	1.0	2	99	66
4	0.5	3	96	65
5	0.2	4	95	70
6	0.1	4	92	69
7	0.05	5	81	58
8	0.01	5	62	50

a) Two eq of **4b** were used.

The size of the alkoxy group of **4** was also one of the influencing factors. The reaction of **4a** bearing the less bulky isopropoxy group was catalyzed by 0.2 eq of **3a** to afford (−)-**6** in 65% ee, which was higher than 45% ee of the reaction using 2.6 eq of **3a** (entries 13, 14). On the other hand, the reaction of **4c** bearing the bulkiest alkoxy group gave **6** in a significantly lower 12% ee than the 58% ee obtained by the stoichiometric reaction (entries 15, 16). These clearly indicate that a complex formation of lithium enolate **4** with **3** and other species is dependent on the bulkiness of components and is likely when bulkiness is moderate as shown by **4a**,**b** and **3a**.

Unexpected Dependency of Enantioselectivity on the Amount of 3a We then examined the same reaction of **4b** with **5** using varying amounts of **3a** from 4 eq to 0.01 eq. It was surprising to find that 0.01 eq of **3a** catalyzed the reaction at −20 °C for 5 h, producing **6** in 50% ee higher than that obtained using 4 eq of **3a** (Table 2, entries 1, 8). The ee of **6** increased up to 70% from 37% along with a decrease of **3a** from 4.0 to 0.2 eq. It was maintained at 70—65% with 1 to 0.1 eq of **3a**, and then with 0.05 eq dropped to 58%, which was still higher than that obtained using 2.6 eq of **3a**. It is also important to note that prolonged reaction time was required for the completion of the reaction with a catalytic amount of **3a**.

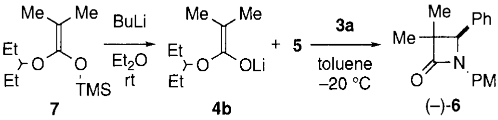

Fig. 2. Generation of Lithium Enolate from **7** and Condensation

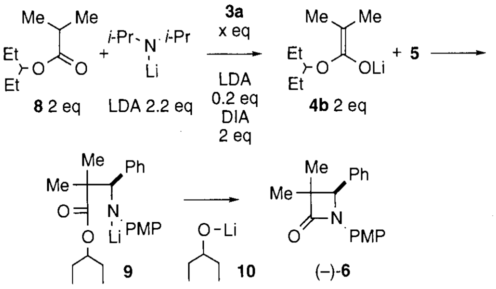

Fig. 3. Controlling Factors of Asymmetric Reaction of Lithium Enolate

Some Factors Affecting the Catalytic Asymmetric Reaction The unexpected behavior of Box ligand **3a** was apparently governed by the steric factors of **3** and **4**. A lesser catalytic amount of **3a** than of **4b** gave **6** in the higher ee than did equal equivalents of **3a** and **4b**. Therefore, it is probable that the reactive enolate species involve **3**, **4**, and several other components such as a small amount of LDA, diisopropylamine (DIA), product **6**, the resulting lithium amide intermediate **9**, and lithium alkoxide **10** generated *in situ* through the reaction. Then, we compared %ees of **6** obtained in the stoichiometric reactions quenched after 2 h and 0.05 h, respectively, and found that there was no significant difference in them (Table 3, entries 1, 2). However, in the catalytic reactions, at the 27% conversion we observed the lower 65% ee than 70% ee at the full conversion (entries 3, 4). These results suggest involvement of some co-products as components of the reactive enolate species.

The previously developed ternary complex reagent, for example, **3a**–**4b**–LDA,[8] was ruled out. The reaction using 2 eq of **4b**, 2.6 eq of **3a**, and 2.4 eq of LDA afforded **6** in lower 43% ee than that obtained in the absence of such large excess of LDA (Table 3, entry 8). The effects of LDA and diisopropylamine on enantioselectivity were determined using a lithium enolate generated free from these species. Thus, the enolate **4b** was generated from a silyl enol ether **7** by treatment with butyllithium in diethyl ether. The stoichiometric and catalytic reactions of **4b** thus generated afforded **6** in

Table 3. Asymmetric Reaction of **4b** with **5** Catalyzed by Box **3a** Giving (−)-**6**[a]

Entry	Type	**3a** eq	LDA eq	DIA eq	**10** eq	**6** eq	Time h	Yield %	Ee %
1	Box–enolate	2.6	0.2	2.0	0	0	2	99	56
2		2.6	0.2	2.0	0	0	0.05[b]	47	54
3		0.2	0.2	2.0	0	0	4	96	70
4		0.2	0.2	2.0	0	0	0.1[b]	27	65
5		2.6	0	0	0	0	2	99	53[c]
6		0.2	0	0	0	0	4	86	48[c]
7		0.2	0.2	2.0	0	0	4	97	37[d]
8	Box–enolate–LDA	2.6	2.4	2.0	0	0	1	75	43
9	Box–enolate–alkoxide	2.6	0.2	2.0	2.4	0	2	86	42
10	Box–enolate–lactam	0.2	0.2	2.0	0	0.2	0.5[b]	80	62
11	Enolate–lactam	0	0.2	2.0	0	0.2	4	50	1

a) Initial equivalent was described. *b*) The reaction was quenched at the indicated reaction time. *c*) The lithium enolate **4b** was generated by treating a silyl enol ether **7** with butyllithium in ether. Therefore, the solvent for asymmetric reaction was a mixture of toluene and ether (8 : 3). *d*) Toluene–ether (8 : 3).

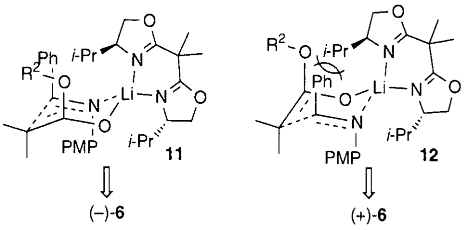

Fig. 4. Models for Probable Asymmetric Induction

53% and 48% ees, respectively (entries 5, 6). Since the reaction of **4b**, generated by LDA treatment of **8**, in the same toluene–ether (8 : 3) mixture as a solvent gave **6** in lower 37% ee than that obtained above, LDA and diisopropylamine unfavorably affect efficiency (entry 7). The effect of lithium alkoxide **10** was also unfavorable to afford **6** in 42% ee (entry 9). Co-existence of **6** significantly affected the efficiency. In the presence of 0.2 eq of optically pure (−)-**6**, the catalytic reaction proceeded rapidly to afford, after 0.5 h, 80% yield of **6** in 62% ee (entries 3, 10). In the absence of Box **3a**, the reaction was not rapid, and after 4 h gave 50% yield of **6** in marginal ee (entry 11). These reactions suggest that a ternary type complex reagent, constituted from **3a**, **4a**, and **6**, is involved in the asymmetric reaction as a reactive species.

Probable Models for Asymmetric Induction The asymmetric reaction mediated by chiral Box ligands **3** always gave (−)-**6**. Although the reactive enolate species are not simple, it is useful to present a fundamental mechanistic model for the sense of asymmetric induction. Models **11** and **12** are constructed based on a six-membered transition state hypothesis for an enolate–imine condensation. Two nitrogen atoms of Box **3a** coordinate to the lithium atom of the enolate to form a six-membered chelate. The stereocontrolling isopropyl group of a Box interferes with a phenyl group of **5** as shown in **12**. While, model **11** is free from such severe steric interference. Based on the above working hypothesis, model **11** predicts production of (−)-**6** that is really the preferential product of the asymmetric reaction.

Conclusion

Although the enantioselectivity is not extremely high, we believe that the catalytic asymmetric reaction demonstrated here will a promising approache to the efficient catalytic carbon–carbon bond forming reaction. Further studies directed toward structural elucidation of the active species and structural tuning of the chiral ligand for the improvement of efficiency are in progress in our laboratories.

Experimental[14]

Catalytic Asymmetric Reaction Controlled by 3a (Table 1, Entry 9) A solution of 3-pentyl 2-methylpropionate **8** (316 mg, 2.0 mmol) and **3a** (53 mg, 0.2 mmol) in toluene (3 ml) was added to a solution of LDA (2.2 mmol) in toluene (5 ml) at −78 °C. The mixture was stirred for 1 h and to this solution was then added a solution of imine **5** (211 mg, 1.0 mmol) in toluene (3 ml). After stirring at −20 °C for 4 h, brine (20 ml) was added and the mixture was extracted with EtOAc (30 ml×3). The organic layer was washed with brine and then dried over Na_2SO_4. Concentration followed by purification through silica gel column chromatography (ether/hexane=1/5, then ether) gave (−)-**6** (268 mg, 96%) as a colorless solid of mp 97—99 °C and $[\alpha]_D^{25}$ −95.3 (c=1.02, $CHCl_3$). Ee was determined by HPLC analysis to be 70% (Daicel Chiralcel OD-H, hexane–iso-PrOH (100 : 1), 1.0 ml/min, 250 nm, 12 min (85%, R): 15 min (15%, S)). Spectral data were identical with those reported previously.[8c]

[1-(1-Ethylpropoxy)-2-methylpropenyloxy]trimethylsilane (7) Chlorotrimethylsilane (8.28 ml, 65 mmol) was added to a cooled solution of LDA, prepared from 8.22 ml (59 mmol) of diisopropylamine and 34.1 ml of butyllithium solution in hexane (55 mmol), in 80 ml of THF at −78 °C. Then, 3-pentyl 2-methylpropionate (8.0 g, 51 mmol) in THF (5.5 ml) was added dropwise over 5 min, and the mixture was allowed to warm up to room temperature. The whole was stirred for 1 h and then diluted with 1.6 l of pentane. Concentration followed by distillation (69 °C, 9 mmHg) gave **7** as a pale yellow oil (8.74 g, 93%). ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 0.20 (9H, s, TMS), 0.90 (6H, t, J=7.5 Hz, Me), 1.30—1.73 (10H, m), 3.85 (1H, quint, J=6.3 Hz). ^{13}C-NMR ($CDCl_3$, 67.8 MHz) δ: 0.16 (q, TMS), 9.31 (q, Me), 16.70 (q, Me), 17.20 (q, Me), 24.78 (t, CH_2), 74.24 (d, CH), 91.95 (s), 146.42 (CO). IR (neat): 1710 cm^{-1}. MS m/z: 230 (M^+). HR-MS m/z: Calcd for $C_{12}H_{26}O_2Si$: 230.1703. Found. 230.1716.

Asymmetric Reaction Starting from Silyl Enol Ether 7 Controlled by 3a (Table 3, entry 5) To silyl enol ether **7** (506 mg, 2.2 mmol) in Et_2O (3 ml) was added butyllithium (1.24 ml, 1.61 N, 2.0 mmol) at −78 °C. The mixture was stirred for 1 h at room temperature. To this suspension was added a solution of **3a** (691 mg, 2.6 mmol) in toluene (5 ml) at −78 °C. After stirring for 1 h at −20 °C, a solution of **5** (211 mg, 1.0 mmol) in toluene (3 ml) was added at −78 °C. After stirring of pale yellow solution for 2 h at −20 °C, brine (20 ml) was added and the whole was extracted with EtOAc (20 ml×3). The organic layer was washed with brine (10 ml), and then dried over Na_2SO_4. Concentration followed by purification through silica gel column chromatography (ether/hexane=1/5, then ether) gave (−)-**6** (281 mg, 99%) as a colorless solid of mp 93—98 °C and $[\alpha]_D^{25}$ −72.7 (c=1.35, $CHCl_3$). Ee was determined to be 53%.

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14) ^{1}H- and ^{13}C-NMR were recorded in CDCl$_3$ unless otherwise noted. Chemical shift was presented in ppm downfield from tetramethylsilane (TMS). Data were reported as follows: integration, multiplicity (br=broad, s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet), coupling constants (Hz), and assignments where relevant. Mass spectra were recorded under electron impact (EI) conditions.

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Preparation of Novel Synthons, Uniquely Functionalized Tetrahydrofuran and Tetrahydropyran Derivatives

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The dianion of the acetoacetic ester reacts with epibromohydrin derivatives to afford a mixture of (*Z*)-2-alkoxycarbonylmethylidenetetrahydrofuran derivative and (*E*)-2-alkoxycarbonylmethylidenetetrahydropyran derivative. The selective formation of the tetrahydrofuran derivative is achieved by the use of $LiClO_4$ as the additive. The preparation of the optically active tetrahydrofuran derivatives and tetrahydropyran derivatives is also examined, and the optical purity and absolute configuration of the products is elucidated.

Key words tetrahydrofuran; tetrahydropyran; acetoacetic ester; dianion; epibromohydrin; epoxide

Since many bioactive natural products, for example, nucleoside antibiotics, polyether antibiotics, and Annonaceous acetogenins,[1)] contain a tetrahydrofuran (THF) and/or a tetrahydropyran (THP) as a part of their gross structure, the development of a synthesis of THF and THP derivatives has been very important. From this standpoint, we have already reported a high-yielding and highly selective one-pot synthesis of THF, THP derivatives by the reaction of commercially available acetoacetic esters with readily available epibromohydrin derivatives.[2)]

In this paper we wish to report the full description of our one-pot synthesis of chiral synthons, uniquely functionalized THF, THP derivatives starting from acetoacetic esters and epibromohydrin derivatives.

The dianion of the β-diketo compound was first reported by Hauser and Harris,[3)] and the dianion of the acetoacetic ester has been a popular reagent in organic synthesis since Huckin and Weiler reported an effective generation method of dianions of acetoacetic esters.[4)] The numerous reports and reviews[5)] of organic synthesis using the dianion of the acetoacetic ester to date suggest its great utility. Among the many reactions of the dianion of the acetoacetic ester, a very useful one is the construction of cyclic compounds by a one-pot reaction utilizing the property of the dianion.[5,6)] Hence, we have examined the one-pot reaction of the dianion of the acetoacetic esters with epibromohydrin derivatives.[7)]

The reaction of the dianion of the acetoacetic ester with an epibromohydrin derivative has not been examined so far as we know. The dianion **1** is expected to react with the epibromohydrin derivative **2** at its γ position to form the intermediate **3** (Table 1), which subsequently cyclizes to afford the *O*-alkylated products and/or the *C*-alkylated products.[5)] However, the ratio of these products was expected to be controlled by the choice of the reaction conditions.[4)]

First, the dianion **1a** generated by the standard procedure[4)] was reacted with the (*E*)-epibromohydrin derivative **2**. After adding **2** to the solution of **1a** at −60 °C, the reaction mixture was warmed to −40 °C and stirred for 6 h. The completion of the alkylation of the dianion **1a** at its γ position was checked by TLC, and further stirring of the reaction mixture at room temperature for 8 h afforded the products, the THF derivative **4a** and the THP derivative **5a** in 84% combined and isolated yield in the ratio of 1 : 1.6 (Table 1, entry 1).[8)] No formation of the *C*-alkylated products was observed.

It is interesting that the alkene in the products **4** and **5** is (*Z*)-form and (*E*)-form, respectively, and no isomeric product was formed. Prolonged reaction time at room temperature (72h) resulted in a change in the ratio of **4a**/**5a** to 1/2.6 (entry 2). This change in the ratio was supposed to arise from the isomerization of the alkoxide **4a′** corresponding to **4a** to the alkoxide **5a′** corresponding to **5a**. To activate the epoxide in the cyclization step, Lewis acid was used as the additive, but no reaction occurred in the case of $ZnCl_2$, $MgBr_2$, $MgClO_4$ (entries 3, 4, 5). On the other hand, the ratio of **4a**/**5a** was improved to 4.4/1 when LiCl was used (entry 6), and by the use of the more acidic $LiClO_4$[9)] the reaction time was reduced and the ratio was further improved to 8.4/1 (entry 7). Finally, the products were obtained in 99% yield in the ratio of **4a**/**5a**=17/1 when the reaction was carried out in the presence of $LiClO_4$ at −10 °C (entry 8). The effect of $LiClO_4$ was supposed to not only activate the epoxide in the cyclization step, but also inhibit **4a′** from isomerizing to **5a′** by the formation of a stable chelate complex of **4a′** with the metal cation.

Reactions of the dianion **1b**, which possesses Me group at its α position, were further carried out and the same results as described above were obtained (entries 11, 12, 13).

To examine the effect of $LiClO_4$ as described above, we carried out the reaction of Eq. 1 in the presence of Hexamethylphosphoric triamide, a well-known solvent having strong solvating property. The results of the reactions under some conditions are summarized in Table 1. Though in the presence of HMPA the reaction proceeded somewhat slower than when $LiClO_4$ was used, the formation of THP derivatives increased as expected (entries 9, 14). Furthermore, when HMPA was added to the reaction mixture and left for 8 h after the formation of the products **4a** and **5a** in the presence of $LiClO_4$ (entry 10), the same result as entry 2 was obtained. By considering these experiments using HMPA, one effect of $LiClO_4$ that is strongly proposed is that $LiClO_4$ prevents the initially formed THF derivatives from isomerizing to the corresponding THP derivatives. However, the possibility that THP derivatives are directly derived from the intermediate **3a** in the reaction of Eq. 1 can not be ruled out by

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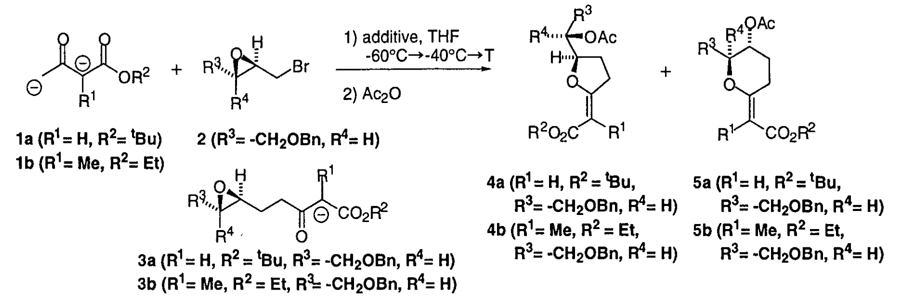
Dedicated to the memory of Dr. Kyosuke Tsuda.

Table 1. Reactions of Acetoacetic Esters **1a**, **1b** with Epibromohydrin Derivative **2**



1a (R^1= H, R^2= ${}^tBu$)
1b (R^1= Me, R^2= Et)
2 (R^3= $-CH_2OBn$, R^4= H)
3a (R^1= H, R^2= ${}^tBu$, R^3= $-CH_2OBn$, R^4= H)
3b (R^1= Me, R^2= Et, R^3= $-CH_2OBn$, R^4= H)
4a (R^1= H, R^2= ${}^tBu$, R^3= $-CH_2OBn$, R^4= H)
4b (R^1= Me, R^2= Et, R^3= $-CH_2OBn$, R^4= H)
5a (R^1= H, R^2= ${}^tBu$, R^3= $-CH_2OBn$, R^4= H)
5b (R^1= Me, R^2= Et, R^3= $-CH_2OBn$, R^4= H)

Eq. (1)

Entry[a)]	1	Additive[b)]	Time[c)] (h)	T (°C)	Yield[d)] (%)	4/5[e)]	(ee of 4/ee of 5)[f),g)]
1	**1a**	—	8	r.t.	84	1/1.6	—[h)]
2	**1a**	—	72	r.t.	63	1/2.6	—[h)]
3	**1a**	Z		r.t.	NR[e)]		—
4	**1a**	M		r.t.	NR[e)]		—
5	**1a**	MP		r.t.	NR[e)]		—
6	**1a**	L	8	r.t.	87	4.4/1	—[h)]
7	**1a**	LP	5	r.t.	91	8.4/1	(86*RS*/98*RS*)
8	**1a**	LP	48	−10	99	17/1	—[h)]
9	**1a**	H	8	r.t.	80	1/3.8	(94*RS*/92*RS*)
10	**1a**	LP	5	r.t.			
		then H	8	r.t.	86	1/4.1	—[h)]
11	**1b**	—	12	r.t.	69	5.4/1	—[h)]
12	**1b**	—	72	r.t.	91	1/1.6	—[h)]
13	**1b**	LP	5	r.t.	64	13/1	(86*RS*/88*RS*)
14	**1b**	H	8	r.t.	83	1/1.2	(88*RS*/89*RS*)

a) **2** (88%ee) was used. *b*) Z: $ZnCl_2$ (2.3 eq), M: $MgBr_2$ (2.3 eq), MP: $MgClO_4$ (2.0 eq), L: LiCl (2.0 eq), LP: $LiClO_4$ (2.0 eq), H: HMPA (3.0 eq). *c*) The reaction time at r.t. *d*) The combined isolated yield of **4** and **5**. *e*) The ratio was determined by ^{1}H-NMR analysis. *f*) Optical purity was determined by HPLC. For the condition of analysis, see Experimental Section. *g*) *RS* means (5*R*,1′*S*) in **4** or (5*R*,6*S*) in **5**. *h*) Optical purity and absolute configuration were not determined.

these experiments.

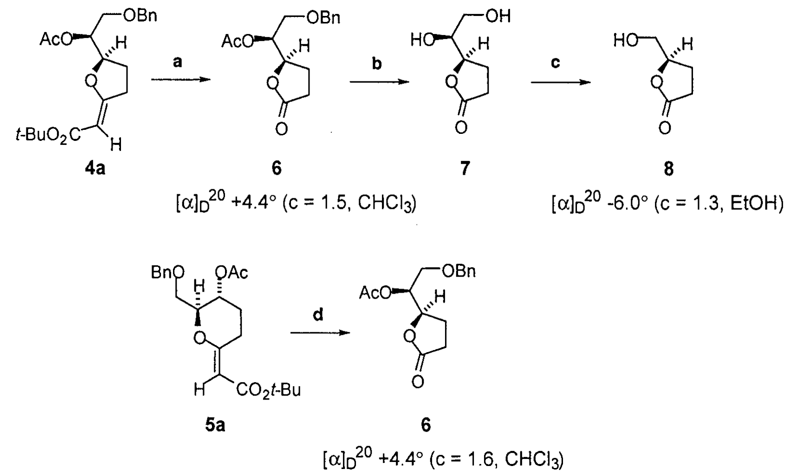
Hence, we next focused on the stereochemical relationship between the starting material **2** and the products **4**, **5** because we thought the reaction pathway of Eq.1 could be rationally explained by analyzing the optical purity and absolute configuration of these compounds.

We used optically active epoxides **2** (88%ee, (2*S*, 3*S*)),[10)] for the reaction of Eq. 1, and the reaction was quenched with Ac_2O to suppress the isomerization of initially formed products. The products, **4a**, **4b**, **5a**, **5b** were subjected to HPLC analysis to determine the ratio and optical purity (Table 1),[10)] and transforming them to the known compounds (Chart 1) elucidated the absolute configuration of the products. Thus, **4a** was first converted to γ-lactone **6** by ozonolysis, followed by removal of the protective groups, 1, 2-diol cleavage with $NaIO_4$, and $NaBH_4$ reduction of the resulting aldehyde to afford the known compound **8**.[11)] Comparing the specific rotation of **8** ($[\alpha]_D^{20}$ −6.0° (c=1.3, EtOH)) with the reported value of (*R*)-**8** ($[\alpha]_D^{20}$ −33.5° (c=3.12, EtOH),[11)] the absolute configuration of **4a** was elucidated as (5*R*,1′*S*). On the other hand, ozonolysis of **5a** resulted in unexpected isomerization to afford **6**, and the specific rotation of this compound ($[\alpha]_D^{20}$ +4.4° (c=1.6, $CHCl_3$) had the same sign of (5*R*,1′*S*)-**6** ($[\alpha]_D^{23}$ +4.4° (c=1.5, $CHCl_3$) derived from **4a**; therefore, the absolute configuration of **5a** was elucidated as (4*R*,5*S*). The optical purity and absolute configuration of the products (entries 7, 9, 13, 14) were determined as above and are summarized in Table 1.

Based on the results thus obtained that the optical purity of the products were almost as same as the starting material **2** and the absolute configuration of the asymmetric center in **2** had been retained through the reaction of Eq. 1, the reaction pathway of the reaction of Eq. 1 could be summarized as Chart 2. That is, dianion **1** reacts with epibromohydrin **2** to afford intermediate **3a**, followed by the intramolecular *O*-alkylation to give the THF derivative **4a′** exclusively, and then **4a′** isomerizes to the THP derivative **5a′** under an appropriate condition. Thus, the possibility that THP derivatives initially form and then isomerize to THF derivatives is nearly ruled out by the experiment described above using the optically active **2**. The comparison of the ^{1}H-NMR of the products with the quite similar compounds[12)] and nuclear Overhauser effect technique determined the geometry of the products and, interestingly, no *Z*-form of **5** was observed in all cases. The reason for this *E* to *Z* selective transformation is not explained clearly yet.

In conclusion, a highly efficient one-pot preparation method of some novel chiral synthons, THF and THP derivatives, was successfully developed. The optical purity of the products obtained by this method is almost the same as that of the epibromohydrins used or better, and the absolute configuration of the products is predictable. Hence, some chiral synthons described in this paper are useful for the synthesis of bioactive natural products, and the reaction of Eq. 1 would offer a convenient one-pot method for the preparation of other similar THF, and THP derivatives with high optical purity.

In our laboratory we are now examining utility of these



Reagents and Conditions; a. O_3, CH_2Cl_2, −78 °C; then Zn, AcOH, 81%; b. MeOLi (1.0 eq), MeOH, 0 °C; then H_2, Pd/C, AcOEt, r.t., 52% (2 steps); c. $NaIO_4$, silica gel, H_2O, CH_2Cl_2, r.t.; then $NaBH_4$ (1.0 eq), MeOH, 0 °C, 35% (2 steps); d. O_3, CH_2Cl_2, −78 °C; then Zn, AcOH, 72%.

Chart 1

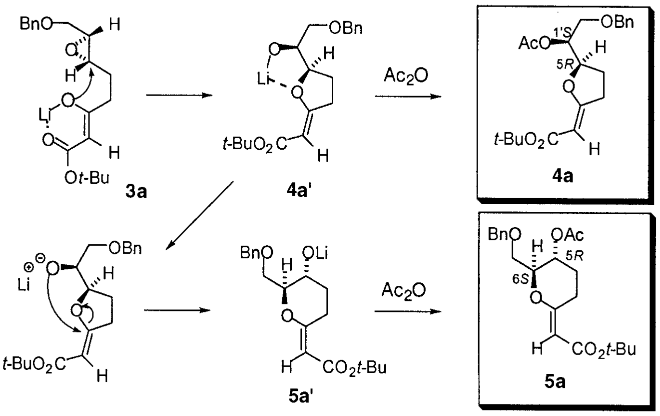


Chart 2. Proposed Reaction Pathway of the Reaction of Eq. 1

chiral synthons, THF and THP derivatives, for natural product synthesis, and our progress will be reported in due course.

Experimental

All reactions were carried out under an argon atmosphere with dry, freshly distilled solvents under anhydrous conditions, unless otherwise noted. THF was distilled from sodium–benzophenon, and methylene chloride (CH_2Cl_2), benzene, and toluene from calcium hydride. Yields refer to chromatographically and spectroscopically (^{1}H-NMR) homogenous materials, unless otherwise stated. All solutions used in workup procedures are saturated unless otherwise noted. All reagents were purchased at highest commercial quality and used without further purification unless otherwise stated.

All reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as visualizing agent and phosphomolybdic acid and heat as developing agents. E. Merck silica gel (60, particle size 0.040—0.063 mm) was used for flash column chromatography. Preparative TLC (PTLC) separations were carried out on self-made 0.3 mm E. Merck silica gel plates (60F-254).

NMR spectra were recorded on JEOL EX-270, AL-400, Lambda500, or Brucker AVANCE600 instruments and calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; band, several overlapping signals; br, broad. IR spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer. Optical rotations were recorded on a JASCO DIP-1000 polarimeter. High-resolution mass spectra (HR-MS) were recorded on a JMS-SX102A mass spectrometer under fast atom bombardment (FAB) conditions. Melting points (mp) are uncorrected, recorded on a Yamato capillary melting point apparatus. HPLC was carried out using JASCO PU-980 HPLC pump and UV-970 Detector. Epibromohydrin derivative **2** was prepared according to the published procedure (ref. 2a).

(5*R*)-2-(*Z*)-*tert*-Butoxycarbonylmethylidene-5-[(1*S*)-1-acetyloxy-2-benzyloxyethyl]tetrahydrofuran 4a, (5*R*,6*S*)-5-Acetyloxy-6-benzyloxymethyl-2-(*E*)-*tert*-butoxycarbonylmethylidenetetrahydropyran 5a (Table 1, Entry 7) To an agitated suspension of NaH (123.4 mg, 2.3 eq) in dry THF (30.0 ml) using a magnetic stirring bar and stirrer was added *tert*-butyl acetoacetate (0.78 ml, 2.1 eq) dropwise at 0 °C under Ar atmosphere and the resulted solution was stirred for 10 min. Then *n*-BuLi (2.85 ml, 1.57 M soln. in hexane, 2.0 eq) was added slowly to the reaction mixture at 0 °C and the stirring was continued further for 10 min. The resulted pale yellow dianion solution was cooled to −60 °C and to this solution was added first $LiClO_4$ (4.47 ml, 1.00 M soln. in Et_2O, 2.0 eq), and then **2** (575.2 mg, 1.0 eq) in dry THF (5.0 ml) dropwise *via* a canula. The flask of the solution of **2** was washed with dry THF (5.0 ml) and the washings were added to the reaction mixture *via* a canula (×2). After the addition of **2**, the reaction temperature was raised to −40 °C and the reaction mixture was stirred for 12 h. At this point **2** almost disappeared. Then the reaction mixture was warmed to r.t. and the stirring was continued for 5 h to complete the reaction. To the reaction mixture was added *ca.* 1.0 ml of Ac_2O to stop the reaction, and then the reaction was quenched with sat. NH_4Cl aq. The aqueous layer was extracted with ether, then CH_2Cl_2 (×2). The combined organic layer was washed with sat. $NaHCO_3$ aq., then with brine, dried with anhydrous Na_2SO_4, concentrated and dried under vacuum. Purification by silica gel chromatography (hexane : EtOAc = 8 : 1) afforded 682.7 mg (81%) of **4a** and 80.3 mg (9.6%) of **5a**.

4a: $[\alpha]_D^{25}$ +37.5° (c=1.2, $CHCl_3$); IR (thin film) ν_{max}: 2980, 2932, 2876, 1750, 1710, 1688, 1656, 1498, 1478, 1454, 1434, 1366, 1300 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.36—7.26 (5H, m), 5.22 (1H, dt, J=4.9, 5.1 Hz), 4.78 (1H, dt, J=5.1, 6.8 Hz), 4.77 (1H, t, J=1.7 Hz), 4.56 (1H, d, J=12.2 Hz), 4.54 (1H, d, J=12.2 Hz), 3.73 (2H, d, J=4.9 Hz), 2.76—2.58 (2H, m), 2.13—1.90 (2H, m), 2.07 (3H, s), 1.46 (9H, s); FAB-HR-MS m/z: 377.1962 (Calcd for $C_{21}H_{28}O_6+H^+$: 377.1964); HPLC Analysis (Daicel Chiral Cell OD-H 0.46 cmϕ×25 cm; hexane : isopropanol = 49 : 1; flow rate = 0.4 ml/min); retention time: 56 min for (5*R*,1*S*), 60 min for (5*S*,1*R*).

5a: $[\alpha]_D^{21}$ −27.0° (c=1.0, $CHCl_3$); IR (thin film) ν_{max}: 2980, 2936, 2876, 1750, 1702, 1642, 1562, 1498, 1480, 1456, 1428, 1370, 1328, 1304 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.34—7.28 (5H, m), 5.23 (1H, t, J=1.7 Hz), 5.18 (1H, ddd, J=4.9, 5.1, 5.1 Hz), 4.60 (1H, dt, J=5.1, 7.1 Hz), 4.54 (1H, d, J=12.5 Hz), 4.53 (1H, d, J=12.5 Hz), 3.64 (1H, dd, J=4.9, 10.7 Hz), 3.59 (1H, dd, J=5.1, 10.7 Hz), 3.20 (1H, dddd, J=1.7, 5.1, 9.3, 18.3 Hz), 2.95 (1H, dddd, J=1.7, 8.2, 9.8, 18.3 Hz), 2.18—1.95 (2H, m), 2.07 (3H, s), 1.46 (9H, s); FAB-HR-MS m/z: 377.1988 (Calcd for $C_{21}H_{28}O_6+H^+$: 377.1964); HPLC analysis (Daicel Chiral Cell OD-H 0.46 cmϕ×25 cm; hexane : isopropanol = 9 : 1; flow rate = 0.4 ml/min); retention time: 19 min for (5*R*,6*S*), 33 min for (5*S*,6*R*).

(5*R*)-2-[(*Z*)-Ethoxycarbonyl-1-ethylidene]-5-[(1*S*)-1-acetyloxy-2-ben-

zyloxyethyl]tetrahydrofuran 4b, (5*R*,6*S*)-5-Acetyloxy-6-benzyloxymethyl-2-[(*E*)-*tert*-butoxycarbonyl-1-ethylidene]tetrahydropyran 5b (Table 1, Entry 13) To an agitated suspension of NaH (90.4 mg, 2.3 eq) in dry THF (20.0 ml) using a magnetic stirring bar and stirrer was added ethyl 2-methylacetoacetate (0.50 ml, 2.1 eq) dropwise at 0 °C under Ar atmosphere and the resulted solution was stirred for 10 min. Then *n*-BuLi (2.05 ml, 1.60 M soln. in hexane, 2.0 eq) was added slowly to the reaction mixture at 0 °C and the stirring was continued further for 10 min. The resulted pale yellow dianion solution was cooled to −60 °C and to this solution was added first $LiClO_4$ (3.27 ml, 1.00 M soln. in Et_2O, 2.0 eq), and then **2** (421.0 mg, 1.0 eq) in dry THF (5.0 ml) dropwise *via* a canula. The flask of the solution of **2** was washed with dry THF (5.0 ml) and the washings were added to the reaction mixture *via* a canula (×2). After the addition of **2**, the reaction temperature was raised to −40 °C and the reaction mixture was stirred for 12 h. At this point **2** almost disappeared. Then the reaction mixture was warmed to r.t. and the stirring was continued for 5 h to complete the reaction. To the reaction mixture was added *ca.* 0.8 ml of Ac_2O to stop the reaction, and then the reaction was quenched with sat. NH_4Cl aq. The aqueous layer was extracted with ether, then CH_2Cl_2 (×2). The combined organic layer was washed with sat. $NaHCO_3$ aq., then with brine, dried with anhydrous Na_2SO_4, concentrated and dried under vacuum. Purification by silica gel chromatography (hexane : EtOAc=8 : 1) afforded 353.8 mg (59.4%) of **4b** and 27.2 mg (4.6%) of **5b**.

4b: $[\alpha]_D^{23}$ +19.0° (c=1.4, $CHCl_3$); IR (thin film) ν_{max}: 2984, 2944, 1744, 1714, 1680, 1650, 1560, 1498, 1456, 1374, 1334, 1304 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$) δ: 7.36—7.27 (5H, m), 5.16 (1H, dt, J=4.4, 6.6 Hz), 4.71 (1H, dt, J=6.6, 6.8 Hz), 4.55 (1H, d, J=12.2 Hz), 4.54 (1H, d, J=12.2 Hz), 4.19 (2H, q, J=7.1 Hz), 3.73 (2H, d, J=4.4 Hz), 2.82—2.62 (2H, m), 2.17—1.90 (2H, m), 2.07 (3H, s), 1.76 (3H, t, J=1.7 Hz), 1.27 (3H, t, J=7.1 Hz); FAB-HR-MS *m/z*: 363.1763 (Calcd for $C_{20}H_{26}O_6+H^+$: 363.1807); HPLC analysis (Daicel Chiral Cell OD-H 0.46 cmϕ×25 cm; hexane : isopropanol= 49 : 1; flow rate=0.4 ml/min); retention time: 64 min for (5*R*,1*S*), 69 min for (5*S*,1*R*).

5b: $[\alpha]_D^{22}$ −22.7° (c=1.1, $CHCl_3$); IR (thin film) ν_{max}: 2984, 2936, 2872, 1750, 1702, 1642, 1540, 1498, 1456, 1370, 1294 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$) δ: 7.36—7.27 (5H, m), 5.19 (1H, ddd, J=4.9, 5.1, 5.1 Hz), 4.61 (1H, dt, J=5.1, 7.1 Hz), 4.57 (1H, d, J=12.0 Hz), 4.53 (1H, d, J=12.0 Hz), 4.15 (2H, q, J=7.1 Hz), 3.66 (1H, dd, J=4.9, 10.7 Hz), 3.63 (1H, dd, J=5.1, 10.7 Hz), 3.19 (1H, dddd, J=1.7, 5.1, 8.1, 18.3 Hz), 2.94 (1H, dddd, J=1.7, 8.1, 9.5, 18.3 Hz), 2.18—1.91 (2H, m), 2.07 (3H, s), 1.77 (3H, t, J=1.7 Hz), 1.27 (3H, t, J=7.1 Hz); FAB-HR-MS *m/z*: 363.1798 (Calcd for $C_{20}H_{26}O_6+H^+$: 363.1807) HPLC analysis (Daicel Chiral Cell OD-H 0.46 cmϕ×25 cm; hexane : isopropanol=9 : 1; flow rate=0.4 ml/min); retention time: 18 min for (5*S*,6*R*), 23 min for (5*R*,6*S*).

(5*R*)-5-[(1*S*)-1-Acetyloxy-2-benzyloxyethyl]butyrolactone 6 A solution of **4a** (844.0 mg, 1.0 eq) in CH_2Cl_2 (5 ml) was cooled to −78 °C, and a flow of O_3 was introduced into the solution with stirring. After the reaction completed (1 h; The disappearance of **4** was checked by TLC.), O_3 remaining in the solution was purged off by passing O_2, and then zinc dust (*ca.* 200 mg) and 50% acetic acid (1.5 ml) was added to the reaction mixture. The mixture was warmed to room temperature with stirring and the stirring was continued for further 1 h. Cold water was added to the mixture, and the aqueous layer was extracted with ether, then CH_2Cl_2 (×2). The combined organic layer was washed with sat. $NaHCO_3$ aq., then with brine, dried with anhydrous Na_2SO_4, concentrated and dried under vacuum. Purification by silica gel chromatography (hexane : EtOAc=8 : 1) afforded 526.0 mg (81%) of **6**.

6: $[\alpha]_D^{22}$ +4.4° (c=1.6, $CHCl_3$); IR (thin film) ν_{max}: 2948, 2872, 1782, 1744, 1500, 1456, 1420, 1374, 1334, 1234 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$) δ: 7.37—7.28 (5H, m), 5.19 (1H, ddd, J=4.8, 4.8, 5.1 Hz), 4.76 (1H, ddd, J=4.8, 7.0, 7.3 Hz), 4.53 (1H, d, J=12.1 Hz), 4.51 (1H, d, J=12.1 Hz), 3.68 (1H, dd, J=4.8, 10.6 Hz), 3.63 (1H, dd, J=5.1, 10.6 Hz), 2.61—2.45 (2H, m), 2.35—2.16 (2H, m), 2.09 (3H, s); FAB HRMS *m/z*: 279.1198 (Calcd for $C_{15}H_{18}O_5+H^+$: 279.1232).

(5*R*)-5-[(1*S*)-1,2-Dihydroxyethyl]butyrolactone 7 A solution of MeOLi in MeOH, prepared from *n*-BuLi (0.95 ml, 1.57 M soln. in hexane, 1.0 eq) and MeOH, was added dropwise to a stirred solution of **6** (413.7 mg) in MeOH (10 ml) at 0 °C. After 1 h the reaction was quenched with sat. NH_4Cl aq. The aqueous layer was extracted with ether, then CH_2Cl_2 (×2). The combined organic layer was washed with brine, dried with anhydrous Na_2SO_4 concentrated and dried under vacuum. The crude alcohol thus obtained was used for next step without purification.

The crude alcohol obtained as described above was dissolved in ethyl acetate (30 ml) and the solution was stirred with 10% palladium on carbon (catalytic amount) at room temperature under an atmosphere of hydrogen for 90 min. After the completion of the reaction, the reaction mixture was filtered and the filtrate was concentrated to afford crude **7** as colorless oil. Purification by silica gel chromatography (CH_2Cl_2 : MeOH=10 : 1) afforded 113.0 mg (52%, 2 steps) of pure **7**.

7: $[\alpha]_D^{22}$ −15.1° (c=1.2, $CHCl_3$); IR (thin film) ν_{max}: 3412, 2948, 1776, 1762, 1462, 1418, 1350, 1306 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$) δ: 4.54 (1H, dt, J=4.9, 7.1 Hz), 3.92 (1H, dddd, J=3.9, 4.9, 5.1, 6.3 Hz), 3.75 (1H, ddd, J=3.9, 6.3, 11.5 Hz), 3.63 (1H, ddd, J=5.1, 6.3, 11.5 Hz), 3.44 (1H, d, J=6.3 Hz), 2.78 (1H, t, J=5.1 Hz), 2.62 (1H, ddd, J=7.5, 15.6, 18.1 Hz), 2.55 (1H, ddd, J=8.3, 9.5, 18.1 Hz), 2.31—2.25 (2H, m); FAB-HR-MS *m/z*: 147.0636 (Calcd for $C_6H_{10}O_4+H^+$: 147.0657).

(5*R*)-5-Hydroxymethyl-2-oxotetrahydrofuran 8 A solution of $NaIO_4$ (110.6 mg, 1.0 eq) in water (1.0 ml) was added dropwise to a vigorously stirred suspension of silica gel (1.0 g) in CH_2Cl_2 (8.0 ml) at room temperature. Then a solution of **7** (75.5 mg, 1.0 eq) in CH_2Cl_2 (1.0 ml) was added dropwise to the suspension at 0 °C. After the reaction was completed, the reaction mixture was filtered and the residue was washed with CH_2Cl_2. The filtrate was combined, concentrated and dried under vacuum. The crude aldehyde obtained was used for next step immediately.

To the crude aldehyde dissolved in MeOH (10 ml) was added $NaBH_4$ (21.7 mg, 1.0 eq) portionwise at 0 °C. After 10 min, the reaction was quenched with sat. $KHSO_4$ aq., and the reaction mixture was evaporated under vacuum to remove MeOH. After adding dry THF to the residue, the resulted suspension was filtered and the filtrate was concentrated to afford crude **8** as colorless oil. Purification by silica gel chromatography (CH_2Cl_2 : MeOH=20 : 1) afforded 21.0 mg (35% 2 steps) of **8**.

8: $[\alpha]_D^{22}$ −6.03° (c=1.3, EtOH); IR (thin film) ν_{max}: 3488, 2944, 1772, 1462, 1420, 1358, 1294 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$) δ: 4.64 (1H, dddd, J=2.9, 4.6, 6.9, 7.6 Hz), 3.91 (1H, ddd, J=2.9, 6.3, 12.5 Hz), 3.66 (1H, ddd, J=4.6, 6.3, 12.5 Hz), 2.63 (1H, ddd, J=5.9, 10.0, 17.8 Hz), 2.56 (1H, ddd, J=8.3, 9.8, 17.8 Hz), 2.49 (1H, t, J=6.3 Hz), 2.28 (1H, dddd, J=5.9, 7.6, 9.8, 18.3 Hz), 2.16 (1H, dddd, J=6.9, 8.3, 10.0, 18.3 Hz); FAB HRMS *m/z*: 117.0573 (Calcd for $C_5H_8O_3+H^+$: 117.0552).

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A Catalytic Asymmetric Strecker-Type Reaction Promoted by Lewis Acid–Lewis Base Bifunctional Catalyst

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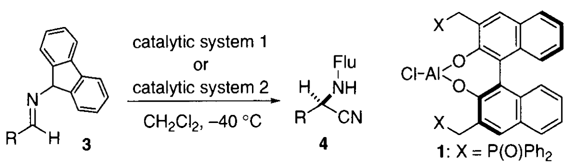
A general asymmetric Strecker-type reaction is reported, catalyzed by the Lewis acid–Lewis base bifunctional catalyst 1. The reaction of trimethylsilyl cyanide (TMSCN) with various fluorenyl imines, including *n*-aldimines and α,β-unsaturated imines, proceeds with good to excellent enantioselectivities in the presence of a catalytic amount of phenol as additive (20 mol%) (catalytic system 1). The products were successfully converted to the corresponding amino acid derivatives in high yields without loss of enantiomeric purity. Furthermore, hydrogenation or dihydroxylation of the products from α,β-unsaturated imines afforded saturated or functionalized aminonitriles also without loss of enantiomeric purity. The absolute configuration of the products and a control experiment using catalyst 2 supported the proposed dual activation of the imine and TMSCN by the Lewis acid (Al) and the Lewis base moiety (phosphine oxide) of 1. From the mechanistic studies including kinetic and NMR experiments of the catalytic species, the role of PhOH seems to be a proton source to protonate the anionic nitrogen of the intermediate. Specifically, we have found that TMSCN is more reactive than HCN in this catalytic system, probably due to the activation ability of the phosphine oxide moiety of 1 toward TMSCN. This fact prompted us to develop the novel catalytic system 2, consisting of 1 (9 mol%), TMSCN (20 mol%) and HCN (1.2 mol eq). This new system afforded comparable results with obtained by system 1 (1 (9 mol%)–TMSCN (2 mol eq)–PhOH (20 mol%)).

Key words asymmetric catalysis; Strecker-type reaction; bifunctional catalyst; Lewis acid; Lewis base; amino acid

The catalytic asymmetric Strecker-type reaction[1] is one of the most direct and efficient methods for the asymmetric synthesis of natural and unnatural α-amino acids. Recently, several excellent reactions of this type have been reported, reflecting the importance of this field.[2] We have been involved in this area, since we found that the Lewis acid–Lewis base bifunctional catalysis could provide a fundamental concept to design an asymmetric catalyst for cyanosilylation of aldehydes.[3] The bifunctional catalyst **1** promoted the addition of trimethylsilyl cyanide (TMSCN) to a variety of aldehydes with high enantioselectivities. The origin of the highly enantioselective catalysis by **1** is the simultaneous activation of the aldehydes and TMSCN by the Lewis acid (Al) and the oxygen atom of the phosphine oxide, respectively. Therefore, it seemed to be a rational extension to apply this catalyst to the development of an asymmetric Strecker-type reaction (the addition of TMSCN to imines). In this paper, we report that **1** is indeed a general catalyst for the asymmetric Strecker-type reaction (Chart 1). The reaction proceeds with good to excellent enantioselectivities toward various imines, including *n*-aldimines and α,β-unsaturated imines, in the presence of a catalytic amount of phenol (20 mol%) (catalytic system 1). Furthermore, we have found that TMSCN is more reactive than HCN in the reaction catalyzed by **1**, which led to the idea to develop a novel catalytic system consisting of **1** (9 mol%), TMSCN (20 mol%) and HCN (120 mol%) (catalytic system 2). Based on these reactions, α-amino acid derivatives, including those containing functionalized side chains, can be prepared efficiently.[4]

Optimization of the Reaction Using Catalytic System 1 Starting this project, we observed a dramatic effect of the substituent on the nitrogen atom of imines on the enantioselectivity (Table 1). Although the reaction of TMSCN (2 mol eq) with the *N*-allyl benzaldehyde imine catalyzed by 9 mol% of **1** at −40 °C gave the product with only 4% ee in 67% yield (entry 1), the reaction of *N*-benzhydryl imine gave the product with 78% ee in 84% yield (entry 2). The ee was further increased up to 95% (97% yield) by the reaction with *N*-fluorenyl imine **3a** (entry 4). More bulky *N*-triphenylmethyl imine did not afford the product even at room temperature (entry 6). As a result, the best substituent on the nitrogen atom for this reaction seemed to be the fluorenyl group, which is also effective for the aliphatic pivalaldehyde imine **3m** to give the corresponding aminonitrile **4m** in 75% ee in 94% yield. As will be discussed later, the substituent on the nitrogen atom should be close to the naphthyl moiety of the catalyst in the transition state (see **7** in Chart 2). The fluorenyl group might be important for stabilizing this desired transition state possibly by π stacking interaction, thus giving the best ee's.



1: X = P(O)Ph_2
2: X = $CHPh_2$

catalytic system 1: **1** (9 mol %), TMSCN (2 mol eq)
PhOH (20 mol %, slow addition over 17 h)
catalytic system 2: **1** (9 mol %), TMSCN (20 mol %)
HCN (1.2 mol eq, slow addition over 24 h)

Chart 1

In order to increase the reaction efficiency by facilitating the reaction rate, we investigated the effect of additives and found that protic additives such as alcohols and phenol afforded a beneficial effect on the reaction rate (Table 2).[5] Thus, by slowly (12 h) adding[6] 110 mol% of MeOH, iso-

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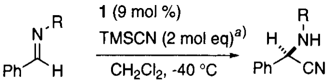
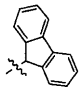
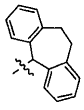
Dedicated to the memory of Dr. Kyosuke Tsuda.

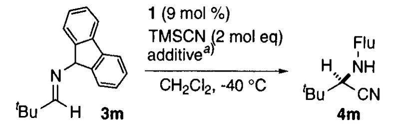
Table 1. Effect of the Substituent of Nitrogen

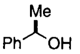
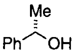


Entry	R	Time (h)	Yield (%)$^{b)}$	ee (%)$^{c)}$
1	Allyl	62	67	4$^{d)}$
2	$CHPh_2$	85	84	78
3	$CH(PhOMe\text{-}p)_2$	90	80	70$^{d)}$
4		111	97	95
5		85	73	65$^{d)}$
6	CPh_3	>170	Trace	—

a) Slowly added over 10 h. *b*) Isolated yield. *c*) Determined by HPLC analysis. *d*) The absolute configuration was not determined.

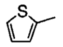
Table 2. Effect of the Proton Source



Entry	Additive (eq)	Time (h)	Yield (%)$^{b)}$	ee (%)$^{c)}$
1	—	192	94	75
2	MeOH (1.1)	21	94	66
3	Iso-PrOH (1.1)	40	96	68
4	*tert*-BuOH (1.1)	21	97	72
5	PhOH (1.1)	22	99	78
6	PhOH (0.2)	44	97	78
7	 (1.1)	13	90	64
8	 (1.1)	13	91	67

a) Slowly added over 12 h. *b*) Isolated yield. *c*) Determined by HPLC analysis.

Table 3. Catalytic Asymmetric Strecker-Type Reaction of Various Imines$^{a)}$

Entry	R	3a—m	System 1			Entry	System 2		
			Time (h)	Yield (%)$^{b)}$	ee (%)$^{c)}$		Time (h)	Yield (%)$^{b)}$	ee (%)$^{c)}$
1	Ph	**a**	44	92	95	14	36	92	95
2	*p*-ClPh	**b**	44	92	95				
3	*p*-MeOPh	**c**	44	93	93				
4	1-Naphthyl	**d**	68	95	89				
5	2-Furyl	**e**	44	93	79				
6	3-Furyl	**f**	44	92	90	15	36	92	87
7		**g**	58	90	89				
8	*trans*-PhCH=CH	**h**	41	80	96	16	36	78	92
9	*trans*-$CH_3(CH_2)_3CH{=}CH$	**i**	24	66	86$^{d)}$				
10	$CH_3(CH_2)_5$	**j**	24	80	80$^{e)}$	17	36	75	81
11	CH_3CH_2	**k**	44	84	70				
12	Iso-Pr	**l**	44	89	72	18	36	92	71
13	*tert*-Bu	**m**	44	97	78	19	36	96	77

a) The method for preparation of the catalyst and the general procedure of the reaction, see Experimental Section. *b*) Isolated yield. *c*) Determined by HPLC analysis. *d*) 50 mol% of PhOH was used. The aminonitrile was isolated as the corresponding trifluoroacetamide. *e*) Without PhOH.

PrOH, *tert*-BuOH, or PhOH, all reactions using **3m** were completed after 22 h, giving the product **4m** in more than 94% yield and with 66, 68, 72, and 78% ee, respectively (entries 2—5). Although the ee of the product varied from 66 to 78% depending on the additive, the following results suggested that the additive does not play a major role in the enantioface selection step. First, when the chiral alcohol 2-phenylethanol was used as an additive, both the *R* and *S* isomer afforded *R*-**4m** in almost the same enantioselectivity (64 and 67% ee, respectively) and yield (90, 91%) (entries 7, 8). Second, the ^{31}P-NMR spectra of the catalyst in the absence or presence of PhOH (20 mol%) was exactly the same under the reaction conditions (δ=42.2, 51.2 ppm), thus suggesting a negligible interaction between the catalyst and the additive. Therefore, the protic additive seems to facilitate the reaction without changing the catalytic species.

We next investigated the possibility of promoting the reaction using a catalytic amount of the best additive (PhOH) without diminishing the synthetic utility of this reaction. Gratifyingly, even when the amount of PhOH was reduced to 20 mol%, the reaction was completed after 44 h to give **4m** in 97% yield with 78% ee (entry 6). Consequently, the effective reaction conditions were found to involve the slow addition (17 h) of PhOH (20 mol%) to a mixture of **1** (9 mol%), the imine and TMSCN (2 mol eq) (catalytic system 1).

A variety of *N*-fluorenyl aldimines were examined as substrates for this optimized catalytic asymmetric Strecker-type reaction, and the results are shown in Table 3 (entries 1—13). Aromatic aldimines including heterocyclic aldimines, α,β-unsaturated aldimines, as well as aliphtic aldimines can be converted to Strecker products in excellent yields with good to excellent enantioselectivities.

Mechanistic Studies In order to gain some insight into the reaction mechanism, the kinetic profile of the reaction was investigated. After adding PhOH in one portion,[7] the reaction was monitored by observing the disappearance of the imine proton of **3a** (δ=8.58 ppm). The results are shown in Fig. 1. The initial reaction rate in the presence of 20 mol% of PhOH (●, initial steep dotted line) was 82 times faster than in the absence of PhOH (■). After about 20% consumption of the starting imine (*ca.* 80 min), which corresponded to the complete consumption of PhOH to TMSOPh, the reaction

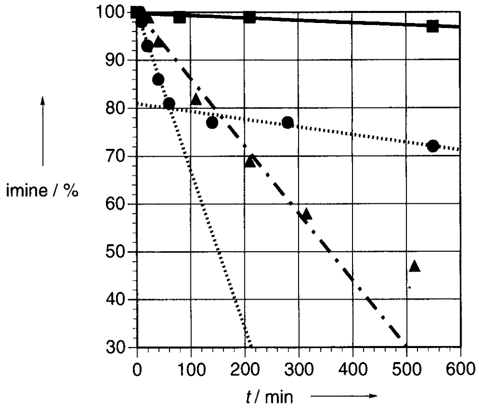


Fig. 1. Initial Reaction Rate of **3a** in the Presence of Various Amounts of PhOH

The disappearance of **3a** was traced by ^{1}H-NMR: the reaction of TMSCN in the absence (■) and in the presence of 20 mol% (●) of PhOH, and the reaction of HCN (▲).

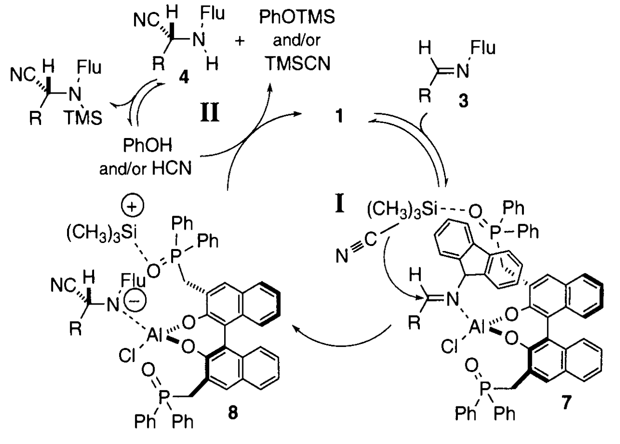


Chart 2. Working Model for the Catalytic Cycle

entered a slower phase (●, after 80 min). However, even in this slower phase, the reaction rate was *ca.* 2 times faster than in the absence of PhOH. This may be due to the re-generation of a very small amount of PhOH from TMSOPh and the product amine **4a**. This re-generation of the proton source was also suggested from the fact that the initial reaction rate in the presence of 20 mol% of **4a** and TMSOPh was *ca.* 2 times faster than in the absence of these additives.

Since other precedents used HCN or Bu_3SnCN as the nucleophile, we were interested in determining the active nucleophile in this catalytic system. Adding PhOH in one portion, the generation of HCN which could work as the active nucleophile, was observed by ^{1}H-NMR. However, when HCN (2 mol eq) was added in one portion, in the absence of TMSCN, the initial reaction rate was 0.4 times slower (Fig. 1, ▲) than when TMSCN was used in the presence of 20 mol% of PhOH, and the ee value of the product **4a** was only 53%. Furthermore, under the slow addition (26 h) conditions of HCN, which should better represent the best reaction conditions using TMSCN and slow addition of PhOH, **4a** was obtained in 54% yield with 28% ee after 85 h. These results reveal the reactive nucleophile to be TMSCN. Although HCN may be generated under the reaction conditions by the reaction of TMSCN with PhOH,[8] the highly enantioselective pathway with TMSCN as an active nucleophile predominates, since the reaction rate with TMSCN is faster than with HCN in this catalytic system. Thus, the described reaction is the first example of a catalytic asymmetric Strecker-type reaction with TMSCN as an active nucleophile. This unique feature of the catalytic system using **1** may be derived from the activation ability of the Lewis basic phosphine oxide moiety of the bifunctional catalyst **1** toward TMSCN.[3] These mechanistic studies suggest that PhOH and/or HCN should work as a proton source to protonate the negative charge on the nitrogen atom which is generated by the addition of CN to the imine, thus accelerating the formation of **4** (Chart 2). A small amount of the proton source would be re-generated *via* the cycle II, thus significantly accelerating the reaction rate by using even a catalytic amount of PhOH.

Meanwhile, the dual activation mechanism by the bifunctional catalyst **1** seems to be supported by the following results. The absolute configuration of the products can be explained from the working model shown as **7** in Chart 2. The Lewis acid (Al) and the Lewis base (phosphine oxide) activate the imine and TMSCN, respectively, at defined positions thus affording *R*-products. Furthermore, a control catalyst **2**, containing the diphenylmethyl group which should work only as a steric hindrance, afforded the opposite enantiomer *S*-**4a** with 15% ee in 100% yield (42 h), using 20 mol% of PhOH. Therefore, in the case of **1**, TMSCN seems to attack the activated imine from the side of the phosphine oxide moiety.

Catalytic Asymmetric Reaction Using System 2 Taking the advantage of the intriguing reactivity difference between TMSCN and HCN in this catalytic reaction, we expected that, if HCN was used instead of PhOH, it would be possible to reduce the amount of TMSCN. After the attack of TMSCN and protonation of the resulting intermediate **8** in Chart 2 by HCN, TMSCN should be re-generated, which should again work as the nucleophile. Since the reaction of HCN with the imine might compete with the desired reaction of TMSCN as the nucleophile, we expected that it would be important to keep the concentration of HCN low enough by slow addition of HCN. Thus, using 20 mol% of TMSCN and slowly adding the solution of HCN (120 mol%) in CH_2Cl_2 (system 2), we could obtain the products with comparable results as by the TMSCN–PhOH system (system 1) (Table 3, entries 14—19). These results demonstrate the great potential for the application of this catalytic asymmetric reaction to a large-scale Strecker-type synthesis using HCN as a stoichiometric cyanide source, optimizing the amount of TMSCN and the addition time of HCN.

Conversion to α-Amino Acid Derivatives Since the fluorenyl group has not often been used as a protecting group for the nitrogen atom, we did some studies to find a procedure to deprotect this group without racemization. Our idea was to oxidize the amine to fluorenone-derived imine, followed by hydrolysis (Chart 3). However, when the aromatic aminonitrile **4a** was directly treated under oxidation conditions (MnO_2, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), ceric ammonium nitrate (CAN), or *N*-bromosuccinimide (NBS)), the target imine was not obtained at all. So, we first hydrolyzed **4a** (95% ee) to the amide **9** (HCl (g)/HCO_2H, r.t., 1 h). Recrystallization from tetrahydrofuran (THF)/ether gave the enantiomerically pure **9** in 90% yield. Oxidation of **9** by DDQ gave the corresponding fluorenone-derived imine **10**, which was converted to the amide **11** in

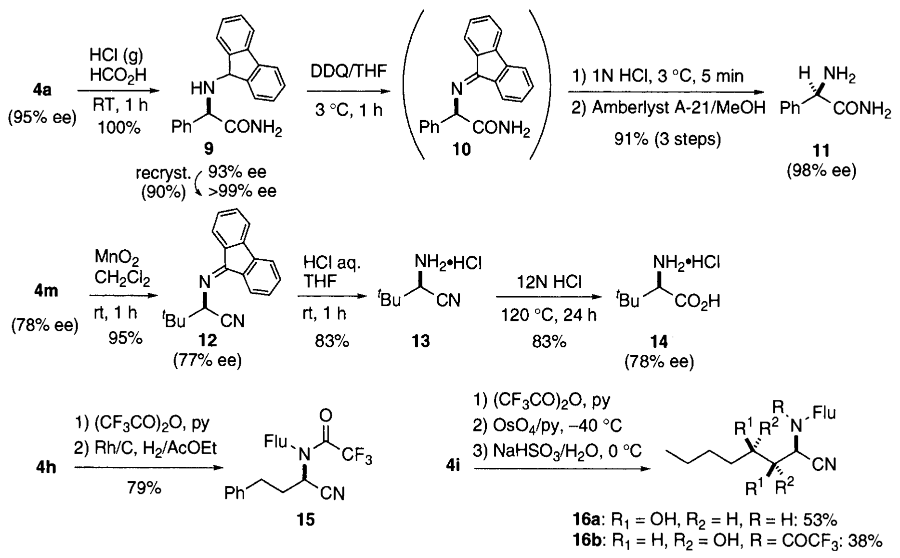


Chart 3. Conversion to α-Amino Acid Derivatives and Functionalization of α,β-Unsaturated Strecker-Products

91% yield by acid hydrolysis. Enantiomeric purity of **11** was determined to be 98% ee after conversion to the corresponding urethane by *p*-nitrobenzyloxycarbonyl chloride. In the case of the aliphatic aminonitrile **4m** (78% ee), direct oxidation by activated MnO_2 gave the imine **12** in 95% yield with 77% ee. After acid hydrolysis to the corresponding aminonitrile **13**, the nitrile group was hydrolyzed to the amino acid **14**. The enantiomeric purity of **14** was determined after conversion to the protected form (*N*-9-fluorenylmethoxycarbonyl (*N*-Fmoc), methyl ester) to be 78%. Therefore, efficient routes for the conversion of the Strecker products were established. In the same way, **4k** was converted to the α-amino acid derivative. The absolute configurations were determined by comparing the optical rotations with the reported values.

We were able to perform other important conversions as the hydrogenation and the dihydroxylation of α,β-unsaturated aminonitriles **4h** and **4i**. Direct hydrogenation of **4h** (96% ee) catalyzed by Pd/C afforded the corresponding saturated aminonitrile with complete racemization. This result should be due to the migration of the olefin to the thermodynamically more stable enamine, followed by reduction. When **4h** was treated with other transition metals such as $(Ph_3P)_3RhCl$ or Raney Ni under hydrogen atmosphere, elimination of HCN and hydrogenation of the resulting imine occurred to give the secondary amine. Therefore, we tried the hydrogenation after protection of the amine as the trifluoroacetamide. Fortunately, hydrogenation of the protected amidonitrile by Rh/C afforded the saturated **15** in 79% yield (2 steps) without any loss of enantiomeric purity. In addition, dihydroxylation of the trifluoroacetamide, derived from **4i**, afforded the amidonitriles **16a** and **16b** with functionalized side chains. These results clearly demonstrate the utility of this catalytic asymmetric Strecker-type reaction to synthesize a wide variety of natural and unnatural α-amino acid derivatives.

Conclusion

In summary, the Lewis acid–Lewis base bifunctional catalyst **1** is shown to be a general catalyst for the catalytic asymmetric Strecker-type reaction. Products were efficiently converted to α-amino acid derivatives without loss of enantiomeric purities. Specifically, α,β-unsaturated aminonitriles with a high enantiomeric excess were obtained and successfully converted to the *n*- or functionalized aminonitriles. It was found that TMSCN is more reactive than HCN in the presence of 20 mol% of PhOH, which made it possible the unique catalytic system 2 using a catalytic amount of TMSCN and stoichiometric amount of HCN. The preliminary studies to elucidate the reaction mechanism using the control catalyst **2** suggested that the bifunctional catalyst **1** should promote the reaction *via* the dual activation of the imine and TMSCN by Al and the oxygen atom of the phosphine oxide, respectively. These results demonstrate that the bifunctional catalysis should become a fundamental concept to design asymmetric catalysts.

Experimental

General NMR spectra were recorded on a JEOL JNM-LA500 spectrometer, operating at 500 MHz for ^1H-NMR and 125.65 MHz for ^{13}C-NMR. Chemical shifts in $CDCl_3$ were reported downfield from tetramethylsilane (TMS) (=0) for ^1H-NMR. For ^{13}C-NMR, chemical shifts were reported in the scale relative to $CHCl_3$ (77.00 ppm for ^{13}C-NMR) as an internal reference. Optical rotations were measured on a JASCO P-1010 polarimeter. Column chromatography were performed with silica gel Merck 60 (230—400 mesh ASTM). The enantiomeric excess (ee) was determined by HPLC analysis. HPLC analysis was performed on JASCO HPLC systems consisting of the following: pump, 880-PU or PU-980; detector, 875-UV or UV-970, measured at 254 nm; column, Daicel CHIRALPAK AS, AD, or Daicel CHIRALCEL OJ, OD; mobile phase, hexane–2-propanol. In general, reactions were carried out in dry solvents under an argon atmosphere, unless noted otherwise. Dichloromethane (CH_2Cl_2) was distilled from calcium hydride. Diethylaluminum chloride in hexane (1 M) was purchased from Kanto Chemical. Co., Inc., Tokyo.

General Procedure for the Preparation of Imines The starting material 9-fluorenylamine was prepared by washing CH_2Cl_2 solution of 9-aminofluorene hydrogen chloride with sat. aq $NaHCO_3$ before use. Into a mixture of 9-fluorenylamine and molecular sieves (MS) 4A in toluene was added the starting material aldehyde (1.1 eq) at ambient temperature (in the case of aliphatic and α,β-unsaturated imines, the reaction was carried out at 0 °C). After stirring for 1 h, MS 4A was filtered off and the resulting solution was evaporated at 25 °C to give the desired imines quantitatively. When the product was solid, further purification was carried out by recrystallization from hexane. However, in the case of unstable imines such as aliphatic and α,β-unsaturated ones, the resulting imine was directly used as a CH_2Cl_2 solution.

Benzaldehyde 9-Fluorenyl Imine (**3a**): ^1H-NMR ($CDCl_3$) δ: 8.77 (s, 1H), 7.88—7.78 (m, 2H), 7.75 (d, J=7.3 Hz, 2H), 7.47—7.34 (m, 7H), 7.33—7.25 (m, 2H), 5.41 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 163.4, 144.8, 141.1, 136.0, 131.0, 128.6, 128.5, 128.4, 127.4, 125.3, 120.1, 74.7; IR (KBr) ν: 3060, 2843, 1630, 1449 cm^{-1}; MS electron impact ionization (EI) *m/z* 269 (M$^+$); high resolution mass spectrometry (HRMS) Calcd for $C_{20}H_{15}N$ 269.1204, Found 269.1204.

p-Chlorobenzaldehyde 9-Fluorenyl Imine (**3b**): ^{1}H-NMR ($CDCl_3$) δ: 8.71 (s, 1H), 7.78—7.71 (m, 4H), 7.44—7.34 (m, 6H), 7.29 (dt, *J*=7.4, 1.0 Hz, 2H), 5.41 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 161.9, 144.5, 141.0, 137.0, 134.5, 129.7, 128.9, 128.5, 127.5, 125.2, 120.2, 74.5; IR (KBr) ν: 3023, 2819, 1637, 1449 cm^{-1}; MS (EI) *m/z* 303 (M$^+$); HRMS Calcd for $C_{20}H_{14}ClN$ 303.0815, Found 303.0806.

p-Methoxybenzaldehyde 9-Fluorenyl Imine (**3c**): ^{1}H-NMR ($CDCl_3$) δ: 8.70 (s, 1H), 7.77—7.71 (m, 4H), 7.42—7.35 (m, 4H), 7.28 (dt, *J*=7.7, 1.2 Hz, 2H), 6.94—6.88 (m, 2H), 5.37 (s, 1H), 3.82 (s, 3H); ^{13}C-NMR ($CDCl_3$) δ: 162.8, 161.9, 145.0, 141.0, 130.1, 129.0, 128.4, 127.4, 125.2, 120.1, 114.0, 74.7, 55.4; IR (KBr) ν: 3005, 2840, 1633, 1450 cm^{-1}; MS (EI) *m/z* 299 (M$^+$); HRMS Calcd for $C_{21}H_{17}NO$ 299.1310, Found 299.1310.

1-Naphthaldehyde 9-Fluorenyl Imine (**3d**): ^{1}H-NMR ($CDCl_3$) δ: 9.43 (s, 1H), 8.89 (d, *J*=8.6 Hz, 1H), 7.98 (d, *J*=7.4 Hz, 1H), 7.91 (d, *J*=8.3 Hz, 1H), 7.87 (d, *J*=7.4 Hz, 1H), 7.78 (d, *J*=7.6 Hz, 2H), 7.57—7.38 (m, 7H), 7.31 (t, *J*=7.3 Hz, 2H), 5.51 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 162.9, 145.0, 141.1, 133.8, 131.5, 131.4, 129.1, 128.6, 128.4, 127.5, 127.3, 126.1, 125.3, 125.2, 124.3, 120.1, 75.6; IR (KBr) ν: 3057, 2829, 1631, 1449 cm^{-1}; MS (EI) *m/z* 319 (M$^+$); HRMS Calcd for $C_{24}H_{17}N$ 319.1361, Found 319.1363.

2-Furaldehyde 9-Fluorenyl Imine (**3e**): ^{1}H-NMR ($CDCl_3$) δ: 8.51 (s, 1H), 7.72 (d, *J*=7.7 Hz, 2H), 7.50 (d, *J*=1.3 Hz, 1H), 7.45—7.33 (m, 4H), 7.28 (dt, *J*=7.3, 0.6 Hz, 2H), 6.83 (d, *J*=3.4 Hz, 1H), 6.47 (dd, *J*=3.4, 1.9 Hz, 1H), 5.42 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 151.5, 151.4, 145.0, 144.5, 141.1, 128.5, 127.4, 125.4, 120.0, 114.5, 111.8, 74.2; IR (KBr) ν: 3078, 2856, 1639, 1448 cm^{-1}; MS (EI) *m/z* 259 (M$^+$); HRMS Calcd for $C_{18}H_{13}NO$ 259.0997, Found 259.1001.

3-Furaldehyde 9-Fluorenyl Imine (**3f**): ^{1}H-NMR ($CDCl_3$) δ: 8.69 (s, 1H), 7.80 (s, 1H), 7.74 (d, *J*=7.6 Hz, 2H), 7.45—7.35 (m, 5H), 7.34—7.26 (m, 2H), 6.84 (d, *J*=1.5 Hz, 1H), 5.34 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 155.0, 145.6, 144.7, 144.1, 141.0, 128.4, 127.4, 125.5, 125.2, 120.1, 108.2, 74.8; IR (KBr) ν: 3090, 2834, 1643, 1447 cm^{-1}; MS (EI) *m/z* 259 (M$^+$); HRMS Calcd for $C_{18}H_{13}NO$ 259.0997, Found 259.0998.

2-Thiophenecarboxaldehyde 9-Fluorenyl Imine (**3g**): ^{1}H-NMR ($CDCl_3$) δ: 8.79 (s, 1H), 7.73 (d, *J*=7.7 Hz, 2H), 7.45—7.35 (m, 6H), 7.29 (dt, *J*=7.7, 1.3 Hz, 2H), 7.07 (dd, *J*=4.9, 3.7 Hz, 1H), 5.43 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 156.2, 144.6, 142.3, 141.0, 130.9, 129.5, 128.4, 127.4, 127.3, 125.3, 120.1, 74.0; IR (KBr) ν: 3066, 2829, 1623, 1449 cm^{-1}; MS (EI) *m/z* 275 (M$^+$); HRMS Calcd for $C_{18}H_{13}NS$ 275.0769, Found 275.0772.

Cinnamaldehyde 9-Fluorenyl Imine (**3h**): ^{1}H-NMR (C_6D_6) δ: 8.12 (d, *J*=9.0 Hz, 1H), 7.57 (d, *J*=7.4 Hz, 2H), 7.38 (d, *J*=7.4 Hz, 2H), 7.23 (t, *J*=7.7 Hz, 2H), 7.0—7.1 (m, 4H), 6.64 (d, *J*=16.1 Hz, 1H), 5.31 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 164.7, 144.8, 142.2, 141.0, 135.6, 129.3, 128.8, 128.4, 128.2, 127.5, 127.3, 125.2, 120.0, 74.4; IR (KBr) ν: 3023, 2852, 1632, 1448 cm^{-1}; MS (EI) *m/z* 295; HRMS Calcd for $C_{22}H_{17}N$ 295.1361, Found 295.1354.

Propionaldehyde 9-Fluorenyl Imine (**3k**): ^{1}H-NMR ($CDCl_3$) δ: 8.18 (t, *J*=4.9 Hz, 1H), 7.71 (d, *J*=7.7 Hz, 2H), 7.43—7.25 (m, 6H), 5.15 (s, 1H), 2.41 (m, 2H), 1.18 (t, *J*=7.6 Hz, 3H); ^{13}C-NMR ($CDCl_3$) δ: 168.9, 144.8, 141.0, 128.3, 127.3, 125.0, 120.0, 74.3, 29.4, 10.6; IR (KBr) ν: 3039, 2845, 1654, 1449 cm^{-1}; MS (EI) *m/z* 221 (M$^+$); HRMS Calcd for $C_{16}H_{15}N$ 221.1204, Found 221.1204.

Isobutyraldehyde 9-Fluorenyl Imine (**3l**): ^{1}H-NMR ($CDCl_3$) δ: 8.05 (dd, *J*=5.2, 0.6 Hz, 1H), 7.71 (d, *J*=7.6 Hz, 2H), 7.45—7.26 (m, 6H), 5.12 (s, 1H), 2.60 (m, 1H), 1.17 (d, *J*=7.0 Hz, 6H); ^{13}C-NMR ($CDCl_3$) δ: 173.1, 144.9, 141.0, 128.3, 127.3, 124.9, 120.0, 74.3, 34.4, 19.6; IR (KBr) ν: 2965, 2869, 1656, 1448 cm^{-1}; MS (EI) *m/z* 235 (M$^+$); HRMS Calcd for $C_{17}H_{17}N$ 235.1361, Found 235.1360.

Pivalaldehyde 9-Fluorenyl Imine (**3m**): ^{1}H-NMR ($CDCl_3$) δ: 8.06 (s, 1H), 7.71 (d, *J*=7.6 Hz, 2H), 7.45—7.25 (m, 6H), 5.10 (s, 1H), 1.16 (s, 9H); ^{13}C-NMR ($CDCl_3$) δ: 175.6, 145.1, 140.9, 128.2, 127.3, 124.8, 120.0, 74.5, 36.5, 27.2; IR (KBr) ν: 2964, 2869, 1654, 1449 cm^{-1}; MS (EI) *m/z* 249 (M$^+$); HRMS Calcd for $C_{18}H_{19}N$ 249.1517, Found 249.1522.

General Procedure for the Preparation of the Catalyst 1 The chiral ligand (13 mg, 18 μmol) was placed in the flame-dried flask and dissolved in 0.5 ml of CH_2Cl_2. To this solution was added Et_2AlCl (17 μl, 16 μmol, 0.96 M in hexane) under argon. The resulting mixture was stirred at room temperature for 1 h to give the clear solution. This solution was directly used as a catalyst in the catalytic asymmetric Strecker-type reaction.

General Procedure for the Catalytic Asymmetric Strecker-Type Reaction (System 1) To a stirred solution of the above mentioned catalyst (0.5 ml, 16 μmol) was added the solution of the imine (0.17 mmol) in CH_2Cl_2 (0.6 ml) at -40 °C, followed by the addition of TMSCN (45 μl, 0.34 mmol). After 30 min, the solution of phenol (3 μl, 34 μmol) in CH_2Cl_2 (0.2 ml) was slowly added over 17 h. The reaction mixture was allowed to stir for the time shown in Table 3. Saturated aq. $NaHCO_3$ was added for quenching and the mixture was diluted with ether. The organic layer was separated, and the water layer was extracted with ether. The combined organic layer was washed with water and dried over Na_2SO_4. Further purification was performed by flash column chromatography on SiO_2 to afford the desired aminonitrile.

General Procedure for the Catalytic Asymmetric Strecker-Type Reaction (System 2) To the CH_2Cl_2 solution (1.0 ml) of the catalyst (33 μmol) prepared as above, was added the solution of the imine (0.352 mmol) in CH_2Cl_2 (1.0 ml) and TMSCN (70 μmol) at -40 °C. To this mixture, the solution of HCN (0.422 mmol) in CH_2Cl_2 (0.26 ml) was slowly added over 24 h. After 12 h (total 36 h), the reaction was worked up as described above.

2-(9-Fluorenylamino)-2-phenylethanenitrile (**4a**): ^{1}H-NMR ($CDCl_3$) δ: 7.78—7.68 (m, 3H), 7.53—7.26 (m, 10H), 5.14 (s, 1H), 4.57 (s, 1H), 2.34 (br s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 143.6, 143.5, 141.1, 140.7, 135.8, 129.01, 128.98, 128.9, 128.8, 127.6, 127.4, 125.7, 125.0, 120.2, 120.1, 119.6, 62.2, 50.3; IR (KBr) ν: 3346, 3065, 2225, 1447 cm^{-1}; MS (EI) *m/z* 296 (M$^+$); HRMS Calcd for $C_{21}H_{16}N_2$ 296.1313, Found 296.1315; $[\alpha]_D^{24}$ -14.0° (*c*=1.0, $CHCl_3$) (95% ee). HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 90/10, 1.0 ml/min) t_R 13.3 and 25.0 min.

2-(9-Fluorenylamino)-2-(*p*-chlorophenyl)ethanenitrile (**4b**): ^{1}H-NMR ($CDCl_3$) δ: 7.75—7.68 (m, 3H), 7.50—7.26 (m, 9H), 5.12 (s, 1H), 4.47 (s, 1H), 2.39 (br s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 143.3, 143.2, 141.1, 140.8, 135.0, 134.4, 129.1, 129.0, 128.9, 128.8, 127.6, 125.7, 125.0, 120.3, 120.2, 119.3, 62.2, 49.4; IR (KBr) ν: 3317, 3039, 2228, 1447 cm^{-1}; MS (EI) *m/z* 330 (M$^+$); HRMS Calcd for $C_{21}H_{15}ClN_2$ 330.0924, Found 330.0918; $[\alpha]_D^{24}$ -50.8° (*c*=1.0, $CHCl_3$) (95% ee). HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 90/10, 1.0 ml/min) t_R 12.4 and 15.2 min.

2-(9-Fluorenylamino)-2-(*p*-methoxyphenyl)ethanenitrile (**4c**): ^{1}H-NMR ($CDCl_3$) δ: 7.77—7.64 (m, 3H), 7.52—7.22 (m, 7H), 6.84 (d, *J*=8.5 Hz, 2H), 5.08 (s, 1H), 4.52 (s, 1H), 3.76 (s, 3H), 2.29 (br s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 160.0, 143.8, 143.7, 141.0, 140.7, 128.8, 128.7, 127.9, 127.5, 125.7, 125.0, 120.2, 120.1, 119.9, 114.3, 62.1, 55.3, 49.9; IR (KBr) ν: 3312, 3009, 2223, 1448 cm^{-1}; MS (EI) *m/z* 326 (M$^+$); HRMS Calcd for $C_{22}H_{18}N_2O$ 326.1419, Found 326.1414; $[\alpha]_D^{26}$ -26.7° (*c*=1.0, $CHCl_3$) (93% ee). HPLC (Daicel CHIRALPAK AD, hexane/2-propanol 70/30, 1.0 ml/min) t_R 10.4 and 14.3 min.

2-(9-Fluorenylamino)-2-(1-naphthyl)ethanenitrile (**4d**): ^{1}H-NMR ($CDCl_3$) δ: 8.50—7.97 (m, 1H), 7.88—7.81 (m, 3H), 7.74 (d, *J*=7.7 Hz, 1H), 7.69 (d, *J*=7.7 Hz, 1H), 7.64 (d, *J*=7.0 Hz, 1H), 7.55—7.28 (m, 7H), 7.20 (dt, *J*=7.4, 1.0 Hz, 1H), 5.31 (s, 1H), 5.15 (s, 1H), 2.40 (br s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 143.7, 141.0, 140.9, 134.1, 131.1, 130.2, 130.1, 129.0, 128.9, 128.8, 127.6, 127.4, 126.9, 126.5, 126.3, 125.7, 125.2, 125.1, 123.3, 120.3, 120.1, 119.8, 62.1, 49.1; IR (KBr) ν: 3315, 3060, 2225, 1449 cm^{-1}; MS (EI) *m/z* 346 (M$^+$); HRMS Calcd for $C_{25}H_{18}N_2$ 346.1470, Found 346.1473; $[\alpha]_D^{24}$ $+65.5$° (*c*=1.0, $CHCl_3$) (88% ee). HPLC (Daicel CHIRALPAK AD, hexane/2-propanol 90/10, 1.0 ml/min) t_R 20.3 and 25.4 min.

2-(9-Fluorenylamino)-2-(2-furyl)ethanenitrile (**4e**): ^{1}H-NMR ($CDCl_3$) δ: 7.74—7.65 (m, 3H), 7.45—7.32 (m, 5H), 7.26 (dt, *J*=7.6, 1.0 Hz, 1H), 6.30—6.26 (m, 2H), 5.09 (s, 1H), 4.59 (s, 1H), 2.64 (brs, 1H); ^{13}C-NMR ($CDCl_3$) δ: 147.8, 143.45, 143.40, 143.2, 140.85, 140.77, 128.9, 128.7, 127.64, 127.58, 125.6, 124.8, 120.2, 120.1, 117.8, 110.7, 109.0, 61.9, 44.1; IR (neat) ν: 3344, 3065, 2241, 1448 cm^{-1}; MS (EI) *m/z* 286 (M$^+$); HRMS Calcd for $C_{19}H_{14}N_2O$ 286.1106, Found 286.1107; $[\alpha]_D^{24}$ -22.4° (*c*=1.0, $CHCl_3$) (79% ee). HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 90/10, 1.0 ml/min) t_R 14.4 and 22.1 min.

2-(9-Fluorenylamino)-2-(3-furyl)ethanenitrile (**4f**): ^{1}H-NMR ($CDCl_3$) δ: 7.73—7.65 (m, 3H), 7.51 (dd, *J*=7.4, 0.9 Hz, 1H), 7.45 (m, 1H), 7.43—7.31 (m, 4H), 7.28 (dt, *J*=7.6, 1.2 Hz, 1H), 5.40 (m, 1H), 5.06 (s, 1H), 4.40 (s, 1H), 2.40 (br s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 144.0, 143.5, 143.4, 141.0, 140.7, 128.86, 128.84, 127.57, 127.55, 125.7, 124.9, 121.9, 120.2, 120.1, 119.4, 109.3, 62.0, 42.2; IR (neat) ν: 3345, 3065, 2231, 1448 cm^{-1}; MS (EI) *m/z* 286 (M$^+$); HRMS Calcd for $C_{19}H_{14}N_2O$ 286.1106, Found 286.1110; $[\alpha]_D^{24}$ $+16.2$° (*c*=1.0, $CHCl_3$) (90% ee). HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 70/30, 1.0 ml/min) t_R 8.1 and 14.6 min.

2-(9-Fluorenylamino)-2-(2-thiophenyl)ethanenitrile (**4g**): ^{1}H-NMR ($CDCl_3$) δ: 7.77—7.68 (m, 3H), 7.61 (d, *J*=7.4 Hz, 1H), 7.47—7.26 (m, 5H), 7.11 (m, 1H), 6.93 (dd, *J*=4.9, 3.4 Hz, 1H), 5.16 (s, 1H), 4.63 (s, 1H), 2.72 (br s, 1H); ^{13}C-NMR ($CDCl_3$) δ: 143.1, 143.0, 141.1, 140.7, 139.7, 129.0, 127.7, 127.6, 126.9, 126.8, 126.1, 125.8, 125.1, 120.24, 120.15, 119.0, 62.2, 45.5; IR (KBr) ν: 3338, 3066, 2225, 1447 cm^{-1}; MS (EI) *m/z* 302 (M$^+$); HRMS

Calcd for $C_{19}H_{14}N_2S$ 302.0878, Found 302.0877; $[\alpha]_D^{26}$ −36.3° (c=1.0, $CHCl_3$) (89% ee). HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 70/30, 1.0 ml/min) t_R 7.7 and 13.8 min.

(*E*)-2-(9-Fluorenylamino)-4-phenyl-3-butenenitrile (**4h**): ^{1}H-NMR ($CDCl_3$) δ: 7.56 (m, 1H), 7.50 (dd, J=2.0, 6.0 Hz, 1H), 7.45 (d, J=7.5 Hz, 1H), 7.38 (d, J=7.5 Hz, 1H), 7.22 (m, 2H), 7.16 (m, 1H), 7.0—7.1 (m, 6H), 6.51 (dd, J=1.2, 15.5 Hz, 1H), 5.67 (dd, J=5.5, 15.5 Hz, 1H), 4.67 (s, 1H), 3.72 (d, J=5.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$) δ: 145.0, 144.3, 141.2, 141.0, 135.9, 133.4, 128.9, 128.8, 128.7, 128.5, 128.3, 127.7, 127.6, 127.1 (overlapped), 126.1 125.4, 124.3, 120.3, 120.2, 119.2, 62.3, 48.4; IR (KBr) ν: 3349, 3061, 2220, 1448 cm^{-1}; MS (EI) m/z 322 (M$^+$); HRMS Calcd for $C_{23}H_{18}N_2$ 322.1470, Found 322.1472; $[\alpha]_D^{24}$ −89.7° (c=0.465, $CHCl_3$) (96% ee). HPLC (Daicel CHIRALPAK AD, hexane/2-propanol 90/10, 0.8 ml/min) t_R 20.2 and 22.6 min.

(*E*)-2-[*N*-(9-Fluorenyl)-*N*-trifluoroacetylamino)-3-octenenitrile (**4i**): ^{1}H-NMR ($CDCl_3$) δ: 7.71 (d, J=2.5 Hz, 1H), 7.70 (d, J=2.8 Hz, 1H), 7.54 (d, J=7.3 Hz, 1H), 7.48—7.36 (m, 4H), 7.30 (d, J=7.75 Hz, 1H), 7.26 (d, J=7.3 Hz, 1H), 5.93 (s, 1H), 5.37 (dd, J=15.3, 7.3 Hz, 1H), 5.10 (ddd, J=7.7, 7.3, 6.7 Hz, 1H), 3.52 (d, J=7.0 Hz, 1H), 1.82—1.72 (m, 2H), 1.15—1.05 (m, 4H), 0.76 (t, J=6.7 Hz, 3H); ^{13}C-NMR ($CDCl_3$) δ: 156.8 (q, J=37 Hz), 140.9, 140.7, 140.4, 138.7, 138.6, 130.4, 130.3, 128.9, 128.2, 126.0, 125.9, 120.8, 119.0, 115.1, 115.0, 62.8, 48.2, 31.5, 29.7, 22.0, 13.7; IR (KBr) ν: 2959, 2246, 1702, 1451 cm^{-1}; MS (EI) m/z 398 (M$^+$); HRMS Calcd for $C_{23}H_{21}F_3N_2O$ 398.1606, Found 398.1607; $[\alpha]_D^{21}$ −12.6 (c=5.3, $CHCl_3$) (85% ee). HPLC (Daicel CHIRALPAK AD, hexane/2-propanol 200/1, 0.7 ml/min) t_R 15.6 and 26.5 min.

2-(9-Fluorenylamino)-*n*-octanenitrile (**4j**): ^{1}H-NMR ($CDCl_3$) δ: 7.63 (d, J=6.7 Hz, 2H), 7.62 (d, J=6.4 Hz, 2H), 7.5 (d, J=7.3 Hz, 1H), 7.49 (d, J=7.7 Hz, 2H), 7.34—7.23 (m, 4H), 5.0 (s, 1H), 3.29 (t, J=7.1 Hz, 1H), 1.65—1.54 (m, 2H), 1.46—1.32 (m, 6H), 0.78 (t, J=6.7 Hz, 3H); ^{13}C-NMR ($CDCl_3$) δ: 143.9, 143.9, 140.6, 140.1, 128.7, 127.5, 127.4, 125.5, 124.9, 121.1, 120.1, 120.0, 62.2, 46.5, 34.9, 31.4, 28.6, 25.3, 22.43, 13.9; IR (KBr) ν: 3335, 2937, 2225, 1449 cm^{-1}; MS (EI) m/z 304 (M$^+$); HRMS Calcd for $C_{21}H_{24}N_2$ 304.1939, Found 304.1938; $[\alpha]_D^{24}$ +27.2 (c=3.9, $CHCl_3$) (80% ee); HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 9/1, 1.0 ml/min) t_R 8 and 11 min.

2-(9-Fluorenylamino)-*n*-butanenitrile (**4k**): ^{1}H-NMR ($CDCl_3$) δ: 7.72—7.64 (m, 2H), 7.62 (d, J=7.3 Hz, 1H), 7.55 (d, J=7.4 Hz, 1H), 7.42—7.25 (m, 4H), 4.99 (s, 1H), 3.28 (t, J=6.8 Hz, 1H), 2.06 (br s, 1H), 1.67 (m, 2H), 1.00 (t, J=7.7 Hz, 3H); ^{13}C-NMR ($CDCl_3$) δ: 143.93, 143.87, 140.8, 140.6, 128.7, 127.5, 127.4, 125.6, 124.9, 120.9, 120.1, 120.0, 62.2, 47.8, 28.3, 10.0; IR (KBr) ν: 3332, 2972, 2221, 1445 cm^{-1}; MS (EI) m/z 248 (M$^+$); HRMS Calcd for $C_{17}H_{16}N_2$ 248.1313, Found 248.1321; $[\alpha]_D^{24}$ +66.6° (c=1.0, $CHCl_3$) (70% ee). HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 95/5, 1.0 ml/min) t_R 10.8 and 14.2 min.

2-(9-Fluorenylamino)-3-methylbutanenitrile (**4l**): ^{1}H-NMR ($CDCl_3$) δ: 7.74—7.67 (m, 2H), 7.64 (d, J=7.4 Hz, 1H), 7.59 (d, J=7.6 Hz, 1H), 7.44—7.28 (m, 4H), 5.04 (s, 1H), 3.15 (d, J=5.2 Hz, 1H), 2.07 (br s, 1H), 1.85 (m, 1H), 1.05 (d, J=6.7 Hz, 3H), 0.98 (d, J=6.7 Hz, 3H); ^{13}C-NMR ($CDCl_3$) δ: 143.8, 140.9, 140.6, 128.72, 128.70, 127.5, 127.4, 125.7, 125.2, 120.2, 120.1, 120.0, 62.4, 52.7, 32.9, 18.9, 17.9; IR (KBr) ν: 3344, 2966, 2225, 1448 cm^{-1}; MS (EI) m/z 262 (M$^+$); HRMS Calcd for $C_{18}H_{18}N_2$ 262.1470, Found 262.1472; $[\alpha]_D^{24}$ +62.5° (c=1.0, $CHCl_3$) (72% ee). HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 95/5, 1.0 ml/min) t_R 7.6 and 11.0 min.

2-(9-Fluorenylamino)-3,3-dimethylbutanenitrile (**4m**): ^{1}H-NMR ($CDCl_3$) δ: 7.74—7.67 (m, 2H), 7.65 (d, J=7.3 Hz, 1H), 7.63 (d, J=7.3 Hz, 1H), 7.44—7.37 (m, 2H), 7.34 (dt, J=7.3, 1.3 Hz, 1H), 7.31 (dt, J=7.3, 1.0 Hz, 1H), 5.05 (s, 1H), 2.91 (s, 1H), 2.08 (br s, 1H), 1.01 (s, 9H); ^{13}C-NMR ($CDCl_3$) δ: 143.7, 143.5, 141.0, 140.6, 128.72, 128.67, 127.4, 127.3, 125.9, 125.7, 120.6, 120.1, 119.9, 62.8, 56.7, 35.1, 26.0; IR (KBr) ν: 3344, 2961, 2228, 1448 cm^{-1}; MS (EI) m/z 276 (M$^+$); HRMS Calcd for $C_{19}H_{20}N_2$ 276.1626, Found 276.1627; $[\alpha]_D^{26}$ +58.3° (c=1.0, $CHCl_3$) (78% ee). HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 95/5, 1.0 ml/min) t_R 6.5 and 12.7 min.

2-(9-Fluorenylamino)-2-phenylacetamide (9) To a solution of **4a** (100 mg, 0.34 mmol) in formic acid (10 ml), HCl gas was bubbled through until the solution was saturated. The mixture was allowed to stir at room temperature for 1 h and the solvent was removed *in vacuo*. The resulting residue was triturated in saturated $NaHCO_3$ (50 ml) and extracted with CH_2Cl_2 (50 ml×3). The organic layer was washed with water (50 ml×2) and dried over $MgSO_4$. After the removal of the solvent, the crude product (108 mg, 100%, 93% ee) was purified by recrystallization from THF/diethyl ether (96 mg, 90%, 100% ee). ^{1}H-NMR (CD_3OD) δ: 7.77—7.68 (m, 1H), 7.46 (m, 1H), 7.39 (t, J=7.3 Hz, 1H), 7.35 (t, J=7.3 Hz, 1H), 7.30 (dt, J=7.3, 1.2 Hz, 1H), 7.28—7.18 (m, 6H), 4.93 (s, 1H), 3.96 (s, 1H); ^{13}C-NMR (dimethylsulfoxide (DMSO)-d_6) δ: 174.2, 145.7, 145.6, 141.1, 140.1, 140.0, 127.9, 127.8, 127.2, 127.02, 126.97, 126.9, 125.3, 125.1, 119.79, 119.76, 62.3, 61.5; IR (KBr) ν: 3355, 1654, 1448 cm^{-1}; MS (EI) m/z 314 (M$^+$); HRMS Calcd for $C_{21}H_{18}N_2O$ 314.1419, Found 314.1420; $[\alpha]_D^{24}$ −62.5° (c=1.0, MeOH) (100% ee). HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 70/30, 1.0 ml/min) t_R 21.6 and 27.0 min.

2-Amino-2-phenylacetamide (11) To a solution of DDQ (202 mg, 0.89 mmol) in THF (14 ml) was added **9** (140 mg, 0.45 mmol) in THF (10 ml) at 3 °C. After stirring for 1 h at the same temperature, the reaction was quenched by addition of 1 N HCl (0.89 ml) and allowed to warm up to room temperature. The solvents were removed *in vacuo* and the resulting residue was triturated in diethyl ether. The suspended residue was filtered and washed with diethyl ether (5 ml×5) to afford HCl salt of **11**. A soluton of the HCl salt in MeOH (3 ml) was passed through a plug of Amberlyst A-21 (OH form) with MeOH (20 ml) and concentrated *in vacuo* to afford a white powder of **11** (61 mg, 91%). $[\alpha]_D^{24}$ −108° (c=1.26, EtOH) (98% ee) (lit.[9] $[\alpha]_D^{22}$ −103° (c=1.2, EtOH) for *R* enantiomer). The enantiomeric excess was determined by HPLC after the conversion to *p*-nitrobenzyloxycarbonyl derivative: ^{1}H-NMR (acetone-d_6) δ: 8.15—7.95 (m, 2H), 7.53 (d, J=8.3 Hz, 2H), 7.37 (d, J=7.3 Hz, 2H), 7.33—7.13 (m, 3H), 6.98 (br s, 1H), 6.86 (br s, 1H), 6.65—6.20 (m, 1H), 5.18 (d, J=7.6 Hz, 1H), 5.15—5.04 (m, 2H); ^{13}C-NMR (acetone-d_6) δ: 172.3, 155.9, 148.4, 145.9, 140.0, 129.3, 128.9, 128.7, 128.2, 124.2, 65.5, 59.3; IR (KBr) ν: 3392, 3297, 1660 cm^{-1}; HPLC (Daicel CHIRALCEL OJ, hexane/2-propanol 70/30, 1.0 ml/min) t_R 20.2 and 25.6 min; $[\alpha]_D^{21}$ −99.0° (c=1.0, THF) (98% ee).

2-(9-Fluorenylideneamino)-3,3-dimethylbutyronitrile (12) To a solution of **4m** (150 mg, 0.54 mmol) in CH_2Cl_2 (10 ml), manganese oxide (555 mg, 5.43 mmol) was added at room temperature. After stirring for 1 h, manganese oxide was filtered off and washed with CH_2Cl_2 (5 ml×4). Solvent was evaporated *in vacuo*, and the residue was purified by preparative TLC (hexane : acetone = 9 : 1) to give a yellowish solid (142 mg, 95%, 77.4% ee). ^{1}H-NMR ($CDCl_3$) δ: 7.87 (d, J=7.6 Hz, 1H), 7.81 (d, J=7.3 Hz, 1H), 7.64 (d, J=7.3 Hz, 1H), 7.54 (d, J=7.6 Hz, 1H), 7.46 (t, J=7.3 Hz, 1H), 7.41 (dt, J=7.3, 1.0 Hz, 1H), 7.36—7.25 (m, 2H), 4.80 (s, 1H), 1.24 (s, 9H); ^{13}C-NMR ($CDCl_3$) δ: 167.1, 144.3, 141.2, 138.1, 132.4, 131.8, 131.1, 128.6, 128.3, 127.9, 123.2, 120.8, 119.5, 117.5, 61.1, 36.2, 26.0; IR (KBr) ν: 2968, 2233, 1647, 1450 cm^{-1}; MS (EI) m/z 274 (M$^+$); HRMS Calcd for $C_{19}H_{18}N_2$ 274.1470, Found 274.1469; $[\alpha]_D^{18}$ +282.3° (c=1.0, $CHCl_3$) (77.4% ee). HPLC (Daicel CHIRALCEL OD, hexane/2-propanol 90/10, 1.0 ml/min) t_R 7.4 and 14.1 min.

2-Amino-3,3-dimethylbutyronitrile HCl Salt (13) To a solution of **12** (200 mg, 0.73 mmol) in THF (15 ml) was added 1 N HCl (1.5 ml) and stirred at room temperature for 1 h. After the solvents were removed *in vacuo*, the resulting residue was triturated in diethyl ether followed by filtrating and washing with diethyl ether (5 ml×5) to give a white powder of **13** (90 mg, 83%). $[\alpha]_D^{18}$ +16.4° (c=1.0, MeOH) (77.4% ee).

Fmoc–*tert*-leucine A solution of **13** (80 mg, 0.54 mmol) in conc. HCl (2 ml) was heated to 120 °C in a sealed tube for 24 h. After cooling to ambient temperature, the solvent was removed *in vacuo* to afford a 1 : 1 mixture of *tert*-leucine HCl salt **14** and ammonium chloride. To a solution of the mixture in 50% aq. acetone (4 ml) were added K_2CO_3 (140 mg, 1.0 mmol) and Fmoc–ONSu (370 mg, 1.1 mmol) and the whole was stirred at room temperature for 4 h. After the removal of acetone *in vacuo*, the resulting precipitate was filtered off and washed with 5% K_2CO_3 (10 ml×3). The combined aqueous layer was washed with diethyl ether (20 ml), acidified with 1 N HCl and then extracted with diethyl ether (20 ml×3). The combined organic layer was washed with brine (50 ml), dried over $MgSO_4$ and concentrated *in vacuo* to afford Fmoc–*tert*-leucine (185 mg, 100%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 7.78—7.68 (m, 2H), 7.62—7.50 (m, 2H), 7.38 (t, J=7.1 Hz, 2H), 7.30 (t, J=7.3 Hz, 2H), 6.12 (m, 0.1H), 5.38 (d, J=9.8 Hz, 0.9H), 4.55—4.33 (m, 2H), 4.30—4.15 (m, 1.8H), 3.87 (m, 0.2H), 1.80—0.90 (m, 9H); ^{13}C-NMR ($CDCl_3$) δ: 176.4, 156.3, 143.8, 143.7, 141.3, 127.7, 127.1, 125.0, 120.0, 67.1, 62.1, 47.2, 34.6, 26.5; $[\alpha]_D^{21}$ +9.3° (c=1.0, MeOH) (78% ee). (lit.[10] $[\alpha]_D^{20}$ −11.0° (c=1, MeOH) for *S* enantiomer). The enantiomeric excess was determined by HPLC after conversion to the methyl ester (MeI, $NaHCO_3$, *N*,*N*-dimethylformamide (DMF)): ^{1}H-NMR ($CDCl_3$) δ: 7.76 (d, J=7.6 Hz, 2H), 7.60 (d, J=7.0 Hz, 2H), 7.40 (t, J=7.3 Hz, 2H), 7.31 (t, J=7.3 Hz, 2H), 5.37 (d, J=9.4 Hz, 0.9H), 5.05 (m, 0.1H), 4.50—4.32 (m, 2H), 4.26—4.16 (m, 2H), 3.74 (s, 3H), 1.10—0.80 (m, 9H); ^{13}C-NMR ($CDCl_3$) δ: 172.2, 156.1, 143.9, 143.8, 141.3, 127.7, 127.0, 125.06, 125.04, 119.97, 119.95, 67.0, 62.1, 51.8, 47.2, 34.7, 26.4; IR (neat) ν: 3431, 2964, 1730 cm^{-1}; MS (EI) m/z 367 (M$^+$); HRMS Calcd for $C_{22}H_{25}NO_4$ 367.1783, Found 367.1789; HPLC (Daicel CHIRALCEL OD, hexane/2-propanol 70/30, 1.0 ml/min) t_R 7.7 and 10.4 min.

(*E*)-2-[*N*-(9-Fluorenyl)-*N*-trifluoroacetylamino]-4-phenyl-3-butenenitrile (15) Protection of a nitrogen atom of **4h** (84% ee) as trifluoroacetoamide was achieved by the usual method (trifluoroacetic anhydride (TFAAA), prydine/CH_2Cl_2, 0 °C) without racemization. ^{1}H-NMR ($CDCl_3$) δ: 7.82 (d, J=8.0 Hz, 2H), 7.80 (d, J=8.3 Hz, 2H), 7.65 (d, J=7.4 Hz, 1H), 7.56—7.53 (m, 2H), 7.49—7.46 (m, 2H), 7.32 (dt, J=0.6, 7.3 Hz, 2H), 7.29—7.24 (m, 2H), 7.13—7.10 (m, 2H), 6.07 (dd, J=7.3, 15.9 Hz, 1H), 6.06 (s, 1H), 5.29 (d, J=15.9 Hz, 1H), 3.82 (d, J=7.3 Hz, 1H); ^{13}C-NMR ($CDCl_3$) δ: 156.9 (q, J=37 Hz), 141.0, 140.7, 138.7, 138.6, 138.1, 134.5, 130.6, 130.4, 129.0, 128.9, 128.6, 128.0, 127.0, 126.2, 125.5, 120.9, 120.9, 117.8, 114.6, 116.3 (q, J=286 Hz), 62.8, 53.4, 48.3, 29.7; IR (KBr) ν: 2924, 2252, 1699, 1446 cm^{-1}; MS (EI) *m*/*z* 418 (M^+); HRMS Calcd for $C_{25}H_{17}F_3N_2O$ 418.12931, Found 418.1290; HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 9/1, 1.0 ml/min) t_R 9 and 14 min. To a solution of the protected **4h** (28 mg, 0.067 mmol, 84% ee) in ethyl acetate (1 ml) was added Rh–C (14 mg). The reaction mixture was allowed to stir vigorously at room temperature for 12 h under 1 atom pressure of hydrogen. Then, Rh–C was removed by filtration, and the filtrate was evaporated under reduced pressure. The resulting residue was purified on preparative TLC (hexane : ethyl acetate=9 : 1) to give **15** (22 mg, yield 79%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 7.63—7.32 (m, 8H), 7.10—6.90 (m, 3H), 6.58 (d, J=7.0 Hz, 2H), 5.87 (s, 1H), 2.81 (dd, J=4.0, 11.0 Hz 1H), 2.73 (m, 1H), 2.58 (m, 1H), 2.2 (ddd, J=4.3, 10.0, 14.3 Hz 1H), 1.47 (m, 1H); ^{13}C-NMR ($CDCl_3$) δ: 157.1 (q, J=36 Hz), 140.8, 140.5, 138.3, 138.2, 137.8, 130.29, 130.24, 128.7, 128.5, 128.0, 127.5, 126.1, 125.0, 121.0, 120.6, 116.2 (q, J=287 Hz), 115.3, 62.7, 45.8, 31.6, 31.3; HPLC (Daicel CHIRALPAK AS, hexane/2-propanol 9/1, 1.0 ml/min) t_R 5 and 10 min.

2-(9-Fluorenylamino)-3,4-dihydroxy-*n*-octanenitrile (16a) and 2-[(9-Fluorenyl)-trifluoroacetyl-amino]-3,4-dihydroxy-*n*-octanenitrile (16b) **4i** (30 mg, 0.075 mmol, 65% ee) was dissolved in pyridine (0.9 ml) and the resulting solution was cooled down to −40 °C. To this mixture was added a solution of osmium tetroxide in *tert*-BuOH (0.96 ml, 0.96 mmol, 0.1 M). After stirring for 3 h at the same temperature, 25% aq. $NaHSO_3$ (2 ml) was added for quenching, followed by dilution with ether (2 ml). The resulting mixture was allowed to stir at 0 °C for 10 h. The *N*-trifluoroacetyl group was deprotected in this reduction step. The organic layer was separated and the water layer was extracted with ethyl acetate (5 ml×2). The combined organic layer was washed with sat. aq. $CuSO_4$, water and brine. After drying this solution over Na_2SO_4, the solvent was evaporated under reduced pressure. Further purification was performed by flash column chromatography (hexane : acetone=20 : 1 to 8 : 1) to afford **16a** and **16b** in 53% and 38% yield, respectively.

16a: ^{1}H-NMR ($CDCl_3$) δ: 7.72 (d, J=7.6 Hz, 2H), 7.66 (d, J=7.6 Hz, 1H), 7.58 (d, J=6.6 Hz, 1H), 7.46—7.33 (m, 4H), 5.1 (s, 1H), 3.61 (ddd, J=1.2, 7.8, 8.3 Hz, 1H), 3.52 (dd, J=1.2, 6.1 Hz, 1H), 3.33 (d, J=6.1 Hz, 1H), 1.5—1.2 (m, 6H), 0.82 (t, J=7.0 Hz, 3H); ^{13}C-NMR ($CDCl_3$) δ: 142.5, 142.4, 141.3, 140.7, 129.2, 129.1, 127.8, 127.7, 125.7, 124.9, 120.3, 118.9, 76.7, 73.2, 71.2, 62.0, 48.8, 33.7, 27.7, 22.5, 13.9; MS *m*/*z* 336 (M^+), 291; HPLC (Daicel CHIRALPAK AD, hexane/2-propanol 90/10, 0.7 ml/min) t_R 25 and 34 min. The relative configuration was determined by the X-ray crystallography of the corresponding cyclic carbonate.

16b: ^{1}H-NMR ($CDCl_3$) δ: 7.8 (d, J=7.7 Hz, 2H), 7.6 (d, J=7.6 Hz, 1H), 7.59—7.44 (m, 4H), 7.37 (dd, J=7.3, 7.3 Hz, 1H), 6.05 (s, 1H), 3.57 (dd, J=2.4, 2.4 Hz, 1H), 3.30 (d, J=2.4 Hz, 1H), 2.6 (m, 1H), 1.2—1.0 (m, 6H), 0.83 (t, J=7.0 Hz, 3H); ^{13}C-NMR ($CDCl_3$) δ: 140.6, 138.4, 130.9, 130.7, 129.1, 128.2, 125.8, 125.0, 121.3, 121.2, 120.3 (q), 74.4, 72.8, 63.2, 52.5, 32.4, 27.6, 22.3, 13.9; IR (neat) ν: 3429, 1692, 1453 cm^{-1}; MS *m*/*z* 432 (M^+), 276, 165; HPLC (Daicel CHIRALCEL OD, hexane/2-propanol 95/5, 0.7 ml/min) t_R 33 and 57 min.

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Construction of Chiral 1,2-Cycloalkanopyrrolidines from L-Proline Using Ring Closing Metathesis (RCM)

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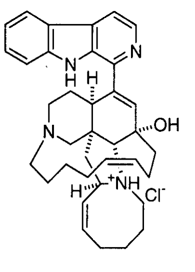
An efficient synthetic method for the preparation of optically active pyrroloazocine, pyrroloazepine, quinolizidine, indolizidine using ring closing olefin metathesis (RCM) is described.

Key word 1,2-cycloalkanopyrrolidine; ring closing metathesis (RCM); azocine; azepine; (−)-coniceine; (*S*)-pyrrolam

Biologically active alkaloids having an azabicyclic framework are abundant in nature. The pyrroloazocine, pyrroloazepine, quinolizidine, indolizidine alkaloids fall into this category and have attracted considerable attention from the synthetic viewpoint due to their biological activity.[1,2] As a part of our program[3] directed toward the synthesis of manzamine A (**1**) (Chart 1), the ring closing metathesis (RCM) strategy for the construction of optically active 1,2-cyclooctanopyrrolidine (**10**) corresponding to the CD ring in **1** and related azabicycles was investigated. We have previously reported the RCM of L-proline derived dienes.[4] In this paper, we report more fully on the synthesis of optically active azacyclic compounds from L-proline including the synthesis of **10**.

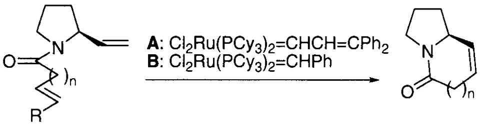
Results and Discussion

The overall procedure consisted of the RCM reaction of chiral dienes (**2**—**6**) in the presence of Grubbs' catalyst (A, B) to form the corresponding bicyclic lactam (**7**—**10**) (Chart 2). Dienes **2**—**6** were prepared from L-proline in good yield as shown in Chart 3 and subjected to RCM. RCM reaction of these dienes is quite interesting, since expected azabicyclic compounds offer tremendous utility as reaction intermediates in organic synthesis. As indicated in Table 1, when **2** was treated with 5 mol% of catalyst A in degassed benzene (0.02 M) at r.t. for 3 d, indolizidine **7** was obtained in 93% yield (entry 1). The reaction of **2** using commercially available catalyst B, on the other hand, proceeded more rapidly and **7** was obtained in 66% yield (entry 2). However, introduction of a substituent to diene **2** suppressed the RCM and no cyclized products were formed from dienes **11a**—**c**, **12**, and **13** (Chart 4) under similar reaction conditions. Under similar conditions using 5 mol% of A, cyclization of a 3 to 7 membered ring system required prolonged reaction time (Table 2). After 15 h, only 29% yield of the expected lactam **8** was obtained and 58% of starting material **3** was recovered (entry 1). A longer reaction period improved the yield (58%,



manzamine A

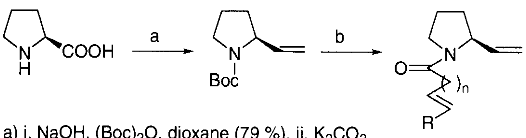
Chart 1



2: n = 1, R = H
3: n = 2, R = H
4: n = 0, R = H
5: n = 0, R = Me
6: n = 3, R = H

7: n = 1
8: n = 2
9: n = 0
10: n = 3

Chart 2

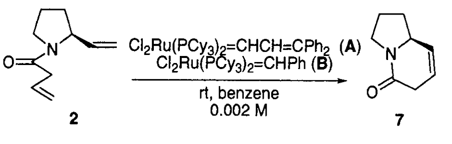


a) i. NaOH, $(Boc)_2O$, dioxane (79 %), ii. K_2CO_3, MeI, DMF (96 %), iii. DIBAH, toluene, -78 °C (93 %), iv. $KN(TMS)_2$, Ph_3PCH_3Br, THF (73 %); b) i. CF_3COOH, CH_2Cl_2, ii. $CH_2{=}CH(CH_2)_nCOCl$

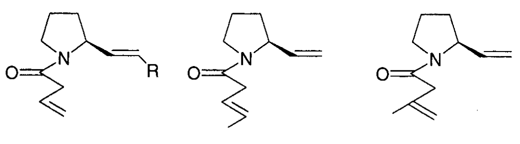
2: n = 1, R = H
3: n = 2, R = H
4: n = 0, R = H
5: n = 0, R = Me
6: n = 3, R = H

Chart 3

Table 1. Construction of chiral Pyrrolizidine Ring Using RCM



Entry	Ru catalyst (mol%)	Time	Yield (%)
1	A (5)	3 d	93
2	B (5)	18 h	66



11a: R = Me
11b: R = Ph
11c: R = CO_2Me

12

13

Chart 4

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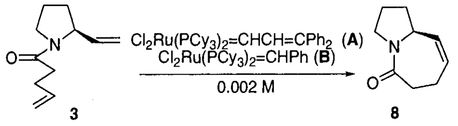
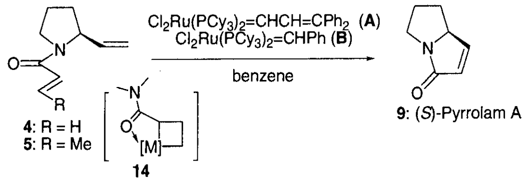
Dedicated to the memory of Dr. Kyosuke Tsuda.

Table 2. Construction of Chiral Pyrroloazepine Ring Using RCM



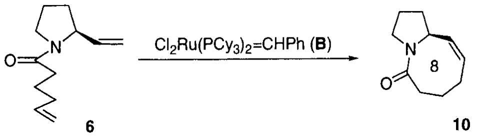
Entry	Ru catalyst (mol%)	Solvent	Temp. (°C)	Time	Yield (%)	Recovery (%)
1	A (5)	Benzene	r.t.	15 h	29	58
2	A (5)	Benzene	r.t.	11.5 d	58	31
3	A (10)	Benzene	r.t.	23 d	91	
4	A (10)	Benzene	r.t.	15 h	34	
5	A (2)	Benzene	50	15 h	11	46
6	A (2)	Benzene	80	7 h	0	
7	B (5)	Benzene	r.t.	4 d	73	
8	B (10)	Benzene	r.t.	15 h	60	
9	B (10)	CH_2Cl_2	r.t.	15 h	73	

Table 3. Construction of Chiral Indolizidine Ring Using RCM



Entry	Diene	Ru catalyst (mol%)	Temp.	Time	Yield (%)
1	**4**	A (5)	r.t.	15 h	0
2	**4**	A (10)	r.t.	14 d	0
3	**5**	A (10)	r.t.	18 d	0
4	**5**	A (5)	50 °C	20 h	29
5	**5**	B (5)	r.t.	8 d	0
6	**5**	B (5)	50 °C	3 h	30

Table 4. Construction of Chiral Pyrroloazocin Ring Using RCM



Entry	Ru catalyst (mol%)	Solvent	Conc. (M)	Temp. (°C)	Time	Yield (%)
1	10	Benzene	0.020	r.t.	23 d	0
2	50	CH_2Cl_2	0.020	r.t.	88 h	0
3	50	CH_2Cl_2	0.002	r.t.	41 h	Trace
4	25	CH_2Cl_2	0.002	r.t.	111 h	24
5	25	CH_2Cl_2	0.002	50	20 h	45
6	10	CH_2Cl_2	0.002	50	20 h	10
7	25	CH_2Cl_2	0.001	50	20 h	35

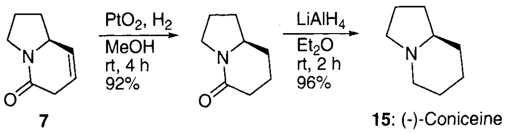


Chart 5

entry 2). However, the yield of **8** increased to 91% when 10 mol% of the catalyst A was used, and stirring was continued for 23 d (entry 3). Cyclization of **3** to **8** also proceeded more rapidly using catalyst B and **8** was obtained in 73 and 60% yield, respectively (entries 7, 8). To improve the yield, the reaction was carried out at 50 °C and at 80 °C. However, the yield of **8** was decreased (at 50 °C) and no product was obtained at an elevated temperature (80 °C) (entries 5, 6), probably due to decomposition of the catalyst. When the solvent was changed to CH_2Cl_2, the yield increased to 73% (entry 9).

Initial attempts to cyclize a diene **4** to 5 membered lactam **9** under similar conditions (0.002 M, r.t.) using either 5 or 10 mol% of A in benzene were unsuccessful (Table 3, entries 1, 2). These results were perhaps due to the formation of stable chelated species such as **14**.[5] Accordingly, we carried out RCM of **5** under conditions summarized in Table 3 and the expected cyclization to **9** was found to occur under a forced condition (50 °C) to give (*S*)-pyrrolam (**9**) in 29% yield. Similar cyclization in the presence of B also proceeded at 50 °C more quickly than that using A, giving **9** in 30% yield. The low yield observed in the formation of **9** appeared to be due to the instability of the product under reaction conditions.

Our next stage is to explore the feasibility of construction of a medium ring such as **10**, which corresponds to the CD ring in **1**. Although in many cases acyclic ring closure did not proceed effectively in the 8 membered series,[6] several groups[7—11] have succeeded in obtaining an 8 membered ring system by RCM methodology. In our preliminary investigation,[4] the cyclization of a diene **6** to 8 membered ring system was not successful (Table 4, entries 1, 2). The above results indicated that the catalyst B seems to be more effective and CH_2Cl_2 is a choice for the solvent. Therefore, we carried out the reaction of **6** in the presence of an excess of B (50 mol%) at r.t., but expected cyclization was not observed (entry 2). Encouraged by detection of a trace amount of **10** by similar reaction carried out under highly diluted conditions, we next reduced the amount of catalyst to 25 mol% and the mixture was treated for 111 h at r.t. and 24% yield of **10** was formed (entry 4). Heating the reaction at 50 °C increased the yield of **10** up to 45% (entry 5).

Lactam **7** was successfully converted to (−)-coniceine (**15**), the simplest indolizidine alkaloid *via* stepwise reductions as shown in Chart 5. Further work on the total synthesis of natural products with the azocine ring system is currently in progress.

Experimental

All melting points are uncorrected. Infrared (IR) absorption spectra (cm^{-1}) were recorded using a KBr pellet. ^{1}H-NMR (and ^{13}C-NMR) spectra were recorded in $CDCl_3$, unless otherwise noted, at 400 or 500 MHz with tetramethylsilane (TMS) as an internal standard. Kanto Chemical Co., Inc. Silica gel 60 N (Spherical, neutral) for column chromatography and E. Merck precoated TLC plates, Silica gel F_{254}, for preparative TLC were used. The organic layers were dried with anhydrous Na_2SO_4. $Cl_2(PCy_3)_2$Ru=CHCH=CHPh was synthesized according to Grubbs' method.[5] $Cl_2(PCy_3)_2$Ru=CHPh is commercially available.

Preparation of (*S*)-*N*-(*tert*-Butoxycarbonyl)-2-vinylpyrrolidine To a stirred solution of L-proline (20.7 g, 180 mmol) in 1 N NaOH (180 ml) was added a solution of di-*tert*-butyldicarbonate ($(Boc)_2O$) (43.2 g, 198 mmol, 1.10 eq) in dioxane (90.0 ml) at 0 °C and the whole was stirred at r.t. for 17 h. To the reaction mixture, 20% H_2SO_4 was added until pH became 2, and the organic compounds were extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated. Crystallization gave *N*-(*tert*-butoxycarbonyl)-L-proline (30.4 g, 79%) as a colorless crystal. To a stirred solution of *N*-[(1,1′-dimethylethoxy)carbonyl]-L-proline (21.5 g, 100 mmol) in *N*,*N*-dimethylformamide (DMF) (250 ml) was added K_2CO_3 (16.5 g, 120 mmol, 1.2 eq) at 0 °C and the whole was stirred at 0 °C for 10 min. MeI (7.50 ml, 120 mmol, 1.2 eq) was added and it was stirred at r.t. for 1 d. The reaction mixture was filtered and to the mother liquor H_2O was added and the organic compounds were extracted with AcOEt, washed with brine, dried over Na_2SO_4, filtered and concentrated. Column chromatography (SiO_2 60, *n*-hexane : AcOEt=4 : 1) gave *N*-[(1,1′-dimethylethoxy)-carbonyl]-L-proline methyl ester (22.1 g, 96%) as a colorless oil. To a stirring solution of *N*-[(1,1′-dimethylethoxy)carbonyl]-L-proline methyl ester (460 mg, 2.00 mmol) in toluene (10.0 ml) was added dropwise a 1.01 M solution of diisobutylaluminum hydride (DIBAH) in toluene (3.79 ml, 3.83 mmol, 1.7 eq) at −78 °C over 15 min. The whole was stirred at −78 °C for 2 h, MeOH (0.500 ml) was added and the mixture was stirred at 0 °C for 20 min. Aqueous sat. Rochelle salt was added and stirring continued at r.t. for 1 h. The organic compounds were extracted with ether/AcOEt and the organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. Column chromatography (SiO_2 60, *n*-hexane : AcOEt=4 : 1) gave *N*-[(1,1′-dimethylethoxy)carbonyl]-L-prolinal (370 mg, 93%) as colorless oil. To a solution of methyltriphenylphosphonium bromide (16.8 g, 47.0 mmol, 2.00 eq) in tetrahydrofuran (THF) (340 ml) was added potassium bis(trimethylsilyl)amide ($KN(TMS)_2$) (9.38 g, 47.0 mmol, 2.00 eq) at r.t. and the whole was stirred at r.t. for 1 h and −78 °C for 10 min. A solution of *N*-[(1,1′-dimethylethoxy)carbonyl]-L-prolinal (4.61 g, 23.2 mmol) in THF (50.0 ml) was then added and the whole was stirred at −78 °C for 10 min and r.t. for 2 h. MeOH (42.0 ml), and aq. sat. Rochelle salt were added and stirring continued at r.t. for 1 h. The organic compounds were extracted with ether and the organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. Column chromatography (SiO_2 60, *n*-hexane : AcOEt=4 : 1) gave (*S*)-*N*-(*tert*-butoxycarbonyl)-2-vinylpyrrolidine[12] (3.34 g, 73%) as colorless oil. $[\alpha]_D^{23}$ −10.8° (c=1.09, $CHCl_3$), lit.[12] $[\alpha]_D^{26-28}$ −13.4° ($CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 1.44 (9H, s), 1.69—2.03 (4H, m), 3.39 (2H, m), 4.29 (1H, br s), 5.04 (2H, d, J=10.0 Hz), 5.73 (1H, br s); IR (KBr): 2960, 1690, 1480, 1450, 1390, 1370, 1250, 1170, 910.

Preparation of Diene (General Procedure) To a stirred solution of (*S*)-*N*-(*tert*-butoxycarbonyl)-2-vinylpyrrolidine in CH_2Cl_2 was added trifluoroacetic acid at 0 °C and the mixture was stirred at r.t. for 1 h. The solvents were removed under reduced pressure and to the residue CH_2Cl_2, triethylamine (5.0 eq) and corresponding acid chloride (1.2 eq) were added and stirred at r.t. for 1 h. Aqueous sat. NH_4Cl was added to the reaction mixture and the whole was extracted with CH_2Cl_2, washed with brine, filtered, dried over Na_2SO_4, and concentrated. The crude residue was subjected to column chromatography to give diene.

(*S*)-*N*-(3-Butenoyl)-2-vinylpyrrolidine (**2**): 73% from (*S*)-*N*-(*tert*-butoxycarbonyl)-2-vinylpyrrolidine. Yellow oil. $[\alpha]_D^{18}$ −10.9° (c=1.02, $CHCl_3$); ^{1}H–NMR (400 MHz), $CDCl_3$) δ (ppm) 1.76—2.11 (4H, m), 3.08 (2H, m), 3.48 (2H, m), 4.38 (1/2H, br s), 4.67 (1/2H, br s), 5.02—5.19 (4H, m), 5.78 (1H, m), 5.97 (1H, m); ^{13}C-NMR (100 MHz), $CDCl_3$) δ (ppm) 21.52, 23.52, 30.26, 32.49, 39.39, 40.16, 46.05, 46.77, 58.37, 59.53, 114.05, 115.03, 117.34, 117.63, 131.45, 131.92, 137.23, 138.07, 169.20, 170.10; IR (KBr) 3100, 2990, 1650, 1440, 1300, 1280, 1200, 995, 910 cm^{-1}; Low resolution mass spectra (LRMS) fast atom bombardment (FAB) *m*/*z* 166 (M^++1); High resolution mass spectra (HRMS) (FAB) Calcd for $C_{10}H_{16}NO$ 166.1232 (M^++1), Found 166.1230.

(*S*)-*N*-(4-Pentenoyl)-2-vinylpyrrolidine (**3**): 88% from (*S*)-*N*-(*tert*-butoxycarbonyl)-2-vinylpyrrolidine. Yellow oil. $[\alpha]_D^{24}$ −16.9° (c=1.03, $CHCl_3$); ^{1}H-NMR (400 MHz), $CDCl_3$) δ (ppm) 1.95 (4H, m), 2.38 (4H, m), 3.50 (4H, m), 4.51 (1H, m), 4.99 (4H, m), 5.82 (2H, m); IR (neat) 2950, 1640, 1420, 1240, 990, 910; LRMS (FAB) *m*/*z* 180 (M^++1).

(*S*)-*N*-(*E*-2-Butenoyl)-2-vinylpyrrolidine (**5**): 82% from (*S*)-*N*-(*tert*-butoxycarbonyl)-2-vinylpyrrolidine. Yellow oil. $[\alpha]_D^{18}$ −32.7° (c=1.07, $CHCl_3$); ^{1}H-NMR (400 MHz), $CDCl_3$) δ (ppm) 1.84 (3H, d, J=6.2 Hz), 1.76—2.18 (4H, m), 3.58 (2H, m), 4.45 (1/2H, br s), 4.72 (1/2H, br s), 5.07—5.18 (2H, m), 5.80 (1H, m), 6.10 (1H, d, J=14.9 Hz), 6.92 (1H, m); ^{13}C-NMR (100 MHz), $CDCl_3$) δ (ppm) 17.90, 17.97, 21.48, 23.56, 30.24, 32.40, 46.03, 46.68, 58.37, 59.13, 113.96, 115.08, 123.00, 123.34, 137.33, 138.42, 140.47, 141.15, 164.54, 165.41; IR (KBr) 3082, 2970, 2877, 1664, 1448, 1305, 1261, 1197, 964, 909 cm^{-1}; LRMS (FAB) *m*/*z* 166 (M^++1); HRMS (FAB) Calcd for $C_{10}H_{16}NO$ 166.1232 (M^++1), Found 166.1239.

(*S*)-*N*-(5-Hexenoyl)-2-vinylpyrrolidine (**6**): 85% from (*S*)-*N*-(*tert*-butoxycarbonyl)-2-vinylpyrrolidine. Colorless oil. $[\alpha]_D^{23}$ −18.5° (c=0.09, $CHCl_3$); ^{1}H-NMR (400 MHz), $CDCl_3$) δ (ppm) 5.84—5.71 (2H, m), 5.15—4.93 (4H, m), 4.67, 4.34 (1H, t-like), 3.55—3.40 (2H, m), 2.37—1.70 (10H, m); ^{13}C-NMR (100 MHz), $CDCl_3$) δ (ppm) 172.23, 171.32, 138.23, 137.54, 137.54, 138.23, 114.85, 113.93, 113.93, 114.85, 59.58, 58.31, 46.81, 45.95, 33.38, 33.46, 30.32, 32.51, 30.34, 23.71, 23.83, 23.60, 21.58; IR (neat) 2950, 1640, 1420, 1350, 1320, 1200, 990, 910 cm^{-1}; LRMS (FAB) *m*/*z* 194 (M^++1); HRMS (FAB) Calcd for $C_{12}H_{20}NO$ 194.1542 (M^++1), Found 194.1529.

RCM Reaction. 2,3,6,8a-Tetrahydro-(*S*)-5(1*H*)-indolizinone (7) To a stirred solution of diene **2** (480 mg, 2.90 mmol) in benzene (100 ml), $Cl_2(PCy_3)_2Ru{=}CHCH{=}CHPh$ (131 mg, 0.150 mmol) was added. The mixture was degassed three times by the F.P.T. (freeze pump thaw cycle) method and the reaction mixture was stirred at r.t. for 3 d. The solvent was removed under reduced pressure to give a residue which was purified by silica gel column chromatography (AcOEt) to give **7** (368 mg, 93%) as brown oil. $[\alpha]_D^{21}$ −59.5° (c=1.02, $CHCl_3$); ^{1}H-NMR (400 MHz), $CDCl_3$) δ (ppm) 1.53—2.16 (4H, m), 2.92 (2H, m), 3.43 (1H, m), 3.74 (1H, m), 4.05 (1H, m), 5.80 (1H, m), 5.88 (1H, dd, J=10.0, 1.0 Hz); ^{13}C-NMR (100 MHz), $CDCl_3$) δ (ppm) 21.94, 31.92, 32.71, 43.91, 58.72, 122.76, 125.11, 166.30; IR (KBr) 2910, 2840, 1630, 1450, 1340, 1310, 1210, 1160, 1000 cm^{-1}; LRMS (FAB) *m*/*z* 137 (M^++1); HRMS (FAB) Calcd for $C_8H_{11}NO$ 137.0841 (M^++1), Found 137.0843.

1,2,3,6,7,9a-Hexahydro-(*S*)-pyrrolo[1,2-*a*]azepin-5(1*H*)-one (8) To a stirred solution of diene **3** (54.5 mg, 0.304 mmol) in benzene (10.0 ml), $Cl_2(PCy_3)_2Ru{=}CHCH{=}CHPh$ (27.2 mg, 0.0304 mmol) was added. The mixture was degassed three times by the F.P.T. method and the reaction mixture was stirred at r.t. for 23 d. The solvent was removed under reduced pressure to give a residue which was purified by silica gel column chromatography (AcOEt) to give **8** (41.7 mg, 91%) as brown oil. $[\alpha]_D^{20}$ −37.0° (c=0.356, $CHCl_3$); ^{1}H-NMR (400 MHz), $CDCl_3$) δ (ppm) 1.43—1.80 (1H, m), 1.68—1.91 (2H, m), 2.21—2.35 (1H, m), 2.21—2.49 (2H, m), 2.40—2.49 (1H, m), 2.91 (1H, J=13.7, 3.9 Hz), 3.45 (1H, m), 3.65 (1H, m), 4.55 (1H, br s), 5.52 (1H, dd, J=11.45, 2.2 Hz), 5.69 (1H, m); ^{13}C-NMR (100 MHz), $CDCl_3$) δ (ppm) 23.08, 24.79, 34.40, 34.91, 46.67, 55.07, 129.87 130.11, 172.78; IR (neat) 2960, 2880, 1640, 1460, 1350, 1320, 1245, 1160, 1030, 900, 860, 850, 785, 740, 720 cm^{-1}; LRMS (FAB) *m*/*z* 152 (M^++1); HRMS (FAB) Calcd for C9$H_{14}NO$ 152.1067 (M^++1), Found 152.1075.

(*S*)-Pyrrolam A (9)[13] To a stirred solution of diene **5** (289 mg, 1.80 mmol) in benzene (60.0 ml), $Cl_2(PCy_3)_2Ru{=}CHPh$ (74.0 mg, 0.0900 mmol) was added. The mixture was degassed three times by the F.P.T. method and the reaction mixture was stirred at 50 °C for 3 h. The solvent was removed under reduced pressure to give a residue which was purified by silica gel column chromatography (AcOEt) to give **9** (64.8 mg, 30%) as brown oil. $[\alpha]_D^{21}$ +26.2° (c=0.97, $CHCl_3$); ^{1}H-NMR (400 MHz), $CDCl_3$) δ (ppm) 1.18 (1H, m), 2.09 (1H, m), 2.20—2.39 (2H, m), 3.28 (1H, m), 3.48 (1H, m), 4.26 (1H, m), 6.04 (1H, dd, J=5.8, 1.6 Hz), 7.20 (1H, dd, J=5.8, 1.8 Hz); ^{13}C-NMR (100 MHz), $CDCl_3$) δ (ppm) 29.0, 29.9, 41.8, 67.7, 128.5, 148.8, 175.5; IR (KBr) 2968, 2884, 1670, 1387, 812 cm^{-1}; LRMS (EI) *m*/*z* 123 (M^+); HRMS (EI) Calcd for C_7H_9NO 123.0684 (M^+), Found 123.0672.

2,3,6,7,8,10a-Hexahydro-[*S*-(*Z*)]pyrrolo[1,2-*a*]azocin-5(1*H*)-one (10) To a stirred solution of diene **6** (30.0 mg, 0.155 mmol) in CH_2Cl_2 (80.0 ml), $Cl_2(PCy_3)_2Ru{=}CHPh$ (32.0 mg, 0.0390 mmol) was added. The mixture was degassed three times by the F.P.T. method and the reaction mixture was stirred at 50 °C for 20 h. The solvent was removed under reduced pressure to give a residue which was purified by silica gel column chromatography (AcOEt) to give **10** (32.0 mg, 45%) as brown oil. $[\alpha]_D^{23}$ −36.6° (c=0.30, $CHCl_3$); ^{1}H-NMR (400 MHz), $CDCl_3$) δ (ppm) 5.65—5.57 (1H, m), 5.40—5.36 (1H, m), 4.39 (1H, s), 3.61—3.55 (1H, m), 3.44—3.38 (1H, m), 2.65—0.78 (10H, m); ^{13}C-NMR (100 MHz), $CDCl_3$) δ (ppm) 172.74, 132.78, 127.36, 59.57, 46.17, 33.66, 33.17, 25.81, 23., 22.79; IR (KBr) 2950, 1640, 1420, 1350, 1320, 1200, 990, 910 cm^{-1}; LRMS (FAB) *m*/*z* 166 (M^++1); HRMS (FAB) Calcd for $C_{10}H_{16}NO$ 166.1232 (M^++1), Found 166.1230.

Preparation of (−)-Coniceine (15) To a stirred solution of **7** (477 mg, 3.50 mmol) in MeOH (69.0 ml) was added $PtO_2 \cdot 1$—$3H_2O$ (46.5 mg) and H_2 (balloon pressure), and the whole was stirred at r.t. for 4 h. The reaction mixture was filtered using celite and the obtained mother liquor was subjected to column chromatography (AcOEt : MeOH=10 : 1) to give amide (447 mg, 92%) as colorless oil. The obtained amide (97.4 mg, 0.700 mmol) was reacted with $LiAlH_4$ (39.9 mg, 1.05 mmol) in Et_2O (10.0 ml) at r.t. for 2 h. The reaction was quenched by 10% NaOH (0.150 ml). Organic compounds were extracted with Et_2O, dried over $CaCO_3$, filtered and concentrated under reduced pressure to give **15** (83.7 mg, 96%) as colorless oil.

15: $[\alpha]_D^{21}$ −20.5° (c=0.98, EtOH), lit.[14] $[\alpha]_D^{23}$ −10.2±0.6° (c=1.76, EtOH); ^{1}H-NMR (400 MHz), $CDCl_3$) δ (ppm) 1.18—1.26 (2H, m), 1.34—1.44 (1H, m), 1.52—1.69 (3H, m), 1.73—1.87 (5H,m), 1.97 (1H, td, J=11.4, 3.4 Hz), 2.08 (1H, q, J=9.0 Hz), 3.06 (1H, m), 3.11 (1H, m); ^{13}C-NMR (100 MHz), $CDCl_3$) δ (ppm) 29.0, 29.9, 41.8, 67.7, 128.5, 148.8, 175.5; IR

(KBr) 20.58, 24.43, 25.37, 30.39, 30.91, 52.98, 54.16, 64.41 cm^{-1}; LRMS (FAB) m/z 126 (M^{+}+1); HRMS (FAB) Calcd for $C_8H_{16}N$ 126.1271 (M^{+}+1), Found 126.1283.

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