Antiandrogenic Effect of 16-Substituted, Non-substituted and D-Homopregnane Derivatives

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The pharmacological activities of 12 pregnane derivatives (4—15) were determined on gonadectomized male hamster flank organs and seminal vesicles as antiandrogens and as 5α-reductase inhibitors. The results from this study indicate that subcutaneous injection of testosterone for 3 d increased the diameter of the pigmented spot in the flank organs, whereas finasteride when injected with testosterone decreased the size of the spot significantly when steroids 4—15 were injected together with testosterone, the diameter of the flank organs of gonadectomized male hamsters, decreased significantly ($p<0.005$) compared to testosterone. Compound 11 was the most active steroid and reduced the diameter of the pigmented spot more than the other synthesized steroids or finasteride. Subcutaneous injections of testosterone to gonadectomized animals restore the seminal vesicle size lost upon castration. Injection of testosterone plus finasteride decreased significantly the weight of these glands ($p<0.005$). Steroids 5—15 when injected with testosterone decreased the weight of the seminal vesicles compared to testosterone. Finasteride is a good inhibitor of the conversion of testosterone to dihydrotestosterone (DHT) (low formation of DHT) measured as pmole of DHT/g of protein/h. Steroids 6—15 inhibited the conversion of testosterone to DHT as compared to testosterone however finasteride and 10 appeared to be the most effective compounds. Castration increases the protein content of the seminal vesicles (control) expressed as μg/mg of tissues. Testosterone tends to decrease it significantly, as did compounds 4, 5, 7, 9, and 15. We demonstrated that DHT as well as cyproterone acetate and steroids 5, 6, 8, 9, 11, and 14 at increasing non radioactive steroid concentration, inhibited the binding of [^{3}H]DHT to cytosolic androgen receptor (AR), as indicated by its K_i values. However, 4, 7, 10, 12, and 13 did not have any inhibitory effect.

Key words seminal vesicle; 5α-reductase; testosterone conversion; D-homo steroid

Antiandrogens offer a potentially useful treatment for androgen mediated diseases such as prostate cancer, benign prostatic hyperplasia, seborrhea, androgenic alopecia, acne and precocious puberty.[1] The Health Secretary[2] reports that prostate carcinoma is an important cause of death in Mexico. The mortality rate per 100000 is about 72.2 for aged men. The potential use of antiandrogens in the treatment of prostate cancer represents an alternative to prostatic operation, which is associated with several side effects and is probably the surgery men fear most.[3]

It has been reported that progesterone (P_4) and deoxycorticosterone inhibit dihydrotestosterone (DHT) (**2**) formation by competing with the Δ^4-3-keto site of testosterone (T) (**1**) for the 5α-reductase enzyme.[4] Previous studies carried out in our laboratory demonstrated that a bromine atom at the C-6 position of the P_4 skeleton increases the inhibitory power of the P_4 molecule on the conversion of T to DHT.[5] Furthermore, a C-17 benzoyloxy moiety in a 4-bromoprogesterone skeleton also contributes to the antiandrogenic activity as measured by reduction in the weight of seminal vesicles, and by the amount of produced DHT. These steroids also showed a much higher inhibitory activity on the conversion of T to DHT than the presently used finasteride (**3**).[6]

Compound **3** is a steroid that has successfully been used for the treatment of benign and malign prostatic hyperplasia.[7] This compound inhibits the activity of 5α-reductase enzyme type 2 that is present in the prostate, and has also a limited inhibitory effect on type 1 enzyme found in the skin.[8]

In this study, we report the antiandrogenic activity of 12 different steroidal compounds **4**—**15** that were synthesized in our laboratory. The antiandrogenic activity was determined in flank organs, seminal vesicles and also *in vitro* by measuring the amount of DHT expressed as pmole of DHT/g of protein/h. In addition, these compounds were evaluated as antagonists for the androgen receptor (AR).

Flank organs are two pigmented nodules located in the dorsal skin surface of hamsters. In female hamsters, the diameter of the pigmented spot measures 2 mm, whereas in males it is 8 mm. In males, these nodules shrink upon castration and they resemble the nodules of the females.[9] However, daily injections of T (**1**) or 5α-DHT (**2**) restore their original size.[10,11] Many steroidal and non-steroidal compounds have been evaluated as antiandrogens using flank organ as a model.[12,13]

Seminal vesicles are male accessory organs that are also androgen-dependent. These organs are capable of reducing T to DHT in both intact and gonadectomized animals and have also been used for evaluation of steroidal and non-steroidal compounds as antiandrogens.[14]

In this study, we evaluated the following compounds: 20-ethylenedioxy-16β-phenylpregn-5-ene-3β,17α-diol (**4**) (Fig. 1); 3β,17α-dihydroxy-17β-methyl-16β-phenyl-D-homopregn-5-en-17a-one (**5**); 17α-hydroxy-17β-methyl-16β-phenyl-D-homopregna-4,6-diene-3,17a-dione (**6**); 17α-acetoxy-17β-methyl-16β-phenyl-D-homopregna-4,6-diene-3,17a-dione (**7**); 17α-hydroxy-16β-methylpregna-1,4,6-triene-3,20-dione (**8**); 16β-methyl-17α-propionyloxypregna-1,4,6-trienediene-3,20-dione (**9**); 17α-acetoxy-16β-methylpregna-1,4,6-triene-3,20-dione (**10**); 17α-acetoxy-4-bromopregna-1,4-diene-3,20-dione (**11**); 3β-acetoxypregna-5,16-dien-20-one (**12**); 3β-hydroxy-16α,17α-epoxypregn-5-ene-20-one (**13**); 3β-

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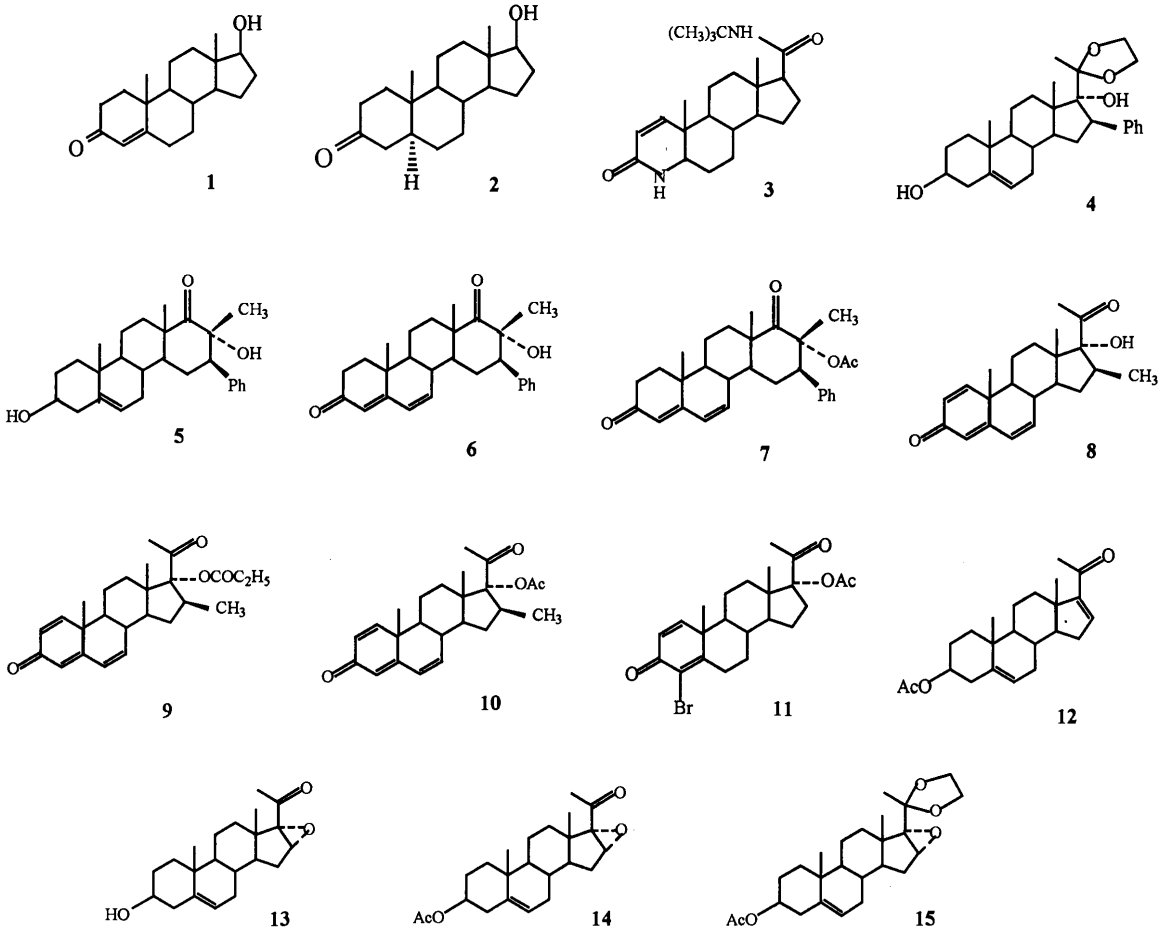

Fig. 1. Steroidal Antiandrogens

acetoxy-16α,17α-epoxypregn-5-ene-20-one (**14**) and 16α,17α-epoxy-20-ethylenedioxypregn-5-ene-3β-yl acetate (**15**).

Methods and Results

Effect of Synthesized Steroids on Flank Organs, Seminal Vesicles, DHT Formation and Protein Synthesis

Flank Organ Test After castration, the diameter of the pigmented spot on the flank organs (control) decreased significantly ($p<0.05$) compared to that of the uncastrated animals. Subcutaneous injections of vehicle alone did not change the size of the organs, however treatment with T (**1**) restored the diameter of the pigmented spot (Table 1). When T (**1**) and **3** were injected together, the diameter of the pigmented spot on the flank organs decreased significantly (2 mm) as compared to that of the gonadectomized animals treated with T (**1**) (8 mm). All synthesized steroids **4**—**15** when injected with T (**1**) decreased the diameter of the spot as compared to the control T. The most effective compounds in this study were steroids **6** and **11**, which reduced the diameter of the flank organs to 2.0 and 1.5 mm respectively. This data suggests that steroids **6** and **11** have similar inhibitory activity towards the enzyme 5α-reductase as compared to the standard **3**.

Seminal Vesicles After castration, the weight of the male hamster seminal vesicles decreased significantly ($p<0.05$) (control) compared to that of the normal glands. Treatment with vehicle alone did not change this condition (Table 1) whereas subcutaneous injections of 200 μg of T (**1**) (T) for 3 d significantly increased ($p<0.05$) the weight of the seminal vesicles in castrated male hamsters.

When T (**1**) and **3** were injected together, the weight of the seminal vesicles decreased significantly ($p<0.005$) as compared to the experiment in which the animals were treated with T only (Table 1).

The injection of steroids **5**—**15** together with T (**1**), decreased ($p<0.005$) the weight of the seminal vesicles as

Table 1. The Diameter of Flank Organs, and the Weight and Protein Content of Seminal Vesicles Were Measured in Animals Which Received Subcutaneous Treatment of the Synthesized Steroids±Standard Deviations

Treatment	Diameter of flank organs (mm)	Weight of seminal vesicles (mg)	Protein content (μg/mg of tissue)
Control	2.0±0.00	91.5±13.8	50.99±4.0
T	8.0±0.00	382.4±10.8	28.10±4.2
T+**3**	2.0±0.02	226.2±30	41.20±9.2
T+**4**	5.5±0.50	350±50	25.00±5
T+**5**	2.5±0.50	173±26	22.82±1.1
T+**6**	2.0±0.00	127±14	37.62±1.1
T+**7**	3.5±0.50	214.3±13.4	22.40±1.9
T+**8**	2.5±0.50	154±31	35.79±1.4
T+**9**	3.0±0.00	120±10	18.00±5.0
T+**10**	3.0±0.00	121.5±23	35.97±1.4
T+**11**	1.5±0.70	111.6±5.4	41.33±1.4
T+**12**	3.0±0.00	111.4±1	52.18±2.0
T+**13**	3.0±0.00	186.3±19	59.18±8.6
T+**14**	2.5±0.60	199.5±24	56.58±2.6
T+**15**	4.0±0.00	179.2±4	25.22±1.0

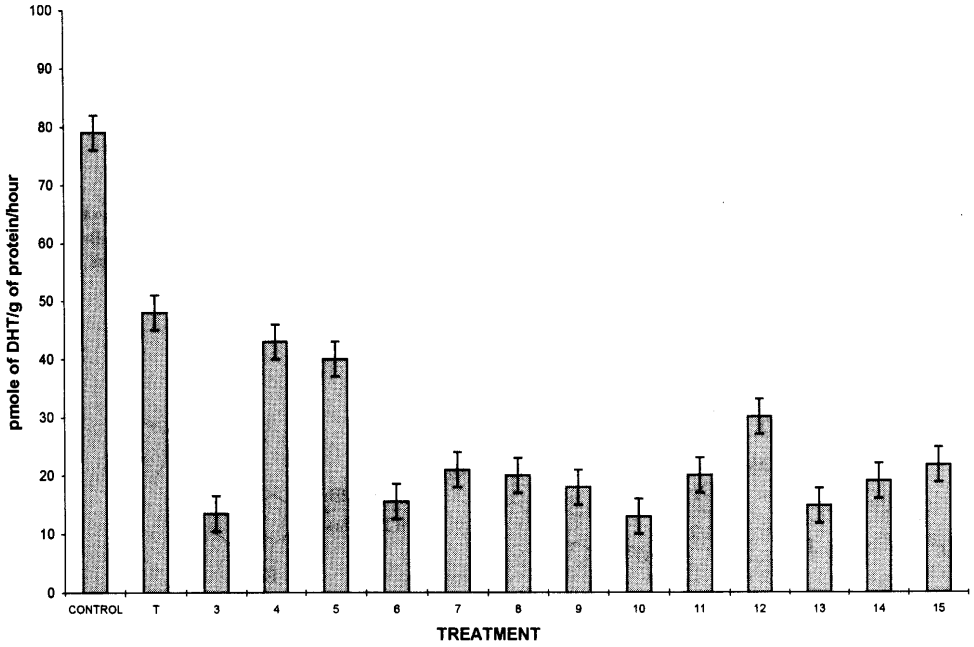

Fig. 2. The Effect of the Synthesized Steroidal Antiandrogens on T to DHT Conversion in Seminal Vesicles Expressed as pmol of DHT/g of Protein/h± Standard Deviation

compared to the T and **4** treated animals. The most effective compounds for decreasing the weight of the seminal vesicles were steroids **6**, **9**—**12**.

Conversion of T to DHT The extracts from castrated male hamster seminal vesicles were subjected to TLC analysis. The zone corresponding to the DHT (**2**) standard (*Rf* value 0.34) of each experimental chromatogram was eluted and the radioactivity determined.

The results from Fig. 2 obtained from two separate experiments performed in duplicate showed that in the seminal vesicles of control animals, a significant conversion of T to DHT took place.

Figure 2 shows the significant difference ($p<0.05$) in the conversion of [^{3}H]T (**1**) to [^{3}H]DHT (**2**) between the control animals treated with T and those treated with T plus **3**. The data from this study clearly indicate that **3** is a good inhibitor of the conversion of T (**1**) to DHT (**2**) at pH 6.

The effect of different steroidal structures **4**—**15** on the rate of DHT formation expressed as pmoles of DHT/g of protein/h is shown in Fig. 2. Compounds **4** and **5** showed a higher *in vitro* conversion of T to DHT ($p<0.05$) than the other synthesized steroids. On the other hand, **6**—**15** exhibited a lower DHT conversion than the control and T-treated animals. Compounds **6**, **10** and **13** demonstrated the same inhibitory effect as **3**.

Antagonistic Activity of the Synthesized Steroids for ARs The effect of increasing non-radioactive natural and synthetic steroid concentrations upon [^{3}H]DHT binding to ARs from seminal vesicles are presented in Fig. 5. The K_i for the synthesized steroids showed the following order of affinity to ARs : DHT>CA>8>11>5>9>14>6 (see Table 2).

Protein Content The protein content was determined using the well known Bradford dye-binding method.[15]

In seminal vesicles the protein content under different treatments is shown in Table 1. Castration tends to increase the protein content (control) expressed as μg/mg of tissue whereas T (**1**) decreased it, as previously reported by our group.[5,6]

Table 2. Inhibition Constants (K_i) of Different Steroids

Steroid	K_i
DHT	10.0
CA	10.8
P_4	NA
4	NA
5	17.2
6	41.0
7	NA
8	16.0
9	19.0
10	NA
11	16.5
12	NA
13	NA
14	22.0

Results are given in [nM]. NA; no affinity for androgen receptors.

Discussion

This study reports the antiandrogenic effect of a variety of 16-phenylsubstituted-D-homo compounds **5**—**7**, 16-methyl-substituted steroids **8**—**10**, a 4-bromo compound **11** without a methyl group at C-16 and the epoxy compounds **13**—**15**.

Compounds **5**—**10**, **13**—**15** were prepared from the commercially available 16-dehydropregnenolone acetate (**12**). Epoxidation of the double bond at C-16 in **12** with hydrogen peroxide and sodium hydroxide afforded the epoxy derivative **13** (Fig. 4). Acetylation of **13** in the usual manner yielded the acetoxy compound **14**. Protection of the carbonyl group in **14** was effected with ethylene glycol, trimethyl orthoformate and *p*-toluenesulfonic acid. The resulting dioxolane derivative **15** was treated with phenylmagnesium bromide in tetrahydrofuran (THF) at reflux to give C-16 phenyl derivative **4** (Fig. 3). Hydrolysis of the dioxolane ring of **4** to recover the carbonyl moiety was carried out with perchloric

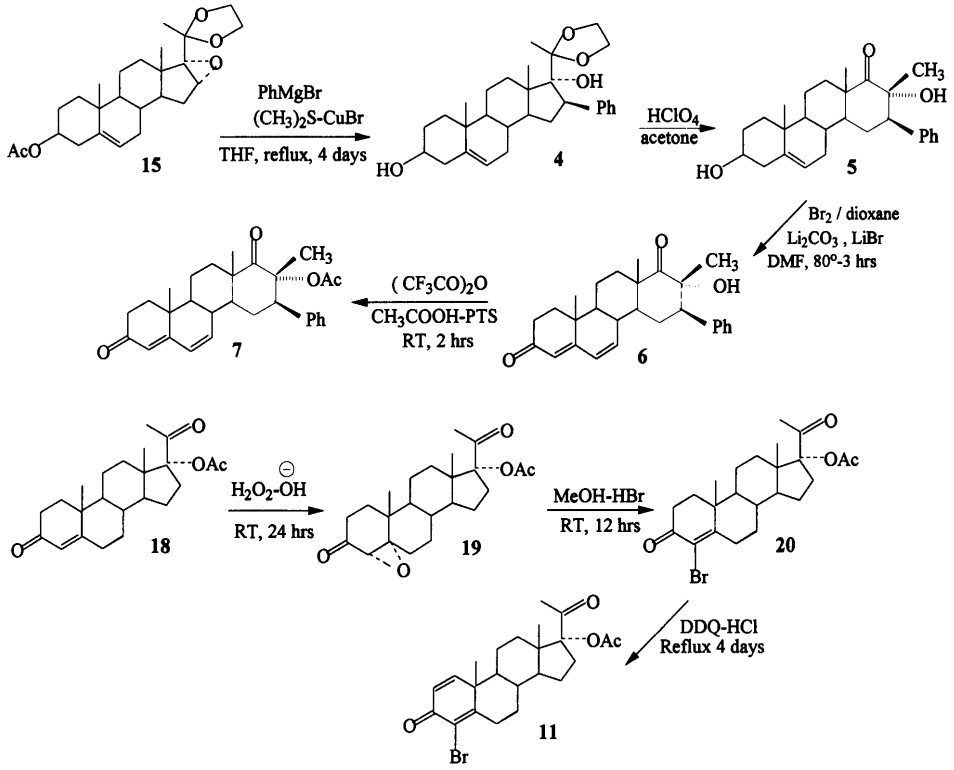

Fig. 3. Synthesis of the Steroidal Compounds from the Intermediate 16α,17α-Epoxy-20-ethylenedioxypregn-5-en-3β-yl Acetate

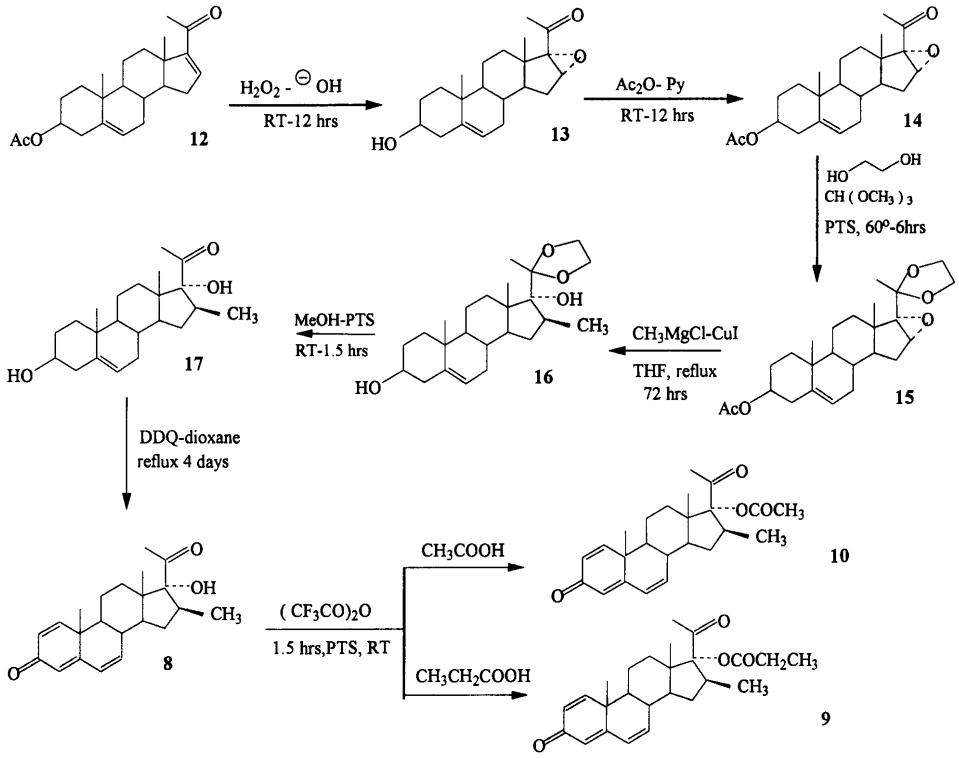

Fig. 4. Synthesis of the Steroidal Compounds from the Commercially Available 16-Dehydropregnenolone Acetate

acid in acetone. In this reaction, expansion of the D-ring took place thus forming D-homosteroid **5**.[16] Treatment of **5** with lithium carbonate, lithium bromide and bromine in *N*,*N*-dimethylformamide afforded the 4,6-diene-3-one moiety **6**. Acetylation of the hydroxyl group in **6** with trifluoroacetic anhydride and acetic acid yielded the desired acetoxy derivative **7**.

Compounds **8**—**10** were synthesized from the intermediate **15** (Fig. 4). Reaction of **15** with methylmagnesium chloride afforded the 16β-methyl derivative **16**. Treatment of **16** with methanol and *p*-toluenesulfonic acid yielded the C-20 carbonyl derivative **17** (ref. 3 describes the synthesis of these compounds). Upon reflux with DDQ (dichlorodicyanobenzoquinone), **17** afforded the steroid **8**. Esterification of **8** with

acetic acid–trifluoroacetic anhydride yielded the desired acetoxy derivative **10**. On the other hand, treatment of **8** with propionic acid–trifluoroacetic anhydride afforded the propionyloxy derivative **9**.

The 4-bromo derivative **11** (Fig. 3) was synthesized from the commercially available 17α-acetoxyprogesterone (**18**). Epoxidation of the C-4 double bond in **18** afforded the 4,5-epoxy compound **19**. Opening of the oxirane ring in **19** was carried out with methanol and hydrogen bromide. The resulting 4-bromo derivative **20** (described in ref. 6) was treated with DDQ in dioxane to afford desired compound **11**.

As can be seen from Table 1 (diameter of flank organs), steroid **6** has the same inhibitory effect as the standard compound **3**, whereas **11** has a higher inhibitory activity for the enzyme 5α-reductase than commercially available **3**. This compound has a bromine atom at C-4 and is very similar to compounds having a bromine atom at C-6 previously synthesized in our laboratory, which also showed potent inhibitory activity towards 5α-reductase, as well as antiandrogenic activity.[5] Flank organs have the capacity to convert T to DHT[11] and in the skin[8] the 5α-reductase enzyme type 1 is more abundant than type 2. Compound **3** is a typical inhibitor for type 2 5α-reductase enzyme, however it can also inhibit the type 1 enzyme albeit to a smaller degree. In our study, all compounds (**4**—**15**) reduced the size of the pigmented spot as compared to T; (diameter 8 mm), however this does not exclude the presence of type 2 enzyme in this tissue. These data also suggest that compounds **4**—**15** could also inhibit the FAR-17A gene.[17]

Table 1 shows the antiandrogenic effect of steroids **4**—**15** related to the weight of the seminal vesicles of castrated male hamsters treated with T (**1**) or a combination of T (**1**) with the synthesized steroids. These data clearly indicate that compounds **5**—**15** showed a higher antiandrogenic effect (lower weight of the seminal vesicles) than the finasteride treated animals. These effects are not only the result of inhibition of conversion of T to DHT[18]; but could also involve inhibition of the synthesis and/or release of pituitary gonadotropins.

Figure 2 shows the conversion of [^{3}H]T to [^{3}H]DHT expressed as pmole of protein per hour. In the control animals, conversion of T to DHT was higher than in the T (**1**) treated animals. This can be explained since the control animals were treated with vehicle only, whereas in all other experiments, they were treated with T (**1**) and the synthesized steroid, which could dilute the radiolabeled marker in the seminal vesicles. It is also possible that the 5α-reductase enzyme was synthesized in a higher amount in the control as compared to the T treated animals.

Compound **3** reduces the conversion of T to DHT substantially and therefore can be considered a good inhibitor of the *in vitro* T to DHT conversion at pH 6 (Fig. 2). These results are in agreement with those obtained by other authors, who reported the inhibitory effect of finasteride and similar compounds on 5α-reductase type 2-activity.[8] All synthesized steroids, with the exception of **4** and **5**, inhibited the conversion of T to DHT at pH 6 in seminal vesicles homogenates from castrated male hamsters treated with a dose of 200 μg as compared to T (**1**). Compounds **10** and **3** showed similar inhibition of the T to DHT conversion.

Compound **5** decreased the weight of the seminal vesicles,

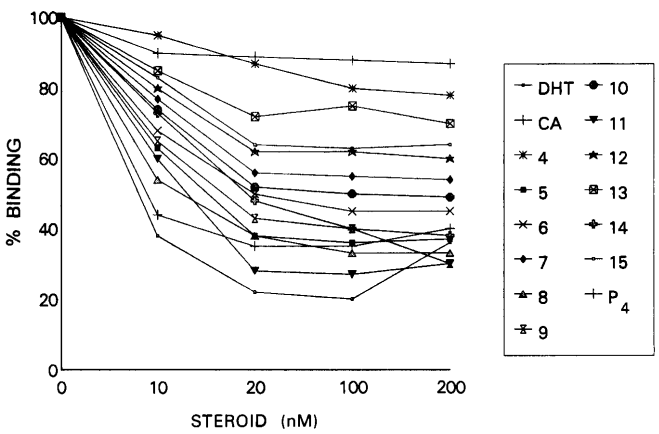

Fig. 5. Binding Specificity

Gonadectomized male hamster seminal vesicle cytosol incubated in the presence of 1 nM [^{3}H]DHT and increasing concentrations of radio-inert steroids. Points represent the mean of duplicate determinations of two different experiments, standard deviations are too small to be represented. DHT, P_4, CA (cyproterone acetate) and the synthesized steroids **4**—**15** are also represented in this figure.

when compared to the T treated animals (Table 1), however it did not show a similar effect in the T to DHT conversion (Fig. 2). These data suggest that **5** is probably an agonist of the AR.

In this study we demonstrated that radio-inert DHT, as well as increasing CA concentrations, were able to inhibit the binding of [^{3}H]DHT to the AR present in the cytosolic fraction, as shown by the K_i values of 10 and 10.8 respectively. Furthermore, some radio-inert synthetic steroids (**5**, **6**, **8**, **9**, **11**, **14**) also inhibited the binding of [^{3}H]DHT to cytosolic AR, as indicated by the respective K_i values (Table 2). In contrast, P_4 as well as the steroids **4**, **7**, **10**, **12** and **13**, did not have any inhibitory effect on cytosolic AR, as shown in Table 2 and Fig. 5.

The data on protein content in the seminal vesicles (Table 1) show that castration (control) increases the protein content, whereas T and compounds **4**, **5**, **7**, **9** and **15** tend to decrease it. These results can be explained by taking into consideration the possibility that T as well as compounds **4**, **5**, **7**, **9** and **15** may downregulate 5α-reductase synthesis and thus control the weight and functioning of the seminal vesicles. This hypothesis could be corroborated by the fact that the control, as well as compounds **6**, **8**, **10**—**14** and **3**, apparently upregulate 5α-reductase synthesis and thus increase the protein content. This however does not explain the behavior of compounds **7** and **9** (Fig. 2). This phenomenon strongly suggests that these compounds may reduce synthesis of ARs.

In the future, several functional group modifications will be carried out with the most active compounds with the purpose of improving their antiandrogenic effect and also increasing their inhibitory activity for the enzyme 5α-reductase.

Experimental

Chemicals and Radioactive Material Solvents were laboratory grade or better. Melting points were determined on a Fisher–John melting point apparatus and are uncorrected. ^{1}H- and ^{13}C-NMR were taken on Varian Gemini 200s and VRX-300 spectrometers, respectively. Chemical shifts are given in ppm relative to Me_4Si (δ=0) in $CDCl_3$. The abbreviations of the signal patterns are as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra were obtained with a HP5985-B spectrometer. IR spectra were taken on a Perkin Elmer 549B and the UV spectra were recorded on a Perkin Elmer 200s spectrometer.

New England Nuclear Co. (Boston, MA, U.S.A.) provided (15, 16 ^{3}H)T ([^{3}H]T, specific activity: (85—100) Ci/mmol) and (1, 2, 6, 7-^{3}H)DHT ([^{3}H]DHT, specific activity: (110) Ci/mmol). Steraloids, Inc. (Wilton, NH, U.S.A.) supplied radio-inert T, 5α-DHT, P_4, and CA. Sigma Chemical Co. (St. Louis, MO, U.S.A.) provided $NADPH^+$.

20-Ethylenedioxy-16β-phenylpregn-5-ene-3β,17α-diol (4) Compound **15** (1 g, 24 mmol) (the preparation of this compound is described in ref. 5), dimethylsulfide–cuprous bromide complex (300 mg) and a 1 mol solution of phenylmagnesium bromide in THF (14 ml) were refluxed for 4 d under a nitrogen atmosphere. The reaction mixture was then poured into saturated aqueous ammonium chloride solution (15 ml), and the mixture extracted 3 times with chloroform. The organic phase was washed with water and dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The resulting crude product was recrystallized from methanol to give 0.71 g, 1.57 mmol (65%) of the pure compound **4** (Fig. 3). mp 220—222 °C. IR (KBr) cm^{-1}: 3400, 1600, 1450, 1048, 900, 766, 698. ^{1}H-NMR ($CDCl_3$) δ: 1.0 (3H, s), 1.10 (3H, s), 2.21 (3H, s), 3.1 (1H, m), 3.3 (2H, m), 3.52 (2H, m), 5.39 (1H, dd, J_1=4, J_2=2 Hz), 7.26 (5H, m). ^{13}C-NMR ($CDCl_3$) δ: 14.5 (C-18), 20 (C-19), 22 (C-21), 60 (C-16), 61, 64 (–OCH_2–CH_2–O–), 71 (C-17), 72 (C-3), 90 (C-20), 121 (C-6), 125—128 (C-16, Ph-), 143 (C-5). MS (*m/z*): 452 (M^+).

3β,17α-Dihydroxy-17β-methyl-16β-phenyl-D-homopregn-5-en-17a-one (5) A solution of steroid **4** (1.0 g, 2.2 mmol) in acetone (80 ml) and perchloric acid (1 ml) (Fig. 3) was stirred at room temperature for 3 h. A saturated aqueous sodium bicarbonate solution (100 ml) was added to neutralize the acid. The crude compound **5** precipitated; it was filtered and recrystallized from methanol. Yield 0.87 g, 2.1 mmol (96%), mp 209—211 °C. IR (KBr) cm^{-1}: 3482, 3426, 1692, 1600, 1050. ^{1}H-NMR ($CDCl_3$) δ: 1.0 (3H, s), 1.10 (3H, s), 1.2 (3H, s), 2.9 (1H, m), 5.39 (1H, dd, J_1=4, J_2=2 Hz), 7.26 (5H, m). ^{13}C-NMR ($CDCl_3$) δ: 16 (C-18), 19 (C-19), 24 (CH_3 at C-17), 54 (C-16), 71 (C-3), 78 (C-17), 218 (C-17a). MS (*m/z*): 409 (M^+).

In the hydrolysis of the ketal **4** an expansion of the 5-membered D-ring to a 6-membered D-homo compound took place.[16]

17α-Hydroxy-17β-methyl-16β-phenyl-D-homopregna-4,6-diene-3,17a-dione (6) To a suspension of **5** (1.0 g, 2.4 mmol), lithium carbonate (3.0 g) and lithium bromide (2.0 g) in *N,N*-dimethylformamide (14 ml) was added dropwise a solution containing bromine (0.3 ml) dissolved in dioxane (8.5 ml). The mixture was then kept a 75 °C for 2 h. The reaction mixture was allowed to cool to room temperature and the inorganic salt was filtered off. To the filtrate, a sodium bicarbonate–sodium bisulfite solution (180 ml, 0.3 g $NaHCO_3$; 0.3 g $NaHSO_3$) was added to eliminate the unreacted bromine. Upon cooling the crude product **6** (Fig. 3) precipitated. Recrystallization from acetone afforded 0.59 g, 1.46 mmol (61%) of the pure compound **6**. mp 276—280 °C. UV (nm): 282 (ε=26500). IR (KBr) cm^{-1}: 3446, 3028, 1694, 1656, 1616, 1126, 878. ^{1}H-NMR ($CDCl_3$) δ: 1.1 (3H, s), 1.2 (3H, s), 1.4 (3H, s), 3.0 (1H, d, J=3 Hz), 5.7 (1H, s), 6.2 (1H, dd, J_1=10, J_2=2 Hz), 6.3 (1H, dd, J_1=10, J_2=2 Hz), 7.26 (5H, m). ^{13}C-NMR ($CDCl_3$) δ: 116.2 (C-18), 16.4 (C-19), 24 (CH_3 at C-17), 54 (C-16), 79 (C-17), 124 (C-4), 165 (C-5), 199 (C-3), 217 (C-17a). MS (*m/z*): 404 (M^+).

17α-Acetoxy-17β-methyl-16β-phenyl-D-homopregna-4,6-diene-3,17a-dione (7) A solution containing steroid **6** (1 g, 2.4 mmol), *p*-toluenesulfonic acid (50 mg), trifluoroacetic anhydride (0.6 ml) and glacial acetic acid (1.26 ml) was stirred for 1.5 h at room temperature (nitrogen atmosphere). The reaction mixture was diluted with chloroform (10 ml) and was neutralized with an aqueous sodium bicarbonate solution to pH 7. The organic phase was separated and dried over anhydrous sodium sulfate and the solvent eliminated *in vacuo* and the crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (8 : 2) eluted 750 mg, 1.68 mmol (70%) of the pure product **7**. mp 278—280 °C. UV (nm): 284 (ε=23400). IR (KBr) cm^{-1}: 1725, 1705, 1660, 1616, 1282, 875. ^{1}H-NMR ($CDCl_3$) δ: 0.8 (3H, s), 1.0 (3H, s), 1.3 (3H, s), 4.0 (1H, m), 5.7 (1H, s), 6.2 (1H, dd, J_1=10, J_2=2 Hz), 6.4 (1H, dd, J_1=10, J_2=2 Hz), 7.2 (2H, m), 7.3 (3H, m). ^{13}C-NMR ($CDCl_3$) δ: 16 (C-18), 18 (C-19), 23 (CH_3 at C-17), 50 (C-16), 84 (C-17), 122 (C-4), 160 (C-5), 165 (CH_3COO), 195 (C-3), 215 (C-17a). MS (*m/z*): 446 (M^+).

17α-Hydroxy-16β-methylpregna-1,4,6-triene-3,20-dione (8) This compound was prepared from 16-dehydropregnenolone acetate **12** (Fig. 4). The exact procedure for the preparation of intermediates **12**—**17** is described in ref. 5.

A solution of steroid **17** (1 g, 2.88 mmol) and DDQ (2.2 g) in dioxane (50 ml) was allowed to reflux for 4 d. Upon cooling, the precipitated 2,3-dichloro-5,6-dicyanohydroquinone was filtered off. To the filtrate was added 3% aqueous sodium hydroxide solution (100 ml) and chloroform (100 ml) and the mixture was stirred for 5 min. The organic phase was washed 3 times with 3% aqueous sodium hydroxide solution and water. It was then dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (6 : 4) eluted 640 mg, 1.88 mmol (65%) of the pure product **8**. mp 197—199 °C. UV (nm): 223, 258, 303 (ε=14800, 12800, 18100). IR (KBr) cm^{-1}: 3389, 1707, 1651, 1600. ^{1}H-NMR ($CDCl_3$) δ: 0.77 (3H, s), 1.2 (3H, d, J=2 Hz), 1.3 (3H, s), 2.3 (3H, s), 6 (1H, d), 6.2 (1H, s), 6.3 (1H, d), 6.4 (1H, J=5 Hz), 7 (1H, d, J=5 Hz). ^{13}C-NMR ($CDCl_3$) δ: 14 (C-18), 18 (C-19), 50 (CH_3, C-16), 78 (OH, C-17), 186 (C-20), 217 (C-3). MS (*m/z*): 340 (M^+).

16β-Methyl-17α-propionyloxypregna-1,4,6-triene-3,20-dione (9) A solution containing steroid **8** (200 mg, 0.5 mmol), *p*-toluenesulfonic acid (10 mg), trifluoroacetic anhydride (1.2 ml) and propionic acid (0.9 ml) was stirred for 5 h at room temperature (nitrogen atmosphere). The reaction mixture was then diluted with chloroform (10 ml) and neutralized with an aqueous sodium bicarbonate solution to pH 7. The organic phase was separated and dried over anhydrous sodium sulfate and solvent removed *in vacuo* and the crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (8 : 2) eluted 163 mg, 0.41 mmol (80%) of the pure product **9** (Fig. 4). mp 218—220 °C. UV (nm): 223, 225, 298 (ε=14800, 12600, 17800). IR (KBr) cm^{-1}: 1725, 1707, 1604. ^{1}H-NMR ($CDCl_3$) δ: 1.1 (3H, s), 1.2 (3H, S), 1.3 (3H, d, J=2 Hz), 2.2 (3H, s), 2.4 (2H, m), 6 (1H, s), 6.2 (1H, s), 6.3 (1H, J=4 Hz), 7.1 (1H, d, J=4 Hz). ^{13}C-NMR ($CDCl_3$) δ: 12 (C-18), 18 (C-19), 28 (C-21), 47 (CH_3, C-16), 83 (C-17), 173 (CH_3COO), 200 (C-20), 212 (C-3). MS (*m/z*): 398 (M^+).

17α-Acetoxy-16β-methylpregna-1,4,6-triene-3,20-dione (10) A solution containing steroid **8** (1 g, 2.5 mmol), *p*-toluenesulfonic acid (50 mg), trifluoroacetic anhydride (6 ml) and glacial acetic acid (1.2 ml) was stirred for 5 h at room temperature (nitrogen atmosphere). The reaction mixture was diluted with chloroform (10 ml) and neutralized with an aqueous sodium bicarbonate solution to pH 7. The organic phase was separated and dried over anhydrous sodium sulfate and the solvent removed *in vacuo* and the crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (8 : 2) eluted 820 mg, 2.14 mmol of the pure product **10** (Fig. 4), (80%). mp 209—210 °C. UV (nm): 222, 253, 296 (ε=14900, 12700, 18000). IR (KBr) cm^{-1}: 1728, 1700, 1610. ^{1}H-NMR ($CDCl_3$) δ: 1.0 (3H, s), 1.2 (3H, S), 1.4 (3H, d, J=3 Hz), 2.2 (3H, s), 2.4 (3H, s), 6.2 (1H, s), 6.3 (1H, s), 6.4 (1H, d, J=3 Hz), 7.2 (1H, d, J=3 Hz). ^{13}C-NMR ($CDCl_3$) δ: 14 (C-18), 20 (C-19), 27 (C-21), 48 (CH_3, C-16), 85 (C-17), 175 (CH_3COO), 190 (C-20), 210 (C-3). MS (*m/z*): 384 (M^+).

17α-Acetoxy-4-bromopregna-1,4-diene-3,20-dione (11) This compound was synthesized from 17α-acetoxyprogesterone (**18**) (Fig. 3). The preparation of the corresponding intermediates **19** and **20** is given in ref. 6.

A solution of steroid **20** (1 g, 2.2 mmol) and DDQ (3 g) in dioxane (30 ml) was stirred at room temperature. Hydrogen chloride was bubbled through the solution for 20 min and stirring was continued for 2 d at room temperature and 1 d under reflux. The reaction mixture was cooled to 0 °C and the precipitated 2,3-dichloro-5,6-dicyanohydroquinone was filtered off. To the filtrate was added 3% aqueous sodium hydroxide solution (50 ml) and chloroform (50 ml) and the mixture was stirred for 10 min. The organic phase was separated, washed 3 times with water, dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (8 : 2) eluted 300 mg, 0.66 mmol (30%) of the pure product **11**. mp 203—205 °C. UV (nm): 242 (ε=15200). IR (KBr) cm^{-1}: 1730, 1705, 1671, 630. ^{1}H-NMR ($CDCl_3$) δ: 0.7 (3H, s), 1.1 (3H, s), 2.0 (3H, s), 6.5 (1H, d, J=6 Hz), 6.9 (1H, d, J=6 Hz). ^{13}C-NMR ($CDCl_3$) δ: 13 (C-18), 16 (C-19), 25 (C-21), 80 (C-17), 122 (C-2), 128 (C-1), 160 (C-5), 175 (CH_3COO), 182 (C-3), 217 (C-20). MS (*m/z*): 448 (M^+).

16α,17α-Epoxy-3β-hydroxypregn-5-en-20-one (13), 3β-Acetoxy-16α,17α-epoxypregn-5-ene-20-one (14), 16α,17α-Epoxy-20-ethylenedioxypregn-5-en-3β-yl Acetate (15) These compounds were prepared from 16-dehydropregnenolone acetate (**12**) (Fig. 4). The detailed preparation of these compounds is described in ref. 5, however this publication does not report the pharmacological evaluation of these compounds.

Animals and Tissues Adult male Syrian Golden hamsters (150—200 g) were obtained from Metropolitan University-Xochimilco, Mexico. The animals were kept in a room with controlled temperature (22 °C) and light–dark periods of 12 h. Food and water were provided *ad libitum*.

Gonadectomies were performed under light ether anesthesia 15 d before treatment. Animals were sacrificed by ether anesthesia. The seminal vesicles were immediately removed, blotted, and weighed prior to use. Tissues used in the metabolic experiments were homogenized with a tissue homogenizer (model 985—370 variable speed 5000—30000 rpm, Biospec Products, Inc.).

Flank Organ Tests The flank organ test was performed as previously

reported.[17] The effect of the steroids on the flank organs of male hamsters, which were gonadectomized 15 d before the experiments, was determined on 15 groups of 4 animals/experiment, selected at random.

Daily subcutaneous injections of 200 μg of steroids **4**—**15** (Fig. 1) dissolved in 20 μl of sesame oil were administered for 3 d together with 200 μg of T. Three groups of animals were kept as controls, one was injected with 20 μl of sesame oil, the second with 200 μg of T for 3 d and the third with 200 μg of finasteride.[5,6]

After treatment, the animals were sacrificed by ether anesthesia. Both flank organs of the animal were shaven and the diameter of the pigmented spot was measured. The results were analyzed using one-way analysis of variance applying the Turkey test for comparison of the results. EPISTAT software was used for this evaluation.

Seminal Vesicles Test The effect of steroids on seminal vesicles from castrated male hamsters was determined after sacrificing the animals. The seminal vesicles were dissected out and weighed on a balance. Two separate experiments were performed for each group of steroid-treated animals. The results were analyzed using one-way analysis of variance with EPISTAT software.

***In Vitro* Metabolic Studies with Seminal Vesicles** Homogenates from male hamster seminal vesicles (4.2±0.02 mg protein) were prepared from intact adult male animals, using Krebs–Ringer-phosphate buffer solution, at pH 6. Tissue preparations were incubated[17] in duplicate with 4.14 mM [^{3}H]T in the presence of 1 μM $NADPH^+$, 0.013 μM of unlabeled T, **3**, and compounds **6**—**15** in a Dubnoff metabolic incubator at 37 °C for 60 min with O_2/CO_2 (95/5) as the gas phase. The final incubation volume was 1 ml. Incubations without tissues were used as controls. Incubations were terminated by addition of dichloromethane, and [^{3}H]steroid was extracted (4×) using 3 vol. of dichloromethane. The solvent was removed under vacuum and the resulting extract was washed with hexane to remove the remaining lipids. The protein content of the homogenates was determined by Bradford's dye-binding method[15] using bovine serum albumin (BSA) as the standard.

Isolation and purity assessment of radioactive DHT was carried out by the reverse isotope dilution technique. The isolated compound was purified with steroid carriers (T, DHT) in a thin-layer chromatographic system (chloroform–acetone, 9 : 1). The radioactive conversion product was identified on chromatographic plates by autoradiography, while non-radioactive steroid carriers were detected using phosphomolybdic acid reagent and a UV lamp (254 nm). Radioactivity was determined in a Packard 3255 liquid scintillation spectrometer, using Riafluor (Dupont, Boston, MA) as the counting solution. The counting efficiency was ^{3}H 67%. The losses of radioactivity during the procedure were calculated in agreement with the results obtained from control experiment without tissue. The conversion to DHT was calculated and expressed as pmol of DHT/g protein /h.

Antagonistic Activity of the Synthesized Steroids for ARs The antagonistic effect of steroids on the ARs of castrated male hamster seminal vesicles was determined on 100 animals (150—200 g), selected at random and gonadectomized 8 d before the experiments. The animals were kept in a room with controlled temperature (22 °C) and light–dark periods of 12 h. Food and water were provided *ad libitum*.

Cytosol Preparations for AR Competition Analysis Tissue homogenization was performed as described above using TEDAM 1 : 1 (20 mM Tris–HCl, pH 7.4 at 4 °C, 1.5 mM EDTA, 0.25 mM dithiothreitol, and 10 mM sodium molybdate) containing 10% glycerol (v/v). The homogenate was centrifuged at 105000×***g*** for 1 h at 2 °C in a SW 50.1 rotor (Beckman Instruments, Palo Alto, CA). Cytosol protein content was determined by Bradford's dye-binding method[15] using BSA as the standard.

AR competition studies were performed as described by Cabeza *et al.*[19] and are briefly summarized below.

Competition Studies For competition experiments, tubes contained 1 nM[20] [^{3}H]DHT (specific activity 110 Ci/mmol) plus a range of increasing concentrations (20—200 nM) of 5-α DHT, CA, and steroids **5**—**15**. Aliquots (150 μl) of cytosol were added and incubated (in duplicate) for 18—20 h at 2—4 °C in the tubes described above. In another experiment, parallel sets of tubes containing identical concentrations of [^{3}H]DHT plus a 100-fold excess of radio inert steroids were used dextran-coated charcoal in TEDAM buffer 800 μl was added and the mixture was incubated for 10 min at 4 °C. The dextran was agitated during 30 min before addition of the charcoal to the mixture. The tubes were vortexed and immediately centrifuged at 800×***g*** for 10 min; aliquots (200 μl) were subsequently submitted and the radioactivity was evaluated. Specific binding was determined by subtracting the mean disintegration per minute (dpm) in the presence of excess unlabeled steroids from the mean dpm of corresponding tubes containing only [^{3}H]DHT.

Results were analyzed using one-way analysis of variance with EPISTAT software. The inhibition constant (K_i) of each compound was calculated according to the procedures described by Cheng and Prusoff.[21]

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Synthesis of Sialyl Lewis X-Polysaccharide Conjugates

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Sialyl Lewis X (SLe^X) is well known as a ligand of the cell adhesion molecule E-selectin which is specifically expressed at inflammatory lesion sites. We have synthesized several SLe^X–polysaccharide conjugates and examined their potential for drug delivery to inflammatory lesions. The *AUC* (area under the blood concentration–time curve) 0—24 h of SLe^X–CMCht (1), SLe^X–CMPul (2) and SLe^X–DSH (3) at the inflammatory lesion was about 60-, 300-, and 30-fold higher than that of the monovalent SLe^X (7), respectively. Moreover, 1 showed 2-fold higher accumulation in the inflammatory lesion than SLN–CMCht (4), and 2 showed 2.5-fold higher accumulation than SLN–CMPul (5).

Key words sialyl Lewis X; E-selectin; carboxymethyl chitosan; carboxymethyl pullulan; *N*-desulfated heparin; active-targeting drug delivery system

Carbohydrate recognition has been shown to be involved in various biological processes and should be useful for an active-targeting DDS (Drug Delivery System). However, carbohydrate recognition has not yet been applied to targeting delivery to tissues or lesions, except for a galactose ligand for the asialoglycoprotein receptor expressed in liver.[2,3] Also, there are disadvantages for targeting delivery using galactose derivatives. For example, in the case of hepatic tumors, anti-tumor drugs modified with galactose derivatives could not be delivered selectively to tumor sites because the asialoglycoprotein receptor is expressed throughout the whole liver containing non-tumor sites. Therefore, it is not a tissue-specific but a lesion-specific active-targeting DDS that is required.

Recently, attention has been directed to cell adhesion molecules called selectins which are involved in various aspects of immune cell trafficking. We have focused on the interaction between E-selectin and sialyl Lewis X (SLe^X, Neu5Acα2→3Galβ1→4(Fucα1→3)GlcNAc).[4] E-selectin is expressed on the endothelial cells stimulated by cytokines, such as IL-1β or TNF-α at inflammatory sites, and plays an important role in the transport of neutrophils to inflammatory sites. The tetrasaccharide SLe^X is distributed on the surface of neutrophils, and has been shown to be a ligand recognized by E-selectin. As the interaction between SLe^X and E-selectin is essential for the initial stage of neutrophil infiltration of the inflammatory site, SLe^X and its derivatives, which block this interaction, should be useful as new anti-inflammatory agents.[5]

SLe^X and its derivatives may be effective homing devices for active-targeting DDS to inflammatory lesions because E-selectin is only expressed on such lesions. Thus, DDS using SLe^X derivatives as homing devices may be effective for drug delivery not to normal tissue but directly to inflammatory lesions. However, to the best of our knowledge, there have been no examples using SLe^X as a homing device for DDS. The reason may be that the affinity of carbohydrate for their protein has been shown to be relatively weak and carbohydrates are generally sensitive to glycosidase *in vivo*. Moreover, oligosacharides such as SLe^X should be rapidly filtered at the glomerulus because of its high hydrophilicity and low molecular weight.[6] One way to solve these problems would be to support SLe^X on a macromolecule[7] which would stabilize the sugar moiety and enhance multivalent interaction of SLe^X with E-selectin expressed at the inflammatory lesion. In our research laboratories, we have studied DDS using liposomes,[3] polypeptides,[8] or polysaccharides[9,10] as carriers and have already reported that some polysaccharides of molecular size above 70 kDa, such as carboxymethylchitosan (CMCht)[9] and carboxymethylpullulan (CMPul),[10] are useful as carriers for a passive-targeting DDS to tumors. The doxorubicin–CMPul conjugate dramatically enhances the therapeutic index of the antitumor effects.[10] This type of conjugate is highly biocompatible and is retained in the circulating blood.[11] Moreover, these conjugates have been found to accumulate in tissues with enhanced vascular permeability such as tumor and inflammatory sites, where E-selectin is expressed, and thus, a synergic effect can be expected between SLe^X and polysaccharides.

These findings prompted us to synthesize SLe^X–polysaccharide (CMCht and CMPul) conjugates and evaluate their usefulness as an active-targeting device for delivery to inflammatory lesions.

The sulfated polysaccharide heparin, or *N*-desulfated heparin (DSH), is an important drug used to prevent cardiovascular diseases and it has been reported to have many unique biological activities.[12] Since these polysaccharides are also expected to be useful as drug carriers of DDS, because of their price and ease of acquisition, we synthesized a conjugate of SLe^X with *N*-desulfated heparin (DSH) and evaluated

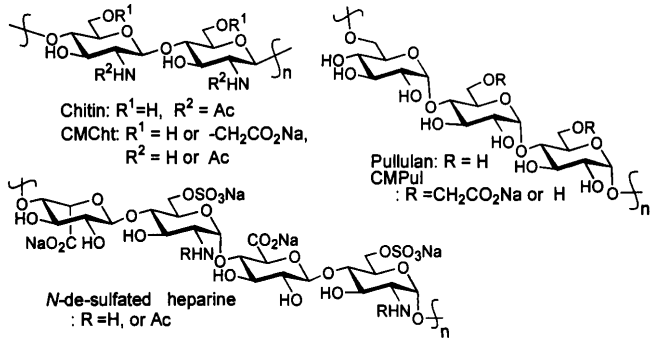

Fig. 1. Structure of Polysaccharides

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its pharmacokinetics.

We also synthesized SLN(Neu5Acα2→Galβ1→4Glc-NAc)–polysaccharide conjugates as the negative control. SLN is a trisaccharide without the fucose moiety of SLe^X and it has been reported not to support E-selectin-mediated adhesion.[13]

Synthesis of SLe^X–Polysaccharide Conjugates It was planned to prepare a SLe^X–CMCht conjugate (**1**) by *N*-alkylation using the amino group of CMCht and the bromo group of **6**. The synthesis of SLe^X–bromide (**6**)[14] is described in Chart 1. The bromo alcohol (**8**) was prepared in two steps from commercially available hexaethyleneglycol [i) MsCl/Py., ii) LiBr/2-butanone, 40%]. 2-Methyl-4,5-(3,4,6-tri-*O*-acetyl-2-deoxy-a-D-glucopyrano)-2-oxazoline (**9**),[15] which is readily available from *N*-acetyl-D-glucosamine, was reacted with bromo alcohol (**8**) in the presence of TMSOTf to give a β-glycoside (**10**, 80%), which was converted to the glycosyl acceptor (**11**) in two steps [i) NaOMe/MeOH, ii) $PhCH(OMe)_2$/CSA/DMF (65%)]. Compound **11** was glycosylated with thioglycoside (**12**)[16] using $Me_2SSMe \cdot OTf$[17] in CH_2Cl_2 at 0 °C to afford the disaccharide **13**, as a mixture of α- and β-glycosides (95%, $\alpha:\beta=15:1$, separation of the α- and β-glycosides was difficult). By means of the regioselective ring opening reaction of benzylidene acetal using $NaBH_3CN$–HCl,[18] **13** was converted to the 6-*O*-benzyl ether with the 4-hydroxy unsubstituted compound **14** (77%). However, $NaBH_3CN$ is a hazardous chemical and difficult to use safely on a large scale, so we investigated the ring opening reaction using alkylsilane derivatives[19] in place of $NaBH_3CN$ as a reducing reagent and found Et_3SiH–TfOH to be useful (83%). On the other hand, when $PhBCl_2$ was used instead of TfOH, 4,6-*O*-benzylidene acetal was converted to the corresponding 4-*O*-benzyl ether with the 6-hydroxy unsubstituted compound in excellent yield, and regioselectivity was complete. Our improved method has proved to be useful[20] not only for this substrate but other carbohydrates having the 4,6-*O*-benzylidene ring and therefore should be useful for the synthesis of various oligosaccharides.

Glycosylation of **14** with sialyl-galactose imidate (**15**)[21] in the presence of $BF_3 \cdot OEt_2$ led to the tetrasaccharide **16** (61%), which was hydrogenated with 10% Pd–C in MeOH to afford **17** (73%) and **18** (16%). When this hydrogenation was carried out for a long period (over *ca.* 12 h), **18** was obtained as a major product. Deacylation of **17** followed by saponification of the methyl ester group gave the bromide (**6**, 93%).

CMCht was prepared from chitin according to a known procedure,[9] and had the following characteristics: M.W., about 100 kDa; degrees of substitution (ds) of *N*-acetyl group, 0.47; ds of amino group, 0.45; ds of carboxymethyl group, 0.7.

By means of *N*-alkylation of CMCht with bromide (**6**) in 0.5% $NaHCO_3$–H_2O for 160 h at 60 °C, a SLe^X–CMCht conjugate (**1**) was obtained after dialysis against distilled water (M.W. cut: 12000—13000). The SLe^X content of this conjugate was determined by the resorcinol–HCl method for the quantitative analysis of sialic acid,[22] and the result was 33 wt% (the ds of SLe^X was 0.17 per glucosamine residue of CMCht).

The SLe^X–CMPul conjugate (**2**) was prepared by condensation of CMPul and the SLe^X derivative (**21**) with an amino group. The synthesis is described in Chart 2. Treatment of **14** with NaN_3 afforded **19** (93%), which was coupled with imidate **15** ($BF_3 \cdot OEt_2/CH_2Cl_2$) to give **20** (52%). Hydrogenation of **20** with 10% Pd–C in THF–HCl gave amine **21** (87%).

Condensation of amine **21** with CMPul[10] (M.W. about 190 kDa, ds of carboxymethyl group was 0.6 per glucose residue), which was prepared from commercially available pullulan by carboxymethylation (aq. $NaOH/ClCH_2CO_2H$)

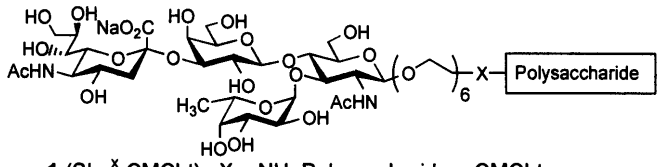
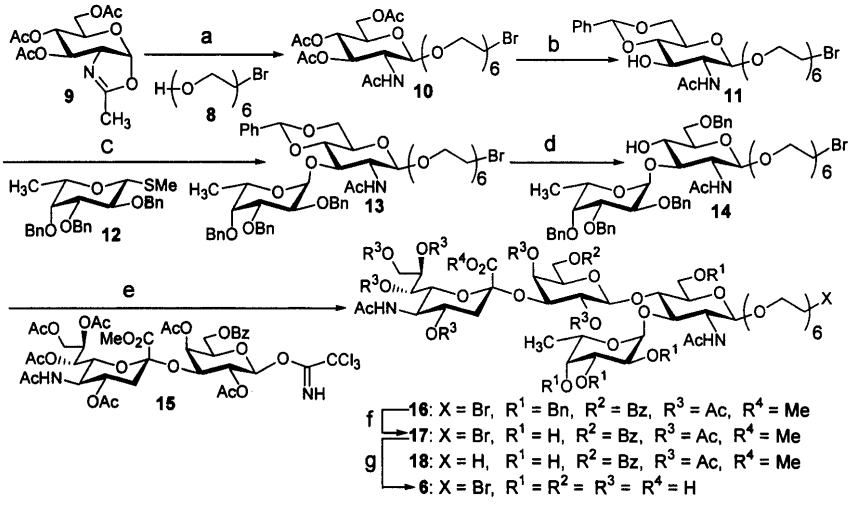


Fig. 2. Structure of SLe^X–Polysaccharide Conjugates



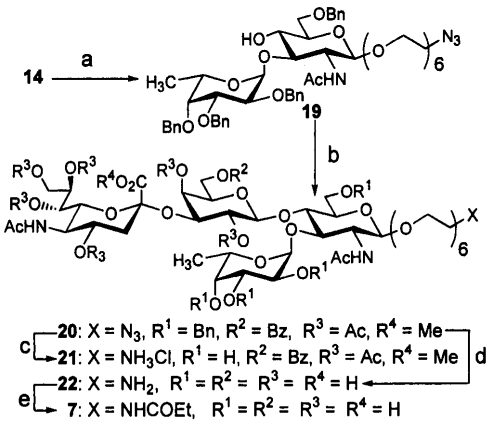
a) TMSOTf / $C_2H_4Cl_2$ / 50°C (80%) b) 1) NaOMe / MeOH / rt, 2) $PhCH(OMe)_2$ / CSA / DMF (65%)
c) Me_2SSMe•OTf / CH_2Cl_2 / 0°C (95%, $\alpha:\beta$ =15:1) d) Et_3SiH / TfOH / CH_2Cl_2 / MS4A / -78°C (83%)
e) BF_3•OEt_2 / CH_2Cl_2 / MS4A / 0°C (61%) f) Pd-C / H_2 / MeOH / rt (**17**:73%, **18**:16%)
g) 1) NaOMe / MeOH / rt, 2) aq.NaOH / rt (90%)

Chart 1

using 1-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in DMF–H_2O at 40 °C, and subsequent deprotection with 1 N aq. NaOH, gave a SLe^X–CMPul conjugate (**2**). The SLe^X content of **2** was determined to be 31 wt% by the same method used for **1**.[22] (The degree of substitution of SLe^X was 0.13 per glucose residue of CMPul.) ^{1}H-NMR spectroscopy showed no benzoyl or *O*-acetyl signals confirming deprotection by 1 N aq. NaOH to be complete.

The SLe^X–DSH conjugate (**3**) was prepared by *N*-alkylation as for the SLe^X–CMCht conjugate (**1**).

Commercially available *N*-de-sulfated heparin (DSH, M.W. about 40 kDa) was treated with $NaBH_4$ to reduce the terminal hemiacetal group, and this polysaccharide was reacted with bromide (**6**) in 0.5% $NaHCO_3$–H_2O at 60 °C, to afford a SLe^X–DSH conjugate (**3**, SLe^X contents of **3**: 24 wt%).



a) NaN_3 / DMF (93%) b) **15** / BF_3•OEt_2 / CH_2Cl_2 / MS4A / 0°C (52%) c) Pd-C / H_2 / THF-HCl / rt (87%)
d) 1) NaOMe / MeOH / rt, 2) aq.NaOH / rt, 3) Pd-C / H_2 / MeOH / *p*TsOH•H_2O (72%) e) $EtCO_2Su$ / MeOH / NMM (79%)

Chart 2

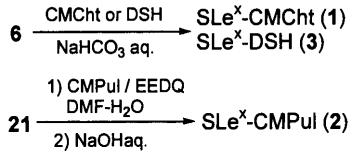


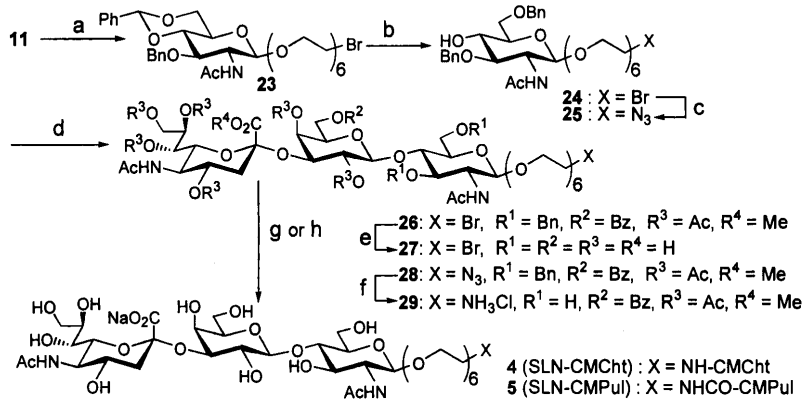
Chart 3

A SLN–CMCht conjugate (**4**) and a SLN–CMPul conjugate (**5**) were synthesized according to a procedure similar to that described for **1** and **2** (**4**, ds=0.17, **5**, ds=0.13, Chart 4).

Accumulations of SLe^X–Polysaccharide Conjugates (1—3) and Monomeric SLe^X (7) in Inflammatory Lesions

To examine the pharmacokinetics of **1**—**5** and **7**, radiolabelled versions of compounds **1**—**5** and **7** were prepared. CMCht derivatives (**1**, **4**) and SLe^X–DSH (**3**) were radiolabelled using *N*-succinimidyl [2,3-^{3}H]-propionate, and CMPul derivatives (**2**, **5**) were radiolabeled using [^{3}H]-glycine and EEDQ (radioactivity: 5—15 mCi/mg). Radiolabelled monovalent SLe^X (**7**) was prepared from **22** using *N*-succinimidyl [2,3-^{3}H]-propionate. In order to evaluate the ability to actively target the inflammatory lesion *in vivo*, we used arachidonic acid-induced ear edema in mice, a model of acute-phase inflammatory disease. First, SLe^X–polysaccharide conjugates (**1**—**3**) and monovalent SLe^X (**7**) were compared with respect to their accumulation in inflammatory lesions (dose: 1 mg/mg, i.v.). The plasma concentration of monovalent SLe^X (**7**) decreased rapidly and could not be detected one hour later, and the concentration in the inflamed ear remained low. On the other hand, SLe^X–polysaccharide conjugates (**1**—**3**) were retained in blood circulation as expected, and subsequent maked enhancement of accumulation in the targeted lesion was observed. The *AUC* (area under the blood concentration–time curve) 0—24 h of macromolecules **1**, **2**, and **3** at the inflammatory lesion was about 60-, 300-, and 30-fold higher than that of the monovalent SLe^X (**7**), respectively. The *AUC* 0—24 h of **3** in plasma was lower than that of **1** or **2**. Thus, compound **3** underwent glomerular filtration before arriving at the inflammatory lesion faster than **1** or **2**, and the accumuation of **3** in the inflammatory lesion was considered to be lower. Reduced accumulation of CMCht conjugate **1** compared with **2** was due to the degradation of CMCht by lysozyme *in vivo*. These findings show that supporting SLe^X on polysaccharide is an effective way to enhance accumulation in inflammatory lesions.

Secondly, the accumulation of SLe^X–polysaccharide conjugates (**1**, **2**) in inflammatory lesions was compared with that of SLN–polysaccharide conjugates (**4**, **5**). Compound **1**



a) BnBr / BaO / $Ba(OH)_2$•$8H_2O$ / DMF / rt (85%) b) Et_3SiH / TfOH / CH_2Cl_2 / MS4A / -78°C (87%)
c) NaN_3 / DMF (98%) d) **15** / BF_3•OEt_2 / CH_2Cl_2 / MS4A / 0°C (**26**: 72%, **28**:48%)
e) 1) Pd-C / H_2 / MeOH / rt, 2) NaOMe / MeOH / rt, 3) aq.NaOH / rt (85%) f) Pd-C / H_2 / THF-HCl / rt (73%) g) CMCht / **27** / aq.$NaHCO_3$ / 60°C h) 1) CMPul / **29** / EEDQ / DMF-H_2O / rt 2) aq.NaOH

Chart 4

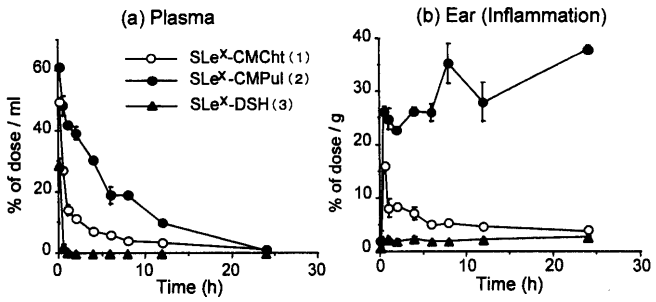


Fig. 3. Concentration–Time Profile of SLe^X–Polysaccharide Conjugates
Data represents mean±S.D. of three mice.

showed a 2-fold higher accumulation in the inflammatory lesion than **3**, and **2** showed a 2.5-fold higher accumulation than **4**. These differences in distribution are thought to be due to differences in the ability to bind to E-selectin.

These results indicate that the interaction between SLe^X and E-selectin could be applied to targeting DDS, and SLe^X–polysaccharide conjugates was useful as drug carriers to inflammatory lesions.

SLe^X–CMPul (**2**) showed a 16-fold higher accumulation in the spleen than SLN–CMPul (**4**). Although the reason is not clear, the accumulation dose not involve E-selectin because it was observed in non-treated normal mice. These are interesting results because the pharmacokinetics of macromolecules such as CMPul change markedly simply following changes in the sugar residues in the macromolecule.

In conclusion, we established a method of synthesizing SLe^X–polysaccharide conjugates, and these conjugates have been shown to be a useful novel system for active-targeting DDS to inflammatory lesions. In the future, we plan to introduce an anti-inflammatory agent to our system and evaluate the subsequent therapeutic effects.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus and were not corrected. ^{1}H-NMR spectra were measured on a Varian VXR-500S (500 MHz) spectrometer, unless otherwise specified. Chemical shifts (δ) are given in ppm relative to tetramethylsilane as an internal standard. IR spectra were measured on a Shimadzu FT-IR-4300. Optical rotations were determined with a Perkin-Elmer 430 polarimeter. FAB-Mass spectra were recorded on a Hitachi M-90 instrument.

17-Bromo-3,6,9,12,15-pentaoxa-1-heptadecanol (8) To a stirred solution of hexaethyleneglycol (23.8 g, 84.3 mmol) in CH_2Cl_2 (200 ml) were added Et_3N (14.1 ml, 101 mmol), and methanesulfonylchloride (6.52 ml, 84.3 mmol) at 0 °C. After being stirred at 0 °C for 1 h, the mixture was diluted with CH_2Cl_2, washed with 2% aqueous citric acid and saturated aqueous $NaHCO_3$, dried over $MgSO_4$ and concentrated *in vacuo*.

The crude mesylate was dissolved in 2-butanone (300 ml), and then LiBr (36.6 g, 422 mmol) was added . After stirring for 1 h under reflux, the mixture was diluted with CH_2Cl_2, washed with saturated brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography [SiO_2 500 g, CH_2Cl_2 : MeOH=20 : 1] to give **8** (11.8 g, 40%) as a colorless oil. IR ($CHCl_3$) cm^{-1}: 3500. ^{1}H-NMR ($CDCl_3$) δ: 3.48 (2H, t, J=6.3 Hz), 3.60—3.63 (2H, m), 3.64—3.70 (8H, m), 3.70—3.75 (2H, m), 3.82 (2H, t, J=6.3 Hz).

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (10) To a stirrred mixture of **9**[15] (1.85 g, 5.62 mmol), **8** (1.56 g, 4.52 mmol) and powdered molecular sieve 4 Å (MS4A, 1.2 g) in 1,2-dichloroethane (12 ml), was added trimethylsilyl trifluomethanesulfonate (835 μl, 4.30 mmol) at room temperature, and the stirring was continued for 1.5 h at 50 °C. The mixture was cooled to room temperature, and then triethylamine (1.40 ml, 10.0 mmol) was added. After stirring for 10 min at room temperature, the resultant mixture was filtered, and the filtrate poured into CH_2Cl_2. The organic phase was washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography [SiO_2 150 g, toluene : acetone : MeOH=500 : 300 : 8] to give **10** (2.45 g, 80%) as a colorless syrup. $[\alpha]_D^{27}$ −14.7° (c=1.18, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3622, 1747, 1678. ^{1}H-NMR ($CDCl_3$) δ: 1.97, 2.09 (each 3H, s), 2.01 (6H, s), 3.48 (2H, t, J=6.3 Hz), 3.58—3.75 (18H, m), 3.76—3.86 (2H, m), 3.81 (2H, t, J=6.3 Hz), 3.90 (1H, m), 4.10 (1H, m, H-2 of GlcNAc), 4.17 (1H, dd, J=12.2, 2.4 Hz, H-6 of GlcNAc), 4.26 (1H, dd, J=12.2, 4.6 Hz, H-6 of GlcNAc), 4.79 (1H, d, J=8.5 Hz, H-1 of GlcNAc), 5.10—5.11 (2H, m, H-3, 4 of GlcNAc), 6.61 (1H, d, J=9.3 Hz, NH). *Anal.* Calcd for $C_{26}H_{44}NO_{14}Br \cdot 0.5H_2O$: C, 45.69; H, 6.64; N, 2.05. Found: C, 45.63; H, 6.68; N, 2.29.

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl 2-Acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (11) To a stirred solution of **10** (13.0 g, 19.3 mmol) in MeOH (30 ml) at room temperature was added 28% sodium methoxide in MeOH (0.3 ml) and the stirring was continued at room temperature for 30 min. The reaction mixture was neutralized with Dowex 50W×8 (H^+ form) and filtered. The filtrate was concentrated *in vacuo*.

The residue was dissolved in *N*,*N*′-dimethylformamide (50 ml), and then were added benzaldehyde dimethyl acetal (10.9 ml), and 10-camphor sulfonic acid (125 mg). After stirring for 3 h at 55 °C under reduced pressure (45 mmHg), the mixture was neutralized with anion exchange resin (AG-1(OH^-)) and filtered. The filtrate was concentrated *in vacuo*, and the crude product was purified by silica gel column chromatography (SiO_2 150 g, toluene : acetone : MeOH=20 : 30 : 1) to give **11** (7.57 g, 65%) as a colorless syrup. $[\alpha]_D^{28}$ −55.4° (c=1.04, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3352, 1666. ^{1}H-NMR ($CDCl_3$) δ: 2.07 (3H, s), 3.44 (2H, t, J=6.2 Hz), 3.45 (1H, m, H-5 of GlcNAc), 3.57—3.72 (17H, m), 3.75 (2H, t, J=6.2 Hz), 3.77—3.94 (5H, m), 4.33 (1H, dd, J=10.5, 4.9 Hz, H-6 of GlcNAc), 4.75 (1H, d, J=8.1 Hz, H-1 of GlcNAc), 5.57 (1H, s), 7.15 (1H, br d, J=6.3 Hz, NH), 7.32—7.38 (3H, m), 7.47—7.52 (2H, m). *Anal.* Calcd for $C_{27}H_{42}NO_{11}Br$: C, 50.95; H, 6.65; N, 2.20. Found: C, 51.07; H, 6.70; N, 2.20.

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl *O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→3)-2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (13) To a solution of **11** (636 mg, 1.00 mmol) and **12**[15] (697 mg, 1.50 mmol) in CH_2Cl_2 (20 ml), was added powdered MS4A (10 g), and the mixture was stirred for 2 h at room temperature. Dimethyl(methylthio)sulfonium triflate (DMTST, 1.16 g, 4.50 mmol) was added to the stirred mixture at 0 °C, and stirring was continued for 30 min at 0 °C. MeOH (2 ml), and Et_3N (1 ml) were added, and then the resultant mixture was filtered. The filtrate was diluted with CH_2Cl_2, washed with H_2O, dried over $MgSO_4$, and concentrated *in vacuo*.

The crude product was chromatographed on a silica gel column (SiO_2 70 g, CH_2Cl_2 : MeOH=50 : 1) to give **13** (896 mg, 95%) as an inseparable mixture of anomers (α : β=15 : 1). IR ($CHCl_3$) cm^{-1}: 1677. ^{1}H-NMR ($CDCl_3$) δ: 0.82 (3H, d, J=6.3 Hz, H-6 of Fuc), 1.75 (3H, s), 3.45 (2H, t, J=6.3 Hz), 3.45—3.52 (2H, m), 3.56 (1H, m, H-4 of Fuc), 3.58—3.70 (20H, m), 3.72—3.80 (2H, m), 3.78 (2H, t, J=6.3 Hz), 3.94 (1H, dd, J=10.3, 2.7 Hz, H-3 of Fuc), 4.04 (1H, dd, J=10.3, 3.7 Hz, H-2 of Fuc), 4.11 (1H, q, J=6.3 Hz, H-5 of Fuc), 4.22 (1H, dd, J=9.5, 9.5 Hz, H-3 of GlcNAc), 4.33 (1H, dd, J=10.5, 4.9 Hz, H-6 of GlcNAc), 4.57, 4.71, 4.79, 4.91 (each 1H, d, J=11.7 Hz), 4.70, 4.78 (each 1H, d, J=11.5 Hz), 4.92 (1H, d, J=8.3 Hz, H-1 of GlcNAc), 5.17 (1H, d, J=3.7 Hz, H-1 of Fuc), 5.50 (1H, s), 6.05 (1H, d, J=8.1 Hz, NH), 7.24—7.39 (18H, m), 7.42—7.45 (2H, m). *Anal.* Calcd for $C_{54}H_{74}NO_{15}Br$: C, 61.35; H, 7.05; N, 1.32. Found: C, 61.41; H, 6.79; N, 1.19.

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl *O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→3)-2-acetamido-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (14) Powdered MS4A (1 g) was placed in a 20 ml flask, and dried at 140 °C over 4 h under vacuum (*ca.* 0.1 mmHg). After cooling to room temperature, **13** (210 mg, 0.20 mmol), and CH_2Cl_2 (3.5 ml) were added. After stirring for 1 h at room temperature, the mixture was cooled to −78 °C, and then Et_3SiH (112 μl, 0.70 mmol) and TfOH (46 μl, 0.60 mmol) were added, successively. After stirring for 1 h at −78 °C, Et_3N (1 ml) and MeOH (1 ml) were added, successively, and the mixture was diluted with $CHCl_3$, washed with aqueous $NaHCO_3$, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by a silica gel column (20 g, $CHCl_3$: MeOH=100 : 1) to give **14** (175 mg, 83 %) as a colorless syrup. $[\alpha]_D^{27}$ −50.8° (c=0.51, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3631, 3450, 1674. ^{1}H-NMR ($CDCl_3$+D_2O) δ: 1.14 (3H, d, J=6.3 Hz, H-6 of Fuc), 1.66 (3H, s), 3.45 (2H, t, J=6.3 Hz), 3.41—3.52 (3H, m), 3.57—3.84 (23H, m), 3.78 (2H, t, J=6.3 Hz), 3.95 (1H, m), 3.97 (1H, m, H-3 of Fuc), 4.06 (1H, dd, J=10.3, 3.7 Hz, H-2 of Fuc), 4.13 (1H, q, J=6.3 Hz, H-5 of Fuc), 4.58, 4.62 (each 1H, d, J=12.2 Hz), 4.61, 4.95 (each 1H, d, J=11.2 Hz), 4.67, 4.75, 4.79, 4.81 (each 1H, d, J=11.7 Hz), 4.84 (1H, d, J=8.3 Hz, H-1 of GlcNAc), 4.97

(1H, d, J=3.7 Hz, H-1 of Fuc), 6.14 (1H, d, J=7.8 Hz, NH), 7.25—7.41 (20H, m). *Anal.* Calcd for $C_{54}H_{72}NO_{15}Br$: C, 61.47; H, 6.87; N, 1.32. Found: C, 61.27; H, 6.75; N, 1.20.

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2,4-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-[(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→3)]-2-acetamido-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (16) To a solution of **14** (1.07 g, 1.01 mmol) and **15**[19] (400 mg, 0.406 mmol) in CH_2Cl_2 (6 ml), was added powdered MS4A (2 g), and the mixture was stirred for 2 h at room temperature. To the stirred mixture, $BF_3 \cdot OEt_2$ (100 ml, 0.812 mmol) was added at 0 °C, and the stirring was continued for 2 h at 0 °C. The reaction mixture was filtered, and the filtrate was diluted with CH_2Cl_2, washed with saturated aqueous $NaHCO_3$, dried over $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (SiO_2 120 g, CH_2Cl_2 : MeOH=30 : 1) to give **16** (460 mg, 61%) as a colorless powder. $[\alpha]_D^{28}$ −34.0° (c=0.63, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 1744, 1688. ^{1}H-NMR ($CDCl_3$) δ: 1.10 (3H, d, J=6.3 Hz, H-6 of Fuc), 1.71 (1H, dd, J=12.4, 12.0 Hz, H-3 of Sia), 1.85, 1.89, 1.96, 1.97, 2.01, 2.07, 2.09, 2.22 (each 3H, s), 2.56 (1H, dd, J=12.4, 4.6 Hz, H-3 of Sia), 3.46 (2H, t, J=6.3 Hz), 3.48—3.67 (21H, m), 3.73—3.87 (5H, m), 3.75 (3H, s), 3.79 (2H, t, J=6.3 Hz), 3.87 (1H, dd, J=10.3, 2.4 Hz, H-3 of Fuc), 3.91 (1H, dd, J=10.0, 4.7 Hz), 3.94 (1H, dd, J=7.3, 7.1 Hz, H-5 of Gal), 3.98 (1H, dd, J=12.4, 5.6 Hz, H-9 of Sia), 4.01 (1H, m), 4.04 (1H, m, H-5 of Sia), 4.05—4.10 (2H, m), 4.17 (1H, dd, J=11.0, 7.3 Hz, H-6 of Gal), 4.20 (1H, m, H-5 of Fuc), 4.24 (1H, dd, J=11.0, 7.1 Hz, H-6 of Gal), 4.30 (1H, dd, J=12.4, 2.7 Hz, H-9 of Sia), 4.44, 4.57, 4.75, 4.77 (each 1H, d, J=12.2 Hz), 4.63, 4.94 (each 1H, d, J=11.7 Hz), 4.67 (1H, dd, J=10.0, 3.7 Hz, H-3 of Gal), 4.70, 4.81 (each 1H, d, J=12.9 Hz), 4.74 (1H, d, J=7.3 Hz, H-1 of GlcNAc), 4.81 (1H, d, J=7.8 Hz, H-1 of Gal), 4.90 (1H, ddd, J=12.0, 10.3, 4.7 Hz, H-4 of Sia), 4.97 (1H, dd, J=10.0, 8.1 Hz, H-2 of Gal), 5.04 (1H, br d, H-4 of Gal), 5.06 (1H, br d, NH), 5.21 (1H, d, J=3.7 Hz, H-1 of Fuc), 5.37 (1H, dd, J=9.3, 2.7 Hz, H-7 of Sia), 5.69 (1H, ddd, J=9.3, 5.6, 2.7 Hz, H-8 of Sia), 6.23 (1H, m, NH), 7.22—7.36 (20H, m), 7.37 (2H, m), 7.50 (1H, m), 7.97 (2H, m). *Anal.* Calcd for $C_{91}H_{117}N_2O_{35}Br \cdot H_2O$: C, 57.62; H, 6.32; N, 1.47. Found: C, 57.64; H, 6.32; N, 1.55.

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2,4-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (17) and 3,6,9,12,15-Pentaoxaheptadecyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2,4-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (18) To a solution of **16** (200 mg) in tetrahydrofuran (15 ml) was added Pd–C (10%, 100 mg), and then hydrogenation was carried out for 24 h at room temperature. The mixture was filtered, and the filtrate was concentrated *in vacuo*. The crude product was purified by preparative thin-layer chromatography (CH_2Cl_2 : MeOH=8 : 1) to give **17** (118 mg, 73%), and **18** (25 mg, 16%).

17: Colorless powder. $[\alpha]_D^{27}$ −53.1° (c=0.66, $CHCl_3$). IR (KBr) cm^{-1}: 3450, 1749, 1665. ^{1}H-NMR ($CDCl_3+D_2O$) δ: 1.27 (3H, d, J=6.6 Hz, H-6 of Fuc), 1.71 (1H, dd, J=12.7, 12.0 Hz, H-3 of Sia), 1.85, 2.01, 2.04, 2.06, 2.11, 2.24 (each, 3H, s), 2.14 (6H, s), 2.59 (1H, dd, J=12.7, 4.6 Hz, H-3 of Sia), 3.48 (2H, t, J=6.3 Hz), 3.60—3.73 (25H, m), 3.77 (3H, s), 3.81 (2H, t, J=6.3 Hz), 3.85 (1H, m), 3.90—4.15 (7H, m), 4.23 (1H, dd, J=11.0, 6.8 Hz, H-6 of Gal), 4.42—4.38 (2H, m), 4.43 (1H, dd, J=12.4, 2.9 Hz, H-9 of Sia), 4.63 (1H, dd, J=10.3, 3.4 Hz, H-3 of Gal), 4.66 (1H, d, J=5.1 Hz, H-1 of GlcNAc), 4.75 (1H, d, J=8.1 Hz, H-1 of Gal), 4.89 (1H, ddd, J=12.0, 10.7, 4.6 Hz, H-4 of Sia), 4.98 (1H, dd, J=10.3, 8.1 Hz, H-2 of Gal), 5.05 (1H, d, J=3.9 Hz, H-1 of Fuc), 5.06 (1H, d, J=2.7 Hz, H-4 of Gal), 5.33 (1H, dd, J=9.3, 2.7 Hz, H-7 of Sia), 5.61 (1H, m), 7.48 (2H, m), 7.59 (1H, m), 8.04(2H, m). *Anal.* Calcd for $C_{63}H_{93}N_2O_{35}Br \cdot H_2O$: C, 49.25; H, 6.23; N, 1.82. Found: C, 49.15; H, 6.40; N, 1.71.

18: Colorless powder. $[\alpha]_D^{28}$ −56.5° (c=0.34, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3452, 1749, 1663. ^{1}H-NMR ($CDCl_3+D_2O$) δ: 1.21 (3H, t, J=7.1 Hz), 1.26 (3H, d, J=6.6 Hz, H-6 of Fuc), 1.72 (1H, dd, J=12.7, 12.0 Hz, H-3 of Sia), 1.86, 2.01, 2.05, 2.06, 2.11, 2.14, 2.14, 2.24 (each, 3H, s), 2.59 (1H, dd, J=12.7, 4.4 Hz, H-3 of Sia), 3.53 (2H, q, J=7.1 Hz), 3.57—3.75 (23H, m), 3.76—3.87 (3H, m), 3.78 (3H, s, $-OCH_3$), 3.89—4.16 (7H, m), 4.23 (1H, dd, J=11.2, 6.8 Hz, H-6 of Gal), 4.39 (1H, dd, J=11.0, 6.8 Hz, H-6 of Gal), 4.42 (1H, dd, J=12.5, 2.9 Hz, H-9 of Sia), 4.39 (1H, m), 4.63 (1H, dd, J=10.3, 3.4 Hz, H-3 of Gal), 4.66 (1H, d, J=4.9 Hz, H-1 of GlcNAc), 4.74 (1H, d, J=8.3 Hz, H-1 of Gal), 4.89 (1H, m, H-4 of Sia), 4.98 (1H, dd, J=10.3, 8.3 Hz, H-2 of Gal), 5.04—5.07 (2H, m, H-4 of Gal, H-1 of Fuc), 5.33 (1H, dd, J=9.1, 2.7 Hz, H-7 of Sia), 5.61 (1H, m), 7.48 (2H, m), 7.59 (1H, m), 8.04 (2H, m). *Anal.* Calcd for $C_{63}H_{94}N_2O_{35}\ H_2O$: C, 51.92; H, 6.64; N, 1.92. Found: C, 51.72; H, 6.70; N, 1.74.

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (6) To a solution of **17** (220 mg) in MeOH (5.0 ml) was added 3% sodium methoxide (200 ml), and the mixture was stirred for 30 min at room temperature. The reaction mixture was neutralized with Dowex 50W×8 (H^+), the mixture filtered, and the filtrate concentrated *in vacuo*.

The residue was dissolved in 0.1 N NaOH aq. (3.0 ml), and then the mixture was stirred for 10 min at room temperature. The reaction mixture was neutralized with Dowex 50W×8 (H^+), the mixture filtered, and the filtrate concentrated *in vacuo*.

The crude product was chromatographed on a column of Sephadex LH-20 (50 g, MeOH) to give **6** (150 mg, 90%) as a colorless powder. $[\alpha]_D^{27}$ −39.4° (c=0.66, MeOH). IR (KBr) cm^{-1}: 3460, 1653. ^{1}H-NMR (CD_3OD) δ: 1.16 (3H, d, J=6.6 Hz, H-6 of Fuc), 1.85 (1H, dd, J=13.3, 11.5 Hz, H-3 of Sia), 1.97, 2.00 (each 3H, s), 2.80 (1H, dd, J=12.4, 4.4 Hz, H-3 of Sia), 3.45—3.41 (2H, m), 3.50 (1H, dd, J=9.0, 1.2 Hz, H-6 of Sia), 3.51 (2H, t, J=6.1 Hz), 3.56 (1H, dd, J=9.5, 7.8 Hz, H-2 of Gal), 3.60—3.79 (28H, m), 3.81 (2H, t, J=6.1 Hz), 3.83—3.95 (8H, m), 4.00 (1H, dd, J=12.2, 3.7 Hz), 4.03 (1H, dd, J=9.5, 2.9 Hz, H-3 of Gal), 4.50 (1H, d, J=7.6 Hz), 4.52 (1H, d, J=7.8 Hz), 4.83—4.85 (1H, m, H-5 of Fuc), 5.03 (1H, d, J=3.9 Hz, H-1 of Fuc). *Anal.* Calcd for $C_{43}H_{75}N_2O_{28}Br \cdot 3H_2O$: C, 42.96; H, 6.79; N, 2.33. Found: C, 43.11; H, 6.84; N, 2.52.

Synthesis of a SLe^X–CMCht Conjugate (1) To a solution of CMCht (40 mg, M.W.: 100 kDa) in 0.5% aq. $NaHCO_3$ (3 ml) were added **20** (276 mg, 0.24 mmol), and powdered $NaHCO_3$ (20 mg). After stirring for 160 h at 60 °C, the reaction mixture was poured into 99.5% EtOH (35 ml) and the entire mixture centrifuged. The precipitate was successively washed with 95% EtOH, acetone, and Et_2O, then dried *in vacuo* to give almost pure **1**. After dissolving the product in H_2O (10 ml), the solution was dialyzed using membrane tubing (M.W. cut off; 12000—13000, Spectra) against deionized H_2O (10000 ml) for 12 h, and lyophilized to give a SLe^X–CMCht conjugate (**1**, 47 mg).

It was confirmed by a GPC analysis that low M.W. molecules derived from **6** were completely excluded from **1**, and the structure of **1** was confirmed by ^{1}H-NMR.

17-Azido-3,6,9,12,15-pentaoxaheptadecyl *O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→3)-2-acetamido-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (19) To a solution of **14** (750 mg) in *N*,*N*′-dimethylformamide (3 ml) was added sodium azide (92.5 mg, 1.42 mmol). After stirring for 1 h at 70 °C, the mixture was diluted with CH_2Cl_2, washed with saturated brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography [SiO_2 150 g, CH_2Cl_2 : MeOH=100 : 1] to give **19** (674 mg, 93%) as a colorless syrup. $[\alpha]_D^{26}$ −31.0° (c=1.07, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3450, 2106, 1674. ^{1}H-NMR ($CDCl_3+D_2O$) δ: 1.14 (3H, d, J=6.3 Hz, H-6 of Fuc), 1.66 (3H, s), 3.36 (2H, t, J=5.0 Hz), 3.43—3.52 (3H, m), 3.56—3.76 (23H, m), 3.77—3.85 (2H, m), 3.95 (1H, m), 3.97 (1H, m, H-3 of Fuc), 4.06 (1H, dd, J=10.2, 3.4 Hz, H-2 of Fuc), 4.13 (1H, q, J=6.6 Hz, H-5 of Fuc), 4.58, 4.62 (each 1H, d, J=12.2 Hz), 4.61, 4.95 (each 1H, d, J=11.5 Hz), 4.67, 4.75, 4.79, 4.81 (each 1H, d, J=11.7 Hz), 4.84 (1H, d, J=8.5 Hz, H-1 of GlcNAc), 4.98 (1H, d, J=3.4 Hz, H-1 of Fuc), 6.13 (1H, d, J=7.6 Hz, NH), 7.26—7.41 (20H, m). *Anal.* Calcd for $C_{54}H_{72}N_4O_{15}$: C, 63.76; H, 7.13; N, 5.50. Found: C, 63.65; H, 7.06; N, 5.48.

17-Azido-3,6,9,12,15-pentaoxaheptadecyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2,4-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-[(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→3)]-2-acetamido-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (20) Compound **19** was converted to **20** (52%) using the procedure described for **16**. Colorless powder. $[\alpha]_D^{28}$ −27.9° (c=0.55, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 2108, 1744, 1688. ^{1}H-NMR ($CDCl_3$) δ: 1.10 (3H, d, J=6.3 Hz, H-6 of Fuc), 1.71 (1H, dd, J=12.7, 12.0 Hz, H-3 of Sia), 1.85, 1.89, 1.96, 1.97, 2.01, 2.07, 2.08, 2.22 (each 3H, s), 2.56 (1H, dd, J=12.7, 4.6 Hz, H-3 of Sia), 3.37 (2H, t, J=5.1 Hz), 3.48—3.65 (21H, m), 3.75 (3H, s), 3.79—3.87 (4H, m), 3.87 (1H, dd, J=10.3, 2.7 Hz, H-3 of Fuc), 3.90 (1H, m), 3.98 (1H, dd, J=12.4, 5.6 Hz, H-9 of Sia), 4.01 (1H, m), 4.04 (1H, m, H-5 of Sia), 4.08 (1H, dd, J=10.0, 3.7 Hz, H-2 of Fuc), 4.08 (1H, m), 4.16 (1H, dd, J=11.0, 7.3 Hz, H-6 of Gal), 4.23 (1H, q, J=6.6 Hz, H-5 of Fuc), 4.24 (1H, dd, J=11.0, 6.8 Hz, H-6 of Gal),

4.29 (1H, dd, J=12.4, 2.7 Hz, H-9 of Sia), 4.36, 4.48, 4.61 (each 1H, d, J=12.0 Hz), 4.67 (1H, dd, J=10.0, 3.7 Hz, H-3 of Gal), 4.44, 4.58, 4.70, 4.75, 4.77, 4.81 (each 1H, d, J=12.0 Hz), 4.63, 4.92 (each 1H, d, J=11.7 Hz), 4.74 (1H, d, J=7.3 Hz, H-1 of GlcNAc), 4.81 (1H, d, J=8.1 Hz, H-1 of Gal), 4.90 (1H, ddd, J=12.0, 10.5, 4.6 Hz, H-4 of Sia), 4.97 (1H, dd, J=10.0, 8.1 Hz, H-2 of Gal), 5.04 (1H, d, J=3.7 Hz, H-4 of Gal), 5.07 (1H, d, J=10.3 Hz, NH), 5.22 (1H, d, J=3.7 Hz, H-1 of Fuc), 5.37 (1H, dd, J=9.3, 2.7 Hz, H-7 of Sia), 5.59 (1H, ddd, J=9.3, 5.6, 2.7 Hz, H-8 of Sia), 6.25 (1H, br.s, NH), 7.16—7.49 (22H, m), 7.50 (1H, m), 7.97 (2H, m). *Anal.* Calcd for $C_{91}H_{117}N_5O_{35}\cdot H_2O$: C, 58.79; H, 6.45; N, 3.76. Found: C, 58.50; H, 6.46; N, 3.69.

17-Amino-3,6,9,12,15-pentaoxaheptadecyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2,4-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside, Hydrochloride Salt (21) To a solution of **20** (200 mg) in MeOH (15 ml) were added Pd–C (10%, 150 mg) and 1 N HCl (330 μl), then hydrogenation was carried out at 3.5×10^4 kg/m^2 (50 psi) for 12 h. The mixture was filtered, and the filtrate concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (SiO_2 45 g, CH_2Cl_2 : MeOH : H_2O=65 : 35 : 10 (lower phase)) to give **21** (140 mg, 87%) as colorless powder. $[\alpha]_D^{27}$ −58.3° (c=0.52, $CHCl_3$). IR (KBr) cm^{-1}: 3430, 1749, 1663. ^{1}H-NMR (CD_3OD) δ: 1.27 (3H, d, J=6.3 Hz, H-6 of Fuc), 1.53 (1H, dd, J=12.4, 12.2 Hz, H-3 of Sia), 1.81, 1.97, 1.99, 2.04, 2.07, 2.08, 2.16, 2.26 (each 3H, s), 2.58 (1H, dd, J=12.4, 4.6 Hz, H-3 of Sia), 3.22 (1H, ddd, J=13.7, 10.0, 5.1 Hz), 3.22 (1H, ddd, J=13.7, 6.1, 4.6 Hz), 3.42 (1H, m), 3.62—3.76 (22H, m), 3.74 (3H, s), 3.79 (5H, m), 3.85 (1H, m), 3.89 (1H, dd, J=12.0, 4.9 Hz, H-6 of GlcNAc), 3.95 (1H, dd, J=10.5, 10.5 Hz, H-5 of Sia), 3.99—4.09 (4H, m), 4.15 (1H, dd, J=10.5, 8.5 Hz), 4.41 (1H, dd, J=12.7, 2.9 Hz, H-9 of Sia), 4.45 (1H, d, J=8.5 Hz, H-1 of GlcNAc), 4.52 (1H, dd, J=10.5, 5.9 Hz, H-6 of Gal), 4.81—4.86 (1H, m, H-5 of Fuc), 4.87 (1H, m, H-4 of Sia), 4.91 (1H, d, J=8.3 Hz, H-1 of Gal), 4.98 (1H, dd, J=10.0, 8.3 Hz, H-2 of Gal), 5.06 (1H, d, J=3.9 Hz, H-1 of Fuc), 5.17 (1H, d, J=3.4 Hz, H-4 of Gal), 5.37 (1H, dd, J=9.3, 2.7 Hz, H-7 of Sia), 5.60 (1H, ddd, J=9.3, 5.6, 2.9 Hz, H-8 of Sia), 7.51 (2H, t-like), 7.63 (1H, t-like), 8.05 (2H, d-like). FAB-MS: 1454 (M+H), 1476 (M+Na). *Anal.* Calcd for $C_{63}H_{94}N_3O_{35}Cl\cdot1.5H_2O$: C, 49.92; H, 6.45; N, 2.77; Cl, 2.34. Found: C, 49.88; H, 6.58; N, 3.08; Cl, 2.08.

17-Amino-3,6,9,12,15-pentaoxaheptadecyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (22) To a solution of **20** (185 mg, 0.100 mmol) in MeOH (4 ml) was added 28% sodium methoxide (400 μl), and the mixture was stirred for 30 min at room temperature. The reaction mixture was neutralized with Dowex 50W×8 (H^+), the mixture was filtered, and then the filtrate concentrated *in vacuo*.

The residue was dissolved in a solution of 0.1 N aq. NaOH (4 ml) and 1,4-dioxane (2 ml), and then the mixture was stirred for 10 min at room temperature. The reaction mixture was neutralized with Dowex 50W×8 (H^+), the mixture was filtered, and then the filtrate concentrated *in vacuo*.

The crude product was chromatographed on a column of Sephadex LH-20 (70 g, MeOH), to give tetrabenzyl ether (139 mg, 94%) as a colorless powder. $[\alpha]_D^{26}$ −46.9° (c=0.33, MeOH). IR (KBr) cm^{-1}: 3377, 2110, 1655. ^{1}H-NMR (CD_3OD) δ: 1.15 (3H, d, J=6.6 Hz, H-6 of Fuc), 1.83 (1H, t-like, H-3 of Sia), 2.00, 2.01 (each 3H, s), 2.83 (1H, m, H-3 of Sia), 3.21 (1H, dd, J=7.8, 3.4 Hz, H-6 of Sia), 3.33 (2H, t, J=5.1 Hz), 3.50—3.56 (4H, m), 3.59—3.70 (25H, m), 3.73—4.01 (13H, m), 4.00 (1H, dd, J=10.0, 2.7 Hz, H-3 of Fuc), 4.04—4.11 (2H, m), 4.16 (1H, dd, J=9.3, 9.3 Hz, H-3 of GlcNAc), 4.44 (1H, d, J=8.3 Hz, H-1 of GlcNAc or Gal), 4.47 (1H, d, J=7.6Hz, H-1 of GlcNAc or Gal), 4.56 (1H, d, J=11.0 Hz), 4.55, 4.57 (each 1H, d, J=12.0 Hz), 4.75 (1H, d, J=11.7 Hz), 4.78—4.88 (5H, m), 5.35 (1H, d, J=3.7 Hz, H-1 of Fuc), 7.22—7.44 (20H, m).

To a solution of tetrabenzyl ether (30 mg, 20.6 mmol) in MeOH (10 ml) was added Pd–C (10%, 60 mg), and 0.1 M *p*-TsOH–MeOH solution (210 ml, 21.0 mmol), then hydrogenation was carried out at 3.5×10^4 kg/m^2 (50 psi) for 24 h. The mixture was filtered, and the filtrate concentrated *in vacuo*. The crude product was chromatographed on a column of Sephadex LH-20 (50 g, MeOH), to give **22** (17 mg, 72% from **22**) as colorless powder. ^{1}H-NMR (CD_3OD) δ: 1.16 (3H, d, J=6.6 Hz, H-6 of Fuc), 1.71 (1H, m, H-3 of Sia), 1.98, 2.01 (each 3H, s), 2.89 (1H, dd, J=12.4, 4.2 Hz, H-3 of Sia), 3.41—3.45 (2H, m), 3.50 (1H, dd, J=9.0, 1.2 Hz, H-6 of Sia), 3.51 (2H, t, J=6.1 Hz), 3.56 (1H, dd, J=9.5, 7.8 Hz, H-2 of Gal), 3.60—3.79 (28H, m), 3.81 (2H, t, J=6.1 Hz), 3.83—3.95 (8H, m), 4.00 (1H, dd, J=12.2, 3.7 Hz), 4.03 (1H, dd, J=9.5, 2.9 Hz, H-3 of Gal), 4.50 (1H, d, J=8.5 Hz), 4.51 (1H, d, J=7.8 Hz), 5.05 (1H, d, J=3.9 Hz, H-1 of Fuc).

3,6,9,12,15-Pentaoxa-17-propionylamino-heptadecyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (7) Compound **22** (6 mg, 5.54 mmol) was dissolved in 20 mM *N*-succinimidyl propionate–MeOH solution (560 μl, 11.2 mmol), and then 0.1 M *N*-methyl morpholine–MeOH solution (168 μl, 16.8 mmol) was added. After stirring for 12 h at room temperature, the reaction mixture was concentrated. The residue was dissolved in H_2O (3 ml), and the solution was treated with Dowex 50W×8 (H^+ form). The resultant solution was filtrated, and the filtrate concentrated. The crude product was purified by silica gel column chromatography (SiO_2 1 g, CH_2Cl_2 : MeOH : H_2O= 6 : 4 : 1), and gel filtration chromatography (Sephadex LH-20, 20 g, MeOH) to give **7** (5 mg, 79%) as a colorless powder. $[\alpha]_D^{27}$ −26.7° (c=0.20, MeOH). IR (KBr) cm^{-1}: 3400, 1650. ^{1}H-NMR (CD_3OD) δ: 1.12 (3H, t, J=7.6 Hz), 1.15 (3H, d, J=6.6 Hz, H-6 of Fuc), 1.71 (1H, m, H-3 of Sia), 1.96, 2.01 (each 3H, s), 2.21 (2H, q, J=7.6 Hz), 2.87 (1H, dd, J=12.5, 3.9 Hz, H-3 of Sia), 3.36 (2H, t, J=5.6 Hz), 3.42—3.48 (3H, m), 3.52—3.74 (27H, m), 3.54 (2H, t, J=5.6 Hz), 3.75 (1H, dd, J=11.5, 7.1 Hz, H-6 of Gal), 3.82—3.96 (9H, m), 4.00 (1H, dd, J=12.0, 3.7 Hz), 4.04 (1H, dd, J=9.8, 3.2 Hz, H-3 of Gal), 4.50 (1H, d, J=8.3 Hz, H-1 of GlcNAc or H-1 of Gal), 4.50 (1H, d, J=7.8 Hz, H-1 of GlcNAc or H-1 of Gal), 4.82 (1H, m, H-5 of Fuc), 5.03 (1H, d, J=3.9 Hz, H-1 of Fuc). *Anal.* Calcd for $C_{46}H_{81}N_3O_{26}\cdot2.5H_2O$: C, 46.62; H, 7.31; N, 3.55. Found: C, 46.32; H, 7.51; N, 3.40.

Synthesis of a SLe^X–CMPul Conjugate (2) To a solution of CMPul (50 mg) in H_2O (2 ml) were added *N*,*N*′-dimethyl formamide (2 ml), **21** (184 mg, 0.124 mmol) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihyroquinoline (EEDQ, 610 mg). After stirring for 160 h at 40 °C, the reaction mixture was concentrated. The residue was dissolved in 1 N NaOH (7 ml), and the mixture was stirred for 12 h at room temperature. The resultant mixture was poured into 99.5% EtOH (35 ml) and the entire mixture centrifuged. The precipitate was successively washed with 95% EtOH, acetone, and Et_2O, and dried *in vacuo* to give almost pure **2**. After dissolving the product in H_2O (10 ml), the solution was dialyzed using membrane tubing (M.W. cut off: 12000—13000 Spectra) against deionized H_2O (10000 ml) for 12 h, and lyophilized to give a SLe^X–CMPul conjugate (**2**, 57 mg).

It was confirmed by GPC Analysis that low M.W. molecules derived from **21**, were completely excluded from **2**, and the structure of **2** was confirmed by ^{1}H-NMR.

Synthesis of a SLe^X–DSH Conjugate (3) To a solution of DSH (300 mg, M.W.: 40 kDa, Sigma Co. Ltd) in H_2O (20 ml) was added $NaBH_4$ (100 mg). After stirring for 19 h at room temperature, the solution was adjusted to pH 5.0 with AcOH at 0 °C. After stirring for 5 min at 0 °C, the solution was adjusted to pH 8.5 with 1 N NaOH at room temperature, and then the reaction mixture was poured into 99.5% EtOH (35 ml) and the entire mixture was centrifuged. The precipitate was successively washed with 95% EtOH, acetone, and Et_2O, and dried *in vacuo* to give a DSH derivative which was reduced the terminal hemiacetal group (278 mg).

To a solution of the DSH derivative (40 mg) in 0.5% aq. $NaHCO_3$ (3 ml) were added **6** (276 mg, 0.24 mmol), and powdered $NaHCO_3$ (20 mg). After stirring for 160 h at 60 °C, the reaction mixture was poured into 99.5% EtOH (35 ml) and the entire mixture was centrifuged. The precipitate was successively washed with 95% EtOH, acetone, and Et_2O, and dried *in vacuo* to give almost pure **3**. After dissolving the product in H_2O (10 ml), the solution was dialyzed using membrane tubing (M.W. cut off: 12000—13000 Spectra) against deionized H_2O (10000 ml) for 12 h, and lyophilized to give a SLe^X–DSH conjugate (**3**, 47 mg).

It was confirmed by a GPC analysis that low M.W. molecules derived from **6**, were completely excluded from **3**, and the structure of **3** was confirmed by ^{1}H-NMR.

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl 2-Acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (23) To a solution of **11** (240 mg, 0.377 mmol) in *N*,*N*′-dimethylformamide (3 ml) were added BaO (127 mg, 0.754 mmol), $Ba(OH)_2\cdot8H_2O$ (24 mg, 0.0754 mmol) and benzyl bromide (90 μl). After stirring for 12 h at 55 °C, MeOH (3 ml) and 28% NaOMe–MeOH (150 μl) were added and the stirring was contined for 20 min. The mixture was diluted with CH_2Cl_2, washed with saturated brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (SiO_2 30 g, CH_2Cl_2 : MeOH= 50 : 1) to give **23** (233 mg, 85%) as a colorless syrup. $[\alpha]_D^{27}$ −13.4° (c=1.03, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3460, 3340, 1674. ^{1}H-NMR ($CDCl_3$) δ: 1.95 (3H, s), 3.44 (2H, t, J=6.3 Hz), 3.73—3.46 (22H, m), 3.77 (2H, t, J= 6.3 Hz), 3.80 (1H, m), 3.90 (1H, m), 4.05 (1H, dd, J=9.8, 9.5 Hz, H-3 of GlcNAc), 4.35 (1H, dd, J=10.5, 5.1 Hz, H-6 of GlcNAc), 4.66, 4.90 (each

1H, d, J=12.0 Hz), 4.95 (1H, d, J=8.1 Hz, H-1 of GlcNAc), 5.57 (1H, s), 6.36 (1H, d, J=8.1 Hz, NH), 7.22—7.42 (8H, m), 7.47—7.51 (2H, m). *Anal.* Calcd for $C_{34}H_{48}NO_{11}Br$: C, 56.19; H, 6.65; N, 1.92. Found: C, 56.18; H, 6.56; N, 1.81.

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl 2-Acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (24) Compound **23** was converted to **24** (87%) using the procedure described for **14**. Colorless syrup. $[\alpha]_D^{24}$ −14.8° (c=1.11, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3460, 3350, 1674. ^{1}H-NMR ($CDCl_3$) δ: 1.95 (3H, s), 2.76 (1H, brs, OH), 3.45 (2H, t, J=6.3 Hz), 3.51 (1H, m), 3.57—3.71 (19H, m), 3.77 (2H, t, J=6.3 Hz), 3.72—3.80 (5H, m), 3.90 (1H, m), 4.56, 4.61 (each 1H, d, J=12.0 Hz), 4.71, 4.77 (each 1H, d, J=11.5 Hz), 4.81 (1H, d, J=8.3 Hz, H-1 of GlcNAc), 6.36 (1H, d, J=7.3 Hz, NH), 7.24—7.38 (10H, m). *Anal.* Calcd for $C_{34}H_{50}NO_{11}Br$: C, 56.04; H, 6.92; N, 1.92. Found: C, 55.89; H, 6.99; N, 1.84.

17-Azido-3,6,9,12,15-pentaoxaheptadecyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (25) Compound **24** was converted to **25** (98%) using the procedure described for **19**. Colorless syrup. $[\alpha]_D^{26}$ −14.3° (c=1.02, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 2108, 1674. ^{1}H-NMR ($CDCl_3$) δ: 1.90 (3H, s), 2.15 (1H, d, J=2.2 Hz, OH), 3.36 (2H, t, J=5.1 Hz), 3.51 (1H, dt, J=9.5, 4.9 Hz), 3.57—3.71 (22H, m), 3.72—3.80 (4H, m), 3.89 (1H, m), 4.57, 4.61 (each 1H, d, J=12.2 Hz), 4.71, 4.77 (each 1H, d, J=11.5 Hz), 4.82 (1H, d, J=8.3 Hz, H-1 of GlcNAc), 6.33 (1H, d, J=7.3 Hz, NH), 7.37—7.26 (10H, m). *Anal.* Calcd for $C_{34}H_{50}N_4O_{11}$: C, 59.12; H, 7.30; N, 8.11. Found: C, 59.21; H, 7.29; N, 8.01.

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2,4-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (26) Compound **24** was converted to **26** (72%) using the procedure described for **16**. Colorless powder. $[\alpha]_D^{24}$ −21.8° (c=0.38, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3300, 1751, 1662. ^{1}H-NMR ($CDCl_3$) δ: 1.72 (1H, dd, J=12.7, 12.0 Hz, H-3 of Sia), 1.85, 1.96, 1.96, 2.01, 2.11, 2.22 (each 3H, s), 2.07 (6H, s), 2.58 (1H, dd, J=12.7, 4.7 Hz, H-3 of Sia), 3.45 (2H, t, J=6.3 Hz), 3.56—3.70 (22H, m), 3.75 (3H, s), 3.79 (2H, t, J=6.3 Hz), 3.84 (1H, t-like, H-5 of Gal), 3.88—3.95 (4H, m), 3.97 (1H, dd, J=12.2, 6.1 Hz, H-9 of Sia), 4.04 (1H, t-like), 4.05 (1H, q-like, H-5 of Sia), 4.11 (1H, dd, J=11.0, 7.1 Hz, H-6 of Gal), 4.16 (1H, dd, J=11.0, 6.6 Hz, H-6 of Gal), 4.32 (1H, dd, J=12.4, 2.4 Hz, H-9 of Sia), 4.51, 4.59 (each 1H, d, J=12.0 Hz), 4.66 (1H, dd, J=10.3, 3.7 Hz, H-3 of Gal), 4.67, 4.77 (each 1H, d, J=11.5 Hz), 4.71 (1H, d, J=6.1 Hz, H-1 of GlcNAc), 4.85 (1H, d, J=7.8 Hz, H-1 of Gal), 4.88 (1H, ddd, J=12.0, 10.5, 4.7 Hz, H-4 of Sia), 5.03 (1H, brd, H-4 of Gal), 5.04 (1H, brd, NH), 5.04 (1H, dd, J=10.3, 8.1 Hz, H-2 of Gal), 5.36 (1H, dd, J=9.3, 2.7 Hz, H-7 of Sia), 5.60 (1H, ddd, J=9.3, 6.1, 2.9 Hz, H-8 of Sia), 6.30 (1H, brd, NH), 7.18—7.34 (10H, m), 7.38 (2H, m), 7.53 (1H, m), 7.96 (2H, m). *Anal.* Calcd for $C_{71}H_{95}N_2O_{31}Br\ H_2O$: C, 54.30; H, 6.22; N, 1.78. Found: C, 54.41; H, 6.22; N, 1.81.

17-Bromo-3,6,9,12,15-pentaoxaheptadecyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (27) Compound **26** was converted to **27** (85%) using the procedure described for **6** and **17**. Colorless powder. $[\alpha]_D^{24}$ −12.2° (c=1.02, MeOH). IR (KBr) cm^{-1}: 3446, 1735, 1655. ^{1}H-NMR (CD_3OD) δ: 1.87 (1H, t-like, H-3 of Sia), 1.98, 2.00 (each 3H, s), 2.79 (1H, dd, J=12.9, 4.2 Hz, H-3 of Sia), 3.40 (1H, m), 3.50 (1H, m, H-6 of Sia), 3.51 (2H, t, J=6.1 Hz), 3.55—3.95 (36H, m), 3.81 (2H, t, J=6.1 Hz), 4.05 (1H, dd, J=9.8, 2.9 Hz, H-3 of Gal), 4.45 (1H, d, J=7.8 Hz, H-1 of Gal), 4.50 (1H, d, J=8.3 Hz, H-1 of GlcNAc). *Anal.* Calcd for $C_{37}H_{65}N_2O_{24}Br\cdot 2.5H_2O$: C, 42.45; H, 6.74; N, 2.68. Found: C, 42.46; H, 6.82; N, 2.93.

17-Azido-3,6,9,12,15-pentaoxaheptadecyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2,4-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (28) Compound **25** was converted to **28** (48%) using the procedure described for **20**. Colorless powder. $[\alpha]_D^{24}$ −20.9° (c=1.05, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 2100, 1744, 1682. ^{1}H-NMR ($CDCl_3$) δ: 1.72 (1H, dd, J=12.7, 12.3 Hz, H-3 of Sia), 1.96, 2.07 (each 6H, s), 1.85, 2.01, 2.10, 2.22 (each 3H, s), 2.58 (1H, dd, J=12.7, 4.6 Hz, H-3 of Sia), 3.37 (2H, t, J=5.1 Hz), 3.56—3.70 (22H, m), 3.75 (3H, s), 3.78 (2H, t, J=6.3 Hz), 3.84 (1H, t-like, H-5 of Gal), 3.86—3.95 (4H, m), 3.97 (1H, dd, J=12.7, 6.1 Hz, H-9 of Sia), 4.03—4.04 (1H, m), 4.05 (1H, q-like, H-5 of Sia), 4.11 (1H, dd, J=11.0, 7.3 Hz, H-6 of Gal), 4.16 (1H, dd, J=11.0, 6.8 Hz, H-6 of Gal), 4.31 (1H, brd, H-9 of Sia), 4.51, 4.59 (each 1H, d, J=12.0 Hz), 4.66 (1H, dd, J=10.3, 3.7 Hz, H-3 of Gal), 4.67, 4.77 (each 1H, d, J=11.5 Hz), 4.71 (1H, d, J=6.1 Hz, H-1 of GlcNAc), 4.85 (1H, d, J=7.8 Hz, H-1 of Gal), 4.89 (1H, ddd, J=12.0, 10.7, 4.6 Hz, H-4 of Sia), 5.00—5.09 (3H, m, H-2,4 of Gal, NH), 5.36 (1H, dd, J=9.3, 2.7 Hz, H-7 of Sia), 5.60 (1H, ddd, J=9.3, 6.1, 2.7 Hz, H-8 of Sia), 6.32 (1H, m, NH), 7.18—7.35 (10H, m), 7.38 (2H, m), 7.53 (1H, m), 7.96 (2H, m). *Anal.* Calcd for $C_{71}H_{95}N_5O_{31}\ H_2O$: C, 55.64; H, 6.38; N, 4.57. Found: C, 55.46; H, 6.47; N, 4.44.

7-Amino-3,6,9,12,15-pentaoxaheptadecyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2,4-di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside, Hydrochloride salt (29) Compound **28** was converted to **29** (73%) using the procedure described for **21**. Colorless powder. $[\alpha]_D^{26}$ −3.4° (c=1.04, $CHCl_3$). IR (KBr) cm^{-1}: 3480, 3420, 1743, 1688. ^{1}H-NMR (CD_3OD) δ: 1.54 (1H, dd, J=12.5, 12.0 Hz, H-3 of Sia), 1.81, 1.97, 1.97, 2.03, 2.07, 2.12, 2.14, 2.31 (each 3H, s), 2.58 (1H, dd, J=12.4, 4.6 Hz, H-3 of Sia), 3.11 (1H, ddd, J=13.2, 5.9, 3.4 Hz), 3.22 (1H, ddd, J=13.7, 7.1, 3.9 Hz), 3.41 (1H, m), 3.52—3.83 (25H, m), 3.80 (3H, s), 3.89 (1H, d-like, H-6 of Sia), 3.93 (1H, m), 3.97 (1H, dd, J=10.3, 10.3 Hz, H-5 of Sia), 3.99 (1H, dd, J=12.2, 5.9 Hz, H-9 of Sia), 4.05 (1H, m), 4.17 (1H, t-like, H-5 of Gal), 4.30 (1H, dd, J=11.2, 7.3 Hz, H-6 of Gal), 4.36 (1H, dd, J=11.2, 5.8 Hz, H-6 of Gal), 4.39 (1H, dd, J=12.7, 2.7 Hz, H-9 of Sia), 4.40 (1H, d, J=8.5 Hz, H-1 of GlcNAc), 4.73 (1H, dd, J=10.3, 3.2 Hz, H-3 of Gal), 4.85 (1H, d, J=8.3 Hz, H-1 of Gal), 4.87 (1H, m, H-4 of Sia), 5.00 (1H, dd, J=10.0, 8.3 Hz, H-2 of Gal), 5.17 (1H, d, J=3.2 Hz, H-4 of Gal), 5.36 (1H, dd, J=9.5, 2.4 Hz, H-7 of Sia), 5.63 (1H, ddd, J=9.3, 5.9, 2.7 Hz, H-8 of Sia), 7.48 (2H, t-like), 7.62 (1H, t-like), 8.07 (2H, d-like).

Syntheses of Radiolabeled 1 and Radiolabeled 3 To a solution of a SLe^x–CMCht conjugate (**1**, 2 mg) in 1% aq. $NaHCO_3$ (200 μl) was added 100 μl (0.98 nmol) *N*-succinimidyl [2,3-^{3}H] propionate-toluene solution (100 μCi, 1 mCi/ml, Amersham International plc.). After stirring for 24 h at room temperature, the reaction mixture was poured into 99.5% EtOH (1.4 ml) and the entire mixture centrifuged. The precipitate was washed with 95% EtOH (1.4 ml), and dissolved in H_2O. The resultant solution was applied to a PD-10 column (M.W. cut off: 5000, Pharmacia-LKB, Uppsala, Sweden) equibrated with saline and the void fraction was pooled. Radioactivity was determined to be 5 μCi/mg by liquid scintillation counting.

Radiolabeled **3** was prepared by a similar procedure to that used for radiolabeled **1**.

Synthesis of Radiolabeled 2 To a SLe^X–CMPul conjugate (**2**, 2 mg) in a reaction vessel were added 300 μl [2-^{3}H] glycine–water solution (300 μCi, 1 mCi/ml, Amersham International plc.), and 200 μl 1% (w/v) 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ)-*N*,*N*′-dimethylformamide solution. The reaction mixture was stirred for 12 h at room temperature and applied to a PD-10 column (M.W. cut off: 5000, Pharmacia-LKB, Uppsala, Sweden) equilibrated with saline and the void fraction was pooled. Radioactivity was determined to be 15 μCi/mg by liquid scintillation counting.

Radiolabeled **4** and radiolabeled **5** were prepared by a similar procedure to that used for radiolabeled **1** and radiolabeled **2**, respectively.

Synthesis of Radiolabeled 7 To a solution of **22** (2 mg) in H_2O (400 μl) were added 400 μl (3.92 nmol) *N*-succinimidyl [2,3-^{3}H] propionate–toluene solution (400 μCi, 1 mCi/ml, Amersham International plc.), 20 mM non-labelled *N*-succinimidyl propionate–H_2O solution (187 μl, 3.92 mmol), and 0.1 M *N*-methyl morpholine–H_2O solution (56 μl, 5.61 μmol). After stirring for 20 h at room temperature, the reaction mixture was washed with $CHCl_3$ to remove the remaining unreacted *N*-succinyl propionate and *N*-succinimidyl [2,3-^{3}H] propionate, and the H_2O phase was treated with Dowex 50W×8 (H^+ form). The resultant solution was purified by gel filtration chromatography on Bio-Gel P-2 (1×50 cm) equilibrated with 50 mM pyridine/AcOH (pH 5.0) to give radiolabeled **7**. The radioactivity and fucose content were measured by liquid scintillation counting and Gibbon's method, respectively.

Tissue Distribution Experiment Using the Mouse Ear Edema Model Male ICR mice (27—33 g body weight; age 5—6 weeks) were obtained from Japan SLC, Inc., and allowed free access to food and water (standard laboratory chow). Mice were anesthetized with diethyl ether. ^{3}H-labeled monovalent SLe^X (**7**) or oligosaccharide (SLe^X or SLN)–polysaccharide conjugates were administered intravenously at a dose of 365 nmol/kg with respect to the concentration of saccharide. Immediately after administration, arachidonic acid (Sigma Chemical Co.) in acetone (1 mg/20 μl) was applied to both surfaces of the right ear. At various intervals after administration, the mice were anesthetized again and exsanguinated through the heart or femoral artery. The lung, spleen, kidney, liver, right ear, and left ear were then excised, rinsed with saline, and weighed. After drying the plasma and tissues had been dried on combustion cones (Parkard Instrument Co., Inc.) at room temperature, the ^{3}H in each sample was collected as ^3H_2O by the

combustion method (Automatic Sample Combustion System, Aloka ASC-113, Tokyo, Japan). The ^{3}H radioactivity was measured with a liquid scintillation counter (Aloka LSC-350) using a liquid scintillation cocktail (Aquasol-II, New England Nuclear Research).

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References and Notes

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Inclusion Complex of 3,9-Bis(*N*,*N*-dimethylcarbamoyloxy)-5*H*-benzofuro[3,2-c]quinoline-6-one (KCA-098) with Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin: Interaction and Dissolution Properties

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Interactions of KCA-098 with heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CyD) in solution and in the solid state were studied by the solubility method, UV and fluorescence spectroscopy, powder X-ray diffractometry, and thermal analysis. The KCA-098/DM-β-CyD system showed an A_L type solubility diagram with stability constants of 5870 and 2220 M^{-1} in aqueous and 10% methanol solutions, respectively. Following the addition of DM-β-CyD, the maximum UV wavelength of KCA-098 was shifted to a longer wavelength and the fluorescence intensity was decreased. A similar spectral change was observed when KCA-098 was dissolved in less polar solvents, especially in proton-acceptor solvents, such as acetone and dimethylsulfoxide, suggesting that KCA-098 interacts with DM-β-CyD through not only a hydrophobic interaction but also hydrogen bonding. The solid complex of KCA-098 with DM-β-CyD in a molar ratio of 1 : 1 was prepared by the kneading method and the solvent evaporation method, using organic solvents. Powder X-ray diffractometric and differential scanning calorimetric studies indicated that KCA-098 was dispersed as microparticles on the DM-β-CyD complex in the solid state prepared by the solvent evaporation method although it dispersed as crystals in the sample prepared by the kneading method. The dissolution of KCA-098 from the solid complex prepared by the former method was markedly faster than that prepared by the latter method, although it slowed down with the passage of time. The reduced dissolution of KCA-098 was explained by crystallization to the hydrate form in the medium. These data indicate that poorly water-soluble KCA-098 interacts with DM-β-CyD in water and in the solid state and that a fast-dissolving form of KCA-098 can be obtained by evaporating with DM-β-CyD using organic solvents.

Key words KCA-098; DM-β-CyD; complex; dissolution

A new benzofuroquinoline derivative, 3,9-bis(*N*,*N*-dimethyl-carbamoyloxy)-5*H*-benzofuro[3,2-c]quinoline-6-one (KCA-098, Fig. 1), has a pharmacological action useful for the treatment of osteoporosis, such as inhibition of bone resorption and stimulation of bone formation.[1—3] We previously reported that KCA-098 has three different anhydrous crystal forms, forms 1, 2, and 3, which occur at 260, 152, and 93 °C from the hydrate form.[4] Forms 1 and 2 have the same dissolution profile, whereas form 3, a metastable form, has a faster dissolution rate. However, because of the transformation to the hydrate, all anhydrous crystal forms of KCA-098 have the same solubility as the hydrate. Because form 2 crystals are physicochemically stable and their preparation is easy, we selected form 2 as suitable for pharmaceutical formulation. However, some pharmaceutical manipulation is required to increase the dissolution rate of form 2 in order to obtain a sufficiently high oral bioavailability for poorly water-soluble KCA-098.

Cyclodextrins (CyDs), which are cyclic oligosaccharides, and their derivatives are useful pharmaceutical excipients. The molecular structure of these glucose derivatives, which resembles a truncated cone or torus, generates a hydrophilic exterior surface and a nonpolar interior cavity. CyDs can interact with molecules of appropriate size to form inclusion complexes. These noncovalent complexes offer a variety of physicochemical advantages over the original drugs, including the possibility of increased aqueous solubility and physicochemical stability.[5] There are several methods for preparing solid complexes depending on the physicochemical properties of the "guest" drug molecules: (a) precipitation based on the phase solubility,[6] (b) neutralization,[7] (c) spray-drying,[8] (d) freeze-drying,[9] (e) kneading,[10] (f) solvent evaporation,[11] and (g) ball-milling and sealed-heating.[12] Precipitates are easily prepared with a water-soluble guest, but such preparations are difficult with guests which are slightly soluble in water. In this study, we selected two preparation methods, *i.e.*, the kneading and solvent evaporation methods, to synthesize the solid complex, because KCA-098 is practicably insoluble in water as previously reported.[4] Generally, ethanol[11] and acetone[13] have been chosen as an organic solvent for the evaporation method. In our preliminary study, KCA-098 was extremely soluble in halogenated solvents, *e.g.*, dichloromethane, and relatively soluble in alcohol. Further, dichloromethane has been widely used as a solvent for preparing solid dispersions.[14] In view of the solubility of KCA-098, we selected a mixture of dichloromethane and ethanol as a solvent for complex formation.

In this study, we found that KCA-098 interacted with heptakis-(2,6-di-*O*-methyl)-β-CyD (DM-β-CyD) in water and/or an aqueous organic solvent through hydrogen bonding as well as *via* hydrophobic interaction and that KCA-098 dispersed in solid complexes prepared by the solvent evaporation method. Furthermore, rapid dissolution of KCA-098 in

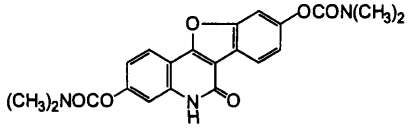

Fig. 1. Structure of KCA-098

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solid complexes was obtained by the solvent evaporation method.

Experimental

Materials KCA-098 was synthesized by Kissei Pharmaceutical Co., Ltd. Form 2 of KCA-098 was prepared by the previously reported method.[4] DM-β-CyD and other CyDs were provided by Toshin Chemical Co. (Tokyo, Japan), and used without further purification. All other chemicals and solvents were of analytical reagent grade.

Phase Solubility Studies The solubility studies were performed according to the method reported by Higuchi and Connors.[15] KCA-098, in an amount that exceeded its aqueous solubility, was accurately weighed in individual 100-ml glass-stoppered flasks to which was added 50 ml water containing various concentrations of CyDs. In addition, the solubility of KCA-098 with DM-β-CyD was also studied in 10% methanol solution. These flasks were shaken at 37 °C in a thermostatically controlled water-bath incubator for 2 h. This short period of shaking was chosen intentionally, because polymorphic transition of the drug from form 2 to the hydrate was observed after 3 h under these conditions as previously reported.[4] The samples were filtered through a 0.45-μm membrane filter, and an internal standard (diphenylamine) was added to the filtrate, and then the KCA-098 content was measured by HPLC as described previously.[4] The apparent 1 : 1 stability constant, *Kc*, was calculated from the initial straight-line portion of the phase solubility diagram, using the equation:

$$Kc = \text{slope}/[\text{intercept} \times (1 - \text{slope})] \quad (1)$$

UV Spectrometry The UV absorption of KCA-098 was recorded at various DM-β-CyD concentrations in 4—50% methanol aqueous solutions or chloroform using a Shimadzu model UV-2500PC spectrophotometer.

Fluorescence spectrometry Fluorescence spectra were obtained by spectrofluorometry (JASCO FP-770, Tokyo, Japan) at an excitation wavelength of 244 nm and scanning the emission over the range 300—500 nm. The fluorescence spectrum of KCA-098 was measured in the presence of DM-β-CyD in 4 and 10% methanol aqueous solutions and in organic solvents.

Preparation of Solid Complexes. Kneading Method KCA-098 and DM-β-CyD in a molar ratio of 1 : 1 were physically mixed and wetted with a few drops of a mixture of dichloromethane and ethanol (2 : 1) to give a paste which was then kneaded using a pestle and motor. The product obtained was dried at 30 °C for 15 h under reduced pressure. The solid mass obtained was then ground and the particles able to pass through a 355 μm sieve were used for further study. For comparison, DM-β-CyD was treated in the same way.

Solvent Evaporation Method KCA-098 and DM-β-CyD at a molar ratio of 1 : 1 were dissolved in a mixture of dichloromethane and ethanol (2 : 1), and sonicated. The solvent was then evaporated (at about 50 °C) under reduced pressure in a rotating evaporator. The resulting solid mass was kept in a desiccator under vacuum at 30 °C for 24 h, and then ground to reduce the particle. Particles passing through a 355 μm sieve were used for further study. A physical mixture of KCA-098 and DM-β-CyD was also prepared using each compound treated in the same solvent.

Determination of Dichloromethane and Ethanol in Solid Complexes by Gas Chromatography Samples were dissolved in dimethylformamide and 1 μl of each solution was subjected to GC analysis. A Shimadzu model GC 14A gas chromatograph, equipped with a FID detector, was used. The column was 2 m×3.0 mm i.d. glass tubing filled with Gasukuropack 54 60/80 mesh (GL Science Ltd.). The operating temperatures used were 180 °C for the column and 250 °C for the injection port. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. The detection limits of dichloromethane and ethanol were 600 and 300 ppm, respectively.

Thermal Analysis Studies Thermal curves were recorded by simultaneous differential scanning calorimetry (DSC) and thermogravimetry (TG) with a thermal analyzer (Model TAS-100, TG-8110, Rigakudenki, Japan). Samples (about 5 mg) were scanned at 10 °C/min over the range 50—300 °C, with an empty aluminium pan as a reference.

Powder X-Ray Diffraction Studies Powder X-ray diffraction patterns of KCA-098 solid complexes were recorded by X-ray diffractometry (Model RINT-1400, Rigakudenki, Japan) using monochromatic Cu-*Kα* radiation at room temperature. The operating conditions were the following: voltage, 30 kV; current, 100 mA; time constant, 1s; diffraction angle (2θ), range of 3—40°; scanning speed, 2°/min.

Dissolution Studies *In vitro* dissolution of the drug was determined according to the procedure described in the Japanese Pharmacopoeia XIII (the paddle method). Solid complex powder equivalent to 50 mg KCA-098 was placed in a vessel with 900 ml water at 37 °C and the paddle was rotated at 50 rpm. Two milliliters aliquots of each sample solution were withdrawn at given intervals, and equal volumes of water at 37 °C were added to each sample to maintain a constant volume of dissolution medium. Each sample solution was filtered through a 0.45-μm membrane filter, and then the filtrate was assayed by the HPLC method described previously.[4]

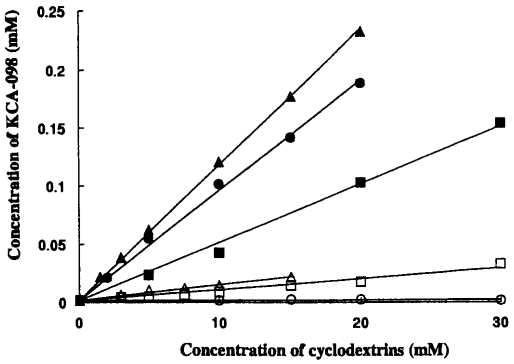

Fig. 2. Phase Solubility Diagrams of KCA-098/CyD Systems in Water and in 10% Methanol Solution at 37 °C

KCA-098 in water with α-CyD (○), β-CyD (△), γ-CyD (□), DM-β-CyD (●), HP-β-CyD (■). KCA-098 in 10 % methanol with DM-β-CyD (▲).

Results and Discussion

Interaction between KCA-098 and DM-β-CyD in Aqueous and Organic Media The solubility method is useful for investigating an inclusion complexation of drugs with CyDs in water because it gives not only the solubilizing ability of the host molecules but also the stability constant of complexes by analysis of the solubility curve.[16] Figure 2 shows the phase solubility diagrams obtained for KCA-098 with various CyDs in water. The extremely low solubility (1.65×10^{-6} M at 37 °C) of KCA-098 increased with a rise in DM-β-CyD and 2HP-β-CyD concentrations; *i.e.*, the solubility of KCA-098 increased 115- and 94-fold in the presence of 20 mM DM-β-CyD and 30 mM 2HP-β-CyD, respectively. However, the other CyDs used in this study had only a slight effect on drug solubility. In the case of DM-β-CyD and 2HP-β-CyD, the phase solubility diagrams showed an A_L type, as defined by Higuchi and Connors,[15] indicating that the aqueous solubility of KCA-098 increased linearly as a function of the CyD concentration and that a soluble complex was formed. The apparent 1 : 1 stability constant, *Kc*, for the complex of KCA-098 with DM-β-CyD and with 2HP-β-CyD was estimated to be 5870 and 3040 M^{-1}, respectively. These values are within the range of 200—5000 M^{-1}, and hence the stability constant obtained by DM-β-CyD and 2HP-β-CyD is considered adequate for the formation of an inclusion complex which may contribute to improving the bioavailability of poorly water-soluble drugs.[17] Furthermore, a solubility study of the KCA-098/DM-β-CyD system was conducted in 10% methanol solution. The system in this solution also exhibited typical A_L type solubility, as shown in Fig. 2. The stability constant calculated by Eq. 1 from the initial straight line was 2220 M^{-1}. This stability constant was smaller than that in water. This phenomenon could be explained as follows: the high concentration of methanol interacted with DM-β-CyD in a competitive manner and addition of alcohol to the aqueous solution reduced the polarity of the solvent and, thus, decreased the hydrophobicity difference between the solvent and the DM-β-CyD cavity.

Figure 3 shows the effect of DM-β-CyD on the UV spectrum of KCA-098 in 4% methanol solution at 37 °C. For the spectral measurement, methanol was added to solubilize KCA-098. Both maxima at 326 and 341 nm shifted to longer wavelengths, and the maximum absorbance decreased with the addition of DM-β-CyD up to 0.61 mM, and then increased in the presence of more concentrated DM-β-CyD. The isosbestic point was observed at 299, 328, 337, and 343 nm. No change in the isosbestic points was observed with the CyD concentrations used, indicating the formation of only one species of complex. Similar spectral changes were observed for the inclusion complex of pyrene with β-CyD in aqueous solution.[18] The UV spectrum of KCA-098 with the highest concentration of DM-β-CyD (2.4×10^{-3} M) might be identical to that of the complex, because 90% of the drug was in the complex when 1 : 1 complex formation was assumed ($Kc=4720\ M^{-1}$, calculated from the linear plot of methanol concentration *vs.* log *Kc*). The UV spectra obtained with various concentrations of DM-β-CyD agreed with the sum of the UV absorption calculated from the fraction of each spectrum of KCA-098 only and its complex with DM-β-CyD. A similar spectral change in KCA-098 caused by DM-β-CyD was observed in 4—30% methanol solution, but the degree of this change was smaller at higher methanol concentrations. In 50% methanol solution, the maximum absorbance decreased upon adding DM-β-CyD with no red shift. The plot of $1/\Delta A$ *vs.* 1/[CD] at 343 nm was linear, suggesting the presence of a 1 : 1 complex. The apparent 1 : 1 stability constant, *Kc*, was determined by the Benesi–Hildebrand plot,[19] and was $90\ M^{-1}$ at 50% methanol concentration. In 20 and 30% methanol solutions, the *Kc* values were similarly calculated to be 1760 and $850\ M^{-1}$, respectively. A reduction in the stability constant in methanol solution was observed, as in the solubility study described above.

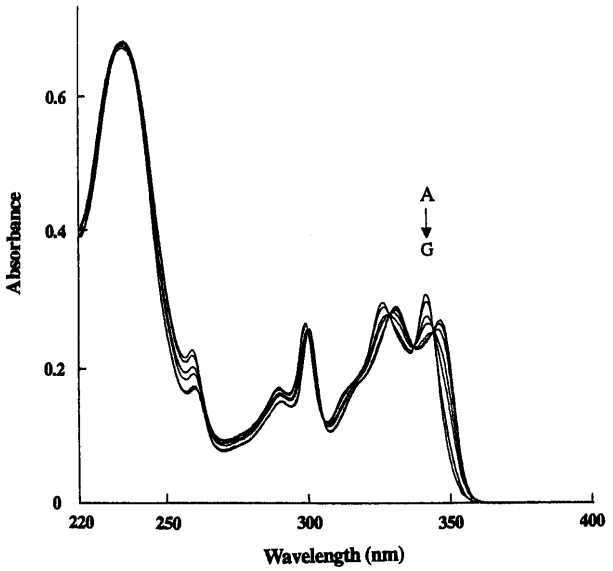

Fig. 3. Effect of DM-β-CyD Concentration on UV Absorption Spectrum of KCA-098 in 4 % Methanol Solution at 37 °C

From A to G ($\times10^{-3}$ M); 0, 0.12, 0.37, 0.61, 1.2, 1.8, 2.4. The concentration of KCA-098 was 1.3×10^{-5} M.

The effect of DM-β-CyD on the fluorescence spectrum of KCA-098 is shown in Fig. 4. The fluorescence intensity of both peak maxima (349.5 and 366 nm) decreased with the addition of DM-β-CyD and shifted to a longer wavelength, from 349.5 nm to 354.5 nm and from 366 nm to 369 nm in 4% methanol solution. The fluorescence spectral change in KCA-098 caused by the addition of DM-β-CyD was smaller in 10% methanol solution than in 4% methanol due to a weaker interaction in the higher concentration of methanol. On the other hand, no change in the fluorescence spectrum of KCA-098 in 4 or 10% methanol solution was seen when 3-*O*-methyl glucose was added in place of DM-β-CyD. These data suggest that KCA-098 interacts with cyclic glycosides. We also measured the fluorescence spectrum of KCA-098 in various solvents. Table 1 lists the maximum wavelength and fluorescence intensity of KCA-098 in these solvents. The fluorescence intensity of KCA-098 decreased with increasing methanol concentration, *i.e.*, with a decrease in dielectric constant (ε). Further, the spectral change in ethanol ($\varepsilon=24.5$) was similar to that in methanol ($\varepsilon=32.7$), where methanol and ethanol were proton-donor solvents. On the other hand, a dramatic reduction in the fluorescence intensity and a pronounced red shift in the maximum wavelength were observed in proton-acceptor solvents, such as acetone ($\varepsilon=20.6$), dimethylsulfoxide ($\varepsilon=46.5$) and dioxane ($\varepsilon=2.2$). The fluorescence spectral change in KCA-098 caused by the addition of DM-β-CyD was similar to that in proton-acceptor solvents in comparison with proton-donor solvents. The above data suggest that KCA-098 is included in the cavity of DM-β-CyD,

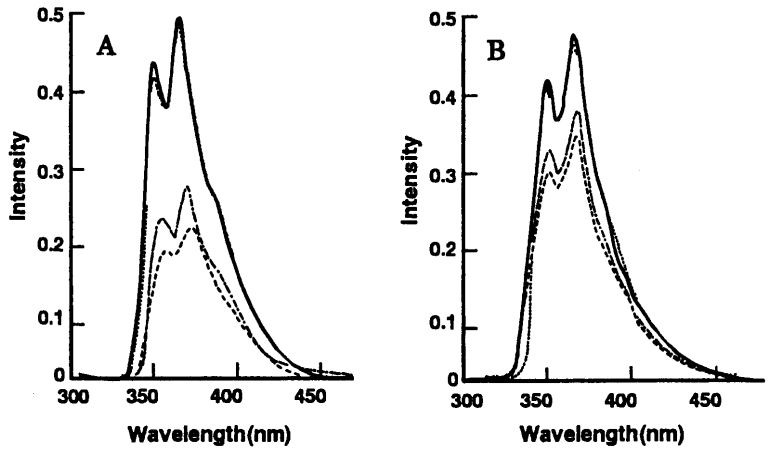

Fig. 4. Effect of DM-β-CyD Concentration on Fluorescence Spectrum of KCA-098 in 4% (A) and in 10% (B) Methanol Solution at 37 °C

—, KCA-098 alone; ·····, KCA-098 with 3-*O*-methyl-D-glucose; —·—, KCA-098 with DM-β-CyD (6.1×10^{-6} M); - - -, KCA-098 with DM-β-CyD (2.4×10^{-4} M). The concentration of KCA-098 was 1.22×10^{-6} M.

Table 1. Maximum Fluorescence Intensity and Wavelength of KCA-098 in Various Organic Solvents and Methanol Aqueous Solutions

Solvent	Wavelength (nm)	Intensity
4% Methanol solution	349.5	0.4314
	366	0.4906
10% Methanol solution	350	0.4194
	366.5	0.4769
50% Methanol solution	351	0.4076
	368	0.454
100% Methanol solution	353	0.3214
	370.5	0.3528
Ethanol	354	0.329
	371	0.3522
Acetone	354	0.0065
	354	0.0068
Dioxane	357	0.0608
	375	0.0614
Dimethylsulfoxide	359	0.0131
	377	0.0134
Chloroform	357	0.0570
	374	0.0608

by donating a proton of the amide group (see Fig. 1) to DM-β-CyD through hydrogen bonding. It has been reported that the driving force for drug interactions with methylated cyclodextrins, *e.g.*, DM-β-CyD and trimethyl-β-CyD, in organic solvents is mainly hydrogen bonding.[20—22] Hydrogen bonding may play an important role in the interaction of the drug with methylated cyclodextrins as well as the hydrophobic interaction.

Characterization of Solid Complexes From the results of the above experiments, we concluded that KCA-098 formed the most water-soluble complex with DM-β-CyD among the five kinds of CyDs used in this study. Because the phase solubility diagram of KCA-098 with DM-β-CyD was of the A_L type and, thus, a precipitate caused by complexation could not be obtained by adding a high concentration of DM-β-CyD (Fig. 2). Therefore, we used two different methods, a kneading method and a solvent evaporation method, in order to prepare the solid complex. A mixture of dichrolomethane and ethanol was used in the preparation by the solvent evaporation method because of the low boiling point of this solvent mixture and the high solubility of KCA-098 in it. DM-β-CyD was also readily soluble in this mixture.

The powder X-ray diffractograms of KCA-098 and its solid complexes are shown in Fig. 5. KCA-098 and DM-β-CyD showed different crystal states by the kneading and solvent evaporation treatments. After the kneading treatment, KCA-098 maintained the form 2 crystal, whereas KCA-098, after treatment with a mixture of dichloromethane and ethanol in the same manner as used for the preparation of the solid complex, showed a different diffraction pattern (trace (b)) from that of form 2. In the case of DM-β-CyD, peaks at 8.4°, 10.1° and 11.4—11.7° were observed after kneading (trace (c)), and peaks at 7.2°, 8.2° and 10.3° appeared when the solvent evaporation method was used (trace (d)). The differences in the powder X-ray pattern for KCA-098 and DM-β-CyD following the two types of treatment might be explained by the rearrangement of crystals after dissolution in dichloromethane and ethanol in the solvent evaporation method. The diffraction patterns of both physical mixtures

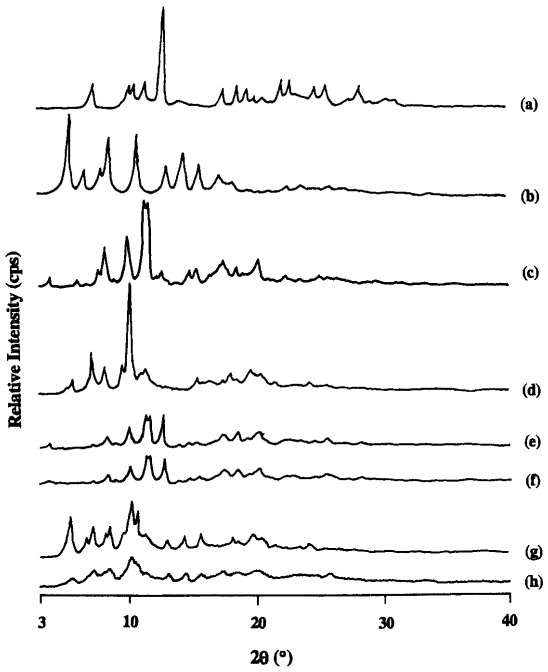

Fig. 5. Powder X-ray Diffraction Patterns of Form 2

(a), New crystal of KCA-098 (b), DM-β-CyD after kneading (c), DM-β-CyD after solvent evaporation (d), physical mixture of form 2 and DM-β-CyD after kneading (e), solid sample produced by kneading (f), physical mixture of new crystal of KCA-098 and DM-β-CyD after solvent evaporation (g), solid complex produced by solvent evaporation (h).

(Fig. 5., trace (e) and (g)) corresponded to the superimposed diffractograms of KCA-098 (form 2 and the new crystal form) and DM-β-CyD obtained by the same treatment. The diffraction pattern of the kneaded sample (trace (f)) was nearly the same as that of the physical mixture (trace (e)), *i.e.*, one characterized by a peak for form 2 of KCA-098 at 12.7°. These data suggest that KCA-098 and DM-β-CyD separately dispersed as crystalline forms. On the other hand, the complex prepared by the solvent evaporation method showed a small and broad peak, indicating the reduced crystallinity of KCA-098 and DM-β-CyD.

DSC was employed to evaluate the crystal state of KCA-098 with DM-β-CyD. The obtained DSC curves are shown in Fig. 6. The thermogram of intact KCA-098 (form 2) showed an endothermic peak at 269 °C, corresponding to the melting point with a transition peak from form 2 to form 1 at 256 °C (trace (a)). The new crystals of KCA-098, obtained by the solvent evaporation method, showed an exothermic peak at 130 °C corresponding to the transformation to form 2 (trace (b)). The crystal forms of DM-β-CyD, before and after treatment in organic solvent, showed no peak. In the case of the physical mixture of kneaded samples, the broad endothermic peak of KCA-098 was found around 261 °C (trace (e)) with the transition peak at 248 °C. The DSC thermogram of the kneaded sample (trace (f)) showed the same peaks as that of the physical mixture. These results indicate that KCA-098 is dispersed as a crystal in the kneaded sample, in agreement with the conclusion made from the powder X-ray diffractogram. However, the solid complex obtained by the solvent evaporation method (trace (h)) showed a weak and broad fusion peak, although the physical mixture (trace (g)) showed the characteristic peak of the new crystal of KCA-098. Moreover, this broad endothermic peak was slightly shifted to a

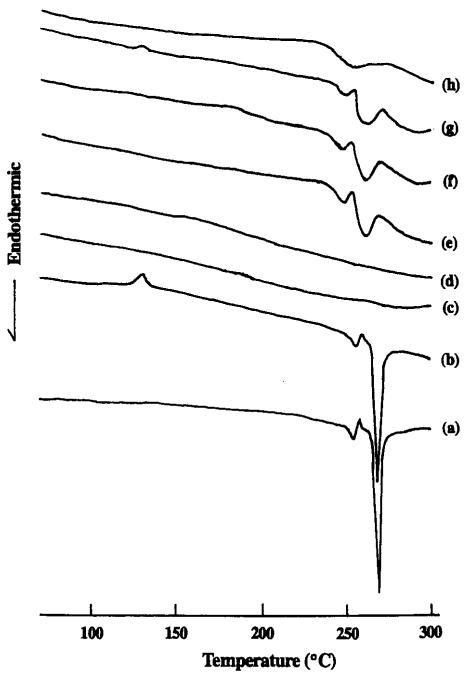

Fig. 6. DSC Curves of Form 2

(a), New crystal form of KCA-098 (b), DM-β-CyD after kneading (c), DM-β-CyD after solvent evaporation (d), physical mixture of form 2 and DM-β-CyD after kneading (e), solid sample produced by kneading (f), physical mixture of new Crystal of KCA-098 and DM-β-CyD after solvent evaporation (g), and solid complex produced by solvent evaporation (h).

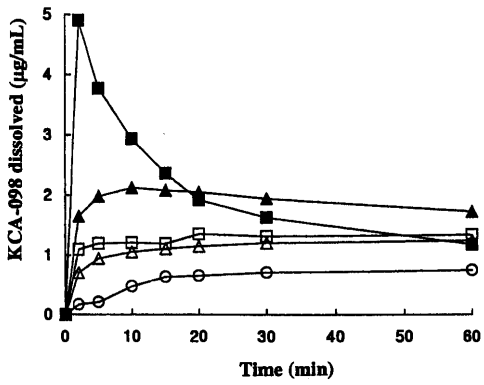

Fig. 7. Dissolution Profiles of KCA-098 from KCA-098/DM-β-CyD Solid Complex

○, form 2; △, physical mixture of form 2 and DM-β-CyD after kneading; ▲, physical mixture of new crystal of KCA-098 and DM-β-CyD after solvent evaporation; □, solid sample produced by kneading; ■, solid complex produced by solvent evaporation.

lower temperature (254 °C), which can be explained by an interaction between the drug and CyD.[23] These data suggest that microparticle dispersion of each component was achieved by the solvent evaporation method and that some of the KCA-098 may have formed a complex with DM-β-CyD.

Dissolution Studies The dissolution profiles of the solid complex samples prepared by the two methods were compared with those of the physical mixture and KCA-098 alone. As shown in Fig. 7, the release profile of the kneaded sample was not improved, compared with that of the physical mixture whose dissolution rate was faster than that of the drug alone. On the other hand, dramatically more rapid dissolution was obtained with the complex prepared by the solvent evaporation method, reflecting the microparticle dispersion. The maximum concentration, which was reached within 2 min, was about 2.5 times higher than the maximum dissolution level of the physical mixture. After reaching the highest dissolution level, the concentration of KCA-098 dissolved from the complex decreased and became less than that of the physical mixture after 60 min. After the dissolution study, the precipitate was filtered and its crystal form was characterized by powder X-ray diffractometry. The precipitate, after dissolution of the complex prepared by the solvent evaporation method, showed the hydrate crystal structure of KCA-098, which has the lowest solubility (0.2 μg/ml) among the five known crystal forms.[4] In contrast, both the physical mixture and the kneaded sample maintained the crystal form (form 2) of the drug up to 60 min after the dissolution study. From these results, the reduction in the drug concentration of KCA-098 dissolved from the solid complex prepared by solvent evaporation was ascribed to transformation to the hydrate, although a few percent of the dissolved KCA-098 interacted with DM-β-CyD to form a complex in accord with the stability constant ($5870\,M^{-1}$). The relatively small increase in the dissolution rate observed for the physical mixture may be explained by the wetting effect of the DM-β-CyD on the drug particle surface. Since DM-β-CyD dissolves more rapidly in the dissolution medium than the pure drug, it can be assumed that, in the early stages of the dissolution process, the DM-β-CyD molecule will operate locally on the hydrodynamic layer surrounding the drug particles, this action resulting in an *in situ* inclusion process, which increases of the amount of dissolved drug.[24] Moreover, the dissolution rate of KCA-098 was faster for the new crystal treated by solvent evaporation than for form 2. Thus, the increase in the dissolution rate was found to be dependent on the preparation method, since the evaporated solid complex exhibited the highest initial dissolution rate. This enhancement can be attributed to microparticle dispersion and complexation in the solid state and to a reduction in the crystallinity of the product, as confirmed by powder X-ray and DSC studies. Moreover, the content of ethanol and dichloromethane in the solid complex prepared by the solvent evaporation method was 0.12% and less than 0.06%, respectively, suggesting that solvent evaporation is a useful and safe method for rapid-dissolving formulations of poorly water-soluble drugs.

In conclusion, DM-β-CyD can interact with KCA-098 in aqueous and methanol solution through hydrogen bonding as well as by hydrophobic interaction, and the solid complex prepared in organic solvent showed a rapid dissolution rate due to microparticle dispersion of the drug.

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Non-glutamate Type Pyrrolo[2,3-*d*]pyrimidine Antifolates. III. Synthesis and Biological Properties of N^{ω}-Masked Ornithine Analogs[1)]

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The glutamic acid moiety of *N*-[4-[3-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-L-glutamic acid (1b, TNP-351) and the related compound (1a), was replaced with various N^{ω}-acyl-, sulfonyl-, carbamoyl- and aryl-2,ω-diaminoalkanoic acids, and the inhibitory effects of the resulting products (9, 11, 14, 18, 21, 23, 25, 30, 36) on dihydrofolate reductase (DHFR), the growth of murine fibrosarcoma Meth A cells, and methotrexate-resistant human CCRF-CEM cells, were examined. Compounds (9a—f) acylated with a hemiphthaloyl group were efficiently synthesized by coupling pyrrolo[2,3-*d*]pyrimidine carboxylic acids (7a,b) and N^{ω}-phthaloyl 2,ω-diaminoalkanoic acid methyl esters (6a—c) and subsequent hydrolysis. The other N^{ω}-acyl- and sulfonyl-ornithine analogs (21, 23, 25) were synthesized by acylation of free amino intermediates (19a,b) derived from *tert*-butoxycarbonyl-ornithine analogs (17a,b). A free ornithine analog (18) did not strongly inhibit Meth A cell growth, whereas all N^{ω}-acyl-, sulfonyl-, carbamoyl- and aryl-ornithine analogs (9, 11, 21, 23, 25, 30, 36) exhibited much more potent inhibitory activities against both DHFR and Meth A cell growth. In particular, compounds 9c, 21k and 36a also showed remarkable growth-inhibitory activities against methotrexate-resistant CCRF-CEM cells. These results demonstrate that the potent inhibitory activities of N^{ω}-masked ornithine analogs against the growth of Meth A cells and methotrexate-resistant CCRF-CEM cells, results from effective uptake *via* reduced folate carrier and their potent DHFR inhibition.

Key words pyrrolo[2,3-*d*]pyrimidine; antifolate; N^{ω}-masked ornithine; dihydrofolate reductase inhibition; methotrexate-resistant tumor; TNP-351

The antifolate methotrexate (MTX) has been clinically useful for treating acute lymphocytic leukemia and choriocarcinoma for more than 40 years. Unfortunately, MTX is active in only a narrow group of solid tumors, and continued therapy induces acquired resistance.[2)] A large number of derivatives of MTX have been synthesized in an attempt to obtain more effective compounds against solid tumors than MTX.[3)] These efforts have led to the discovery of several promising compounds[4a)] such as trimetrexate, edatrexate, lometrexol and tomudex (ZD-1694).[4b)]

We previously reported that *N*-[4-[3-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-L-glutamic acid (**1b**, TNP-351), a novel dihydrofolate reductase (DHFR) inhibitor characterized by the presence of a pyrrolo[2,3-*d*]pyrimidine ring, showed potent antitumor activities against not only leukemia cells but also various solid tumor cells both *in vitro*[5)] and *in vivo*.[6)] Biological evaluation of TNP-351 has demonstrated that it is more efficiently taken up by tumor cells *via* the reduced folate carrier (RFC) system and is much more rapidly converted to polyglutamates than MTX,[6)] and that the formed polyglutamates were potent inhibitors of aminoimidazolecarboxamide ribonucleotide transformylase[7)] as well as DHFR.[8)] As an extension of our research, we have made modifications to the glutamic acid moiety of TNP-351, as shown in Fig. 1. Replacement of the α- or γ-carboxyl group of the glutamic acid by a tetrazolyl group suggested that the α-carboxyl group plays an important role in effective uptake *via* the RFC-mediated transport system, and that the γ-carboxyl group moiety is important for tight binding to DHFR.[9)] In our previous paper, we reported that non-glutamate type antifolates containing N^5-substituted glutamines (**2**) were potent inhibitors of DHFR and active against some murine tumor cells and MTX-resistant human CCRF-CEM cells.[1)] Based on our previous results, we designed N^{ω}-masked ornithine analogs (**9**, **11**, **14**, **18**, **21**, **23**, **25**, **30**, **36**) of TNP-351 and its related compound (**1a**) as novel non-glutamate type antifolates, that were expected to be transported effectively into cells *via* the RFC due to the existence of a α-carboxyl group and to be potent inhibitors of DHFR because of optimization of the γ-carboxyl group moiety of the glutamic acid residue.

In this paper, we describe the preparation of novel non-glutamate type pyrrolo[2,3-*d*]pyrimidine antifolates (**9**, **11**, **14**, **18**, **21**, **23**, **25**, **30**, **36**) with N^{ω}-masked 2,ω-diaminoalkanoic acid in place of glutamic acid, together with their inhibitory effects on DHFR and the growth of murine Meth A cells and some MTX-resistant human CCRF-CEM cells in culture.

Chemistry Pyrrolo[2,3-*d*]pyrimidine carboxylic acids **7a**, **b** were synthesized by our previously reported method.[10)] Most pyrrolo[2,3-*d*]pyrimidine antifolates containing acylornithines were prepared by one of two methods; the first is coupling of **7a**, **b** and N^{δ}-acylornithine esters (**6**, **12**, **16**) and subsequent hydrolysis (method A), and the other is acylation of free amino ornithinate analogs **19**, key intermediates derived from N^{δ}-*tert*-butoxycarbonyl (Boc)-ornithine antifolates (**17**) and subsequent hydrolysis (method B) (Table 1). Synthesis of hemiphthaloyl ornithine analogs (**9**) and benzyloxycarbonyl (Z)- or Boc-ornithine analogs (**14**, **18**) were performed using method A, and the other acylornithine analogs (**21**) and sulfonyl analogs (**23**, **25**) were prepared using method B.

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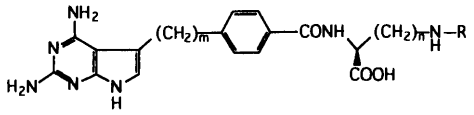
Table 1. Preparation of Novel Pyrrolo[2,3-*d*]pyrimidine Antifolates Containing Ornithine Derivatives



Compd.	*m*	*n*	R	Method[a]	Yield (%)	mp (°C)	SI-MS (MH^+)	Formula	*Anal.* Calcd (Found) C	H	N	S
18	2	3	COO^{t}Bu	A	85	175—176.5	512	$C_{25}H_{33}N_7O_5 \cdot 3.2H_2O$	52.75 (52.60	6.98 6.47	17.22 17.04)	
14	3	3	COOBzl	A	89	138—139	560	$C_{29}H_{3}3N_7O_5 \cdot 1.0H_2O$	60.30 (60.27	6.11 6.30	16.97 16.74)	
15	3	3	H	C	99	180—182	448	$C_{21}H_{26}N_7O_3Na \cdot 2.2H_2O$	51.78 (51.81	6.29 6.40	20.13 19.85)	
21a	2	3	$COCH_2CH_2COOH$	B	37	164—166.5	512	$C_{24}H_{29}N_7O_6 \cdot 1.0H_2O$	54.44 (54.69	5.90 6.27	18.52 18.50)	
21b	2	3	COCH=CHCOOH	B	43	225—230	510	$C_{24}H_{27}N_7O_6 \cdot 4.0H_2O$	49.57 (49.74	6.07 5.59	16.86 16.29)	
9a	2	2	COPh-2-COOH	A	82	191—192.5	546	$C_{27}H_{27}N_7O_6 \cdot 1.2H_2O$	57.18 (57.09	5.22 5.34	17.29 17.22)	
9b	3	2	COPh-2-COOH	A	99	184—185	560	$C_{28}H_{29}N_7O_6 \cdot 2.0H_2O$	56.46 (56.54	5.58 5.72	16.46 16.46)	
9c	2	3	COPh-2-COOH	A	54	186.5—189	560	$C_{28}H_{29}N_7O_6 \cdot 2.5H_2O$	55.62 (55.63	5.67 5.60	16.22 16.19)	
9d	3	3	COPh-2-COOH	A	87	176—179	574	$C_{29}H_{31}N_7O_6 \cdot 2.5H_2O$	56.30 (56.57	5.87 5.65	15.85 15.63)	
9e	2	4	COPh-2-COOH	A	77	178.5—179.5	574	$C_{29}H_{31}N_7O_6 \cdot 2.0H_2O$	57.14 (57.40	5.79 5.78	16.08 16.13)	
9f	3	4	COPh-2-COOH	A	73	173.5—174.5	588	$C_{30}H_{33}N_7O_6 \cdot 2.5H_2O$	56.95 (57.10	6.05 5.75	15.50 15.63)	
11	2	3	COPh-2-$CONC_4H_8$	A	68	177.5—179.5	613	$C_{32}H_{36}N_8O_5 \cdot 2.5H_2O$	58.44 (58.29	6.28 6.10	17.04 16.94)	
21c	2	3	COPh-4-COOH	B	56	206.5—208.5	560	$C_{28}H_{29}N_7O_6 \cdot 1.5H_2O$	57.33 (57.60	5.50 5.51	16.71 16.59)	
21d	2	3	COPh-2-OH	B	42	166.5—168.5	532	$C_{27}H_{29}N_7O_5 \cdot 1.6H_2O$	57.88 (58.12	5.79 5.75	17.50 16.96)	
21e	2	3	COPh-4-NHAc	B	52	194—195.5	573	$C_{29}H_{32}N_8O_5 \cdot 1.7H_2O$	57.74 (57.78	5.91 6.13	18.58 18.52)	
21f	2	3	COPh-3,4-OCH_2O–	B	51	172.5—173.5	560	$C_{28}H_{29}N_7O_6 \cdot 1.5H_2O$	57.33 (57.21	5.50 5.67	16.71 16.53)	
21g	2	3	COPh-3,4,5-$(OMe)_3$	B	54	174—175	606	$C_{30}H_{35}N_7O_7 \cdot 1.5H_2O$	56.95 (56.98	6.05 6.05	15.50 15.35)	
21h	2	3	COPh-2,6-Me_2	B	25	178—180	544	$C_{29}H_{33}N_7O_4 \cdot 2.0H_2O$	59.99 (60.27	6.60 6.50	16.89 16.63)	
21i	2	3	CO-5-Py-2-COOH	B	37	197—200.5	561	$C_{27}H_{28}N_8O_6 \cdot 1.7H_2O$	54.85 (55.03	5.35 5.88	18.95 18.88)	
21j	2	3	CO-1-Napht	B	31	180—184	566	$C_{31}H_{31}N_7O_4 \cdot 3.0H_2O$	60.09 (59.77	6.02 5.68	15.82 15.69)	
21k	2	3	CO-2-Napht-3-COOH	B	46	201—205	610	$C_{32}H_{31}N_7O_6 \cdot 3.3H_2O$	57.44 (57.33	5.66 5.28	14.65 14.87)	
21l	3	3	CO-2-Napht-3-COOH	B	24	183.5—186	624	$C_{33}H_{33}N_7O_6 \cdot 3.0H_2O$	58.49 (58.56	5.80 5.70	14.17 14.20)	
23a	2	3	SO_2Ph-4-Me	D	58, 88	167—169	566	$C_{27}H_{31}N_7O_5S \cdot 2.4H_2O$	53.26 (53.03	5.93 5.85	16.10 16.47	5.27 5.18)
23b	2	3	SO_2Ph-2-COOH	D	55, 79	205—208	596	$C_{27}H_{29}N_7O_7S \cdot 1.5H_2O$	52.08 (52.24	5.18 5.44	15.75 15.87	5.15 4.99)
23c	2	3	SO_2Ph-3,5-$(COOH)_2$	D	29, 83	213—215	640	$C_{28}H_{29}N_7O_9S \cdot 2.0H_2O$	49.77 (49.99	4.92 4.88	14.51 14.75	4.75 4.46)
25	2	3	CONHPh-4-F	E	71, 77	183—185	549	$C_{27}H_{29}N_8O_4F \cdot 2.5H_2O$	54.63 (54.84	5.77 5.13	18.88 18.67)	
30a	2	1	CONHPh-3-COOH	A	100, 85	212 (dec.)	547	$C_{26}H_{26}N_8O_6 \cdot 4.0H_2O$	50.48 (50.27	5.54 4.40	18.11 17.64)	
30b	2	2	CONHPh-3-COOH	A	99, 94	213—218	561	$C_{27}H_{28}N_8O_6 \cdot 3.0H_2O$	52.76 (52.63	5.58 5.01	18.23 18.16)	
30c	2	2	CONHPh-3-$B(OH)_2$	A	68, 92	>260 (dec.)	561	$C_{26}H_{29}N_8O_6B \cdot 2.2H_2O$	52.05 (52.04	5.61 5.65	18.68 18.49)	
36a	2	3	Ph-3-COOH	A	93, 98	191—194	532	$C_{27}H_{29}N_7O_5 \cdot 1.3H_2O$	58.43 (58.48	5.74 5.95	17.67 17.69)	
36b	3	3	Ph-3-COOH	A	73,100	181—183	546	$C_{28}H_{31}N_7O_5 \cdot 2.5H_2O$	56.94 (57.04	6.14 5.88	16.60 16.50)	
36c	2	3	$CH(COOH)CH_2CH_2COOH$	A	100, 75	182—183.5		$C_{25}H_{31}N_7O_7 \cdot 1.9H_2O$	52.15 (52.13	6.09 5.97	17.03 16.87)	

a) A: i) **7a**, **b**/amino acid derivatives/DEPC; ii) Alkaline hydrolysis, B: i) **17a**/TFA; ii) DEPC, Et_3N, RCOOH; iii) hydrolysis, C: hydrogenation, D: i) **17a**/TFA; ii) DEPC, Et_3N, RSO_2Cl; iii) alkaline hydrolysis, E: i) **17a**/TFA; ii) isocyanate/Et_3N; iii) alkaline hydrolysis.

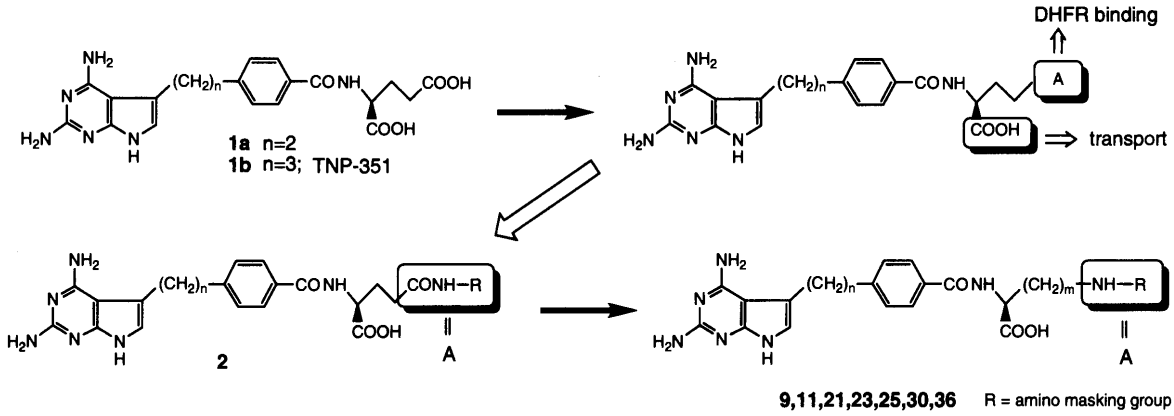


Fig. 1

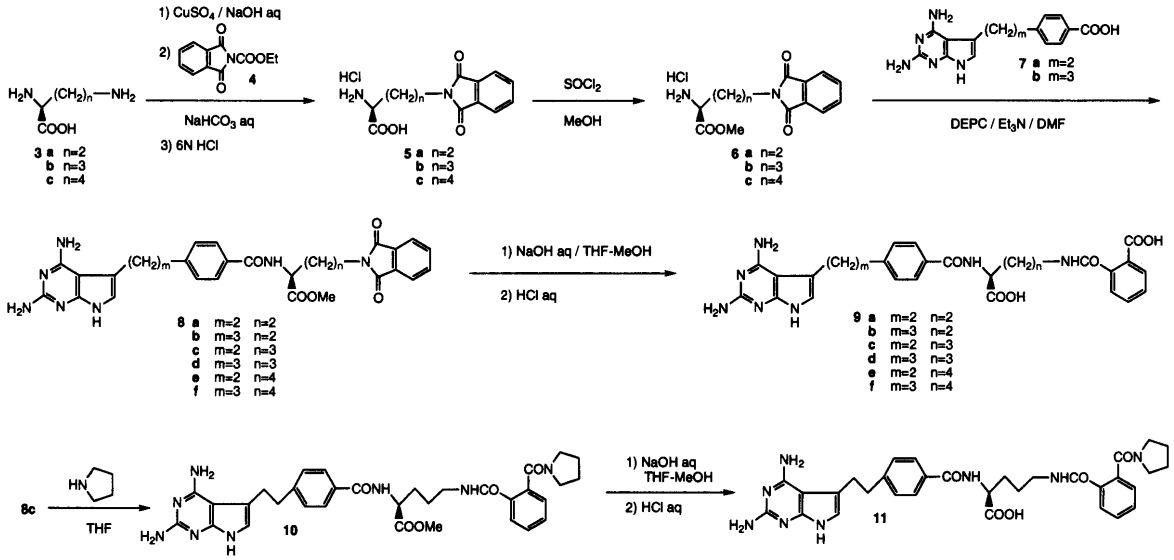
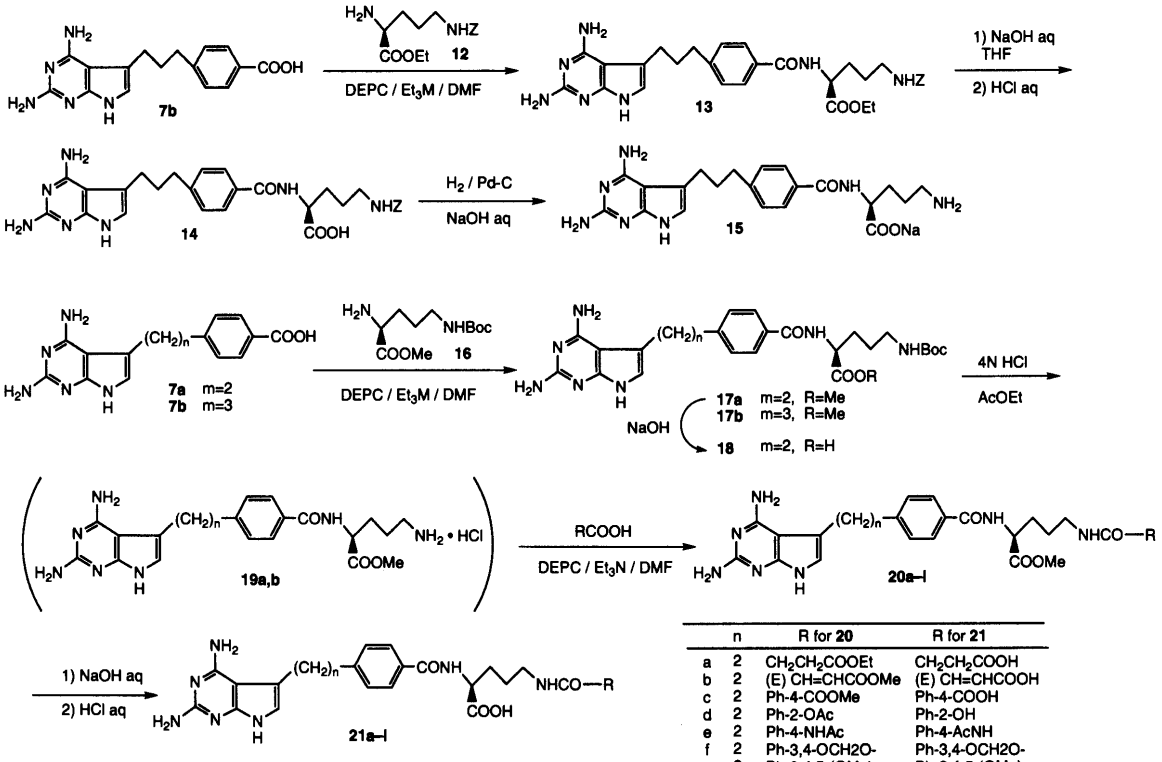


Chart 1

In the case of hemiphthaloyl ornithine analogs (**9**), the hemiphthaloyl group was prepared by hydrolysis of the phthaloyl group at the same time as hydrolysis of the methyl ester. N^{δ}-Phthaloyl ornithine methyl ester (**6b**) was prepared from ornithine (**3b**) by a common method, as shown in Chart 1. The α-amino and carboxyl groups of ornithine were blocked by forming a chelate with Cu^{2+} ion and phthaloylation of the ω-amino group was carried out using *N*-ethoxycarbonylphthalimide (Nefkens' reagent: **4**).[11] Removal of Cu was performed by treatment with 6 N HCl and the resulting compound **5b** was esterified with dimethyl sulfite prepared from thionyl chloride and methanol to give **6b**. The N^{ω}-phthaloyl methyl esters of lysine and 2,4-diaminobutyric acid (**6c**, **6a**) were also prepared by the same procedure. Condensation of **7a**, **b** and **6a**—**c** in the presence of diethyl phosphorocyanidate (DEPC) and triethylamine in *N*,*N*-dimethylformamide (DMF) gave the corresponding antifolates containing N^{ω}-phthaloyl methyl esters (**8a**—**f**). Alkaline hydrolysis of the esters **8a**—**f** afforded antifolates with N^{ω}-hemiphthaloyl 2,4-diaminobutyric acid, ornithine or lysine (**9a**—**f**). Ring-opening of the phthaloyl group was also carried out with pyrrolidine instead of aqueous sodium hydroxide. Treatment of **8c** with pyrrolidine in tetrahydrofuran (THF) at room temperature for 36 h gave **10** in 85% yield. Hydrolysis of **10** with 1 eq of aqueous sodium hydroxide in MeOH provided **11** in 68% yield.

N^{δ}-Z-Ornithine and free ornithine analogs (**14**, **15**) were synthesized as shown in Chart 2. N^{δ}-Z-Ornithine ethyl ester (**12**) was prepared from ornithine by protection of the α-amino and carboxyl groups using chelation with Cu^{2+} ion as described above, followed by introduction of a Z-group at the δ-amino group and subsequent esterification with 10% HCl in EtOH. Antifolate N^{δ}-Z-ornithine ethyl ester (**13**) was synthesized by coupling **7b** and **12** in the presence of DEPC and triethylamine in DMF. Alkaline hydrolysis of **13** gave an antifolate with N^{δ}-Z-ornithine (**14**) in 89% yield, and the catalytic hydrogenation of **14** over palladium carbon in aqueous NaOH solution afforded the free ornithine-containing antifolate (**15**) in 99% yield.

Antifolates with N^{δ}-Boc-ornithine methyl ester (**17a**, **b**), key intermediates for preparing various N^{δ}-acylornithine analogs **21**, were also synthesized by condensation of **7a**, **b** and N^{δ}-Boc-ornithine methyl ester (**16**) using DEPC. Compound **16** was prepared from commercially available N^{α}-Z-N^{δ}-Boc-ornithine by esterification with diazomethane and removal of the Z group by catalytic hydrogenation over palladium carbon. Alkaline hydrolysis of **17a** gave N^{δ}-Boc-ornithine analog (**18**). Most methyl N^{ω}-acylornithinate-containing antifolates (**20**) were prepared by acylation of free amino ornithine antifolate methyl esters (**19**), key intermedi-



	n	R for **20**	R for **21**
a	2	CH_2CH_2COOEt	CH_2CH_2COOH
b	2	(E) CH=CHCOOMe	(E) CH=CHCOOH
c	2	Ph-4-COOMe	Ph-4-COOH
d	2	Ph-2-OAc	Ph-2-OH
e	2	Ph-4-NHAc	Ph-4-AcNH
f	2	Ph-3,4-OCH2O-	Ph-3,4-OCH2O-
g	2	Ph-3,4,5-$(OMe)_3$	Ph-3,4,5-$(OMe)_3$
h	2	Ph-2,6-Me_2	Ph-2,6-Me_2
i	2	5-Py-2-COOMe	5-Py-2-COOH
j	2	1-Napht	1-Napht
k	2	2-Napht-3-COO^{t}Bu	2-Napht-3-COOH
l	3	2-Napht-3-COO^{t}Bu	2-Napht-3-COOH

Chart 2

ates derived from **17** by removal of the Boc-protecting group in the presence of HCl in AcOEt, using DEPC as a condensation reagent in the presence of triethylamine in DMF. The resulting esters (**20**) were transformed to the desired N^{ω}-acylornithine-containing antifolates (**21**) by alkaline hydrolysis. N^{ω}-Sulfonyl or carbamoyl ornithine-containing antifolates (**23**, **25**) were also prepared from **19** by sulfonylation or reaction with isocyanate and hydrolysis.

In the case of carbamoyl as a masking group for the ω-amino group of the ornithine, the length of the side chain of amino acid seemed to be rather long. Therefore, shorter side chain compounds **30a**—**c** were synthesized. The amino acid moieties **28a**—**c** were synthesized from Boc-L-Glu-OMe (**26b**) and Boc-L-Asp-OMe (**26a**) by adding the intermediate isocyanate, prepared by Curtius reaction using diphenyl phosphoryl azide (DPPA), with anilines and subsequent deprotection of the Boc group. N^{δ}-Alkyl and aryl substituted ornithine analogs (**36a**—**c**) were also synthesized. Compound **36c**, as a non-glutamate type γ-diglutamate, was expected to show potent activity. The amino acid moieties **34a**, **b** were synthesized from **26b**. Coupling of **26b** with ethyl chloroformate in the presence of triethylamine in THF gave a mixed acid anhydride, which was converted to alcohol **31** by reduction using $NaBH_4$. Mesylation of **31** gave the mesylated analog, which was transformed to a more reactive iodide **32** using NaI in acetone. Reaction of **32** with aryl or alkyl amines and subsequent deprotection of the Boc group yielded the amino acids **34a**, **b**. (Chart 3)

Biological Activity and Discussion Inhibitory activities of the synthesized antifolates (**9**, **11**, **14**, **15**, **18**, **21**, **23**, **25**, **30**, **36**) against DHFR and murine fibrosarcoma Meth A cell growth were examined and compared with those of TNP-351 (**1b**), the parent compound with a glutamic acid residue (Table 2). Inhibition of DHFR is thought to be the primary mode of action of these compounds for three main reasons; (1) they have two amino groups on the pyrimidine ring,[3b] (2) they can not be converted to polyglutamates because antifolates with acylornithine are not substrates for folylpolyglutamate synthetase (FPGS),[12] and (3) all are weak inhibitors of thymidylate synthase, a representative folate related enzyme, with IC_{50} of $>10\,\mu M$ (data not shown).

A free ornithine analog **15** was found to be a moderate inhibitor of DHFR comparable to TNP-351, while **15** showed much less inhibitory activity against Meth A cell growth. These results are consistent with previous observations[13] that antifolates with a positively charged δ-free amino ornithine are not efficiently transported *via* the RFC, which is known as an anion exchange transporter.[14] On the other hand, all prepared N^{ω}-masked ornithine analogs (**9**, **11**, **14**, **18**, **21**, **23**, **25**, **30**, **36**) were found to be much more potent DHFR inhibitors, and strongly inhibited the growth of Meth A cells. These findings suggest that N^{ω}-masked ornithine analogs could be efficiently transported into target cells *via* the RFC because they have no positive charge on the amino acid moiety, and that they should strongly inhibit DHFR in the cells. With regards to the length of the spacer between the pyrrolo-[2,3-*d*]pyrimidine ring and the benzene ring, ethylene analogs (**9a**, **c**, **e**, **21k**) more potently inhibited DHFR and Meth A cell growth than trimethylene analogs (**9b**, **d**, **f**, **21l**). In the case of hemiphthaloyl ornithine analogs (**9a**—**f**), we

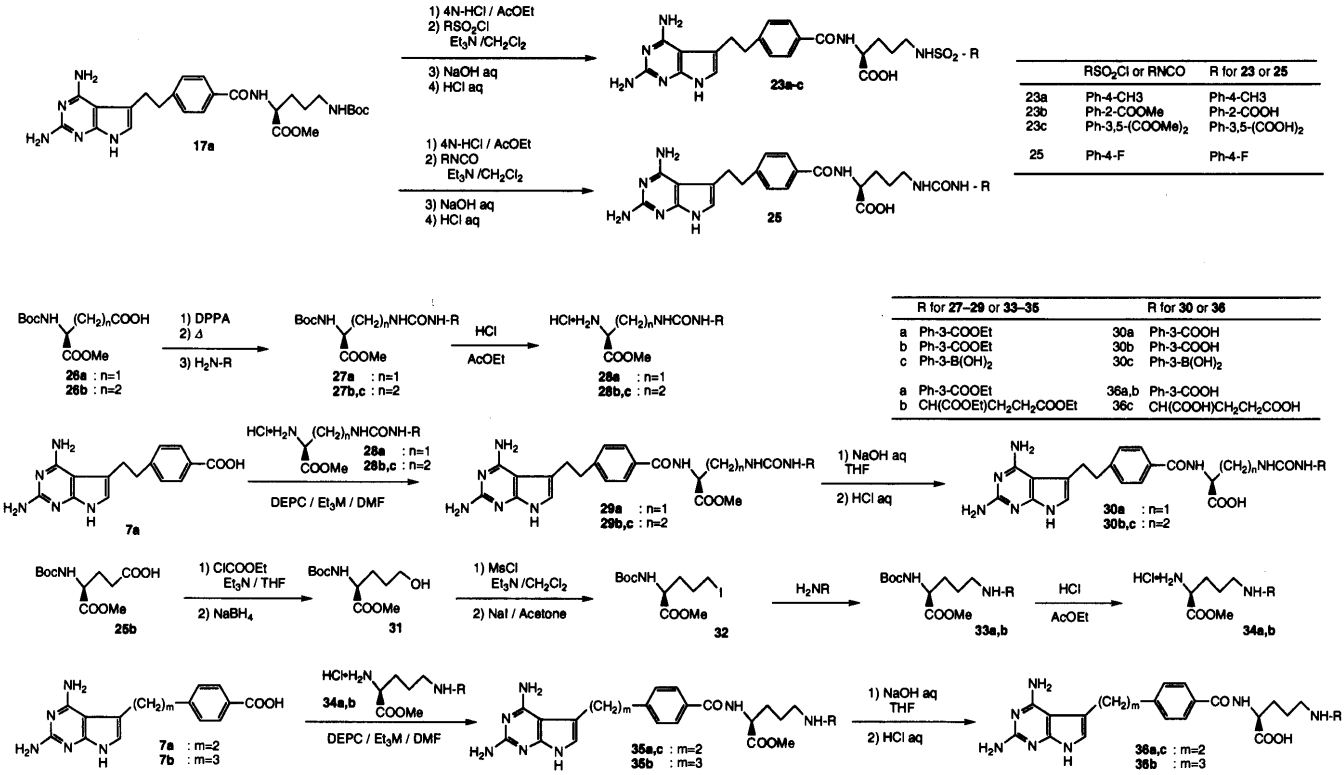


Chart 3

have synthesized compounds with two to four methylenes in the side chain in order to determine the optimal length of the amino acid side chain. Trimethylene compounds (**9c, d**) were found to show more potent activities against DHFR than ethylene or tetramethylene compounds (**9a, b, e, f**). Regarding masking groups for the ω-amino group of amino acids, carboxamide types (**9, 11, 21**) were found to have better activity than carbamate (**14, 18**), sulfonamide (**23**) and urea types (**25**). Among them, we noted the tendency that antifolates with an acidic group such as a carboxylic acid or a phenol on the acyl group of the ornithine show tight binding to DHFR and more potent growth inhibitory activity. These acidic functional groups may play important roles in efficient transport *via* the RFC, anion exchange transporter, and binding to the polyglutamate region of DHFR. Surprisingly, the aryl substituted ornithine analog (**36a**), but not the alkyl substituted analog (**36c**), was found to show very strong Meth A growth-inhibitory activity, even though the free ornithine analog was not strongly active due to poor uptake. This suggests that the RFC may allow transportation of ornithine analogs with low basicity and high lipophilicity such as aniline type compounds. In particular, analogs (**9a, c, 21k**) having ortho-carboxy aryl on the masking group showed the best inhibitory activities against Meth A cell growth. Antifolates containing an acylated ornithine with naphthalene or pyridine as an acyl group have never been prepared. In this study, 3-carboxy-2-naphthoyl ornithine analog (**21k**) was found to be twice as potent against Meth A cells as the corresponding hemiphthaloyl analog (**9c**).

As shown in Table 3, the inhibitory effects of some typical compounds (**9c, 21k, 23b, 36a**) on the growth of MTX-sensitive and resistant CCRF-CEM cells in culture were examined in comparison with MTX in order to confirm whether the prepared antifolates were active against MTX-resistant tumor cells. The MTX-resistant cell lines used were CCRF-CEM R_1,[15] CCRF-CEM R_{BO}[16] and CCRF-CEM $R_{30/6}$[17] which were resistant to MTX due to an increase of DHFR level, defective transport through the RFC and decreased polyglutamylation by FPGS, respectively. All MTX-resistant CCRF-CEM cells were much less resistant to the non-glutamate antifolates compared with MTX. In particular, the *o*-carboxyaroyl analogs (**9c, 21k**) exhibited little resistance against CCRF-CEM R_{BO} cells. These findings are consistent with previous studies showing that non-glutamate derivatives of MTX, such as PT523, showed less resistance to MTX-resistant cells with defective transport than MTX, and showed that a non-polyglutamylatable antifolate may have effective potency against MTX-resistant tumors with decreased polyglutamylation.[12]

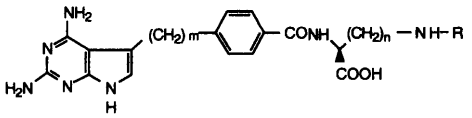
In our previous report we suggested that it might be possible to obtain novel DHFR inhibitors by replacing the γ-carboxyl group with other appropriate groups while leaving the α-carboxyl group intact. In this work, we could obtain novel potent DHFR inhibitors by designing non-glutamate type antifolates with a N^{ω}-masked ornithine analog in place of glutamic acid.

Experimental

Melting points were determined on Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were taken on JASCO IR-810 spectrometer. ^{1}H-NMR spectra were recorded on Varian Gemini-200 spectrometers; chemical shifts are given in ppm with tetramethylsilane as the internal standard and coupling constants (*J*) are measured in hertz (Hz). Secondary ion mass spectra (SI-MS) were determined by Takeda Analytical Laboratories (Osaka, Japan) on a Hitachi M-80B instrument. Column chromatography was carried out on Silica gel 60 (E. Merck, Darmstadt, Germany).

N^{δ}-Phthaloyl-L-ornithine Hydrochloride (2a) A solution of $CuSO_4$·

Table 2. *In Vitro* Activity of Novel Antifolates



Compd.	*m*	*n*	R	IC_{50} (μM)	
				DHFR	Meth A
18	2	3	COO^{t}Bu	0.022	0.055
14	3	3	COOBzl	0.30	0.018
15	3	3	H	0.1	0.12
21a	2	3	$COCH_2CH_2COOH$	0.0086	0.0047
21b	2	3	COCH=CHCOOH (*E*)	0.0083	0.0031
9a	2	2	COPh-2-COOH	0.0097	0.00094
9b	3	2	COPh-2-COOH	0.047	0.0080
9c	2	3	COPh-2-COOH	0.0069	0.0015
9d	3	3	COPh-2-COOH	0.025	0.0051
9e	2	4	COPh-2-COOH	0.0075	0.0065
9f	3	4	COPh-2-COOH	0.030	0.0090
11	2	3	$COPh\text{-}2\text{-}CONC_4H_8$	0.012	0.0013
21c	2	3	COPh-4-COOH	0.0051	0.0035
21d	2	3	COPh-2-OH	0.0099	0.0016
21e	2	3	COPh-4-NHAc	0.0077	0.0065
21f	2	3	$COPh\text{-}3,4\text{-}OCH_2O\text{-}$	0.0070	0.0014
21g	2	3	$COPh\text{-}3,4,5\text{-}(OMe)_3$	0.017	0.0043
21h	2	3	$COPh\text{-}2,6\text{-}Me_2$	0.0094	0.0028
21i	2	3	CO-5-Py-2-COOH	0.0059	0.015
21j	2	3	CO-1-Napht	0.015	0.0036
21k	2	3	CO-2-Napht-3-COOH	0.0075	0.00064
21l	3	3	CO-2-Napht-3-COOH	0.012	0.0039
23a	2	3	$SO_2Ph\text{-}4\text{-}Me$	0.032	0.019
23b	2	3	$SO_2Ph\text{-}2\text{-}COOH$	0.011	0.0030
23c	2	3	$SO_2Ph\text{-}3,5\text{-}(COOH)_2$	0.0070	0.088
25	2	3	CONHPh-4-F	0.013	0.0053
30a	2	1	CONHPh-3-COOH	0.015	0.0022
30b	2	2	CONHPh-3-COOH	0.0085	0.0026
30c	2	2	$CONHPh\text{-}3\text{-}B(OH)_2$	0.012	0.0038
36a	2	3	Ph-3-COOH	0.0062	0.00087
36b	3	3	Ph-3-COOH	0.019	0.0070
36c	2	3	$CH(COOH)CH_2CH_2COOH$	0.0094	0.033
1b	TNP-351			0.37	0.0006

Table 3. Inhibitory Effects of N^{ω}-Substituted Ornithine-Containing Antifolates on MTX-Reistant CCRF-CEM Cell Lines

Compd.	IC_{50} (μM)			
	S (wild)	R_1 (DHFR ↑)	R_{BO} (transport ↓)	$R_{30/6}$ (FPGS ↓)
		Resistance[a]		
9c	0.0075	0.11	0.0094	0.00091
		14.7	1.25	0.12
21k	0.0090	0.21	0.011	0.0014
		23.3	1.22	0.16
23b	0.057	0.60	0.27	0.011
		10.5	4.74	0.19
36a	0.0074	0.12	0.046	0.00089
		16.2	6.2	0.12
MTX	0.010	0.46	2.0	0.0096
		46.0	200.0	0.96

Cells (5×10^4/ml) were incubated for 72 h with various concentrations of the indicated test compounds and the cell density was determined with a Coulter counter. *a*) (IC_{50} value for resistant cell lines)/(IC_{50} value for parent cell line).

$5H_2O$ (3.75 g) in H_2O (40 ml) was added to a solution of L-ornithine (**1a**) (5.0 g) and NaOH (2.4 g) in H_2O (45 ml). $NaHCO_3$ (3.0 g) and *N*-ethoxycarbonylphthalimide (7.5 g) were added and the mixture was stirred for 20 h. The resulting precipitate was collected by filtration, washed with 5 N HCl and dried *in vacuo*. The crude **2a** was purified by recrystallization from MeOH–AcOEt to give **2a** (7.08 g, 79%) as colorless needles.

N^{ε}-Phthaloyl-L-lysine Hydrochloride (2b) Compound **2b** (6.01 g, 70%) as colorless needles was prepared from L-lysine hydrochloride (**1b**) (5.0 g) by the same method as that described for **2a**.

(*S*)-2-Amino-4-phthaloylaminobutyric Acid Hydrochloride (2c) Compound **2c** (4.44 g, 59%) as colorless needles was prepared from L-2,4-diaminobutyric acid hydrochloride (**1c**) (5.0 g) by the same method as that described for **2a**.

Methyl N^{δ}-Phthaloyl-L-ornithinate Hydrochloride (3a) Thionyl chloride (2.6 ml) was added dropwise to MeOH (15 ml) at −10 °C and the mixture was stirred for 10 min. **2a** (3.0 g) was added to the mixture and the reaction mixture was stirred at room temperature for 4 d and concentrated *in vacuo*. The residue was triturated with Et_2O, collected by filtration and dried *in vacuo* to give **3a** (3.09 g, 99%) as colorless needles. ^{1}H-NMR dimethyl sulfoxide (DMSO-d_6) δ: 1.60—1.90 (4H, m), 3.60 (2H, m), 3.73 (3H, s), 4.03 (1H, m), 7.80—7.90 (4H, m), 8.51 (3H, br s).

Methyl N^{ε}-Phthaloyl-L-lysinate Hydrochloride (3b) Compound **3b** (3.23 g, 100%) as colorless needles was synthesized from **2b** (2.76 g) by the same method as that described for **3a**. ^{1}H-NMR (DMSO-d_6) δ: 1.20—1.55 (2H, m), 1.61 (2H, m), 1.81 (2H, m), 3.58 (2H, t, J=6.8 Hz), 3.73 (3H, s), 4.01 (1H, m), 7.80—7.95 (4H, m), 8.48 (3H, br s).

Methyl (*S*)-2-Amino-4-phthaloylaminobutyrate Hydrochloride (3c) Compound **3c** (2.83 g, 95%) as colorless needles was synthesized from **2c** (2.83 g) by the same method as that described for **3a**.

General Procedure for Preparation of Antifolates with N^{ω}-Phthaloyl-L-Diamino Acid (8) Et_3N (3.5 mmol) was added dropwise to a stirred solution of 4-[ω-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)alkyl]benzoic acid (**7a** or **7b**) (1 mmol), N^{ω}-phthaloyldiamino acid methyl ester **6** (1.1 mmol) and DEPC (1.5 mmol) in dry DMF (15 ml) under ice cooling. The reaction mixture was stirred at room temperature for 4 h, then concentrated *in vacuo*, and the residue was dissolved in AcOEt (100 ml). The AcOEt solution was successively washed with H_2O and brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$: 1% NH_3 in EtOH, 20 : 1→10 : 1) to give **8**.

The following compounds **8a**—**f** were prepared.

Methyl 2(*S*)-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoyl]amino-4-phthaloylaminobutyrate (**8a**): Yield, 99%; pale green solid. IR (KBr): 3370, 3200, 2950, 1770, 1735, 1710, 1660, 1610, 1575, 1545, 1500, 1430, 1400, 1200, 1185, 720 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.00—2.35 (2H, m), 2.97 (4H, m), 3.61 (3H, s), 3.72 (2H, q, J=6.4 Hz), 4.44 (1H, m), 5.57 (2H, br s), 6.20 (2H, br s), 6.41 (1H, s), 7.32 (2H, d, J=8.2 Hz), 7.75 (2H, d, J=8.2 Hz), 7.83 (4H, s), 8.74 (1H, d, J=7.6 Hz), 10.49 (1H, br s).

Methyl 2(*S*)-[4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]-benzoyl]amino-4-phthaloylaminobutyrate (**8b**): Yield, 60%; pale green solid. IR (KBr): 3380, 2970, 1740, 1710, 1610, 1570, 1540, 1435, 1395, 720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.00 (2H, m), 2.40 (2H, m), 2.65 (2H, t, J=7.8 Hz), 2.76 (2H, t, J=7.2 Hz), 3.54 (3H, s), 3.87 (2H, m), 4.63 (2H, br s), 4.85—5.00 (3H, m), 6.49 (1H, s), 7.27 (2H, d, J=8.2 Hz), 7.45 (1H, d, J=8.2 Hz), 7.72 (2H, dd, J=5.6, 3.2 Hz), 7.85 (2H, dd, J=5.6, 3.2 Hz), 7.86 (2H, d, J=8.2 Hz), 8.54 (1H, br s). SI-MS *m/z*: 556 (MH^+).

Methyl N^{α}-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoyl]-N^{δ}-phthaloyl-L-ornithinate (**8c**): Yield, 99%; pale green solid. IR (KBr): 3370, 1735, 1710, 1610 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.70—2.10 (4H, m), 3.01 (4H, br s), 3.74 (2H, m), 3.78 (3H, s), 4.70 (2H, br s), 4.88 (1H, m), 5.08 (2H, br s), 6.41 (1H, s), 6.80 (1H, d, J=7.0 Hz), 7.22 (2H, d, J=8.2 Hz), 7.65—7.80 (4H, m), 7.84 (2H, dd, J=5.4, 3.2 Hz), 8.25 (1H, br s). SIMS *m/z*: 556 (MH^+).

Methyl N^{α}-[4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]-benzoyl]-N^{δ}-phthaloyl-L-ornithinate (**8d**): Yield, 74%; pale green solid. IR (KBr): 3390, 1735, 1710, 1610 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.70—2.10 (6H, m), 2.50—2.75 (4H, m), 3.72 (2H, m), 3.76 (3H, s), 4.60 (2H, br s), 4.83 (1H, m), 5.10 (2H, br s), 6.45 (1H, s), 6.91 (1H, d, J=8.0 Hz), 7.17 (2H, d, J=8.0 Hz), 7.60—7.85 (6H, m), 9.13 (1H, br s). SI-MS *m/z*: 570 (MH^+).

Methyl N^{α}-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoyl]-N^{ε}-phthaloyl-L-lysinate (**8e**): Yield, 99%; pale green solid. IR (KBr): 3370, 3200, 2950, 1740, 1710, 1605, 1570, 1545, 1495, 1435, 1395, 1200, 750, 720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.20—1.55 (2H, m), 1.55—1.80 (2H, m), 1.80—2.05 (2H, m), 2.92 (4H, m), 3.66 (2H, t, J=6.2 Hz), 3.74

Table 4. Spectral Data of Novel Pyrrolo[2,3-*d*]pyrimidine Antifolates (**9**, **11**, **14**, **15**, **18** and **21**)

Compd.	IR (KBr): cm^{-1}	^{1}H-NMR (DMSO-d_6) δ (ppm): J (Hz)
9a	3330, 3200, 2920, 1640, 1540, 1380	1.75—2.20 (2H, m), 2.96 (4H, brs), 3.32 (2H, m), 4.51 (1H, m), 5.77 (2H, brs), 6.41 (3H, brs), 7.32 (2H, d, *J*=8.0 Hz), 7.35—7.55 (3H, m), 7.70—7.85 (3H, m), 8.31 (1H, m), 8.53 (1H, d, *J*=7.4 Hz), 10.59 (1H, brs).
9b	3325, 3200, 2930, 1640, 1545, 1380	1.75—2.20 (4H, m), 2.70 (4H, m), 3.28 (2H, m), 4.49 (1H, m), 5.84 (2H, brs), 6.41 (2H, brs), 6.51 (1H, s), 7.29 (2H, d, *J*=8.2 Hz), 7.30—7.55 (3H, m), 7.76 (1H, dd, *J*=7.0, 1.8 Hz), 7.81 (2H, d, *J*=8.2 Hz), 8.30 (1H, m), 8.53 (1H, d, *J*=7.2 Hz), 10.68 (1H, brs).
9c	3330, 3200, 2920, 1640, 1545	1.50—2.00 (4H, m), 2.95 (4H, m), 3.21 (2H, m), 4.40 (1H, m), 5.62 (2H, brs), 6.26 (2H, brs), 6.40 (1H, s), 7.31 (2H, d, *J*=8.2 Hz), 7.35—7.55 (3H, m), 7.73 (1H, dd, *J*=7.0, 1.8 Hz), 7.81 (2H, d, *J*=8.2 Hz), 8.31 (1H, t, *J*=8.2 Hz), 8.46 (1H, d, *J*=7.6 Hz), 10.50 (1H, brs).
9d	3330, 3200, 1660, 1640, 1540	1.60 (2H, m), 1.83 (4H, m), 2.50—2.75 (4H, m), 3.30 (2H, m), 4.37 (1H, m), 5.60 (2H, brs), 6.21 (2H, brs), 6.47 (1H, s), 7.29 (2H, d, *J*=8.2 Hz), 7.30—7.55 (3H, m), 7.73 (1H, dd, *J*=7.0, 1.8 Hz), 7.82 (2H, d, *J*=8.2 Hz), 8.32 (1H, m), 8.48 (1H, d, *J*=8.2 Hz), 10.54 (1H, brs).
9e	3330, 3200, 2920, 1640, 1540	1.35—1.60 (4H, m), 1.75—1.90 (2H, m), 2.94 (4H, m), 3.18 (2H, m), 4.34 (1H, m), 5.97 (2H, brs), 6.45 (1H, s), 6.64 (2H, brs), 7.30 (2H, d, *J*=8.2(1H,), 7.35 (1H, m), 7.40—7.55 (2H, m), 7.75 (1H, m), 7.79 (2H, d, *J*=8.2 Hz), 8.27 (1H, t, *J*=5.4 Hz), 8.44 (1H, d, *J*=7.8 Hz), 10.73 (1H, brs).
9f	3330, 3200, 2930, 1640, 1540, 1380	1.35—1.60 (4H, m), 1.70—1.95 (4H, m), 2.60—2.80 (4H, m), 3.20 (2H, m), 4.35 (1H, m), 5.50 (2H, brs), 6.08 (2H, brs), 6.45 (1H, s), 7.27 (2H, d, *J*=8.2 Hz), 7.36 (1H, m), 7.46 (2H, m), 7.73 (1H, dd, *J*=7.0, 2.6 Hz), 7.80 (2H, d, *J*=8.2 Hz), 8.34 (1H, m), 8.44 (1H, d, *J*=7.6 Hz), 10.47 (1H, brs).
11	3330, 1610, 1570, 1540, 1490, 1450,	1.50—2.00 (8H, m), 2.97 (4H, brs), 3.09 (2H, t, *J*=6.4 Hz), 3.22 (2H, m), 3.38 (2H, m), 4.39 (1H, m), 5.63 (2H, brs), 6.28 (2H, brs), 6.40 (1H, s), 7.32 (2H, d, *J*=8.2 Hz), 7.25—7.65 (4H, m), 7.82 (2H, d, *J*=8.2 Hz), 8.36 (1H, t, *J*=5.0 Hz), 8.51 (1H, d, *J*=7.8 Hz), 10.52 (1H, brs).
14	3340, 3200, 2940, 1710, 1650, 1635, 1540, 1500, 1450, 1400, 1340, 1250	1.40—1.60 (2H, m), 1.70—1.95 (4H, m), 2.60—2.80 (4H, m), 3.02 (2H, q, *J*=6.0 Hz), 4.34 (1H, m), 5.00 (2H, s), 5.40 (2H, brs), 5.98 (2H, s), 6.42 (1H, d, *J*=1.8 Hz), 7.29 (2H, d, *J*=8.2 Hz), 7.33 (5H, s), 7.80 (2H, d, *J*=8.2 Hz), 8.46 (1H, d, *J*=8.0 Hz), 10.41 (1H, s).
15	3400, 3200, 2940, 1640, 1600, 1540, 1495, 1450, 1380, 1335, 1240	1.30—1.55 (2H, m), 1.60—1.90 (4H, m), 2.55—2.95 (6H, m), 3.99 (1H, m), 5.36 (2H, s), 5.90 (2H, brs), 6.42 (1H, s), 7.27 (2H, d, *J*=8.0 Hz), 7.71 (2H, d, *J*=8.0 Hz), 7.90 (1H, d, *J*=6.6 Hz), 10.40 (1H, brs).
18	3335, 3200, 2930, 1705, 1645, 1620, 1540, 1500, 1460, 1400, 1330, 1255	1.37 (9H, s), 1.40—1.90 (4H, m), 2.90—3.10 (6H, m), 4.34 (1H, m), 6.31 (2H, brs), 6.51 (1H, s), 6.80 (1H, t, *J*=7.0 Hz), 6.95 (2H, brs), 7.33 (2H, d, *J*=8.2 Hz), 7.81 (2H, d, *J*=8.2 Hz), 8.48 (1H, d, *J*=7.8 Hz), 10.94 (1H, brs).
21a	3330, 3200, 2930, 1640, 1545, 1500, 1455, 1400	1.40—1.90 (4H, m), 2.20—2.50 (4H, m), 2.96 (4H, brs), 3.05 (2H, m), 4.34 (1H, m), 5.58 (2H, brs), 6.22 (2H, brs), 6.39 (1H, s), 7.33 (2H, d, *J*=8.2 Hz), 7.80 (2H, d, *J*=8.2 Hz), 7.86 (1H, t, *J*=7.6 Hz), 8.49 (1H, d, *J*=7.4 Hz), 10.48 (1H, brs).
21b	3325, 3200, 2925, 1645, 1620, 1565, 1540, 1500, 1460, 1390, 1330, 1190, 1090	1.40—1.90 (4H, m), 2.96 (4H, brs), 3.16 (2H, m), 4.32 (1H, m), 5.48 (2H, brs), 6.12 (2H, brs), 6.37 (1H, s), 6.51 (1H, d, *J*=15.4 Hz), 6.83 (1H, d, *J*=15.4 Hz), 7.32 (2H, d, *J*=8.0 Hz), 7.78 (2H, d, *J*=8.0 Hz), 8.38 (1H, d, *J*=7.4 Hz), 8.47 (1H, m), 10.42 (1H, brs).
21c	3330, 3200, 2930, 1640, 1570, 1540, 1500, 1455, 1385, 1290, 1190	1.60—2.00 (4H, m), 2.96 (4H, brs), 3.30 (2H, m),4.41 (1H, m), 5.45 (2H, brs), 6.09 (2H, brs), 6.37 (1H, s), 7.33 (2H, d, *J*=8.2 Hz), 7.80 (2H, d, *J*=8.2 Hz), 7.92 (2H, d, *J*=8.6 Hz), 8.00 (2H, d, *J*=8.6 Hz), 8.51 (1H, d, *J*=8.0 Hz), 8.66 (1H, t, *J*=5.8 Hz), 10.41 (1H, brs).
21d	3340, 3200, 2930, 1640, 1595, 1540, 1490, 1450, 1390, 1300, 1250	1.50—1.95 (4H, m), 2.96 (4H, brs), 3.33 (2H, m), 4.41 (1H, m), 5.73 (2H, brs), 6.37 (2H, brs), 6.41 (1H, s), 6.80—6.95 (2H, m), 7.32 (2H, d, *J*=8.0 Hz), 7.39 (1H, m), 7.80 (2H, d, *J*=8.0 Hz), 7.84 (1H, m), 8.53 (1H, d, *J*=7.8 Hz), 8.84 (1H, m), 10.57 (1H, brs).
21e	3320, 3200, 2930, 1640, 1530, 1500, 1460, 1400, 1370, 1315, 1260, 1180	1.45—1.95 (4H, m), 2.06 (3H, s), 2.96 (4H, brs), 3.27 (2H, m), 4.39 (1H, m), 5.83 (2H, brs), 6.43 (1H, s), 6.50 (2H, brs), 7.33 (2H, d, *J*=8.0 Hz), 7.63 (2H, d, *J*=8.4 Hz), 7.79 (2H, d, *J*=8.4 Hz), 7.81 (2H, d, *J*=8.0 Hz), 8.36 (1H, m), 8.53 (1H, d, *J*=7.8 Hz), 10.16 (1H, s), 10.65 (1H, brs).
21f	3340, 3200, 2930, 1640, 1540, 1500, 1485, 1440, 1400, 1360, 1300, 1260, 1040	1.45—1.95 (4H, m), 2.96 (4H, brs), 3.25 (2H, m), 4.39 (1H, m), 5.83 (2H, brs), 6.09 (2H, s), 6.43 (1H, s), 6.47 (2H, brs), 6.97 (1H, d, *J*=8.4 Hz), 7.33 (2H, d, *J*=8.0 Hz), 7.38 (1H, s), 7.43 (1H, d, *J*=8.4 Hz), 7.81 (2H, d, *J*=8.0 Hz), 8.33 (1H, brt, *J*=5.2 Hz), 8.53 (1H, d, *J*=7.6 Hz), 10.63 (1H, brs).
21g	3340, 3200, 2940, 1640, 1590, 1540, 1460, 1410, 1335, 1235, 1185, 1125, 1000	1.50—2.00 (4H, m), 2.95 (4H, brs), 3.29 (2H, m), 3.69 (3H, s), 3.81 (6H, s), 4.38 (1H, m), 5.45 (2H, brs), 6.08 (2H, brs), 6.36 (1H, s), 7.17 (2H, s), 7.32 (2H, d, *J*=8.2 Hz), 7.80 (2H, d, *J*=8.2 Hz), 8.40—8.55 (2H, m), 10.40 (1H, brs).
21h	3330, 3200, 2925, 1640, 1600, 1560, 1540, 1500, 1460, 1400, 1300	1.50—2.00 (4H, m), 2.19 (6H, s), 2.96 (4H, brs), 4.38 (1H, m), 5.45 (2H, brs), 6.07 (2H, brs), 6.36 (1H, s), 7 .01 (2H, d, *J*=7.8 Hz), 7.16 (1H, dd, *J*=8.7, 6.4 Hz), 7.33 (2H, d, *J*=8.0 Hz), 7.79 (2H, d, *J*=8.0 Hz), 8.29 (1H, t, *J*=5.0 Hz), 8.51 (1H, d, *J*=7.7 Hz), 10.40 (1H, brs).
21i	3330, 3200, 2930, 1640, 1540, 1500, 1460, 1390, 1315, 1285, 1250, 1190	1.45—1.95 (4H, m), 2.96 (4H, brs), 3.34 (2H, m), 4.40 (1H, m), 5.63 (2H, brs), 6.37 (2H, brs), 6.40 (1H, s), 7.33 (2H, d, *J*=7.8 Hz), 7.81 (2H, d, *J*=7.8 Hz), 8.10 (1H, d, *J*=8.0 Hz), 8.31 (1H, d, *J*=8.0 Hz), 8.52 (1H, d, *J*=7.6 Hz), 8.83 (1H, m), 9.06 (1H, s), 10.55 (1H, brs).
21j	3370, 3200, 2930, 1640, 1590, 1545, 1500, 1400, 1300	1.60—2.05 (4H, m), 2.96 (4H, brs), 3.35 (2H, m), 4.44 (1H, m), 5.58 (2H, brs), 6.21 (2H, brs), 6.38 (1H, s), 7.33 (2H, d, *J*=8.0 Hz), 7.50—7.60 (4H, m), 7.81 (2H, d, *J*=8.0 Hz), 7.90—8.05 (2H, m), 8.17 (1H, m), 8.50—8.60 (2H, m), 10.48 (1H, brs).
21k	3350, 3220, 2930, 1705, 1650, 1540, 1500, 1460, 1380, 1300	1.60—2.05 (4H, m), 2.96 (4H, brs), 3.35 (2H, m), 4.43 (1H, m), 5.77 (2H, brs), 6.42 (3H, brs), 7.32 (2H, d, *J*=8.0 Hz), 7.60—7.70 (2H, m), 7.83 (2H, d, *J*=8.0 Hz), 7.97 (1H, s), 7.95—8.15 (2H, m), 8.36 (1H, s), 8.40—8.60 (2H, m), 10.62 (1H, brs).
21l	3370, 3210, 2930, 1705, 1650, 1545, 1500, 1460, 1380, 1300	1.60—2.00 (6H, m), 2.69 (4H, brs), 3.30 (2H, m), 4.41 (1H, m), 6.09 (2H, brs), 6.55 (1H, s), 6.71 (2H, brs), 7.30 (2H, d, *J*=8.2 Hz), 7.60—7.75 (2H, m), 7.84 (2H, d, *J*=8.2 Hz), 7.97 (1H, s), 7.95—8.15 (2H, m), 8.37 (1H, s), 8.47 (1H, m), 8.53 (1H, d, *J*=8.6 Hz), 10.84 (1H, brs).

(3H, s), 4.74 (1H, m), 4.82 (2H, br s), 5.22 (2H, br s), 6.36 (1H, s), 6.91 (1H, d, J=8.0 Hz), 7.14 (2H, d, J=8.2 Hz), 7.60—7.80 (6H, m), 8.80 (1H, br s).

Methyl N^{α}-[4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]-benzoyl]-N^{ε}-phthaloyl-L-lysinate (**8f**): Yield, 60%; pale green solid. IR (KBr): 3370, 2930, 1740, 1710, 1610, 1575, 1400 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.20—1.80 (4H, m), 1.90—2.10 (4H, m), 2.60—2.80 (4H, m), 3.70 (2H, m), 3.78 (3H, s), 4.57 (2H, br s), 4.81 (1H, m), 4.88 (2H, br s), 6.50 (1H, s), 6.80 (1H, d, J=8.0 Hz), 7.27 (2H, d, J=8.2 Hz), 7.65—7.85 (6H, m), 8.25 (1H, br s). SI-MS *m/z*: 584 (MH^+).

N^{α}-[4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-N^{δ}-hemiphthaloyl-L-ornithine (9d). Typical Procedure 1 N NaOH aqueous solution (1 ml) was added to a stirred solution of **8d** (70 mg) in MeOH–THF (2 : 1, 3 ml) at room temperature. The reaction mixture was stirred for 3 h, concentrated *in vacuo* and dissolved in water, and 1 N HCl (1 ml) was added to the mixture. The resulting precipitate was collected by filtration, washed with H_2O and dried *in vacuo* to give **9d** (80 mg, 85%) as a colorless solid.

Compounds **9a**—**f** were prepared in a similar manner as described for the preparation of **9d**. Physical properties and spectral data for compounds **9a**—**f** are listed in Tables 1 and 4.

Methyl N^{α}-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoyl]-N^{δ}-(2-pyrrolidinocarbonylbenzoyl)-L-ornithinate (10) Pyrrolidine (56 mg) was added to a stirred solution of **8c** (124 mg) in THF (3 ml). The reaction mixture was stirred at room temperature for 36 h and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$: 1% NH_3 in EtOH, 15 : 1—5 : 1) to give **10** (119 mg, 85%) as a pale green solid. ^{1}H-NMR ($CDCl_3$) δ: 1.55—2.05 (8H, m), 2.84 (4H, m), 3.12 (2H, t, J=6.4 Hz), 3.36 (2H, q, J=6.0 Hz), 3.51 (2H, t, J=6.8 Hz), 3.70 (3H, s), 4.71 (1H, m), 5.10 (2H, br s), 5.33 (2H, br s), 6.29 (1H, s), 7.04 (2H, d, J=8.2 Hz), 7.10—7.50 (4H, m), 7.60—7.80 (4H, m), 9.35 (1H, br s).

N^{α}-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]amino-N^{δ}-(2-pyrrolidinocarbonylbenzoyl)-L-ornithine (11) 0.1 N NaOH aqueous solution (1.9 ml) was added to a stirred solution of **10** (119 mg) in MeOH (5 ml) at room temperature. The reaction mixture was stirred for 24 h, concentrated *in vacuo* and dissolved in H_2O, and 0.1 N HCl aqueous solution (1.9 ml) was added to the mixture. The resulting precipitate was collected by filtration, washed with H_2O and dried *in vacuo* to give **11** (79 mg, 68%) as a colorless solid. Physical properties and spectral data for **11** are listed in Tables 1 and 4.

Ethyl N^{α}-[4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]-benzoyl]-N^{δ}-benzyloxycarbonyl-L-ornithinate (13) Triethylamine (219 mg) was added dropwise to a stirred solution of **7b** (224 mg), ethyl N^{δ}-benzyloxycarbonyl-L-ornithinate (**12**) (250 mg) and DEPC (124 mg) in dry DMF (10 ml) under ice cooling. The reaction mixture was stirred at room temperature for 4 h, then concentrated *in vacuo*, and the residue was dissolved in AcOEt (50 ml). The AcOEt solution was successively washed with H_2O and brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$: 8% NH_3 in EtOH, 20 : 1) to give **13** (323 mg, 76%) as an off-white solid. IR (KBr): 3345, 2940, 2860, 1735, 1715, 1635, 1600, 1570, 1540, 1500, 1420, 1250, 1210, 1180, 1015 cm^{-1}. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.30 (3H, t, J=7.2 Hz), 1.70—2.10 (6H, m), 2.66 (2H, t, J=7.2 Hz), 2.76 (2H, t, J=7.4 Hz), 3.24 (2H, m), 4.24 (2H, q, J=7.2 Hz), 4.79 (1H, dd, J=6.8, 5.2 Hz), 5.09 (2H, s), 6.48 (1H, s), 7.26 (2H, d, J=8.4 Hz), 7.34 (5H, s), 7.75 (2H, d, J=8.4 Hz).

N^{α}-[4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-N^{δ}-benzyloxycarbonyl-L-ornithine (14) Compound **14** (223 mg, 89%) was synthesized from **13** (265 mg) by the same method as that described for **9a**. Physical properties and spectral data for **14** are listed in Tables 1 and 4.

Sodium N^{α}-[4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-propyl]benzoyl]-L-ornithinate (15) A solution of **14** (196 mg) in 0.1 N NaOH (3.3 ml) and MeOH–THF (1 : 1, 10 ml) was hydrogenated over 10% Pd–C (65 mg) for 12 h. After the catalyst was removed, the reaction mixture was concentrated *in vacuo*. The residue was dissolved in H_2O and lyophilized to give **15** (156 mg, 99%) as a colorless powder. Physical properties and spectral data for **15** are listed in Tables 1 and 4.

Methyl N^{δ}-(*tert*-Butoxycarbonyl)-N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-ornithinate (17a) Triethylamine (3.0 g) was added dropwise to a stirred solution of **7a** (1.53 g), methyl N^{δ}-(*tert*-butoxycarbonyl)-L-ornithinate (**16**; 1.29 g) and DEPC (1.40 g) in dry DMF (20 ml) under ice cooling. The reaction mixture was stirred for 1 h at room temperature then concentrated *in vacuo*, and the residue was dissolved in AcOEt (50 ml). The AcOEt solution was successively washed with H_2O and brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$: 8% NH_3 in EtOH, 20 : 1) to give **17a** (2.30 g, 85%) as a pale yellow amorphous solid. ^{1}H-NMR (DMSO-d_6) δ: 1.37 (9H, s), 1.40—1.60 (2H, m), 1.70—1.85 (2H, m), 2.90—3.15 (6H, m), 3.64 (3H, s), 4.40 (1H, m), 6.61 (1H, s), 6.81 (1H, t, J=7.0 Hz), 6.97 (2H, br s), 7.33 (2H, d, J=8.2 Hz), 7.63 (2H, br s), 7.80 (2H, d, J=8.2 Hz), 8.63 (1H, d, J=7.4 Hz), 11.35 (1H, br s).

Methyl N^{δ}-(*tert*-Butoxycarbonyl)-N^{α}-[4-[3-(2,4-diamino-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-L-ornithinate (17b) Compound **17b** (968 mg, 46%) as a pale yellow amorphous solid was prepared from **7b** (1.23 g) and **16** (968 mg) by the same method as that described for **17a**.

N^{δ}-(*tert*-Butoxycarbonyl)-N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-ornithine (18) Compound **18** (100 mg, 85%) was synthesized from **17a** (121 mg) by the same method as that described for **9a**. Physical properties and spectral data for **18** are listed in Tables 1 and 4.

Synthesis of N^{δ}-Acyl Ornithine Antifolates (21a—l). General Procedure A 4 N HCl solution of AcOEt (1 ml) was added to a stirred solution of **17** (0.5 mmol) in AcOEt (1 ml) at 0 °C, the reaction mixture was stirred at room temperature for 20 min and concentrated *in vacuo*. The residue was dissolved in DMF and carboxylic acid (0.55 mmol) and DEPC (0.60 mmol) were added to the mixture. After 10 min, Et_3N (1.5 mmol) was added at 0 °C. The reaction mixture was stirred at room temperature for 2—5 h and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$: 8% NH_3 in EtOH, 15 : 1) to give the methyl N^{δ}-acyl-L-ornithinate. The ester was dissolved in MeOH (6 ml), and 1 N NaOH (1 ml) was added. The mixture was stirred at room temperature for 5 h, concentrated *in vacuo* and dissolved in H_2O, and 1 N HCl (1 ml) was added. The resulting precipitate was collected by filtration, washed with H_2O, and dried *in vacuo* to give **21a—l** as a colorless solid. Physical properties and spectral data for compounds **21a—l** are listed in Tables 1 and 4.

N^{δ}-(2-Carboxybenzenesulfonyl)-N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-ornithine (23b), Typical Procedure A 4 N HCl solution of AcOEt (1.5 ml) was added to a solution of **17a** (200 mg) in THF (3 ml), and the reaction mixture was stirred for 10 min and concentrated *in vacuo*. The residue and 2-methoxycarbonylbenzenesulfonyl chloride (89 mg) were dissolved in dry DMF (4 ml) and Et_3N (1 ml) was added dropwise to the mixture at 0 °C. The reaction mixture was stirred at room temperature for 30 min and concentrated *in vacuo*. The residue was dissolved in $CHCl_3$, and the solution was washed with H_2O and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$: MeOH, 15 : 1) to give methyl N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{δ}-(2-methoxycarbonylbenzenesulfonyl)-L-ornithinate (**22b**, 130 mg; 55%) as a colorless solid. IR (KBr): 3400, 1730, 1610, 1580, 1550, 1500, 1435, 1330, 1300, 1160, 760 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.35—1.55 (2H, m), 1.65—1.90 (2H, m), 2.86 (2H, m), 2.96 (4H, br s), 3.62 (3H, s), 3.83 (3H, s), 4.34 (1H, m), 5.35 (2H, br s), 5.97 (2H, br s), 6.35 (1H, s), 7.33 (2H, d, J=8.0 Hz), 7.47 (1H, t, J=8.2 Hz), 7.60—7.90 (6H, m), 8.59 (1H, d, J=7.2 Hz), 10.34 (1H, br s). SI-MS *m/z*: 624 (MH^+).

1 N NaOH aqueous solution (1 ml) was added to a stirred solution of **22b** (120 mg) in MeOH (6 ml) at room temperature. The reaction mixture was stirred for 5 h and concentrated *in vacuo*. The residue was dissolved in H_2O, and 1 N HCl (1 ml) was added to the mixture. The resulting precipitate was collected by filtration, washed with H_2O and dried *in vacuo* to give **23b** (90 mg; 79%) as a colorless solid. IR (KBr): 3340, 3200, 2930, 1660, 1640, 1540, 1500, 1450, 1380, 1320, 1220, 1190, 1160, 1120, 1080, 1015, 760, 610 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.40—1.55 (2H, m), 1.60—1.85 (2H, m), 2.80 (2H, m), 2.95 (4H, m), 4.30 (1H, m), 6.40 (2H, br), 6.51 (1H, m), 6.84 (1H, br s), 7.03 (1H, br), 7.28 (2H, d, J=8.0 Hz), 7.40—7.90 (6H, m), 8.42 (1H, d, J=8.0 Hz), 11.03 (1H, br s).

N^{α}-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{δ}-(3,5-dicarboxybenzenesulfonyl)-L-ornithine (23c) Methyl N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{δ}-(3,5-dimethoxycarbonylbenzenesulfonyl)-L-ornithinate (**22c**, 45 mg, 29%) as a colorless solid was synthesized from **17a** (120 mg) and 3,5-dimethoxycarbonylbenzenesulfonyl chloride (67 mg) by the same method as that described for **22b**. IR (KBr): 3380, 3200, 2950, 1730, 1610, 1580, 1550, 1500, 1430, 1325, 1250, 1160, 750 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.30—1.55 (2H, m), 1.60—1.85 (2H, m), 2.77 (2H, m), 2.95 (4H, br s), 3.60 (3H, s), 3.93 (6H, s), 4.30 (1H, m), 5.35 (2H, s), 5.96 (2H, s), 6.35 (1H, s), 7.31 (2H, d, J=7.8 Hz), 7.72 (2H, d, J=7.8 Hz), 8.00 (1H, t, J=6.0 Hz), 8.52 (2H, s), 8.56 (1H, d, J=7.6 Hz), 8.60 (1H, s), 10.34 (1H, br s). SI-MS *m/z*: 682 (MH^+).

Compound **23c** (32 mg, 83%) was synthesized from **22c** (41 mg) by the

same method as that described for **23b**. IR (KBr): 3390, 3200, 2930, 1700, 1650, 1640, 1540, 1460, 1380, 1330, 1310, 1180, 1150, 1100, 760, 670, 590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.35—1.95 (4H, m), 2.85 (2H, m), 3.00 (4H, m), 4.28 (1H, m), 6.56 (3H, br s), 6.71 (2H, br s), 7.28 (2H, d, J=7.6 Hz), 7.74 (2H, d, J=7.6 Hz), 7.91 (1H, m), 8.42 (1H, d, J=7.6 Hz), 8.48 (2H, s), 8.65 (1H, s), 10.84 (1H, br s).

N^{α}-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{δ}-(4-toluenesulfonyl)-L-ornithine (23a) Methyl N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{δ}-(4-toluenesulfonyl)-L-ornithinate (**22a**, 64 mg; 58%) as a colorless solid was synthesized from **17a** (100 mg) and 4-toluenesulfonyl chloride (36 mg) by the same method as that described for **22b**. IR (KBr): 3380, 3200, 2930, 1740, 1610, 1580, 1545, 1495, 1430, 1320, 1215, 1155, 1090, 750, 660, 550 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 1.45—1.65 (2H, m), 1.70—2.05 (2H, m), 2.38 (3H, s), 2.88 (2H, t, J=6.6 Hz), 3.04 (4H, br s), 3.71 (3H, s), 4.51 (1H, dd, J=9.0, 4.8 Hz), 6.38 (1H, s), 7.27 (2H, d, J=8.0 Hz), 7.32 (2H, d, J=8.0 Hz), 7.70 (2H, d, J=8.0 Hz), 7.73 (2H, d, J=8.0 Hz). SI-MS *m/z*: 580 (MH^+).

Compound **23a** (43 mg, 88%) was synthesized from **22a** (50 mg) by the same method as that described for **23b**. IR (KBr): 3400, 3200, 2930, 1640, 1565, 1540, 1500, 1450, 1395, 1320, 1300, 1155, 1090, 810, 660, 550 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.35—1.55 (2H, m), 1.60—1.90 (2H, m), 2.35 (3H, s), 2.73 (2H, m), 2.96 (4H, br s), 4.29 (1H, m), 5.82 (2H, br s), 6.43 (3H, br s), 7.32 (2H, d, J=8.2 Hz), 7.35 (2H, d, J=8.2 Hz), 7.50 (1H, t, J=5.6 Hz), 7.65 (2H, d, J=8.2 Hz), 7.77 (2H, d, J=8.2 Hz), 8.43 (1H, d, J=7.6 Hz), 10.63 (1H, br s).

N^{α}-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{δ}-(4-fluorophenylcarbamoyl)-L-ornithine (25) A 4 N HCl solution of AcOEt (1.5 ml) was added to a solution of **17a** (200 mg) in THF (3 ml), and the reaction mixture was stirred for 10 min and concentrated *in vacuo*. The residue and 4-fluorophenyl isocyanate (53 mg) were dissolved in dry DMF (4 ml) and Et_3N (1 ml) was added dropwise to the mixture at 0 °C. The reaction mixture was stirred at room temperature for 30 min and concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ and the solution washed with H_2O and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$: MeOH, 20 : 1) to give methyl N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{δ}-(4-fluorophenylcarbamoyl)-L-ornithinate (**24**, 152 mg, 71%) as a colorless solid. IR (KBr): 3330, 2950, 1740, 1610, 1570, 1550, 1510, 1430, 1405, 1385, 1310, 1215, 1155, 830, 760 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.40—1.60 (2H, m), 1.70—1.90 (2H, m), 2.96 (4H, m), 3.11 (2H, m), 3.64 (3H, s), 4.44 (1H, m), 5.34 (2H, br s), 5.96 (2H, s), 6.17 (1H, t, J=6.0 Hz), 6.35 (1H, s), 7.03 (2H, t, J=9.0 Hz), 7.30—7.45 (4H, m), 7.80 (2H, d, J=7.8 Hz), 8.46 (1H, br s), 8.67 (1H, d, J=7.0 Hz), 10.34 (1H, br s). SI-MS *m/z*: 563 (MH^+).

1 N NaOH aqueous solution (1 ml) was added to a stirred solution of **24** (143 mg) in MeOH (6 ml) at room temperature. The reaction mixture was stirred for 5 h and concentrated *in vacuo* and the residue was dissolved in H_2O, and 1 N HCl (1 ml) was added to the mixture. The resulting precipitate was collected by filtration, washed with water and dried *in vacuo* to give **25** (108 mg, 77%) as a colorless solid. IR (KBr): 3330, 3200, 2930, 1660, 1640, 1610, 1560, 1545, 1510, 1455, 1400, 1305, 1210, 830 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.40—1.60 (2H, m), 1.70—1.90 (2H, m), 2.96 (4H, br s), 3.10 (2H, m), 4.36 (1H, m), 5.69 (2H, br s), 6.16 (1H, t, J=7.0 Hz), 6.32 (2H, br s), 6.40 (1H, s), 7.03 (2H, t, J=8.8 Hz), 7.32 (2H, d, J=8.2 Hz), 7.37 (2H, dd, J=8.8, 5.0 Hz), 7.80 (2H, d, J=8.2 Hz), 8.43 (1H, s), 8.52 (1H, d, J=8.0 Hz), 10.55 (1H, br s).

Ethyl 3-[[(3*S*)-(*tert*-Butoxycarbonylamino)-3-(methoxycarbonyl)propyl]carbamoyl]aminobenzoate (27b), Typical Procedure A solution containing Boc-L-Glu-OMe (**26b**) (532 mg), DPPA (607 mg) and Et_3N (0.56 ml) in dry toluene (20 ml) was stirred at 80 °C for 1 h, and then ethyl 3-aminobenzoate (330 mg) was added to the mixture. The reaction mixture was stirred at 80 °C for 20 h and concentrated *in vacuo*. The residue was chromatographed on silica gel (AcOEt : hexane, 2 : 3) to give **27b** (349 mg, 41%) as a colorless syrup. IR (KBr): 3360, 3150, 3100, 2980, 2950, 2930, 2880, 1740, 1715, 1640, 1605, 1590, 1550, 1515, 1485 ,1435, 1390, 1365, 1300, 1285, 1235, 1165, 1125, 1100 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.38 (3H, t, J=7.2 Hz), 1.44 (9H, s), 2.13 (1H, m), 3.02 (1H, m), 3.40—3.80 (2H, m), 3.73 (3H, s), 4.36 (2H, q, J=7.2 Hz), 4.36 (1H, m), 5.38 (1H, d, J=8.6 Hz), 5.81 (1H, m), 7.05 (1H, s), 7.35 (1H, t, J=8.0 Hz), 7.72 (2H, m), 7.90 (1H, t, J=2.0 Hz).

3-[[(3*S*)-(*tert*-Butoxycarbonylamino)-3-(methoxycarbonyl)propyl]carbamoyl]-aminobenzeneboric Acid (27c) Compound **27c** (321 mg, 41%) as a colorless syrup was synthesized from **26b** (532 mg) and 3-aminobenzeneboric acid (372 mg) by the same method as that described for **27b**. IR (neat): 3330, 2980, 2930, 2880, 2850, 2820, 1750, 1710, 1555, 1480, 1440, 1395, 1370, 1305, 1285, 1230, 1160, 1090, 1070, 1020, 995, 850, 780, 750, 720, 710, 660, 650 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.41 (9H, s), 1.80—2.35 (2H, m), 2.95—3.40 (2H, m), 3.78 (3H, s), 4.40 (1H, m), 5.52 (1H, t, J=4.8 Hz), 5.95—6.25 (3H, m), 7.25 (1H, t, J=7.6 Hz), 7.35—7.55 (2H, m), 7.60 (1H, s).

Ethyl 3-[[(2*S*)-(*tert*-Butoxycarbonylamino)-2-(methoxycarbonyl)ethyl]carbamoyl]aminobenzoate (27a) Compound **27a** (200 mg, 17%) as a colorless solid was synthesized from Boc-L-Asp-OMe (**26a**) (1.29 g) and ethyl 3-aminobenzoate (826 mg) by the same method as that described for **27b**. ^{1}H-NMR ($CDCl_3$) δ: 1.37 (3H, t, J=7.2 Hz), 1.41 (9H, s), 3.64 (2H, m), 3.76 (3H, s), 4.31 (3H, q, J=7.2 Hz), 5.67 (1H, br s), 5.74 (1H, t, J=7.4 Hz), 7.33 (1H, t, J=8.0 Hz), 7.43 (1H, br s), 7.69 (1H, d, J=8.0 Hz), 7.73 (1H, d, J=8.0 Hz), 7.86 (1H, s).

N^{γ}-(3-Carboxyphenylcarbamoyl)-N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-2,4-diaminobutyric Acid (30b), Typical Procedure A 2 N HCl solution of AcOEt (10 ml) was added to **27b** (340 mg) and the mixture was stirred for 2 h and concentrated *in vacuo* to give **28b**. Et_3N (0.56 ml) was added dropwise to a solution of **28b**, **7a** (368 mg) and DEPC (143 mg) in dry DMF (4 ml) at 0 °C. The reaction mixture was stirred at room temperature for 16 h and concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ and the solution washed with H_2O and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel pretreated with NH_3 ($CHCl_3$: MeOH, 15 : 1) to give methyl N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{γ}-(3-ethoxycarbonylphenylcarbamoyl)-L-2,4-diaminobutyrate (**29b**, 415 mg; 99%) as a colorless solid. IR (KBr): 3425, 2930, 2855, 1710, 1635, 1610, 1575, 1550, 1490, 1435, 1350, 1300, 1285, 1235, 1200, 1100, 1080, 1020, 660 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.31 (3H, t, J=7.0 Hz), 1.99 (2H, m), 2.97 (4H, m), 3.02 (1H, m), 3.28 (1H, m), 3.65 (3H, s), 4.30 (2H, q, J=7.0 Hz), 4.53 (1H, m), 5.44 (2H, s), 6.06 (2H, s), 6.30 (1H, t, J=6.0 Hz), 6.38 (1H, s), 7.34 (2H, d, J=8.0 Hz), 7.35 (1H, t, J=7.6 Hz), 7.49 (1H, d, J=7.6 Hz), 7.61 (1H, d, J=7.6 Hz), 7.82 (2H, d, J=8.0 Hz), 8.08 (1H, s), 8.71 (1H, d, J=7.6 Hz), 8.85 (1H, s), 10.40 (1H, s).

1 N NaOH aqueous solution (1 ml) was added to a stirred solution of **29b** (415 mg) in MeOH–THF (1 : 2, 6 ml) at room temperature. The reaction mixture was stirred for 4 h, concentrated *in vacuo* and dissolved in H_2O, and 1 N HCl (1 ml) was added. The resulting precipitate was collected by filtration, washed with H_2O and dried *in vacuo* to give **30b** (363 mg, 94%) as a colorless solid. IR (KBr): 3350, 3200, 2930, 2850, 1660, 1645, 1610, 1500, 1440, 1385, 1300, 1280, 1240, 1165, 1090, 800, 760, 680, 650 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.00 (1H, m), 3.30 (4H, m), 3.00—3.40 (2H, m), 4.45 (1H, m), 5.91 (2H, s), 6.31 (1H, t, J=1.2 Hz), 6.44 (1H, s), 6.56 (2H, s), 7.32 (1H, t, J=7.6 Hz), 7.34 (2H, d, J=8.0 Hz), 7.47 (1H, d, J=7.6 Hz), 7.59 (1H, d, J=7.6 Hz), 7.83 (2H, d, J=8.0 Hz), 8.05 (1H, s), 8.56 (1H, d, J=6.4 Hz), 8.82 (1H, s), 10.68 (1H, s).

N^{α}-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{γ}-[3-(dihydroxyboryl)phenylcarbamoyl]-L-2,4-diaminobutyric Acid (30c) Methyl N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{γ}-[3-(dihydroxyboryl)phenylcarbamoyl]-L-2,4-diaminobutyrate (**29c**, 234 mg; 68%) as a colorless solid was synthesized from **27c** (310 mg) and **7a** (315 mg) by the same method as that described for **29b**. IR (KBr): 3400, 2980, 2950, 2930, 2855, 1735, 1635, 1610, 1550, 1500, 1430, 1340, 1275, 1230, 1160, 1110, 1080, 1050, 950, 795, 760, 710 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.98 (2H, m), 2.97 (4H, m), 3.10—3.50 (2H, m), 3.64 (3H, s), 4.53 (1H, m), 5.61 (2H, br s), 6.23 (1H, m), 6.30 (2H, br s), 6.39 (1H, s), 7.16 (1H, t, J=7.8 Hz), 7.33 (3H, d, J=8.0 Hz), 7.58 (1H, m), 7.82 (2H, d, J=8.0 Hz), 8.01 (1H, s), 8.46 (1H, s), 8.72 (1H, d, J=7.6 Hz), 10.52 (1H, s).

Compound **30c** (204 mg, 92%) as a colorless solid was synthesized from **29c** (230 mg) by the same method as that described for **30b**. IR (KBr): 3330, 3200, 2930, 2850, 1640, 1545, 1500, 1420, 1335, 1275, 1230, 1185, 760, 700 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.80—2.15 (2H, m), 2.96 (4H, m), 3.15—3.40 (2H, m), 4.43 (1H, m), 5.99 (2H, s), 6.26 (1H, m), 6.45 (1H, s), 6.62 (3H, br s), 7.16 (1H, t, J=7.6 Hz), 7.33 (3H, d, J=8.2 Hz), 7.56 (2H, s), 7.82 (2H, d, J=8.2 Hz), 7.95 (1H, br s), 8.46 (1H, s), 8.57 (1H, d, J=8.0 Hz), 10.73 (1H, s).

N^{β}-(3-Carboxyphenylcarbamoyl)-N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-2,3-diaminopropionic Acid (30a) Methyl N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-N^{β}-(3-ethoxycarbonylphenylcarbamoyl)-L-2,3-diaminopropionate (**29a**, 228 mg; 100%) as a colorless solid was synthesized from **27a** (200 mg) and **7a** (188 mg) by the same method as that described for **29b**. IR (KBr): 3380, 2930, 2850, 1660, 1630, 1610, 1550, 1490, 1430, 1365, 1300, 1285,

1235, 1200, 1130, 1115, 830, 800, 755, 720 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.31 (3H, t, J=7.0 Hz), 2.97 (4H, m), 3.35—3.85 (2H, m), 3.66 (3H, s), 4.30 (2H, q, J=7.0 Hz), 4.50 (1H, m), 5.80 (2H, s), 6.42 (4H, br s), 7.34 (2H, d, J=8.0 Hz), 7.36 (1H, t, J=7.6 Hz), 7.50 (1H, dt, J=1.2, 7.6 Hz), 7.61 (1H, dt, J=1.2, 7.6 Hz), 7.80 (2H, d, J=8.0 Hz), 8.07 (1H, t, J=1.2 Hz), 8.78 (1H, d, J=7.2 Hz), 9.00 (1H, s), 10.61 (1H, s).

Compound **30a** (166 mg, 85%) as a colorless solid was synthesized from **29a** (212 mg) by the same method as that described for **30b**. IR (KBr): 3380, 3200, 2920, 2850, 1640, 1545, 1500, 1435, 1380, 1300, 1280, 1235, 755 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.48 (1H, m), 3.69 (1H, m), 4.47 (1H, m), 6.09 (1H, s), 6.44 (1H, br s), 6.47 (1H, s), 6.73 (2H, br s), 7.33 (1H, t, J=6.2 Hz), 7.35 (2H, d, J=8.0 Hz), 7.49 (1H, d, J=6.2 Hz), 7.59 (1H, d, J=6.2 Hz), 7.82 (2H, d, J=8.0 Hz), 8.07 (1H, s), 8.68 (1H, d, J=7.0 Hz), 9.00 (1H, s), 10.80 (1H, s).

Methyl (2*S*)-(*tert*-Butoxycarbonylamino)-5-hydroxypentanoate (31) Triethylamine (1.68 ml) was added dropwise to a stirred solution of **26b** (1.566 g) and ethyl chloroformate (1.30 g) in dry THF (10 ml) at −5 °C and the mixture was stirred for 30 min. The resulting precipitate was filtered off and the filtrate was added to a solution of $NaBH_4$ (1.0 g) in H_2O (6 ml). The reaction mixture was stirred for 30 min, then adjusted to pH 7, and diluted with CH_2Cl_2 (20 ml). The organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel pretreated with NH_3 (AcOEt : hexane, 1 : 10) to give **31** (840 mg, 57%) as a colorless syrup. IR (neat): 3370, 2980, 2950, 2875, 1740, 1710, 1695, 1520, 1455, 1440, 1390, 1365, 1280, 1250, 1210, 1165, 1050, 1020 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.38 (9H, s), 1.39—1.85 (4H, m), 3.37 (2H, q, J=5.0 Hz), 3.62 (3H, s), 3.94 (1H, m), 4.41 (1H, t, J=5.0 Hz), 7.22 (1H, d, J=7.8 Hz).

Methyl (2*S*)-(*tert*-Butoxycarbonylamino)-5-iodopentanoate (32) Methanesulfonyl chloride (MsCl) (506 mg) was added dropwise to a stirred solution of **31** (840 mg) and Et_3N (0.71 ml) in dry dichloromethane (16 ml) at 0 °C and the mixture was stirred for 30 min then poured into saturated $NaHCO_3$ aqueous solution. The organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give crude methyl (2*S*)-(*tert*-butoxycarbonylamino)-5-methanesulfonyloxypentanoate (1.118 g; 100%) as a colorless syrup. IR (neat): 3380, 2980, 2930, 1745, 1710, 1515, 1450, 1395, 1355, 1245, 1215, 1170, 1050, 1020, 975, 960, 930, 830, 780, 530 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.45 (9H, s), 1.62—2.10 (4H, m), 3.02 (3H, s), 3.77 (3H, s), 4.26 (2H, t, J=5.8 Hz), 4.35 (1H, m), 5.08 (1H, br s).

A solution containing NaI (2.0 g) and the above prepared mesylate (1.118 g) in acetone (4 ml) was stirred at room temperature for 16 h. The reaction mixture was poured into H_2O and extracted with Et_2O. The Et_2O solution was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give **32** (1.103 g, 90%) as a colorless syrup. ^{1}H-NMR (DMSO-d_6) δ: 1.38 (9H, s), 1.50—1.90 (4H, m), 3.22 (2H, dd, J=6.7, 5.9 Hz), 3.63 (3H, s), 3.97 (1H, m), 7.29 (1H, d, J=7.8 Hz).

Ethyl 3-[[(4*S*)-(*tert*-Butoxycarbonylamino)-4-(methoxycarbonyl)butyl]-amino]benzoate (33a) A solution containing **32** (535 mg) and ethyl 3-aminobenzoate (1.0 g) was stirred at 70 °C for 10 h and the reaction mixture was diluted with CH_2Cl_2 (25 ml). The CH_2Cl_2 solution was successively washed with 2% AcOH, saturated $NaHCO_3$ aqueous solution, H_2O and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel pretreated with NH_3 (AcOEt : hexane, 1 : 5) to give **33a** (411 mg; 76%) as a colorless syrup. IR (neat): 3380, 2975, 2930, 2860, 1740, 1715, 1605, 1585, 1515, 1490, 1475, 1450, 1435, 1390, 1650, 1335, 1280, 1240, 1165, 1100, 1020, 750 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.30 (3H, t, J=7.0 Hz), 1.38 (9H, s), 1.45—1.85 (4H, m), 3.00 (2H, m), 3.62 (3H, s), 3.98 (3H, s), 3.99 (1H, m), 4.27 (2H, q, J=7.0 Hz), 5.89 (1H, t, J=5.8 Hz), 6.77 (1H, dt, J=1.2, 5.4 Hz), 7.05—7.35 (4H, m).

Diethyl *N*-[(4*S*)-(*tert*-Butoxycarbonylamino)-4-(methoxycarbonyl)-butyl]-L-glutamate (33b) A solution containing **32** (357 mg) and diethyl L-glutamate (620 mg) was stirred at 60 °C for 16 h and the reaction mixture was diluted with CH_2Cl_2 (25 ml). The CH_2Cl_2 solution was successively washed with 2% AcOH, saturated $NaHCO_3$ aqueous solution, water, and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on silica gel pretreated with NH_3 (AcOEt : hexane, 3 : 2) to give **33b** (270 mg, 63%) as a colorless syrup. IR (neat): 3370, 3320, 2980, 2930, 2870, 1740, 1710, 1520, 1450, 1420, 1390, 1370, 1275, 1250, 1200, 1170, 1100, 1045, 1030 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.30 (6H, t, J=7.0 Hz), 1.44 (9H, s), 1.50—2.20 (4H, m), 2.20—2.60 (4H, m), 3.00 (1H, m), 3.62 (1H, m), 3.74 (3H, s), 4.19 (2H, m), 4.23 (4H, q, J=7.0 Hz), 5.10 (1H, br s), 5.85 (1H, br s).

N^{δ}-(3-Carboxyphenyl)-N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-ornithine (36a), Typical Procedure A 2 N HCl solution of AcOEt (12 ml) was added to **33a** (411 mg) and the mixture was stirred for 2 h then concentrated *in vacuo* to give **34a**. Triethylamine (0.67 ml) was added dropwise to a solution of the prepared **34a**, **7a** (428 mg) and DEPC (172 mg) in dry DMF (8 ml) at 0 °C. The reaction mixture was stirred at room temperature for 2 h and concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ and the solution was washed with H_2O and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel pretreated with NH_3 ($CHCl_3$: MeOH, 10 : 1) to give methyl N^{α}-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoyl]-N^{δ}-(3-ethoxycarbonylphenyl)-L-ornithinate (**35a**, 430 mg; 93%) as a colorless solid. IR (KBr): 3370, 2920, 2850, 1730, 1710, 1605, 1570, 1540, 1490, 1425, 1365, 1280, 1235, 1180, 1100, 750 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.29 (3H, t, J=7.0 Hz), 1.50—2.05 (4H, m), 2.96 (4H, m), 3.06 (2H, t, J=5.8 Hz), 3.64 (3H, s), 4.26 (2H, q, J=7.0 Hz), 4.48 (1H, m), 5.34 (2H, s), 5.92 (1H, t, J=5.2 Hz), 5.96 (2H, s), 6.35 (1H, d, J=1.8 Hz), 6.78 (1H, dt, J=1.6, 8.4 Hz), 7.05—7.25 (3H, m), 7.32 (2H, d, J=8.2 Hz), 7.79 (2H, d, J=8.2 Hz), 8.65 (1H, d, J=7.6 Hz), 10.33 (1H, d, J=1.8 Hz).

1 N NaOH (1 ml) was added to a stirred solution of **35a** (415 mg) in MeOH–THF (1 : 2, 6 ml) at room temperature. The reaction mixture was stirred for 2 h, concentrated *in vacuo*, and dissolved in H_2O. 1 N HCl (1 ml) was added to the mixture and the resulting precipitate collected by filtration, washed with H_2O and dried *in vacuo* to give **36a** (386 mg, 98%) as a colorless solid. IR (KBr): 3390, 3200, 2920, 2850, 1640, 1610, 1540, 1495, 1450, 1435, 1380, 1335, 1270, 1190, 760 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.55—2.10 (4H, m), 2.96 (4H, m), 3.10 (2H, m), 4.40 (1H, m), 5.45 (2H, br s), 5.85 (1H, br s), 6.09 (2H, s), 6.37 (1H, s), 6.76 (1H, dt, J=7.6, 2.0 Hz), 7.05—7.25 (3H, m), 7.32 (2H, d, J=8.2 Hz), 7.80 (2H, d, J=8.2 Hz), 8.49 (1H, d, J=7.8 Hz), 10.40 (1H, s).

N^{δ}-(3-Carboxyphenyl)-N^{α}-[4-[3-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-L-ornithine (36b) Methyl N^{α}-[4-[3-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-N^{δ}-(3-ethoxycarbonylphenyl)-L-ornithinate (**35b**, 316 mg; 73%) as a colorless solid was synthesized from **33a** (295 mg) and **7b** (230 mg) by the same method as that described for **35a**. IR (KBr): 3380, 3180, 2930, 2850, 1740, 1710, 1610, 1575, 1550, 1535, 1490, 1425, 1370, 1325, 1280, 1240, 1180, 1105, 1020, 990, 800, 755 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.29 (3H, t, J=7.0 Hz), 1.67 (1H, m), 1.86 (4H, m), 3.04 (2H, m), 3.64 (3H, s), 4.27 (2H, q, J=7.0 Hz), 4.46 (1H, m), 5.31 (2H, s), 5.92 (1H, t, J=8.0 Hz), 6.42 (1H, s), 6.76 (1H, dd, J=1.4, 3.8 Hz), 7.13 (3H, m), 7.30 (2H, d, J=8.2 Hz), 7.80 (2H, d, J=8.2 Hz), 8.65 (1H, d, J=7.2 Hz), 10.36 (1H, s).

Compound **36b** (297 mg, 100%) as a colorless solid was synthesized from **35b** (310 mg) by the same method as that described for **36a**. IR (KBr): 3400, 3200, 2930, 2850, 1645, 1610, 1540, 1500, 1385, 1335, 1265, 1230, 1185, 755 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.69 (2H, m), 1.85 (4H, m), 2.70 (4H, m), 3.46 (2H, m), 4.41 (1H, m), 5.72 (2H, br s), 5.86 (1H, br s), 6.29 (2H, s), 6.49 (1H, s), 6.76 (1H, dt, J=7.4, 2.2 Hz), 7.05—7.23 (3H, m), 7.30 (2H, d, J=8.4 Hz), 7.82 (2H, d, J=8.4 Hz), 8.51 (1H, d, J=7.8 Hz), 10.61 (1H, s).

***N*-[(4*S*)-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoylamino]-4-carboxybutyl]-L-glutamic Acid (36c)** Diethyl *N*-[(4*S*)-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoylamino]-4-methoxycarbonylbutyl]-L-glutamate (**35c**, 291 mg, 100%) as a colorless syrup was synthesized from **33b** (270 mg) and **7a** (192 mg) by the same method as that described for **35a**. IR (KBr): 3330, 3200, 2990, 2950, 1740, 1690, 1680, 1615, 1575, 1545, 1500, 1455, 1430, 1200, 1130, 830, 800, 720 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.81 (6H, t, J=7.2 Hz), 1.52 (2H, m), 1.72 (2H, m), 1.93 (1H, m), 2.27 (3H, m), 2.96 (5H, m), 3.51 (1H, m), 3.64 (3H, s), 4.10 (1H, m), 4.12 (4H, q, J=7.2 Hz), 4.30 (1H, m),4.42 (1H, m), 5.98 (2H, br s), 6.46 (1H, s), 6.61 (2H, br s), 7.34 (2H, d, J=8.0 Hz), 7.79 (2H, d, J=8.0 Hz), 8.62 (1H, d, J=8.0 Hz), 10.74 (1H, s).

Compound **36c** (167 mg, 75%) as a colorless solid was synthesized from **35c** (286 mg) by the same method as that described for **36a**. IR (KBr): 3400, 3330, 3180, 2920, 2860, 1720, 1640, 1540, 1500, 1460, 1420, 1385, 1335, 1280, 1230, 1200, 1145, 1105, 1090, 730, 650 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.56 (2H, m), 1.74 (2H, m), 1.95 (1H, m), 2.25 (3H, m), 2.95 (4H, m), 3.53 (2H, m), 4.16 (1H, m), 4.37 (1H, m), 6.38 (2H, br s), 6.53 (1H, s), 7.03 (2H, s), 7.31 (2H, d, J=8.2 Hz), 7.79 (2H, d, J=8.2 Hz), 8.48 (1H, d, J=7.4 Hz), 10.99 (1H, s).

Physical properties for compounds **23a**—**c**, **25**, **30a**—**c** and **36a**—**c** are listed in Table 1.

Cell Lines The parent CCRF-CEM human lymphoblastic leukemia cell line and the MTX-resistant sublines CCRF-CEM R_1[15] characterized by increased DHFR activity, CCRF-CEM R_{BO}[16] which has normal DHFR levels but impaired MTX transport and CCRF-CEM $R_{30/6}$[17] characterized by impaired polyglutamylation of MTX were routinely cultured in RPMI 1640

medium supplemented with 10% horse serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 atmosphere.

Cell Growth Inhibition Assay Meth A cells were grown in MEM (minimum essential medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 at 37 °C. Logarithmically growing cells (4×10^4) in 2.0 ml of medium were seeded in 12-well plates. Test drugs were added at various concentrations prior to the cell seeding. Cells were incubated for 72 h, and the cell number was counted with a Coulter Counter, Model ZM (Coulter Electronics Ltd., Luton, England). Exponentially growing CCRF-CEM cells were prepared at a density of $3—5\times10^4$ cells/ml and distributed in duplicate 5-ml portions into tissue culture tubes to which 0.05 ml of drug solutions at various concentrations was added. The cells were incubated at 37 °C for 72 h and cell density was determined with a model B Coulter counter (Coulter Electronics, Hialeah, FL).

DHFR Inhibition Assay DHFR activity was measured photometrically by a modification of the method of Bertino.[18] The reaction was carried out at 30 °C in flat-bottomed 96-well plates (Nunc-Immunoplate Maxisorp) pretreated with 1 mg/ml bovine serum albumin utilizing 0.25 μg protein/ml (1.9 μU/ml) bovine liver DHFR, various concentrations of dihydrofolic acid and 300 μl of a reaction buffer containing 0.1 M Tris–HCl (pH 7.5), 150 mM KCl, 15 mM 2-mercaptoethanol and 125 mM NADPH. The reaction was started by adding a mixture of NADPH and dihydrofolic acid to the reaction buffer containing DHFR and drugs. Changes in the absorbance of NADPH and dihydrofolic acid were measured at 340 nm using a Titertek Multiskan MCC/340 (Labsystems, Finland) controlled by a personal computer for 2 min at 2 s intervals.

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Studies on the Constituents of *Broussonetia* Species. VII. Four New Pyrrolidine Alkaloids, Broussonetines M, O, P, and Q, as Inhibitors of Glycosidase, from *Broussonetia kazinoki* SIEB.

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Four new pyrrolidine alkaloids, broussonetines M, O, P, and Q, were isolated from the branches of *Broussonetia kazinoki* SIEB. (Moraceae). Broussonetines M, O, P, and Q were formulated as (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-[(10*S*)-10,13-dihydroxy-tridecyl]pyrrolidine (1), (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-[(*E*)9-oxo-13-hydroxy-3-tridecenyl]pyrrolidine (2), (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-[(*E*)10-oxo-13-hydroxy-3-tridecenyl)pyrrolidine (3), and (2*R*,3*S*,4*R*,5*R*)-2-hydroxymethyl-3-hydroxy-4-(β-D-glucopyranosyloxy)-5-[10-oxo-13-(β-D-glucopyranosyloxy)tridecyl]pyrrolidine (4) respectively, by spectroscopic and chemical methods. 1—4 inhibited β-glucosidase, β-galactosidase and β-mannosidase.

Key words pyrrolidine alkaloid; glycosidase inhibitor; *Broussonetia kazinoki*; Moraceae

Recently, we reported the structures of thirteen pyrrolidine or pyrrolizidine alkaloids, broussonetines A—H, K, L, N and broussonetinines A and B, as glycosidase inhibitors and two pyrrolidinyl piperidine alkaloids, broussonetines I and J, from *Broussonetia kazinoki* SIEB. (Moraceae).[1—6] In our latest work, we obtained four new pyrrolidine alkaloids, broussonetines M (**1**), O (**2**), P (**3**) and Q (**4**) (Fig. 1), from the same tree. The present report deals with the isolation, structural elucidation including absolute stereostructures and inhibitory activity on some glycosidases.

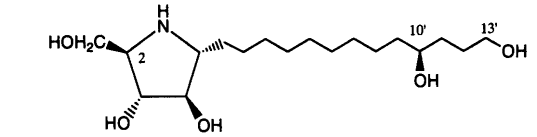
The branches of this tree were extracted with hot water and the alkaloid constituents were concentrated as described in the Experimental section. Compounds **1**—**4** were isolated by preparative HPLC of the concentrated alkaloids.

Compound **1** was obtained as a colorless powder, $[\alpha]_D$ +5.9° (c=0.30, MeOH), showing a yellowish spot on TLC when sprayed with ninhydrin reagent followed by heating on a hot plate (ninhydrin reaction). The molecular formula was determined as $C_{18}H_{37}NO_5$ on the basis of positive high resolution secondary ion mass spectroscopy (pos. HR-SI-MS) (m/z: 348.2741, $[M+H]^+$, error, −0.7 mmu). The IR spectrum showed a strong OH and NH band at 3275 cm^{-1}.

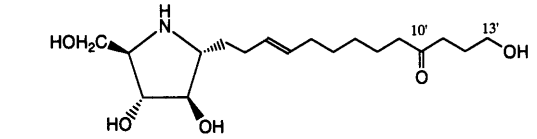
The ^{1}H-NMR spectrum of **1** suggested the presence of seven methylene groups [δ: 1.16—1.66 (14H, m)], two oxymethylene groups [δ: 4.22 (1H, dd, J=11.0, 4.1 Hz), 4.15 (1H, dd, J=11.0, 5.9 Hz), 3.95 (2H, t, J=6.4 Hz)], three oxymethine groups [δ: 4.62 (1H, t, J=6.4 Hz), 4.36 (1H, t, J=6.4 Hz), 3.90 (1H, m)], and two methine groups attached to a nitrogen atom [δ: 3.75 (1H, m), 3.45 (1H, m)]. These data were strikingly similar to those of broussonetine C,[1] except for an additional oxymethine signal and disappearance of two signals due to methylene groups attached to a carbonyl group. Thus, **1** was concluded to be the compound following reduction of the carbonyl group of broussonetine C. The ^{1}H- and ^{13}C-NMR signals were assigned by ^{1}H–^{1}H correlated spectroscopy (^{1}H–^{1}H COSY), total correlation spectroscopy (TOCSY), heteronuclear signal quantum coherence (HSQC), distortionless enhancement by polarization transfer (DEPT), and heteronuclear multiple bond correlation (HMBC), as shown in Tables 1 and 2.

The relative stereochemistry of the pyrrolidine moiety in **1** was disclosed by the vicinal coupling constants ($J_{2,3}$= $J_{3,4}$=$J_{4,5}$=6.4 Hz) and nuclear Overhauser effects (NOEs) in the nuclear Overhauser enhancement and exchange spectroscopy (NOESY) spectrum: NOEs were observed between H-2 and H-4, and H-3 and H-5 to establish the 2β-hydroxymethyl-3α,4β-dihydroxy-5α-alkylpyrrolidine structure.

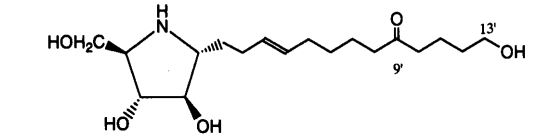
The absolute stereochemistry of the pyrrolidine moiety and C-10′ in **1** was determined by a new modification of



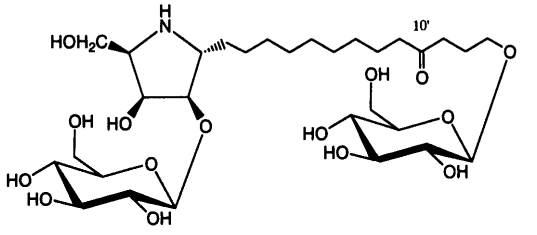
broussonetine M (**1**)



broussonetine O (**2**)



broussonetine P (**3**)



broussonetine Q (**4**)

Fig. 1. Structures of **1**—**4**

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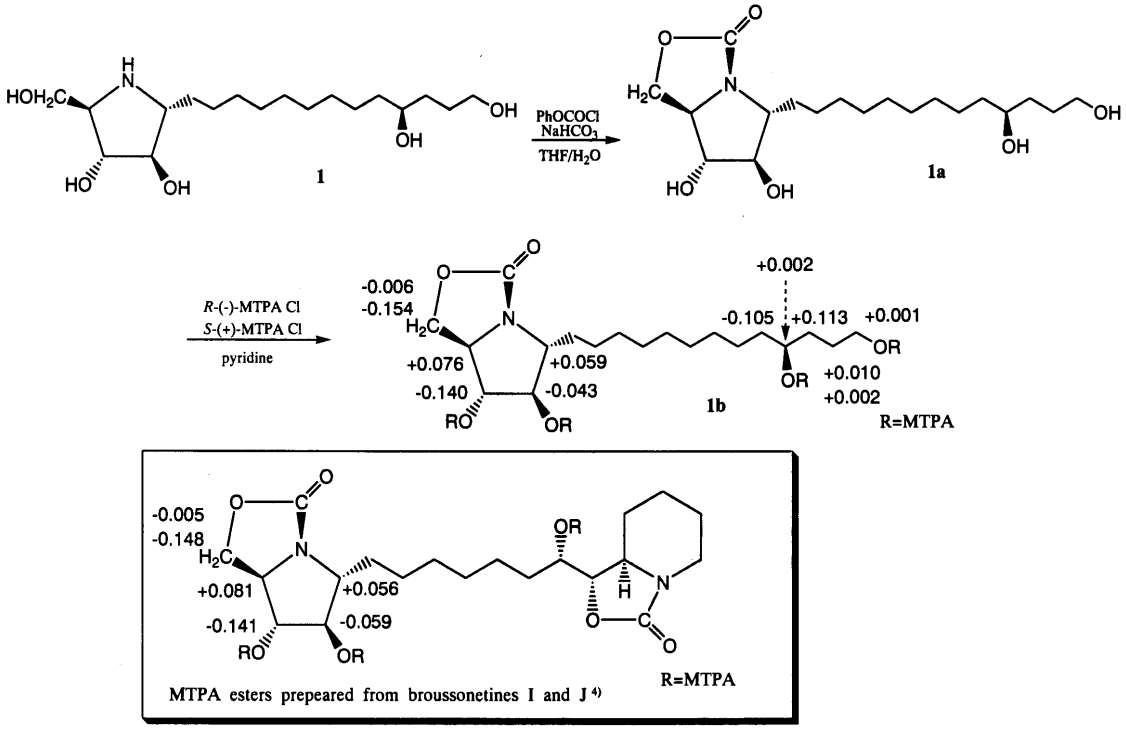

Fig. 2. $\Delta\delta$ Values Obtained for the MTPA Esters of **1**

Mosher's method.[7] A cyclic carbamate (**1a**) was prepared from **1** by reaction with phenyl chlorocarbonate in teterahydrofuran (THF) : H_2O (7 : 3). ^{1}H-NMR signals of the tetra (*S*)- and (*R*)-2-methoxy-2-phenyl-2-(trifluoromethyl) acetic acid (MTPA) esters (**1b***S*, **1b***R*) prepared from **1a** were assigned by analyzing the ^{1}H–^{1}H COSY (500 MHz) spectra, and the $\Delta\delta$ ($=\delta_S-\delta_R$) values were measured respectively: these values established the (*S*) configuration at C-10′ (Fig. 2). In addition, the $\Delta\delta$ values of the pyrrolidine moiety in **1b** coincided with those in the tri-MTPA esters prepared from broussonetines I and J.[4]

Thus, the absolute stereostructure of **1** was formulated as (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-[(10*S*)-10,13-dihydroxy-tridecyl]pyrrolidine.

Compound **2** was obtained as a colorless powder, $[\alpha]_D$ +22.7° (c=0.37, MeOH), showing a brownish spot on TLC with the ninhydrin reaction, and the molecular formula was determined as $C_{18}H_{33}NO_5$ on the basis of pos. HR-SI-MS (*m/z*: 344.2435, $[M+H]^+$, error, 0.0 mmu). The IR spectrum showed a strong OH and NH band at 3406 cm^{-1} and a carbonyl band at 1706 cm^{-1}. The ^{1}H-NMR spectrum was strikingly similar to that of broussonetine C,[1] except for two additional olefin proton signals [δ: 5.53 (1H, m), 5.45 (1H, m)]. These signals were assigned as in **1** and summarized in Tables 1 and 2. The vicinal coupling constant (J=15.3 Hz), which was shown by decoupling a methylene signal [δ: 1.93 (2H, dd)], of C′-3 and C′-4 in **2** established the (*E*) conformation of this olefin.

Compound **3** was obtained as a colorless powder, $[\alpha]_D$ +28.8° (c=0.96, MeOH), showing a brownish spot on TLC with the ninhydrin reaction, and the molecular formula was determined as $C_{18}H_{33}NO_5$ on the basis of pos. HR-SI-MS (*m/z*: 344.2442, $[M+H]^+$, error, +0.7 mmu). The IR spectrum showed a strong OH and NH band at 3308 cm^{-1} and a carbonyl band at 1705 cm^{-1}.

The ^{1}H-NMR spectrum was strikingly similar to **2** except for the signals due to a partial structure, $CO(CH_2)_3CH_2OH$ instead of $CO(CH_2)_2CH_2OH$. The ^{1}H- and ^{13}C-NMR signals were assigned as above and summarized in Tables 1 and 2.

The absolute stereochemistry of the pyrrolidine ring moieties in **2** and **3** was established by comparison of the values of $[\alpha]_D$ +22.7° (**2**) and +28.8° (**3**), with that of broussonetine C (+25.0°).

Thus, **2** and **3** were formulated as (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-[(*E*) 9-oxo-13-hydroxy-3-tridecenyl]pyrrolidine and (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-[(*E*)10-oxo-13-hydroxy-3-tridecenyl)pyrrolidine.

Compound **4** was obtained as a colorless powder, $[\alpha]_D$ +8.8° (c=0.25, MeOH), showing a brownish spot on TLC with the ninhydrin reaction, and the molecular formula was determined as $C_{30}H_{55}NO_{15}$ on the basis of pos. HR-SI-MS (*m/z*: 670.3664, $[M+H]^+$, error, +1.7 mmu). The IR spectrum showed a strong OH and NH band at 3402 cm^{-1} and a carbonyl band at 1705 cm^{-1}.

The ^{1}H-NMR spectrum of **4** showed two anomeric protons [δ: 4.79 (1H, d, J=7.8 Hz), 4.94 (1H, d, J=7.8 Hz)]. Hydrolysis of **4** with 1 N HCl provided a genuine aglycone (**4a**) and D-glucose ($[\alpha]_D$ +40.5°). The ^{1}H- and ^{13}C-NMR spectra of **4a** were identical with those of broussonetinine A.[2,6]

The structure of **4** was concluded to be 4-*O*-β-D-glucopyranosyl 13′-*O*-β-D-glucopyranosyl broussonetinine A: the glucosylation shift was 9.02 and 7.92 ppm between the C-4, 13′ of **4** and that of **4a** (Table 1) and the HMBC spectrum of **4** showed long-range correlations between H-4, 13′ and two anomeric carbons (δ: 105.35, 104.41), and two anomeric protons and C-4, 13′, respectively.

Thus, the structure of **4** was formulated as (2*R*,3*S*,4*R*,5*R*)-2-hydroxymethyl-3-hydroxy-4-(β-D-glucopyranosyloxy)-5-[10-oxo-13-(β-D-glucopyranosyloxy)tridecyl]pyrrolidine.

Table 1. ^{1}H-NMR Spectral Data for **1**—**4** (500 MHz, Pyridine-d_5)

	1	**2**	**3**	**4**
2	3.75 m	3.72 m	3.74 m	3.75[a]
3	4.62 t (6.4)	4.62 t (6.4)	4.61 t (6.4)	4.75 t (4.0)
4	4.36 t (6.4)	4.36 t (6.4)	4.36 t (6.4)	4.07 dd (3.5, 4.5)
5	3.45 m	3.49 m	3.48 m	3.66[a]
1′	1.70[a], 2.00[a]	1.82 m, 2.09[a]	1.83[a], 2.09 m	1.59[a], 1.93 m
2′	1.16—1.66	2.29 m, 2.39[a]	2.26 m, 2.36[a]	1.39 m, 1.57[a]
3′	1.16—1.66	5.53 m	5.50 m	1.16—1.66
4′	1.16—1.66	5.45 m	5.43 m	1.16—1.66
5′	1.16—1.66	1.93 dd (13.5, 7.3)	1.93 dd (14.0, 7.3)	1.16—1.66
6′	1.16—1.66	1.26[a]	1.29 quin (7.3)	1.16—1.66
7′	1.16—1.66	1.22[a]	1.57 quin (7.3)	1.16—1.66
8′	1.16—1.66	1.57 quin (7.3)	2.36 t (7.3)	1.53[a]
9′	1.63[a], 1.70[a]	2.39 t (7.3)	—	2.35 t (7.3)
10′	3.90 m	—	2.46 t (7.3)	—
11′	1.86 m	2.65 t (7.3)	1.83	2.62 t (7.3)
12′	2.00[a], 2.12 m	2.06	1.72 m	2.02 quin (7.3)
13′	3.95 t (6.4)	3.85 t (6.4)	3.83 t (6.4)	3.66[a], 4.15[a]
CH_2OH	4.15 dd (11.0, 5.9)	4.14 dd (11.0, 5.8)	4.14 dd (11.0, 5.9)	4.23[a]
	4.22 dd (11.0, 4.1)	4.19 dd (11.0, 4.2)	4.21 dd (11.0, 4.0)	4.31[a]
Glc				
1″				4.94 d (7.8)
2″				4.01[a]
3″				4.24[a]
4″				4.17[a]
5″				3.98[a]
6″				4.31[a], 4.52[a]
Glc				
1‴				4.79 d (7.8)
2‴				4.01[a]
3‴				4.24[a]
4‴				4.17[a]
5‴				3.94[a]
6‴				4.31[a], 4.48[a]

a) Overlapped signals.

Table 2. ^{13}C-NMR Spectral Data for **1**—**4** and **4a** (Pyridine-d_5)

	1[a]	**2**[a]	**3**[a]	**4**[a]	**4a**[b]
2	64.71	64.98	64.57	61.42	62.12
3	80.02	80.22	79.77	73.18	73.42
4	84.01	84.24	83.76	88.95	79.93
5	62.55	62.24	61.90	59.83	61.48
1′	35.44	35.68	35.17	35.36	35.32
2′		30.40	30.13		
3′	30.10, 30.07	130.83	130.69	30.10, 29.85	30.05, 29.88
4′	29.94, 29.88	130.48	130.05	29.69, 29.65	29.76, 29.61
5′	29.83, 27.22	32.71	32.45	29.40, 27.56	29.35, 27.61
6′	26.29	29.63	29.12		
7′		28.96	23.43		
8′		24.02	42.33	24.06	24.07
9′	38.27	42.78	210.79	42.78	42.72
10′	70.94	210.75	42.37	210.99	210.67
11′	34.93	39.52	20.69	39.31	39.40
12′	30.17	27.81	32.69	24.44	27.42
13′	62.34	61.24	61.44	68.98	61.06
CH_2OH	63.45	63.54	63.14	62.13	61.84
1″				105.35	
2″				74.92[c]	
3″				78.16[c]	
4″				71.47[c]	
5″				78.28[c]	
6″				62.56[c]	
1‴				104.41	
2‴				74.62[c]	
3‴				77.97[c]	
4‴				71.18[c]	
5‴				78.22[c]	
6‴				62.23[c]	

a) Measured at 125 MHz. *b*) Measured at 75 MHz. *c*) Assignments may be interchangeable.

Table 3. Concentration of Inhibitor Required to Produce 50% Inhibition of Enzyme Activity

	Inhibitors						
	1	2	3	4	DNJ	DGJ	DMJ
α-Glucosidase (from yeast)	NI	NI	NI	NI	0.93	—	—
β-Glucosidase (from sweet almond)	NI	1.4	2.4	1.4	0.58	—	—
β-Galactosidase (from bovine liver)	8.1	0.17	0.20	0.60	—	0.13	—
α-Mannosidase (from Jack beans)	NI	NI	NI	NI	—	—	0.94
β-Mannosidase (from snail acetone powder)	NI	8.2	7.6	20.0	—	—	0.81

NI: up to 100 μM or no inhibition. (μM)

The inhibitory activities of **1**—**4**, 1-deoxynojirimycin (DNJ),[8,9] 1-deoxygalactonojirimycin (DGJ), and 1-deoxymannojirimycin (DMJ)[8,9] were assayed with respect to α-glucosidase, β-glucosidase, β-galaclosidase, α-mannosidase and β-mannosidase by the methods described in the Experimental section and the results are summarized in Table 3.

Broussonetines M (**1**) inhibited β-galactosidase and broussonetines O (**2**), P (**3**), and Q (**4**) inhibited β-glucosidase, β-galactosidase and β-mannosidase.

Experimental

General The instruments used in this work were: a JASCO digital polarimeter (for specific rotation, measured at 25 °C); a Perkin-Elmer 1720X-FTIR spectrometer (for IR spectra); a Hitachi M-80 spectrometer (for MS spectra); a Varian Mercury 300, unity Inova-500 (for NMR spectra, measured in pyridine-d_5, on the δ scale using tetramethylsilane as an internal standard); a Shimadzu spectrophotometer UV 1200 (for enzyme assay).

Column chromatography was carried out on an ion exchange resin (Amberlite CG-50, Amberlite IRA-67/Orugano Company and Dowex 50W-X4/the Dow Chemical Company), and silica-gel (Chromatorex DM1020/Fuji Silysia Chemical Ltd.). HPLC was conducted using a Gilson 305 pump or a JASCO PU 980 equipped with a JASCO 830-RI or UV-970 as a detector. Silica-gel 60 F_{254} (Merck) precoated TLC plates were used, developed in a $CHCl_3$–MeOH–AcOH–H_2O (20 : 10 : 7 : 5) solvent system, and detection was carried out with the ninhydrin reagent followed by heating.

Isolation of 1—4 Dried branches of *Broussonetia kazinoki* (7.5 kg, collected in Takatsuki City (Osaka) in 1998) were cut finely and then extracted with hot water (40 l×3) for 2 h. The extracted solution was chromatographed on an Amberlite CG-50 (H^+-form) column (8 l, i.d. 6.5−30 cm, repeated 8 times). After washing the column with water and then 50% MeOH, the adsorbed material was eluted with 50% MeOH–28% ammonia solution (9 : 1). The eluted fraction was concentrated *in vacuo* to give a basic fraction (35.3 g). This fraction was chromatographed on a Dowex 50W-X4 column (200—400 mesh, 500 ml, i.d. 5.0×30 cm) pretreated with formic acid–ammonium formate buffer (0.2 M ammonia formate, adjusted to pH 5.7 with 1 N formic acid), with gradient elution (H_2O(2.0 l)→H_2O–28% ammonia solution (9 : 1, 2.0 l)). The fractions containing **1**, **2**, **3** and **4** were rechromatographed on silica-gel (Chromatorex DM1020) using $CHCl_3$ and MeOH, followed by preparative HPLC [column: Asahipak ODP 5E (i.d. 10× 250 mm); solvent: CH_3CN–H_2O (15 : 85), adjusted to pH 12.0 with ammonia solution; flow rate: 1.5 ml/min; column temperature : ambient]. **1** (20 mg), **2** (10 mg), **3** (20 mg) and **4** (10 mg) were finally obtained.

Broussonetine M (**1**): Colorless powder, ninhydrin reaction: positive (a yellow spot on TLC), $[\alpha]_D$ +5.9° (c=0.30, MeOH), $C_{18}H_{37}NO_5$, pos. HR-SI-MS m/z: 348.2741 ($[M+H]^+$) error: −0.7 mmu, IR ν (KBr) cm^{-1}: 3275 (OH, NH), ^{1}H- and ^{13}C-NMR (pyridine-d_5): Tables 1, 2.

Broussonetine O (**2**): Colorless powder, ninhydrin reaction: positive (a brownish spot on TLC), $[\alpha]_D$ +22.7° (c=0.27, MeOH), $C_{18}H_{33}NO_5$, pos. HR-SI-MS m/z: 344.2435 ($[M+H]^+$) error: 0.0 mmu, IR ν (KBr) cm^{-1}: 3406 (OH, NH), 1706 (CO), ^{1}H- and ^{13}C-NMR (pyridine-d_5): Tables 1, 2.

Broussonetine P (**3**): Colorless powder, ninhydrin reaction: positive (a brownish spot on TLC), $[\alpha]_D$ +28.8° (c=0.96, MeOH), $C_{18}H_{33}NO_5$, pos. HR-SI-MS m/z: 344.2442 ($[M+H]^+$) error: +0.7 mmu, IR ν (KBr) cm^{-1}: 3308 (OH, NH), 1705 (CO), ^{1}H- and ^{13}C-NMR (pyridine-d_5): Tables 1, 2.

Broussonetine Q (**4**): Colorless oil, ninhydrin reaction: positive (a brownish spot on TLC), $[\alpha]_D$ +8.8° (c=0.25, MeOH), $C_{30}H_{55}NO_{15}$, pos. HR-SI-MS m/z: 670.3664 ($[M+H]^+$) error: +1.7 mmu, IR ν (KBr) cm^{-1}: 3402 (OH, NH), 1705 (CO), ^{1}H- and ^{13}C-NMR (pyridine-d_5): Tables 1, 2.

Carbamate (1a) **1** (10 mg) was treated with phenyl chloroformate (1.5 ml) in THF–H_2O (7 : 3) (10 ml) and $NaHCO_3$ (0.5 g) at 2 °C for 3 h followed by warming to room temperature for 36 h. The reaction products were subject-ed to HPLC [column, Asahipak ODP-5E (i.d. 6.0×250 mm); solvent, CH_3CN–H_2O (30 : 70), adjusted to pH 12.0 with ammonia solution; flow rate, 1.0 ml/min; detection, RI; column temperature, ambient]. Carbamate (**1a**) was obtained as a colorless oil (8 mg).

(*S*)-(−)-MTPA Ester (1b*S*) **1a** (4 mg) was treated with (*R*)-(−)-MTPA-Cl (20 μl) in pyridine (300 μl) at room temperature overnight, and then *N*,*N*-dimethyl-1,3-propanediamine was added. The reaction products were subjected to HPLC [column, Cosmosil C18-AR-300 (i.d. 4.6×150 mm); solvent, CH_3CN–H_2O (20 : 80→100 : 0 40 min); flow rate, 1.0 ml/min; detection, UV 230 nm; column temperature, 40 °C]. **1b***S* was obtained as a colorless oil (1.5 mg). **1b***S*: $C_{59}H_{63}NO_{14}F_{12}$ pos. SI-MS m/z: 1238 $(M+H)^+$, 189 (base peak). ^{1}H-NMR ($CDCl_3$) δ: 1.150*—1.730* (CH_2), 1.545* (9′-H), 1.606* (12′-H), 1.651* (11′-H), 1.688* (12′-H), 3.488 (3H, s, OCH_3), 3.510*—3.547* (9H, OCH_3), 3.962 (1H, m, 2-H), 4.020 (1H, m, 5-H), 4.200* (1H, 13′-H), 4.231 (1H, C$\underline{H}_2$O), 4.279* (1H, 13′-H), 4.586 (1H, m, C$\underline{H}_2$O), 4.870 (1H, m, 3-H), 5.034 (1H, m, 10′-H), 5.202 (1H, m, 4-H), 7.350—7.550 (20H, m, MTPA-Ar H). *: overlapped signals.

(*R*)-(+)-MTPA Ester (1b*R*) **1b** (4 mg) was treated with (*S*)-(+)-MTPA-Cl (20 μl) in pyridine (300 μl) at room temperature overnight, and then *N*,*N*-dimethyl-1,3-propanediamine was added. The reaction products were subjected to HPLC [column, Cosmosil C18-AR-300 (i.d. 4.6×150 mm); solvent, CH_3CN–H_2O (20 : 80→100 : 0 40 min); flow rate, 1.0 ml/min; detection, UV 230 nm; column temperature, 40 °C]. **1b***R* was obtained as a colorless oil (1.5 mg). **1b***R*: $C_{59}H_{63}NO_{14}F_{12}$ pos. SI-MS m/z: 1238 $(M+H)^+$, 189 (base peak). ^{1}H-NMR ($CDCl_3$) δ: 1.130*—1.720* (CH_2), 1.538* (11′-H), 1.596* (12′-H), 1.650* (9′-H), 1.686* (12′-H), 3.466 (3H, s, OCH_3), 3.503*—3.543* (9H, OCH_3), 3.886 (1H, m, 2-H), 3.961 (1H, m, 5-H), 4.200* (1H, 13′-H), 4.278 (1H, m, 13′-H), 4.385 (1H, dd, J=9.7, 4.2 Hz, C$\underline{H}_2$O), 4.592 (1H, m, C$\underline{H}_2$O), 5.010* (1H, 3-H), 5.032* (1H, 10′-H), 5.245 (1H, m, 4-H), 7.350—7.540 (20H, m, MTPA-Ar H). *: overlapped signals.

Hydrolysis of 4 with 1 N HCl **4** (8 mg) was dissolved in 1 N HCl (4 ml) and the solution was refluxed on a water bath for 2 h. After cooling, the reaction mixture was passed through an Amberlite IRA-67 (OH^- form) column (i.d. 2.0×5.0 cm) to neutralize it. The resulting solution was chromatographed on a Sep-Pak C-18 column (Waters), and elution with water afforded D-glucose (1.3 mg), $[\alpha]_D$ +40.5° (c=0.13, H_2O), which was identified by HPLC (t_R=12.2 min) [column, Shodex NH_2 P-50 4E (i.d. 4.6×250 mm); solvent, CH_3CN–H_2O (75 : 25); flow rate, 0.8 ml/min; detection, RI; column temperature, 40 °C], ^{1}H- and ^{13}C-NMR. Elution with MeOH afforded an aglycone, broussonetinine A (**4a**) (3 mg), as a colorless powder.

Enzyme Assays Materials α-Glucosidase (from Bakers yeast), β-galactosidase (from bovine liver), α-mannosidase (from Jack beans), and β-mannosidase (from snail acetone powder) were obtained from Sigma Chemical Company (St. Louis, U.S.A.), and β-glucosidase (from sweet almond) was obtained from Toyobo Company (Osaka, Japan). *p*-Nitrophenyl-α-D-glucopyranoside, -β-D-glucopyranoside, -α-D-mannopyranoside, and -β-D-galactopyranoside were obtained Nacalai Tesque, Inc (Osaka, Japan), *p*-nitrophenyl-β-D-mannopyranoside from Sigma Chemical Company, and DNJ, DGJ, and DMJ from Funakoshi Company (Tokyo, Japan).

Assay of β-Galactosidase Inhibition The reaction mixture consisted of 475 μl 0.1 M acetate buffer (pH 5.0), 250 μl 20 mM *p*-nitrophenyl-β-D-glucopyranoside and 250 μl β-galactosidase solution (a stock solution of 1.0 mg/ml in 10 mM acetate buffer (pH 5.0) was diluted 5 times with the same buffer, pH 5.0, just before assay), with the substrates **1**, **2**, **3**, **4** or DGJ (25 μl solution, concentration: 20—0.1 mg/ml). After incubation for 20 min at 37 °C, the reaction was interrupted by the addition of 1 ml 0.2 M sodium carbonate, and the amount of *p*-nitrophenol liberated was measured colorimetrically at 400 nm (ODtest). The rates of inhibition (%) were calculated from the formula 100−100×(ODtest−ODblank)/(control ODtest−control ODblank) and the IC_{50} values were obtained from the inhibition curves . The IC_{50} values were 8.1 μM for **1**, 0.17 μM for **2**, 0.20 μM for **3** and 0.60 μM for **4**, while that of DGJ was 0.13 μM. Assays of α-glucosidase, β-glucosidase, β-mannosidase and α-mannosidase were carried out as above using *p*-nitro-

phenyl-α-D-glucopyranoside, -β-D-glucopyranoside, -β-D-mannopyranoside and -α-D-mannopyranoside as substrates. The IC_{50} values are shown in Table 3.

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Phenolic Constituents of Licorice. VIII.[1] Structures of Glicophenone and Glicoisoflavanone, and Effects of Licorice Phenolics on Methicillin-Resistant *Staphylococcus aureus*

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Two new phenolic compounds, glicophenone (1) and glicoisoflavanone (2), were isolated from commercial licorice, and their structures were elucidated on the basis of spectroscopic data. Antibacterial assays of licorice phenolics for *Staphylococcus aureus*, including four strains of methicillin-resistant *S. aureus* (MRSA), and also for *Escherichia coli* K12 and *Pseudomonas aeruginosa* PAO1, were then examined. Two compounds among them, 8-(γ,γ-dimethylallyl)-wighteone (21) and 3′-(γ,γ-dimethylallyl)-kievitone (28), showed remarkable antibacterial effects [minimum inhibitory concentrations (MICs), 8 μg/ml] on the MRSA strains and methicillin-sensitive *S. aureus*. Licochalcone A (14), gancaonin G (20), isoangustone A (24), glyasperins C (30) and D (31), glabridin, (32), licoricidin (33), glycycoumarin (34) and licocoumarone (40) showed antibacterial effects on the MRSA strains with MIC values of 16 μg/ml. Effects on the β-lactam resistance of the MRSA strains were also examined, and licoricidin (33) noticeably decreased the resistance of the MRSA strains against oxacillin, as shown by the reduction in the MICs of oxacillin (lower than 1/128—1/1000 in the presence of 8 μg/ml of 33, and 1/8—1/32 in the presence of 4 μg/ml of 33). Mechanistic study suggested that 33 does not inhibit the formation of penicillin-binding protein 2′ (PBP2′), but affects the enzymatic function of PBP2′.

Key words licorice; glicophenone; glicoisoflavanone; licoricidin; methicillin-resistant *Staphylococcus aureus*; oxacillin

Much of the recent research on licorice constituents has indicated the pharmacological importance of phenolic compounds, together with saponins, in the medicinal use of licorice.[2—6] We also reported inhibitory effects on oxidative enzymes, radical-scavenging effects and the antiviral effect of licorice phenolics.[7]

Since the antibiotic-resistance of bacteria is one of the most serious problems in clinical medicine today, development of new drugs against the drug-resistant bacteria or suppression of the drug-resistance in bacteria is desired. Previously we reported that some low-molecular-weight phenolics (rhubarb anthraquinones and aglycones of naphthalene glycosides of cassia seeds) showed antibacterial effects on methicillin-resistant *Staphylococcus aureus* (MRSA).[8] On the other hand, the effects of licorice phenolics on various microbes have been reported.[9—13]

We therefore examined the effects of licorice phenolics on MRSA. In the course of this study, we isolated two new compounds named glicophenone (**1**) and glicoisoflavanone (**2**), along with known compounds, and found that various licorice phenolics have potent antibacterial effects on MRSA and methicillin-sensitive *S. aureus* (MSSA). Among the compounds which showed potent antibacterial effects on the MRSA strains, an isoflavan noticeably reduced the resistance of MRSA against a β-lactam antibiotic oxacillin. This paper deals with the structural elucidation of the new compounds, and the antibacterial effects of licorice phenolics, especially those on MRSA.

Results and Discussion

Structures of Glicophenone and Glicoisoflavanone The licorice used in this study is a commercial variety, and its source plant was tentatively assigned to be *Glycyrrhiza uralensis* on the basis of similarity of the high-performance liquid chromatography (HPLC) profile to that reported for *G. uralensis*.[14] An ethyl acetate extract from the licorice was subjected to centrifugal partition chromatography (CPC)[15] and/or column chromatography, and the fractions were further purified by preparative TLC or preparative HPLC to give glicophenone (**1**), glicoisoflavanone (**2**), and 32 known compounds.

Glicophenone (**1**) was obtained as colorless needles. High-resolution electron-impact (EI) MS indicated its molecular formula, $C_{20}H_{22}O_6$. The UV spectrum is similar to that of licoriphenone (**3**).[12] The ^{1}H-NMR spectrum indicated the presence of tri-substituted [δ: 6.31 (1H, d, J=2.5 Hz; H-3), 6.43 (1H, dd, J=2.5, 8.5 Hz; H-5), 8.01 (1H, d, J=8.5 Hz; H-6)] and penta-substituted [δ: 6.29 (1H, s; H-5′)] benzene rings. The spectrum also showed signals due to methylene [δ: 4.21 (2H, s; H-8)] and methoxyl protons [δ: 3.62 (3H, s)], along with those attributable to a γ,γ-dimethylallyl group [δ: 3.25 (2H, br d, J=7 Hz; H-1″), 5.18 (1H, br t, J=7 Hz; H-2″), 1.63, 1.73 (3H each, br s; *gem*-dimethyl at C-3″)]. The pattern of these signals is very similar to that for licoriphenone (**3**), except for the number of methoxyl signals. The EI-MS showed fragment ion peaks at *m/z* 137 and 221, indicating the presence of a methoxyl group on the B-ring (Fig. 1). The ^{13}C chemical shifts of B-ring carbons [δ: 107.9 (C-1′), 159.5 (C-2′), 114.0 (C-3′), 156.2 (C-4′), 99.7 (C-5′), 155.3 (C-6′)] of **1** were similar to those of the corresponding carbons of glicoricone (**4**)[16] [δ: 106.2 (C-1′), 159.1 (C-2′), 114.2 (C-3′), 157.7 (C-4′), 100.9 (C-5′), 156.6 (C-6′)], suggesting the same substitution pattern of this benzene ring as that of **4**. The nuclear Overhauser effect spectroscopy (NOESY) measurement of **1** showed cross peaks due to the nuclear Overhauser effects (NOEs) of the methoxyl group with H-8 (methylene), H-1″ and H-2″ (γ,γ-dimethylallyl group), as indicated by the arrows in the formula. The loca-

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tion of the methoxyl group in **1** was thus determined to be at C-2′.

Glicoisoflavanone (**2**) was obtained as colorless needles. Its molecular formula $C_{22}H_{24}O_6$ was indicated by its high-resolution EI-MS. The UV spectrum was characteristic of isoflavanone. The ^{1}H-NMR spectrum also showed signals of the isoflavanone skeleton as follows: δ: 4.38 (1H, dd, J=6, 13 Hz; H-2), 4.90 (1H, dd, J=10.5, 13 Hz; H-2), 4.33 (1H, dd, J=6, 10.5 Hz; H-3) (C-ring); 7.76 (1H, d, J=2.5 Hz; H-5), 6.56 (1H, dd, J=2.5, 8.5 Hz; H-6), 6.40 (1H, d, J=8.5 Hz; H-8) (tri-substituted benzene ring); 6.33 (1H, s; H-5′) (penta-substituted benzene-ring). Signals due to a γ,γ-dimethylallyl group [δ: 3.25 (2H, m; H-1″), 5.18 (1H, br t, J=5 Hz; H-2″), 1.64, 1.73 (3H each, br s; *gem*-dimethyl at C-3″)] and two methoxyl groups [δ: 3.66, 3.74 (3H each, s)] were also shown. The fragment ions *m/z* 136 and 247 shown in the EI-MS suggested that the tri-substituted and penta-substituted benzene rings are respectively attributed to the A and B rings of the isoflavanone structure (Fig. 1), and the two methoxyl groups are on the B-ring. The ^{13}C chemical shifts of the A-ring [δ: 115.9 (C-4a), 130.0 (C-5), 110.9 (C-6), 164.7 (C-7), 103.5 (C-8), 164.5 (C-8a)] and B-ring [δ: 108.9 (C-1′), 159.7 (C-2′), 114.9 (C-3′), 159.2 (C-4′), 96.5 (C-5′), 155.7 (C-6′)] carbons of **2** are closely similar to the corresponding carbons of the A-ring of 4′-methoxy-7-hydroxyisoflavanone (**5**)[17] [δ: 115.3 (C-4a), 130.2 (C-5), 111.4 (C-6), 165.1 (C-7), 103.4 (C-8), 164.4 (C-8a)] and the B-ring of licoriphenone (**3**) [δ: 108.6 (C-1′), 159.2 (C-2′), 114.9 (C-3′), 158.7 (C-4′), 96.1 (C-5′), 155.7 (C-6′), in acetone-d_6], respectively. The NOESY spectrum of **2** showed cross peaks of the methoxyl signal at δ 3.66 with H-2 (C-ring), H-1″ and H-2″ (γ,γ-dimethylallyl group) (indicated by the arrows in the formula). On the other hand, the methoxyl signal at δ 3.74 showed cross peaks with H-5′ and protons of the γ,γ-dimethylallyl group in the NOESY spectrum. These NOEs substantiated the substitution pattern of the B-ring. The circular dichroism (CD) spectrum of **2** showed a positive Cotton effect at 328 nm, indicating[18] the *R*-configuration at C-3. Structure **2** was thus assigned to glicoisoflavanone.

Antibacterial Effects of Licorice Phenolics on MRSA and MSSA Antibacterial effects on four strains of MRSA and a strain of MSSA, and also on *Escherichia coli* K12 and *Pseudomonas aeruginosa* PAO1, were evaluated for licorice phenolics of various types using the liquid dilution method.[8] Minimum inhibitory concentrations (MICs) of the tested compounds are shown in Table 1.

Flavanones and Chalcones Among the tested compounds, flavanones **6**—**9** did not show antibacterial effects on MRSA and MSSA (MIC >128 μg/ml). Most of the chalcones such as licochalcone B (**15**) and tetrahydroxymethoxychalcone (**12**) showed weak or negligible effects (MIC 64, 128 or >128 μg/ml). However, licochalcone A (**14**) showed antibacterial effects on both MRSA and MSSA with MICs of 16 μg/ml.

Isoflavones, Isoflavanones and Isoflavans An isoflavone, 8-(γ,γ-dimethylallyl)-wighteone (**21**), and an isoflavanone, 3′-(γ,γ-dimethylallyl)-kievitone (**28**), showed potent antibacterial effects on MRSA and MSSA (MIC 8 μg/ml). Isoflavones, gancaonin G (**20**) and isoangustone A (**24**), and isoflavans, glyasperins C (**30**) and D (**31**), glabridin (**32**) and licoricidin (**33**), showed antibacterial effects on MRSA and MSSA with MICs of 16 μg/ml, while isowighteone (**22**) (isoflavone) had MICs of 16 and 32 μg/ml for MSSA and MRSA, respectively. The MICs of licoisoflavanone (**27**) (isoflavanone) for MRSA and MSSA were 32 μg/ml. The other isoflavones, isoflavanones and isoflavans, including glicoisoflavanone (**2**), showed MICs of 32—128 μg/ml for MRSA and MSSA, or negligible effects (MIC >128 μg/ml).

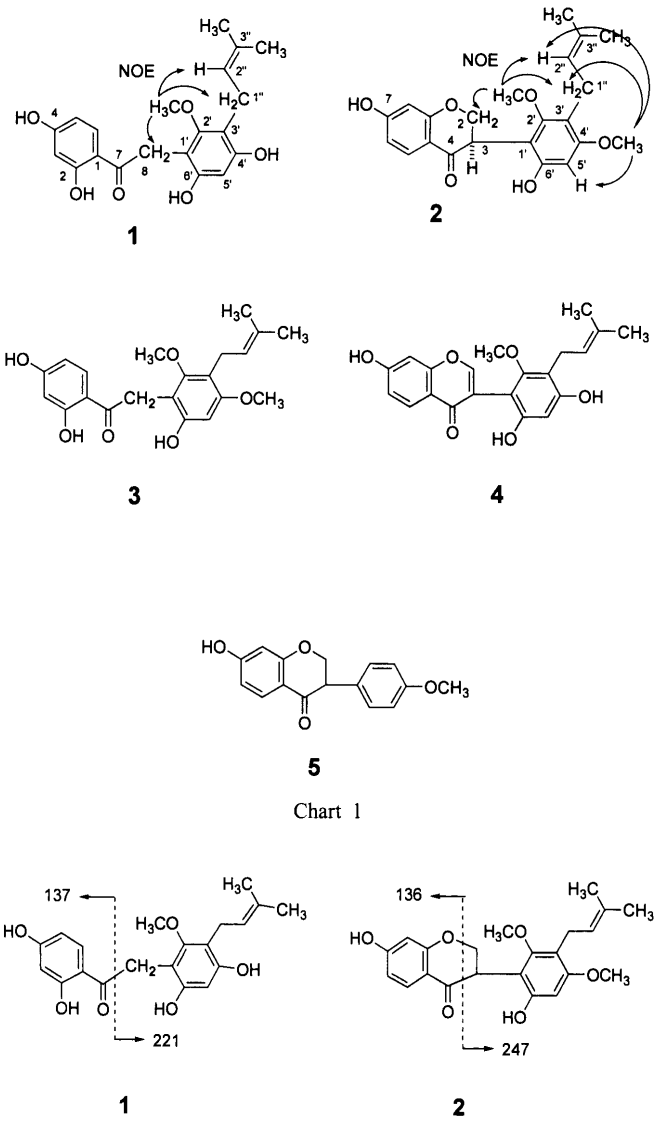

Chart 1

Fig. 1. Mass Fragmentation of **1** and **2** in Their EI-MS

3-Arylcoumarins and Others Among the 3-arylcoumarins and other phenolic compounds, glycycoumarin (**34**), licocoumarone (**40**) (MIC 16 μg/ml), licoarylcoumarin (**36**), licoriphenone (**3**) (MIC 16—32 μg/ml), and glicophenone (**1**) (MIC 32 μg/ml) showed antibacterial effects on MSSA and MRSA.

Structure–Activity Relationships Antibacterial effects of flavanones isolated from leguminous plants on MRSA have been reported,[19] and potent anti-MRSA activity was correlated with the presence of an aliphatic or lavandulyl group, in addition to the substitution pattern of the phenolic hydroxyl groups. Compounds **21** and **28**, which showed MIC values of 8 μg/ml for MSSA and MRSA, have two γ,γ-dimethylallyl groups, and all of the compounds with the MICs of 16 μg/ml have at least one γ,γ-dimethylallyl or equivalent (α,α-dimethylallyl or dimethylpyrane) group. On the other

Table 1. MICs of Licorice Phenolics for MRSA, MSSA, *Escherichia coli* and *Pseudomonas aeruginosa* (μg/ml)

Compounds	MRSA OM481	MRSA OM505	MRSA OM584	MRSA OM623	MSSA 209P	*E. coli* K12	*P. aeruginosa* PAO1
Flavanones							
Liquiritigenin (**6**)	>128	>128	>128	>128	>128	>128	>128
Liquiritin (**7**)	>128	>128	>128	>128	>128	>128	>128
6″-*O*-Acetylliquiritin (**8**)	>128	>128	>128	>128	>128	>128	>128
Naringenin (**9**)	>128	>128	>128	>128	>128	>128	>128
Chalcones							
Isoliquiritin apioside (**10**)	>128	>128	>128	>128	>128	>128	>128
Isoliquiritin (**11**)	>128	>128	>128	>128	>128	>128	>128
Tetrahydroxymethoxychalcone (**12**)	>128	>128	>128	>128	>128	>128	>128
Echinatin (**13**)	128	64	64	64	64	>128	>128
Licochalcone A (**14**)	16	16	16	16	16	>128	>128
Licochalcone B (**15**)	128	128	128	128	128	>128	>128
Isoliquiritigenin (**16**)	128	128	128	128	128	>128	>128
Isoflavones							
Glycyrrhisoflavone (**17**)	64	64	32	32	32	>128	>128
Semilicoisoflavone B (**18**)	64	64	64	32	32	>128	>128
Genistein (**19**)	>128	>128	>128	>128	>128	>128	>128
Glicoricone (**4**)	64	64	64	64	64	>128	>128
Gancaonin G (**20**)	16	16	16	16	16	>128	>128
8-(γ,γ-Dimethylallyl)-wighteone (**21**)	8	8	8	8	8	>128	>128
Isowighteone (**22**)	32	32	32	32	16	>128	>128
Glisoflavone (**23**)	64	64	64	64	64	>128	>128
Isoangustone A (**24**)	16	16	16	16	16	>128	>128
Isoflavanones							
Glycyrrhisoflavanone (**25**)	64	64	32	32	32	>128	>128
Glyasperin F (**26**)	64	64	64	64	32	>128	>128
Licoisoflavanone (**27**)	32	32	32	32	32	>128	>128
Glicoisoflavanone (**2**)	64	64	32	32	32	>128	>128
3′-(γ,γ-Dimethylallyl)-kievitone (**28**)	8	8	8	8	8	>128	>128
Isoflavans							
(3*R*)-Vestitol (**29**)	128	128	128	128	128	>128	>128
Glyasperin C (**30**)	16	16	16	16	16	>128	>128
Glyasperin D (**31**)	16	16	16	16	16	>128	>128
Glabridin (**32**)	16	16	16	16	16	>128	>128
Licoricidin (**33**)	16	16	16	16	16	>128	>128
3-Arylcoumarins							
Glycycoumarin (**34**)	16	16	16	16	16	>128	>128
Licopyranocoumarin (**35**)	>128	>128	128	128	>128	>128	>128
Licoarylcoumarin (**36**)	32	32	32	32	16	>128	>128
Glycyrin (**37**)	128	128	128	128	128	>128	>128
Isolicopyranocoumarin (**38**)	>128	>128	>128	>128	>128	>128	>128
Glycyrin permethyl ether (**39**)	>128	>128	>128	>128	>128	>128	>128
Others							
Licocoumarone (**40**)	16	16	16	16	16	>128	>128
Glicophenone (**1**)	32	32	32	32	32	>128	>128
Licoriphenone (**3**)	32	32	32	16	16	>128	>128
Glycyrol (**41**)	>128	>128	>128	>128	>128	>128	>128
Isoglycyrol (**42**)	>128	>128	>128	>128	>128	>128	>128

hand, the glycosides tested exhibited negligible effects on MRSA and MSSA. These results implied participation of their lipophilicity in the antibacterial effects of the phenolic compounds on *S. aureus* strains.

The order of strength of the antibacterial activity, glycycoumarin (**34**)>glycyrin (**37**)>glycyrin permethyl ether (**39**), suggested requirements of phenolic hydroxyl groups in the molecule for the antibacterial effects. A difference in the antibacterial activity between glycycoumarin (**34**) (MIC 16 μg/ml) and glycyrol (**41**) (MIC >128 μg/ml) is also attributable to the difference in the number of phenolic hydroxyl groups. However, in this case, rigidity in the bond between the coumarin structure and B-ring of glycyrol may be related to the decrease of the activity.

Antibacterial properties of various types of licorice phenolics described above may suggest some usefulness of licorice in the treatment of diseases responsible for *S. aureus*, at least in the intestines.

Effects of Licorice Phenolics on *E. coli* and *P. aeruginosa* None of the tested compounds showed antibacterial effects on *E. coli* K12 and *P. aeruginosa* PAO1 (MIC >128 μg/ml).

Effects of Licorice Phenolics on the Resistance of MRSA against Oxacillin Recently, the suppression of bacterial resistance against β-lactam antibiotics by several phenolic compounds,[20—22] including tea catechins and hydrolyzable tannins, have been shown. We therefore examined the effects of licorice phenolics on the MICs of oxacillin for MRSA.

Oxacillin in the absence of the phenolic compounds

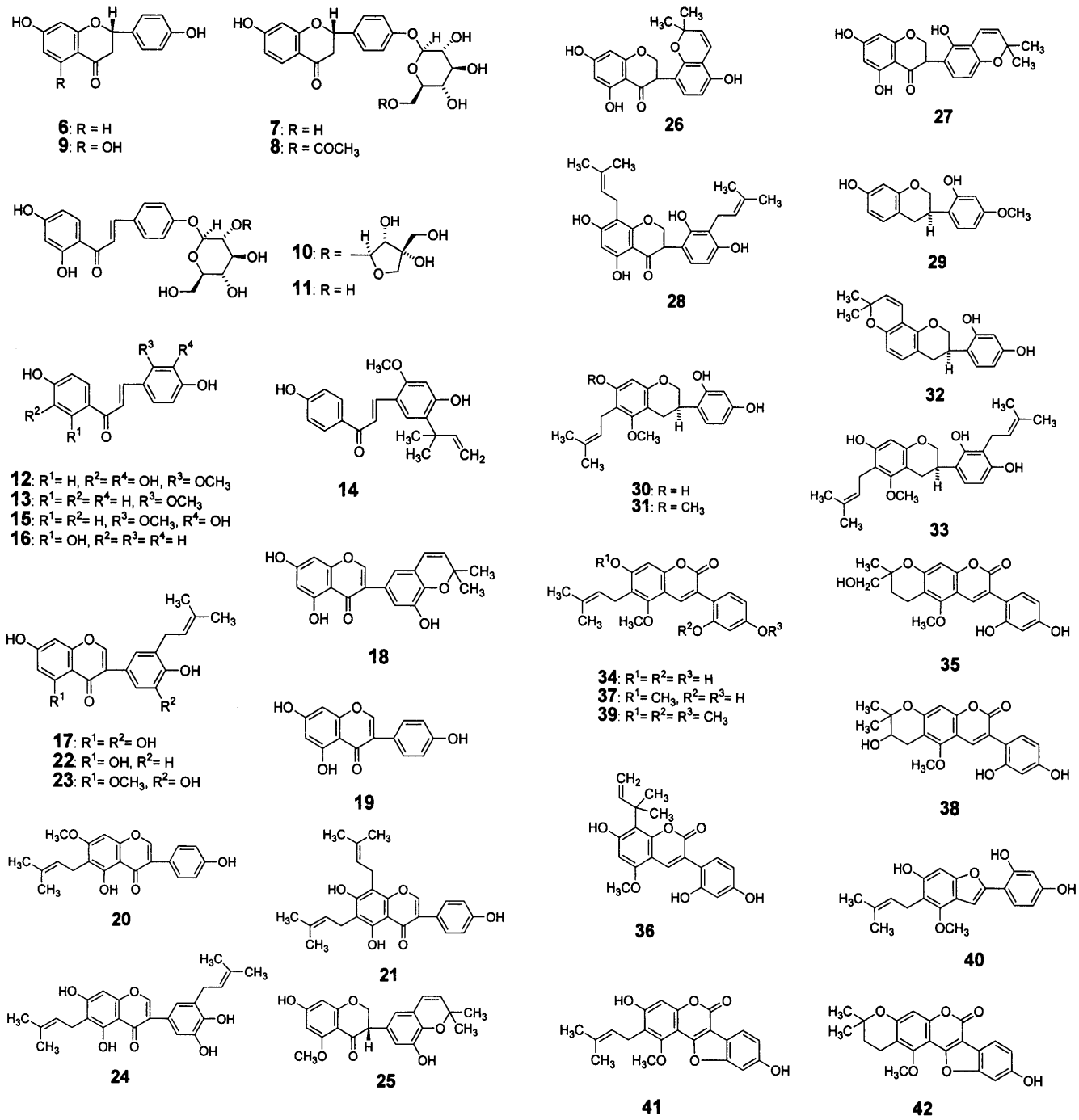

Chart 2

showed MICs of 64—512 μg/ml for the four MRSA strains, while the MIC for MSSA 209P was <0.5 μg/ml. However, in the presence of 16 μg/ml of glicophenone (**1**), one of the newly isolated compounds, the MICs of oxacillin for the MRSA strains decreased to 1/2—1/8 of the values in the absence of glicophenone (**1**). Isowighteone (**22**) (16 μg/ml) reduced the MICs of oxacillin to 1/4—1/8, and isoangustone A (**24**) reduced them to 1/2—1/4. Other tested licorice phenolics, except for glycycoumarin (**34**), had an analogous effect on at least two MRSA strains (Table 2).

However, the effects of licoricidin (**33**) were much stronger. In the presence of 8 μg/ml of licoricidin, the MICs of oxacillin decreased to lower than 1/128—1/1000 of the values in the absence of the compound. Even the presence of 4 μg/ml of licoricidin decreased the MICs of oxacillin to 8—16 μg/ml.

The effect of licoricidin (**33**) on the growth curve of one of the MRSA strains, OM481, was then examined. As shown in Fig. 2, the amount of the bacterium after 24 h incubation in the presence of both of oxacillin (10 μg/ml) and licoricidin (8 μg/ml) was about 1/100 of that in the absence of them (control), while oxacillin alone (10 μg/ml) or licoricidin alone (8 μg/ml) did not cause such an inhibition of the bacterial growth.

In order to clarify the mechanism of the reduction of MICs of oxacillin, the effect of licoricidin (**33**) on the formation of penicillin-binding protein 2′ (PBP2′) was examined, since the formation of the enzymatic protein PBP2′, which cat-

alyzes cell wall construction, causes the resistance of MRSA against the β-lactams.

The MRSA strain OM481 was incubated in the presence of licoricidin at a concentration of 8 μg/ml, where licoricidin showed the reduction of the MICs of oxacillin. After the incubation of MRSA, the bacterium was subjected to the slide latex agglutination assay to examine whether PBP2′ was formed.

As a result, the agglutination due to the formation of PBP2′ was observed analogously to that in the absence of licoricidin or in the presence of oxacillin (1 μg/ml) (Fig. 3). Therefore, this compound restored the antibacterial effect of oxacillin without affecting the PBP2′ formation. Although the mechanism for the restoring effect is still unclear, licoricidin may affect the enzymatic function of PBP2′. Assuming that the other PBPs are still available for the bacteria in the presence of licoricidin, oxacillin might work well leading the marked decrease of its MIC. However, other possible mechanisms such as an increase in the affinity of oxacillin to PBP2′ by licoricidin may not be excluded.

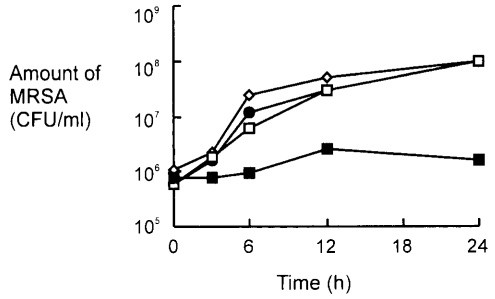

Fig. 2. Growth Curves for MRSA OM481 in the Absence (—◇—) and in the Presence of Oxacillin (1 μg/ml) (—●—), Licoricidin (8 μg/ml) (—□—) or Oxacillin (1 μg/ml) Plus Licoricidin (8 μg/ml) (—■—)

Experimental

^{1}H- and ^{13}C-NMR spectra were measured on a Varian VXR-500 instrument (500 MHz for ^{1}H and 125.7 MHz for ^{13}C) in acetone-d_6. Chemical shifts are given in δ values (ppm) based on the chemical shifts of solvent signals (δ_H 2.04, δ_C 29.8).

Isolation of Phenolic Compounds from Licorice Licorice (2 kg) (purchased from Tochimoto-tenkai-do, Osaka) was pulverized and extracted with *n*-hexane (6 l×3) and ethyl acetate (6 l×3), successively. The ethyl acetate extract (87 g) was subjected to counter-current distribution ($CHCl_3$–MeOH–H_2O, 7 : 13 : 8, n=3, r=3) to separate six fractions, S1—S6. A portion (20 g) of fraction S6 (the fraction containing compounds of the lowest polarity among the six fractions) (75.9 g) was subjected to CPC ($CHCl_3$–MeOH–H_2O, 7 : 13 : 8, reversed-phase development). Fractions

Table 2. Effects of Licorice Phenolics on the MICs of Oxacillin for MRSA Strains (μg/ml)

Compounds	MRSA OM481	MRSA OM505	MRSA OM584	MRSA OM623	MSSA 209P
Oxacillin alone	512	64	256	512	<0.5
Oxacillin plus					
Chalcones					
Licochalcone A (**14**) (8 μg/ml)	128	128	64	128	<0.5
Licochalcone B (**15**) (64 μg/ml)	128	64	16	64	<0.5
Isoflavones					
Glicoricone (**4**) (32 μg/ml)	512	64	64	128	<0.5
Isowighteone (**22**) (16 μg/ml)	64	16	64	64	<0.5
Glisoflavone (**23**) (32 μg/ml)	128	64	32	128	<0.5
Isoangustone A (**24**) (8 μg/ml)	256	32	128	128	<0.5
Isoflavanones					
3′-(γ,γ-Dimethylallyl)-kievitone (**28**) (4 μg/ml)	256	64	64	256	<0.5
Isoflavans					
Glabridin (**32**) (8 μg/ml)	256	128	128	128	<0.5
Licoricidin (**33**) (8 μg/ml)	<0.5	<0.5	<0.5	<0.5	<0.5
Licoricidin (**33**) (4 μg/ml)	16	8	16	16	<0.5
3-Arylcoumarins					
Glycycoumarin (**34**) (8 μg/ml)	1024	64	256	256	<0.5
Others					
Glicophenone (**1**) (16 μg/ml)	256	32	64	64	<0.5

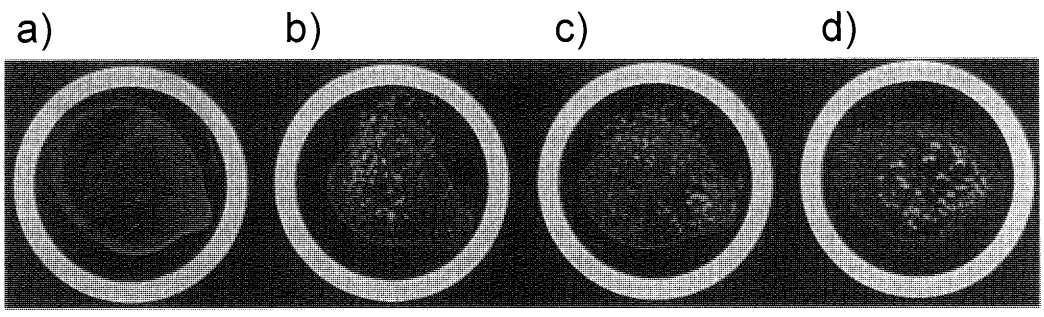

Fig. 3. Formation of PBP2′ in MRSA OM481 in the Absence and in the Presence of Oxacillin or Licoricidin

a) MSSA 209P, b) MRSA OM481 in the absence of oxacillin and licoricidin, c) MRSA OM481 in the presence of oxacillin (1 μg/ml), d) MRSA OM481 in the presence of licoricidin (8 μg/ml). Detection of PBP2′ was effected with anti-PBP2′ monoclonal antibody-sensitized latex (MRSA Screen, Denka Seiken). The MSSA 209P did not show the formation of PBP2′, while the other three showed the formation of PBP2′.

from CPC were chromatographed on Fuji gel ODS G3 and MCI gel CHP-20P, and further purified by preparative TLC on silica gel and preparative HPLC [YMC-A324 (5 μm, 10 mm i.d.×300 mm), CH_3CN–H_2O–AcOH (60 : 35 : 5)] to give glycycoumarin (**34**),[23] glisoflavone (**23**),[24] glycyrol (**41**),[25] licoisoflavanone (**27**),[26] isowighteone (**22**),[27] glycyrin (**37**),[28] isoangustone A (**24**),[12] glyasperin D (**31**),[29] glicophenone (**1**), licoricidin (**33**)[30] and 3′-(γ,γ-dimethylallyl)-kievitone (**28**).[31] A part of fraction S6 was chromatographed on YMC-gel SIL-120-S50 and Fuji gel ODS G3, then purified by preparative TLC to give glyasperin F (**26**).[32] Yields of the phenolics from the ethyl acetate extract were: **34** (0.26%), **23** (0.059%), **41** (0.19%), **27** (0.023%), **22** (0.009%), **37** (0.012%), **24** (0.18%), **31** (0.029%), **1** (0.004%), **33** (0.15%), **28** (0.072%) and **26** (0.026%).

Analogous treatments of commercial licorice in separate experiments gave isoglycyrol (**42**)[25] (0.008% from an ethyl acetate extract), licoriphenone (**3**)[12] (0.016%), glycyrrhisoflavanone (**25**)[23] (0.015%), glycyrrhisoflavone (**17**)[23] (0.052%), glicoisoflavanone (**2**) (0.007%), liquiritigenin (**6**)[33] (0.006%), glyasperin C (**30**)[29] (0.014%), licopyranocoumarin (**35**)[24] (0.032%), glicoricone (**4**)[16] (0.001%), semilicoisoflavone B (**18**)[1] (0.29%), liquiritin (**7**)[34] (0.21%), isoliquiritin apioside (**10**)[35] (0.016%), isoliquiritin (**11**)[35] (0.019%), 6″-*O*-acetylliquiritin (**8**)[36] (0.022%), tetrahydroxymethoxychalcone (**12**)[1] (0.007%), naringenin (**9**)[1] (0.023%), genistein (**19**)[16] (0.016%), echinatin (**13**)[16] (0.021%), licocoumarone (**40**) (0.022%),[24] (3*R*)-vestitol (**29**)[1] (0.024%), gancaonin G (**20**)[37] (0.042%), 8-(γ,γ-dimethylallyl)-wighteone (**21**)[38] (0.008%), in addition to the compounds described above.

Glicophenone (**1**): Colorless needles, mp 145 °C. EI-MS *m/z*: 358 (M^+, 38%), 221 (74%), 165 (24%), 137 (100%). High-resolution EI-MS *m/z*: 358.1460 (M^+; Calcd for $C_{20}H_{22}O_6$, *m/z* 358.1416). UV λ_{max}^{MeOH} nm (log ε): 209 (4.64), 230 (sh, 4.21), 276 (4.13), 313 (3.63). ^{1}H-NMR: see text. ^{13}C-NMR δ: 17.9 (CH_3 at C-3″), 23.5 (C-1″), 25.8 (CH_3 at C-3″), 34.4 (C-8), 61.6 (OCH_3), 99.7 (C-5′), 103.5 (C-3), 107.9 (C-1′), 108.6 (C-5), 112.4 (C-1), 114.0 (C-3′), 125.4 (C-2″), 130.3 (C-3″), 133.6 (C-6), 155.3 (C-6′), 156.2 (C-4′), 159.5 (C-2′), 165.3 (C-2), 166.1 (C-4), 204.3 (C-7).

Glicoisoflavanone (**2**): Colorless needles, mp 102 °C. $[\alpha]_D$ −3° (*c*=2, MeOH). CD (MeOH) [θ] (nm): +9200 (215), +5800 (227), −6500 (300), +3500 (328). EI-MS *m/z*: 384 (M^+, 40%), 366 ($[M-H_2O]^+$, 100%), 247 (5%), 136 (7%), 115 (21%). High-resolution EI-MS *m/z* 384.1630 (M^+; Calcd for $C_{22}H_{24}O_6$, 384.1573). ^{1}H-NMR: see text. ^{13}C-NMR δ: 17.9 (CH_3 at C-3″), 23.6 (C-1″), 25.8 (CH3 at C-3″), 45.3 (C-3), 55.8 ($2OCH_3$ at C-4′), 62.3 ($-OCH_3$ at C-6′), 70.6 (C-2), 96.5 (C-5′), 103.5 (C-8), 108.9 (C-1′), 110.9 (C-6), 114.9 (C-3′), 115.9 (C-4a), 125.1 (C-2″), 130.0 (C-5), 130.6 (C-3″), 155.7 (C-6′), 159.2 (C-4′), 159.7 (C-2′), 164.5 (C-8a), 164.7 (C-7), 191.2 (C-4).

Estimation of Antibacterial Effects of Licorice Phenolics on MRSA Strains Four MRSA strains used in this study are clinical isolates from Okayama University hospital.[21] Phenolic compounds of which the isolation procedure is not described here, licochalcones A (**14**)[23] and B (**15**),[23] isoliquiritigenin (**16**),[1] glabridin (**32**),[11] licoarylcoumarin (**36**)[24] and isolicopyranocoumarin (**38**),[1] were obtained as described in previous reports. Glycyrin permethyl ether (**39**)[28] was prepared from glycycoumarin (**34**). The MICs of tested compounds for the bacterial strains were determined using 10^4 colony forming unit (CFU)/well of bacterial solution on 96-well plates in a way reported previously.[8]

Effects of Licorice Phenolics on the MICs of Oxacillin for MRSA Strains In the presence of each phenolic compound at concentrations lower than its MIC value, the lowest concentration of oxacillin which did not cause turbidity due to bacterial proliferation was estimated in a way analogous to that described above.

Effects of the Addition of Licoricidin on the Inhibitory Activity of Oxacillin against the Growth of MRSA OM481 The MRSA OM481 strain, which was maintained in the laboratory of the Department of Microbiology, was precultured overnight in a Mueller–Hinton (MH) medium containing Ca^{2+} (50 mg/l) and Mg^{2+} (25 mg/l) ions. A bacterial solution (0.2 ml) of absorbance 0.6—0.7 at 650 nm, prepared upon incubation of the precultured bacteria, was diluted with the MH medium (1.8 ml), and 50 μl portions of the solution were then added to the MH medium (5 ml each) in test tubes. The bacterial solution (*ca.* 10^6 CFU/ml) in the tubes was incubated with and without licoricidin (**33**) and/or oxacillin at 32 °C for 24 h. The bacteria in each tube was incubated on Nutrient agar plates at 32 °C for 24 h to estimate the amounts of the bacteria.[21]

Detection of PBP2′ in MRSA OM481 in the Presence of Oxacillin or Licoricidin A portion (0.2 ml) of the precultured solution of MRSA OM481 was added to the MH medium (4.8 ml) containing Ca^{2+} (50 mg/l) and Mg^{2+} (25 mg/l) ions, and the solution was incubated until the absorbance at 650 nm attained 0.6—0.7 in the presence of oxacillin (1 μg/ml) or licoricidin (8 μg/ml). The bacterial solution was then centrifuged at 10000 rpm for 5 min, and the precipitated bacteria was washed with 0.05 M phosphate buffer (pH 7.0) twice. A part of the bacterial mass was extracted with 1 M NaOH (0.2 ml) in a boiling-water bath for 3 min, then left to stand at room temperature. A solution of 0.5 M KH_2PO_4 (0.05 ml) was added, and the mixture was centrifuged at 4500 rpm for 5 min. The supernatant was diluted with 9-fold water, and 50 μl of the resulting solution was treated with anti-PBP2′ monoclonal antibody-sensitized latex (MRSA Screen, Denka Seiken Co.) (15 μl) on a test card for 3 min to show the presence/absence of PBP2′ by agglutination on the card.[22]

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Interactions between Local Anesthetics and Na^+ Channel Inactivation Gate Peptides in Phosphatidylserine Suspensions as Studied by ^{1}H-NMR Spectroscopy

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Interactions between local anesthetics and a sodium channel inactivation gate peptide (Ac–GGQDIFMTEEQK–NH_2, MP-1A), which was dissected from the cytoplasmic linker between domains III and IV of the sodium channel α-subunit (G1484—K1495 in rat brain type IIA), have been studied by ^{1}H-NMR spectroscopy. Changes in ^{1}H-NMR chemical shifts of the aromatic proton resonances of dibucaine (pH 7.0) and lidocaine (pH 6.0 and 9.0) in phosphatidylserine (PS) suspensions were observed. The effects of substitution of glutamine (F1489Q; MP-2A) or D-phenylalanine (MP-1A′) for L-phenylalanine (F1489) in MP-1A and the effects of substitution of neutral amino acid residues for the corresponding acidic amino acid residues (D1487N, MP-1NA; E1492Q, MP-1QEA; E1493Q, MP-1EQA) in MP-1A, on the aromatic ^{1}H-NMR cheimcal shift changes of dibucaine and lidocaine were also investigated. From these results it was concluded that: the aromatic ring of phenylalanine of MP-1A and the aromatic ring of the cationic form of dibucaine or lidocaine are interacting by π–π stacking; the tertiary amine nitrogen of dibucaine is interacting electrostatically with D1487, whereas that of lidocaine is interacting with E1492.

Key words ^{1}H-NMR; dibucaine; lidocaine; phosphatidylserine; Na^+ channel; inactivation gate peptide

Local anesthetics are chemicals that block action potentials in excitable membranes.[1,2] Their receptor sites are now considered to be within a sodium channel α-subunit.[3,4] This hypothesis is called a specific receptor theory.[4] However, there still remains the hypothesis that considers the receptor site to be located within the lipid membrane,[5,6] because a wide variety of different chemicals which include neutral and both negatively and positively charged molecules can act as local anesthetics.[5,7] There are good correlations between the membrane concentrations of anesthetics and their potencies,[8] suggesting that local anesthetics are binding at a hydrophobic region of the lipid membrane. This hypothesis is called a non-specific general perturbation theory.[4] Since both theories have their own rationalizations, a new concept which involves both theories is necessary for elucidating the molecular mechanisms of local anesthesia.

The sodium channel α-subunit consists of four homologous domains, I—IV, each with six transmembrane segments, S1—S6, (Fig. 1).[9] The cytoplasmic linker between domains III and IV of the sodium channel α-subunit (III–IV linker) is known to play a decisive role in a fast inactivation process.[10,11] The inactivation gate is considered to close by hydrophobic interactions between the three adjacent hydrophobic amino acids, Ile-Phe-Met (I1488-F1489-M1490, Fig. 1), and their receptors which are considered to be composed of the short S4–S5 loops of both domains III[12] and IV.[13—15] Bennett *et al.* have demonstrated that removal of the fast inactivation by mutations in the IFM motif to QQQ in human heart sodium channels (hH1) results in loss of a high-affinity inactivated-state block of the channel by lidocaine.[16] Their results suggest that the III–IV linker, especially the IFM motif, is a determinant of the local anesthetic binding site. In our previous paper,[17] we addressed the III–IV linker, especially the three adjacent hydrophobic amino acids (IFM) moiety as a docking site of charged amine type local anesthetics, because there exist negatively charged amino acids only on both sides of the IFM motif (D1487, E1492, E1493) in the III–IV linker (Fig. 1). We have proposed a new concept that can satisfy the requirements from both the specific receptor theory and the non-specific general perturbation theory: a local anesthetic molecule, located at the polar headgroup region of the so-called boundary lipids in the vicinity of the Na^+ channel pore, is also interacting by π-π stacking interactions with the phenylalanine residue in the III-IV linker, and by electrostatic interactions with one of the negatively charged amino acid residues.[17] We have studied interactions between a model peptide MP-1 (Ac–GGQDIFMTEEQK–OH), which was dissected from the III–IV linker (G1484–K1495, Fig. 1), and dibucaine (Fig. 2) by ^{1}H-NMR sepctroscopy. We found that the quinoline ring of dibucaine can interact with the aromatic ring of Phe by π–π stacking of the rings. However, we could not specify which negatively charged amino acid residues are interacting electrostatically with the tertiary amine nitrogen of dibucaine. In the present study, in order to specify the negatively charged amino acid residue, we have synthesized MP-1A (Ac–GGQDIFMTEEQK–NH_2), which was amidated at the C-terminus of MP-1, and some related peptides in which some acidic amino acid residues (Asp, Glu) are substituted by the corresponding neutral amino acid residues (Asn, Gln), and investigated the interactions with dibucaine in sonicated phosphatidylserine (PS) suspensions by ^{1}H-NMR spectroscopy. In order to obtain information on the role of the cationic charge at the tertiary amine nitrogen of a drug, we also studied the interactions between the peptides and lidocaine at both pH 6.0 (cationic form) and pH 9.0 (neutral form), since we could not use dibucaine under alkaline conditions on account of its low solubility.

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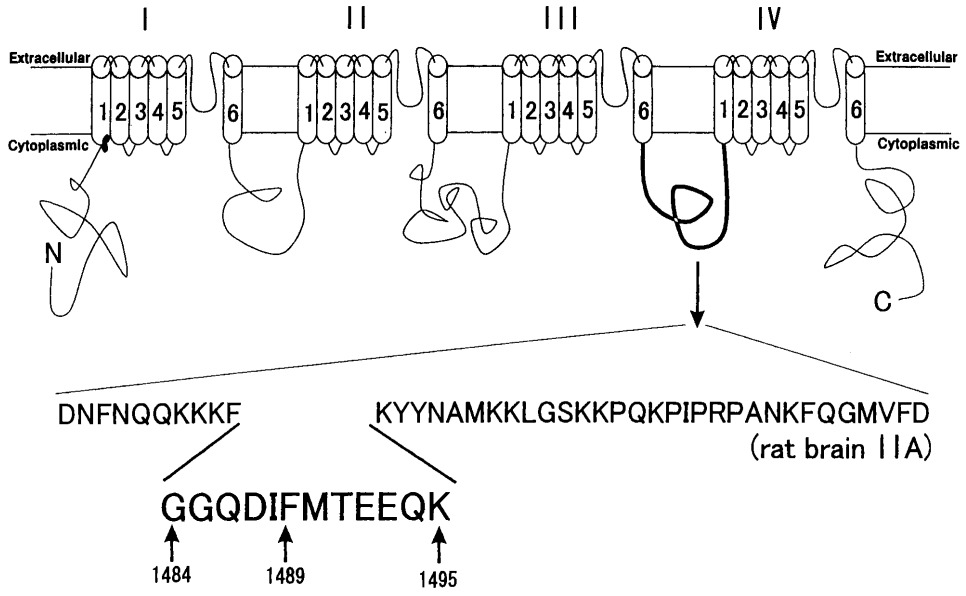

Fig. 1. Schematic Representation for the Sodium Channel α Subunit

The amino acid sequence of the cytoplasmic linker between domains III and IV of the rat brain type IIA sodium channel is shown at the bottom using one-letter symbol; the amino acid sequence corresponding to MP-1A is between G1484 and K1495.

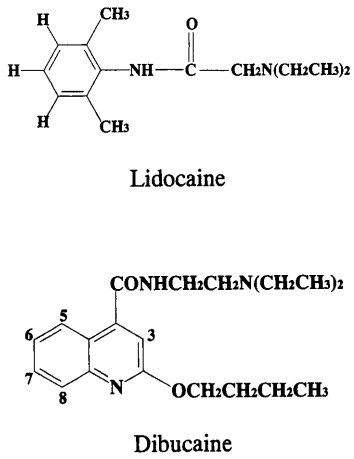

Fig. 2. Structures of Dibucaine and Lodocaine

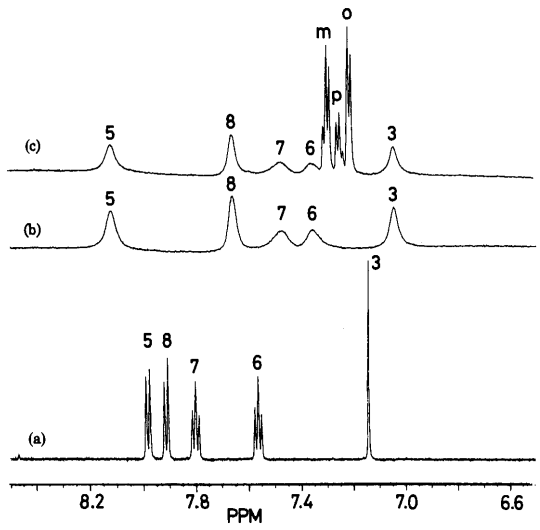

Fig. 3. ^{1}H-NMR Spectra of (a) Dibucaine in a Phsophate Buffer (pH 7.0), (b) Dibucaine in PS Suspensions (pH 7.0), and (c) Dibucaine and MP-1A in PS Suspensions (pH 7.0)

Experimental

Materials Dibucaine hydrochloride, lidocaine hydrochloride and bovine brain L-α-PS were obtained from Sigma and used without further purification. All the peptides were synthesized automatically by the solid phase method using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 433A peptide synthesizer; their N-termini were acetylated (denoted by Ac–) and their C-termini were amidated (denoted by–NH_2). They were purified on a reverse-phase C_{18} high-performance liquid chromatography column using a gradient of 90% A, 10% B to 70% A, 30% B, where A is 0.1% trifluoroacetic acid (TFA) in water and B is 0.1% TFA in acetonitrile; the rate of decrease in A was 20%/40 min. They were characterized by ion spray mass spectrometry. The amino acid sequences of the peptides were: MP-1A, Ac–GGQDIFMTEEQK–NH_2; MP-1A′, Ac–GGQDI[F]MTEEQK–NH_2 ([F] means D-phenylalanine); MP-2A, Ac–GGQDIQMTEEQK–NH_2; MP-1NA, Ac–GGQNIFMTEEQK–NH_2; MP-1QEA, Ac–GGQDIFMTQEQK–NH_2; MP-1EQA, Ac-GGQDIFMTEQQK-NH_2.

Preparation of Sample Solutions Single bilayer vesicles (liposomes) were prepared by ultrasonic irradiation of an isotonic (310 mOsm, 150 mM) phosphate buffer (in D_2O) suspension of dried PS for 20 min, cooling in an ice/water bath and bubbling with nitrogen gas. A weighed amount of a drug (3 mM) dissolved in the buffer and/or a peptide (3 mM) was added to the suspension of the pre-formed vesicles (15 mM).

Measurements The ^{1}H-NMR experiments were carried out on a Bruker AM-600 (600.13 MHz) spectrometer with a digital resolution of 0.25 Hz per point. Ambient probe temperature was 27 °C. Chemical shifts were referenced to internal TSP (3-trimethylsilylpropionic acid-d_4).

Results and Discussion

Figure 3 shows a typical example of ^{1}H-NMR spectra of (a) dibucaine in a phsophate buffer (pH 7.0), (b) dibucaine in PS suspensions (pH 7.0), and (c) dibucaine and MP-1A in PS suspensions (pH 7.0), respectively. At pH 7.0, most of the dibucaine molecules exist as a cationic form (pK_a=8.0).[18] Since the peaks due to the drug, PS, and the peptide overlapped with one another at a lower frequency region than that of the HDO resonance, we followed the changes in the chemical shifts of the aromatic proton resonances of the drug and the phenylalanine residue of the peptide. The assignments for the quinoline ring proton resonances were 3, 6, 7, 8, and 5, respectively, from low to high frequency.[17,19] The spin-coupled sharp resonances at around 7.2—7.3 ppm in Fig. 3c

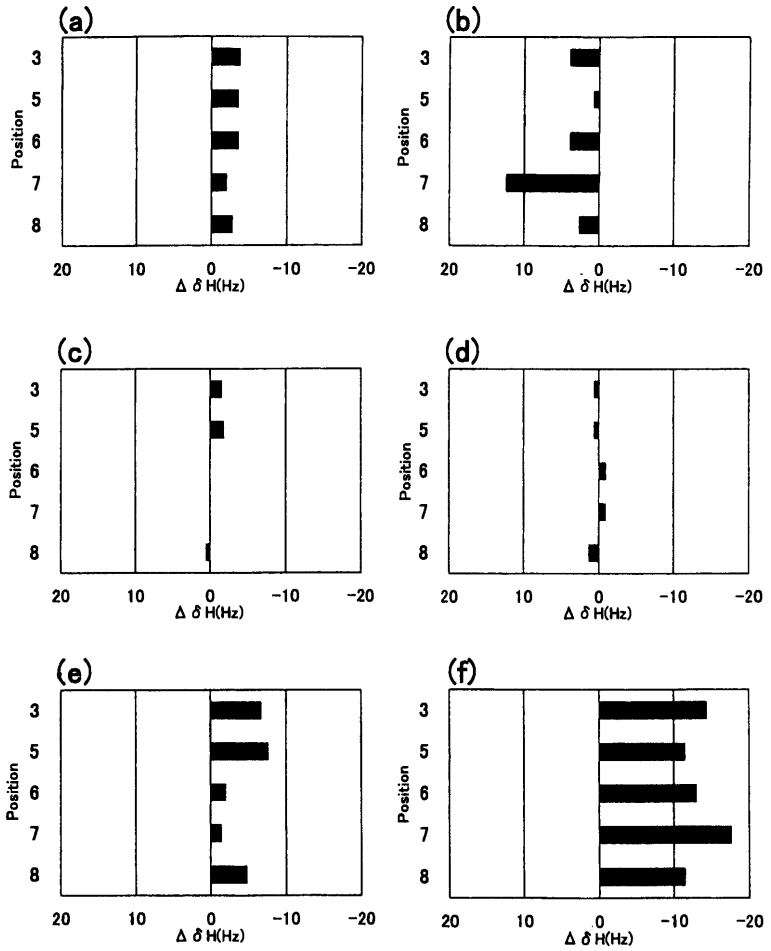

Fig. 4. Changes in Chemical Shifts (in Units of Hz at 600.13 MHz) of the Quinoline Ring Proton Resonances (at 3, 5, 6, 7, and 8 Positions) of Dibucaine as Caused by the Peptides

(a) MP-1A, (b) MP-2A, (c) MP-1A′, (d) MP-1NA, (e) MP-1QEA, and (f) MP-1EQA.

were due to the aromatic ring protons of the phenylalanine residue of MP-1A; the assignments were *ortho*, *para*, and *meta* protons from low to high frequency, respectively. The quinoline ring proton resonances were broadened by the presence of PS suspensions (Fig. 3b), indicating that dibucaine partitioned into the lipid bilayer. The resonance at the 5 position shifted to higher frequency, whereas all the remaining resonances (8, 7, 6, and 3 positions) shifted to lower frequency in PS suspensions. All these quinoline ring proton resonances shifted slightly to lower frequencies when MP-1A was added (spectrum c). Since in the PS suspensions, the resonance at the 5 position and those at the 8, 7, 6, and 3 positions shifted to the opposite direction with each other as compared to those in the phosphate buffer solution, the changes in the chemical shifts from spectrum b to spectrum c are not due to the change in the partition of dibucaine into the PS bilayers which might be caused by the peptide. Observed changes in chemical shifts (in units of Hz at 600.13 MHz) by MP-1A are summarized graphically in Fig. 4 together with the results by the other peptides.

It can be seen that: 1) the quinoline ring proton resonances of dibucaine were shifted to a low frequency by MP-1A (Fig. 4a), whereas to a high frequency by MP-2A (Fig. 4b); 2) MP-1A′ (Fig. 4c) caused smaller chemical shift changes than did MP-1A; 3) the magnitudes of the changes in chemical shifts were the smallest by MP-1NA (Fig. 4d), while the largest were by MP-1EQA (Fig. 4f); 4) MP-1EQA caused chemical shift changes more than twice as much as those done by MP-1A (Fig. 4a) or by MP-1QEA (Fig. 4e). Observation 1 suggests that the aromatic ring of the phenylalanine residue of MP-1A and the quinoline ring of dibucaine are interacting by π–π stacking with each other. Observation 2 supports this view and suggests that to interact by the π–π stacking, an appropriate stereochemical configuration is required. In our previous paper,[17] we also deduced this π–π stacking interaction by comparing the changes in chemical shifts of the quinoline ring proton resonances caused by the peptides, MP-1 and MP-2; MP-2 is the F1489Q substituted peptide of MP-1. In the case of MP-1, all the quinoline ring proton resonances are shifted to a low frequency in a similar manner as in MP-1A, but to a much larger extent than by MP-1A. Moreover, MP-2 also caused a low frequency shift to the quinoline ring proton resonances, although the magnitudes were smaller than those caused by MP-1.[17] Since the C-termini of both MP-1 and MP-2 are not amidated, the C-terminal carboxyl group appears to induce the low frequency shift to the quinoline ring proton resonances of dibucaine. Observation 3, especially for MP-1NA is rather dramatic and means that D1487 is indispensible for the interaction with dibucaine, while observation 4 means that E1493 is interfering with the binding of dibucaine.

In Fig. 5, we summarized the changes in chemical shifts of

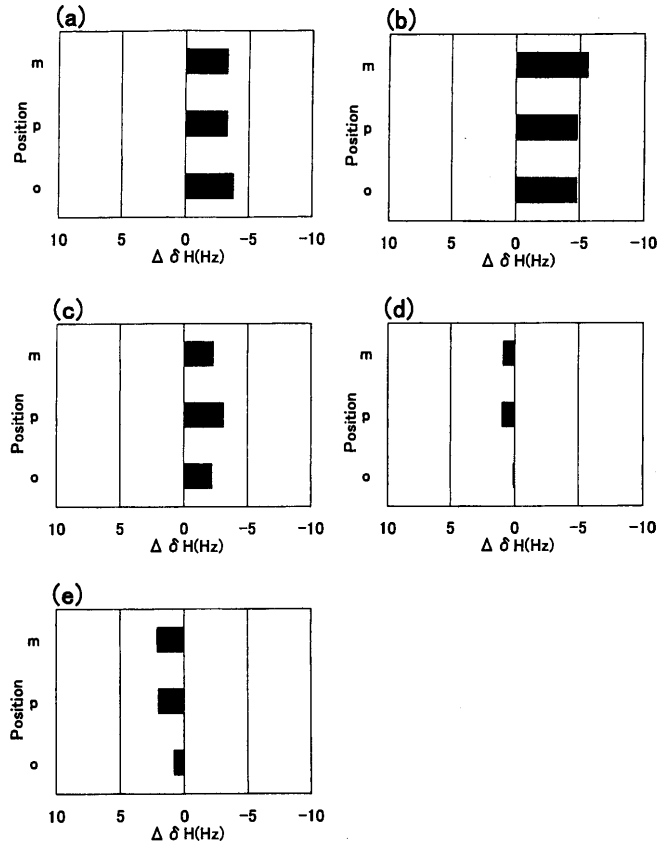

Fig. 5. Changes in Chemical Shifts (in Units of Hz at 600.13 MHz) of the Aromatic Ring Proton Resonances of F1489 as Caused by Dibucaine

m: *meta*, p: *para*, and o: *ortho* positions. (a) MP-1A, (b) MP-1A′, (c) MP-1NA, (d) MP-1QEA, and (e) MP-1EQA.

the aromatic proton resonances of the phenylalanine residues of (a) MP-1A, (b) MP-1A′, (c) MP-1NA, (d) MP-1QEA, and (e) MP-1EQA in PS suspensions as caused by dibucaine. The aromatic proton resonances were shifted to a low frequency by dibucaine in MP-1A, MP-1A′, and MP-1NA, whereas to a high frequency in MP-1QEA and MP-1EQA. All these chemical shift changes may include those due to changes in the strength of the interaction of the peptides with PS suspensions as caused by dibucaine. The changes in chemical shifts due to the interaction of a peptide with PS suspensions were found to be as large as −1.8 Hz for all the peptides. Thus, the observed high frequency shifts of MP-1QEA (Fig. 5d) and MP-1EQA (Fig. 5e), especially of MP-1EQA can be considered to be due to relatively strong interaction of dibucaine with the peptide, which is also seen in Fig. 4f.

Figure 6 shows a typical example of ^{1}H-NMR spectra of a) lidocaine in a phsophate buffer (pH 9.0), b) lidocaine in PS suspensions (pH 9.0), and c) lidocaine and MP-1A in PS suspensions (pH 9.0), respectively. Aromatic proton resonance regions are shown here for brevity. At pH 6.0, the overall spectral features were the same as in Fig. 6 except that spectrum c contained some amide proton resonances due to the peptide. The lidocaine molecules exist as a cationic form at pH 6.0 and a neutral form at pH 9.0 (pK_a=7.86).[20] The *meta* and *para* ring proton resonances of lidocaine (Fig. 2), which were observed as sharp spin-coupled peaks at around 7.2 ppm (spectrum a), became a broad single peak and were shifted to a low frequency by the interaction with PS liposomes. The broad peak in spectrum b shifted to a high frequency in the presence of MP-1A (spectrum c). Thus in the case of lidocaine, the changes in chemical shifts of the aromatic proton resonances from spectrum b to spectrum c may include changes in the partitioning of lidocaine into PS liposomes which are caused by the peptide. All the observed changes in chemical shift of lidocaine by MP-1A and also by the other peptides at pH 6.0 and 9.0 are summarized in Fig. 7. It is seen that: 1) in all the cases (Figs. 7a—f) and at both pH 6.0 and 9.0, the aromatic proton resonances of lidocaine were shifted to a high frequency by the peptides; 2) these high frequency shifts were much larger than those observed

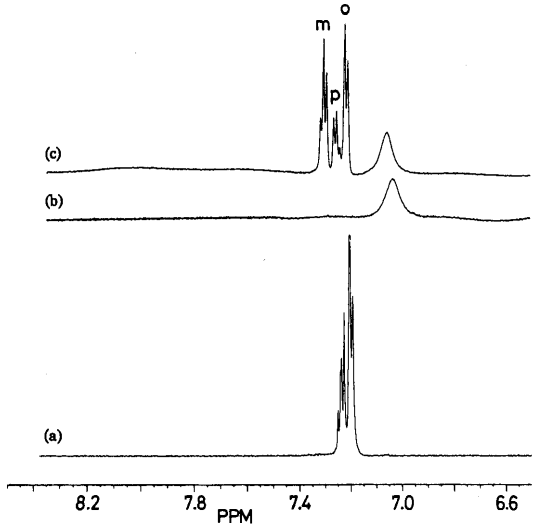

Fig. 6. ^{1}H-NMR Spectra of (a) Lidocaine in a Phsophate Buffer (pH 9.0), (b) Lidocaine in PS Suspensions (pH 9.0), and (c) Lidocaine and MP-1A in PS Liposomes (pH 9.0)

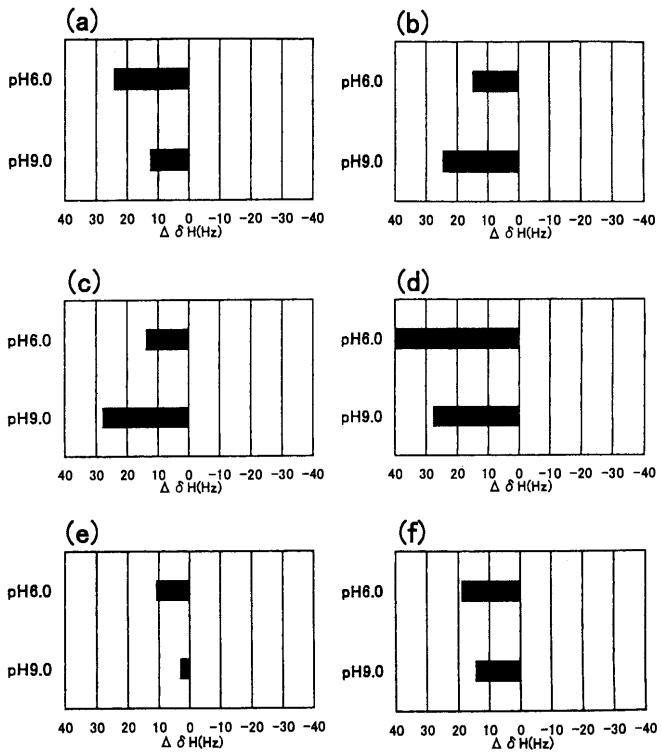

Fig. 7. Changes in Chemical Shifts (in units of Hz at 600.13 MHz) of the Aromatic Ring Proton Resonances of Lidocaine as Caused by the Peptides

(a) MP-1A, (b) MP-2A, (c) MP-1A′, (d) MP-1NA, (e) MP-1QEA, and (f) MP-1EQA.

in dibucaine (Fig. 5); 3) MP-1NA (Fig. 7d) caused the largest chemical shift changes, whereas MP-1QEA (Fig. 7e) caused the least changes at both pH 6.0 and 9.0; 4) MP-1A′ (Fig. 7c) caused smaller chemical shift changes than did MP-1A (Fig. 7a) at pH 6.0; 5) in MP-1A, MP-1NA, and MP-1EQA, each of the low frequency shifts was larger at pH 6.0 than the corresponding shift change at pH 9.0; 6) at pH 9.0, the shift changes by MP-2A (Fig. 7b) and MP-1A′ (Fig. 7c) were larger than by MP-1A (Fig. 7a). Although it is difficult to rule out the possibility that the large high frequency shift (observations 1 and 2) was due to the decrease in the partition of lidocaine into PS liposomes, observed variations in the magnitude of the changes in chemical shifts appear to indicate the differences in the strength and the mode of the interaction between lidocaine and the peptides. Observation 3 can thus be considered to mean that D1487 interfered with the interaction, while E1492 played an important role in the interaction with lidocaine. Moreover, observation 4 appears to be a manifestation of the importance of the stereochemical configuration around F1489 for the interaction. Observation 5 suggests that the electrostatic interaction between the positive charge at the tertiary amine nitrogen of lidocaine and the negative charge at E1492 strengthened the binding of lidocaine with the peptide. Although both MP-1A (Fig. 7a) and MP-2A (Fig. 7b) caused a high frequency shift, the magnitude was larger in MP-1A than in MP-2A at pH 6.0. Thus the π–π stacking interaction may be present between the aromatic ring of F1489 and that of the cationic form of lidocaine. However at pH 9.0, since observation 6 indicates that the presence of F1489 and the stereochemical configuration around F1489 are not important for the binding, the π–π stacking interaction is not operating or weak, if any, in the interaction for the neutral form of lidocaine. The ^{2}H-NMR quadrupole splittings of the aromatic ring of tetracaine indicate that charged and uncharged tetracaine occupy different sites in the PS bilayer;[21] the latter binds at a more ordered environment than the former. In analogy with tetracaine, uncharged lidocaine may also be binding with PS at a more ordered environment than its charged counterpart, probably a little deeper into the PS bilayer. This may be the reason why the π–π stacking interaction was weakened in the interaction with the peptide for the neutral form of lidocaine.

Figure 8 shows changes in the chemical shifts of the aromatic proton resonances of the phenylalanine residues of (a) MP-1A, (b) MP-1A′, (c) MP-1NA, (d) MP-1QEA, and (e) MP-1EQA in PS suspensions as caused by lidocaine and at pH 6.0 and 9.0. It is seen that: 1) except for MP-1QEA (Fig. 8d), the aromatic protons of the phenylalanine residues shifted to a high frequency at pH 6.0 and oppositely at pH 9.0; 2) the magnitudes were smaller than those observed with interactions for dibucaine (Fig. 5); 3) at pH 6.0, MP-1NA showed the largest high frequency shift; 4) at pH 9.0, in all the cases (Figs. 8a—e), the phenylalanine ring protons showed a very small low frequency shift. Observation 3) again means that at pH 6.0, D1487 is interfering with the interaction for lidocaine. The fact that MP-1QEA showed an opposite chemical shift change as compared to the other peptides means that E1492 had been playing a key role in the interaction. Finally observation 4) implies that the neutral form of lidocaine does not interact with F1489 as discussed above.

In conclusion, the cationic forms of dibucaine and lidocaine interact differently with MP-1A. The tertiary amine nitrogen of dibucaine interacts electrostatically with the negative charge of D1487, while its quinoline ring interacts with the aromatic ring of F1489. On the other hand, the tertiary amine nitrogen of lidocaine interacts electrostatically with the negative charge of E1492, while its aromatic ring interacts with the aromatic ring of F1489.

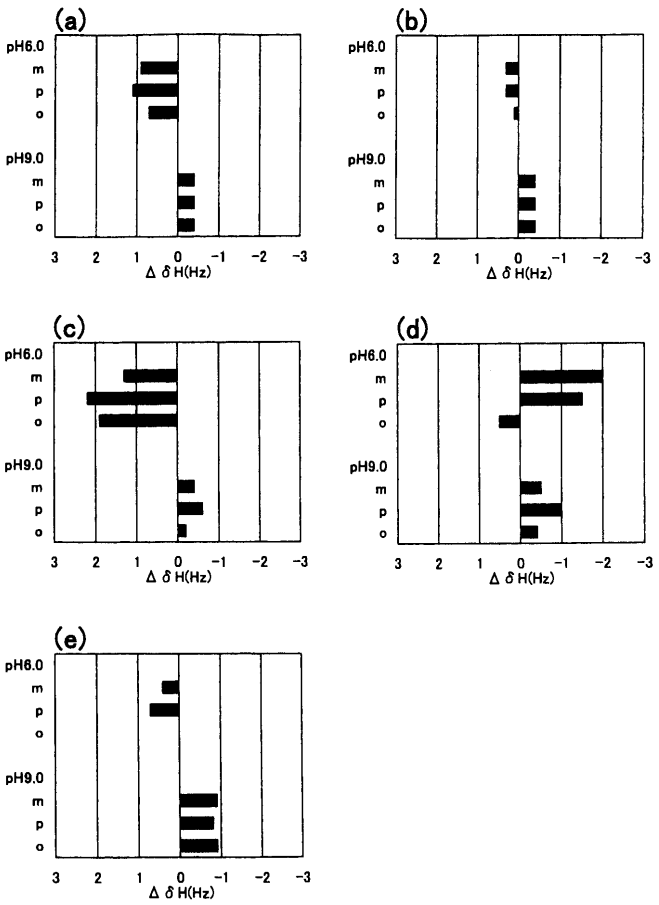

Fig. 8. Changes in Chemical Shifts (in Units of Hz at 600.13 MHz) of the Aromatic Ring Proton Resonances of F1489 as Caused by Lidocaine at pH 6.0 and pH 9.0

(a) MP-1A, (b) MP-1A′, (c) MP-1NA, (d) MP-1QEA, and (e) MP-1EQA.

Finally, it should be added that since the absolute magnitudes of the presently observed changes in chemical shifts of the aromatic proton resonances of the local anesthetics and those of the phenylalanine residues of the peptides were small, other explanations not including the π–π stacking interactions cannot be excluded for explaining the differences in the magnitudes of the chemical shift changes among the peptides. Differences in the magnetic anisotropy effects between the side-chains of the amino acids and/or the conformational changes due to substitution of one of the amino acids in a peptide can be a candidate for explaining the reason for the differences in the observed chemical shift changes. To verify these hypotheses and for a more thorough discussion, determination of the structures of the peptides in solution is required. Structural studies on the peptides including the IMF motif in trifluoroethanol solutions and in micelles are currently being undertaken and some results have been reported elsewhere.[22]

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Degradation Products Generated by Sonication of Benzyl Alcohol, a Sample Preparation Solvent for the Determination of Residual Solvents in Pharmaceutical Bulks, on Capillary Gas Chromatography

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Benzyl alcohol used as the sample preparation solvent in the determination of residual solvents in pharmaceutical bulks yielded benzene, toluene, and benzaldehyde on capillary gas chromatography (GC) by sonication. The factors responsible for compounds generated are discussed. The quality of benzyl alcohol and the type of sonicator were not involved in the generation of benzene, toluene, and benzaldehyde, whereas matrix contributions were observed. The degradation profiles of benzyl alcohol and its analogous compounds obtained by pyrolysis-GC/mass spectrometric analysis were similar to those obtained by sonication, suggesting that benzyl alcohol is degraded by the high local heat generated by sonication. Consequently, no matter how long it may take to dissolve bulk substances in benzyl alcohol completely, we do not recommend the use of a sonicator in sample preparation for the determination of residual solvents in pharmaceutical bulks.

Key words benzyl alcohol; residual solvent; pharmaceutical bulk; capillary gas chromatography; sonication; pyrolysis-GC/mass spectrometric analysis

The determination of residual solvents in pharmaceutical bulks is very important for the development of pharmaceuticals due to the toxicity of some types of solvent. In addition to this main reason, residual solvents may affect the physicochemical properties and stability of pharmaceutical products. For the determination of residual solvents, gas chromatography (GC) as described in standards USP 24 and EP 3 has been widely used as the most appropriate of a number of generalized methods. In the ICH guidelines, limits for residual solvents in pharmaceutical bulks are described in detail, although the determination method is not described.[1] Furthermore, JP 13 Supplement No. 2, newly revised to include a residual solvent test, does not include a definitive determination method.

Sampling techniques in GC contain direct injection (USP) and headspace sampling (USP and EP). Direct injection is the preferred method because it is simple and requires standard GC equipment. Dissolution of a bulk substance in a suitable solvent and direct injection of this solution onto the GC column are both rapid and convenient, and can readily be automated. Benzyl alcohol is generally used as a solvent because its boiling point is higher than that of the objective residual solvents used as analytes.[2—5]

To dissolve bulk substances into benzyl alcohol rapidly, they are usually irradiated with ultrasonic waves. We have found that benzene and toluene are generated by sonicating benzyl alcohol solution of bulk substances in the residual solvent test. This appears to be a serious hindrance for the performance of the test.

This report describes the identification of compounds generated by the sonication of benzyl alcohol and speculation on the mechanism by which these compounds are generated based on the degradation profile obtained by pyrolysis-GC/mass spectrometric analysis.

Experimental

Materials and Reagents All pharmaceutical bulks (compounds A—E) were prepared in-house by Takeda Chemical Industries, Ltd. (Osaka, Japan). All solvents were of reagent grade and obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) unless stated otherwise. Benzyl alcohol obtained was of 97.0 and 99.5% purity as determined by GC, in addition to the reagent grade (99.0%).

Gas Chromatographic Analysis Quantitative analysis was performed on a Shimadzu Model GC-15A gas chromatograph equipped with a flame ionization detector and a Shimadzu Model AOC-17 auto injector. Samples were injected into the gas chromatograph in the direct mode. The column used was a 30 m×0.53 mm i.d. fused silica capillary column coated with 5.0 μm film of 5% diphenyl, 95% dimethylsiloxane (SPB-5, Supelco Co., Ltd., Bellefonte, PA, U.S.A.) coupled to a 5 m×0.53 mm i.d. fused silica column coated with 0.5 μm film of SPB-5 as a guard column. The carrier gas was helium, and the average column linear velocity determined by injections of methane was about 35 cm/sec. Nitrogen was used as the make-up gas at a flow rate of 50 ml/min. The injector and detector temperatures were 140 °C and 260 °C, respectively. The column temperature was programmed at 35 °C for 10 min, increased to 175 °C at 4 °C/min, then to 260 °C at 35 °C/min, and finally maintained at 260 °C for at least 16 min. The injection volume was 1.0 μl.

GC/Mass Spectrometry (MS) A Shimadzu Model QP5050A GC/MS system with an electron-impact ion source was used. The column used was a 30 m×0.25 mm i.d. fused silica capillary column coated with a 5.0 μm film of 5% diphenyl, 95% dimethylsiloxane (DB-5, J&W Scientific Co., Ltd., Folsom, CA, U.S.A.). The split-injection mode was used with an approximate splitting ratio of 1 : 50. The carrier gas, avarage column linear velocity, and injection temperature were the same as in the GC (FID) conditions described above. The column temperature was programmed at 35 °C for 5 min, increased to 175 °C at 8 °C/min, then to 260 °C at 35 °C/min, and finally maintained at 260 °C for at least 16 min. Mass spectra were recorded under an electron-impact (EI) ionization mode at 70 eV from *m/z* 35 to 200, and the ion source was maintained at 280 °C.

Pyrolysis-GC/MS A vertical furnace-type Double Shot Pyrolyser (Frontier Lab Co., Ltd., Koriyama, Japan) mounted on top of the GC/MS injection port mentioned above was used. The GC and MS conditions were similar to those in the GC/MS analysis mentioned above. Several tens of micrograms of solvents placed inside the sample holder were pyrolysed at 500 °C for 1 min.

Determination of Residual Solvents Approximately 0.1 g of a bulk substance weighed accurately was placed into a 5-ml volumetric flask, dissolved in and diluted to volume with benzyl alcohol, and mixed. Standard

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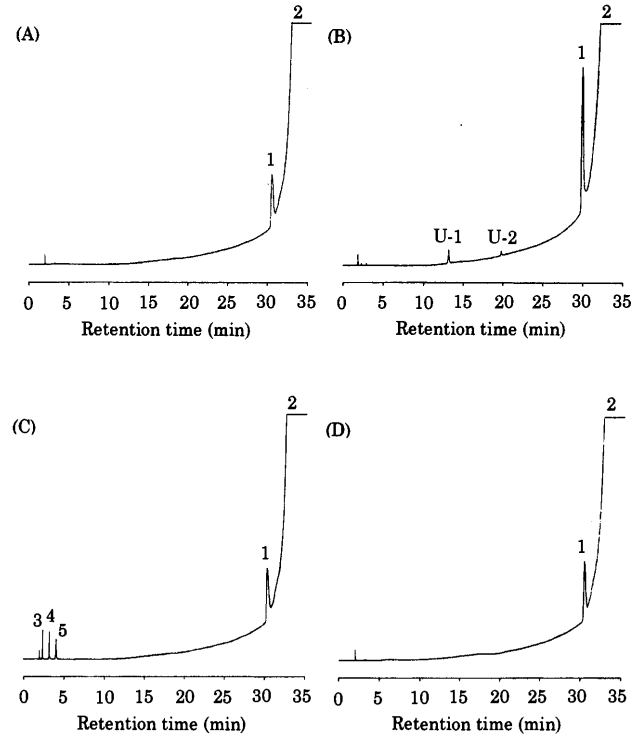


Fig. 1. Gas Chromatographic Profiles from Determination of Residual Solvent in Pharmaceutical Bulk

(A) Sample prepared by dissolving pharmaceutical bulk in benzyl alcohol with shaking; (B) sample prepared with sonication; (C) standard containing methanol, ethanol, and 2-propanol 250 ppm each; (D) benzyl alcohol (sample preparation solvent). 1: Benzaldehyde; 2: benzyl alcohol; 3: methanol; 4: ethanol; 5: 2-propanol.

solvents were prepared by accurately weighing approximately 1.0 g of appropriate solvents into a 20-ml volumetric flask, diluting to volume with benzyl alcohol, and mixing. These solutions were accurately diluted 10000 times with benzyl alcohol, which corresponds to solvent level of 250 ppm by weight in the bulk substance. An equal volume (about 1 μl) of the sample and standard was separately subjected to GC or GC/MS analysis.

Ultrasonic Irradiation Five milliliters solvent or solution of bulk substances was placed into a Pyrex test tube clamped in the center of the sonicators (SONO Cleaner Za 200 [38 kHz, 200 W], Za 100a [38 kHz, 100 W], or Za 100 [25 kHz, 100 W], Kaijo Co., Ltd., Tokyo, Japan) and irradiated with ultrasonic waves. The level of the solution in the tube was maintained at the same as that of water in the sonicator to obtain reproducible sonochemical yields. The temperature of the water in the sonicator was maintained at approximately 25 °C. Standard samples were prepared in the same manner described for the determination of residual solvents, which corresponds to a solvent level of 250 ppm by weight in the bulk substance. An equal volume (about 1 μl) of the sonicated sample or solvent and standard was separately subjected to GC or GC/MS analysis. The amount of generated compounds corresponding to the concentration (ppm) in the bulk substance was calculated using the following equation:

$$\text{concentration of compounds generated (ppm)} = \frac{A_{Ti}}{A_{Si}} \times W_{Si} \times 250$$

where A_{Ti} and A_{Si} represent the peak area responses of the compounds in the sample and standard, respectively. W_{Si} is the sampling amount of intended compounds in the standard.

Results and Discussion

Figure 1 shows typical gas chromatograms of samples and standard along with benzyl alcohol as the sample preparation solvent. Figures 1A and 1B are chromatograms of samples prepared by dissolving a pharmaceutical bulk in benzyl alcohol with shaking and sonicating (38 kHz, 200 W), respectively. Figure 1C is a chromatogram of the standard containing methanol, ethanol, and 2-propanol used for purification and recrystallization of the bulk. Although none of these solvents in the standard was detected in either sample, two unknown peaks, U-1 and U-2, were observed in the sonicated sample. This phenomenon was also seen when benzyl alcohol was sonicated alone, as shown in Fig. 2.

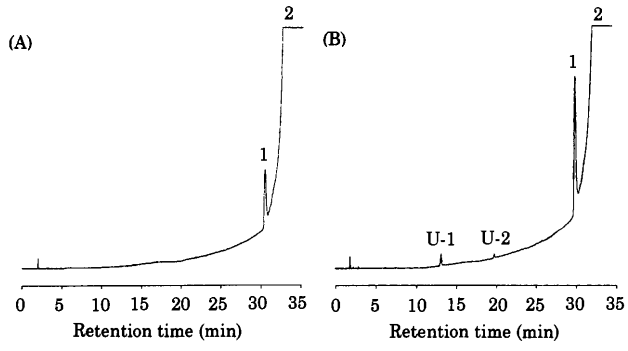


Fig. 2. Gas Chromatographic Profiles of Benzyl Alcohol

(A) Intact; (B) sonicated. 1: Benzaldehyde; 2: benzyl alcohol.

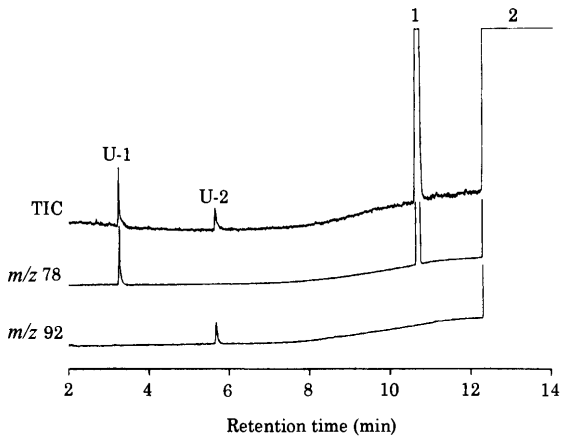


Fig. 3. Mass Chromatograms of Total Ions, *m/z* 78, and *m/z* 92 for Sonicated Benzyl Alcohol

1: Benzaldehyde; 2: benzyl alcohol.

The chemical structures of U-1 and U-2 were elucidated by EI-MS. The mass spectra showed characteristic ion peaks due to ionization and fragmentation, and U-1 and U-2 were easily identified as benzene and toluene, respectively. Furthermore, these mass spectra were in good agreement with those of the benzene and toluene standard, respectively (Figs. 3 and 4).

Figure 5 shows the time courses of concentrations of these compounds generated by the sonication of benzyl alcohol. Five replicate experiments revealed that benzene, toluene, and benzaldehyde were generated almost in proportion to the sonication period and reached 110 ppm, 38 ppm, and 1030 ppm, respectively, at 30 min. Therefore, to study the generation of these compounds as described below, the operating conditions were set at 38 kHz and 200 W for 30 min as standard conditions.

Factors in Generation Initially, the effect of the purity of benzyl alcohol on the amount of compounds generated by sonication was examined. As shown in Table 1, no difference in the amount of compound generated was observed by sonication of three different commercially available grades of benzyl alcohol under the standard conditions.

Table 2 shows the results of effect of bulk matrices in ben-

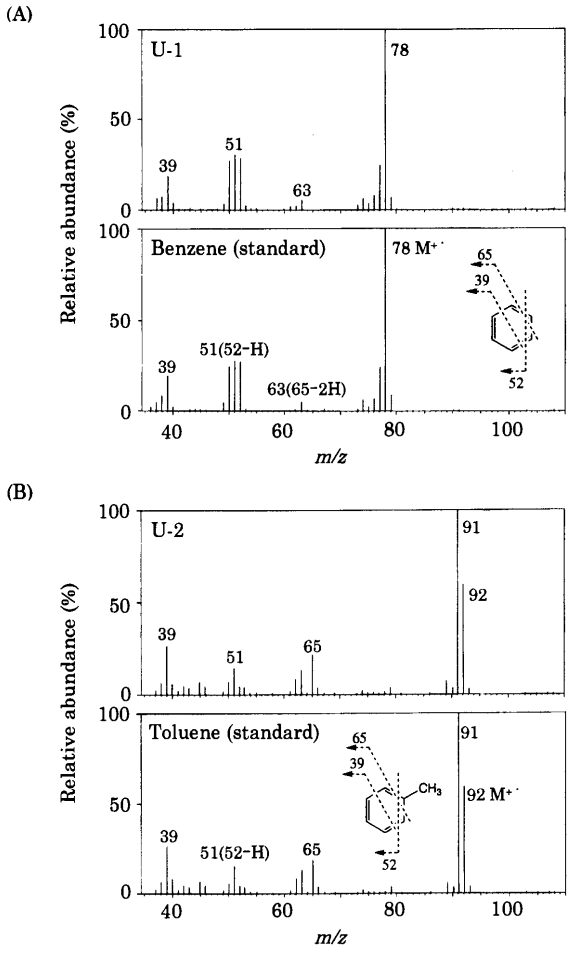


Fig. 4. Mass Spectra of U-1 (A) and U-2 (B) Generated by Sonication of Benzyl Alcohol

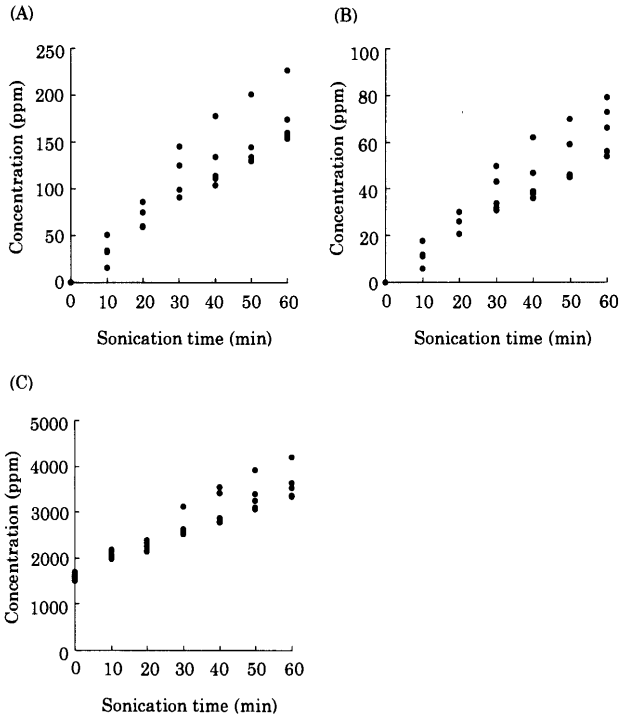


Fig. 5. Time Course of Concentration of Generated Compounds
(A) Benzene; (B) toluene; (C) benzaldehyde.

zyl alcohol on the amount of compounds generated. The amount of compounds generated by the sonication of benzyl alcohol in the presence of bulk substances tended to decrease compared with that in the absence of drug matrix. In addition, the amount of compounds generated was independent of the type of sonicator with power of 28 to 38 kHz and 100 to 200 W, as shown in Table 3.

Generation Mechanism Commercially available benzyl alcohol may contain impurities undetected by GC, with the exception of benzaldehyde. Therefore several approaches were used to determine whether benzyl alcohol itself or its impurities degrade into benzene and toluene. The sonication of benzaldehyde under the standard conditions yielded neither benzene nor toluene. Furthermore, the amount of compounds generated by the sonication of benzyl alcohol containing 1% benzaldehyde was not greater than that of benzyl alcohol alone. These results indicate that degradation products were not derived from benzaldehyde (Chart 1). Additionally, when benzyl alcohol containing 1% toluene was sonicated, the amount of compounds generated was not greater than that of benzyl alcohol alone, indicating that benzene is not a second degradation product *via* toluene but a primary degradation product of benzyl alcohol (Chart 1). Table 4 summarizes the amount of each degradation product generated by the sonication of some of the solvents mentioned above.

It is accepted that ultrasonic irradiation of a liquid medium

Table 1. Concentration of Compounds Generated by the Sonication of Various Grades of Benzyl Alcohol

Grade	Compound generated (ppm)		Av. (min.—max.)[a]
	Benzene	Toluene	Benzaldehyde
97.0% Purity	115 (69—193)	33 (24—49)	712 (443—1187)
99.0% Purity	110 (91—145)	38 (31—50)	1029 (816—1473)
99.5% Purity	130 (60—254)	37 (18—74)	771 (380—1146)

a) Results of five determinations.

Table 2. Concentration of Compounds Generated by the Sonication of Benzyl Alcohol Containing Various Drug Substances

Matrix	Compound generated (ppm)		Av. (min.—max.)[a]
	Benzene	Toluene	Benzaldehyde
No bulk substance	110 (91—145)	38 (31—50)	1029 (816—1473)
Bulk substance A	100 (83—135)	29 (25—37)	602 (464—842)
Bulk substance B	78 (58—122)	32 (23—61)	458 (353—573)
Bulk substance C	71 (53—116)	22 (17—27)	459 (288—550)
Bulk substance D	68 (42—95)	23 (19—28)	622 (501—775)
Bulk substance E	62 (47—84)	26 (20—34)	422 (316—503)

a) Results of five determinations.

Table 3. Concentration of Compounds Generated by the Sonication of Benzyl Alcohol with Various Sonicators

Sonicator	Compound generated (ppm)		Av. (min.—max.)[a]
	Benzene	Toluene	Benzaldehyde
38 kHz, 200 W	110 (91—145)	38 (31—50)	1029 (816—1473)
38 kHz, 100 W	110 (68—135)	31 (22—38)	1079 (716—1359)
28 kHz, 100 W	93 (66—115)	29 (25—33)	1186 (747—1609)

a) Results of five determinations.

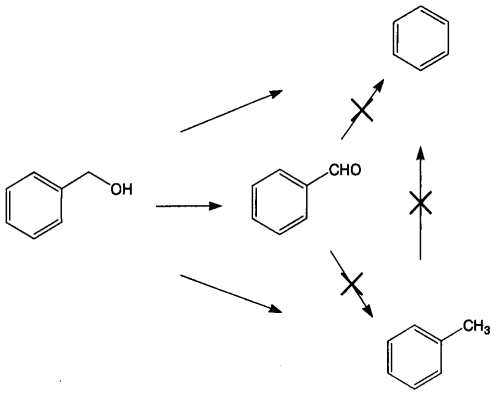
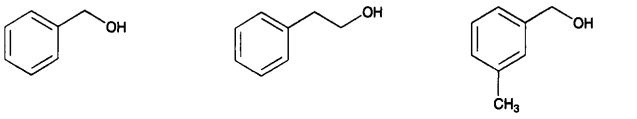


Chart 1. Estimated Degradation Pathway of Benzyl Alcohol by Sonication

Table 4. Concentration of Compounds Generated by the Sonication of Various Solvents

Solvent	Compound generated (ppm)		Av. (min.—max.)[a]
	Benzene	Toluene	Benzaldehyde
Benzyl alcohol	110 (91—145)	38 (31—50)	1029 (816—1473)
Benzaldehyde	0 (0—0)	0 (0—0)	—
1% Benzaldehyde[b]	68 (39—101)	18 (9—32)	—
1% Toluene[b]	22 (12—32)	—	1362 (1110—1850)

a) Results of five determinations. *b*) Added to benzyl alcohol. Data indicated by — are not applicable.



Benzyl alcohol 2-Phenylethanol 3-Methylbenzyl alcohol

Chart 2. Structure of Benzyl Alcohol Analogues

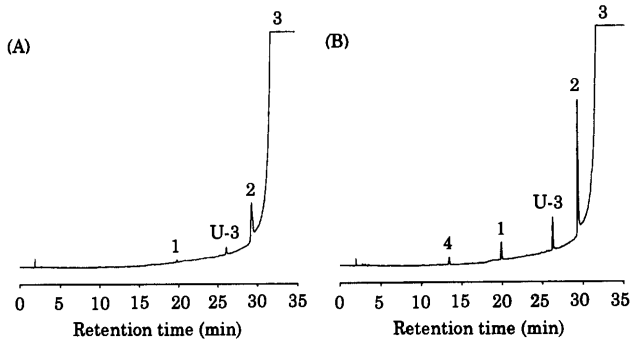


Fig. 6. Gas Chromatographic Profiles of 2-Phenylethanol

(A) Intact; (B) sonicated. 1: Toluene; 2: benzaldehyde; 3: 2-phenylethanol; 4: benzene.

causes acoustic cavitation: the formation, growth, and implosive collapse of bubbles. When the cavity implodes, an enormous amount of local heat energy is generated, and peak temperatures of several thousands of degrees Celsius have been predicted.[6—8] Many synthetic or degradation studies of organic compounds in liquid medium using this energy have been reported,[9—12] although there are only a few reports on the degradation of organic solvents themselves.[13]

To investigate the orientation of substances, 2-phenylethanol and 3-methylbenzyl alcohol (Chart 2), which have structures similar to that of benzyl alcohol, were sonicated under the standard conditions. When 2-phenylethanol was sonicated under the standard conditions, toluene, benzaldehyde, and the unknown compound U-3 in trace amounts were observed, as shown in Fig. 6. U-3 was identified as styrene by its mass spectrum (Fig. 7). On the other hand, sonication of 3-methylbenzyl alcohol yielded only toluene, which is

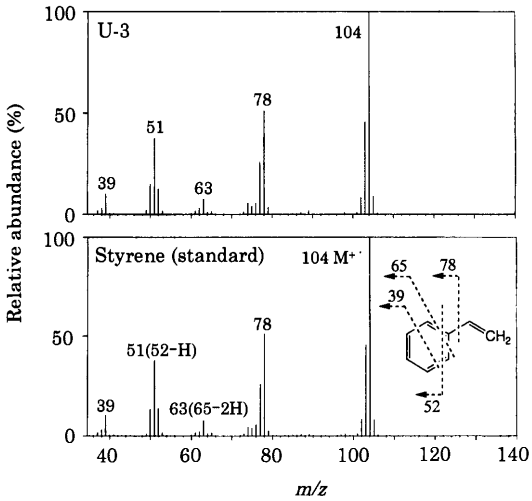


Fig. 7. Mass Spectrum of U-3 Generated by Sonication of 2-Phenylethanol

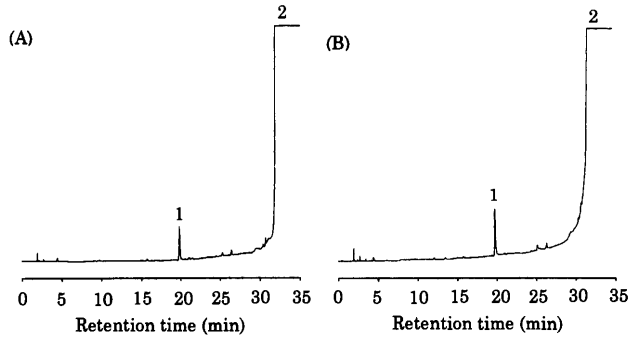


Fig. 8. Gas Chromatographic Profiles of 3-Methylbenzyl Alcohol

(A) Intact; (B) sonicated. 1: Toluene; 2: 3-methylbenzyl alcohol.

Table 5. Concentration of Compounds Generated by the Sonication of 2-Phenylethanol and 3-Methylbenzyl Alcohol

Solvent	Compound generated (ppm)		Av. (min.—max.)[a]	
	Benzene	Toluene	Styrene	Benzaldehyde
2-Phenylethanol	127 (87—161)	229 (142—300)	145 (54—220)	203 (115—309)
3-Methylbenzyl alcohol	0 (0—0)	51 (29—84)	0 (0—0)	—

a) Results of five determinations. Datum indicated by — is not applicable because the benzaldehyde peak overlapped with the 3-methylbenzyl alcohol peak.

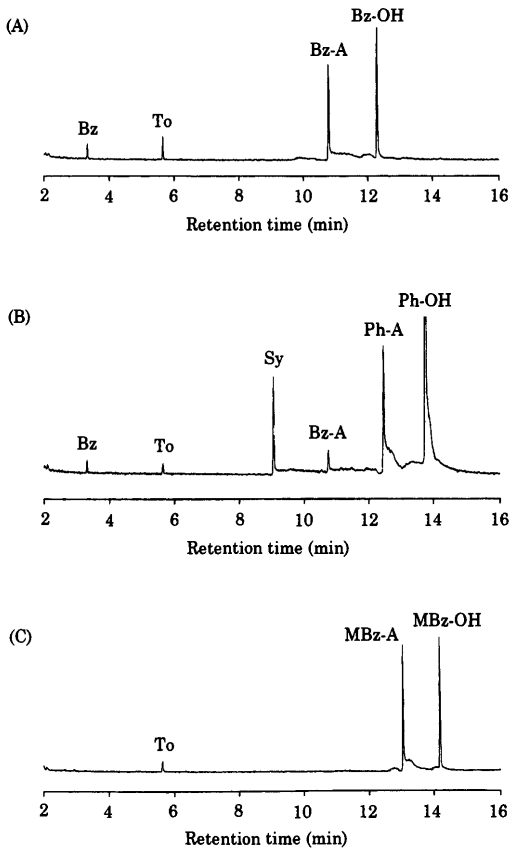


Fig. 9. Pyrograms of Benzyl Alcohol (A), 2-Phenylethanol (B), and 3-Methylbenzyl Alcohol (C)

Bz: Benzene; To: toluene; Bz-A: benzaldehyde; Bz-OH: benzyl alcohol; Sy: styrene; Ph-A: phenylacetaldehyde; Ph-OH: 2-phenylethanol; MBz-A: 3-methyl benzaldehyde; MBz-OH: 3-methylbenzyl alcohol.

contained initially (Fig. 8). The amount of each degradation product is summarized in Table 5.

These results indicate that not only benzyl alcohol but also 2-phenylethanol and 3-methylbenzyl alcohol yield degradation products characterized by each parent structure. This suggests that benzene, toluene, and benzaldehyde generated by the sonication of benzyl alcohol are degradation products of benzyl alcohol itself.

Pyrolysis-GC/MS Figure 9 shows the pyrograms for benzyl alcohol, 2-phenylethanol, and 3-methylbenzyl alcohol. The mass spectra of each peak revealed that the pyrolytic products of benzyl alcohol were benzene, toluene, and benzaldehyde (Fig. 9A), which were identical with the products generated by the sonication of benzyl alcohol. In addition, the pyrolysis of 2-phenylethanol and 3-methylbenzyl alcohol yielded the corresponding products by sonication, expect for phenylacetaldehyde and 3-methyl benzaldehyde, which overlapped with 2-phenylethanol and 3-methylbenzyl alcohol, respectively, in the GC (FID) conditions (Figs. 9B and 9C). The good agreement between the products generated by sonication and pyrolysis suggests that the enormous amount of local heating generated by sonication is one factor responsible for the degradation of benzyl alcohol as well as of 2-phenylethanol and 3-methylbenzyl alcohol.

Conclusions

Sonochemical degradation of benzyl alcohol, the sample preparation solvent for the determination of residual solvents in pharmaceutical bulks, was studied using capillary GC. Benzene, toluene, and benzaldehyde were generated at about 110 ppm, 38 ppm, and 1030 ppm, respectively, by the sonication of benzyl alcohol at 38 kHz and 200 W for 30 min. Although the amount of these compounds was independent of the quality of benzyl alcohol and the type of sonicator (28 to 38 kHz and 100 to 200 W), it increased in proportion to the length of the sonication period and decreased slightly in the presence of bulk matrices. Good agreement between the degradation products of the sonication and pyrolysis of benzyl alcohol as well as its structurally analogous compounds was observed, suggesting that these compounds are degraded by the enormous local heat generated by sonication. According to the ICH guidelines for residual solvents,[1] benzene belongs to the Class 1 toxic solvents which should be avoided, and its limit is set at 2 ppm. Consequently, no matter how long it may take to dissolve bulk substances in benzyl alcohol completely, we do not recommend the use of a sonicator for the sample preparation in the determination of residual solvents in pharmaceutical bulks.

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Direct Identification of a Novel Disulfide Bond Linkage System of New Isolated Isomer (Isomer V) in Recombinantly Produced h-IGF-I[1)]

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Marine Technical College,[a] Faculty of Liberal Arts and Science, Ashiya, Hyogo 659–0026, Japan, Fujisawa Pharmaceutical Co., Ltd.,[b] Research Laboratories, Yodogawa-ku, Osaka 532–0031, Japan, and The University of Tokushima,[c] Institute of Medicinal Resources, Tokushima 770–0044, Japan. Received April 12, 2000; accepted May 29, 2000

Insulin-like growth factor I (IGF-I or somatomedin C) is a serum polypeptide with three intramolecular disulfide bonds. In the course of synthesis by the recombinant DNA method, three disulfide bond isomers, all of which have Cys^{18}–Cys^{61} with three combinations of two disulfide bonds formed by Cys^{6}, Cys^{47}, Cys^{48} and Cys^{52}, were identified. Natural type, isomer II, was proved to have a Cys^{6}–Cys^{48}, Cys^{18}–Cys^{61}, Cys^{47}–Cys^{52} disulfide bond system. Now, the fourth isomer, isomer V which doesn't have Cys^{18}–Cys^{61} disulfide, has been isolated, and its novel disulfide bond linkage system was identified by a chemical synthetic method. The supposed conformation constrained in 3D structure for isomer V would be discussed for its biological activity.

Key words insulin-like growth factor I; somatomedin C; disulfide bond linkage system; disulfide bond isomer; manual solid phase peptide synthesis

Insulin-like growth factor I (IGF-I or somatomedin C) is a serum factor with growth promoting and metabolic activities,[4)] which circulates through the body bound by several IGF binding proteins (IGFBPs). It is considered to mediate the action of longitudinal growth promotion by growth hormone secreted from the pituitary. It also has intrinsic activity of glucose uptake promotion through its IGF receptor. Based on these biological activities, IGF-I has been approved in Japan to treat growth hormone resistant dwarfism and diabetes mellitus caused by a genetic insulin receptor defect. IGF-I consists of 70 amino acid residues with three internal disulfide bonds formed by six internal cysteines; the primary structure was determined by Rinderknecht and Humbel in 1978 (Fig. 1).[5)]

During the course of IGF-I preparation by the recombinant DNA method,[6,7)] we found the presence of several IGF-I disulfide bond isomers (Fig. 2). In previous reports,[8—12)] we reported the isolation of three disulfide bond isomers, isomer I, II, and IV, and the determination of disulfide bond linkages. In all respects of the analyses, isomer II was identified as natural IGF-I. Isomer I has a disulfide bond linkage of Cys^{6}–Cys^{47}, Cys^{18}–Cys^{61} and Cys^{48}–Cys^{52}, isomer II has a linkage of Cys^{6}–Cys^{48}, Cys^{18}–Cys^{61} and Cys^{47}–Cys^{52}, while isomer IV has a linkage of Cys^{6}–Cys^{52}, Cys^{18}–Cys^{61} and Cys^{47}–Cys^{48}. In spite of the speculated conformational similarity between isomer II and I from the disulfide linkages, a bioassay of growth promoting activity by ^{3}H-thymidine uptake revealed the activity order as isomer II>IV>I.[8,13)]

Here, we report the isolation of a new disulfide bond isomer which doesn't have Cys^{18}–Cys^{61} disulfide, common to the previously identified IGF-Is. Also, we report in detail[14,15)] the determination of its disulfide linkage system by a synthetic chemical method in a manner similar to that the applied for isomer I and II.[10—12)] This approach of determining the position in the coordinate of key residues should provide hints for synthesizing protein mimics in the future.

Cell growth promoting activity and binding activity to IGFBP-2 and -3 are also reported with the discussion of the conformation in 3D structures for the isomers.

A reduced form of recombinant h-IGF-I in 6 M guanidine HCl—10 mM Tris–HCl (pH 8.0) with 10-fold 10 mM Tris–HCl buffer (pH 8.0) was subjected to air-oxidation to cause refolding. Four main peaks, designated isomer I, II, IV, and V(**1**) (Fig. 2), were isolated by preparative RP-HPLC.

An outline for the structural determination of a new disulfide isomer of h-IGF-I, isomer V(**1**), is shown in Fig. 3. **1** was digested in 0.1 M $(NH_4)HCO_3$ with chymotrypsin at RT for 2 h. All fragments were isolated by RP-HPLC, and the

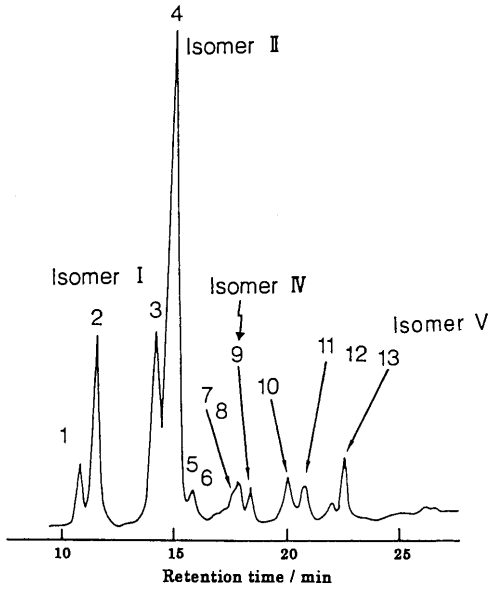

Fig. 2. HPLC Pattern of Refolding and Air-Oxidation Mixture of h-IGF-I by a Recombinant DNA Method

The mixture was loaded on a YMC-Pack AP-302 column (4.6×150 mm, ODS 200 Å s-5) and eluted with 0.08% TFA *vs.* acetonitrile, 27.5—37.5%, over a period of 0—30 min. Flow rate, 1 ml/min. Detection was at 214 nm.

1 6 18
H-Gly-Pro-Glu-Thr-Leu-Cys-Gly-Ala-Glu-Leu-Val-Asp-Ala-Leu-Gln-Phe-Val-Cys-

Gly-Asp-Arg-Gly-Phe-Tyr-Phe-Asn-Lys-Pro-Thr-Gly-Tyr-Gly-Ser-Ser-Ser-Arg-

47 48 52
Arg-Ala-Pro-Gln-Thr-Gly-Ile-Val-Asp-Glu-Cys-Cys-Phe-Arg-Ser-Cys-Asp-Leu-

61 70
Arg-Arg-Leu-Glu-Met-Tyr-Cys-Ala-Pro-Leu-Lys-Pro-Ala-Lys-Ser-Ala-OH

Fig. 1. Primary Structure of h-IGF-I (Somatomedin C)

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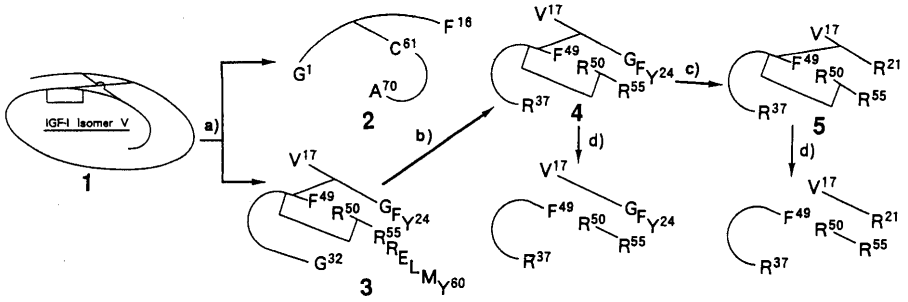

Fig. 3. Strategy for the Structural Determination of a New Disulfide Bond Isomer of h-IGF-I, Isomer V(**1**)

(a) α-Chymotrypsin (0.1 M $(NH_4)HCO_3$), (b) trypsin (0.1 M $(NH_4)HCO_3$), (c) trypsin (0.2 M phosphate buffer (pH 7.2)), (d) 2-ME.

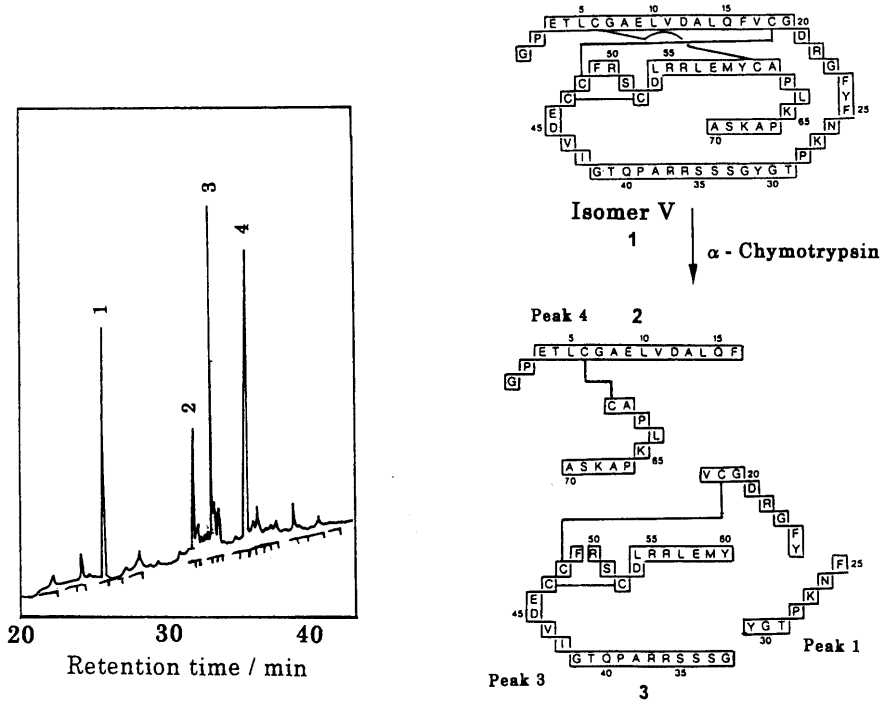

Fig. 4. Peptide Map on the C-18 RP-HPLC of Isomer V(**1**) Digested with α-Chymotrypsin

Conditions: column, YMC-Pak ODS AP302-5 μm (4.6×150 mm); detection, 210 nm; flow rate, 1.0 ml/min; elution solvent, 0.1% TFA with a 0—60% CH_3CN linear gradient.

structure of **2**, which contains the disulfide bond between Cys^6–Cys^{61}, was determined by amino acid analysis (Fig. 4) and MS spectral measurements. **3** obtained by chymotrypsin digestion was identified to contain Cys^{18}, Cys^{47}, Cys^{48}, and Cys^{52} (Fig. 4), and afforded **4** by the treatment with trypsin in 0.1 M $(NH_4)HCO_3$ at RT for 70 min. **4** was further digested with trypsin in 0.2 M phosphate buffer (pH 7.2) at RT for 3 h to give **5**.

For direct identification of the disulfide bond linkages of **5**, chemical synthesis of two possible fragments with Cys^{18}–Cys^{47}, Cys^{48}–Cys^{52} (form I), and Cys^{18}–Cys^{48}, Cys^{47}–Cys^{52} (form II) were attempted by the selective removal of protecting groups, Acm and MBzl, on the Cys residues.

The protected peptide resins, fully protected 18-Acm h-IGF-I (17—21)-resin, 47-Acm h-IGF-I (37—55)-resin, and 48-Acm h-IGF-I (37—55)-resin, were assembled manually by Boc/Bzl-based solid phase peptide synthesis using Merrifield resin, on Boc–Arg(Tos)–$OCH_2C_6H_4$-resin, as shown in Fig. 5.

All amino acids were protected at their amino groups with a Boc group, and the side chain protecting groups were OBzl for Ser and Thr, Tos for Arg, OcHx for Asp and Glu, Acm or

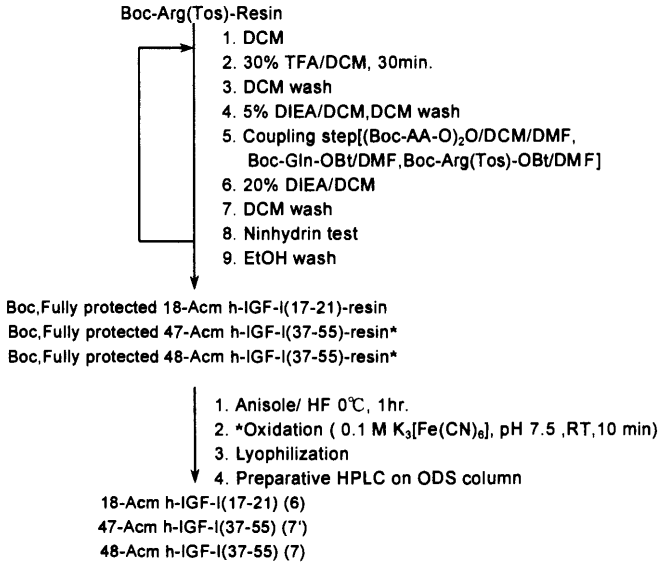

Fig. 5. General Procedure for the Synthesis of the Key Compounds, **6**, **7'**, and **7**, by Manual Solid Phase Peptide Synthesis

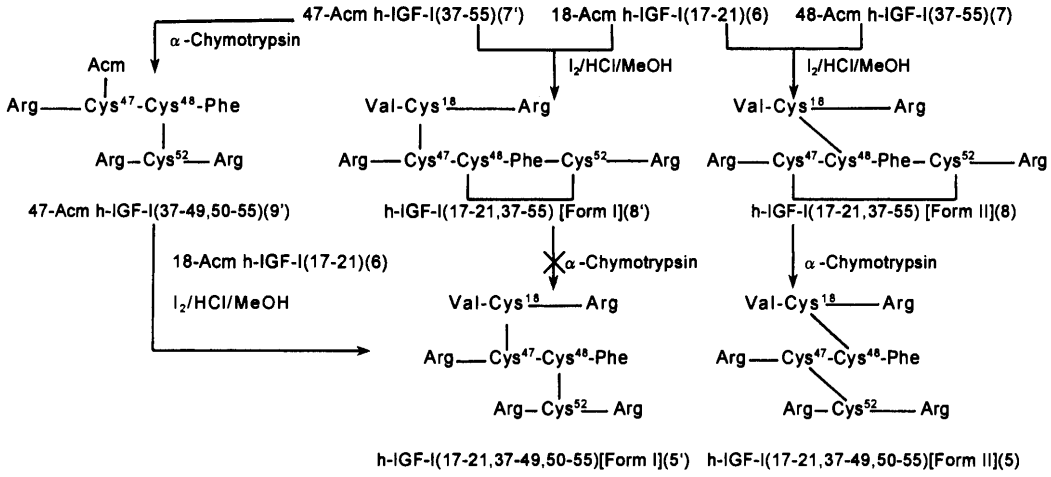

Fig. 6. Synthetic Scheme for Two Possible Disulfide Containing Fragment Peptides of Isomer V(**1**), h-IGF-I (17—21, 37—49, 50—55) [Form I] (**5′**) and [Form II] (**5**)

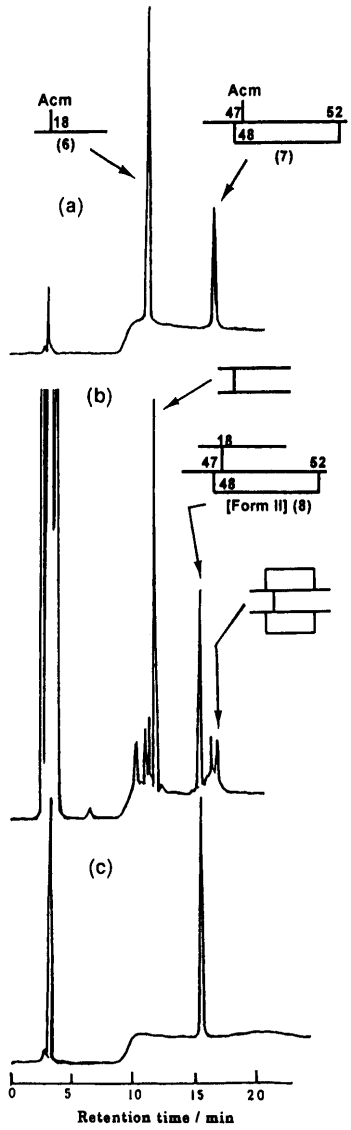

Fig. 7. HPLC Analysis of Heterodimerization Reaction for Form II (**8**)

The peptides and the reaction mixture were loaded on a Shim-pack CLC-ODS 120 Å column (6×150 mm) and eluted with 0.01 M TFA *vs.* acetonitrile, 0—60% over a period of 0—15 min. Flow rate, 1 ml/min. Detection was at 214 nm. a) 18-Acm h-IGF-I(17—21) (**6**) and 48-Acm h-IGF-I (37—55) (**7**). b) I_2-Oxidized reaction mixture of (a). c) Synthetic h-IGF-I (17—21, 37—55) [form II] (**8**) after purification with preparative HPLC (YMC-Pack ODS-AP S-5 μm, 200 A, 10×300 mm).

MBzl for Cys. Coupling reactions were carried out with a 2-fold excess of pre-prepared Boc amino acid anhydride,[16] and in the case of the coupling reactions for Boc–Gln and Boc–Arg(Tos), with a 2-fold excess of their HOBt esters which were pre-prepared from Boc amino acid, HOBt, and DCC. After the cleavage from the resin and deprotection, except for the Cys(Acm) group with anhydrous liquid HF containing 10% anisole, **6** was obtained, while the intrachain disulfide bonds of **7** and **7′** were formed from the fully protected peptide resin by oxidation in 0.1 M $K_3[Fe(CN)_6]$ following cleavage from the resin and deprotection procedure as for **6** (Fig. 5). The purity of synthetic peptides was confirmed by analytical HPLC on a C_{18} column and by amino acid analysis with a Waters Pico-Tag system.

Coupling, interchain disulfide bond formation, of **6** with **7′** or **7** under Kamber's condition by I_2/HCl in MeOH[17] produced the heterodimer **8′** or **8**, along with the respective homodimers (Figs. 6, 7).

After the purification of produced heterodimers by RP-HPLC, **8′** and **8** were digested by chymotrypsin in 0.1 M $(NH_4)HCO_3$ at RT for 60 min to obtain the form I (**5′**) and II (**5**). **8** afforded **5**, but chymotrypsin digestion of **8′** to obtain the form I (**5′**) failed because of steric hindrance of the cyclic domain containing a disulfide linkage with –Phe^{49}–Arg^{50}– for the enzyme in **8′**. So, in the case of the preparation of **5′**, before the heterodimeric reaction between **6** and **7′**, we tried to digest the bond of –Phe^{49}–Arg^{50}– in **7′** by chymotrypsin to form **9′** in good yield. Interchain disulfide bond formation, of **6** with **9′** in a similar manner as in the synthesis of **8′** or **8** by I_2/HCl in MeOH, produced the heterodimer **5′** along with the respective homodimers (Fig. 8). **5′** and **5** were identified by comparing the retention time on RP-HPLC (Fig. 9), amino acid analyses, measurements of FAB-MS (Found $m/z=2732\pm1$, Calcd 2731.9), and mapping of 2-ME reduced peptides, and its amino acid analysis as **5** obtained from h-IGF-I isomer V(**1**). Thus, the new disulfide linkage system of **1** was absolutely determined to be Cys^6–Cys^{61}, Cys^{18}–Cys^{48}, and Cys^{47}–Cys^{52}.

Although **1** (isomer V) and isomer I have similar affinity, 0.1—0.2% of IGF-I, to IGFBP-2 and -3, **1** showed distinctive activity in the cell growth promoting assay from isomer I; **1** retains only 11.1% activity of isomer I (Table 1).

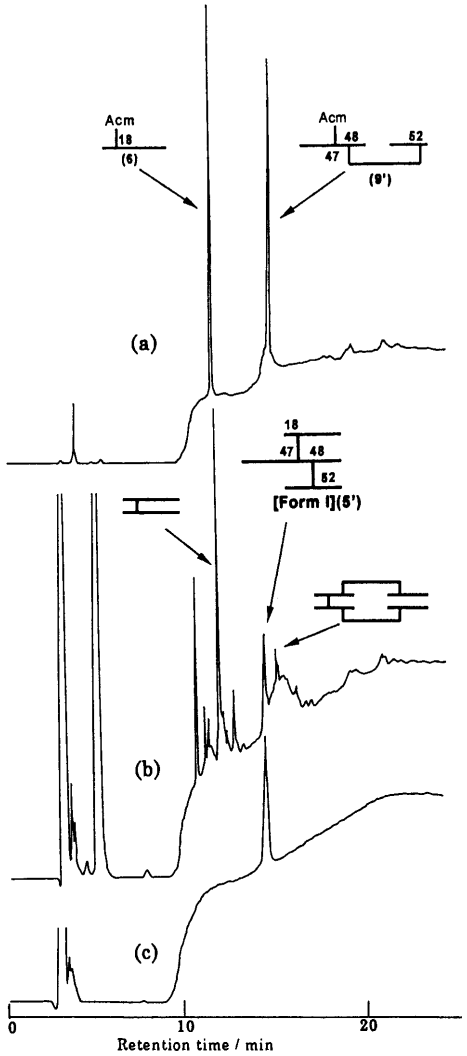

Fig. 8. HPLC Analysis of the Heterodimerization Reaction for the Synthesis of h-IGF-I (17—21, 37—49, 50—55) [Form I] (**5'**)

The peptide was eluted on a DAISO-PAK SP-200-5-ODS-AP column (6×150 mm) with a H_2O–CH_3CN linear gradient containing 0.01 M TFA (0 to 60% CH_3CN over a period of 0 to 15 min). Flow rate, 1 ml/min. Detection was 214 nm. a) 18-Acm h-IGF-I (17—21) (**6**) and 47-Acm h-IGF-I (37—49, 50—55) (**9'**). b) I_2-Oxidized reaction mixture of (a). c) Synthetic h-IGF-I (17—21, 37—49, 50—55) [form I] (**5'**) after purification with HPLC (the conditions were the same as those shown above).

3D structures of isomer I and isomer II have been determined by NMR NOE data and distance geometry algorithm calculation in hopes of explaining the conformation activity relationship.[18—20] Isomer II (IGF-I) was revealed to have three α-helix domains (helix I: Ala^8–Cys^{18}, helix II: Gly^{42}–Cys^{48} and helix III: Leu^{54}–Cys^{61}), whereas isomer I lost the helix II domain. From the comparison of calculated 3D structure simulation models of the isomers using BioGraf software, isomer IV, having three α-helix domains, also showed much closer conformation to isomer II than isomer I. The supposed 3D structure for isomer V, which doesn't have Cys^{18}–Cys^{61} disulfide bond and has Cys^6–Cys^{61} with Cys^{18}–Cys^{48} to form the domain containing the anti-parallel peptide chain instead of the parallel peptide chain by Cys^{18}–Cys^{61} with Cys^6–Cys^{48} (isomer II) or Cys^6–Cys^{47} (isomer I) or Cys^6–Cys^{52} (isomer IV) in three former isomers, is quite different from the isomers. The conformational changes in simulated 3D structures were coincident with the order of biological activities of these four isomers. Studies along these lines are now in progress in our laboratory.

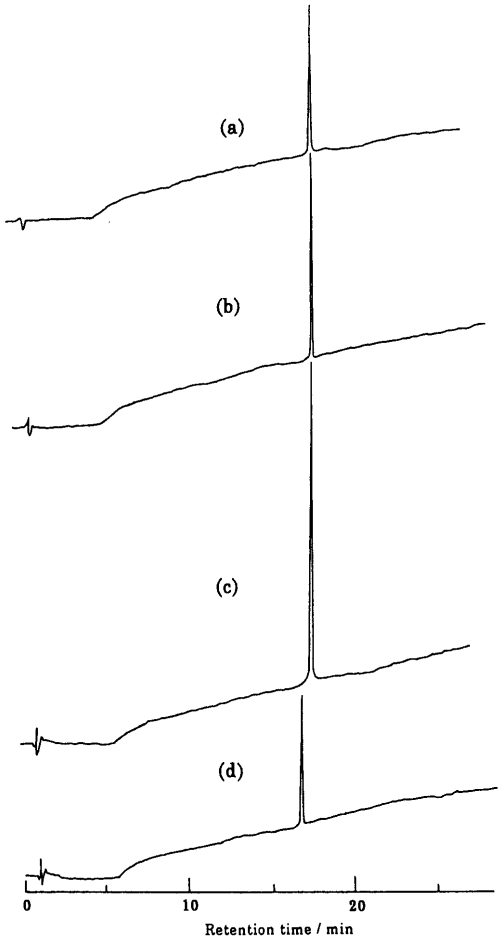

Fig. 9. HPLC Profile for Determination of the Disulfide Structure between the Cys^{18}, Cys^{47}, Cys^{48} and Cys^{52} of Isomer V(**1**)

The peptide was loaded on YMC ODS 200 Å column (4.6×150 mm) and eluted with 0.01 M TFA *vs.* acetonitrile, 0—60% over a period of 0—50 min. Flow rate, 1 ml/min. Detection was at 214 nm. a) Synthetic h-IGF-I (17—21, 37—49, 50—55) [form II] (**5**). b) Chymotrypsin–trypsin digested peptide fragment (**5**) of recombinantly produced isomer V(**1**). c) Mixture of (a) and (b). d) Synthetic h-IGF-I (17—21, 37—49, 50—55) [form I] (**5'**) ((c) in Fig. 8).

Table 1. Cell Growth Activities and Binding Activities to IGF-I Binding Protein-2 and -3 of h-IGF-I Disulfide Bond Isomers

	IGF-bioassay (cell growth)	Binding affinity to h-IGFBP-2	Binding affinity to h-IGFBP-3
Isomer I	0.90%	0.10%	0.20%
Isomer II	100%	100%	100%
Isomer IV	8.20%	1.70%	5.00%
Isomer V	0.10%	0.10%	0.10%

Experimental

Amino acid analysis of each peptide was carried out with Water's Pico-Tag amino acid analysis System (OPA method) after hydrolysis for 24 h in HCl (5.7 mol/l) at 110 °C in an evacuated tube. Amino acid sequences of peptides were determined by Applied Biosystem's Model 476A Sequencer. HPLC were performed by use of a Waters M600 multisolvent delivery system, a Shimadzu LC-6A, and a LC-10AS liquid chromatograph system. FAB-MS spectra were obtained on a Finnigan MAT TSQ-70 spectrometer. TOF-MS spectra were obtained on a Voyager Elite spectrometer (PE Biosystems, Foster City, CA). h-IGF-I was produced by a recombinant DNA technique in our laboratory.[6,7] α-Chymotrypsin and trypsin were from Sigma Chemical (St. Louis, MO, U.S.A.). For the peptide synthesis, Boc amino acid derivatives, Boc–Arg(Tos)-resin, DCM, DMF, TFA, and DIEA were from Watanabe Chemical Industries (Hiroshima, Japan), and DCC and HOBt were from Peptide Institute, Inc. (Osaka, Japan).

Isolation of Isomer V(1) Recombinant IGF-I was produced by *Escherichia coli* and purified as reported.[7] IGF-I (135 mg), a reduced form,[7] was dissolved in 6 M guanidine HCl–10 mM Tris–HCl buffer (pH 8.4) (40 ml), and then diluted with a 10-fold volume of 10 mM Tris–HCl buffer (pH 8.4) to cause refolding and air-oxidation. The mixture was analyzed by RP-HPLC (Fig. 2), and then IGF-I and its disulfide bond isomers containing isomer V(**1**) were isolated from the refolding mixture by preparative RP-HPLC with a linear gradient of CH_3CN from 27 to 38% in 30 min in 0.01 M TFA in a similar manner to the case of the reported IGF-I.[7]

Enzyme Digestion of Isomer V(1) to Obtain the Disulfide Fragment (5) for the Determination of the Disulfide Bond Linkage System of Isomer V(1) **1** was digested with α-chymotrypsin at the E/S ratio of 1/100 in 0.1 M $(NH_4)HCO_3$ (pH 8.0) at RT for 2 h. The product was analyzed by amino acid analysis and measurements of MS spectra and all fragments. Peaks 1, 3, and 4, as shown in Fig. 4, were isolated by RP-HPLC. The structure of peak 4 (FAB-MS: $m/z=2646\pm1$ $(M+H)^+$), which contains the disulfide bond between Cys^6–Cys^{61}, was determined to be compound **2** (mol.wt: 2646.1), and the structure of peak 3 (TOF-MS: $m/z=4267\pm1$ $(M+H)^+$), which contains Cys^{18}, Cys^{47}, Cys^{48}, and Cys^{52}, was determined to be compound **3** (mol.wt: 4266.9) except for the disulfide bond positions. **3** afforded **4** by treatment with trypsin at the E/S ratio of 1/50 in 0.1 M $(NH_4)HCO_3$ (pH 8.0) at RT for 70 min. **4** was further digested with trypsin at the E/S ratio of 1/50 in 0.2 M phosphate buffer (pH 7.2) at RT for 3 h to give **5**. FAB-MS: $m/z=2732\pm1$ $(M+H)^+$ (Calcd 2731.9). Amino acid analysis: Arg 3.6(4), Ala 1.0*(1), Pro 0.8(1), Thr 0.8(1), Gly 1.9(2), Ile 0.6(1), Val 1.4(2), Asx 2.1(3), Glx 1.8(2), Cys_2 1.6(2), Phe 0.8(1), Ser 0.8(1), Leu 1.0(1).

Peptide Synthesis of the Possible Disulfide Fragments Form I (5′) and Form II(5) of Isomer V(1). Synthesis of Three Key Intermediate Peptides (18-Acm h-IGF-I (17—21) (6), 47-Acm h-IGF-I (37—55) (7′), and 48-Acm h-IGF-I (37—55) (7)). Boc, Fully Protected 18-Acm h-IGF-I (17—21)-Resin After pre-swelling Boc–Arg(Tos)-resin (0.278 meq/g, 4.889 g) with DCM (30 ml) for 4 h in a glass reaction vessel, elongation of the peptide chain was carried out by manual solid-phase-synthesis, as shown in Fig. 5. The resin was treated with 30% TFA in DCM (v/v) (1 min×1 and 30 min×1), washed with DCM (1 min×3), treated with 5% DIEA in DCM (1 min×1 and 10 min×1) and then washed with DCM (1 min×8). The solution of a 2-fold excess of Boc amino acid anhydride,[18] pre-prepared with Boc amino acid and DCC in DCM–DMF, was added to the resin and the mixture was shaken for 15 min as the coupling step. The solution of 20% DIEA in DCM was added, and the mixture was shaken for 15 min. After washing of the resin with DCM (1 min×3), the coupling efficiency was checked by Kaiser's Ninhydrin test,[21] which indicated the disappearance of the free amino group. In the case of an incomplete condensation reaction at the N-terminus, observed in the free amino group by the above test, the resin was recoupled by the same manner until the test indicated the disappearance of the free amino group. The resin was washed with DCM (1 min×3), then subjected to the next condensation cycle. After introducing the last Boc amino acid, Boc–Val, the resin was washed with EtOH (1 min×2) and filtered, washed by DCM, DCM–EtOH (1/1, v/v), and EtOH, then dried *in vacuo* to yield Boc, fully protected 18-Acm h-IGF-I (17—21)-resin (Yield 5.832 g, weight of increase 0.943 g, 73.88%).

Boc, Fully Protected 47-Acm h-IGF-I (37—55)-Resin Starting from Boc-Arg(Tos)-resin (0.5 meq/g, 1.500 g), Boc, fully protected 47-Acm h-IGF-I (37—55)-resin was prepared in a similar manner as described above and as shown in Fig. 5. In the case of the introduction of Boc–Gln and Boc–Arg(Tos), a 2-fold excess of their HOBt esters pre-prepared with Boc amino acid, HOBt, and DCC were used instead of their anhydrides. (Yield 3.585 g, weight of increase 2.085 g, 89.4%).

Boc, Fully Protected 48-Acm h-IGF-I (37—55)-Resin Starting from Boc–Arg(Tos)-resin (0.5 meq/g, 1.500 g), Boc, fully protected 48-Acm h-IGF-I (37—55)-resin was prepared in a similar manner as described above and as shown in Fig. 5. (Yield 3.406 g, weight of increase 1.906 g, 79.33%).

18-Acm h-IGF-I (17—21) (6) Boc, fully protected 18-Acm h-IGF-I (17—21)-resin (2.7085 g) was treated with anhydrous HF (30 ml) and anisole (2.5 ml) at 0 °C for 60 min (Fig. 5). After evaporation of the HF, ether (30 ml) was added to the mixture, and the precipitate obtained was washed with ether (30 ml×3), then extracted with water (10 ml×5). The aqueous solution was washed with ether (50 ml×2) and the aqueous layer was applied to a column of Dowex 1×2 (AcO^-, 2×10 cm). The passed solution (100 ml) and washings (1 M AcOH, 200 ml) were combined to give crude peptide (130 mg) after lyophilization. This was purified on RP-HPLC (column: YMC pack ODS-AP-324 s-5 μm 200 Å, conditions: linear gradient of 0—60% CH_3CN/0.01 M TFA in 15 min, flow rate 4.0 ml/min, detection 214 nm) to yield 18-Acm h-IGF-I (17—21) (**6**) in a pure state. MALDI-TOF-MS: $m/z=620.40$ $(M+H)^+$ (Calcd 619.65). Amino acid analysis: Val 1.0(1), Cys 0.4(1), Gly 0.9(1), Asp 0.6(1) Arg 1.0*(1). The amino acid sequence was determined by an Applied Bio-systems Model 476A Sequencer.

47-Acm h-IGF-I (37—55) (7′) Boc, fully protected 47-Acm h-IGF-I (37—55)-resin (1.728 g) was treated with anhydrous HF (30 ml) and anisole (2.5 ml) at 0 °C for 60 min (Fig. 5). After evaporation of the HF, ether (30 ml) was added to the mixture, and the precipitate obtained was washed with ether (30 ml×3), then extracted with water (10 ml×5). The aqueous solution was washed with ether (50 ml×2) and the aqueous layer was applied to a column of Dowex 1×2 (AcO^-, 2×10 cm). The passed solution (100 ml) and washings (1 M AcOH, 200 ml) were combined, and were then adjusted to pH 7.5 by 2 M NaOH, followed by intramolecular disulfide bond formation by 0.1 M $K_3[Fe(CN)_6]$ (10 ml) for 10 min at RT. The reaction mixture was acidified at pH 5.0 by AcOH, treated with an Amberlite IRA 94s (Cl^- type) column (1.8×13 cm) to remove the color and SEP-PAK (C_{18}) to remove the inorganic salt, and lyophilized to give crude 47-Acm h-IGF-I (37—55) (**7′**) (602 mg). This was purified on RP-HPLC (column: YMC pack ODS-AP-324 s-5 μm 200 Å, conditions: linear gradient of 0—60% CH_3CN/0.01 M TFA in 15 min, flow rate 4.0 ml/min, detection 214 nm) to yield **7′** as a pure state. MALDI-TOF-MS: $m/z=2237.93$ $(M+H)^+$ (Calcd 2238.42). Amino acid analysis: Arg 2.6(3), Ala 1.0*(1), Pro 0.8(1), Thr 0.9(1), Gly 0.9(1), Ile 0.5(1), Val 0.5(1), Asp 1.3(2), Glx 1.7(2), Phe 0.9(1), Ser 0.7(1), Leu 0.9(1). The amino acid sequence was determined by an Applied Bio-systems Model 476A Sequencer.

48-Acm h-IGF-I (37—55) (7) Boc, fully protected 48-Acm h-IGF-I (37—55)-resin (1.663 g) was treated with anhydrous HF (30 ml) and anisole (2.5 ml) at 0 °C for 60 min (Fig. 5). After evaporation of the HF, crude 48-Acm h-IGF-I (37—55) (**7**) (541 mg) was obtained in a similar manner as described above, as shown in the case of the synthesis of **7′**. This was purified on RP-HPLC (column: YMC pack ODS-AP-324 s-5 μm 200 Å, conditions: linear gradient of 0—60% CH_3CN/0.01 M TFA in 15 min, flow rate 4.0 ml/min, detection 214 nm) to yield **7** as a pure state. MALDI-TOF-MS: $m/z=2238.15$ $(M+H)^+$ (Calcd 2238.42). Amino acid analysis: Arg 2.5(3), Ala 1.0*(1), Pro 0.8(1), Thr 0.9(1), Gly 0.9(1), Ile 0.5(1), Val 0.5(1), Asp 0.9(2), Glx 1.6(2), Phe 0.9(1), Ser 0.7(1), Leu 0.9(1). The amino acid sequence was determined by an Applied Bio-systems Model 476A Sequencer.

h-IGF-I (17—21, 37—55) [Form II] (8) 18-Acm h-IGF-I (17—21) (**6**) (150 μg) and 48-Acm h-IGF-I (37—55) (**7**) (150 μg) were dissolved in a mixture (200 μl) of MeOH and H_2O (4 : 1, v/v). To this solution, a mixture (40 μl) of MeOH and 1 M HCl (4 : 1, v/v) containing I_2 (40 eq of the peptides) was added with stirring to create heterointer-molecular disulfide bond formation, together with the removal of Acm groups. After incubation at room temperature for 15 min, 0.25 M L-ascorbic acid in H_2O was added to stop the iodine oxidation reaction. The resulting solution was subjected to a RP-HPLC column using an acetonitrile gradient containing 0.01 M TFA, from 0 to 60% in 30 min, as shown in Fig. 7, to yield **8** in a pure state. FAB-MS: $m/z=2714\pm1$ $(M+H)^+$ (Calcd 2713.9). Amino acid analysis: Arg 3.7(4), Ala 1.0*(1), Pro 0.9(1), Thr 0.9(1), Gly 1.9(2), Ile 0.6(1), Val 1.3(2), Asx 2.0(3), Glx 1.7(2), Cys_2 1.7(2) Phe 0.9(1), Ser 0.8(1), Leu 0.9(1).

h-IGF-I (17—21, 37—55) [Form I] (8′) 18-Acm h-IGF-I (17—21) (**6**) (150 μg) and 47-Acm h-IGF-I (37—55) (**7′**) (150 μg) in a mixture (200 μl) of MeOH and H_2O (4 : 1, v/v) were subjected heterointer-molecular disulfide bond formation with I_2 (40 eq of the peptides) in a mixture (40 μl) of MeOH and 1 M HCl (4 : 1, v/v) to obtain **8′** in a manner similar to that described above, as shown in case of the synthesis of **8**. FAB-MS: $m/z=2714\pm1$ $(M+H)^+$ (Calcd 2713.9). Amino acid analysis: Arg 3.6(4), Ala 1.0*(1), Pro 0.8(1), Thr 0.9(1), Gly 1.9(2), Ile 0.5(1), Val 1.2(2), Asx 1.9(3), Glx 1.6(2), Cys_2 2.8(2) Phe 0.9(1), Ser 0.7(1), Leu 0.9(1).

h-IGF-I (17—21, 37—49, 50—55) [Form II] (5) h-IGF-I (17—21, 37—55) [form II] (**8**) in 1% $(NH_4)HCO_3$ (0.1 mol/ml, pH 8.0) was digested with α-chymotrypsin at an E/S ratio of 1/100 (w/w) for the enzyme, at 23 °C for 1 h. After the addition of acetic acid (pH 4.0) to stop the reaction, products were purified by RP-HPLC to obtain h-IGF-I (17—21, 37—49, 50—55) [form II] (**5**) as a single peak on RP-HPLC. FAB-MS: $m/z=2732\pm1$ $(M+H)^+$ (Calcd 2731.9). Amino acid analysis: Arg 3.6(4), Ala 1.0*(1), Pro 0.8(1), Thr 0.8(1), Gly 1.9(2), Ile 0.6(1), Val 1.4(2), Asx 2.1(3), Glx 1.8(2), Cys_2 1.6(2) Phe 0.8(1), Ser 0.8(1), Leu 1.0(1). For identification of the chemical structure of **5**, it was reduced by 2-ME to obtain the three peptides, h-IGF-I (17—21), h-IGF-I (50—55), and h-IGF-I (37—49) after purification with RP-HPLC. Amino acid analysis: h-IGF-I (17—21): Asx 0.6(1), Gly 1.0*(1), Arg 1.0(1), Val 0.8(1), Cys 0.6(1). h-IGF-I (50—55): Asx 0.8(1), Ser 1.0*(1), Arg 1.9(2), Cys 0.5(1), Leu 0.9(1). h-IGF-I (37—49): Asx 0.7(1), Glx 1.8(2), Gly 0.9(1), Arg 0.9(1), Thr 0.8(1), Ala 0.8(1), Val 0.7(1), Cys 1.4(2), Ile 0.6(1), Phe 1.0*(1).

47-Acm h-IGF-I (37—49, 50—55) (9′) 47-Acm h-IGF-I (37—55) (7′) (1.0 mg) in 10% $(NH_4)HCO_3$ (10 μl) and water (90 μl) was digested with α-chymotrypsin (10 μg) in 0.001 M HCl (1.0 ml) (pH 8.0) at an E/S ratio of 1/100 (w/w) for the enzyme, at 23 °C for 2 h. After the addition of acetic acid (pH 4.0) to stop the reaction, products were purified by RP-HPLC to obtain 47-Acm h-IGF-I (37—49, 50—55) (**9′**) as a single peak on RP-HPLC. FAB-MS: $m/z=2257\pm1$ $(M+H)^+$ (Calcd 2256.44). Amino acid analysis: Arg 2.5(3), Ala 1.0*(1), Pro 0.8(1), Thr 0.9(1), Gly 0.9(1), Ile 0.5(1), Val 0.5(1), Asp 0.9(2), Glx 1.6(2), Cys_2 0.8(1) Phe 0.9(1), Ser 0.7(1), Leu 0.9(1).

h-IGF-I (17—21, 37—49, 50—55) [Form I] (5′) 18-Acm h-IGF-I (17—21) (**6**) (50 μg) and 47-Acm h-IGF-I (37—49, 50—55) (**9′**) (50 μg) in a mixture (200 μl) of MeOH and H_2O (4 : 1, v/v) were subjected hetero-intermolecular disulfide bond formation with I_2 (40 eq of the peptides) in a mixture (40 μl) of MeOH and 1 M HCl (4 : 1, v/v) to obtain h-IGF-I (17—21, 7—49, 50—55) [form I] (**5′**) in a manner similar to that described in the case of the synthesis of **8** (Fig. 8). FAB-MS: $m/z=2732\pm1$ $(M+H)^+$ (Calcd 2731.9). Amino acid analysis: Arg 3.7(4), Ala 1.0*(1), Pro 0.9(1), Thr 0.9(1), Gly 1.9(2), Ile 0.6(1), Val 1.3(2), Asx 2.0(3), Glx 1.7(2), Cys_2 2.7(2) Phe 0.9(1), Ser 0.8(1), Leu 0.9(1).

Biological Activities Cell growth promoting activity was measured using BALB/c 3T3 fibroblasts grown under a serum-free condition. The detection of bioactivity was performed colorimetrically.[22] Binding affinities to human IGFBP-2 and IGFBP-3 were evaluated by a competition assay with ^{125}I-IGF-I.

Acknowledgments The authors would like to thank Professor K. Kawasaki and Dr. M. Maeda, Kobe Gakuin University, for use of the HF-reaction apparatus.

References and Notes

1) Abbreviations according to the IUPAC-IUB Commission *Eur. J. Biochem.*, **138**, 9—37 (1984) and "Amino Acids and Peptides Vol. 24" (The Royal Society of Chemistry, 1993) are used throughout. All amino acid symbols denote the L-configuration except glycine. Additional abbreviations are as follows: DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Boc, *tert*-butoxycarbonyl; Bzl, benzyl ether and benzyl ester; Tos, *p*-toluenesulfonyl; OcHx, cyclohexyl ester; Acm, acetamidomethyl; MBzl, *p*-methoxybenzyl; TFA, trifluoroacetic acid; DCM, dichloromethane; DMF, *N,N*-dimethyl formamide; DIEAN, *N,N′*-diisopropylethylamine; 2-ME, 2-mercaptoethanol; h-IGF-I, human insulin like growth factor I; IGFBP, IGF binding proteins; 3D, 3 dimensional; RT, room temperature; E/S, enzyme to substrate ratio; NOE, nuclear Overhauser effect; FAB-MS, fast atom bombardment mass spectrometer; MALD-TOF-MS, matrix-assisted laser desorption ionization-time of flight mass, spectrometer; RP-HPLC, reversed phase HPLC.

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Structure–Activity Relationship of Orally Potent Tripeptide-Based HIV Protease Inhibitors Containing Hydroxymethylcarbonyl Isostere

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We designed and synthesized a new class of peptidomimetic human immunodeficiency virus protease inhibitors containing a unique unnatural amino acid, allophenylnorstatine [Apns; (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid], with a hydroxymethylcarbonyl isostere as the active moiety. From a structure–activity relationship study of HIV-1 protease inhibition, enzyme selectivity for other aspartyl proteases, the antiviral activity and pharmacokinetics in rats, 24c (KNI-227) and 24d (KNI-272, our first clinical candidate) were found to be selective and orally potent HIV protease inhibitors. Moreover, an improvement of the pharmacokinetic features of KNI-272 provided two long-lasting and highly bioavailable compounds (24g: JE-2178, 24h: JE-2179).

Key words HIV protease; inhibitor; AIDS; KNI-272; allophenylnorstatine

The alarming spread of human immunodeficiency virus (HIV), the etiologic agent of AIDS, has initiated an urgent pursuit to comprehend and control this disease. Advances in molecular, viral, and cell biology have defined numerous targets for potential drug intervention. The virally encoded homodimeric aspartyl protease, which is responsible for processing the *gag* and *gag/pol* gene products that allow for the organization of core structural proteins and the release of viral enzymes, is one such target.[1)] Inhibition of this enzyme prevents the maturation and replication of the virus in cell culture. Inhibitors of HIV protease are presently being used in therapy for the treatment of AIDS.[2)] The HIV-1 protease is a member of the aspartic acid family of proteases, such as renin, pepsin and cathepsin D. Mammalian aspartyl proteases are well known to have two characteristic Asp–Thr–Gly sequences at the active center of the enzyme, and both side chain carboxyl groups are important in the catalysis of the peptide bond cleavage. In contrast, the retroviral protease has only one Asp–Thr–Gly sequence and has been shown by X-ray crystallography to be a C-2 symmetrical dimer. The HIV-1 protease can recognize Phe–Pro and Tyr–Pro sequences as the retrovirus-specific cleavage site, whereas mammalian aspartyl proteases such as renin, pepsin, and cathepsin D do not have such specificity. These features provided a basis for the rational design of selective HIV protease-targeted drugs for the treatment of AIDS and related diseases. Previously, we[3—5)] and other research groups[6)] reported a series of peptidomimetic HIV protease inhibitors containing allophenylnorstatine [Apns; (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] with a hydroxymethylcarbonyl (HMC) isostere based on the transition-state mimic concept. Especially the tripeptide compounds containing an Apns–Pro type structure at P1–P1′ showed a potent HIV-1 protease inhibitory activity. Here, we provide the details for the selection of KNI-272 (**24d**, our first clinical candidate, Fig. 1),[5,7)] based on the structure–activity relationship (SAR) of these series of inhibitors, not only in terms of HIV-1 protease inhibitory potency, but also with respect to the enzyme selectivity for other aspartyl proteases, the antiviral activity, and pharmacokinetics in rats. Moreover, an improvement of the pharmacokinetic features of KNI-272, providing two long-lasting and highly bioavailable compounds (**24g**: JE-2178, **24h**: JE-2179, Fig. 1), is reported.

Design of HIV Protease Inhibitor The transition state of amide hydrolysis by an aspartyl protease is proposed as illustrated in Fig. 2. The hydrogen bond between the carboxylic acid of the protease and the hydroxyl group of the substrate transition state is very important in the design of tight binding inhibitors.[8)] The HMC structure (Fig. 2) was reported to be incorporated in renin inhibitors. Therefore, we reasoned that the HMC group at the scissile site would interact at the active site of HIV protease and that the peptide compounds containing this structure would be highly potent HIV-1 protease inhibitors. In order to design substrate-based HIV protease inhibitors, we turned our attention to the *p17/p24* cleavage site region (Table 1).[9)] As described above, Tyr–Pro (or Phe–Pro) is a unique substrate structure for HIV-1 protease, and the synthetic substrates containing this sequence has low *Km* value.[10)] However, the phenolic hydroxyl group of the P1 site and the carboxamide side chain of Gln at the P3 site seem to have no apparent effect on the binding to HIV-1 protease.[11)] On the basis of these specifications, we considered the heptapeptide amide Ser–Phe–Asn–Phe–Pro–Ile–Val–NH_2, a chimeric structure of the *TF/PR* and *p17/p24* sequences (Table 1). Then, we incorporated an unnatural amino acid, phenylnorstatine [Pns;(2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid) or allophenylnorstatine [Apns; (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid),[12)] containing the HMC isostere as a transition-state mimic at the P1 site in this peptide amide (compounds **2a, b**). In order to obtain smaller inhibitors, Ser (P4) was deleted, and Phe (P3) was replaced with the isosteric 3-phenylpropionyl (Pp) group (compounds **3a, b**). Moreover, Val (P3′) was deleted, Ile (P2′) was replaced with the isosteric *tert*-butylamine, and the Pp group was replaced with benzyloxycarbonyl (Z) group (compounds **7a, b**, Fig. 3).

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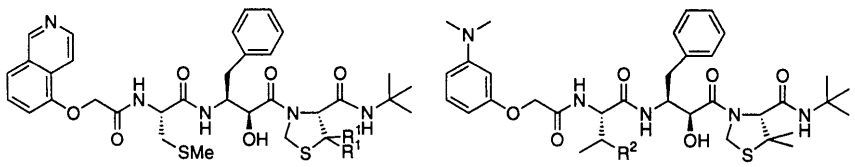


R^1 = Me : KNI-227 (**24c**)
R^1 = H : KNI-272 (**24d**)

R^2 = Me : JE-2178 (**24g**)
R^2 = H : JE-2179 (**24h**)

Fig. 1. Chemical Structures of KNI-227, -272, and JE-2178, -2179

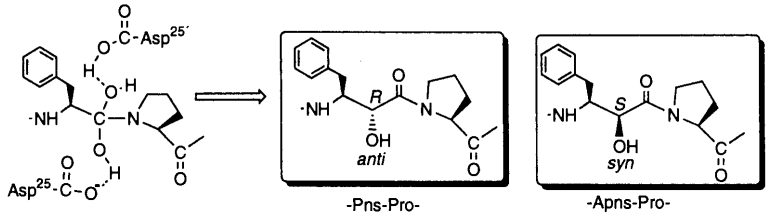


Fig. 2. The Phe–Pro Transition State in HIV-1 Protease, Pns–Pro and Apns–Pro (P1–P1′) with the Hydroxymethylcarbonyl (HMC) Isostere Mimicking the Transition State

Pns=phenylnorstatine=(2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid, Apns=allophenylnorstatine=(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid.

Table 1. Amino Acid Sequences of Cleavage Sites for HIV-1 Protease[9] and Model Substrate

Cleavage site	Amino acid sequence							
	P4	P3	P2	P1	P1′	P2′	P3′	P4′
p17/p24	–Ser	Gln	Asn	Tyr	Pro	Ile	Val	Gln–
p24/p1	–Ala	Arg	Val	Leu	Ala	Glu	Ala	Met–
p1/p9	–Ala	Thr	Ile	Met	Met	Gln	Arg	Glu–
p9/p6	–Pro	Gly	Asn	Phe	Leu	Gln	Ser	Arg–
TF/PR	–Ser	Phe	Asn	Phe	Pro	Gln	Ile	Thr–
PR/RT	–Thr	Leu	Asn	Phe	Pro	Ile	Ser	Pro–
RT/RN	–Ala	Glu	Thr	Phe	Tyr	Val	Asp	Gly–
RN/IN	–Arg	Lys	Ile	Leu	Phe	Leu	Asp	Gly–
Model substrate	Ser	Phe	Asn	Phe	Pro	Ile	Val	NH_2

TF=transframe protein; *PR*=protease; *RT*=reverse transcriptase; *RN*=ribonuclease H; *IN*=integrase.

Chemistry Boc–Apns–OH (**1a**), and Boc–Pns–OH (**1b**) were prepared according to the methods described previously.[12,13] Compounds **2a, b**, and **3a, b** were synthesized by the solid-phase method using Boc strategy on *p*-methylbenzhydrylamine (MBHA) resin.[14] The protected peptide resin thus obtained was treated with anhydrous HF containing *m*-cresol at 0 °C for 60 min, to obtain fully deprotected peptides. The crude peptides were purified by preparative HPLC on an ODS-column. Compounds **7a, b** were synthesized by the solution method in a stepwise manner (Chart 1). The amide bond formation of P1′–P2′ and of P1–P1′ was achieved by use of carbodiimide/1-hydroxybenzotriazol (HOBt) as a condensation reagent. Z-Asn (P3–P2 segment) was incorporated *via* its *p*-nitrophenyl (Np) ester in the presence of HOBt. The inhibitors in Tables 3, 4 (**13a—h**) were obtained as shown in Chart 2. Compounds **13a—e** were prepared by use of the fragment coupling method (P3–P2–P1+P1′–P2′, route A), while compounds **13f, g, h** were obtained by the stepwise elongation method (route B). Boc-protected (*R*)-1,3-thiazolidine-4-carboxylic acid derivatives **8b, c** were prepared from the corresponding L-cysteine analogs by cyclization with formaldehyde, followed by *tert*-butoxycarbonylation with Boc_2O in a one-pot reaction. The amide bonds of P1′–P2′ were formed by use of *N,N′*-dicyclohexylcarbodiimide (DCC)/HOBt or diphenylphosphoryl chloride (DPP-Cl) as a condensation reagent. The mixed anhydride prepared with DPP-Cl was effective for the preparation of Boc-protected 5,5-dimethyl-1,3-thiazolidine-4-carboxamide **9h**. The compounds in Tables 5—8 were synthesized by the following methods (Charts 3—8): Chart 3 shows the preparative method of the aryloxyacetic acids corresponding to the P3 building block. Aryloxyacetic acids **16a—f** were obtained by the reaction of phenols and chloroacetyl esters in the presence of K_2CO_3 or sodium methoxide followed by saponification or hydrogenation. 3-(Dimethylamino)phenoxyacetic acid (**16f**) was isolated as the corresponding crystalline *N*-hydroxy-5-norbornene-2,3-carboxamide (HONB) active ester **17**. Chart 4 illustrates the procedures to synthesize Boc-protected P2–P1–P1′–P2′ intermediates **18a—k**. Compounds **18a, b**, and **h** incorporated asparagine residue at the P2 site were obtained by an active ester method, in order to avoid the contamination of nitrile compounds. Other intermediates **18c—g, i—k** were synthesized under *N*-ethyl-*N′*-[3-(dimethylamino)propyl]carbodiimide (EDC)/HOBt coupling conditions. Removal of the Boc group under acidic conditions and coupling to the corresponding P3 building blocks provided the inhibitors (Charts 5—8). The carbamate compound **21** was synthesized by coupling with the appropriate carbonate **20** derived from *p*-nitrophenyl chloroformate and the corresponding alcohol **19**. The aryloxyacetamide inhibitors in this article except **24g** and **h** were obtained by the coupling to the corresponding aryloxyacetic acids under EDC–HOBt conditions, and compounds **24g** and **h** were prepared by the HONB active ester method (Charts 7—8).

Structure–Activity Relationship against HIV-1 Protease The compounds synthesized in this study were firstly tested for HIV-1 protease inhibitory activity. HIV-1 protease activity was determined by an HPLC method using chemically synthesized [$Ala^{67,95}$] HIV-1 protease (NY-5)[15] and synthetic peptide Ac–Arg–Ala–Ser–Gln–Asn–Tyr–Pro–Val–Val–NH_2[16] as a substrate. Table 2 shows the results of the

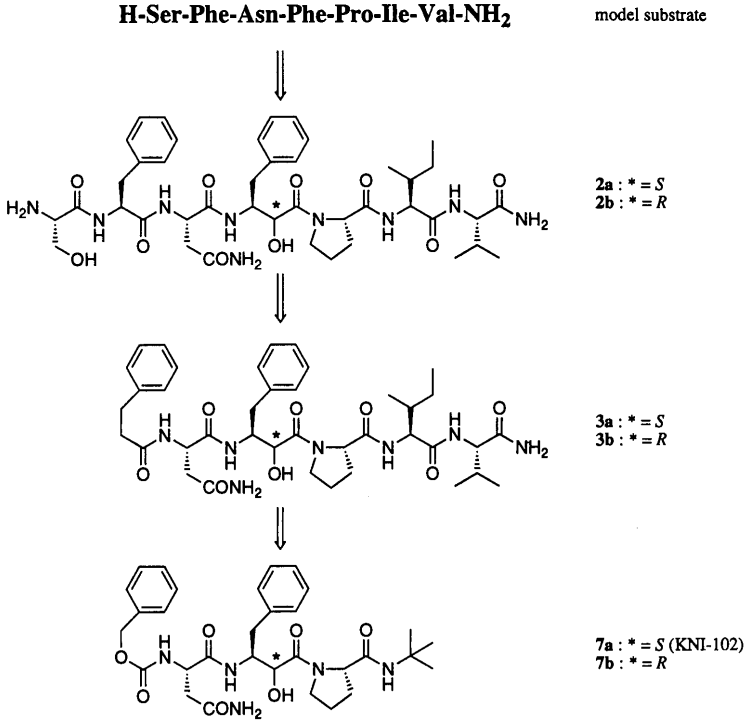
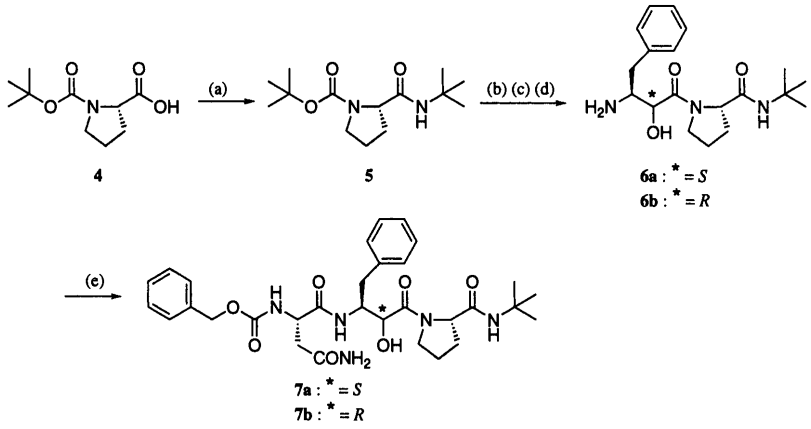
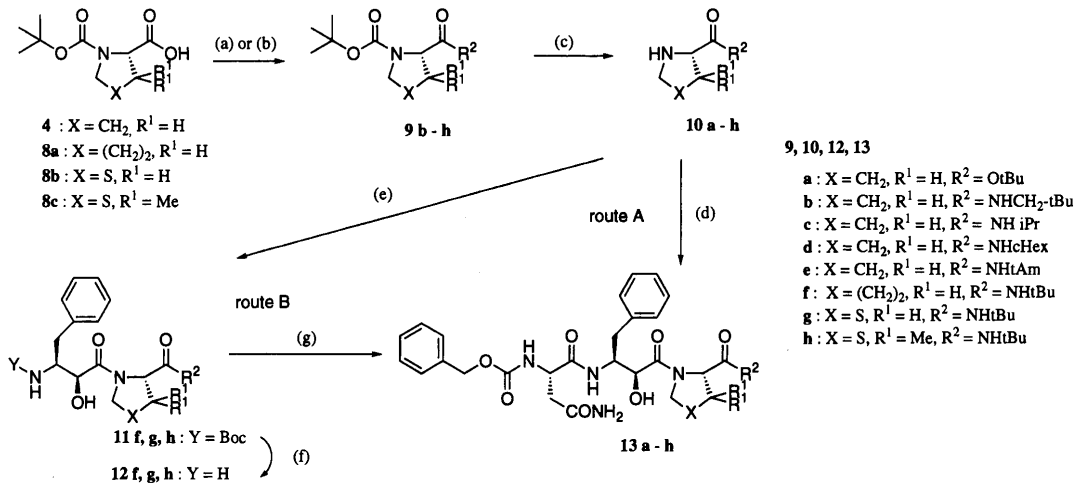


Fig. 3. Design of Substrate-Based HIV Protease Inhibitors



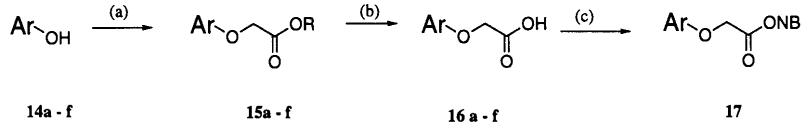
Reagents : (a) EDC-HOBt, *tert*-butylamine, CH_2Cl_2; (b) 4 N HCl/dioxane; (c) **1a** or **1b**, EDC-HOBt, DMF; (d) 4 N HCl/dioxane; (e) Z-Asn-ONp, HOBt, TEA, DMF

Chart 1



Reagents : (a) EDC-HOBt, *tert*-butylamine, CH_2Cl_2; (b) DPP-Cl, TEA, EtOAc, and then *tert*-butylamine; (c) 4 N HCl/dioxane, (d) Z-Asn-Apns-OH[6b], EDC-HOBt, DMF; (e) **1a**, DCC-HOBt, EtOAc; (f) 4 N HCl/dioxane; (g) Z-Asn-ONp, HOBt, TEA, DMF

Chart 2



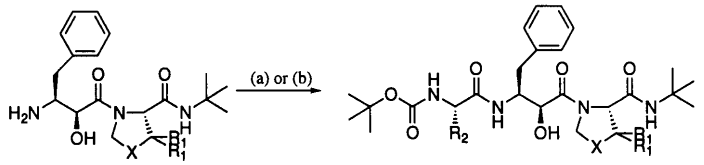
14a - f 15a - f 16 a - f 17

14 - 16 a: Ar = 2-biphenyl, R = Me
b: Ar = 3-biphenyl, R = Me
c: Ar = 4-biphenyl, R = Me
d: Ar = 3-(phenylamino)phenyl, R = Me
e: Ar = 5-isoquinolinyl, R = Me
f: Ar = 3-(dimethylamino)phenyl, R = Bn

Ar = 3-(dimethylamino)phenyl

Reagents : (a) methyl chloroacetate or benzyl chloroacetate , K_2CO_3, DMF; (b) NaOH, MeOH aq, or H_2, Pd/C, MeOH; (c) *N*-hydroxy-5-norbornene-2,3-dicarboximide (HONB), DCC, CH_2Cl_2

Chart 3

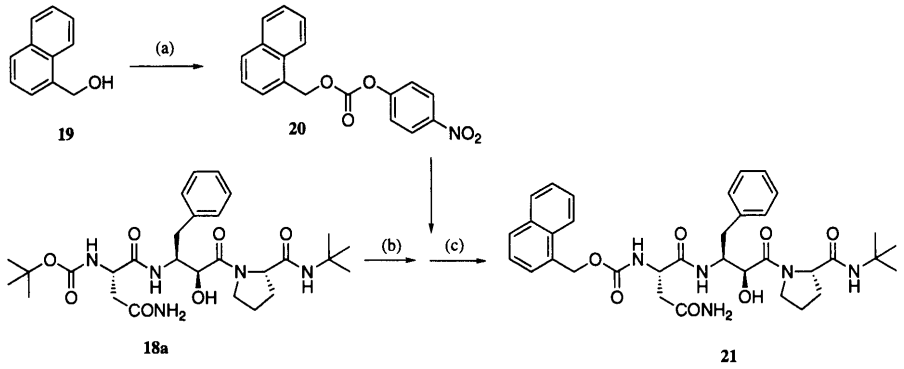


6a: X = CH_2, R^1 = H
12g: X = S, R^1 = H
12i: X = S, R^1 = Me

18a: X = CH_2, R^1 = H, R^2 = CH_2CONH_2
b: X = S, R^1 = H, R^2 = CH_2CONH_2
c: X = S, R^1 = H, R^2 = nPr
d: X = S, R^1 = H, R^2 = iPr
e: X = S, R^1 = H, R^2 = tBu
f: X = S, R^1 = H, R^2 = CH_2SMe
g: X = S, R^1 = H, R^2 = Et
h: X = S, R^1 = Me, R^2 = CH_2CONH_2
i: X = S, R^1 = Me, R^2 = Et
j: X = S, R^1 = Me, R^2 = iPr
k: X = S, R^1 = Me, R^2 = CH_2SMe

Reagents : (a) Boc-Asn-ONp, HOBt, TEA, DMF; (b)Boc-AA-OH, EDC-HOBt, DMF

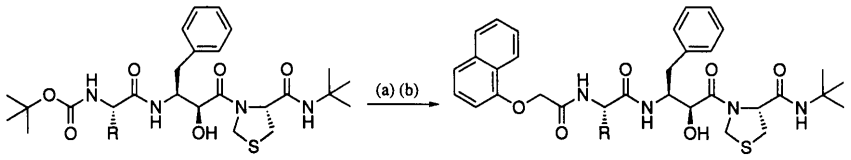
Chart 4



19 20 18a 21

Reagents : (a) 4-nitrophenyl chloroformate, pyridine; (b) 4 N HCl/dioxane; (c) HOBt, TEA, DMF

Chart 5



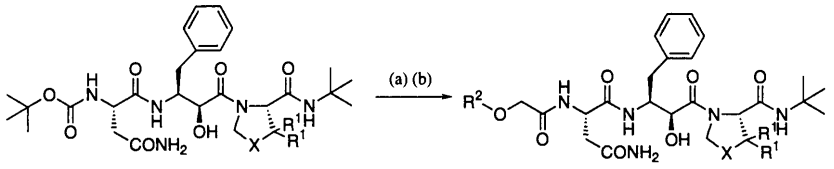
18b - f

22a: R = CH_2CONH_2
b: R = nPr
c: R = iPr
d: R = tBu
e: R = CH_2SMe

Reagents : (a) 4 N HCl/dioxane; (b) 1-naphthoxyacetic acid, EDC-HOBt, DMF

Chart 6

SAR study on the peptide size and stereochemistry of the hydroxyl group in an unnatural amino acid on HIV-1 protease inhibition.[4] Compound **2a** (KNI-93) containing Apns with an HMC isostere exhibited a potent inhibitory activity against HIV-1 protease with an IC_{50} value of 5 nM. Compound **3a**, with the deletion of Ser (P4) and replacement of Phe (P3) by isosteric Pp, also maintained a sufficient inhibitory activity (IC_{50}=468 nM). The Apns-containing tripeptide **7a** (KNI-102), in which Phe (P3) was replaced by its isosteric Z group, Val (P3′) was deleted, and Ile (P2′) was replaced by its isosteric *tert*-butylamine, exhibited a strong activity compared with the pentapeptide **3a**. The stereochemistry of the hydroxyl group is very important for the inhibition of aspartyl proteases. In the case of substrate-based HIV

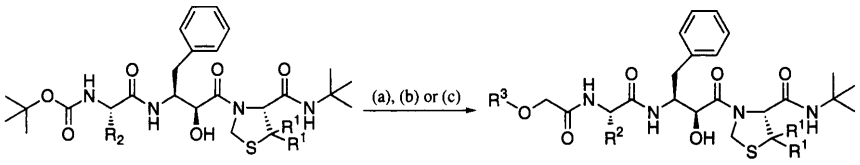


18a: X = CH_2, R^1 = H
18h: X = S, R^1 = Me

23 a: R^2 = 1-naphthyl, X = CH_2, R^1 = H
b: R^2 = 2-naphthyl, X = CH_2, R^1 = H
c: R^2 = 1-naphthyl, X = S, R^1 = Me
d: R^2 = 2-biphenyl, X = S, R^1 = Me
e: R^2 = 3-biphenyl, X = S, R^1 = Me
f: R^2 = 4-biphenyl, X = S, R^1 = Me
g: R^2 = 3-(phenylamino)phenyl, X = S, R^1 = Me
h: R^2 = 5-isoquinolinyl, X = S, R^1 = Me

Reagents : (a) 4 N HCl/dioxane; (b) aryloxyacetic acid in Chart 3, EDC-HOBt, DMF

Chart 7



18d: R^1 = H, R^2 = iPr
f: R^1 = H, R^2 = CH_2SMe
g: R^1 = H, R^2 = Et
i: R^1 = Me, R^2 = Et
j: R^1 = Me, R^2 = iPr
k: R^1 = Me, R^2 = CH_2SMe

24 a: R^1 = Me, R^2 = CH_2SMe, R^3 = 1-naphthyl
b: R^1 = Me, R^2 = CH_2SMe, R^3 = 3-(phenylamino)phenyl
c: R^1 = Me, R^2 = CH_2SMe, R^3 = 5-isoquinolinyl
d: R^1 = H, R^2 = CH_2SMe, R^3 = 5-isoquinolinyl
e: R^1 = H, R^2 = iPr, R^3 = 5-isoquinolinyl
f: R^1 = H, R^2 = Et, R^3 = 5-isoquinolinyl
g: R^1 = Me, R^2 = iPr, R^3 = 3-(dimethylamino)phenyl
h: R^1 = Me, R^2 = Et, R^3 = 3-(dimethylamino)phenyl

Reagents : (a) 4 N HCl/dioxane; (b) aryloxyacetic acid in Chart 3, EDC-HOBt, DMF; (c) **17**, TEA, DMF

Chart 8

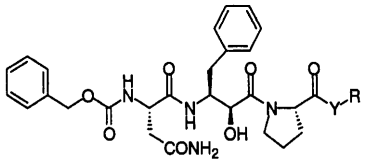
Table 2. Inhibitory Activity against HIV-1 Protease and Anti-HIV-1 IIIB Activity of HIV Protease Inhibitors Containing an Unnatural Amino Acid Such as Apns or Pns

No.		Structure								HIV-1 protease inhibition (IC_{50}, nM)	Anti HIV-1[7] (IC_{50}, μM)
		P4	P3	P2	P1	P1′	P2′	P3′	P4′		
2a	(KNI-93)	Ser	Phe	Asn	Apns	Pro	Ile	Val	NH_2	5.0	>10
2b		Ser	Phe	Asn	Pns	Pro	Ile	Val	NH_2	100	>10
3a			Pp	Asn	Apns	Pro	Ile	Val	NH_2	468	>10
3b			Pp	Asn	Pns	Pro	Ile	Val	NH_2	3000	N.D.
7a	(KNI-102)		Z	Asn	Apns	Pro	NH*t*Bu			89	1.1
7b			Z	Asn	Pns	Pro	NH*t*Bu			>10000	N.D.

Apns=allophenylnorstatine; (2*S*,3*S*)-3-amimo-2-hydroxy-4-phenylbutyryl, Pns=phenylnorstatine; (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyryl, Pp=3-phenylpropionyl, Z=benzyloxycarbonyl, tBu=*tert*-butyl, N.D.=not determined.

protease inhibitors containing HMC–Pro at the scissile peptide bond, the 2*S*-HMC inhibitor (*syn*-diastereomer) containing Apns (**2a**, **3a**, **7a**) was more active than the anti-diastereomer containing Pns (**2b**, **3b**, **7b**) in contrast to the case of the renin inhibitor, which showed a preference of anti-diastereomer over the *syn*-diastereomer.[8] In the case of hydroxyethyl (HE)–Pro type HIV protease inhibitors, the preference shifted from the *syn*-diastereomer in short-chain inhibitors to the anti-diastereomer in long-chain inhibitors.[17,18] The discrepancy between the HMC–Pro inhibitors and the HE–Pro inhibitors seems to be due to the conformational difference between the constrained peptide bond and the relatively flexible methylene-amine bond. On the other hand, HMC–Phe type compounds preferred the anti-hydroxyl group.[4] A preference for the *syn*-hydroxyl group shown in this series of HMC–Pro inhibitors is exceptional among various inhibitors of aspartyl protease such as HIV protease, renin and pepsin, which implies the uniqueness of the HMC–Pro structure. The HIV-1 cytopathic inhibition assay of these HMC compounds was carried out by using $CD4^+$ATH8 cell.[19] The long-chain compounds **2a, b** and **3a**, containing natural peptide bonds, showed no inhibitory activity against the HIV cytopathic effect in spite of their potent HIV-1 protease inhibitory activity (Table 2). These compounds seemed to be decomposed by cellular peptidases, or not to be able to penetrate the cell membrane. In contrast, a small-sized compound **7a**, having no natural peptide bonds, showed a potent anti-viral activity with an IC_{50} value of 1.1 μM.[7] In the case

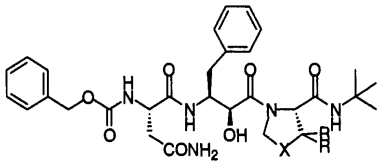
Table 3. HIV-1 Protease Inhibitory Activity (P2′ Site)



Compound	Structure		HIV-1 protease inhibition
	Y	R	IC_{50} (nM)
7a	NH	*t*Bu	89
13a	O	*t*Bu	868
13b	NH	CH_2*t*Bu	520
13c	NH	*i*Pr	320
13d	NH	*c*Hex	572
13e	NH	*t*Am	182

*i*Pr=isopropyl; *c*Hex=cyclohexyl; *t*Am=*tert*-amyl.

Table 4. HIV-1 Protease Inhibitory Activity (P1′ Site)



Compound	Structure			HIV-1 protease inhibition
	X	R		IC_{50} (nM)
7a	CH_2	H	(Pro)	89
13f	$(CH_2)_2$	H		450
13g	S	H	(Thz)	31
13h	S	Me	(Dmt)	3.5

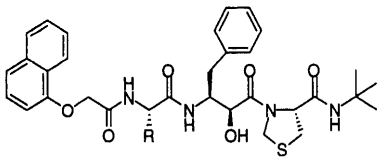
Thz=(*R*)-1,3-thiazolidine-4-carbonyl; Dmt=(*R*)-5,5-dimethyl-1,3-thiazolidine-4-carbonyl.

of our HMC–Pro type compounds, the tripeptide compound was considered to be maximum size required for showing a potent anti-HIV activity.

Table 3 presents the results of the SAR study on the P2′ site. The amide compound **7a** was about 10 fold more preferable than the corresponding ester compound **13a**. According to the result of the analysis of the X-ray crystal structure of KNI-272 complexed to HIV-1 protease,[20] the amide nitrogen of *tert*-butyl (P2′) binds to a water molecule, which forms bridging hydrogen bonds between the amide nitrogen atom of the P2′ group and the backbone nitrogen of Asp29. This causes the 10-fold preference in the HIV protease inhibition of the amide linkage over the ester linkage. The tertiary amides **7a**, **13e**, which might be constrained in their conformation, were more suitable than the primary **13b** or secondary amide **13c**, **d**. Moreover, *tert*-butyl **7a** was more suitable than the bulkier group **13e**.

The SAR study on the P1–P1′ site (P1′ amino acid), which seemed to influence the conformation of the whole compound, is summarized in Table 4. Replacement of the pyrrolidine ring **7a** by the expanded piperidine ring **13f** decreased the HIV-1 protease inhibitory activity; and compound **13g**, with the pyrrolidine ring replaced by the thiazolidine ring (Thz), showed three times greater potency in HIV-1 protease inhibitory activity as compared with compound **7a**. In the case of the hydroxyethylamine (HEA) type inhibitor,

Table 5. HIV-1 Protease Inhibitory Activity (P2 Site)



Compound	Structure		HIV-1protease inhibition
	R		IC_{50} (nM)
22a	$-CH_2CONH_2$	(Asn)	8.8
22b	*n*Pr		6.1
22c	*i*Pr	(Val)	4.1
22d	*t*Bu		12
22e	$-CH_2SMe$	(Mta)	3.2

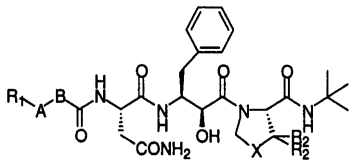
Mta=(*R*)-methylthioalanine.

the replacement of the pyrrolidine ring by the piperidine ring increased the inhibitory activity.[18] As shown in Table 1, HIV-1 protease is known to recognize various sequences as cleavage sites. In addition to Phe–Pro and Tyr–Pro sites, some hydrophobic sites resembling the angiotensinogen cleavage site were recognized.[9] Some compounds, which were designed as renin inhibitors, were also reported to inhibit HIV-1 protease.[21,22] Especially, U-81749, containing ChaΨ[CH(OH)-CH_2]Val at P1–P1′ as a transition-state mimic of the angiotensinogen cleavage site (Leu–Val), exhibited a potent HIV-1 PR inhibitory activity.[22] Accordingly we examined the β-branched amino acid, 5,5-dimethylthiazolidine carboxylic acid (Dmt), a chimeric structure of Thz and Val. The additional dimethyl groups caused an alternate hydrophobic interaction with the S1′ site, and compound **13h** showed a highly potent HIV-1 PR inhibition (IC_{50}=3.5 nM).

Table 5 presents the results of the SAR study on the P2 site. The P2 amino acid of the substrates that have an Xaa–Pro cleavage site, is always Asn, but that of substrates having other cleavage site is a hydrophobic amino acid (Table 1). We investigated some hydrophobic amino acids in addition to Asn at the P2 site of the tripeptide. The structural restriction of this site seemed to be relatively generous in the case of small hydrophobic side chains. The β-branched amino acid **22c** (Val) and a C3 straight chain amino acid **22b** showed good inhibitory activity, but a bulkier group **22d** reduced the inhibitory activity. The introduction of the sulfur atom **22e** (Mta: methylthioalanine) gave more potent interaction, because the bulky sulfur atom helps to fill the subsite volume.[20]

Table 6 presents the results of the SAR study on the P3 site. Replacement of the phenyl group of compound **7a** by the bulkier naphthyl group **21** enhanced the inhibitory activity. Moreover, the 1-naphthoxyacetyl group containing compound **23a** was preferable to the corresponding urethane type compounds **21**. Whereas the 2-naphthoxyacetyl group **23b**, a regioisomer of compound **23a**, reduced the inhibitory activity. The phenyl substituent on the phenoxyacetyl group **23d**—**f** also enhanced the HIV-1 protease inhibitory activity, especially the *m*-substituent **23e** was more suitable than the other regioisomers **23d**, **f**. Although these compounds showed a highly potent inhibitory activity (IC_{50}=2—3 nM), these P3 ligands increased the hydrophobicity, especially in combination with a hydrophobic amino acid at the P2 site

Table 6. HIV-1 Protease Inhibitory Activity (P3 Site)



Compound	Structure						HIV-1 protease inhibition
	P3						
	R^1	A	B		X	R^2	IC_{50} (nM)
7a	Phenyl	CH_2	O	(Z)	CH_2	H	89
21	1-Naphthyl	CH_2	O		CH_2	H	24
23a	1-Naphthyl	O	CH_2	(1-Noa)	CH_2	H	12
23b	2-Naphthyl	O	CH_2		CH_2	H	19
23c	1-Naphthyl	O	CH_2		S	Me	2.8
23d	2-Biphenyl	O	CH_2		S	Me	3.0
23e	3-Biphenyl	O	CH_2	(3-Bpoa)	S	Me	2.2
23f	4-Biphenyl	O	CH_2		S	Me	3.0
23g	3-(Phenylamino)phenyl	O	CH_2	(3-Papoa)	S	Me	2.2
23h	5-Isoquinolinyl	O	CH_2	(5-iQoa)	S	Me	3.6

1-Noa=1-naphthoxyacetyl; 3-Bpoa=3-biphenyloxyacetyl; 3-PaPoa=3-(phenylamino)phenoxyacetyl; 5-iQoa=5-isoquinolinyloxyacetyl.

Table 7. Selectivity against Other Aspartyl Proteases

No.		Structure					HIV-1 PR	Pepsin[a]	Renin[b]	Cathepsin D[c]
		P3	P2	P1	P1′	P2′	IC_{50} (nM)			
7a		Z	Asn	Apns	Pro	NH*t*Bu	87	>80000	>100000	>100000
23a		1-Noa	Asn	Apns	Pro	NH*t*Bu	12.3	>80000	>100000	>100000
22a		1-Noa	Asn	Apns	Thz	NH*t*Bu	8.8	>80000	>100000	18000
23c	(KNI-174)	1-Noa	Asn	Apns	Dmt	NH*t*Bu	2.8	>80000	>100000	6500
23e		3-Bpoa	Asn	Apns	Dmt	NH*t*Bu	2.2	>80000	>100000	2900
23g	(KNI-241)	3-Paoa	Asn	Apns	Dmt	NH*t*Bu	2.2	>80000	>100000	1600
23h		5-iQoa	Asn	Apns	Dmt	NH*t*Bu	3.6	>80000	>100000	>100000
24a		1-Noa	Mta	Apns	Dmt	NH*t*Bu	2.7	18600	>100000	1500
24c	(KNI-227)	5-iQoa	Mta	Apns	Dmt	NH*t*Bu	2.6	12900	>100000	23000
24d	(KNI-272)	5-iQoa	Mta	Apns	Thz	NH*t*Bu	6.6	>80000	>100000	>100000
Pepstatin A							940	2600	1690	62

a) porcine pepsin, *b*) human plasma renin, *c*) bovine cathepsin D; IC_{50} values against HIV-1 PR of this table were determined by an HPLC method using [$Ala^{67,95}$]HIV-1 protease and synthetic peptide Ac–Arg–Ala–Ser–Gln–Asn–Tyr–Pro–Val–Val–NH_2 as a substrate.

(*e.g.* **24a**). This increased hydrophobicity seemed to cause cytotoxicity, and to reduce the oral absorption. Compound **23h**, which incorporated a nitrogen atom at position 6 of the naphthalene ring (corresponding to 5-isoquinoline), reduced hydrophobicity but retained the HIV-1 PR inhibitory activity.

Enzyme Selectivity for Other Aspartyl Proteases Some of the potent inhibitors obtained in this study were examined for enzyme selectivity. The inhibitory activities toward the closely related aspartyl proteases, *i.e.*, pepsin, renin, and cathepsin D, are shown in Table 7. All compounds tested showed more than 500 fold selectivity for HIV-1 PR over other aspartyl proteases, whereas pepstatin A,[23] a typical aspartyl protease inhibitor, showed no selectivity. The high selectivity of these compounds is due to the unique *syn*-configuration of the hydroxyl group of the HMC-Pro like transition state analogue, which is exceptional among various inhibitors of aspartyl proteases. Especially the compounds containing Asn at the P2 site showed higher selectivity over the compounds containing a hydrophobic amino acid (Mta). Increase in HIV-1 protease inhibition by the replacement of P1′ or P3 residues was accompanied by the inhibition of cathepsin D. The relatively hydrophilic 5-isoquinolyloxyacetyl (5-iQoa) group (P3) improved these selectivities, and compounds **23h** and **24d** (KNI-272) showed a high enzyme selectivity without loss of HIV-1 protease inhibitory activity.

Antiviral Activity and Pharmacokinetics *Ki* values, antiviral activity, and pharmacokinetics for selected compounds are shown in Table 8. *Ki* values against HIV-1 protease were determined by an HPLC method using recombinant HIV-1 protease (NY-5) and synthetic peptide H–Ser–Gln–Asn–Tyr–Pro–Ile–Val–OH as a substrate. Antiviral activity was determined in cell culture against HIV-1 strain IIIB in CEM-SS cells.[24]

The compound containing 3-(phenylamino)phenoxyacetyl (3-Papoa) as the P3 ligand preferred Asn (**23g**) to Mta (**24b**) for the P2 residue, whereas 5-iQoa preferred Mta (**24c**) to Asn (**23h**), which was caused by the different binding mode between Asn and Mta for the S2 subsite (hydrogen bond formation and hydrophobic interaction, respectively). Tanaka *et al.*[25] reported that KNI-272-resistant HIV-1 was nearly com-

Table 8. HIV-1 Protease Inhibitory Activity, Anti-HIV Activity, and Pharmacokinetic Profile in Rats

No		Structure					*Ki* (nM)	HIV-1 IIIB IC_{50} (nM)	C_{max} (μM)	T_{max} (min)	$t_{1/2\beta}$ (min)	*AUC* (μM·min)	*F* (%)
		P3	P2	P1	P1′	P2′							
23g	(KNI-241)	3-Papoa	Asn	Apns	Dmt	NH*t*Bu	0.058	50	N.D.	N.D.	70	N.D.	N.D.
23h		5-iQoa	Asn	Apns	Dmt	NH*t*Bu	0.349	225	0.04	10	17	1.6	0.7
24b		3-Papoa	Mta	Apns	Dmt	NH*t*Bu	0.184	6.8	N.D.	N.D.	42	N.D.	N.D.
24c	(KNI-227)	5-iQoa	Mta	Apns	Dmt	NH*t*Bu	0.088	5.7	0.37	30	48	79	29
24d	(KNI-272)	5-iQoa	Mta	Apns	Thz	NH*t*Bu	0.744	25	1.29	60	22	117	28
24e		5-iQoa	Val	Apns	Thz	NH*t*Bu	1.498	31	2.46	30	29	127	32
24f		5-iQoa	Abu	Apns	Thz	NH*t*Bu	2.144	102	1.58	30	28	74	23
24g	(JE-2178)	3-Dapoa	Val	Apns	Dmt	NH*t*Bu	0.318	15	0.70	90	63	157	89
24h	(JE-2179)	3-Dapoa	Abu	Apns	Dmt	NH*t*Bu	0.399	24	1.16	60	110	228	82

Ki values of this table were determined by an HPLC method using recombinant HIV-1 protease (NY-5) and synthetic peptide H–Ser–Gln–Asn–Tyr–Pro–Ile–Val–OH as a substrate. Antiviral activity was determined based on the HIV-1 IIIB-induced cytopathic effects evaluated by the use of the tetrazolium reagent as described in the Experimental Section. *F* (%) which is percent bioavailability *via* i.d. route, was determined by comparing the mean areas under the curves (*AUC*) after i.v. and i.d. doses., 3-Dapoa=3-(dimethylamino)phenoxyacetyl; Abu=(*S*)-2-aminobutyryl; *CL*, plasma clearance rate; V_{dss}, volume of distribution; $t_{1/2\beta}$, plasma half-life; C_{max}, maximum plasma concentration; T_{max}, time of maximum plasma concentration; N.D.=not detected.

pletely sensitive to compound KNI-241 (**23g**). The low resistance profile among these compounds seems to be due to this different binding mode of each compound. Although compound **23g** showed an extremely low *Ki* value, its anti-HIV activity was limited to 50 nM. The compounds bearing Asn at P2 site **23g, h** did not translate their HIV-1 protease inhibitory action into anti-HIV activity in CEM-SS cells. The hydrophilic carboxamide group of Asn, which would be expected to hydrate easily, would interrupt penetration of the drug through the cell membrane. On the other hand, the compounds containing hydrophobic Mta at the P2 site **24b, c** showed a potent anti-HIV activity in CEM-SS cells with IC_{50} values below 10 nM.

These selected compounds were examined for pharmacokinetics when administered by the intraduodenal (i.d.) route to rats as a 50% polyethylene glycol (PEG) solution (10 mg/kg). The plasma levels of the compounds, bearing Asn residue at P2 or the 3-Papoa structure at P3, could not be detected as significant (**23g, h, 24b**). The hydrophilicity of asparagine, or the oxidation sensitive nature of the diphenylamine structure (3-Papoa) would explain these undesirable results. Compound **24c** (KNI-227, Fig. 1), bearing an hydrophobic Mta residue at P2, showed the plasma concentration over 0.3 μM, and had 29% bioavailability after i.d. administration in rats. The replacement of Dmt with Thz at P1′ gave compound **24d** (KNI-272, Fig. 1), having a higher plasma concentration (C_{max}=1.29 μM, Fig. 5) and potent anti-HIV activity. Moreover, the maximum plasma concentration achieved was 4.18 μM when KNI-272 was orally administered at a dose of 15 mg/kg to dogs, and the bioavailability was estimated to be 29%.[26] When we found KNI-272 to be a promising HIV protease inhibitor, there were no known orally potent HIV protease inhibitors available (saquinavir was only several percentage points orally bioavailable in animals[27]); therefore we chose this compound as our first clinical candidate.

Improvement on KNI-272 Clinical trials of KNI-272 were undertaken and the result showed a decrease in the viral load in the blood of patients.[28] Unfortunately, as the relatively short plasma half-life of KNI-272 limits our ability to maintain plasma levels, KNI-272 requires frequent dosing for clinical use. Some HIV protease inhibitors, especially ritonavir,[29] enhanced the plasma concentration of KNI-272 by

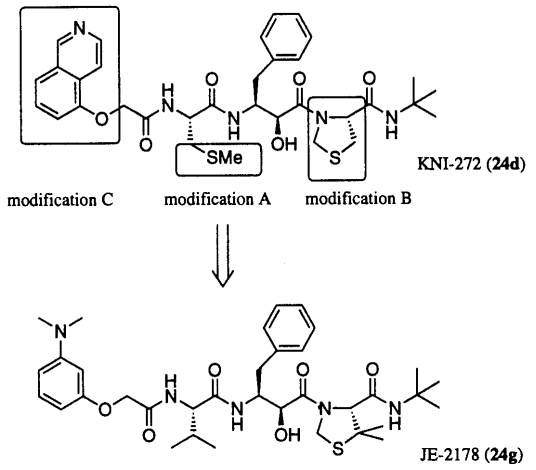


Fig. 4. Chemical Modification of KNI-272 to Improve Pharmacokinetic Profile

Modifications A and B, preventing the oxidation of sulfur atom, modification C, reducing the hydrophobicity.

inhibiting cytochrome P-450 CYP3A4, a major metabolic enzyme of KNI-272. Therefore we tried to co-administer KNI-272 with other HIV protease inhibitors to improve its pharmacokinetic profile.[30] On the other hand, we further tried to improve its pharmacokinetic profile by the structural modifications. We studied the metabolic fate of KNI-272 *in vivo* by means of the distribution of ^{14}C-labeled KNI-272 after i.v. administration to bile-exteriorized dogs and rats. In each case, three major metabolites were produced: the sulfoxide resulting from oxidation at either methylthioalanine or thiazolidine, and the sulfone of thiazolidine. Although minor hydroxyl compounds of the isoquinoline ring were also detected, the structural features responsible for the rapid clearance of KNI-272 were mainly the two oxidized sulfur atoms existing at P2 and P1′ sites.[31] With the above information in hand, we sought structural modifications of KNI-272 that would improve its pharmacokinetic profile without sacrificing its antiviral potency (another approach to the structural modification, *i.e.* reducing the molecular size, was reported in another article.[32]) As shown in Fig. 4, to reduce the rate of oxidation, we carried out the following two structural modifications: 1) replacement of the oxidation-sensitive Mta residue with an aliphatic amino acid residue (modification

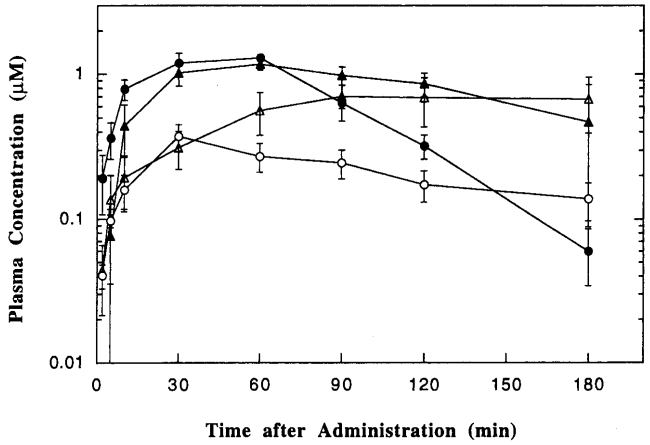


Fig. 5. Plasma Concentration of Selected Compounds after i.d. Administration (10 mg/kg) in Rats

○, compound **24c**; ●, compound **24d**; △, compound **24g**; ▲, compound **24h**.

A), 2) substitution of the sterically hindered dimethyl group on the thiazolidine ring (replacement with Dmt) to avoid the oxidation of the sulfur atom (modification B). The first structural modification (P2) was accomplished in consideration of the following matters: The mutation of Ile84 of HIV-1 protease to Val was widely observed in protease inhibitor-resistant HIV proteases,[33] and the mutation of this site often causes cross-resistance between protease inhibitors. The terminal methyl group of Mta contacts I84 of HIV protease tightly,[20] so KNI-272 is strongly decreased in its inhibitory activity by mutation of this site.[33] Therefore, norvaline, the isosteric analogue of Mta, was avoided, and Val or 2-aminobutyric acid (Abu) was selected as the aliphatic P2 residue. Although the compound **24e** with replacement by Val at the P2 site showed a higher level of maximum plasma concentration (C_{max}=2.46 μM) after i.d. administration than KNI-272, the maintenance of its plasma concentration was not improved; the elimination half-life and *AUC* did not change compared with those for KNI-272. These data suggested that preventing the oxidation of Mta was not sufficient to improve the pharmacokinetic profile of KNI-272. Therefore, the next structural modification (modification B), preventing the oxidation of thiazolidine ring, was expected to improve the plasma half-life (*e.g.* **24c**: $t_{1/2\beta}$=48 min *vs.* **24d**: $t_{1/2\beta}$=22 min). Substitution of dimethyl groups on the thiazolidine ring was also expected to improve the HIV-1 protease inhibitory activity, but this modification seemed to be accompanied by decreased solubility. Although the isoquinoline structure of the P3 site contributed to HIV protease inhibitory activity, the hydrophobicity of this structure limited its absorption especially in basic medium. Therefore, other structural modification at the P3 site was required to compensate for the lack of solubility (modification C). As a result of our SAR study of another series of inhibitors, the 3-(dimethylamino)phenoxyacetyl (3-Dapoa) group was found to be a P3 sunstituent with favorable pharmacokinetics, so we introduced the 3-Dapoa group into these Dmt containing compounds **24g** and **24h** (Fig. 1). These compounds had higher solubility than **24d** in basic medium at pH 7.4 (the solubility of **24g**, **24h** and **24d** were 28.2, 37.2 and 2.7 μg/ml, respectively), and showed a good pharmacokinetic profile in rats. After i.v. administration (10 mg/kg), the elimination half-life of these compounds was 63 min and 110 min, respectively, which was more than 3—5 times longer than that of KNI-272. The bioavailabilty of these compounds after i.d. administration was estimated to be over 80%. Although their maximum plasma concentration after i.d. administration did not reach the level of KNI-272, the duration of their plasma concentration was superior to that of KNI-272 (Fig. 5). Moreover compounds **24g** and **24h** presented a potent antiviral activity in CEM-SS cells compared with KNI-272. Study to obtain additional pharmacokinetic profiles and additional antiviral profile (*e.g.*; antiviral activity in various cell lines, or resistance profiles against HIV) of these compounds are now in progress.

Conclusions

In summary, we designed and synthesized a series of a novel class of substrate-based peptidomimetic HIV protease inhibitors containing Apns based on the transition-state isostere concept. From the SAR study of HIV-1 protease inhibition, enzyme selectivity for other aspartyl proteases, the antiviral activity, and pharmacokinetic study in rats, **24c** (KNI-227) and **24d** (KNI-272, our first clinical candidate) were found to be selective and orally potent HIV protease inhibitors. Although KNI-272 decreased of the viral load in the blood of patients in a clinical trial, the relatively short plasma half-life of this compound did not permit its plasma level to be adequately maintained without an inhibitor of its metabolic enzyme. Therefore, we sought to improve the pharmacokinetic feature of KNI-272, and in doing so found the long lasting ($t_{1/2\beta}$>60 min) and highly bioavailable (F>80%) compounds **24g** (JE-2178) and **24h** (JE-2179). The pharmacokinetic profiles of these compounds indicated that **24g, h** are promising as orally available HIV protease inhibitors.

Experimental

HIV-1 Protease Inhibition 1) HIV-1 protease inhibitory activity of the compounds in Tables 2—7 was determined by the following method using chemically synthesized [Ala^{67,95}]HIV-1 protease (NY5-type sequence): The [Ala^{67,95}]HIV-1 protease was synthesized by the general solid-phase method described below using hydroxymethyl-phenylacetoamidemethyl (PAM) resin (0.3 meq/g). The protease was removed from the resin by treatment with HF/10% *m*-cresol at 0 °C for 1 h, and the product was purified by the gel-filtration (Sephadex G-50, G-75) in 50% AcOH. The crude HIV-1 protease was folded to an active protease by dialysis in 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MES)-NaOH buffer, pH 6.0 containing 1 mM ethylenediaminetetraacetic acid (EDTA)-2Na and 2.5 mM dithiothreitol (DTT). In the inhibition assay, the reaction mixture contained 100 mM MES–NaOH buffer (pH 5.5), 40 mM substrate (Ac–Arg–Ala–Ser–Gln–Asn–Tyr–Pro–Val–Val–NH_2 trifluoroacetate), inhibitors at various concentrations dissolved in dimethylsulfoxide (DMSO), and 9.2 μg of the HIV-1 protease in a total volume of 15 μl. After incubation for 60 min at 37 °C, the reaction was terminated by the addition of 15 μl of acetonitrile; and the C-terminal cleavage fragment (Pro–Val–Val–NH_2) was separated by reversed phase HPLC on a C_{18} column with linear gradient of water to acetonitrile (both solutions containing 0.1% trifluoroacetic acid [TFA]), detected by absorbance at 215 nm, and quantified by comparison with a synthetic product standard. 2) HIV-1 protease inhibitory activity of the compounds in Table 8 was determined by the following method using recombinant HIV-1 protease (NY5-type sequence): Recombinant HIV-1 protease was expressed in *Escherichia coli* and purified to a single band by sodium dodecylsulfate–polyacrylamide gel electrophoresis. In the inhibition assay, 25 μl of 200 mM MES–NaOH buffer, pH 6.0, containing 2 mM DTT and 2 mM EDTA-2Na was mixed with 5 μl of various concentrations of the inhibitor dissolved in DMSO and 10 μl of titrated HIV-1 protease (10.5 nM) in 50 mM MES–NaOH, pH 6.0, containing 2.5 mM DTT, 1 mM EDTA-2Na, 0.2% Nonidet P-40 and 15% glycerol. The mixture of protease and inhibitor was preincubated for 5 min at 37 °C, and the enzymatic reaction was initiated by addition of 10 μl of a 75-mM

substrate solution in the above-described assay buffer. After incubation for 60 min at 37 °C, the reaction was terminated by addition of 75 μl of TFA (4%); and the C-terminal cleavage fragment (Pro–Ile–Val–OH) was separated by reverse phase HPLC on a C_{18} column with a linear gradient of water to acetonitrile (both solutions containing 0.1% TFA), detected by absorbance at 215 nm, and quantified by comparison with a synthetic product standard. The *Ki* values of the inhibitors were analyzed by a mathematical model for tight-binding inhibitors,[34] in which the concentration of inhibitor is less than or approximately equal to the enzyme concentration. The initial velocity data of HIV protease in the presence of various inhibitor concentrations were fitted by nonlinear regression analysis to equation 1, showed below, with KaleidaGraph (Version 3.08 d) for Macintosh, where V is the initial velocity with an inhibitor; V_0 is the measured initial velocity in the absence of the inhibitor; the substrate *Km* is estimated to be 21.4 mM; and *S*, *Et*, and *It* are the concentrations of substrate, active enzyme, and inhibitor, respectively.

$$V=\frac{V_0}{2Et}\left(\left\{\left[Ki\left(1+\frac{S}{Km}\right)+It-Et\right]^2+4Ki\left(1+\frac{S}{Km}\right)Et\right\}^{1/2}-\left[Ki\left(1+\frac{S}{Km}\right)+It-Et\right]\right) \quad (1)$$

Enzyme Selectivity Assay Inhibition of renin (human plasma renin) was measured by a radioimmunoassay using RENIN RIABEAD (Dainabbott).[35] Activities of pepsin (porcine pepsin, Nakalai) and cathepsin D (bovine cathepsin D, Sigma) were measured by a spectorphotomeric assay using albumin-BPB[36] and Phe–Ala–Phe(4-NO_2)-Phe–Val–Leu–OM4P,[37] respectively, as substrate.

Antiviral Activity Antiviral activity of test compounds was determined based on inhibition of HIV-1 IIIB-induced cytopathic effects in CEM-SS cells *in vitro*. The CEM-SS cells (2.5×10^4 cells/ml) were incubated in a total volume of 200 μl of tissue culture medium (RPMI-1640 medium plus 10% fetal calf serum with 50 μg of gentamicin/ml) containing test compound and HIV-1 IIIB for 6 days at 37 °C in a 5% CO_2 incubator. The virus was added to each well as a titer sufficient to give complete cell killing at 6 days post-infection. After incubation, HIV-1 III-induced cytopathic effects were analyzed by staining with the tetrazolium dye XTT.[24] The antiviral activity of a given compound was expressed as the 50% inhibitory concentration (IC_{50}).

Pharmacokinetics Pharmacokinetic parameters of the protease inhibitors were studied in rats and dogs. In the rat i.v. or i.d. administration studies, three male Sprague–Dawley rats (300—400 g) received the compound at 10 mg/kg in 50% PEG (1 ml/kg) under anesthesia in combination with KETARAL (Sankyo Co. Ltd, Tokyo)/SELACTAL (Bayer AG, Germany). The i.v. administration was made *via* a femoral vein. In the i.d. dosing study, rats were incised subphrenically for *ca.* 3 cm along the abdominal median line, a polyethylene tube (INTRAMEDIC, PE10) was inserted into duodenum, and then the test solution was injected into the duodenum through the tube. Heparinized blood samples (0.5 ml) were obtained after dosing at appropriate times, and plasma (0.2 ml) was obtained by immediate centrifugation and kept frozen (−80 °C) until analyzed. A plasma aliquot (0.2 ml) was combined with 4 ml of *tert*-butylmethyl ether containing an appropriate internal standard. Samples were vortexed vigorously for 10 s, shaken for 1 h at room temperature., and then centrifuged at 2500×***g*** for 15 min at 4 °C. The organic layer (3.6 ml) was evaporated to dryness at 40 °C, and then the samples were reconstituted in 0.3 ml of 50% methanol with vortexing. The parent inhibitors and the respective internal standard were separated from plasma contaminants on a CAPCELLPAK C_{18} column (4.6×150 mm; Shiseido Ltd., Tokyo). The elution condition was a linear gradient of 45% to 60% acetonitrile in 0.1% TFA for 12 min at a flow rate of 1.0 ml/min with UV detection at 210 nm. The drug concentration in each plasma sample was calculated by the internal standard method. Standard plasma samples spiked with specified amounts of each compound were analyzed, and the calibration curve was prepared by plotting the concentration of test compound and its ratio to the internal standard. The assays for each inhibitor were linear (correlation coefficients, >0.999) over the concentration range of 0 to 10 μg/ml, and the detection limit of quantification was 0.01 μg/ml. Pharmacokinetic parameters for inhibitors were estimated by a non-compartmental method. Maximum plasma concentration (C_{max}), and time of maximum plasma concentration (T_{max}) were determined by inspection of individual subject concentration–time curves, and the mean area under the plasma concentration–time curve (*AUC*) was determined by the linear trapezoidal rule. The apparent plasma half-life ($t_{1/2}$) was estimated from the slope of the terminal phase fitted to the log plasma concentration–time data by the method of least squares. The apparent distribution volume (V_{dss}) of the inhibitor was determined by the following equation:

$$V_{dss}=\text{Dose i.v.}\times AUMC(0\text{—}\infty)/AUC\ \text{i.v.}(0\text{—}\infty), \quad (2)$$

where *AUMC* (0—∞) is the total area under the first moment of the drug concentration curve from zero to infinity. The plasma clearance (*CL*) was calculated as the dose divided by the *AUC* from zero to infinity $[AUC]_0^\infty$.

Chemistry In general, reagents and solvents were used as purchased without further purification. All compounds except free peptides and resins were routinely checked by TLC with Merck Silica gel $60F_{254}$ precoated plates. Column chromatography was performed on Wakogel C-200 (Wako, 70—150 μm) or Wakogel C-300 (Wako, 45—75 μm). Preparative HPLC were conducted with a Shimadzu LC-4A. Melting points were measured with a Yanagimoto melting point apparatus and left uncorrected. Proton and carbon NMR spectra were recorded on a JEOL GSX270 FT NMR spectrometer. Chemical shifts were expressed in δ ppm from the internal standard tetramethylsilane, and following abbreviations were used: s=singlet, d=doublet, t=triplet, m=multiplet, br=broad, bs=broad singlet, and dd=double doublet. Time-of-flight mass spectrometry (TOF-MS) was recorded on a KOMPACT MALDI III spectrometer. FAB-MS was obtained on a JEOL JMS-SX102A spectrometer equipped with a JMA-DA7000 data system.

General Procedure for Solid-Phase Peptide Coupling *p*-Methylbenzhydrylamine (MBHA) resin or hydroxymethyl-PAM resin was used as a solid support, and standard solid-phase techniques were used for Boc–amino acid coupling, that is, 1) selective deprotection of Boc group using 0.5 M methanesulfonic acid (MSA)/CH_2Cl_2 : 1,4-dioxane (9 : 1), 2% anisole (1 and 20 min), 2) weak-basewash with 2% pyridine/DMF; and 3) coupling using Boc-amino acid (2 eq), benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium-hexafluoro-phosphate (BOP, 2 eq), and triethylamine (TEA, 4 eq) in *N*,*N*-dimethylformamide (DMF, 60 min), and capping with 0.3 M decanoic anhydride in DMF (30 min).

H–Ser–Phe–Asn–Apns–Pro–Ile–Val–NH_2 Trifluoroacetate (2a) Compound **2a** was synthesized by the general solid-phase method on MBHA resin, using **1a**, and removed from the resin by treatment with HF containing 10% *m*-cresol at 0 °C for 1 h. The crude peptide was purified by reverse phase chromatography followed by lyophilization from H_2O. Yield, 38%; purity, >98% by analytical HPLC; HRFAB-MS for $C_{42}H_{61}N_9O_{10}+H_1$: Calcd, 852.4619. Found, 852.4612.

H–Ser–Phe–Asn–Pns–Pro–Ile–Val–NH_2 Trifluoroacetate (2b) Compound **2b** was synthesized in a manner similar to that described for compound **2a**, using **1b**. Yield, 44%; purity, >98% by analytical HPLC; HRFAB-MS for $C_{42}H_{61}N_9O_{10}+H_1$: Calcd, 852.4619. Found, 852.4614.

3-Phenylpropionyl-Asn–Apns–Pro–Ile–Val–NH_2 (3a) Compound **3a** was synthesized in a manner similar to that described for compound **2a**, using **1a**. Yield, 45%; purity, >98% by analytical HPLC; HRFAB-MS for $C_{39}H_{55}N_7O_8+H_1$: Calcd, 750.4190. Found, 750.4183.

3-Phenylpropionyl-Asn–Pns–Pro–Ile–Val–NH_2 (3b) Compound **3b** was synthesized in a manner similar to that described for compound **2a**, using **1b**. Yield, 37%; purity, >98% by analytical HPLC; HRFAB-MS for $C_{39}H_{55}N_7O_8+H_1$: Calcd, 750.4190. Found, 750.4200.

(*S*)-1-*tert*-Butoxycarbonyl-2-*N*-*tert*-butylcarbamoylpyrrolidine (5) To a solution of Boc–Pro–OH (10.0 g, 46.5 mmol) and HOBt (6.30 g, 46.5 mmol) in CH_2Cl_2 (30 ml), EDC·HCl (9.80 g, 51.2 mmol) was added in an ice-bath. After 30 min, *tert*-butylamine (5.86 ml, 55.8 mmol) in CH_2Cl_2 (30 ml) was added dropwise to the reaction mixture and stirred overnight. The reaction mixture was sequentially washed with 3% K_2CO_3, 1 N HCl, and brine, dried over $MgSO_4$, and then evaporated. The obtained solid was recrystallized from *n*-hexane to give 11.7 g of the title compound. Yield, 93%, mp, 118—119 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.25 (s, 9H), 1.35 (s, 9H), 1.6—1.9 (m, 3H), 1.9—2.1 (m, 1H), 3.2—3.4 (m, 2H), 3.9—4.1 (m, 1H), 7.38 (s, 1H); HRFAB-MS *m/z*: 271.2028 for $(M+H)^+$ (Calcd 271.2021 for $C_{14}H_{27}N_2O_3$).

(*S*)-*N*-*tert*-Butyl-1-[(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoyl]-pyrrolidine-2-carboxamide (6a) To a solution of **5** (2.71 g, 10.0 mmol) in CH_2Cl_2 (10 ml), 4 N HCl in dioxane (10 ml) was added and stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF followed by neutralization with TEA (1.39 ml, 10.0 mmol). To this solution, Boc–Apns–OH (**1a**, 2.95 g, 10.0 mmol), HOBt (1.35 g, 10.0 mmol)

and EDC·HCl (2.10 g, 11.0 mmol) were added, and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 1 N HCl were added; and then the organic layer was washed with 3% K_2CO_3 and brine, dried over $MgSO_4$ and evaporated to give the residue. To the solution of this residue in CH_2Cl_2 (10 ml), 4 N HCl in dioxane (10 ml) was added and stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in H_2O, washed with CH_2Cl_2, adjusted to pH 10 with 3 N NaOH, and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated. The residue was recrystallized from *n*-hexane/EtOAc to give 2.62 g of the title compound. Yield, 75%; mp 152—155 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.19 (s, 9H), 1.3—1.4 (br, 2H), 1.7—1.9 (m, 3H), 1.9—2.1 (m, 1H), 2.35 (dd, 1H, J=13.2 Hz, 10.0 Hz), 2.8—3.0 (m, 1H), 3.01 (d, 1H, J=13.2 Hz), 3.5—3.7 (m, 2H), 3.9—4.1 (m, 1H), 4.31 (t, 1H, J=4.2 Hz), 4.96 (d, 1H, J=7.8 Hz), 7.1—7.4 (m, 5H), 7.47 (s, 1H); TOF-MS *m/z*: 348 $(M+H)^+$; *Anal.* Calcd for $C_{19}H_{29}N_3O_3$: C, 65.68; H, 8.41; N, 12.09. Found: C, 65.39; H, 8.51; N, 12.31.

(*S*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-benzyloxycarbonylaminosuccinamyl]amino-4-phenylbutanoyl]pyrrolidine-2-carboxamide (7a) To the solution of **6a** (1.00 g, 2.88 mmol) and HOBt (0.39 g, 2.88 mmol) in DMF (10 ml), Z–Asn–ONp (1.34 g, 3.46 mmol) and TEA (0.40 ml, 3.46 mmol) were added and stirred overnight. To the reaction mixture, CH_2Cl_2 and 3% K_2CO_3 were added, and then the organic layer was washed sequentially with 3% K_2CO_3, 1 N HCl, and brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and recrystallized from *n*-hexane/EtOAc to give 1.47 g of the title compound. Yield, 86%; mp 102—104 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.25 (s, 9H), 1.7—1.9 (m, 2H), 1.9—2.1 (m, 2H), 2.2—2.4 (m, 2H), 2.5—2.8 (m, 2H), 3.5—3.7 (m, 2H), 4.0—4.2 (m, 1H), 4.2—4.4 (m, 3H), 4.91 (d, 1H, J=7.3 Hz), 5.01 (s, 2H), 6.89 (bs, 1H), 7.1—7.4 (m, 12H), 7.54 (s, 1H), 7.89 (d, 1H, J=8.4 Hz); TOF-MS *m/z*: 596 $(M+H)^+$; *Anal.* Calcd for $C_{31}H_{41}N_5O_7$: C, 62.50; H, 6.94; N, 11.76. Found: C, 62.22; H, 7.20; N, 11.77.

(*S*)-*N*-*tert*-Butyl-3-[(2*R*,3*S*)-2-hydroxy-3-[(*S*)-2-benzyloxycarbonylaminosuccinamyl]amino-4-phenylbutanoyl]pyrrolidine-2-carboxamide (7b) To a solution of **5** (271 mg, 1.0 mmol) in CH_2Cl_2 (2 ml), 4 N HCl in dioxane (2 ml) was added and stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF followed by neutralization with TEA (139 μl, 1.0 mmol). To this solution, Boc–Pns–OH (**1b**, 295 mg, 1.0 mmol), HOBt (135 mg, 1.0 mmol) and EDC·HCl (210 mg, 1.1 mmol) were added, and the mixture was then stirred overnight. To the reaction mixture, CH_2Cl_2 and 1 N HCl were added, and then the organic layer was washed with 3% K_2CO_3 and brine, dried over $MgSO_4$ and evaporated. The oily residue thus obtained was dissolved in CH_2Cl_2 (2 ml), to which 4 N HCl in dioxane (2 ml) was added, and the mixture was stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in H_2O, washed with CH_2Cl_2, and adjusted to pH 10 with 3 N NaOH, and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated to give 200 mg of crude **6b**. This oily residue was dissolved in DMF (3 ml), and Z–Asn–ONp (241 mg, 0.68 mmol), HOBt (77 mg, 0.57 mmol), and TEA (95 μl, 0.68 mmol) were added; and the mixture was stirred overnight. To the reaction mixture, EtOAc and 3% K_2CO_3 were added, and then the organic layer was washed sequentially with 3% K_2CO_3, 1 N HCl and brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was recrystallized from *n*-hexane/EtOAc to give 239 mg of the title compound. Yield, 40%; mp, 189—191 °C; (DMSO-d_6) δ (ppm): 1.20 (s, 9H), 1.6—2.0 (m, 4H), 2.2—2.4 (m, 2H), 2.6—2.9 (m, 2H), 3.3—3.4 (m, 2H, overlapped with H_2O), 4.1 (br, 2H), 4.2—4.4 (br, 2H), 4.73 (d, 1H, J=6.8 Hz), 5.02 (s, 2H), 6.91 (bs, 1H), 7.2—7.4 (m, 13H), 7.78 (d, 1H, J=9.2 Hz); TOF-MS *m/z*: 596 $(M+H)^+$; *Anal.* Calcd for $C_{31}H_{41}N_5O_7$: C, 62.50; H, 6.94; N, 11.76. Found: C, 62.26; H, 7.13; N, 11.61.

(*S*)-3-[(2*S*,3*S*)-2-Hydroxy-3-[(*S*)-2-benzyloxycarbonylaminosuccinamyl]amino-4-phenylbutanoyl]pyrrolidine-2-carboxylic Acid *tert*-Butylester (13a) To a solution of H–Pro–OtBu (**10a**, 51 mg, 0.3 mmol), Z–Asn–Apns–OH[6b] (133 mg, 0.3 mmol), HOBt (41 mg, 0.3 mmol) in DMF (2 ml), and EDC·HCl (63 mg, 0.33 mmol) were added, and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 1 N HCl were added, and then the organic layer was washed with 3% K_2CO_3, then with brine, dried over $MgSO_4$, and evaporated. The residue was recrystallized from *n*-hexane/EtOAc to give 136 mg of the title compound. Yield, 76%; mp, 149—150 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.40 (s, 9H), 1.8—2.0 (m, 3H), 2.1—2.4 (m, 3H), 2.6—2.8 (m, 2H), 3.6—3.8 (m, 2H), 4.1—4.2 (br, 1H), 4.2—4.4 (m, 3H), 5.01 (s, 2H), 5.10 (d, 1H, J=7.3 Hz), 6.89 (br, 1H), 7.1—7.4 (m, 12H), 7.96 (d, 1H, J=8.4 Hz); TOF-MS *m/z*: 597 $(M+H)^+$; *Anal.* Calcd for $C_{31}H_{40}N_4O_8$: C, 62.40; H, 6.76; N, 9.39. Found: C, 62.23; H, 6.84; N, 9.32.

(*S*)-1-*tert*-Butoxycarbonyl-2-(*N*-2,2-dimethylethyl)carbamoylpyrrolidine (9b) Compound **9b** was prepared from Boc–Pro–OH and neopentylamine in a similar manner as described for compound **5**. Yield, 46%; mp, 114—116 °C; HRFAB-MS *m/z*: 285.2184 for $(M+H)^+$ (Calcd 285.2178 for $C_{15}H_{29}N_2O_3$).

(*S*)-*N*-2,2-Dimethylethyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-benzyloxycarbonylaminosuccinamyl]amino-4-phenylbutanoyl]pyrrolidine-2-carboxamide (13b) To a solution of **9b** (94 mg, 0.33 mmol) in CH_2Cl_2 (2 ml), 4 N HCl in dioxane (2 ml) was added and stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF followed by neutralization with TEA (46 μl, 0.33 mmol). To this solution, Z–Asn–Apns–OH (133 mg, 0.3 mmol), HOBt (41 mg, 0.3 mmol), and EDC·HCl (63 mg, 0.33 mmol) were added; and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 1 N HCl were added, and then the organic layer was washed with 3% K_2CO_3 and brine, dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and then reprecipitated from *n*-hexane/EtOAc to give 117 mg of the title compound. Yield, 64%; mp, 108—110 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.83 (s, 9H), 1.7—1.9 (m, 2H), 1.9—2.0 (m, 1H), 2.0—2.2 (m, 1H), 2.2—2.4 (m, 2H), 2.6—2.8 (m, 2H), 2.90 (d, 1H, J=6.5 Hz), 3.6—3.7 (br, 2H), 4.0—4.2 (br, 1H), 4.2—4.4 (m, 2H), 4.4—4.5 (m, 1H), 4.96 (d, 1H J=7.0 Hz), 5.01 (s, 2H), 6.88 (bs, 1H), 7.1—7.4 (m, 12H), 7.96 (d, 1H, J=7.0 Hz); TOF-MS *m/z*: 610 $(M+H)^+$; *Anal.* Calcd for $C_{32}H_{43}N_5O_7 \cdot 0.25$EtOAc: C, 62.74; H, 7.18; N, 11.09. Found: C, 62.43; H, 7.33; N, 11.31.

(*S*)-1-*tert*-Butoxycarbonyl-2-*N*-isopropylcarbamoylpyrrolidine (9c) Compound **9c** was prepared from Boc–Pro–OH and isopropylamine in a similar manner as described for compound **5**. Yield, 55%; mp, 132—134 °C; HRFAB-MS *m/z*: 257.1870 for $(M+H)^+$ (Calcd 257.1865 for $C_{13}H_{25}N_2O_3$).

(*S*)-*N*-Isopropyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-benzyloxycarbonylaminosuccinamyl]amino-4-phenylbutanoyl]pyrrolidine-2-carboxamide (13c) Compound **13c** was prepared from Z–Asn–Apns–OH and **9c** in a similarly as described for compound **13b**. Yield, 40%; mp, 160—162 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.03 (d, 3H, J= 6.5 Hz), 1.05 (d, 3H, J=6.2 Hz), 1.7—1.9 (m, 2H), 1.9—2.1 (m, 2H), 2.2—2.4 (m, 2H), 2.6—2.8 (m, 2H), 3.6—3.7 (br, 2H), 3.7—3.9 (m, 1H), 4.0—4.2 (br, 1H), 4.2—4.4 (m, 3H), 4.95 (d, 1H, J=7.6 Hz), 5.01 (s, 2H), 6.89 (bs, 1H), 7.1—7.4 (m, 12H), 7.74 (d, 1H, J=7.8 Hz), 7.87 (d, 1H, J=8.9 Hz); TOF-MS *m/z*: 582 $(M+H)^+$; *Anal.* Calcd for $C_{30}H_{39}N_5O_7$: C, 61.95; H, 6.76; N, 12.04. Found: C, 61.99; H, 6.91; N, 11.78.

(*S*)-1-*tert*-Butoxycarbonyl-2-*N*-cyclohexylcarbamoylpyrrolidine (9d) Compound **9d** was prepared from Boc–Pro–OH and cyclohexylamine in a manner similar to that described for compound **5**. Yield, 65%; mp, 141—143 °C; HRFAB-MS *m/z*: 297.2181 for $(M+H)^+$ (Calcd 297.2178 for $C_{16}H_{29}N_2O_3$).

(*S*)-*N*-Cyclohexyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-benzyloxycarbonylaminosuccinamyl]amino-4-phenylbutanoyl]pyrrolidine-2-carboxamide (13d) Compound **13d** was prepared from Z–Asn–Apns–OH and **9d** similarly as described for compound **13b**. Yield, 42%; mp, 103—105 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.0—1.4 (m, 5H), 1.4—1.6 (m, 1H), 1.6—1.9 (m, 6H), 1.9—2.0 (m, 1H), 2.0—2.1 (m, 1H), 2.2—2.4 (m, 2H), 2.6—2.8 (m, 2H), 3.4—3.5 (br, 1H), 3.5—3.6 (m, 2H), 4.0—4.2 (m, 1H), 4.2—4.4 (m, 3H), 4.95 (d, 1H, J=7.6 Hz), 5.01 (s, 2H), 6.88 (br, 1H), 7.1—7.4 (m, 12H), 7.72 (d, 1H, J=7.8 Hz), 7.88 (d, 1H, J=8.4 Hz); TOF-MS *m/z*: 622 $(M+H)^+$; *Anal.* Calcd for $C_{33}H_{43}N_5O_7 \cdot 0.8$EtOAc: C, 62.81; H, 7.19; N, 10.12. Found: C, 62.49; H, 7.13; N, 10.54.

(*S*)-1-*tert*-Butoxycarbonyl-2-*N*-*tert*-amylcarbamoylpyrrolidine (9e) Compound **9e** was prepared from Boc–Pro–OH and *tert*-amylamine similarly as described for compound **5**. Yield, 68%; mp, 110—112 °C; HRFAB-MS *m/z*: 285.2184 for $(M+H)^+$ (Calcd 285.2178 for $C_{15}H_{29}N_2O_3$).

(*S*)-*N*-*tert*-Amyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-benzyloxycarbonylaminosuccinamyl]amino-4-phenylbutanoyl]pyrrolidine-2-carboxamide (13e) Compound **13e** was prepared from Z–Asn–Apns–OH and **9e** similarly as described for compound **13b**. Yield, 34%; mp, 96—98 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.77 (t, 1H, J=7.3 Hz), 1.19 (s, 6H), 1.6—1.7 (m, 2H), 1.7—1.9 (m, 2H), 1.9—2.2 (m, 2H), 2.2—2.4 (m, 2H), 2.5—2.8 (m, 2H), 3.5—3.7 (m, 2H), 4.0—4.2 (m, 1H), 4.2—4.4 (m, 3H), 4.89 (d, 1H, J=7.3 Hz), 5.01 (s, 2H), 6.89 (s, 1H), 7.1—7.4 (m, 13H), 7.88 (d, 1H, J= 8.4 Hz); TOF-MS *m/z*: 610 $(M+H)^+$; *Anal.* Calcd for $C_{32}H_{43}N_5O_7$ 0.25EtOAc: C, 62.74; H, 7.18; N, 11.09. Found: C, 62.28; H, 7.28; N, 11.06.

(*S*)-1-*tert*-Butoxycarbonyl-2-*N*-*tert*-butylcarbamoylpiperidine (9f) To a solution of Boc-protected L-pipecolinic acid (530 mg, 2.31 mmol) and HOBt (328 mg, 2.43 mmol) in CH_2Cl_2 (10 ml), EDC·HCl (485 mg,

2.54 mmol) was added in an ice-bath. After 30 min, *tert*-butylamine (0.73 ml, 6.93 mmol) was added dropwise to the reaction mixture, which was then stirred overnight. The reaction mixture was washed with 3% K_2CO_3, 1 N HCl, and brine, and dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give 500 mg of the title compound. Yield, 76%; mp, 126—128 °C; HRFAB-MS *m/z*: 285.2174 for $(M+H)^+$ (Calcd 285.2178 for $C_{15}H_{29}N_2O_3$).

(*S*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-benzyloxycarbonylaminosuccinamyl]amino-4-phenylbutanoyl]piperidine-2-carboxamide (13f) To a solution of **9f** (114 mg, 0.40 mmol) in CH_2Cl_2 (2 ml), 4 N HCl in dioxane (2 ml) was added and stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF followed by neutralization with TEA (56 μl, 0.40 mmol). To this solution, Boc–Apns–OH (**1a**, 118 mg, 0.40 mmol), HOBt (54 mg, 0.40 mmol) and EDC·HCl (84 mg, 0.44 mmol) were added, and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 1 N HCl were added, and then the organic layer was washed with 3% K_2CO_3, brine, and dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give 87 mg (0.19 mmol) of the crude compound (**11f**). The crude compound thus obtained was dissolved in CH_2Cl_2 (2 ml), and then 4 N HCl in dioxane (2 ml) was added, and the mixture was stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF followed by neutralization with TEA (26 μl, 0.19 mmol). To the solution, Z–Asn–ONp (81 mg, 0.23 mmol), HOBt (26 mg, 0.19 mmol) and TEA (32 μl, 0.23 mmol) were added and stirred overnight. To the reaction mixture, CH_2Cl_2 and 3% K_2CO_3 were added, and then the organic layer was washed sequentially with 3% K_2CO_3, 1 N HCl, and brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and recrystallized from *n*-hexane/EtOAc to give 63 mg of the title compound. Yield, 26%; mp, 96—98 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.27 (s, 9H), 1.3—1.5 (m, 2H), 1.5—1.7 (br, 3H), 2.0—2.2 (br, 1H), 2.2—2.4 (m, 2H), 2.56 (d, 1H, *J*=6.8 Hz), 2.6—2.8 (m, 1H), 3.5—3.7 (m, 1H), 3.9—4.0 (m, 2H), 4.1—4.2 (m, 1H), 4.2—4.4 (m, 1H), 4.47 (d, 1H, *J*=4.9 Hz), 4.85 (d, 1H, *J*=7.3 Hz), 5.01 (s, 2H), 5.0—5.4 (br, 1H), 6.88 (br, 1H), 7.1—7.4 (m, 13H), 7.87 (d, 1H, *J*=8.4 Hz); TOF-MS *m/z*: 610 $(M+H)^+$; *Anal.* Calcd for $C_{32}H_{43}N_5O_7\cdot$0.5EtOAc: C, 62.46; H, 7.25; N, 10.71. Found: C, 62.04; H, 7.11; N, 11.00.

(*R*)-*N*-*tert*-Butyl-1,3-thiazolidine-4-carboxamide (10g) To a solution of Boc–Thz–OH (11.65 g, 50 mmol) and HOBt (6.75 g, 50 mmol) in CH_2Cl_2 (80 ml), DCC (11.33 g, 55 mmol) was added in an ice-bath. After 30 min, *tert*-butylamine (13.75 ml, 150 mmol) in CH_2Cl_2 (60 ml) was added dropwise to the reaction mixture, which was then stirred overnight. The reaction mixture was washed sequentially with 3% K_2CO_3, 1 N HCl, and brine, dried over $MgSO_4$, and then evaporated. The residue was redissolved in CH_2Cl_2 (125 ml), 4 N HCl in dioxane (125 ml) was added, and the mixture was stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in H_2O and filtered. The filtrate was washed with CH_2Cl_2, adjusted to pH 8 with K_2CO_3, and extracted with CH_2Cl_2. After drying and concentrating, the obtained solid was recrystallized from *n*-hexane/toluene to give 7.87 g of the title compound. Yield, 84%; mp, 63—65 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.26 (s, 9H), 2.75—2.82 (m, 1H), 2.91—2.98 (m, 1H), 3.10—3.21 (m, 1H), 3.62—3.71 (m, 1H), 4.01 (t, 1H, *J*=9.7 Hz), 4.14 (t, 1H, *J*=8.8 Hz), 7.56 (s, 1H); TOF-MS *m/z*: 189 $(M+H)^+$; *Anal.* Calcd for $C_8H_{16}N_2OS$: C, 51.03; H, 8.56; N, 14.88. Found: C, 51.13; H, 8.41; N, 15.02.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (12g) To a solution of **10g** (1.05 g, 5.59 mmol) in DMF (10 ml), Boc–Apns–OH (1.50 g, 5.09 mmol), HOBt (0.69 g, 5.09 mmol), and EDC·HCl (1.07 g, 5.60 mmol) were added and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 1 N HCl were added, and then the organic layer was washed with 3% K_2CO_3 and then with brine, dried over $MgSO_4$, and evaporated to give **11g**. To a solution of **11g** in CH_2Cl_2 (13 ml), 4 N HCl in dioxane (13 ml) was added, and the mixture was stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in H_2O, washed with CH_2Cl_2 and adjusted to pH 8 with K_2CO_3 to give a solid. The obtained solid was washed with hot methanol to give 1.57 g of the title compound. Yield, 85%; mp, 208—210 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.19 (s, 9H), 1.40 (br, 2H), 2.31—2.40 (m, 1H), 2.90 (t, 1H, *J*=8.1 Hz), 3.01—3.07 (m, 2H), 3.16—3.26 (m, 1H), 4.11 (t, 1H, *J*=7.6 Hz), 4.60 (d, 1H, *J*=8.9 Hz), 4.77—4.82 (m, 1H), 4.89 (d, 1H, *J*=8.6 Hz), 5.20 (d, 1H, *J*=7.8 Hz), 7.16—7.31 (m, 5H), 7.57 (s, 1H); TOF-MS *m/z*: 366 $(M+H)^+$; *Anal.* Calcd for $C_{18}H_{27}N_3O_3S$: C, 59.15; H, 7.45; N, 11.50. Found: C, 58.93; H, 7.56; N, 11.12.

(*R*)-*N*-*tert*-Butyl-3-[(2S,3*S*)-2-hydroxy-3-[(S)-2-benzyloxycarbonylaminosuccinamyl]amino-4-phenylbutanoyl]thiazolidine-4-carboxamide (13g) To a solution of **12g** (500 mg, 1.37 mmol) and HOBt (185 mg, 1.37 mmol) in DMF (5 ml), Z–Asn–ONp (636 mg, 1.64 mmol) and TEA (190 μl, 1.37 mmol) were added; and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 3% K_2CO_3 were added, and then the organic layer was washed sequentially with 3% K_2CO_3, 1 N HCl, and brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and recrystallized from toluene to give 690 mg of the title compound. Yield, 82%; mp, 102—104 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.26 (s, 9H), 2.2—2.4 (m, 2H), 2.6—2.7 (br, 2H), 2.98 (dd, 1H, *J*=6.2 Hz, 11.1 Hz), 3.3—3.4 (m, 1H, overlapped with H_2O), 4.0—4.2 (br, 1H), 4.2—4.5 (br, 2H), 4.60(d, 1H, *J*=9.5 Hz), 4.76 (t, 1H, *J*=7.0 Hz), 4.94 (d, 1H, *J*=9.7 Hz), 5.01 (s, 2H), 5.19 (d, 1H, *J*=7.0 Hz), 6.88 (bs, 1H), 7.1—7.4 (m, 12H), 7.67 (s, 1H), 7.94 (d, 1H, *J*=8.4 Hz); TOF-MS *m/z*: 614 $(M+H)^+$; *Anal.* Calcd for $C_{30}H_{39}N_5O_7S\cdot$0.4EtOAc: C, 58.48; H, 6.55; N, 10.79. Found: C, 57.98; H, 6.67; N, 10.58.

(*R*)-*N*-*tert*-Butyl-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (10h) To a solution of Boc–Dmt–OH (5.22 g, 20.0 mmol) and TEA (3.34 ml, 24.0 mmol) in EtOAc (100 ml), DPP-Cl (4.55 ml, 22.0 mmol) was added in an ice-bath, and the mixture was stirred for 1 h. Then to the reaction mixture, *tert*-butylamine (6.30 ml, 60.0 mmol) was added in an ice-bath. After overnight stirring, the reaction mixture was washed sequentially with 1 N HCl, 3% K_2CO_3, and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was redissolved in CH_2Cl_2 (30 ml), to which was added 4 N HCl in dioxane (30 ml), and then stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in H_2O and filtered. The filtrate was washed with CH_2Cl_2, adjusted to pH 8 with K_2CO_3, and extracted with CH_2Cl_2. After drying and concentrating, the obtained solid was recrystallized from *n*-heptane to give 3.01 g of the title compound. Yield, 70%; mp, 75—77 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.16 (s, 3H), 1.27 (s, 9H), 1.52 (s, 3H), 3.16 (d, 1H, *J*=13.2 Hz), 3.46—3.58 (m, 1H), 3.99 (dd, 1H, *J*=11.8 Hz, 9.2 Hz), 4.26 (dd, 1H, *J*=7.3 Hz, 9.2 Hz), 7.47 (s, 1H); TOF-MS *m/z*: 217 $(M+H)^+$; *Anal.* Calcd for $C_{10}H_{20}N_2OS$: C, 55.52; H, 9.32; N, 12.95. Found: C, 55.52; H, 9.16; N, 13.08.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (12h) To a solution of **10h** (2.90 g, 13.43 mmol) in EtOAc (30 ml), Boc–Apns–OH (3.77 g, 12.79 mmol), HOBt (1.73 g, 12.79 mmol) and DCC (3.03 g, 14.71 mmol) were added; and the mixture was stirred overnight. The reaction mixture was then washed with 3% K_2CO_3, 1 N HCl, and brine in this order, dried over $MgSO_4$ and evaporated. The residue was redissolved in CH_2Cl_2 (20 ml), and then 4 N HCl in dioxane (20 ml) was added, and the mixture was stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in H_2O and filtered. The filtrate was washed with CH_2Cl_2, adjusted to pH 8 with K_2CO_3, and extracted with CH_2Cl_2. After drying with $MgSO_4$ and concentrating, the obtained solid was recrystallized from *n*-hexane/EtOAc to give 3.73 g of the title compound. Yield, 74%; mp, 177—180 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.23 (s, 9H), 1.35 (s, 3H), 1.3—1.5 (m, 2H), 1.49 (s, 3H), 2.30—2.38 (m, 1H), 2.88—3.04 (m, 2H), 4.10 (t, 1H, *J*=7.3 Hz), 4.36 (s, 1H), 4.90 (s, 1H), 5.19 (d, 1H, *J*=7.3 Hz), 7.16—7.31 (m, 5H), 7.52 (s, 1H); TOF-MS *m/z*: 394 $(M+H)^+$; *Anal.* Calcd for $C_{20}H_{31}N_3O_3S$: C, 61.04; H, 7.94; N, 10.68. Found: C, 61.03; H, 8.06; N, 10.69.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-benzyloxycarbonylaminosuccinamyl]amino-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (13h) To a solution of **12h** (500 mg, 1.27 mmol) and HOBt (172 mg, 1.27 mmol) in DMF (5 ml), Z–Asn–ONp (591 mg, 1.52 mmol) and TEA (177 μl, 1.27 mmol) were added; and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 3% K_2CO_3 were added; and then the organic layer was washed sequentially with 3% K_2CO_3, 1 N HCl, and brine, and dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and recrystallized from *n*-hexane/EtOAc to give 710 mg of the title compound. Yield, 87%; mp, 113—115 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.27 (s, 9H), 1.39 (s, 3H), 1.48 (s, 3H), 2.2—2.4 (m, 2H), 2.6—2.7 (m, 2H), 4.0—4.2 (br, 1H), 4.2—4.4 (m, 2H), 4.50 (s, 1H), 4.8—5.0 (m, 2H), 5.01 (s, 2H), 5.20 (d, 1H, *J*=7.3 Hz), 6.89 (bs, 1H), 7.1—7.4 (m, 12H), 7.65 (s, 1H), 7.95 (d, 1H, *J*=8.1 Hz); TOF-MS *m/z*: 628 $(M+H)^+$; *Anal.* Calcd for $C_{32}H_{43}N_5O_7S$: C, 59.89; H, 6.75; N, 10.91. Found: C, 59.62; H, 7.03; N, 10.90.

2-Biphenyloxyacetic Acid (16a) To a solution of *o*-phenylphenol (**14a**, 0.85 g, 5.0 mmol) and K_2CO_3 (1.04 g, 7.5 mmol) in DMF (10 ml), methyl chloroacetate (0.52 ml, 6.0 mmol) was added in an ice-bath, and the reaction mixture was stirred overnight. To the reaction mixture, H_2O (30 ml) was

added, followed by extraction with EtOAc. The organic layer was washed with 1 N HCl, and then with brine, and evaporated. The obtained oily residue was dissolved in MeOH (20 ml) and after the addition of 1 N NaOH (7.5 ml, 7.5 mmol), was stirred for 2 h. The reaction mixture was acidified to pH 3 with conc.HCl, and then extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and then evaporated. The obtained solid was recrystallized from *n*-hexane/EtOAc to give 1.02 g of the title compound. Yield, 84%; mp, 92—93 °C; *Anal.* Calcd for $C_{14}H_{12}O_3$: C, 73.67; H, 5.30. Found: C, 74.00; H, 5.38.

3-Biphenyloxyacetic Acid (16b) Compound **16b** was prepared from *m*-phenylphenol (**14b**) in a similar manner as described for compound **16a**. Yield, 82%; mp, 98—100 °C; *Anal.* Calcd for $C_{14}H_{12}O_3$: C, 73.67; H, 5.30. Found: C, 74.00; H, 5.35.

4-Biphenyloxyacetic Acid (16c) Compound **16c** was prepared from *p*-phenylphenol (**14c**) similarly as described for compound **16a**. Yield, 61%; mp, 192—194 °C; *Anal.* Calcd for $C_{14}H_{12}O_3$: C, 73.67; H, 5.30. Found: C, 74.01; H, 5.34.

3-(Phenylamino)phenoxyacetic Acid (16d) To a solution of 3-(phenylamino)phenol (**14a**, 0.93 g, 5.0 mmol) and K_2CO_3 (1.04 g, 7.5 mmol) in DMF (10 ml), methyl chloroacetate (0.52 ml, 6.0 mmol) was added; and the mixture was then stirred 3 h at 80 °C. To the reaction mixture, H_2O (30 ml) was added, followed by extraction with EtOAc. The organic layer was washed with 1 N HCl, then with brine, and evaporated. The obtained oily residue was dissolved in MeOH (20 ml); and 3 N NaOH (7.5 ml, 7.5 mmol) was added to it, followed by stirring for 1 h. To the reaction mixture, H_2O and EtOAc were added, and the aqueous layer was acidified to pH 3 with conc.HCl, and then extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and then evaporated. The obtained solid was recrystallized twice from *n*-hexane/EtOAc to give 374 mg of the title compound. Yield, 31%; mp, 134—135 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.59 (s, 2H), 6.35 (dd, 1H, J=1.6 Hz, 6.4 Hz), 6.56 (s, 1H), 6.64—6.67 (m 1H), 6.82 (t, 1H, J=5.8 Hz), 7.02—7.13 (m, 3H), 7.22 (t, 2H, J=6.3 Hz), 8.17 (s, 1H), 12.95 (br, 1H); *Anal.* Calcd for $C_{14}H_{13}NO_3$: C, 69.12; H, 5.39; N, 5.76. Found: C, 69.45; H, 5.47; N, 5.56.

5-Isoquinolinyloxyacetic Acid (16e) To a solution of 5-hydroxyisoquinoline (2.90 g, 20 mmol) in DMF (40 ml), sodium methoxide (1.10 g, 20.4 mmol) was added in ice bath and stirred 1 h, and then methyl chloroacetate (1.79 ml, 20.4 mmol) was added and stirred overnight. After removal of the solvent, H_2O (30 ml) was added, followed by extraction with EtOAc. The organic layer was washed with H_2O, and then evaporated. The obtained oily residue was dissolved in MeOH (10 ml), to which 3 N NaOH (8.0 ml, 24 mmol) was added; and then the mixture was stirred for 1 h. The reaction mixture was concentrated (MeOH was removed), H_2O (10 ml) and acetone (20 ml) were added, and then the mixture was acidified to pH 3 with conc.HCl to afford the precipitate. This precipitate was filtered and then washed with H_2O and acetone to give 2.96 g of the title compound. Yield, 73%, mp, 218—220 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.99 (s, 2H), 7.20 (d, 1H, J=8.1 Hz), 7.60 (t, 1H, J=8.0 Hz), 7.72 (d, 1H, J=7.8 Hz), 8.59 (d, 1H, J=8.1 Hz), 9.32 (s, 1H), 11.36 (br, 1H); *Anal.* Calcd for $C_{11}H_9NO_3$: C, 65.02; H, 4.46; N, 6.89. Found: C, 65.39; H, 4.53; N, 6.67.

3-(Dimethylamino)phenoxyacetic Acid *N*-Hydroxy-5-norbornene-2,3-dicarboxamide ester (17) To a solution of 3-(dimethylamino)phenol (**14a**, 15.1 g, 110 mmol) and K_2CO_3 (20.7 g, 150 mmol) in DMF (150 ml), benzyl chloroacetate (15.2 ml, 100 mmol) was added and stirred 5 h at 80 °C. To the reaction mixture, H_2O was added; and the product was then extracted with CH_2Cl_2, washed with 1 N HCl, followed with brine, dried over $MgSO_4$, and then evaporated. The oily residue was dissolved in MeOH (150 ml), and 5% Pd/C (2.0 g) was added to the solution, which was then stirred for 2 h at H_2 atmosphere. The reaction mixture was filtered, and the filtrate was evaporated to afford the crude **16f** (19.3 g, yield: 99%). To a solution of **16f** (1.95 g, 100 mmol) and HONB (1.79 g, 100 mmol) in CH_2Cl_2 (20 ml), DCC (2.06 g, 10.0 mmol) was added; and the mixture was stirred overnight. The reaction mixture was filtered, and the filtrate was washed with H_2O and brine, dried over $MgSO_4$, and then evaporated. The obtained residue was recrystallized from methanol to give 1.63 g of the title compound. Yield, 46%; mp, 133—135 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.53 (d, 1H, J=8.9 Hz), 1.61 (d, 1H, J=8.9 Hz), 2.88 (s, 6H), 3.33 (bs, 2H, overlapped with H_2O), 3.53 (bs, 2H), 5.17 (s, 2H), 6.1—6.2 (br, 2H), 6.23—6.26 (m, 2H), 6.36 (d, 1H, J=9,7 Hz), 7.07 (t, 1H, J=8.6 Hz): *Anal.* Calcd for $C_{19}H_{20}N_2O_5$: C, 64.04; H, 5.66; N, 7.86. Found: C, 64.33; H, 5.66; N, 7.70.

(*S*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(*tert*-butoxycarbonyl)aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]pyrrolidine-2-carboxamide (18a) To a solution of **6a** (1.04 g, 3.00 mmol) and HOBt (0.41 g, 3.00 mmol) in DMF (10 ml), Boc–Asn–ONp (1.27 g, 3.60 mmol) and TEA (0.50 ml, 3.60 mmol) were added; and the mixture was then stirred overnight. To the reaction mixture, EtOAc and 3% K_2CO_3 were added, and then the organic layer was washed sequentially with 3% K_2CO_3, 1 N HCl, and brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and recrystallized from *n*-hexane/EtOAc to give 0.79 g of the title compound. Yield, 47%; mp, 120—123 °C; *Anal.* Calcd for $C_{28}H_{43}N_5O_7$: C, 59.87; H, 7.72; N, 12.47. Found: C, 59.59; H, 7.84; N, 12.27.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(*tert*-butoxycarbonyl)aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (18b) To a solution of **12g** (0.73 g, 2.00 mmol) and HOBt (0.27 g, 2.00 mmol) in DMF (10 ml), Boc–Asn–ONp (0.85 g, 2.40 mmol) and TEA (0.33 ml, 2.40 mmol) were added; and the mixture was then stirred overnight. To the reaction mixture, EtOAc and 3% K_2CO_3 were added; and then the organic layer was washed sequentially with 3% K_2CO_3, 1 N HCl, and brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was recrystallized from *n*-hexane/EtOAc to give 0.81 g of the title compound. Yield, 70%; mp, 125—128 °C; *Anal.* Calcd for $C_{27}H_{41}N_5O_7S$: C, 55.94; H, 7.13; N, 12.08. Found: C, 55.98; H, 7.30; N, 12.08.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(*tert*-butoxycarbonyl)aminopentanoyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (18c) To a solution of **12g** (0.53 g, 1.47 mmol), Boc-protected L-norvaline (0.35 g, 1.61 mmol), and HOBt (0.22 g, 1.61 mmol) in DMF (5 ml), EDC·HCl (0.34 g, 1.76 mmol) was added; and the mixture was stirred overnight. To the reaction mixture, 3% K_2CO_3 was added, and the obtained precipitate was washed sequentially with 3% K_2CO_3, 1 N HCl and H_2O. After drying, the crude product was stirred in EtOAc under refluxed conditions, and filtered to give 0.47 g of the title compound. Yield, 57%; mp, 232—234 °C; *Anal.* Calcd for $C_{28}H_{44}N_4O_6S$: C, 59.55; H, 7.85; N, 9.92. Found: C, 59.66; H, 7.78; N, 10.23.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(*tert*-butoxycarbonyl)amino-3-methylbutanoyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (18d) Compound **18d** was prepared from Boc–Val–OH and compound **12g** in a similar manner as described for compound **18c**. Yield, 78%; mp, 235—237 °C; *Anal.* Calcd for $C_{28}H_{44}N_4O_6S$: C, 59.55; H, 7.85; N, 9.92. Found: C, 59.55; H, 7.76; N, 10.22.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(*tert*-butoxycarbonyl)amino-3,3-dimethylbutanoyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (18e) Compound **18e** was prepared from Boc-protected L-*tert*-leucine and compound **12g** in a manner similar to that described for compound **18c**. Yield, 79%; mp, 238—240 °C; *Anal.* Calcd for $C_{29}H_{46}N_4O_6S$: C, 60.18; H, 8.01; N, 9.68. Found: C, 59.80; H, 7.90; N, 9.96.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(*tert*-butoxycarbonyl)amino-3-methylthiopropionyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (18f) Compound **18f** was prepared from Boc–Mta–OH and compound **12g** similarly as described for compound **18c**, except for washing in refluxing MeOH. Yield, 84%; mp, 218—220 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.26 (s, 9H), 1.38 (s, 9H), 1.99 (s, 3H), 2.4—2.7 (m, 4H, overlapped with DMSO), 2.98 (dd, 1H, J=6.5 Hz, 11.6 Hz), 3.29—3.36 (m, 1H, overlapped with H_2O), 4.0—4.2 (m, 2H), 4.4—4.5 (m, 1H), 4.61 (d, 1H, J=9.5 Hz), 4.76 (t, 1H, J=6.8 Hz), 4.94 (d, 1H, J=9.5 Hz), 5.20 (d, 1H, J=6.8 Hz), 6.84 (d, 1H, J=9.5 Hz), 7.13—7.22 (m, 3H), 7.34 (d, 2H, J=6.8 Hz), 7.68 (s, 1H), 7.99 (d, 1H, J=8.4 Hz); *Anal.* Calcd for $C_{27}H_{42}N_4O_6S_2$: C, 55.65; H, 7.26; N, 9.61. Found: C, 55.65; H, 7.17; N, 10.05.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(*tert*-butoxycarbonyl)aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (18h) Compound **18h** was prepared from Boc–Asn–ONp and compound **12h** in a manner similar to that described for compound **18b**. Yield, 71%; mp, 126—128 °C; *Anal.* Calcd for $C_{29}H_{45}N_5O_7S$ 0.5H2O: C, 56.47; H, 7.52; N, 11.35. Found: C, 56,63; H, 7.56; N, 11.62.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(*tert*-butoxycarbonyl)aminobutanoyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (18i) To a solution of **12h** (0.39 g, 1.0 mmol), Boc-protected (*S*)-2-aminobutyric acid (0.20 g, 1.0 mmol) and HOBt (0.14 g, 1.0 mmol) in DMF (6 ml), EDC·HCl (0.21 g, 1.1 mmol) was added; and the mixture was stirred overnight. To the reaction mixture, EtOAc and 3% K_2CO_3 were added, and then the organic layer was washed sequentially with 3% K_2CO_3, 1 N HCl, and brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was recrystallized from *n*-hexane/EtOAc to give 0.56 g of the title compound. Yield, 96%; mp, 196—198 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.66 (t, 3H, J=7.3 Hz), 1.27 (s, 9H), 1.37 (s, 9H), 1.40 (s, 3H), 1.49 (s, 3H), 2.6—2.8 (m, 2H), 3.7—3.9 (br, 1H), 4.1—4.2 (br, 1H), 4.36—4.38 (m, 1H), 4.50 (s, 1H), 4.87 (d, 1H, J=9.2 Hz), 5.01 (d, 1H,

J=8.6 Hz), 5.12 (d, 1H, J=7.3 Hz), 6.73 (d, 1H, J=8.9 Hz), 7.1—7.3 (m, 3H), 7.35 (d, 2H, J=7.0 Hz), 7.65 (s, 1H), 7.87 (d, 1H, J=8.1 Hz); *Anal.* Calcd for $C_{29}H_{46}N_4O_6S$: C, 60.18; H, 8.01; N, 9.68. Found: C, 60.08; H, 7.96; N, 9.99.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(*tert*-butoxycarbonyl)amino-3-methylbutanoyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (18j) Compound **18j** was prepared from Boc–Val–OH and compound **12h** similarly as described for compound **18i**. Yield, 94%; mp, 190—192 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.55 (d, 3H, J=6.5 Hz), 0.65 (d, 3H, J=6.5 Hz), 1.27 (s, 9H), 1.38 (s, 9H), 1.40 (s, 3H), 1.49 (s, 3H), 2.6—2.8 (m, 2H), 3.6—3.8 (m, 1H), 4.1—4.3 (m, 1H), 4.38 (d, 1H, J=5.7 Hz), 4.52 (s, 1H), 4.87 (d, 1H, J=8.6 Hz), 5.06 (d, 2H, J=7.8 Hz), 6.65 (d, 1H, J=9.2 Hz), 7.1—7.3 (m, 3H), 7.36 (d, 2H, J=7.3 Hz), 7.69 (s, 1H), 7,88 (d, 1H, J=8.1 Hz); *Anal.* Calcd for $C_{30}H_{486}N_4O_6S$: C, 60.78; H, 8.16; N, 9.45. Found: C, 60.96; H, 8.37; N, 9.38.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(*tert*-butoxycarbonyl)amino-3-methylthiopropionyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (18k) To a solution of **12h** (0.79 g, 2.0 mmol), Boc–Mta–OH (0.52 g, 2.2 mmol), and HOBt (0.30 g, 2.2 mmol) in DMF (5 ml), EDC·HCl (0.46 g, 2.4 mmol) was added; and the mixture was stirred overnight. To the reaction mixture, EtOAc and 3% K_2CO_3 were added, and then the organic layer was washed with 3% K_2CO_3, 1 N HCl, and brine in this order, and then dried over $MgSO_4$. After removal of the solvent, the residue was recrystallized from *n*-hexane to give 1.09 g of the title compound. Yield, 90%; mp, 175—177 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.27 (s, 9H), 1.38 (s, 12H), 1.48 (s, 3H), 1.99 (s, 3H), 2.4—2.8 (m, 4H, overlapped with DMSO), 4.0—4.2 (m, 2H), 4.3—4.4 (m, 1H), 4.50 (s, 1H), 4.87 (d, 1H, J=8.6 Hz), 4.98 (d, 1H, J=8.6 Hz), 5.20 (d, 1H, J=7.3 Hz), 6.84 (d, 1H, J=8.9 Hz), 7.1—7.3 (m, 3H), 7.33 (d, 2H, J=6.8 Hz), 7.65 (s, 1H), 7.99 (d, 1H, J=8.4 Hz); *Anal.* Calcd for $C_{29}H_{46}N_4O_6S_2$: C, 57.02; H, 7.59; N, 9.17. Found: C, 57.02; H, 7.57; N, 9.40.

4-Nitrophenyl 1-Naphthylmethylcarbonate (20) To a solution of 1-naphthalenemethanol (**19**, 1.00 g, 6.3 mmol) in pyridine (5 ml), 4-nitrophenyl chloroformate (1.27 g, 6.3 mmol) was added in an ice-bath and stirred for 3 h. After removal of the solvent *in vacuo*, the oily residue was extracted with CH_2Cl_2, washed with 1 N HCl and brine, and dried over $MgSO_4$. The solvent was removed *in vacuo*, and the obtained residue was recrystallized from ethanol to give 1.08 g of the title compound. Yield, 53%; mp, 140—141 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 5.79 (s, 2H), 7.5—7.7 (m, 6H), 8.01 (d, 2H, J=8.4 Hz), 8.18 (d, 1H, J=7.8 Hz), 8.29—8.35 (m, 2H); *Anal.* Calcd for $C_{18}H_{13}NO_5$: C, 66.87; H, 4.05; N, 4.33. Found: C, 67.32; H, 4.22; N, 4.27.

(*S*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-(1-naphthylmethyloxycarbonyl)aminosuccinamyl]amino-4-phenylbutanoyl]pyrrolidine-2-carboxamide (21) To a solution of **18a** (169 mg, 0.3 mmol) in CH_2Cl_2 (5 ml), 4 N HCl in dioxane (5 ml) was added and stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF followed by neutralization with TEA (42 μl, 0.3 mmol). To this solution, compound **20** (116 mg, 0.36 mmol), HOBt (41 mg, 0.3 mmol) and TEA (50 μl, 0.36 mmol) were added; and the mixture was stirred overnight. To the reaction mixture, EtOAc and 3% K_2CO_3 were added, and then the organic layer was washed sequentially with 3% K_2CO_3, 1 N HCl, and brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and recrystallized from *n*-hexane/EtOAc to give 31 mg of the title compound. Yield, 16%; mp, 110—112 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.25 (s, 9H), 1.7—1.8 (m, 2H), 1.9—2.1 (m, 2H), 2.2—2.4 (m, 2H), 2.5—2.8 (m, 2H), 3.6—3.8 (br, 2H), 4.0—4.2 (br, 1H), 4.2—4.4 (m, 3H), 4.90 (d, 1H, J=7.3 Hz), 5.48 (s, 1H), 6.8—6.9 (br, 1H), 7.1—7.3 (m, 4H), 7.31—7.34 (m, 3H), 7.4—7.7 (m, 5H), 7.87—8.05 (m, 4H); TOF-MS *m/z*: 647 $(M+H)^+$; *Anal.* Calcd for $C_{35}H_{43}N_5O_7 \cdot 0.5H_2O$: C, 64.20; H, 6.77; N, 10.70. Found: C, 64.08; H, 6.71; N, 10.53.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(1-naphthoxyacetyl)aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (22a) To a solution of **18b** (116 mg, 0.2 mmol) in CH_2Cl_2 (3 ml), 4 N HCl in dioxane (3 ml) was added; and the mixture was stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF followed by neutralization with TEA (28 μl, 0.2 mmol). To this solution, *l*-naphthoxyacetic acid (40 mg, 0.2 mmol), HOBt (27 mg, 0.2 mmol), and EDC·HCl (42 mg, 0.22 mmol) were added; and the mixture was then stirred overnight. To the reaction mixture, CH_2Cl_2 and 3% K_2CO_3 were added, and then the organic layer was washed with 1 N HCl, then with brine, and dried over $MgSO_4$. After removal of the solvent, the residue was repecipitated from *n*-hexane/CH_2Cl_2 to give 61 mg of the title compound. Yield, 46%; mp, 112—115 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.25 (s, 9H), 2.50—2.53 (m, 1H, overlapped with DMSO), 2.6—2.7 (br, 2H), 2.98—3.02 (m, 1H), 3.30—3.37 (m, 1H, overlapped with H_2O), 4.1—4.2 (br, 1H), 4.47—4.50 (m, 1H), 4.62—4.67 (m, 4H), 4.76 (t, 1H, J=6.9 Hz), 4.96 (d, 1H, J=9.5 Hz), 5.20 (d, 1H, J=7.0 Hz), 6.9—7.2 (m, 5H), 7.3—7.6 (m, 7H), 7.68 (s, 1H), 7.90 (d, 1H, J=6.8 Hz), 8.04 (d, 1H, J=8.1 Hz), 8.32 (m, 2H); TOF-MS *m/z*: 665 $(M+H)^+$; *Anal.* Calcd for $C_{34}H_{41}N_5O_7S$: C, 61.52; H, 6.23; N, 10.55. Found: C, 61.16; H, 6.33; N, 10.17.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(1-naphthoxyacetyl)aminopentanoyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (22b) Compound **22b** was prepared from 1-naphthoxyacetic acid and compound **18c** in a similar manner as described for compound **22a** (recrystallization from *n*-hexane/EtOAc). Yield, 45%; mp, 162—164 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.81 (t, 3H, J=7.3 Hz), 1.26 (s, 9H), 1.4—1.6 (br, 2H), 2.6—2.7 (br, 2H), 3.00 (dd, 1H, J=11.6 Hz, 6.8 Hz), 3.15—3.37 (m, 1H, overlapped with H_2O), 4.1—4.2 (br, 1H), 4.3—4.4 (m, 1H), 4.46 (d, 1H, J=5.1 Hz), 4.65—4.71 (m, 3H), 4.77 (t, 1H, J=6.9 Hz), 4.99 (d, 1H, J=9.5 Hz), 5.17 (d, 1H, J=6.8 Hz), 6.88 (d, 1H, J=7.3 Hz), 7.10—7.21 (m, 3H), 7.34—7.42 (m, 3H), 7.50—7.55 (m, 3H), 7.69 (s, 1H), 7.81—7.91 (m, 1H), 7.99 (d, 1H, J=8.1 Hz), 8.18—8.24 (m, 2H); TOF-MS *m/z*: 649 $(M+H)^+$; *Anal.* Calcd for $C_{35}H_{44}N_4O_6S$: C, 64.79; H, 6.84; N, 8.64. Found: C, 64.87; H, 6.88; N, 8.55.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(1-naphthoxyacetyl)amino-3-methylbutanoyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (22c) Compound **22c** was prepared from 1-naphthoxyacetic acid and compound **18d** in a manner similar to that described for compound **22a** (recrystallization from n-hexane/EtOAc). Yield, 27%; mp, 151—153 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.75 (t, 6H, J=6.9 Hz), 1.26 (s, 9H), 1.9—2.1 (m, 1H), 2.6—2.7 (br, 2H), 2.95—3.05 (m, 1H), 3.30—3.37 (m, 1H, overlapped with H_2O), 4.1—4.3 (m, 1H), 4.3—4.5 (m, 1H), 4.67—4.81 (m, 4H), 4.99 (d, 1H, J=9.5 Hz), 5.13 (d, 1H, J=6.8 Hz), 6.89 (d, 1H, J=7.8 Hz), 7.11—7.21 (m, 3H), 7.35—7.42 (m, 3H), 7.50—7.56 (m, 3H), 7.70 (s, 1H), 7.82—7.91 (m, 2H), 8.19—8.24 (m, 2H); TOF-MS *m/z*: 649 $(M+H)^+$; *Anal.* Calcd for $C_{35}H_{44}N_4O_6S$: C, 64.79; H, 6.84; N, 8.64. Found: C, 64.57; H, 6.80; N, 8.85.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(1-naphthoxyacetyl)amino-3,3-dimethylbutanoyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (22d) Compound **22d** was prepared from 1-naphthoxyacetic acid and compound **18e** similarly as described for compound **22a** (recrystallization from *n*-hexane/EtOAc). Yield, 26%; mp, 222—225 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.86 (s, 9H), 1.25 (s, 9H), 2.6—2.7 (br, 2H), 2.99 (dd, 1H, J=11.6 Hz, 6.2 Hz), 3.3—3.4 (m, 1H, overlapped with H_2O), 4.1—4.3 (br, 1H), 4.34 (d, 1H, J=9.2 Hz), 4.48—4.50 (m, 1H), 4.66—4.82 (m, 4H), 5.00—5.08 (m, 2H), 6.87 (d, 1H, J=7.8 Hz), 7.07—7.21 (m, 3H), 7.35—7.41 (m, 3H), 7.49—7.56 (m, 3H), 7.71 (d, 2H, J=11.3 Hz), 7.88—7.91 (m, 1H), 8.15—8.22 (m, 2H); TOF-MS *m/z*: 663 $(M+H)^+$; *Anal.* Calcd for $C_{36}H_{46}N_4O_6S$: C, 65.23; H, 6.99; N, 8.45. Found: C, 65.35; H, 7.12; N, 8.22.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(1-naphthoxyacetyl)amino-3-methylthiopropionyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (22e) Compound **22e** was prepared from 1-naphthoxyacetic acid and compound **18f** in a manner similar to that described for compound **22a** (recrystallization from *n*-hexane/EtOAc). Yield, 30%; mp, 176—178 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.26 (s, 9H), 2.04 (s, 3H), 2.6—2.8 (m, 3H), 2.9—3.0 (m, 1H), 3.3—3.4 (m, 1H, overlapped with H_2O), 4.1—4.2 (br, 1H), 4.46—4.50 (m, 1H), 4.5—4.8 (m, 5H), 4.97 (d, 1H, J=9.5 Hz), 5.24 (d, 1H, J=7.3 Hz), 6.93 (d, 1H, J=7.8 Hz), 7.10 (d, 1H, J=6.8 Hz), 7.18 (t, 2H, J=7.2 Hz), 7.32—7.45 (m, 3H), 7.50—7.55 (m, 3H), 7.69 (s, 1H), 7.88—7.91 (m, 1H), 8.14 (d, 1H, J=8.9 Hz), 8.25—8.33 (m, 2H); TOF-MS *m/z*: 667 $(M+H)^+$; *Anal.* Calcd for $C_{34}H_{42}N_4O_6S_2$: C, 61.24; H, 6.35; N, 8.40. Found: C, 61.37; H, 6.39; N, 8.27.

(*S*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(1-naphthoxyacetyl)aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]pyrrolidine-2-carboxamide (23a) To a solution of **18a** (169 mg, 0.3 mmol) in CH_2Cl_2 (3 ml), 4 N HCl in dioxane (3 ml) was added; and the mixture was stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF followed by neutralization with TEA (42 μl, 0.3 mmol). To this solution, 1-naphthoxyacetic acid (61 mg, 0.3 mmol), HOBt (41 mg, 0.3 mmol), and EDC·HCl (63 mg, 0.33 mmol) were added; and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 3% K_2CO_3 were added, and then the organic layer was washed sequentially with 1 N HCl and brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and reprecipitated from *n*-hexane/EtOAc to give 80 mg of the title compound. Yield, 41%;

mp, 103—106 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.23 (s, 9H), 1.7—1.9 (br, 2H), 1.9—2.1 (br, 2H), 2.5—2.8 (br, 4H, overlapped with DMSO), 3.5—3.7 (br, 2H), 4.1—4.2 (br, 1H), 4.3—4.4 (br, 2H), 4.6—4.7 (br, 3H), 4.90 (d, 1H, J=7.3 Hz), 6.9—7.2 (m, 5H), 7.30—7.53 (m, 8H), 7.89 (d, 1H, J=7.3 Hz), 7.97 (d, 1H, J=8.6 Hz), 8.34 (d, 1H, J=9.5 Hz), 8.43 (d, 1H, J=7.8 Hz); TOF-MS m/z: 647 $(M+H)^+$; *Anal.* Calcd for $C_{35}H_{43}N_5O_7 \cdot 0.5$EtOAc: C, 64.42; H, 6.87; N, 10.15. Found: C, 64.51; H, 6.95; N, 10.20.

(*S*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(2-naphthoxyacetyl)aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]pyrrolidine-2-carboxamide (23b) Compound **23b** was prepared from 2-naphthoxyacetic acid and compound **18a** in a manner similar to that described for compound **23a**. Yield, 45%; mp, 106—109 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.24 (s, 9H), 1.7—1.9 (m, 2H), 1.9—2.1 (m, 2H), 2.5—2.8 (m, 4H, overlapped with DMSO), 3.5—3.7 (br, 2H), 4.1—4.2 (br, 1H), 4.3—4.4 (br, 2H), 4.59—4.64 (m, 3H), 4.89 (d, 1H, J=7.3 Hz), 6.92 (bs, 1H), 7.08—7.54 (m, 11H), 7.80—7.88 (m, 3H), 7.98 (d, 1H, J=8.1 Hz), 8.26 (d, 1H, J=8.4 Hz); TOF-MS m/z: 647 $(M+H)^+$; *Anal.* Calcd for $C_{35}H_{43}N_5O_7 \cdot H_2O$: C, 63.33; H, 6.83; N, 10.55. Found: C, 63.77; H, 6.73; N, 10.48.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(1-naphthoxyacetyl)aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (23c) Compound **23c** was prepared from 1-naphthoxyacetic acid and compound **18h** similarly as that described for compound **23a**. Yield, 64%; mp, 115—118 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.26 (s, 9H), 1.40 (s, 3H), 1.49 (s, 3H), 2.5—2.7 (m, 4H, overlapped with DMSO), 4.1—4.2 (br, 1H), 4.46 (dd, 1H, J=7.3 Hz, 2.4 Hz), 4.51 (s, 1H), 4.61—4.68 (m, 3H), 4.88 (d, 1H, J=8.6 Hz), 5.01 (d, 1H, J=8.6 Hz), 5.20 (d, 1H, J=7.3 Hz), 6.92—7.19 (m, 6H), 7.29—7.56 (m, 6H), 7.66 (s, 1H), 7.89 (d, 1H, J=8.6 Hz), 8.03 (d, 1H, J=8.4 Hz), 8.34 (d, 1H, J=7.3 Hz), 8.41 (d, 1H, J=7.8 Hz); TOF-MS m/z: 692 $(M+H)^+$; *Anal.* Calcd for $C_{36}H_{45}N_5O_7S \cdot 0.5H_2O$: C, 61.69; H, 6.62; N, 9.99. Found: C, 61.52; H, 6.60; N, 9.73.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(2-biphenyloxyacetyl)aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (23d) Compound **23d** was prepared from 2-biphenyloxyacetic acid (**16a**) and compound **18h** similarly as described for compound **23a**. Yield, 33%; mp, 107—109 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.27 (s, 9H), 1.40 (s, 3H), 1.50 (s, 3H), 2.3—2.5 (m, 2H, overlapped with DMSO), 2.5—2.7 (m, 2H), 4.1—4.2 (br, 1H), 4.43—4.59 (m, 5H), 4.87 (d, 1H, J=8.9 Hz), 5.01 (d, 1H, J=8.9 Hz), 5.16 (d, 1H, J=7.6 Hz), 6.92—7.19 (m, 7H), 7.31 (t, 4H, J=7.3 Hz), 7.40 (d, 1H, J=7.3 Hz), 7.55 (d, 1H, J=6.8 Hz), 7.67 (s, 1H), 7.83 (d, 1H, J=7.8 Hz), 8.03 (d, 1H, J=8.4 Hz); TOF-MS m/z: 734 $(M+H)^+$; *Anal.* Calcd for $C_{38}H_{47}N_5O_7S \cdot 0.5H_2O$: C, 62.79; H, 6.66; N, 9.63. Found: C, 62.45; H, 6.59; N, 9.55.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(3-biphenyloxyacetyl)aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (23e) Compound **23e** was prepared from 3-biphenyloxyacetic acid (**16b**) and compound **18h** similarly as described for compound **23a**. Yield, 62%; mp, 110—112 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.26 (s, 9H), 1.39 (s, 3H), 1.49 (s, 3H), 2.4—2.5 (m, 2H, overlapped with DMSO), 2.6—2.8 (m, 2H), 4.0—4.2 (br, 1H), 4.42—4.68 (m, 5H), 4.87 (d, 1H, J=8.9 Hz), 4.99 (d, 1H, J=8.9 Hz), 5.18 (d, 1H, J=7.3 Hz), 6.90—6.98 (m, 2H), 7.06—7.49 (m, 12H), 7.59—7.69 (m, 3H), 8.02 (d, 1H, J=8.9 Hz), 8.18 (d, 1H, J=8.4 Hz); TOF-MS m/z: 734 $(M+H)^+$; *Anal.* Calcd for $C_{38}H_{47}N_5O_7S \cdot 0.5H_2O$: C, 62.79; H, 6.66; N, 9.63. Found: C, 62.82; H, 6.64; N, 9.46.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(4-biphenyloxyacetyl)aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (23f) Compound **23f** was prepared from 4-biphenyloxyacetic acid (**16c**) and compound **18h** similarly as described for compound **23a**. Yield, 66%; mp, 114—116 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.27 (s, 9H), 1.40 (s, 3H), 1.49 (s, 3H), 2.4—2.5 (m, 2H, overlapped with DMSO), 2.5—2.7 (m, 2H), 4.0—4.2 (br, 1H), 4.4—4.7 (m, 5H), 4.87 (d, 1H, J=8.9 Hz), 5.00 (d, 1H, J=8.9 Hz), 5.20 (d, 1H, J=7.3 Hz), 6.92 (bs, 1H), 7.04 (d, 2H, J=8.9 Hz), 7.11—7.22 (m, 3H), 7.30—7.32 (m, 4H), 7.43 (t, 2H, J=7.6 Hz), 7.59—7.66 (m, 5H), 8.01 (d, 1H, J=8.4 Hz), 8.19 (d, 1H, J=8.1 Hz); TOF-MS m/z: 734 $(M+H)^+$; *Anal.* Calcd for $C_{38}H_{47}N_5O_7S \cdot 0.5$EtOAc: C, 63.05; H, 6.75; N, 9.19. Found: C, 63.10; H, 6.68; N, 9.25.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(3-phenylaminophenyloxyacetyl)-aminosuccinamyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (23g) Compound **23g** was prepared from 3-(phenylamino)phenoxyacetic acid (**16d**) and compound **18h** in a manner similar to that described for compound **23a**. Yield, 29%; mp, 109—111 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.27 (s, 9H), 1.39 (s, 3H), 1.48 (s, 3H), 2.4—2.5 (m, 2H, overlapped with DMSO), 2.6—2.8 (m, 2H), 4.0—4.2 (br, 1H), 4.40—4.45 (m, 3H), 4.50 (s, 1H), 4.58—4.64 (m, 1H), 4.86 (d, 1H, J=8.9 Hz), 4.99 (d, 1H, J=8.9 Hz), 5.18 (d, 1H, J=6.8 Hz), 6.41 (d, 1H, J=8.1 Hz), 6.68 (d, 2H, J=7.8 Hz), 6.81—6.90 (m, 2H), 7.07—7.31 (m, 11H), 7.67 (s, 1H), 7.98 (d, 1H, J=8.1 Hz), 8.13 (d, 1H, J=8.4 Hz), 8.19 (s, 1H); TOF-MS m/z: 733 $(M+H)^+$; *Anal.* Calcd for $C_{38}H_{48}N_6O_7S \cdot 0.5$EtOAc: C, 61.84; H, 6.75; N, 10.82. Found: C, 61.66; H, 6.70; N, 10.90.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(5-isoquinolinyloxyacetyl)-aminosuccinamyl] amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (23h) To a solution of **18h** (121 mg, 0.2 mmol) in CH_2Cl_2 (3 ml), 4 N HCl in dioxane (3 ml) was added; and the mixture was stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF followed by neutralization with TEA (28 μl, 0.2 mmol). To this solution, 5-isoquinolinyloxyacetic acid (40 mg, 0.2 mmol), HOBt (27 mg, 0.2 mmol), and EDC·HCl (42 mg, 0.22 mmol) were added; and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 3% K_2CO_3 were added, and then the organic layer was washed with brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and reprecipitated from *n*-hexane/EtOAc to give 78 mg of the title compound. Yield, 57%; mp, 131—133 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.25 (s, 9H), 1.39 (s, 3H), 1.49 (s, 3H), 2.4—2.5 (m, 2H, overlapped with DMSO), 2.4—2.6 (m, 2H), 4.0—4.2 (br, 1H), 4.46 (dd, 1H, J=7.3 Hz, 2.4 Hz), 4.50 (s, 1H), 4.60—4.71 (m, 3H), 4.87 (d, 1H, J=8.9 Hz), 5.01 (d, 1H, J=8.9 Hz), 5.20 (d, 1H, J=6.8 Hz), 6.99—7.22 (m, 5H), 7.31 (d, 2H, J=7.3 Hz), 7.38 (br, 1H), 7.57—7.74 (m, 3H), 8.02 (d, 1H, J=8.1 Hz), 8.15 (d, 1H, J=5.7 Hz), 8.47 (d, 1H, J=7.8 Hz), 8.52 (d, 1H, J=5.9 Hz), 9.30 (s, 1H); TOF-MS m/z: 693 $(M+H)^+$; *Anal.* Calcd for $C_{35}H_{44}N_6O_7S \cdot H_2O$: C, 59.14; H, 6.52; N, 11.82. Found: C, 59.52; H, 6.45; N, 11.32.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(1-naphthoxyacetyl)amino-3-methylthiopropionyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (24a) Compound **24a** was prepared from 1-naphthoxyacetic acid and compound **18k** similarly as described for compound **22a** (recrystallization from *n*-hexane/EtOAc). Yield, 71%; mp, 113—115 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm); 1.27 (s, 9H), 1.40 (s, 3H), 1.49 (s, 3H), 2.04 (s, 3H), 2.6—2.8 (m, 4H), 4.1—4.3 (br, 1H), 4.45 (dd, 1H, J=7.3 Hz, 2.4 Hz), 4.52 (s, 1H), 4.5—4.7 (m, 1H), 4.89 (d, 1H, J=8.9 Hz), 5.05 (d, 1H, J=8.9 Hz), 5.24 (d, 1H, J=7.3 Hz), 6.93 (d, 1H, J=7.3 Hz), 7.10—7.21 (m, 3H), 7.32—7.42 (m, 3H), 7.50—7.56 (m, 3H), 7.67 (s, 1H), 7.87—7.91 (m, 1H), 8.13 (d, 1H, J=8.1 Hz), 8.24—8.32 (m, 2H); TOF-MS m/z: 695 $(M+H)^+$; *Anal.* Calcd for $C_{36}H_{46}N_4O_6S_2$: C, 62.22; H, 6.67; N, 8.06. Found: C, 61.97; H, 6.64; N, 8.03.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(3-phenylaminophenoxyacetyl)-amino-3-methylthiopropionyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (24b) Compound **24a** was prepared from **16d** and compound **18k** in a manner similar to that described for compound **22a** (reprecipitation from *n*-hexane/CH_2Cl_2). Yield, 82%; mp, 103—105 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.27 (s, 9H), 1.40 (s, 3H), 1.49 (s, 3H), 2.01 (s, 3H), 2.59—2.80 (m, 4H), 4.0—4.2 (br, 1H), 4.41—4.53 (m, 5H), 4.87 (d, 1H, J=8.9 Hz), 5.02 (d, 1H, J=8.9 Hz), 5.21 (d, 1H, J=7.3 Hz), 6.41 (d, 1H, J=9.2 Hz), 6.67 (d, 2H, J=7.6 Hz), 6.83 (t, 1H, J=7.4 Hz), 7.07—7.26 (m, 6H), 7.32 (d, 2H, J=6.8 Hz), 7.67 (s, 1H), 7.94 (d, 1H, J=8.6 Hz), 8.18 (s, 1H), 8.26 (d, 1H, J=8.9 Hz); TOF-MS m/z: 736 $(M+H)^+$; *Anal.* Calcd for $C_{38}H_{49}N_5O_6S_2$: C, 62.02; H, 6.71; N, 9.52. Found: C, 62.32; H, 7.07; N, 9.19.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(5-isoquinolinyloxyacetyl)amino-3-methylthiopropionyl]amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (24c) Compound **24c** was prepared from **16e** and compound **18k** similarly as described for compound **22a** (recrystallization from *n*-hexane/ethanol). Yield, 77%; mp, 107—109 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.27 (s, 9H), 1.40 (s, 3H), 1.49 (s, 3H), 2.04 (s, 3H), 2.6—2.9 (m, 4H), 4.1—4.3 (br, 1H), 4.45 (dd, 1H, J=7.3 Hz, 3.0 Hz), 4.52 (s, 1H), 4.5—4.6 (m, 1H), 4.76 (s, 2H), 4.89 (d, 1H, J=8.9 Hz), 5.04 (d, 1H, J=8.9 Hz), 5.24 (d, 1H, J=7.6 Hz), 7.09—7.21 (m, 4H), 7.33 (d, 2H, J=7.3 Hz), 7.57 (t, 1H, J=8.1 Hz), 7.69 (t, 1H, J=7.8 Hz), 8.06 (d, 1H, J=5.9 Hz), 8.21 (d, 1H, J=8.4 Hz), 8.30 (d, 1H, J=8.4 Hz), 8.54 (d, 1H, J=5.9 Hz), 9.29 (s, 1H); TOF-MS m/z: 696 $(M+H)^+$; *Anal.* Calcd for $C_{35}H_{45}N_5O_6S_2$: C, 60.41; H, 6.52; N, 10.06. Found: C, 59.93; H, 6.53; N, 10.31.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-[(*S*)-2-(5-isoquinolinyloxyacetyl)amino-3-methylthiopropionyl]amino-2-hydroxy-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (24d) To a solution of **18f** (600 mg, 1.03 mmol) in CH_2Cl_2 (5 ml), 4 N HCl in dioxane (5 ml) was added; and the mixture was then stirred for 2 h. The reaction mixture was concentrated, and then

the residue was dissolved in DMF followed by neutralization with TEA (143 μl, 1.03 mmol). To this solution, 5-isoquinolinyloxyacetic acid (**16e**, 210 mg, 1.03 mmol), HOBt (139 mg, 1.03 mmol), and EDC·HCl (236 mg, 1.24 mmol) were added; and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 3% K_2CO_3 were added, and then the organic layer was washed with 3% K_2CO_3 followed by brine, and dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and recrystallization from ethanol gave 510 mg of the title compound. Yield, 74%; mp 175 °C,[38] ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.27 (s, 9H), 2.06 (s, 3H), 2.68—2.76 (m, 3H), 2.87 (dd, 1H, J=13.5 Hz, 4.9 Hz), 3.02 (dd, 1H, J=11.9 Hz, 6.5 Hz), 3.32—3.40 (m, 1H, overlapped with H_2O), 4.1—4.3 (br, 1H), 4.52 (dd, 1H, J=7.0 Hz, 2.4 Hz), 4.58—4.84 (m, 4H), 5.00 (d, 1H, J=9.5 Hz), 5.27 (d, 1H, J=7.3 Hz), 7.11—7.23 (m, 3H), 7.37 (d, 1H, J=7.6 Hz), 7.58 (t, 1H, J=8.0 Hz), 7.71—7.73 (m, 2H), 8.09 (d, 1H, J=5.9 Hz), 8.24 (d, 1H, J=8.4 Hz), 8.34 (d, 1H, J=7.8 Hz), 8.56 (d, 1H, J=5.4 Hz), 9.31 (s, 1H); TOF-MS *m/z*: 668 $(M+H)^+$; *Anal.* Calcd for $C_{33}H_{41}N_5O_6S_2 \cdot 0.5H_2O$: C, 58.56; H, 6.25; N, 10.35. Found: C, 58.55; H, 6.23; N, 10.06.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-(5-isoquinolinyloxyacetyl)amino-3-methylbutanoyl]amino-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (24e) Compound **24e** was prepared from **16e** and compound **18d** in a manner similar to that described for compound **22a** (recrystallization from *n*-hexane/EtOAc). Yield, 72%; mp, 172—173 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.73—0.78 (m, 6H), 1.26 (s, 9H), 1.9—2.1 (m, 1H), 2.6—2.7 (m, 2H), 2.95—3.05 (m, 1H), 3.30—3.34 (m, 1H, overlapped with H_2O), 4.1—4.3 (m, 2H), 4.4—4.5 (m, 1H), 4.67—4.81 (m, 4H), 4.99 (d, 1H, J=9.2 Hz), 5.14 (d, 1H, J=7.3 Hz), 7.11—7.22 (m, 4H), 7.36 (d, 2H, J=9.0 Hz), 7.57 (t, 1H, J=7.6 Hz), 7.69—7.72 (m, 2H), 7.93 (d, 1H, J=8.6 Hz), 8.00 (d, 1H, J=5.7 Hz), 8.22 (d, 1H, J=8.4 Hz), 8.54 (d, 1H, J=5.9 Hz), 9.29 (s, 1H); TOF-MS *m/z*: 650 $(M+H)^+$; *Anal.* Calcd for $C_{34}H_{43}N_5O_6S$: C, 62.84; H, 6.67; N, 10.78. Found: C, 63.05; H, 6.76; N, 10.76.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-(5-isoquinolinyloxyacetyl)aminobutanoyl]amino-4-phenylbutanoyl]-1,3-thiazolidine-4-carboxamide (24f) To a solution of **12g** (730 mg, 2.0 mmol), Boc-protected (*S*)-2-aminobutyric acid (406 mg, 2.0 mmol), and HOBt (270 mg, 2.0 mmol) in DMF (5 ml), EDC·HCl (420 mg, 2.2 mmol) was added; and the mixture was stirred overnight. To the reaction mixture, 3% K_2CO_3 was added; and the obtained precipitate was washed sequentially with 3% K_2CO_3, 1 N HCl and H_2O. After drying, the crude product was stirred in 50% aqueous MeOH under refluxing conditions, and filtered to give 760 mg of **18g** (69%). To the solution of **18g** (165 mg, 0.30 mmol) in CH_2Cl_2 (3 ml), 4 N HCl in dioxane (3 ml) was added; and the mixture was stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF followed by neutralization with TEA (50 μl, 0.30 mmol). To this solution, **16e** (61 mg, 0.30 mmol), HOBt (41 mg, 0.30 mmol), and EDC·HCl (63 mg, 0.33 mmol) were added; and the mixture was stirred overnight. To the reaction mixture, CH_2Cl_2 and 3% K_2CO_3 were added, and then the organic layer was washed with 3% K_2CO_3 and then with brine, and dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH), and reprecipitated from *n*-hexane/CH_2Cl_2 to give 110 mg of the title compound. Yield (total), 40%; mp, 152—154 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.77 (t, 3H, J=7.4 Hz), 1.26 (s, 9H), 1.4—1.7 (m, 2H), 2.6—2.7 (m, 2H), 3.00 (dd, 1H, J=6.5 Hz, 11.9 Hz), 3.30—3.37 (m, 1H, overlapped with H_2O), 4.1—4.2 (m, 1H), 4.2—4.3 (m, 1H), 4.4—4.5 (m, 1H), 4.6—4.8 (m, 4H), 4.99 (d, 1H, J=9.2 Hz), 5.19 (d, 1H, J=7.6 Hz), 7.11—7.22 (m, 4H), 7.35 (d, 2H, J=7.0 Hz), 7.57 (t, 1H, J=8.1 Hz), 7.71 (d, 2H, J=7.3 Hz), 8.03 (t, 2H, J=8.1 Hz), 8.21 (d, 1H, J=8.1 Hz), 8.54 (d, 1H, J=5.9 Hz), 9.29 (s, 1H); TOF-MS *m/z*: 636 $(M+H)^+$; *Anal.* Calcd for $C_{33}H_{41}N_5O_6S$: C, 62.34; H, 6.50; N, 11.02. Found: C, 62.09; H, 6.51; N, 10.82.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-(3-dimethylaminophenoxyacetyl)amino-3-methylbutanoyl]amino-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (24g) To a solution of **18j** (1.18 g, 2.0 mmol) in CH_2Cl_2 (10 ml), 4 N HCl in dioxane (10 ml) was added; and the mixture was stirred for 2 h. The reaction mixture was concentrated, and then the residue was dissolved in DMF (8.0 ml) followed by neutralization with TEA (278 μl, 2.0 mmol). To this solution, **17** (748 mg, 2.1 mmol) and TEA (278 μl, 2.0 mmol) were added; and the mixture was stirred overnight. To the reaction mixture, EtOAc and 3% K_2CO_3 were added, and then the organic layer was washed with 3% K_2CO_3 followed with brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH) to give 1.13 g of the title compound. Yield, 84%; mp, 98—100 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.70 (d, 6H, J=6.8 Hz), 1.27 (s, 9H), 1.40 (s, 3H), 1.49 (s, 3H), 1.88—1.95 (m, 1H), 2.5—2.8 (m, 2H), 2.85 (s, 6H), 4.13—4.24 (m, 2H), 4.4—4.7 (m, 4H), 4.89 (d, 1H, J=8.9 Hz), 5.06—5.11 (m, 2H), 6.20—6.23 (m, 2H), 6.31—6.35 (m, 1H), 7.0—7.2 (m, 4H), 7.35 (d, 2H, J=6.8 Hz), 7.58—7.67 (m, 2H), 8.21 (d, 1H, J=8.4 Hz); TOF-MS *m/z*: 670 $(M+H)^+$; *Anal.* Calcd for $C_{35}H_{51}N_5O_6S$: C, 62.75; H, 7.67; N, 10.45. Found: C, 62.66; H, 7.73; N, 10.35.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-2-hydroxy-3-[(*S*)-2-(3-dimethylaminophenoxyacetyl)aminobutanoyl]amino-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (24h) Compound **24h** was prepared from **18i** in a manner similar to that described for compound **24g**. Yield, 50%; mp, 93—96 °C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 0.73 (t, 3H, J=7.3 Hz), 1.27 (s, 9H), 1.40 (s, 3H), 1.49 (s, 3H), 1.3—1.7 (m, 2H), 2.5—2.8 (m, 2H), 2.85 (s, 6H), 4.1—4.3 (m, 2H), 4.4—4.7 (m, 4H), 4.89 (d, 1H, J=8.9 Hz), 5.04 (d, 1H, J=8.9 Hz), 5.16 (d, 1H, J=7.6 Hz), 6.20—6.23 (m, 2H), 6.32—6.35 (m, 1H), 7.0—7.2 (m, 4H), 7.34 (d, 2H, J=7.3 Hz), 7.65—7.75 (m, 2H), 8.19 (d, 1H, J=8.1 Hz); TOF-MS *m/z*: 656 $(M+H)^+$; *Anal.* Calcd for $C_{34}H_{49}N_5O_6S \cdot 0.5H_2O$: C, 61.42; H, 7.58; N, 10.53. Found: C, 61.49; H, 7.46; N, 10.56.

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Bioactive Constituents of Chinese Natural Medicines. V.[1] Radical Scavenging Effect of Moutan Cortex. (1): Absolute Stereostructures of Two Monoterpenes, Paeonisuffrone and Paeonisuffral

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The methanolic extract and the ethyl acetate-soluble and methanol-eluted fractions from Chinese Moutan Cortex, the roots of *Paeonia suffruticosa* ANDREWS, were found to exhibit scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical and superoxide anion radical generated by the xanthine–xanthine oxidase system. Two monoterpenes called paeonisuffrone and paeonisuffral were isolated from the ethyl acetate-soluble fraction. Their absolute stereostructures were elucidated on the basis of chemical and physiochemical evidence, which included the application of a modified Mosher's method and lipase catalyzed debenzoylation reaction.

Key words *Paeonia suffruticosa*; Moutan Cortex; paeonisuffrone; peaonisuffral; monoterpene; radical scavenging effect

The root cortex of *Paeonia suffruticosa* ANDREWS (Paeoniaceae) has been used as a Chinese herbal medicine, Moutan Cortex (牡丹皮 in Japanese). Moutan Cortex is one of the most important herbal medicines known and is used as an analgesic, a sedative, an antiinflammatory agent, and a remedy for female diseases in Chinese traditional medicine. Particularly, this herbal medicine is prescribed in various Chinese preparations used for treatment of "Oketsu" syndrome, which is presumed to be caused by blood stagnation. Extensive chemical and pharmacological studies were made on the constituents of Moutan Cortex to determine its bioactive principles, and the presence of paeonol (**10**) and its glycosides, and monoterpene glycosides, paeoniflorin (**6**), oxypaeoniflorin, benzoyl-paeoniflorin (**7**), and benzoyl-oxypaeoniflorin (**8**), has hitherto been reported.[2]

Active oxygen species and free radicals react with biomolecular constituents (*e.g.* lipids, protein, and DNA) to cause certain clinical diseases, such as atherosclerosis, inflammation, diabetes, and cancer[3] which are regarded as Oketsu syndrome.

During the course of our chemical studies on the bioactive constituents of Chinese herbal medicine,[1,4] the methanolic extract of Chinese Moutan Cortex was found to show scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide anion radical ($\cdot O_2^-$) generated by the xanthine–xanthine oxidase system.

The radical scavenging effect was concentrated in the ethyl acetate-soluble fraction from the methanolic extract. Two then new monoterpenes, paeonisuffrone (**1**) and paeonisuffral (**3**), were isolated from the ethyl acetate-soluble fraction. In this report, we describe a full account of the structural elucidation of **1** and **3**.[5]

Chinese Moutan Cortex was extracted with methanol under reflux. The methanolic extract was found to show DPPH radical scavenging effect and inhibitory effect on the formazan formation from nitroblue tetrazolium (NBT) in the xanthine-xanthine oxidase system, while the extract showed little inhibition for xanthine oxidase activity. These results indicated that the extract scavenged $\cdot O_2^-$ as well as DPPH radical. The methanolic extract was then partitioned into an ethyl acetate and water mixture to give an ethyl acetate-soluble fraction and an aqueous phase. The aqueous phase was subjected to XAD-2 column chromatography to give a water-eluted fraction and a methanol-eluted fraction. As is apparent from Table 1, the ethyl acetate-soluble and methanol-eluted fractions showed scavenging effects on both DPPH radical and $\cdot O_2^-$. Particularly, DPPH radical scavenging effect of the ethyl acetate-soluble fraction was more potent than that of positive control, α-tocopherol. The ethyl acetate-soluble fraction was separated by repeated silica gel column chromatograohy to give **1** (0.0003%), **3** (0.0014%), and 3-*O*-methylpaeonisuffral (**4**, 0.0003%) together with paeoniflorigenone (**5**,[6] 0.0110%), benzoyl-paeoniflorin (**7**,[7] 0.10%), benzoyl-oxypaeoniflorin (**8**,[8] 0.05%), paeonol (**10**,[2,9] 1.3%), resacetophenone (**11**,[2,9] 0.0030%), acetovanillone (**12**,[2,9] 0.0010%), acetoisovanillone (**13**,[2,9] 0.0030%), 2,5-dihydroxy-4-methylacetophenone (**14**,[2,9] 0.0020%), 2,5-dihydroxy-4-methoxyacetophenone (**15**,[2,9] 0.0010%).

Compound **1** was isolated as a white powder with negative optical rotation ($[\alpha]_D^{20}$ −16.8°). The IR spectrum of **1** showed absorption bands ascribable to hydroxyl and ketone functions at 3426 and 1725 cm^{-1}. The molecular formula $C_{10}H_{14}O_4$ of **1** was determined from the quasimolecular ion peaks observed in the positive- and negative-ion FAB-MS and by high-resolution MS analysis. Namely, quasimolecular ion peaks were observed at *m/z* 237 $(M+K)^+$ and *m/z* 221 $(M+Na)^+$ in the positive-ion FAB-MS of **1**, while its nega-

Table 1. DPPH radical and $\cdot O_2^-$ Scavenging Activities of the Methanolic Extract and its Fractions

	DPPH radical	$\cdot O_2^-$	
		Formozan formation	Xanthine oxidase
	SC_{50}	IC_{50}	IC_{50}
MeOH ext.	5.3 μg/ml	12 μg/ml	>50 μg/ml (27%)[c]
AcOEt-soluble fraction	2.5 μg/ml	5.5 μg/ml	>25 μg/ml (40%)[b]
MeOH-eluted fraction	10 μg/ml	11 μg/ml	>50 μg/ml (15%)[c]
H_2O-eluted fraction	17 μg/ml	14 μg/ml	>50 μg/ml (14%)[c]
α-Tocopherol	11 μM	—	—
Gallic acid	3.9 μM	1.8 μM	>10 μM (−7%)[a]
(+)-Catechin	6.0 μM	5.3 μM	>10 μM (−2%)[a]

Values in parentheses represent the inhibition (%) at *a*) 10 μM, *b*) 25 μg/ml, and *c*) 50 μg/ml.

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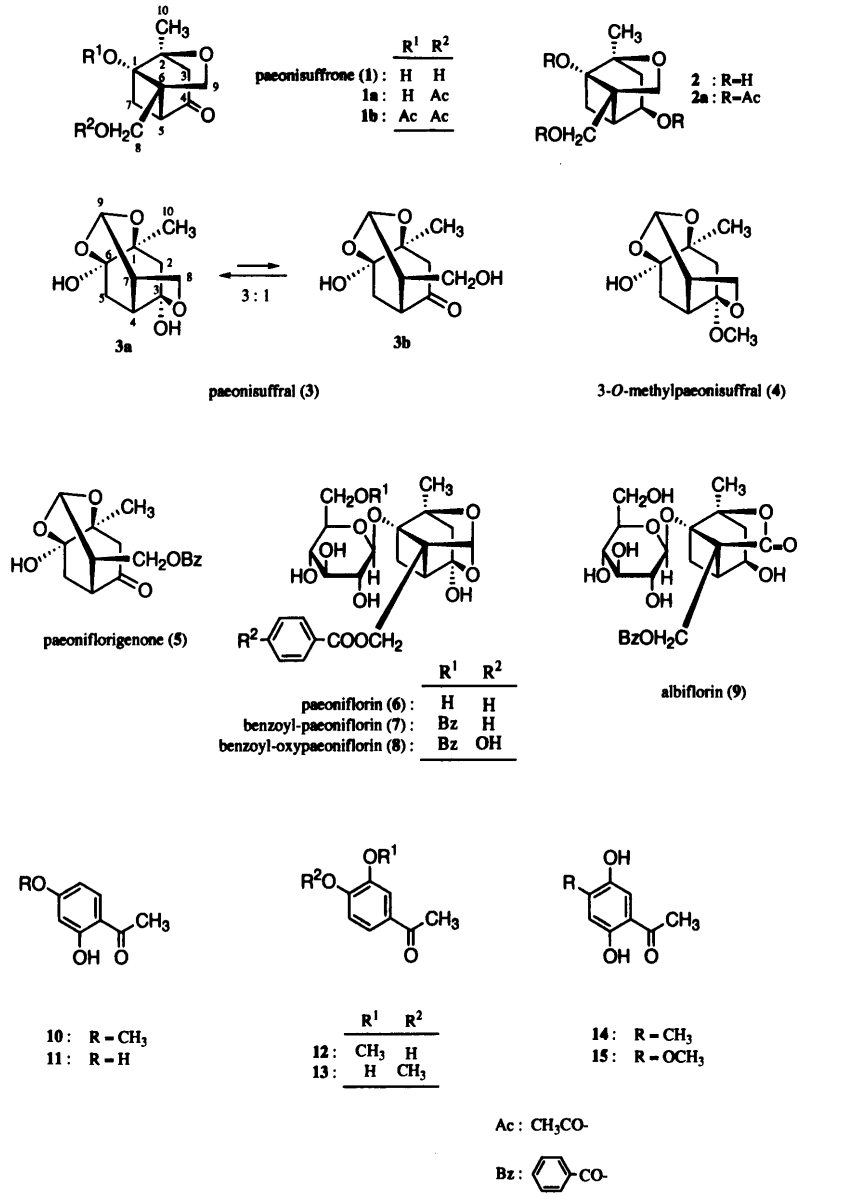


Chart 1

tive-ion FAB-MS showed a quasimolecular ion peak at *m/z* 197 $(M-H)^-$. The ^{1}H-NMR (CD_3OD) and ^{13}C-NMR (CD_3OD, Table 2) spectra of **1**, which were assigned with the aid of various NMR analytical methods,[10] showed the presence of a tertiary methyl [δ 1.31 (s, 10-H_3)], two methylenes [δ 2.31 (d, J=18 Hz), 2.91 (dd, J=1, 18 Hz) (3-H_2); δ 2.21(d, J=11 Hz), 2.47 (ddd, J=1, 7, 11 Hz) (7-H_2)], two methylenes bearing an oxygen function [δ 3.57, 3.88 (ABq, J=10 Hz, 9-H_2); δ 3.84, 3.89 (ABq, J=12 Hz, 8-H_2)], and a methine [δ 2.86 (d, J=7 Hz, 5-H)] together with a ketone carbonyl carbon [δ_C 212.9 (4-C)], two quaternary carbons bearing an oxygen function [δ_C 82.2, 87.6 (1, 2-C)], and another quaternary carbon [δ_C 63.0, (6-C)].

Acetylation of **1** with acetic anhydride (Ac_2O) in pyridine furnished the monoacetate (**1a**), which showed signals assignable to a tertiary methyl [δ 1.42 (s, 10-H_3)], an acetyl [δ 2.10 (s)], two methylenes [δ 2.21 (d, J=11 Hz), 2.50 (dd, J=7, 11 Hz) (7-H_2); 2.54, 2.78 (ABq, J=18 Hz, 3-H_2)], a methine [δ 2.84 (d, J=7 Hz, 5-H)], a methylene bearing an oxygen function [δ 3.70, 3.92 (ABq, J=11 Hz, 9-H_2)], and a methylene bearing an acetoxyl group [δ 4.38, 4.42 (ABq, J=12 Hz, 8-H_2)] in the ^{1}H-NMR ($CDCl_3$) spectrum. On the other hand, acetylation of **1** with Ac_2O in pyridine in the presence of dimethylaminopyridine (DMAP) yielded the diacetate (**1b**), which showed no absorption bands ascribable to a hydroxyl function in the IR spectrum. Comparison of the ^{13}C-NMR data (Table 2) of **1** with those for **1a** and **1b** revealed acylation shifts around the 1 and 8-positions in **1**. The connectivities of the quarternary carbons (C-1, 2, 6) were clarified by heteronuclear multiple bond correlation (HMBC) experiment with **1a**. Thus, long-range correlations were observed between the following carbons and protons of **1a**: 1-C and 3-H_2, 5-H, 8-H_2; 2-C and 3-H_2, 10-H_3; 6-C and 7-H_2, 8-H_2 (Fig. 1).

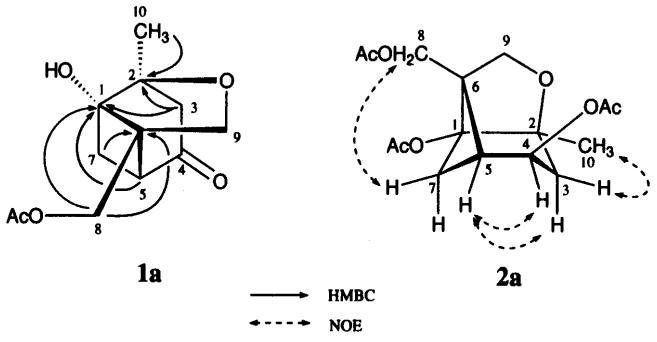
Reduction of **1** with sodium borohydride ($NaBH_4$) in methanol was found to stereoselectively give the 4β-hydroxyl derivative (**2**), which was subjected to acetylation with Ac_2O, pyridine, and DMAP to afford the triacetate (**2a**). The ^{1}H–^{1}H correlation spectroscopy (H–H COSY) experiment on **2** and **2a** indicated the presence of a partial structure (from 3-C to

7-C). In the HMBC experiment, long-range correlations were observed between the following carbons and protons of **2** (1-C and 3-H_2, 5-H, 8-H_2; 2-C and 3-H_2, 10-H_3; 6-C and 4-H, 7-H_2, 8-H_2). Comparison of the ^{1}H-NMR data for **2** and **2a** with those for **1**, **1a**, and **1b** showed the presence of a partial structure from the 3-position to the 7-position. Furthermore, the nuclear Overhauser enhancement and exchange spectroscopy (NOESY) experiment on **2a** showed NOE correlations between the signals of the following proton pairs (3β-H and 10-H_3; 3α-H and 5-H; 4-H and 5-H; 7β-H and 8-H_2) (Fig. 1). On the basis of the above-mentioned evidence and comparison of the NMR data for **1** and **2** with those for paeoniflorin (**6**) and albiflorin (**9**),[7] the relative stereostructures of **1** and **2** were determined to be as shown.

Table 2. ^{13}C-NMR Data for **1**, **1a**, **1b**, **2**, **3a**, **3b**, and **4**

	1[a)]	1[b)]	1a[a)]	1b[a)]	2[b)]	3a[b)]	3b[b)]	4[b)]
C-1	81.7	82.2	81.4	85.6	82.6	79.7	79.7	79.7
C-2	86.1	87.6	86.1	86.0	90.5	45.2	48.1	43.9
C-3	48.6	49.8	48.5	48.9	45.4	107.6	213.0	110.9
C-4	209.9	212.9	209.1	208.2	70.4	45.6	47.9	43.9
C-5	48.5	49.5	48.6	51.0	40.1	32.0	35.7	32.0
C-6	60.4	63.0	59.8	59.0	59.1	102.7	97.3	102.9
C-7	31.2	31.7	31.2	31.9	30.8	44.0	47.3	40.5
C-8	61.2	62.7	69.8	70.5	64.1	67.2	61.2	68.3
C-9	68.8	71.7	63.0	63.1	70.3	101.1	101.0	101.1
C-10	18.9	19.2	19.0	19.8	20.4	22.6	21.9	22.7
$\underline{C}OCH_3$			171.7	171.0				
				169.7				
$CO\underline{C}H_3$			21.0	20.9[c)]				
				21.1[c)]				
$O\underline{C}H_3$								49.3

The spectra were measured *a*) in $CDCl_3$, or *b*) in CD_3OD, and *c*) assignments may be interchangeable.

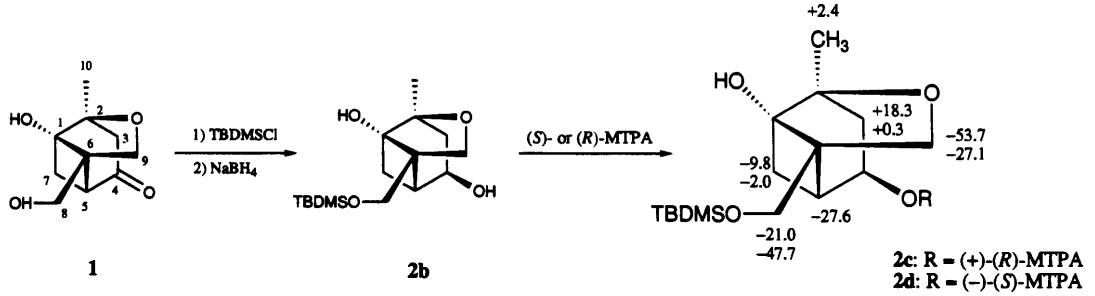


1a **2a**

Fig. 1

The absolute configuration of **1** has been determined by application of a modified Mosher's method.[11] That is, **1** was treated with *t*-butyldimethylsilyl chloride (TBDMS-Cl) and imidazole in dimethylformamide (DMF) to furnish the 8-TBDMS derivative, which was subjected to the stereoselective reduction to provide the alcohol (**2b**). The alcohol (**2b**) was treated with (*R*)- or (*S*)-2-methoxy-2-trifluoromethylphenylacetic (MTPA) acid in the presence of dicyclohexylcarbodiimide (DCC) and DMAP in dichloromethane (CH_2Cl_2) to give the (*R*)-MTPA ester (**2c**) and (*S*)-MTPA ester (**2d**), respectively. As shown in Chart 2, the signals due to protons on the 3 and 10-carbons in the (*R*)-MTPA ester (**2c**) appeared at higher fields than those of the (*S*)-MTPA ester (**2d**) [$\Delta\delta$: positive], whereas the signals due to protons attached to the 5 and 7-carbons of **2c** were observed at lower fields as compared to those of **2d** [$\Delta\delta$: negative]. Consequently, the absolute configuration at the 4-position in **2** has been elucidated to be *R*. On the basis of the above evidence, the absolute structure of a pinane-type monoterpene, **1**, has been characterized as shown.

Compound **3** was also obtained as a white powder with positive optical rotation ($[\alpha]_D^{20}$ −39.7°). The IR spectrum of **3** showed absorption bands ascribable to hydroxyl and ketone functions at 3400, 1719, and 1071 cm^{-1}. The positive-ion FAB-MS of **3** showed a quasimolecular ion peak at *m/z* 215 $(M+H)^+$ and high-resolution MS analysis revealed the molecular formula of **3** to be $C_{10}H_{14}O_5$. However, the ^{1}H-NMR (CD_3OD) and ^{13}C-NMR (Table 2) spectra of **3** showed two pairing signals in *ca.* 3 : 1 ratio. The detailed assignments of the major peaks in the ^{1}H- and ^{13}C-NMR spectra showed the presence of a tertiary methyl [δ 1.14 (s, 10-H_3)], two methylenes [δ 1.77 (d, J=14 Hz), 2.01 (dd, J=1, 14 Hz) (2-H_2); 1.85 (dd, J=1, 13 Hz), 2.13 (dd, J=4, 13 Hz) (5-H_2)], a methylene bearing an oxygen function [δ 3.64 (d, J=8 Hz), 3.96 (dd, J=5, 8 Hz) (8-H_2)], two methines [δ 2.33 (m, 7-H), 2.46 (m, 4-H)], an acetal methine [δ 5.19 (d, J=3 Hz, 9-H)], and two ketal carbons (3, 6-C), whereas the minor signals were found to be superimposable on those for **5**, except for some signals due to the benzoyl group of the latter. Treatment of **3** with silica gel (Merck, 60—230 mesh) in methanol yielded **4**, which showed no absorption due to a ketone function in its IR spectrum. The ^{1}H- and ^{13}C-NMR data of **4** were very similar to the major signals in those of **3**, except for the signals due to the methoxyl group. Finally, debenzoylation of **5** with lipase from *Candida cylindracea* furnished **3**, which was converted to **4** by methanol treatment. On the basis of those findings, **3** was a mixture of the 3-ketal form (**3a**) and



1 **2b**

$\Delta\delta$ values in Hz (= δS–δR, measured in 270 MHz)

Chart 2

3-keto form (**3b**) and the absolute stereostructure of a menthane-type monoterpene, **3**, was clarified as shown.

Experimental

The instruments used for obtaining physical data and the experimental conditions for chromatography were the same as described in our previous paper.[1)]

Extraction and Isolation Moutan Cortex (10.0 kg, cultivated in Anhwei, China and purchased from Koshiro Co. Ltd., Osaka) were finely minced and extracted with MeOH under reflux. Evaporation of the solvent under reduced pressure gave the MeOH extract (1.98 kg). The MeOH extract (1.97 kg) was partitioned in a AcOEt and H_2O (1 : 1) mixture, and removal of the solvent under reduced pressure from the AcOEt- and H_2O-soluble fractions yielded 400 g and 1.55 kg of residues, respectively. The H_2O-soluble portion (960 g) was separated by XAD-2 (Organo Co., Ltd., 2 kg, $H_2O \rightarrow$MeOH) column chromatography to give a H_2O-eluted fraction (781 g) and a MeOH-eluted fraction (179 g). The AcOEt-soluble fraction (200 g) was subjected to repeated ordinary-phase silica gel column chromatography (4 kg, i) *n*-hexane–AcOEt, ii) $CHCl_3$–MeOH–H_2O) to give paeonisuffrone (**1**, 0.0003%), paeonisuffral (**3**, 0.0014%), 3-*O*-methylpaeonisuffral (**4**, 0.0003%), paeoniflorigenone (**5**, 0.0110%), benzoyl-paeoniflorin (**7**, 0.10%), benzoyl-oxypaeoniflorin (**8**, 0.05%), paeonol (**10**, 1.3%), resacetophenone (**11**, 0.0030%), acetovanillone (**12**, 0.0010%), acetoisovanillone (**13**, 0.0030%), 2,5-dihydroxy-4-methylacetophenone (**14**, 0.0020%), and 2,5-dihydroxy-4-methoxyacetophenone (**15**, 0.0010%). Five known compounds (**7**, **8**, **10**, **11**, **12**) were identified by comparison of TLC behavior, ^{1}H- and ^{13}C-NMR spectra with those of authentic samples,[8)] while other known compounds (**5**, **13**, **14**, **15**) were identified by comparison of their physical data with reported values.[6,9)]

Paeonisuffrone (**1**): A white powder, $[\alpha]_D^{20}$ −16.8° (*c*=1.45, MeOH). High-resolution FAB-MS: Calcd for $C_{10}H_{14}O_4Na$ $(M+Na)^+$: 221.0790. Found: 221.0802. IR (KBr): 3426, 1725, 1705, 1597, 1541, 1408 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 1.31 (3H, s, 10-H_3), 2.21 (1H, d, *J*=11 Hz), 2.47 (1H, ddd, *J*=1, 7, 11 Hz) (7-H_2), 2.31 (1H, d, *J*=18 Hz), 2.91 (1H, dd, *J*=1, 18 Hz) (3-H_2), 2.86 (1H, d, *J*=7 Hz, 5-H), 3.57, 3.88 (2H, ABq, *J*=10 Hz, 9-H_2), 3.84, 3.89 (2H, ABq, *J*=12 Hz, 8-H_2); ($CDCl_3$) δ: 1.40 (3H, s, 10-H_2), 2.19 (1H, d, *J*=11 Hz), 2.55 (1H, dd, *J*=7, 11 Hz) (7-H_2), 2.50, 2.75 (2H, ABq, *J*=18 Hz, 3-H_2), 2.66 (1H, d, *J*=7 Hz, 5-H), 3.62, 4.09 (2H, ABq, *J*=10 Hz, 9-H_2), 3.82, 4.07 (2H, ABq, *J*=12 Hz, 8-H_2). ^{13}C-NMR ($CDCl_3$ and CD_3OD) δ_C: given in Table 2. Positive-ion FAB-MS *m/z*: 237 $(M+K)^+$, 221 $(M+Na)^+$. Negative-ion FAB-MS *m/z*: 197 $(M-H)^-$.

Paeonisuffral (**3**): A white powder, $[\alpha]_D^{20}$ +39.7° (*c*=0.6, MeOH). High-resolution FAB-MS: Calcd for $C_{10}H_{14}O_5$ $(M+H)^+$: 215.0920. Found: 215.0898. IR (KBr): 3400, 2936, 1719, 1356, 1071 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: the major signals assignable to the ketal form (**3a**): 1.14 (s, 10-H_3), 1.77 (d, *J*=14 Hz), 2.01 (dd, *J*=1, 14 Hz) (2-H_2), 1.85 (dd, *J*=1, 13 Hz), 2.13 (dd, *J*=4, 13 Hz) (5-H_2), 2.33 (m, 7-H), 2.46 (m, 4-H), 3.64 (d, *J*=8 Hz), 3.96 (dd, *J*=5, 8 Hz) (8-H_2), 5.19 (d, *J*=3 Hz, 9-H); the minor signals assignable to the ketone form (**3b**): 1.22 (s, 10-H_2), 2.01 (m, 7-H), 2.18 (dd, *J*=2, 13 Hz), 2.28 (dd, *J*=3, 13 Hz) (5-H_2), 2.48 (d, *J*=17 Hz), 2.72 (d, *J*=17 Hz) (2-H_2), 2.71 (m, 4-H), 3.29 (dd, *J*=9, 11 Hz), 3.47 (dd, *J*=6, 11 Hz) (8-H_2), 5.37 (br s, 9-H). ^{13}C-NMR (CD_3OD) δ_C: given in Table 2. Positive-ion FAB-MS *m/z*: 215 $(M+H)^+$.

3-*O*-Methylpaeonisuffral (**4**): A white powder, $[\alpha]_D^{20}$ +21.1° (*c*=0.4, MeOH). High-resolution FAB-MS: Calcd for $C_{11}H_{16}O_5Na$ $(M+Na)^+$: 251.0895. Found: 251.0908. IR (KBr): 3419, 2936, 1356, 1073 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 1.15 (3H, s, 10-H_3), 1.76, 2.61 (1H each, both br d, *J*=*ca.* 14 Hz, 2-H_2), 1.76 (1H, d, *J*=13 Hz), 2.07 (1H, dd, *J*=5, 13 Hz) (5-H_2), 2.34 (1H, m, 7-H), 2.70 (1H, m, 4-H), 3.25 (3H, s, OMe), 3.71 (1H, d, *J*=9 Hz), 3.92 (1H, dd, *J*=4, 9 Hz) (8-H_2), 5.21 (1H, d, *J*=3 Hz, 9-H). ^{13}C-NMR (CD_3OD) δ_C: given in Table 2.

Acetylation of 1 with Ac_2O in Pyridine A solution of **1** (20 mg) in pyridine (0.43 ml) was treated with Ac_2O (0.14 ml) and the mixture was stirred at room temperature for 1.5 h. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was washed with 1% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ powder and filtered. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was purified by silica gel column chromatography (0.5 g, *n*-hexane–AcOEt=1 : 3) to give **1a** (1 mg).

1a: A white powder, $[\alpha]_D^{20}$ −48.9° (*c*=0.55, EtOH). High-resolution FAB-MS: Calcd for $C_{12}H_{16}O_5Na$ $(M+Na)^+$: 263.0895. Found: 263.0876. IR (KBr): 3566, 2975, 1732, 1240, 1042 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.42 (3H, s, 10-H_3), 2.10 (3H, s, OAc), 2.21 (1H, d, *J*=11 Hz), 2.50 (1H, dd, *J*=7, 11 Hz) (7-H_2), 2.54, 2.78 (2H, ABq, *J*=18 Hz, 3-H_2), 2.84 (1H, d, *J*=7 Hz, 5-H), 3.70, 3.92 (2H, ABq, *J*=11 Hz, 9-H_2), 4.38, 4.42 (2H, ABq, *J*=12 Hz, 8-H_2). ^{13}C-NMR ($CDCl_3$) δ_C: given in Table 2. Positive-ion FAB-MS *m/z*: 263 $(M+Na)^+$.

Acetylation of 1 with Ac_2O in Pyridine and DMAP A solution of **1** (20 mg) in pyridine (0.28 ml) was treated with Ac_2O (0.10 ml) and DMAP (2 mg) and the mixture was stirred at 40 °C for 12 h. The reaction mixture was poured into ice-water and the whole mixture was extracted with AcOEt. The AcOEt was treated in the usual manner to give a residue, which was purified by silica gel column chromatography (1 g, *n*-hexane–AcOEt) to provide **1b** (19 mg).

1b: A white powder, $[\alpha]_D^{20}$ −9.0° (*c*=0.5, EtOH). High-resolution FAB-MS: Calcd for $C_{14}H_{18}O_6Na$ $(M+Na)^+$: 305.1001. Found: 305.1026. IR (KBr): 2930, 1790, 1744, 1372, 1242, 1134, 1046, 1022 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.36 (3H, s, 10-H_3), 2.09, 2.12 (3H each, both s, OAc×2), 2.54, 2.92 (2H, ABq, *J*= 17 Hz, 3-H_2), 2.76 (2H, s, 7-H_2), 3.00 (1H, m, 5-H), 3.72, 3.92 (2H, ABq, *J*=16 Hz, 9-H_2), 4.36, 4.40 (2H, ABq, *J*=12 Hz, 8-H_2). ^{13}C-NMR ($CDCl_3$) δ_C: given in Table 2. Positive-ion FAB-MS *m/z*: 305 $(M+Na)^+$.

Reduction of 1 with $NaBH_4$ A solution of **1** (13 mg) in EtOH (1.0 ml) was treated with $NaBH_4$ (6 mg) and the mixture was stirred at room temperature for 1.5 h. Excess $NaBH_4$ was quenched with Dowex 50W×8 (H^+ form) and filtered. Removal of the solvent from the filtrate yielded **2** (8 mg).

2: A white powder, $[\alpha]_D^{20}$ −6.2° (*c*=0.33, EtOH). High-resolution FAB-MS: Calcd for $C_{10}H_{16}O_4Na$ $(M+Na)^+$: 223.0946. Found: 223.0927. IR (KBr): 3400, 2930 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 1.22 (3H, s, 10-H_3), 1.54 (1H, d, *J*=10 Hz), 2.03 (1H, dd, *J*=8, 10 Hz) (7-H_2), 1.78 (1H, d, *J*=16 Hz), 2.29 (1H, dd, *J*=8, 16 Hz) (3-H_2), 2.42 (1H, dd, *J*=4, 8 Hz, 5-H), 3.68, 4.62 (2H, ABq, *J*=8 Hz, 9-H_2), 3.80, 3.83 (2H, ABq, *J*=12 Hz, 8-H_2), 4.11 (1H, dd, *J*=4, 8 Hz, 4-H). ^{13}C-NMR (CD_3OD) δ_C: given in Table 2. Positive-ion FAB-MS *m/z*: 223 $(M+Na)^+$.

Acetylation of 2 with Ac_2O in Pyridine and DMAP A solution of **2** (3 mg) in pyridine (0.28 ml) was treated with Ac_2O (0.14 ml) and DMAP (1 mg) and the mixture was stirred at 40 °C for 12 h. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was treated in the usual manner to give a residue, which was purified by silica gel column (0.5 g, *n*-hexane–AcOEt) to furnish **2a** (4.8 mg).

2a: A white powder, $[\alpha]_D^{20}$ −1.5° (*c*=0.3, EtOH). IR(KBr): 2924, 1740, 1250. ^{1}H-NMR ($CDCl_3$) δ: 1.27 (3H, s, 10-H_3), 1.98 (1H, d, *J*=16 Hz, 3α-H), 2.06, 2.07, 2.09 (3H each, all s, OAc×3), 2.23 (1H, d, *J*=12 Hz, 7α-H), 2.43 (1H, dd, *J*=8, 12 Hz, 7β-H), 2.51 (1H, dd, *J*=8, 16 Hz, 3β-H), 2.67 (1H, dd, *J*=4, 8 Hz, 5-H), 3.83, 4.44 (1H each, both d, *J*=9 Hz, 9-H_2), 4.27, 4.39 (2H, ABq, *J*=12 Hz, 8-H_2), 5.12 (1H, dd, *J*=4, 8 Hz, 4-H).

Preparation of 4 from 3 A solution of **3** (10 mg) in MeOH (2 ml) was treated with silica gel (0.5 g, Merck, 60—230 mesh) and the mixture was stirred at room temperature for 12 h. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure to give a residue. The residue was purified by silica gel column chromatography (1 g, $CHCl_3$–MeOH) to give **4** (8 mg), which was identified by comparison of TLC behavior and IR, ^{1}H-NMR, and ^{13}C-NMR spectra with those of natural authentic **4**.

Debenzoylation of 5 Followed by Methanol Treatment A solution of **5** (23.7 mg) in a mixture of phosphate buffer (pH 7.0, 0.5 ml) and acetone (0.5 ml) was treated with 20 mg lipsase (from *Candida cylindracea*, Type VII, Sigma) and the mixture was stirred at 37 °C for 10 h. The reaction mixture was poured into MeOH and filtered. The filtrate was evaporated under reduced pressure to give a residue. The residue was purified by silica gel column chromatography (1 g, $CHCl_3$–MeOH=8 : 1) to give **3** (15.2 mg), which was identical by comparison of TLC behavior and ^{1}H-NMR spectrum with those of natural authentic **3**.

DPPH Radical Scavenging Activity DPPH radical scavenging activities of the MeOH extract and fractions were investigated according to the method of Uchiyama *et al.*[12)] Briefly, a solution of the extract in acetate buffer (pH 5.5, 2.0 ml) and EtOH (2.0 ml) was treated with 2×10^{-4} M DPPH EtOH solution (1.0 ml) and the mixture was incubated at room temperature for 30 min. Reduction of the DPPH radical was measured at 517 nm. Measurements were performed in duplicate, and the concentration required for a 50% reduction (50% scavenging concentration, SC_{50}) of 40 μM DPPH radical was determined graphically. Results are shown in Table 1.

$^{\cdot}O_2^-$ Scavenging Activity The improved assay method for superoxide dismutase described by Imanari *et al.* was used.[13)] Briefly, a reaction mixture containing 100 μM xanthine, 100 μM EDTA, 25 μM NBT, 0.005% bovine

serum albumin, and *ca.* 1.8 mU/ml xanthine oxidase in 33.3 mM sodium carbonate buffer (pH 10.2) was incubated with or without each test sample for 20 min at 25 °C (total volume: 3.0 ml). After incubation, the solution was mixed with 0.1 ml of 6 mM $CuCl_2$ to stop the reaction. The formazan formation was monitored at 560 nm. In addition, inhibitory effect of test samples on xanthine oxidase activity was examined to clarify whether the inhibition of the formazan formation was due to inhibition of xanthine oxidase. The reaction mixture without NBT was incubated in similar conditions described above and 0.1 ml of 2 N HCl was added to stop the reaction. Uric acid formation was monitored at 290 nm. IC_{50} values for the formazan formation and xanthine oxidase activity were determined.

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The Synthesis and Human FP Receptor Binding Affinity of 13,14-Dihydro Prostaglandin $F_{1\alpha}$ Sulfonamides: Potential Treatments for Osteoporosis

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A novel class of saturated prostaglandin $F_{2\alpha}$ sulfonamide analogs have been synthesized and evaluated in the human FP receptor binding assay for potential use in the treatment of osteoporosis. These compounds have been modified at the C_1 carboxylic acid moiety and at the C_{16}—C_{20} region of the prostaglandin. Based on the structure–activity relationships, it was found that at C_1, the aryl sulfonamide analogs possessed greater affinity for the hFP receptor when compared to alkyl sulfonamides. When the sulfonamide was introduced into the C_{16}—C_{20} region (omega chain) of the prostaglandin, a significant reduction in binding was observed. These results are discussed within the framework of a proposed model for the human FP receptor.

Key words osteoporosis; 13,14 dihydro $PGF_{1\alpha}$; sulfonamide; Sulprostone

Prostaglandins of the E-family and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are potent, naturally occurring hormones produced locally upon demand by various stimuli and have been implicated in a myriad of physiological processes, including the growth of new bone in animal models.[1] By design, they are short-lived, and are inactivated rapidly by a variety of chemical and metabolic pathways.[2] As therapeutic agents, their success has been limited primarily due to metabolic instability and a side-effect profile resulting from the indiscriminant binding of individual ligands to multiple receptors. However, with the recent cloning and expression of the individual prostaglandin receptors,[3] the tools are now available to design and synthesize potent prostaglandin agonists and antagonists which may potentially possess enhanced therapeutic benefits based on a greater receptor and/or tissue specificity.

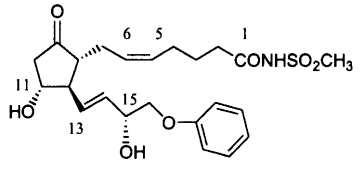
During the course of our work on identifying novel bone anabolic agents for the treatment of osteoporosis, we have recently discovered a family of saturated prostaglandin F ligands, the 13,14-dihydro prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) class, which have proven to be potent and selective agonists for the human FP receptor,[4] and bone anabolic agents *in vivo* in the ovariectomized rat model.[5] In an effort to further our understanding of how prostaglandin structure relates to hFP receptor binding affinity and potential anabolic effect in this series, we explored the use of the sulfonamide moiety in the 13,14 dihydro $PGF_{1\alpha}$ skeleton. This concept is based on previous work in the prostaglandin-E family describing the therapeutic benefits of sulfonamide incorporation; the end result being the fertility control agent Sulprostone (**1**) (Fig. 1).[6] As there is currently no data available on the binding affinity of sulfonamide analogs to any of the individual human prostaglandin receptors,[7] (the earlier Sulprostone work involved animal tissue preparations) we subsequently synthesized a series of 13,14-dihydro $PGF_{1\alpha}$ sulfonamides and screened them at the human FP receptor in a preliminary attempt to better our understanding of the relationship between sulfonamide structure and receptor binding affinity.

Chemistry We focused our efforts for sulfonamide incorporation in two areas of the molecules, the C_1 carboxylic acid of the alpha chain, and the C_{16}—C_{20} region of the omega "tail" of the prostaglandin. For sulfonamide replacement at C_1 we chose the more potent 17-thiophenyl and 17-aminophenyl omega "tails", based on previous work done on the parent carboxylic acid series.[8] Accordingly, the 17-thiophenyl and 17-aminophenyl groups were incorporated into the 13,14-dihydro $PGF_{1\alpha}$ skeleton from the epoxide (**2**) and then converted to the appropriate sulfonamides as outlined in Chart 1.

The epoxide (**2**)[8] was ring-opened with the appropriate thiol or aniline under either basic or Lewis acid conditions[9] to provide, after silylation, the tris-*tert*-butyldimethylsilyl ether (**3**) as a 1 : 1 mixture of diastereomers at C_{15}. The ester was cleaved with base under mild conditions to provide the free acid, which was activated for nucleophilic displacement as the mixed anhydride[10] and then condensed with the appropriate sulfonamide[11] to provide the intermediate (**4**). The final desilylation step was performed with hydrogen fluoride/pyridine to provide the desired sulfonamide (**5**). Careful monitoring of the reaction by TLC was critical in this step, as slow decomposition of the sulfonamide was noted with prolonged reaction times under these conditions. Attempts to separate the C_{15} diastereomers using conventional flash chromatography were unsuccessful and the compounds were tested as the mixture.

For analogs in which the sulfonamide was incorporated in to the C_{16}—C_{20} region (tail), we concentrated on the aryl sulfonamide series, based on the earlier precedent of increased binding affinity for the human FP receptor with the 13,14 dihydro-16-aryl $PGF_{1\alpha}$ skeleton *versus* the aliphatic counterpart.[12] The synthesis of these compounds is outlined in Chart 2.

The epoxide intermediate (**6**) was opened regioselectively with sodium azide and then protected with *tert*-butyldimethylsilyltrifluoromethanesulfonate[13] to give (**7**). The



(1) Sulprostone

Fig. 1

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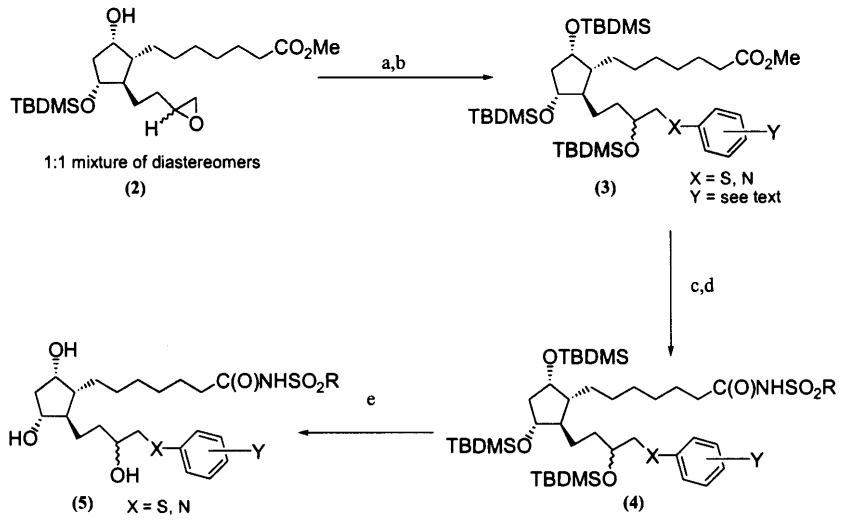
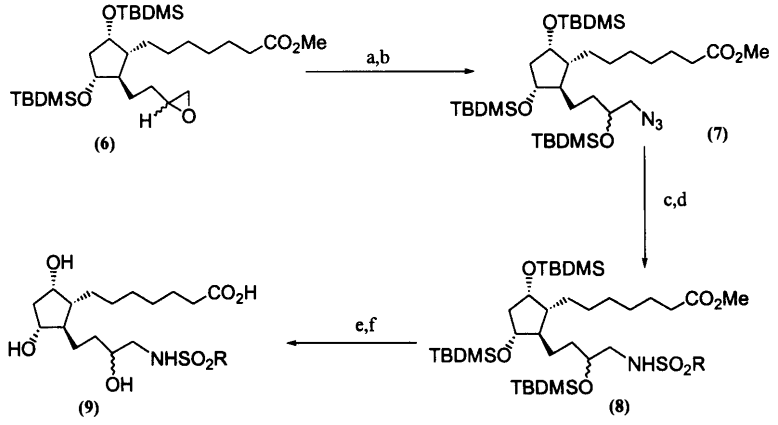


Key:

a) Et_3N, thiophenol or $Mg(ClO_4)_2$, aniline b)TBDMSOTf, 2,6-Lutidine, 75% c) LiOH, THF/H_2O, 74 % d) CDI, THF, DBU, RSO_2NH_2, 35-70% e) HF/pyridine, 30-90 %

Chart 1



Key:

a)NaN_3, $Mg(ClO_4)_2$, CH_3CN, 48% b)TBDMSOTf, 2,6-Lutidine, 75% c)Ph_3P, THF/H_2O, 77 % d) RSO_2Cl, Et_3N, 80% e) HF/pyridine, 90% f) LiOH, THF/H_2O, 10-50 %

Chart 2

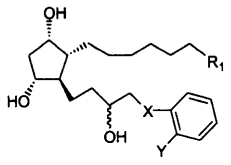
azide was reduced to the amine,[14] and then sulfonylated under standard conditions to provide the protected sulfonamide (**8**). The product was then desilylated to give the triol, which was saponified to provide the omega-chain sulfonamide (**9**).

Results and Discussion

The binding affinity at the human FP receptor for the C_1 sulfonamides is shown in Table 1. The compounds were evaluated for their ability to displace radiolabeled $PGF_{2\alpha}$ in membrane preparations isolated from COS-7 cells transiently transfected with hFP prostaglandin membrane. Data analysis was done on Graph Pad Prism (ver. 3.0) and the resulting IC_{50} values provide a measure of the relative affinity of the compounds for the hFP receptor in the presence of [^{3}H] $PGF_{2\alpha}$ (Fig. 2, see experimental section for details).

Several observations can be made based on the SAR of the sulfonamide substitution at C_1. For alkyl sulfonamides (**10**—

Table 1. Human FP Receptor Binding Data For C_1 Sulfonamides



Compd.	X	Y	R_1	IC_{50} hFP (nM)
1	Sulprostone		$C(O)NHSO_2CH_3$	—
10	S	H	$C(O)NHSO_2CH_3$	1400
11	NH	*o*-F	$C(O)NHSO_2CH_3$	2700
12	S	*o*-F	$C(O)NHSO_2CH_3$	1200
13	S	H	$C(O)NHSO_2CH_2CH_3$	1400
14	S	H	$C(O)NHSO_2CH(CH_3)_2$	1500
15	S	H	$C(O)NHSO_2Ph$	120
16	S	*o*-F	$C(O)NHSO_2Ph$	185
17	NH	*o*-F	$C(O)NHSO_2Ph$	550
18	S	*o*-F	$C(O)NHSO_2$·4-(Me)-Ph	$>10^4$
19	S	*o*-F	$C(O)NHSO_2$·4-(F)-Ph	1600

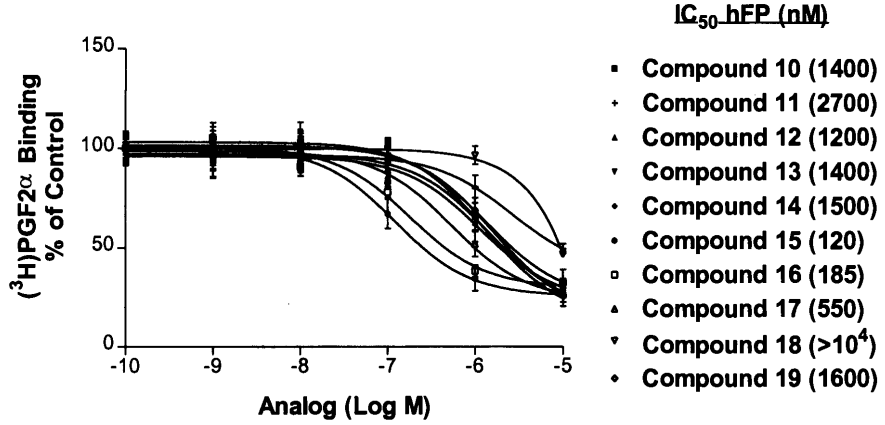


Fig. 2. Binding Affinity of Compounds **10**—**19** at the human FP Receptor

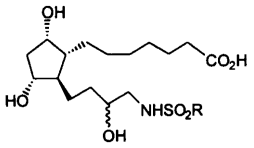
14), there seems to be only a slight apparent difference in binding affinity for the human FP receptor as the size of the alkyl group increases. When the alkyl group is replaced with an aryl group, however, a 10× increase in binding affinity is seen (**10** *vs.* **15**). This result suggests that the steric environment at the C_1 pocket in the human FP receptor can accommodate rather bulky substituents, and that binding affinity may be a function more of differences in sulfonamide acidity at C_1 (aromatic *vs.* aliphatic) rather than in the steric environment at the receptor binding site. This trend seemed to follow in comparing compounds (**11**) *vs.* (**17**) and (**12**) *vs.* (**16**). It is also interesting to note the loss of binding affinity in compound (**18**) (*p*-tolyl), in which the additional steric bulk of the *p*-methyl group may influence binding to a much greater degree, especially when compared to the analog (**19**) in which a fluorine atom (isosteric with H) is substituted at the same position. The phenyl sulfonamides (**15**) and (**16**) showed the best potency of all the compounds tested at 120 and 185 nM, respectively.

In the examples in which the sulfonamide was incorporated into the omega-chain of the prostaglandin, we saw a significant loss in binding affinity to the human FP receptor in all cases (Table 2). These results may be rationalized in terms of a putative human FP receptor model[8] in which the omega-tail of the ligand is residing in a hydrophobic cleft in the receptor populated with aromatic amino acid residues, resulting in a greater binding affinity for 13,14-dihydro $PGF_{1\alpha}$ analogs which possess aromatic rings in the "tail" position. In the case of ligands **20**—**24**, the incorporation of the more polar, hydrophilic sulfonamide moiety may result in a perturbation of binding at this site and loss of binding affinity.

Conclusions

We have designed and synthesized a novel series of sulfonamide analogs of the 13,14 dihydro $PGF_{1\alpha}$ skeleton and have evaluated them for the first time in a human FP receptor binding assay. We have found that when the sulfonamide substitution is at C_1, aryl sulfonamides provided better binding (nanomolar) than the alkyl counterparts (micromolar), suggesting that the steric environment of the C_1 pocket in the hFP receptor can accommodate rather large substituents, and that the p*K*a of the C_1 substituent may play a critical role in binding of the ligand to the receptor. In addition, we have also found that when the sulfonamide is incorporated into the C_{16}—C_{20} region (omega-tail) of the 13,14-dihydro $PGF_{1\alpha}$ prostaglandin skeleton, there is a substantial loss of binding at the human FP receptor, which is in keeping with our previously proposed receptor binding model for ligands of this type. As pharmacokinetics and metabolism have dramatic effects on prostaglandin efficacy *in vivo*, we are continuing to evaluate the potential stability and selectivity advantages of the most potent of these sulfonamide compounds in the ovariectomized rat model (OVX); the results of which will be reported in due course.

Table 2. Human FP Receptor Binding Data For Omega-Chain Sulfonamides



Compound	R	IC_{50} hFP (nM)
20	Ph	3800
21	*m*-F Ph	6700
22	2,4-difluoro Ph	$>10^4$
23	*p*-F Ph	$>10^4$
24	*o*-F Ph	$>10^4$

Experimental

General Methods: ^{1}H-NMR spectra were recorded on a Varian Unity Plus 300 MHz spectrometer and are referenced to either the deuteriochloroform singlet at 7.27 ppm or deuteriomethanol singlet at 4.87 ppm. ^{13}C spectra were obtained on a Varian Unity Plus 300 MHz spectrometer and are referenced at either the center line of the deuteriochloroform triplet at 77.0 ppm or the deuteriomethanol heptet at 49.15 ppm. Infrared absorption spectra were obtained on a Perkin-Elmer Model 197 spectrophotometer and are referenced to polystyrene (1601 cm^{-1}). Mass spectra were obtained on either a Fison Platform-II Quadrupole Mass Spectrometer or a Fison Trio2000 Quadrupole Mass Spectrometer. High Resolution Mass Spectra were obtained from the Procter & Gamble CRD Mass Spectrometry Lab (M. Lacey) or at the Nebraska Center for Mass Spectrometry. Elemental analysis were obtained from the Procter & Gamble EA Lab in Norwich, NY. Melting points were determined in open Pyrex capillary tubes on a Thomas-Hover Unimelt apparatus. Melting points and boiling points are uncorrected. All solvents were purchased anhydrous (Aldrich Chemical) and used without further purification. All air-sensitive reactions were performed under an anhydrous nitrogen atmosphere. Flash chromatography was performed on silica gel (70—230 mesh; Aldrich) or (230—400 mesh; Merck) as appropriate. Thin layer chromatography analysis was performed on glass mounted silica gel plates (200—300 mesh; Baker) and visualized using UV, 5% phosphomolybdic acid in EtOH, or ammonium molybdate/cerric sulfate in 10% aqueous H_2SO_4.

Radioligand Binding Assay COS-7 cells were transiently transfected with a hFP recombinant plasmid using LipofectAMINE Reagent. Forty-eight hours later, the transfected cells were washed with Hanks' Balanced Salt Solution (HBSS, without $CaCl_2$, $MgCl_2$, $MgSO_4$, or phenol red). The cells were detached with versene, and HBSS was added. The mixture was centrifuged at 200 ***g*** for 10 min, at 4 °C to pellet the cells. The pellet was re-suspended in phosphate-buffered saline–EDTA buffer (PBS; 1 mM EDTA; pH 7.4; 4 °C). The cells were disrupted by nitrogen cavitation (Parr model 4639), at 800 psi, for 15 min at 4 °C. The mixture was centrifuged at 1000 ***g*** for 10 min at 4 °C. The supernatant was centrifuged at 100000 ***g*** for 60 min at 4 °C. The pellet was resuspended to 1 mg protein/ml TME buffer (50 mM Tris; 10 mM $MgCl_2$; 1 mM EDTA; pH 6.0; 4 °C) based on protein levels measured using the Pierce BCA Protein Assay kit. The homogenate was mixed using a Kinematica Polytron for 10 s. The membrane preparations were then stored at −80 °C, until thawed for assay use.

The receptor competition binding assays were developed in a 96 well format. Each well (n=3) contained 100 μg of hFP membrane, 5 nM [^{3}H] $PGF_{2\alpha}$, and the various competing compounds in a total volume of 200 μl. The plates were incubated at 23 °C for 1 h. The incubation was terminated by rapid filtration using the Packard Filtermate 196 harvester through Packard UniFilter GF/B filters that were pre-wetted with TME buffer. The filter was washed four times with TME buffer. Packard Microscint 20, a high efficiency liquid scintillation cocktail, was added to the filter plate wells and the plates remained at room temperature for three hours prior to counting. The plates were read on the Packard TopCount Microplate Scintillation Counter.

General Procedure for Compounds 10—19: Synthesis of *N*-(7-{2-[4-(2-Fluoro-phenylsulfanyl)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl}-heptanoyl)-methanesulfonamide (12) To a solution of the tris-protected starting material (**3**) (X=S, Y=*o*-F) (1.89 g, 2.4 mol) in THF (15 ml) and H_2O (5 ml) at room temperature was added LiOH (1.5 eq) and the resulting mixture was stirred overnight. The reaction mixture was poured onto saturated citric acid solution, and the layers were separated. The aqueous layer was extracted with CH_2Cl_2 (3×50 ml), and the organic layers were combined. The combined CH_2Cl_2 extracts were washed with saturated NaCl, dried with Na_2SO_4, concentrated, and purified by flash chromatography (95% CH_2Cl_2/MeOH) to afford 1.35 g (74%) of the free acid. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 0.05—0.10 (m, 18H), 0.90—0.93 (m, 27H), 1.14—1.83 (m, 17H), 2.10—2.19 (m, 1H), 2.31—2.37 (t, 2H, J=10.5 Hz), 2.94—3.08 (m, 2H), 3.80—3.84 (m, 2H), 4.08—4.09 (m, 1H), 7.03—7.09 (m, 2H), 7.18—7.25 (m, 1H), 7.39—7.44 (dt, 1H, J=1.5, 7.5 Hz); MS (+ES) *m/z* (relative intensity): 771 ($M+H^+$, 50), 788 ($M+NH_4^+$, 100).

A solution of the acid (X=S, Y=*o*-F) (0.3 mmol) in dry THF (3 ml) was added dropwise to as stirred solution of carbonyldiimidazole (0.33 mmol) in dry THF (2 ml) under nitrogen. The reaction was stirred 30 min at room temperature, refluxed 30 min, and then allowed to cool to room temperature again. The methanesulfonamide (0.3 mmol) was added in one portion and the reaction was stirred for 10 min before a solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (0.3 mmol) in dry THF (2 ml) was added dropwise. The reaction was stirred overnight, then poured into 1 N HCl (50 ml). The aqueous layer was extracted with CH_2Cl_2 (3×50 ml), and the organic layers were combined. The organic layer was then washed (sat. $NaHCO_3$, then sat. NaCl), dried with Na_2SO_4 and concentrated. Purification *via* flash chromatography on SiO_2 (0.4%MeOH/CH_2Cl_2) gave 81 mg (32%) of product (**4**) (X=S, Y=*o*-F, R=CH_3) as an oil. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 0.03—0.06 (m, 18H), 0.88—0.89 (m, 27H), 1.23—1.76 (m, 17H), 2.03—2.16 (m, 1H), 2.31—2.26 (t, J=7.5 MHz, 2H), 2.92—3.05 (m, 2H), 3.31 (s, 3H), 3.76—3.78 (m, 2H), 4.05—4.06 (m, 1H), 7.03—7.11 (m, 2H), 7.18—7.29 (m, 1H), 7.37—7.42 (dt, J=1.5, 7.8 MHz, 1H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz) δ: −4.8, −4.5, −4.4, −4.3, −4.2, −3.9, −3.8, −3.7, 18.1, 18.2, 24.7, 26.1, 26.6, 25.7, 27.6, 28.0, 29.4, 30.1, 33.4, 33.5, 36.7, 40.6, 41.7, 44.7, 48.1, 50.1, 50.3, 72.0, 72.1, 72.3, 76.9, 76.9, 77.3, 77.7, 115.7, 116.0, 123.9, 124.1, 124.6, 124.7, 128.3, 128.4, 132.2, 132.3, 159.9, 161.0, 163.2, 167.2, 172.5; ^{19}F-NMR ($CDCl_3$), 57.5, 57.5, 57.5, 57.582, 57.617; MS (+ES) *m/z* (relative intensity): 848 ($M+H^+$, 5), 865 ($M+NH_4^+$, 100).

A solution of protected sulfonamide (**4**) (0.197 mmol, 167 mg) in CH_3CN (2 ml) was treated with 0.3 ml HF/pyridine solution at 0 °C under nitrogen. The reaction was stirred at 0 °C for 5 h, then another 0.15 ml of HF/pyridine was added. The reaction was stirred at 0 °C for an additional 3 h. The reaction mixture was poured onto sat. $NaHCO_3$/CH_2Cl_2 solution, extracted with CH_2Cl_2 (3×50 ml), combined, and washed with sat. NaCl. After drying over Na_2SO_4 and concentrating, the residue was chromatographed on silica gel (95% CH_2Cl_2/MeOH) to give 75 mg (78%) of the final product, *N*-(7-{2-[4-(2-fluoro-phenylsulfanyl)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl}-heptanoyl)-methanesulfonamide (**12**). ^{1}H-NMR ($CDCl_3$, 300 MHz) 1.33—1.92 (m, 21H), 2.29—2.34 (t, 2H), 2.86—2.93 (dd, J=8.2, 13.5 Hz, 1H), 3.07—3.12 (dd, J=3.3, 13.2 Hz, 1H), 3.27 (s, 3H), 3.68—3.69 (m, 1H), 3.96 (s, 1H), 4.16 (s, 1H), 7.03—7.13 (m, 2H), 7.21—7.26 (m, 1H), 7.40—7.46 (dt, J=1.8, 7.8 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz) 24.5, 28.0, 28.7, 28.9, 29.5, 29.6, 30.12, 34.3, 34.5, 36.67, 41.6, 41.8, 42.7, 51.6, 52.6, 52.9, 70.1, 70.4, 74.6, 78.4, 78.7, 115.9, 116.2, 122.3, 122.5, 129.2, 129.3, 133.1, 160.2, 163.5, 173.5; ^{19}F-NMR ($CDCl_3$,) 57.9, 57.9, 57.8, 57.8, 57.8; MS (+AP) *m/z* (relative intensity) 505 ($M+H^+$, 5), 528 (M+Na, 20); HRMS Calcd for $C_{23}H_{36}O_6S_2NF$ ($M+H^+$): 506.2046. Found: 506.2052. Compounds **10**, **11** and **13**—**19** were prepared in a similar fashion:

N-(7-{2-[4-(2-Fluoro-phenylsulfanyl)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl}-heptanoyl)-benzenesulfonamide (**16**): 73% ^{1}H-NMR ($CDCl_3$, 300 MHz) 1.21—1.87 (m, 22H), 2.19 (t, 2H, J=7.2 Hz), 2.86—2.93 (dd, 1H, J=8.1, 13.2 Hz), 3.69—3.70 (m, 1H), 3.97 (s, 1H), 4.16 (s, 1H), 7.02—7.11 (m, 2H), 7.10—7.26 (m, 1H), 7.02—7.11 (m, 2H), 7.19—7.26 (m, 1H), 7.39—7.44 (dt, J=1.8, 7.8 Hz), 7.48—7.51 (dd, J=7.2, 7.8 Hz, 2H), 7.58—7.63 (m, 1H), 8.03—8.06 (d, J=7.2 Hz, 2H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz) δ: 24.4, 28.0, 28.6, 28.9, 29.4, 29.7, 30.2, 34.4, 34.5, 36.4, 41.6, 41.8, 42.7, 51.7, 52.6, 52.9, 70.1, 70.5, 74.6, 78.4, 78.7, 115.9, 116.2, 122.3, 122.5, 124.9, 128.4, 129.1, 133.1, 133.9, 139.1, 160.2, 163.5, 172.3; ^{19}F-NMR ($CDCl_3$) 57.9; MS (+AP) *m/z* (relative intensity): 567 ($M+H^+$, 20), 590 ($M+Na^+$, 40); HRMS Calcd for $C_{28}H_{38}O_6S_2NF$ ($M+Na^+$): 590.2022. Found: 590.2008.

N-(7-[3,5-Dihydroxy-2-(3-hydroxy-4-phenylsulfanyl-butyl)-cyclopentyl]-heptanoyl}-methanesulfonamide (**10**): 62% ^{1}H-NMR ($CDCl_3$, 300 MHz) 1.28—1.88 (m, 18H), 2.31—2.36 (t, J=7.5 Hz, 2H), 2.90—2.96 (dd, 1H, J=8.4 Hz, 13.5 MHz), 3.12—3.17 (dd, 1H, J=2.7, 13.5 Hz), 3.27 (s, 3H), 3.67—3.79 (m, 1H), 3.99 (s, 1H), 4.18 (s, 1H), 7.22—7.24 (m, 1H), 7.28—7.33 (ddd, 2H, J=2.1, 6.9, 7.8 Hz), 7.38—7.41 (dd, 2H, J=1.8, 6.9 Hz); ^{13}C-NMR ($CDCl_3$, 75.5 MHz) δ: 24.5, 27.9, 28.7, 28.9, 29.4, 29.7, 30.3, 34.5, 34.6, 36.6, 41.6, 41.9, 42.3, 42.5, 42.7 51.8, 51.9 52.7, 53.0, 69.9, 70.3, 74.7, 78.4, 78.7, 126.1, 129.3, 130.1, 135.6, 173.4; MS (+ES) *m/z* (relative intensity): 470 (M^+-H_2O, 100), 488 ($M+H^+$, 40), 505 ($M+NH_4^+$, 50), 510 ($M+Na^+$, 30); HRMS Calcd for $C_{23}H_{37}O_6S_2N$ ($M+Na^+$): 510.1960. Found: 510.1985.

N-(7-[3,5-Dihydroxy-2-(3-hydroxy-4-phenylsulfanyl-butyl)-cyclopentyl]-heptanoyl}-benzenesulfonamide (**15**): 44% ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.23—1.89 (m, 27H), 2.20—2.25 (t, 2H, J=7.5 Hz), 2.89—2.97 (dd, 1H, J=8.4, 13.5 Hz), 3.11—3.17 (dd, 1H, J=3.3, 13.5 Hz), 3.74 (m, 1H), 3.99 (br s, 1H), 4.17 (br s, 1H), 7.18—7.22 (dd, 1H, J=7.2, 7.2 Hz), 7.26—7.31 (dd, 2H, J=7.2, 7.5 Hz), 7.36—7.39 (d, 2H, J=7.5 Hz), 7.49—7.54 (dd, 2H, J=7.5, 7.8 Hz), 7.59—7.64 (dd, 1H, J=7.2, 7.5 Hz), 8.05—8.07 (d, 2H, J=7.5 Hz), 9.99 (br s, 1H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz) δ: 24.4, 28.049, 29.0, 29.8, 30.3, 34.5, 34.6, 36.4, 41.9, 42.2, 42.6, 42.7, 51.7, 51.8, 52.8, 53.0, 69.9, 70.3, 74.7, 78.5, 78.8, 126.8, 128.5, 129.2, 129.3, 130.0, 134.0, 135.6, 139.1, 171.9; MS (+ES) *m/z* (relative intensity): 567 ($M+NH_4^+$, 30); HRMS Calcd for $C_{28}H_{39}O_6S_2N$ ($M+Na^+$): 572.2117. Found: 572.2138.

N-(7-{2-[4-(2-Fluoro-phenylsulfanyl)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl}-heptanoyl)-4-methyl-benzenesulfonamide (**18**): 90% ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.21—1.87 (m, 22H), 2.18—2.23 (t, 2H, J=6.9 Hz), 2.39 (s, 3H), 2.87—2.94 (dd, 1H, J=8.1, 13.2 Hz), 3.05—3.11 (dd, 1H, J=3.6, 13.2 Hz), 3.69 (br s, 1H), 3.97 (br s, 1H), 4.14 (br s, 1H), 7.01—7.09 (m, 2H), 7.18—7.25 (m, 1H), 7.27—7.30 (d, 2H, J=8.1 Hz), 7.38—7.43 (t, 1H, J=7.5 Hz), 7.90—7.93 (d, 2H, J=8.1 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 21.9, 24.4, 28.1, 28.8, 28.9, 29.5, 30.1, 34.4, 36.4, 41.7, 42.6, 51.3, 52.6, 70.5, 74.5, 76.9, 77.4, 77.8, 78.5, 115.9, 116.2, 122.5, 122.7, 124.9, 125.0, 128.5, 129.1, 129.2, 129.8, 132.9, 136.2, 145.0, 160.2, 163.4, 172.3; MS (+ES) *m/z* (relative intensity): 546 (M^+-2H_2O, 40), 564 (M^+-H_2O, 90), 582 ($M+H^+$, 90), 599 ($M+NH_4^+$, 100); HRMS Calcd for $C_{29}H_{40}NO_6FS_2$ ($M+Na^+$): 604.2179. Found: 604.2186.

N-(7-{2-[4-(2-Fluoro-phenylsulfanyl)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl}-heptanoyl)-4-fluoro-benzenesulfonamide (**19**): 79% ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.21—1.87 (m, 22H), 2.20—2.25 (t, 2H, J=7.2 Hz), 2.87—2.94 (dd, 1H, J=7.8, 13.5 Hz), 3.06—3.12 (dd, 1H, J=3.6, 13.5 Hz), 3.70 (m, 1H), 3.98 (br s, 1H), 4.15 (br s, 1H), 7.01—7.09 (m, 2H), 7.15—7.26 (m, 3H), 7.39—7.43 (t, 1H, J=7.5 Hz), 8.05—8.10 (m, 2H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz) δ: 24.4, 28.1, 28.7, 28.9, 29.5, 30.1, 34.3, 36.4, 41.8, 42.6, 51.6, 52.6, 70.5, 74.6, 78.4, 115.9, 116.2, 116.3, 116.6, 122.4, 122.6, 124.9, 125.0, 129.2, 129.3, 131.5, 131.6, 132.9, 135.1, 160.2, 163.5, 164.2, 167.6, 172.3; MS (+ES) *m/z* (relative intensity): 550 (M^+-2H_2O, 25), 568 (M^+-H_2O, 50), 586 ($M+H^+$, 50), 603 ($M+NH_4^+$, 55), 608 ($M+Na^+$, 20); HRMS Calcd for $C_{28}H_{37}NO_6F_2S_2$ ($M+Na^+$): 608.1928. Found: 608.1947.

Propane-2-sulfonic acid {7-[3,5-dihydroxy-2-(3-hydroxy-4-phenylsulphanyl-butyl)-cyclopentyl]-heptanoyl}-amide (**14**): 69% ^{1}H-NMR ($CDCl_3$,

300 MHz) δ: 1.33—1.86 (m, 22H), 1.38 (S, 3H), 1.40 (S, 3H), 2.29—2.35 (t, 2H, J=7.2 Hz), 2.89—2.96 (dd, 1H, J=8.1, 13.5 Hz), 3.09—3.14 (dd, 1H, J=3.6, 13.5 Hz), 3.67—3.83 (m, 2H), 3.96 (bs, 1H), 4.15 (bs, 1H), 7.17 (m, 1H), 7.26—7.31 (overlapped dd, 2H, J=6.9, 8.1 Hz), 7.36—7.38 (d, 2H, J=7.5 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 16.1, 24.7, 28.1, 28.9, 29.0, 29.6, 29.7, 30.2, 34.5, 34.6, 36.7, 41.9, 42.1, 42.6, 42.7, 51.7, 52.7, 52.9, 53.8, 69.9, 70.3, 74.6, 78.5, 78.7, 126.7, 129.3, 129.9, 135.8, 173.3; MS (+AP) m/z (relative intensity): 462 (M^+-3H_2O, 50), 480 (M^+-2H_2O, 100), 497 (M^+-H_2O, 15), 515 ($M+H^+$, 20), HRMS Calcd for $C_{25}H_{41}NO_6S_2$ ($M+Na^+$): 538.2273. Found: 538.2268.

Ethanesulfonic acid {7-[3,5-dihydroxy-2-(3-hydroxy-4-phenylsulphanyl-butyl)-cyclopentyl]-heptanoyl}-amide (**13**): 82% ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.32—1.37 (t, 3H, J=7.2 Hz), 1.43—1.85 (m, 22H), 2.29—2.33 (t, 2H, J=6.9 Hz), 2.89—2.96 (dd, 1H, J=8.1, 13.5 Hz), 3.09—3.14 (dd, 1H, J=3.9, 13.5 Hz), 3.38—3.47 (q, 2H, J=7.2 Hz), 3.72—3.73 (m, 1H), 3.95 (bs, 1H), 4.14 (bs, 1H), 7.17—7.21 (dd, 1H, J=6.9, 7.5 Hz), 7.26—7.31 (overlapped dd, 2H, J=7.2, 7.8 Hz), 7.35—7.37 (d, 2H, J=7.5 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 8.1, 24.7, 28.1, 28.9, 29.0, 29.6, 29.7, 30.2, 34.5, 34.6, 36.6, 41.2, 42.1, 42.6, 42.7, 47.9, 51.6, 52.6, 52.9, 70.0, 70.3, 74.6, 78.4, 78.7, 126.7, 129.3, 129.9, 135.8, 173.4; MS (+AP) m/z (relative intensity): 448 (M^+-3H_2O, 40), 466 (M^+-2H_2O, 100), 524 ($M+Na^+$, 15); HRMS Calcd for $C_{24}H_{39}NO_6S_2$ ($M+Na^+$): 524.2117. Found: 524.2122.

N-(7-{2-[4-(2-Fluoro-phenylamino)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl}-heptanoyl)-benzenesulfonamide (**17**): 69% ^{1}H-NMR (CD_3OD, 300 MHz) δ: 1.17—1.79 (m, 22H), 2.07—2.14 (m, 1H), 2.16—2.27 (t, 2H, J=7.8 Hz), 3.03—3.10 (dd, 1H, J=7.5, 12.9 Hz), 3.24—3.29 (dd, 1H, J= 4.2, 12.9 Hz), 3.76—3.89 (m, 2H), 4.08 (br s, 1H), 6.59 (m, 1H), 6.74—6.80 (overlapped dd, 1H, J=8.4, 8.8 Hz), 6.90—7.00 (m, 2H), 7.56—7.66 (overlapped dd, 2H, J=7.5, 8.1 Hz), 7.68—7.71 (m, 1H), 8.00—8.03 (dd, 2H, J=1.2, 7.2 Hz); ^{13}C-NMR (CD_3OD, 75 MHz) δ: 25.7, 28.9, 29.3, 29.4, 29.9, 30.0, 30.1, 30.7, 33.9, 34.1, 37.1, 43.9, 44.0, 50.6, 50.7, 51.1, 51.2, 52.4, 52.6, 71.3, 71.5, 73.6, 78.5, 113.6, 115.2, 115.4, 117.5, 117.6, 125.7, 129.1, 130.0, 134.8, 138.2, 138.5, 140.9, 174.0; MS (+AP) m/z (relative intensity): 551 ($M+H^+$, 100); HRMS Calcd for $C_{28}H_{39}O_6SN_2F$ ($M+H^+$): 551.2591. Found: 551.2581.

N-(7-{2-[4-(2-Fluoro-phenylamino)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl}-heptanoyl)-methanesulfonamide (**11**): 31% ^{1}H-NMR (CD_3OD, 300 MHz) δ: 1.37—1.79 (m, 22H), 2.09—2.18 (m, 1H), 2.29—2.34 (t, 2H, J=7.5 Hz), 3.04—3.11 (m, 1H), 3.19—3.33 (m, 1H), 3.24 (s, 3H), 3.79—3.88 (m, 2H), 4.11 (m, 1H), 6.57—6.64 (m, 1H), 6.76—6.82 (ddd, 1H, J=1.5, 7.8, 9 Hz), 6.92—7.01 (m, 2H); ^{13}C-NMR (CD_3OD, 75 MHz) δ: 25.7, 29.0, 29.4, 29.9, 30.1, 30.7, 33.9, 34.1, 37.2, 41.3, 43.9, 50.6, 50.7, 51.1, 51.2, 52.4, 52.6, 71.3, 71.5, 73.6, 78.5, 113.6, 115.1, 115.4, 117.5, 125.7, 151.6, 154.7, 175.3; MS (+AP) m/z (relative intensity): 489 ($M+H^+$, 100); HRMS Calcd for $C_{23}H_{37}O_6SN_2F$ ($M+H^+$): 489.2439. Found: 489.2450.

General Procedure for Compounds 20—24: Synthesis of 7-[2-(4-Benzenesulfonylamino-3-hydroxy-butyl)-3,5-dihydroxy-cylopentyl] heptanoic Acid (20) A solution of the epoxide (**6**) (0.39 mmol) and $Mg(ClO_4)_2$ (0.58 mmol) in CH_3CN (1 ml) was stirred until complete dissolution of the perchlorate salt was achieved. The resulting solution was treated, under N_2 with stirring, with the required amount of NaN_3 (0.58 mmol), at room temperature. The reaction was heated to 80 °C for 5 h and then cooled to room temperature. The reaction was diluted with water, extracted with Et_2O (3×30 ml), and the organic layers were combined. The organic layer was washed with sat. NaCl, dried (Na_2SO_4) and concentrated. After column chromatography on SiO_2 (10% EtOAc/Hexane), 108 mg (48%) of azide was recovered as a yellow oil. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 0.02—0.08 (m, 12H), 0.88—0.89 (m, 18H), 1.20—1.80 (m, 18H), 2.09—2.19 (m, 1H), 2.29—2.34 (t, 2H, J=7.5 Hz), 3.23—3.39 (m, 2H), 3.68 (s, 3H), 3.74—3.81 (m, 2H), 4.04—4.07 (m, 1H); MS (+ES) m/z (relative intensity): 586 ($M+H^+$, 75), 603 ($M+NH_4^+$, 100). The azide was protected under standard conditions[12] to give product (**7**) before being reduced to the free amine.

To the solution of the azide (**7**) (0.16 mmol) in THF (3 ml), was added 3 drops of water. While stirring at room temperature, 84 mg triphenylphosphine (0.32 mmol) was added. The reaction was allowed to stir at room temperature under N_2 overnight. The solvent was then removed under vacuum and the residue chromatographed on SiO_2 (25% EtOAc/hexanes) to obtain 83 mg (77%) of the amine product as a yellow oil. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 0.02—0.08 (m, 18H), 0.87—0.91 (m, 27H), 1.09—1.73 (m, 19H), 2.05—2.16 (m, 1H), 2.28—2.33 (t, 2H, J=7.8 Hz), 2.65—2.74 (m, 2H), 3.57—3.58 (m, 1H), 3.67 (s, 3H), 3.71—3.78 (m, 1H), 4.03—4.07 (m, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: −4.5, 44.1, −3.9, −3.8, 18.1, 18.3, 25.2, 26.1, 27.1, 27.3, 27.6, 28.1, 29.5, 29.9, 32.1, 32.3, 34.3, 44.8, 47.9, 48.2, 50.1, 50.4, 51.6, 71.9, 74.3, 76.9, 77.1, 77.2, 77.3, 77.7; MS (+ES) m/z (relative intensity): 674 ($M+H^+$, 100).

A solution of the amine (0.18 mmol) in CH_2Cl_2 (2 ml) was cooled to 0 °C, and triethylamine (56 μl, 0.40 mmol) and benzenesulfonyl chloride (25 μl, 0.2 mmol) were added while stirring. The reaction was slowly warmed to room temperature and stirred overnight. Methylene chloride (20 ml) was added to the reaction mixture, and the solution was washed with sat. NaCl, and dried (Na_2SO_4). The solvent was concentrated and the resulting oil chromatographed on SiO_2 (30% EtOAc/hexanes) to provide 113 mg (77%) of the product (**8**). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: −0.04—0.04 (m, 18H), 0.83—0.89 (m, 27H), 1.09—1.78 (m, 18H), 2.06—2.15 (m ,1H), 2.29—2.34 (t, 2H, J=7.5 Hz), 2.91—2.96 (m, 2H), 3.67 (s, 3H), 3.69—3.70 (m, 1H), 4.04 (m, 1H), 4.65—4.68 (m, 1H), 7.50—7.62 (m, 3H), 7.85—7.87 (d, 1H, J=7.2 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: −4.5, −4.3, −3.9, −3.8, −3.7, 18.1, 18.2, 18.3, 25.2, 26.0, 26.1, 26.9, 27.6, 28.0, 29.5, 29.9, 32.2, 32.3, 34.3, 44.7, 44.8, 48.2, 48.5, 49.9, 50.2, 51.7, 71.4, 71.7, 71.9, 76.9, 77.1, 77.3, 77.7, 127.3, 129.3, 132.8, 139.9, 174.6.

A solution of the protected sulfonamide (**8**) (0.14 mmol) in CH_3CN (1.5 ml) was cooled to 0 °C, and 0.14 ml (0.14 mmol) of HF/pyridine was added dropwise at 0 °C. The reaction was stirred at 0 °C overnight under N_2, then poured onto sat. $NaHCO_3$ solution. The solution was extracted with CH_2Cl_2 (3×50 ml), and the organic layers were combined and washed with sat. NaCl. The organic layer was dried (Na_2SO_4), concentrated, and chromatographed (4% MeOH/CH_2Cl_2) to yield 57 mg (88%) of product. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.15—1.81 (m, 20H), 1.90—1.91 (m, 1H), 2.279—2.329 (t, 2H, J=7.8 Hz), 2.81—2.88 (m, 1H), 3.01—3.08 (m, 1H), 3.66 (s, 3H), 3.69 (m, 1H), 3.91 (m, 1H), 4.13 (m, 1H), 5.98—6.05 (m, 1H), 7.48—7.59 (m, 3H), 7.84—7.88 (d, 2H, J=6.9 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 25.1, 28.2, 28.6, 28.7, 29.2, 29.7, 29.8, 32.6, 33.1, 34.3, 42.9, 48.9, 51.6, 51.8, 52.3, 70.6, 70.8, 74.0, 74.2, 78.1, 78.7, 127.2, 129.4, 132.9, 140.0, 174.8; MS (+ES) m/z (relative intensity): 436 ($M-2H_2O$, 60), 454 ($M-H_2O$, 20), 472 ($M+H^+$, 100).

A solution of the sulfonamide methyl ester (0.12 mmol) in THF (1.5 ml) and H_2O (0.5 ml) was treated with LiOH (20 mg, 0.48 mmol) at room temperature overnight under N_2. The solvent was removed under vacuum and the residue was chromatographed (10% MeOH/CH_2Cl_2) to give 7 mg (13%) of final product (**20**). ^{1}H-NMR (CD_3OD, 300 MHz) δ: 1.23—1.71 (m, 22H), 2.06—2.15 (m, 1H), 2.29—2.34 (t, 2H, J=6.9 Hz), 2.79—2.86 (dd, 1H, J=6.6, 12.9 Hz), 2.88—2.94 (dd, 1H, J=5.1, 12.9 Hz), 3.56—3.60 (m, 1H), 3.83 (m, 1H), 4.10 (m, 1H), 7.56—7.67 (m, 3H), 7.87—7.90 (m, 2H); MS (+ES) m/z (relative intensity): 404 ($M-3H_2O$, 20), 422 ($M-2H_2O$, 50), 440 ($M-H_2O$, 20), 458 ($M+H^+$, 100), 475 ($M+NH_4^+$, 60). Compounds **21—24** were prepared in a similar fashion.

7-{2-[4-(3-Fluoro-benzenesulfonylamino)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl} heptanoic acid (**21**): 33%; ^{1}H-NMR (CD_3OD, 300 MHz) δ: 1.24—1.72 (m, 22H), 2.06—2.15 (m, 1H), 2.75—2.33 (t, 2H, J= 7.5 MHz), 2.83—2.98 (m, 2H), 3.59—3.87 (m, 1H), 3.83—3.87 (m, 1H), 4.11 (bs, 1H), 7.37—7.43 (m, 1H), 7.59—7.66 (m, 2H), 7.70—7.74 (dt, 1H, J=1.2, 7.8 MHz); ^{13}C-NMR (CD_3OD, 75 MHz) δ: 25.1, 27.9, 28.2, 28.5, 28.6, 29.1, 29.6, 32.1, 32.2, 34.3, 42.8, 50.0, 51.2, 51.3, 70.3, 70.6, 72.5, 77.3, 113.8, 114.1, 119.3, 119.6, 122.9, 131.2, 131.3, 143.1, 161.1, 164.4, 177.3; MS (+AP) m/z (relative intensity): 493.2 ($M+NH_4^+$, 55), 476.2 ($M+H^+$, 40), 440.2 ($M-2H_2O$, 100), 421.1 ($M-3H_2O$, 95); ^{19}F-NMR (CD_3OD): 51.1; HRMS Calcd for $(C_{22}H_{34}O_7NSF+H)^+$: 476.2118. Found: 476.2107.

7-{2-[4-(2,4-Fluoro-benzenesulfonylamino)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl} heptanoic acid (**22**): 48%; ^{1}H-NMR (CD_3OD, 300 MHz) δ: 1.20—1.71 (m, 22H), 2.06—2.15 (m, 1H), 2.27—2.32 (t, 2H, J= 7.5 MHz), 2.89—2.96 (dd, 1H, J=6.9, 13.2 MHz), 2.98—3.04 (dd, 1H, J=5.1, 13.2 MHz), 3.58—3.60 (m, 1H), 3.81—3.86 (m, 1H), 4.10 (m, 1H), 7.12—7.27 (m, 2H), 7.91—7.99 (m, 1H); ^{13}C-NMR (CD_3OD, 75 MHz) δ: 25.0, 27.9, 28.1, 28.5, 28.7, 29.1, 29.6, 32.0, 32.2, 33.9, 42.8, 49.9, 51.2, 51.3, 70.3, 70.6, 72.4, 77.3, 105.1, 105.4, 105.8, 111.6, 111.8, 125.4, 131.9, 132.1, 158.1, 161.2, 164.2, 167.5, 176.7; MS (+ES) m/z (relative intensity): 511.1 ($M+Na^+$,100), 494.1 ($M+H^+$, 85); ^{19}F-NMR (CD_3OD): 56.1, 59.1; HRMS Calcd for $(C_{22}H_{33}O_7NSF_2+Na)^+$: 516.1834. Found: 516.1854.

7-{2-[4-(4-Fluoro-benzenesulfonylamino)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl} heptanoic acid (**23**): 57%; ^{1}H-NMR (CD_3OD, 300 MHz) δ: 1.24—1.72 (m, 22H), 2.07—2.16 (M, 1H), 2.28—2.33 (t, 2H, J=7.5 MHz), 2.81—2.96 (m, 2H), 3.58—3.63 (m, 1H), 3.84—3.85 (m, 1H), 4.11 (bs, 1H), 7.30—7.37 (m, 2H), 7.91—7.97 (m, 2H); ^{13}C-NMR (CD_3OD, 75 MHz) δ: 25.1, 27.9, 28.1, 28.2, 28.5, 28.7, 29.1, 29.6, 32.1, 32.2, 42.8, 49.9, 51.2, 51.3, 70.3, 70.6, 72.4, 77.3, 115.9, 116.2, 129.7, 129.9, 137.1, 163.5, 166.9, 177.1; MS (+AP) m/z (relative intensity): 498.3 ($M+Na^+$, 100), 440.4 ($M-2H_2O$, 85), 422.3 ($M-3H_2O$, 65); ^{19}F-NMR (CD_3OD): 52.1;

HRMS Calcd for $(C_{22}H_{34}O_7NSF+H)^+$: 476.2118. Found: 476.2135.

7-{2-[4-(2-Fluoro-benzenesulfonylamino)-3-hydroxy-butyl]-3,5-dihydroxy-cyclopentyl} heptanoic acid (**24**): 56%; ^{1}H-NMR (CD_3OD, 300 MHz) δ: 1.14—1.71 (m, 22H), 2.06—2.15 (m, 1H), 2.27—2.32 (t, 2H, J=7.2 MHz), 2.90—3.04 (m, 2H), 3.61 (m, 1H), 3.84 (M, 1H), 4.11 (s, 1H), 7.31—7.39 (m, 2H), 7.65—7.72 (m, 1H), 7.87—7.92 (overlapped dd, 1H, J=7.8, 8.4 MHz); ^{13}C-NMR (CD_3OD, 75 MHz) δ: 25.1, 27.9, 28.2, 28.5, 28.7, 29.1, 29.6, 30.0, 32.1, 34.3, 42.8, 50.0, 51.2, 51.3, 70.4, 70.6, 72.5, 77.3, 116.9, 117.1, 124.6, 130.1, 135.0, 135.2, 157.4, 160.8, 177.4; ^{19}F-NMR (CD_3OD) 51.1; MS (+AP) *m/z* (relative intensity): 493.1 ($M+NH_4^+$, 100), 475.1 ($M+H^+$, 25), 457.1 ($M-H_2O$, 17), 440.1 ($M-2H_2O$, 20), 421.1 ($M-3H_2O$, 19); HRMS Calcd for $(C_{22}H_{34}O_7NSF+Li)^+$: 4482.2200. Found: 482.2186.

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Convenient Synthesis of α-Trifluoromethylated Acyloins from α-Hydroxy or α-Amino Acids

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α-Trifluoromethylated acyloins (2 and 6) have been prepared from α-hydroxy acids (1), *N*-acylprolines (5) or *N*-acyl-*N*-alkyl α-amino acids (8) by novel transformation reactions with trifluoroacetic anhydride (TFAA) in the presence of pyridine. The former reaction of 1 could proceed through mesoionic 1,3-dioxolium-4-olates, whereas the latter two reactions of α-amino acids (5 and 8) could involve mesoionic 1,3-oxazolium-5-olates. The reaction of 1 with TFAA shows more potential for practical applications because of the ready availability of the starting materials and ease of manipulation.

Key words acyloin; trifluoromethyl; α-hydroxy ketone; mesoionic compound; trifluoroacetic anhydride

Organic compounds containing fluorine possess special chemical properties[1] and hence often exhibit an interesting physiological profile.[2] Among them, molecules bearing a trifluoromethyl (CF_3) group are of great interest. This has stimulated intensive research and many reliable methods are now available to introduce the CF_3 moiety into organic substrates.[3] Ideally, the fluorinated building block should be easy to handle and relatively readily available. This could mean that the material is available commercially, or can be synthesized from an appropriate compound *via* a short reaction sequence. The reagents should be inexpensive and carry the required CF_3 group.

α-Hydroxy ketones or acyloins are very useful synthons for a variety of organic synthesis,[4] while their fluorine-containing analogues, *e.g.* α-trifluoromethylated acyloins have not been easily accessible in spite of their potential building blocks for CF_3-substituted compounds.[5] Four reactions for the formation of CF_3-acyloins have been recorded: 1) hydrolysis of 3-bromo-1,1,1-trifluoro-3-phenylpropanone with aq. Na_2CO_3 to afford CF_3-acyloin as one example,[6] 2) hydrolytic ring cleavage of 6-trifluoromethyl-1,3,4-oxadiazine derivatives obtained from benzaldehyde,[7] 3) hydrolysis of α-amino CF_3-ketones during the purification by chromatography on SiO_2, resulting in a mixture of regioisomeric α-hydroxy ketones[8] and 4) acid hydrolysis of α-amido CF_3-ketone to give CF_3-acyloin as one example.[9] However, these reactions are limited by the fact that only benzoyl derivatives are accessible or they need too many steps to be efficient in a building block strategy.

Following our first publication[10] on the use of trifluoroacetic anhydride (TFAA) as a useful reagent[11] for the introduction of the CF_3 group into organic compounds, we were interested in other reactions of TFAA leading to new CF_3-substituted compounds[12] and started a study on the interaction of TFAA with α-hydroxy acids (**1**),[13] *N*-acylprolines (**5**), or *N*-acyl-*N*-alkyl α-amino acids (**8**).[14] We now present a full account of these reactions and a new synthesis of CF_3-acyloin derivatives in good yields.

Results and Discussion

Reactions of α-Hydroxy Acids (1) with TFAA A reaction of simple carboxylic acids with TFAA to yield the corresponding trifluoromethyl ketones is not known. However, it is reported that some carboxylic acid chlorides were converted to trifluoromethyl ketones by reaction with pyridine and TFAA, in which the intermediate ketenes undergo the trifluoroacetylation.[15] We examined a reaction of mandelic acid (**1a**) with TFAA in detail, and the results are presented in Table 1. Thus, the reaction of **1a** with TFAA in the presence of pyridine results in the formation of CF_3-acyloin (**2a**) in 87% yield. Pyridine was essential, and the absence of the base lowered the yield (run 1). High temperature (80 °C) was needed to obtain a high yield of **2a**, the reaction at room temperature reducing the yield (9%) (runs 2, 3 and 4). Other α-hydroxy acids (**1b—f**) were subjected to the reaction under optimum conditions and the results are listed in Table 2. *O*-Acetylmandelic acid (**1b**) gave **2a** in a good yield, whereas *O*-benzoylmandelic acid (**1c**) gave **2a** and **3a** in 41% and 42% yields, respectively. However, *O*-methylmandelic acid (**1d**) and α-methylmandelic acid failed to give the corre-

Table 1. Reactions of Mandelic Acid (**1a**) with TFAA in Benzene[a)]

Run	Base (eq)[b)]	Conditions	Yield of **2a** (%)
1	None	Reflux, 6 h	5
2	Pyridine (6)	r. t., 24 h	9
3	Pyridine (6)	Reflux, 1 h	43
4	Pyridine (6)	Reflux, 3 h	87

a) The reactions were carried out on a 2 mmol scale. *b*) Eq. refers to molar equivalents with respect to **1a**.

Table 2. Reactions of α-Hydroxy Acids (**1**) with TFAA

Run	**1**	Product (yield, %)
1	**a**	**2a** (87)
2	**b**	**2a** (88)
3	**c**	**2a** (41), **3a** (42)
4	**d**	—[a)]
5	**e**	**2b** (71)
6	**f**	**2c** (42)
7[b)]	**a**	**4a** (84)
8[c)]	**a**	**4b** (66)

a) Methyl benzoate was isolated in 41% yield. *b*) Pentafluoropropionic anhydride was used instead of TFAA. *c*) Heptafluorobutyric anhydride was used instead of TFAA.

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sponding trifluoroacetyl compounds. In the reaction of **1d**, we obtained in 41% yield the methyl benzoate, which might be formed through decarboxylative oxygenation involving autoxidation.

Pentafluoropropionic and heptafluorobutyric anhydride also reacted readily with **1a** to yield the corresponding α-pentafluoroethyl- (**4a**) or α-heptafluoropropyl-substituted acyloins (**4b**) in good yield, respectively.

The structures of **2** and **3** are supported by spectral and analytical data. The assignments of the carbon signals in **2a** were performed on the basis on the long-range ^{13}C–^{19}F coupling. Thus, $^{1}J_{C-F}$ and $^{2}J_{C-F}$ are measured in ^{1}H-decoupled ^{13}C-NMR spectra: at around δ 123 (CF_3, $^{1}J_{C-F}$=285 Hz) and δ 71 (CH, $^{2}J_{C-F}$=31 Hz).

Reactions of *N*-Acylprolines (5) or *N*-Acyl-*N*-alkylamino Acids (8) with TFAA The reaction of α-amino acids and acetic anhydride in the presence of a base to give α-acetaminoalkyl ketones is known as the Dakin–West (D-W) reaction.[16] Likewise, the D-W reaction of α-amino acids with TFAA yields the corresponding α-amido CF_3-ketones, which have attracted much interest as an important class of inhibitors of a variety of hydrolytic enzymes.[17] Recently, we have reported that the D-W reaction of *N*-alkoxycarbonyl-*N*-alkyl α-amino acids with TFAA affords the corresponding CF_3-ketones.[18] However, our early discovery[10] of the unusual formation of 5-CF_3-oxazoles (**9**) from *N*-acylprolines (**5**) or *N*-acyl-*N*-alkylamino acids (**8**) and TFAA under the D-W reaction conditions prompted us to investigate in detail the D-W reaction of *N*-acyl-*N*-alkyl-α-amino acids (**5** and **8**) with TFAA. We have now found that the reaction of **5** or **8** with TFAA affords CF_3-acyloins (**2** and **6**) as the main products. According to a previous report,[10] the reaction was carried out by the addition of TFAA at 0 °C to a solution of *N*-acylprolines (**5**) in benzene in the presence of pyridine to give 5-CF_3-oxazole derivative (**9**) in good yield. In contrast, when the addition of TFAA was performed at 100 °C, the results were quite different. Thus, TFAA was added to a refluxing solution of **5a** and pyridine in benzene and the mixture was further refluxed for 1 h. After usual work-up, the compound **6a** was isolated in 88% yield. The reason why the reaction affords a completely different product depending on the reaction temperature is still unclear. However, several *N*-acyl derivatives (**5b—d**) were easily converted to the acyloins (**6b—d**) in good yields (Table 3, runs 2—4). The reaction is generally applicable to not only *N*-acylprolines (**5**) but also other *N*-acyl-*N*-alkylamino acids (**8**), as shown in Table 3. In the case of **8c**, the temperature at addition of TFAA does not have a profound effect on the product distribution (Table 3, runs 7 and 8). Among the three *N*-alkyl groups examined (Me, Et, and benzyl), use of the Me group proved to give the best result (Table 3, runs 7, 9, and 11).

Transformation of acyloins (**2**) to oxazoles (**10**) is possible (Chart 2).[19] Thus, reactions of acyloins (**2b** or **6a**) with formamide at 105 °C afforded the corresponding 5-CF_3-oxazoles (**10a** and **b**) in 47% and 27% yields, respectively.

Mechanistic Consideration Plausible mechanisms of the reactions of α-hydroxy acids (**1**) and *N*-acyl amino acids (**5** and **8**) are suggested in Charts 3, 4, and 5, respectively. The reaction of α-hydroxy acids (**1**) could involve mesoionic 1,3-dioxolium-4-olates (**12**), whose existence was supported by the following facts: 1) α-hydroxy carboxylic acid (**1a**), *O*-acetylmandelic acid (**1b**), and *O*-benzoylmandelic acid (**1c**), which can form the mesoionic intermediates, gave the expected product, 2) neither *O*-methylmandelic acid (**1d**) nor α-methylmandelic acid can form the mesoionic intermediate and failed to give the corresponding CF_3-ketones. These results also showed that the mechanism of the reaction of α-hydroxy acids (**1**) with TFAA did not consist with those of the Hunsdiecker reaction[20] or the reaction of carboxylic acid chlorides with pyridine and TFAA to afford CF_3-ketones *via* ketene intermediates.[15] The existence of the mesoionic intermediate (**12**) was postulated for the first time in the 1,3-dipolar cycloaddition reaction with olefinic dipolarophiles.[21] The conversion of **12** to **2** appears to proceed in a similar mechanism to that described in the case of the D-W reaction of *N*-acyl-*N*-alkyl α-amino acids.[10,16]

Table 3. Reactions of *N*-Acyl-*N*-alkyl-α-amino Acids with TFAA

Run	Amino acid	Method[a]	Product (yield, %)
1	**5a**	A	**6a** (88)
2	**5b**	A	**6b** (85)
3	**5c**	A	**6c** (81)
4	**5d**	A	**6d** (62)+**7** (12)
5	**8a**	A	**2a** (68)+**3a** (8)+**9a** (12)
6	**8b**	A	**2a** (30)+**3a** (4)+**9a** (41)
7	**8c**	A	**2b** (54)+**3b** (11)+**9b** (5)
8	**8c**	B	**2b** (50)+**3b** (14)+**9b** (13)
9	**8d**	A	**2b** (27)+**3b** (23)+**9b** (1)
10	**8d**	B	**2b** (17)+**3b** (43)+**9b** (16)
11	**8e**	A	**2b** (41)+**3b** (10)+**9b** (33)
12[b]	**8e**	B	**9b** (88)
13[c]	**8f**	A	**3c** (43)+**9c** (2)
14	**8g**	A	**3d** (23)+**9d** (5)

a) Methods A and B are described in the experimental. *b*) Literature data.[10] *c*) No isolation of **2d** is probably due to the loss during extraction because the product may be water-soluble.

Several observations help to delineate the gross mechanistic details of the way **8** is converted to **2** and **3**, respectively (Chart 4). First, in the reaction of **8e**, benzoic acid (40%), benzylamine (35%, isolated as an *N*-acetyl derivative) and benzyl alcohol (13%) were isolated as the acidic and basic fractions after extraction of the products. Second, it was proved that **2a** and **3a** were not the direct reaction products, but they were formed after hydrolysis of the reaction mixture. On the other hand, oxazole (**9a**) was formed before the acid hydrolysis. Third, it was also proved that **2a** was not derived from the hydrolysis of **3a**, because **3a** was recovered unchanged under the same reaction conditions as **8e**. The reaction involves mesoionic 1,3-oxazolium-5-olates (**18**), commonly known as munchnones, formed through the cyclodehydration of **8** by TFAA. In Chart 4, the key intermediate oxazolium ion (**19**), the postulated common intermediate for the formation of oxazoles,[10] could have three sites which could be attacked by the trifluoroacetate anion. Further addition of the trifluoroacetate anion to intermediate **20** could lead to **21**, and acid hydrolysis of **21** *via* α-amino trifluoromethyl ketones (**22**) may account for the formation of **2**. In the case of *N*-acylprolines (**5**), the formation of **6** could be through hydrolysis of **25** as described in Chart 5.

In summary, our methods make this class of compounds readily accessible for study as building blocks for the synthesis of fluorine-containing compounds. Recently, three methods for the preparation of α-hydroxy-α-trifluoromethyl car-

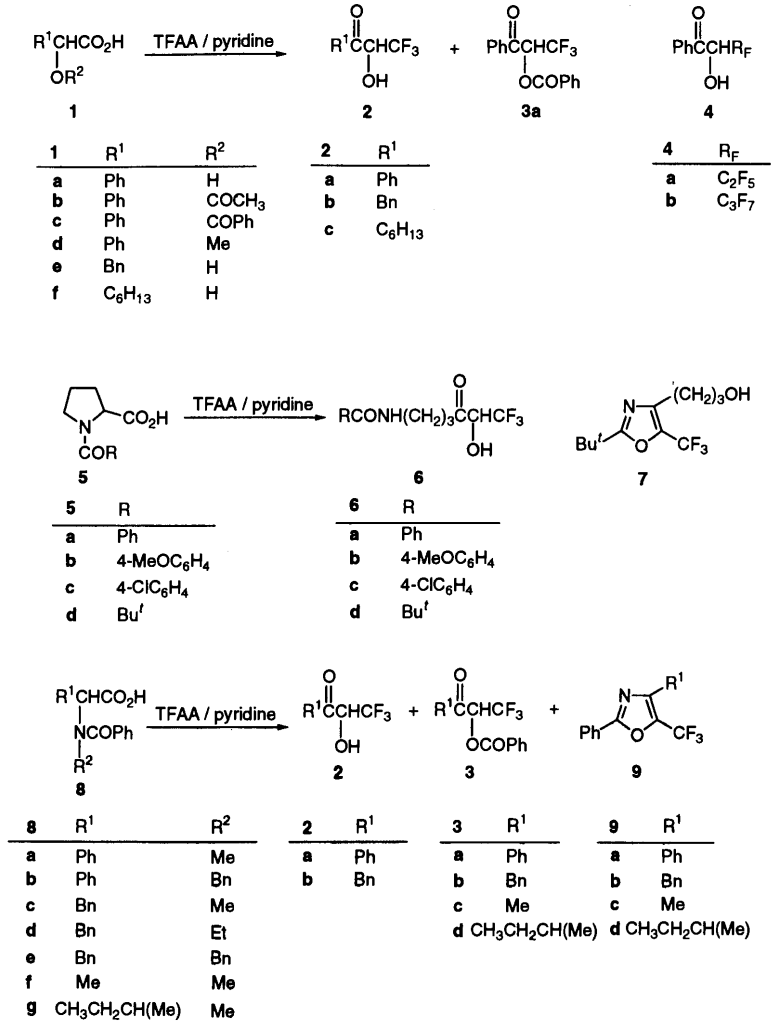

Chart 1

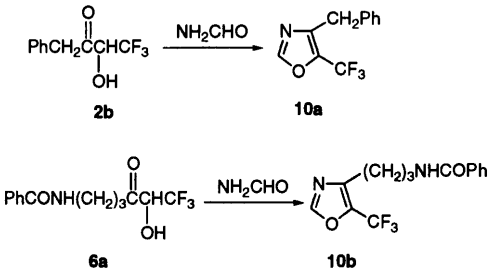

Chart 2

bonyl compounds have been devised: 1) the addition of formaldehyde *N,N*-dialkylhydrazones to trifluoromethyl ketones, followed by hydrazone cleavage,[22] 2) Friedel-Crafts type reactions of trifluoropyruvamides to give trifluoromethylated glycolic acid derivatives[23] and 3) Grignard reactions of trifluoropyruvamides.[24] Recently, we have found that the CF_3-acyloins (**2a, b**) induce apoptotic cell death in human oral tumor cell lines and could be a lead compound of anticancer agents.[25] The reactions of α-hydroxy acids **1** show more potential for practical applications because of the ready availability of the starting materials and ease of manipulation.

Experimental

General Methods All melting points were determined using a Yanagimoto hot-stage melting point apparatus and are uncorrected. ^{1}H-NMR spectra were measured on either a JEOL JNM-PMX60SI, JNM-FX270, or JNM-GSX500 spectrometer with tetramethylsilane (Me_4Si) as an internal reference and $CDCl_3$ as the solvent. ^{13}C-NMR spectra were obtained on a JEOL JNM-FX270 or JNM-GSX500 spectrometer (at 68 or 127 MHz). Both ^{1}H- and ^{13}C-NMR spectral data are reported in parts per million (δ) relative to Me_4Si. Infrared (IR) spectra were recorded on a JASCO IR810 spectrometer. Low- and high-resolution MS were obtained with a JEOL JMS-DX300 spectrometer with a direct inlet system at 70 eV. Combustion analyses were carried out in the microanalytical laboratory of this university. Standard workup means that the organic layers were finally dried over Na_2SO_4, filtered, and concentrated *in vacuo* below 45 °C using a rotary evaporator. In the preparation of *N*-acyl-*N*-alkyl-α-amino acids (**5** and **8**), L-Ala, L-Ileu, L-Phe, DL-phenylglycine, and L-Pro were used as the starting materials.

Materials The following compounds were prepared by reported procedures[26]: *O*-Acetylmandelic acid (**1b**): mp 50—52 °C (mp[26] 52 °C). *O*-Benzoylmandelic acid (**1c**): mp 114—115 °C (mp[26] 114—115 °C). The following compounds were previously synthesized and used in this study as the starting materials[10]: *N*-benzoylproline (**5a**): mp 155—157 °C; *N*-(4-methoxybenzoyl)proline (**5b**): mp 105—108 °C; *N*-(4-chlorobenzoyl)proline (**5c**): mp 122—125 °C; *N*-pivaloylproline (**5d**): mp 131—132 °C; *N*-benzoyl-*N*-methylphenylglycine (**8a**): mp 120—122 °C; *N*-benzoyl-*N*-benzylphenylalanine (**8b**): mp 114—116 °C; *N*-benzoyl-*N*-methylphenylalanine (**8c**): mp 137—138 °C; *N*-benzoyl-*N*-ethylphenylalanine (**8d**): mp 162—164 °C; *N*-benzoyl-*N*-benzylphenylalanine (**8e**): mp 114—116 °C; *N*-benzoyl-*N*-methylalanine (**8f**): mp 131—132 °C.

***N*-Benzoyl-*N*-methylisoleucine (8g)** This was obtained according to a conventional method[10]: Oil. IR (oil) cm^{-1}: 3000 (br), 1730, 1635. ^{1}H-NMR (500 MHz) δ: 0.75—0.88+0.93—1.10 (m, 3H), 1.01+1.09 (d, 3H,

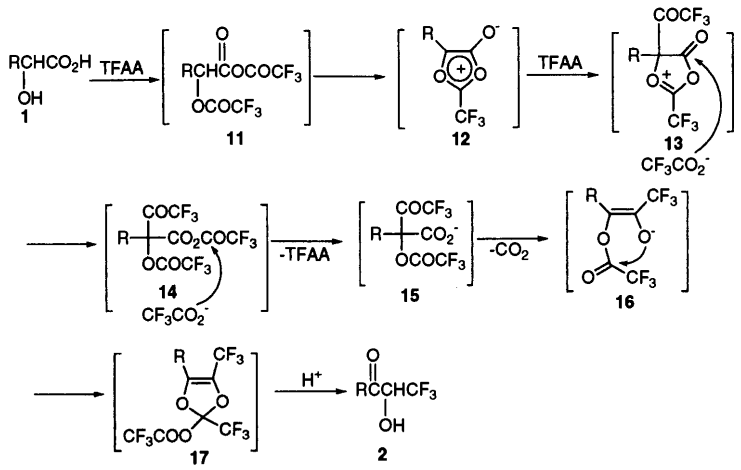

Chart 3

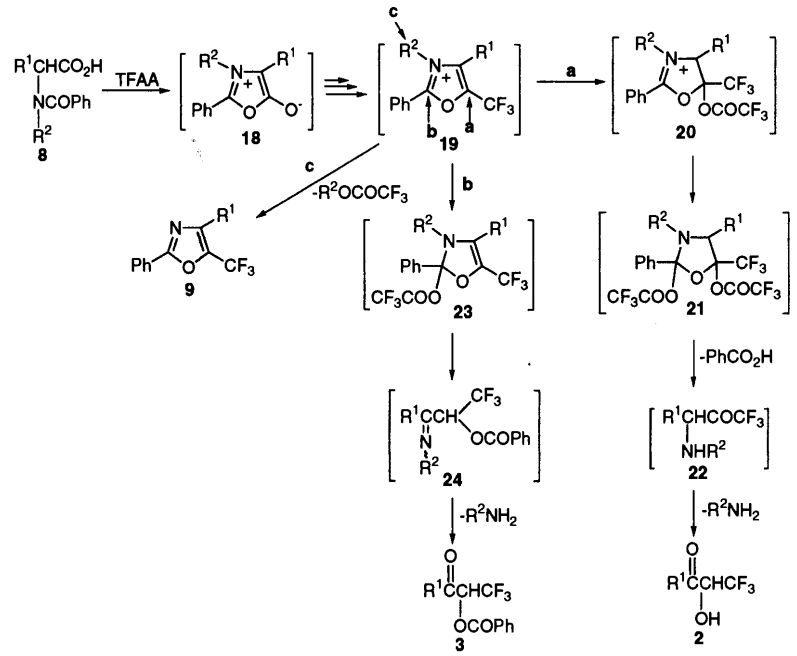

Chart 4

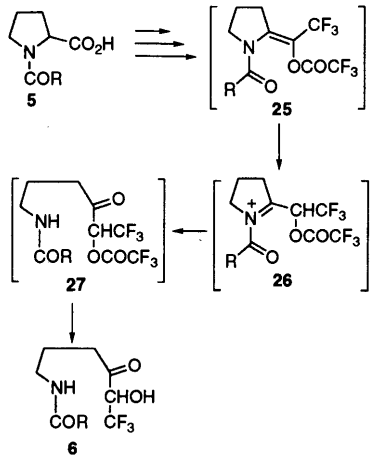

Chart 5

J=6.9 Hz), 1.20—1.65 (m, 2H), 1.95—2.30 (m, 1H), 2.99+3.06 (s, 3H), 4.71—4.84 (m, 1H), 7.35—7.80 (m, 5H), 7.55—8.10 (br, 1H). MS *m/z*: 249 (M^+, 0.4%), 105 (100%). High-resolution MS: Calcd for $C_{14}H_{19}NO_3$: 249.1364. Found: 249.1362.

The following compounds are commercially available: **1a**, **1d**, **1e**, **1f**, and α-methylmandelic acid.

General Procedure for the Reactions of α-Hydroxy Acids with TFAA TFAA (1.2 ml, 8 mmol) was added to a stirred solution of α-hydroxy acid (**1**) (2 mmol) and pyridine (0.97 ml, 12 mmol) in dry benzene (7 ml) at room temperature under Ar atmosphere and the mixture was refluxed for 3 h. Then, 5% HCl (5 ml) was added to the mixture and the solution was stirred at 60 °C for 10 min. After standard workup, the crude product was purified by column chromatography on silica gel eluting with EtOAc–hexane (1 : 4) to give the products (**2** and/or **3**). The results are summarized in Table 2.

3,3,3-Trifluoro-2-hydroxy-1-phenyl-1-propanone (**2a**): mp 80—81 °C (benzene) (mp[6] 80.5—82.5 °C). IR (Nujol) cm^{-1}: 3370, 1685. ^{1}H-NMR (500 MHz) δ: 4.00—4.40 (br, 1H), 5.40 (q, 1H, J=5.8 Hz), 7.40—7.71 (m, 3H), 7.98 (m, 2H). ^{13}C-NMR (126 MHz) δ: 70.97 (CH, $^2J_{C-F}$=31 Hz), 123.34 (CF_3, $^1J_{C-F}$=285 Hz), 129.03 (CH), 129.50 (CH), 133.35 (C), 135.34 (CH), 193.10 (C). CI-MS *m/z*: 205 (M^++1, 100%).

4,4,4-Trifluoro-3-hydroxy-1-phenyl-2-butanone (**2b**): mp 95—96 °C (benzene–hexane). IR (Nujol) cm^{-1}: 3420, 3350, 1735. ^{1}H-NMR (500 MHz) δ: 3.61—4.08 (br, 1H), 3.93 (d, 1H, J=16.3 Hz), 4.04 (d, 1H, J=16.3 Hz), 4.60 (q, 1H, J=7.4 Hz), 7.18—7.21 (m, 2H), 7.31—7.37 (m, 3H). ^{13}C-NMR (126 MHz) δ: 46.41 (CH_2), 73.82 (CH, $^2J_{C-F}$=31 Hz), 122.50 (CF_3, $^1J_{C-F}$=284 Hz), 127.89 (C), 129.10 (CH), 129.54 (CH), 131.49 (C), 200.61 (C). MS *m/z*: 218 (M^+, 6%), 91 (100%). *Anal.* Calcd for $C_{10}H_9F_3O_2$: C,

55.05; H, 4.16. Found: C, 54.95; H, 4.26.

1,1,1-Trifluoro-2-hydroxy-3-nonane (**2c**): Oil. IR (oil) cm^{-1}: 3450, 1725. ^{1}H-NMR (500 MHz) δ: 0.89 (t, 3H, J=7.0 Hz), 1.23—1.37 (m, 6H), 1.62—1.72 (m, 2H), 2.58—2.76 (m, 2H), 4.00—4.20 (br, 1H), 4.50 (q, 1H, J=7.5 Hz). ^{13}C-NMR (126 MHz) δ: 13.99 (CH_3), 22.45 (CH_2), 23.27 (CH_2), 28.62 (CH_2), 31.45 (CH_2), 39.73 (CH_2, $^4J_{C-F}$=2 Hz), 74.81 (CH, $^2J_{C-F}$=31 Hz), 122.53 (CF_3, $^1J_{C-F}$=283 Hz), 203.08 (C). CI-MS m/z: 213 (M^++1, 79%), 113 (100%).

2-Benzoyloxy-3,3,3-trifluoro-1-phenyl-1-propanone (**3a**): mp 87—88 °C (Et_2O–hexane). IR (Nujol) cm^{-1}: 1750, 1700. ^{1}H-NMR (500 MHz) δ: 6.52—6.57 (m, 1H), 7.47—7.62 (m, 4H), 7.62—7.68 (m, 2H), 8.03—8.14 (m, 4H). ^{13}C-NMR (126 MHz) δ: 71.29 (CH, $^2J_{C-F}$=32 Hz), 121.87 (CF_3, $^1J_{C-F}$=282 Hz), 127.73 (C×2), 128.69 (CH), 128.97 (CH×2), 130.29 (CH), 134.26 (CH), 134.58 (CH), 164.66 (C), 188.36 (C). MS m/z: 308 (M^+, 0.1%), 105 (100%). High-resolution MS: Calcd for $C_{16}H_{11}F_3O_3$: 308.0660. Found: 308.0670.

3,3,4,4,4-Pentafluoro-2-hydroxy-1-phenyl-1-butanone (4a) The procedure was the same as described above, except that TFAA was replaced with pentafluoropropionic anhydride: Yield 84%, oil. IR (oil) cm^{-1}: 3450, 1690. ^{1}H-NMR (500 MHz) δ: 3.81—4.22 (br, 1H), 5.52 (dd, 1H, J=2.8, 19.5 Hz), 7.54 (t, 2H, J=8.1 Hz), 7.70 (t, 1H, J=8.1 Hz), 7.96 (d, 2H, J=8.1 Hz). ^{13}C-NMR (126 MHz) δ: 69.81 (CH, $^2J_{C-F}$=25, 28 Hz), 110—120 (CF_2CF_3), 129.03 (CH), 129.46+129.48 (CH), 133.93 (C), 135.33 (CH), 193.52 (C). MS m/z: 253 (M^+−1, 28%), 105 (100%).

3,3,4,4,5,5,5-Heptafluoro-2-hydroxy-1-phenyl-1-pentanone (4b) The procedure was the same as described above, except that TFAA was replaced with heptafluorobutyric anhydride: Yield 66%, oil. IR (oil) cm^{-1}: 3450, 1685. ^{1}H-NMR (500 MHz) δ: 4.28 (d, 1H, J=8.6 Hz), 5.60 (dd, 1H, J=8.6, 20.5 Hz), 7.54 (t, 2H, J=7.8 Hz), 7.69 (t, 1H, J=7.8 Hz), 7.96 (d, 2H, J=7.8 Hz). ^{13}C-NMR (126 MHz) δ: 69.74 (CH, $^2J_{C-F}$=24, 28 Hz), 105—120 ($CF_2CF_2CF_3$), 129.08 (CH), 129.42+129.44 (CH), 134.12 (C), 135.32 (CH), 193.52 (C). CI-MS m/z: 305 (M^++1, 52%), 105 (100%).

General Procedures for the Reactions of *N*-Acylprolines (5) or *N*-Acyl-*N*-alkylamino Acids (8) with TFAA Method A: TFAA (0.64 ml, 4.5 mmol) was added to a refluxing solution of **5** or **8** (1.5 mmol) and pyridine (0.73 ml, 9 mmol) in dry benzene (6 ml) at 100 °C and the mixture was refluxed for 1 h. Then, 5% HCl (6 ml) was added to the mixture and the solution was stirred at 60 °C for 30 min. After standard workup, the crude product was purified by column chromatography on silica gel eluting with EtOAc–hexane (1 : 4) to give the products (**2**, **3** and/or **9**).

Method B: Compound **5** or **8** (1.5 mmol) was added to a stirred solution of TFAA (0.64 ml, 4.5 mmol) and pyridine (0.73 ml, 9 mmol) in dry benzene (6 ml) at 0 °C and the mixture was stirred at 25 °C for 3 h, then refluxed for 5 h. After the reaction, 5% HCl (6 ml) was added to the mixture and the solution was stirred at 60 °C for 30 min. After standard workup, the crude product was purified by column chromatography on silica gel eluting with EtOAc–hexane (1 : 4) to give the products (**2**, **3** and/or **9**). The results are summarized in Table 3.

3-Benzoyloxy-4,4,4-trifluoro-1-phenyl-2-butanone (**3b**): mp 63—64 °C (hexane). IR (Nujol) cm^{-1}: 1745, 1735. ^{1}H-NMR (270 MHz) δ: 3.98 (d, 2H, J=2 Hz), 5.79 (q, 1H, J=7.4 Hz), 7.15—7.19 (m, 2H), 7.27—7.35 (m, 3H), 7.46—7.52 (m, 2H), 7.61—7.68 (m, 1H), 8.07—8.11 (m, 2H). ^{13}C-NMR (68 MHz) δ: 46.85 (CH_2), 74.14 (CH, $^2J_{C-F}$=31 Hz), 121.77 (CF_3, $^1J_{C-F}$=282 Hz), 127.58 (CH), 123.77 (CH), 129.69 (CH), 130.28 (CH×2), 131.63 (C), 134.37 (CH), 164.44 (C), 196.19 (C). MS m/z: 322 (M^+, 1.8%), 91 (100%). *Anal.* Calcd for $C_{17}H_{13}F_3O_3$: C, 63.36; H, 4.07. Found: C, 63.24; H, 4.06.

3-Benzoyloxy-4,4,4-trifluoro-2-butanone (**3c**): Oil. IR (oil) cm^{-1}: 1740 (br), 1730, 1640. ^{1}H-NMR (270 MHz) δ: 2.38 (s, 3H), 5.67 (q, 1H, J=7.4 Hz), 7.50 (t, 2H, J=7.5 Hz), 7.66 (t, 1H, J=7.5 Hz), 8.12 (d, 2H, J=7.5 Hz). ^{13}C-NMR (68 MHz) δ: 27.39 (CH_3), 74.96 (CH, $^2J_{C-F}$=32 Hz), 121.75 (CF_3, $^1J_{C-F}$=282 Hz), 127.71 (C), 128.81 (CH), 130.26 (CH), 134.40 (CH), 164.56 (C), 196.16 (C). MS m/z: 246 (M^+, 9%), 105 (100%). High-resolution MS: Calcd for $C_{11}H_9F_3O_3$: 246.0503. Found: 246.0475.

2-Benzoyloxy-1,1,1-trifluoro-4-methyl-3-hexanone (**3d**): mp 34—35 °C (hexane). IR (Nujol) cm^{-1}: 1745, 1735. ^{1}H-NMR (500 MHz) δ: 0.86+0.96 (t, 3H, J=7.4 Hz), 1.11+1.24 (d, 3H, J=7.1 Hz), 1.36—1.44+1.45—1.52 (m, 1H), 1.71—1.78+1.83—1.90 (m, 1H), 2.84—2.91 (m, 1H), 5.82—5.87 (m, 1H), 7.49—7.52 (m, 2H), 7.63—7.66 (m, 1H), 8.12+8.13 (d, 2H, J=7.6 Hz). ^{13}C-NMR (126 MHz) δ: 11.31+11.47 (CH_3), 15.06+16.57 (CH_3), 25.23+26.18 (CH_2), 44.83+45.26 (CH), 73.61+73.96 (CH, $^2J_{C-F}$=32 Hz), 121.85+121.89 (CF_3, $^1J_{C-F}$=281 Hz), 127.85+127.87 (C), 128.76 (CH), 130.23 (CH), 134.28 (CH), 164.42+164.44 (C), 202.33+202.40 (C). CI-MS m/z: 289 (M^++1, 7%), 105 (100%). *Anal.* Calcd for $C_{14}H_{15}F_3O_3$: C, 58.33; H, 5.24. Found: C, 58.17; H, 5.40. The multiplicity of ^{1}H and ^{13}C NMR peaks is due to the diastereomer of **3d**.

1,1,1-Trifluoro-2-hydroxy-6-pivaloamido-3-hexanone (**6d**): Oil. IR (oil) cm^{-1}: 3350 (br), 1730, 1640. ^{1}H-NMR (270 MHz) δ: 1.18 (s, 9H), 1.78—1.94 (m, 2H), 2.61—2.83 (m, 2H), 3.16—3.30 (m, 2H), 4.53 (q, 1H, J=7.4 Hz), 4.90—5.00 (br, 1H), 6.07 (br s, 1H). ^{13}C-NMR (68 MHz) δ: 24.82 (CH_2), 28.72 (C), 29.03 (CH_3), 37.89 (CH_2), 40.20 (CH_2), 76.77 (CH, $^2J_{C-F}$=31 Hz), 124.30 (CF_3, $^1J_{C-F}$=283 Hz), 181.28 (C), 205.42 (C). MS m/z: 269 (M^+, 5%), 57 (100%). High-resolution MS: Calcd for $C_{11}H_{18}F_3O_3$: 269.1239. Found: 269.1239.

The following products were identified by comparison of the IR and ^{1}H-NMR spectral data with those of authentic samples[10]: 6-Benzamido-1,1,1-trifluoro-2-hydroxy-3-hexanone (**6a**); 1,1,1-trifluoro-2-hydroxy-6-(4-methoxybenzamido)-3-hexanone (**6b**); 6-(4-chlorobenzamido)-1,1,1-trifluoro-2-hydroxy-3-hexanone (**6c**); 3-(2-*tert*-butyl-5-trifluoromethyloxazol-4-yl)propanol (**7**); 5-trifluoromethyl-2,4-diphenyloxazole (**9a**); 4-benzyl-5-trifluoromethyl-2-phenyloxazole (**9b**); 5-trifluoromethyl-4-methyl-2-phenyloxazole (**9c**); 4-*sec*-butyl-5-trifluoromethyl-2-phenyloxazole (**9d**).

General Procedures for the Reactions of Acyloins with Formamide A solution of acyloin (**2b** or **6a**) (1 mmol) in formamide (0.9 ml, 20 mmol) containing c.H_2SO_4 (0.2 ml, 2 mmol) was heated at 105 °C for 3 h. The mixture was diluted with Et_2O (40 ml) and 1% Na_2CO_3 (30 ml). After standard workup, the crude product was purified by column chromatography on silica gel eluting with EtOAc–hexane (1 : 1) to give the products (**10a, b**).

4-Benzyl-5-trifluoromethyloxazole (**10a**): Yield 41%, oil. IR (oil) cm^{-1}: 1630. ^{1}H-NMR (270 MHz) δ: 4.00 (s, 2H), 7.22—7.33 (m, 5H), 7.83 (s, 1H). ^{13}C-NMR (68 MHz) δ: 32.01 (CH_2), 119.71 (CF_3, $^1J_{C-F}$=268 Hz), 126.96 (CH), 128.75 (CH), 129.14 (CH), 133.78 (C, $^2J_{C-F}$=42 Hz), 137.02 (C), 140.95 (C), 151.40 (C). MS m/z: 227 (M^+, 100%). High-resolution MS: Calcd for $C_{11}H_8NF_3O$: 227.0558. Found: 227.0548.

4-(3′-Benzamidopropyl)-5-trifluoromethyloxazole (**10b**): Yield 27%, mp 87—88 °C (Et_2O–hexane). IR (Nujol) cm^{-1}: 3300, 1625. ^{1}H-NMR (270 MHz) δ: 1.94—2.05 (m, 2H), 2.78 (t, 2H, J=6.9 Hz), 3.45—3.52 (m, 2H), 6.80 (br s, 1H), 7.38—7.51 (m, 3H), 7.56—7.80 (m, 2H), 7.89 (s, 1H). ^{13}C-NMR (68 MHz) δ: 23.09 (CH_2), 28.07 (CH_2), 39.13 (CH_2), 119.65 (CF_3, $^1J_{C-F}$=268 Hz), 126.94 (CH), 128.57 (CH), 131.70 (CH), 133.80 (CH, $^2J_{C-F}$=42 Hz), 134.70 (C), 141.39 (C), 151.29 (C), 167.72 (C). MS m/z: 298 (M^+, 8%), 105 (100%). *Anal.* Calcd for $C_{14}H_{13}N_2F_3O_2$: C, 56.38; H, 4.39; N, 9.39. Found: C, 56.42; H, 4.49; N, 9.28.

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Taxane Diterpenoids from Seeds of *Taxus mairei*

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A new 2(3→20) abeotaxane, taxumairone A (1), and a new *cis-p*-coumaroyl myo-inositol have been isolated from the seeds of T*axus mairei* in addition to taxin B (2), taxinine A, taxuspine X, decinnamoyltaxinine E, 5α-cinnamoyloxy-9α,10β,13α-triacetoxy-taxa-4(20)11-diene and 5α-cinnamoyloxy-2α,9α,10β,13α-tetraacetoxy-taxa-4(20)11-diene. The structure of 1 was determined by 2D-NMR spectral analysis and chemical correlation with taxin B (2). Compound 1 exhibited potent cytotoxicity against human colon carcinoma cells with an ED_{50} of 0.1 μg/ml.

Key words *Taxus mairei*; Taxaceae; taxane diterpenes; taxumairones

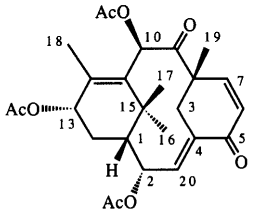
Paclitaxel has proven effective in the treatment of ovarian, breast and lung cancers. However, very little is known about its production and the many steps involved in assembling its unique structure need to be explored.[1,2] The rearranged 2(3→20) abeotaxanes such as taxines A and B, 2-deacetyltaxin B,[3] deaminoacyltaxine A[4] and taxuspines B[5] and W[6] were considered to be derived from isomeric verticilladiene precursors. These particular compounds have been found in stem, twigs and needles in tiny amounts from *Taxus baccata*, *T. cuspidata* and *T. yunnanensis*. Although more than 350 taxane diterpenes have been isolated to date,[7—9] there are still new taxoids awaiting isolation and structural characterization. The isolation of new taxanes might provide important clues in the biosynthesis of paclitaxel, especially for those compounds related to the intermediates of the verticillene pathway.

In a preliminary study, the crude extract of *T. mairei* seeds was found to contain a high amount of taxoids, which might be the reason for their intoxication to birds, animals and human beings. To search for new and bioactive taxanes, we previously isolated taxumairol M and known taxoids from the seeds of *Taxus mairei*.[10] Continued investigation of the *n*-hexane- and *n*-butanol-soluble fractions of the same material has resulted in the isolation of a new 2(3→20) abeotaxane named taxumairone A (**1**), and a new myo-inositol of *cis-p*-coumarate ester (**4**) together with taxin B (**2**), taxuspine X,[11] decinnamoyltaxinine E,[12] taxinine A,[13] and a mixture of 5α-cinnamoyloxy-9α,10β,13α-triacetoxy-taxa-4(20)11-diene and 5α-cinnamoyloxy-2α,9α,10β,13α-tetraacetoxy-taxa-4(20)11-diene,[14] and the *p*-coumarate ester of myo-inositol. In this communication, we wish to report the isolation and structure determination of the novel compound **1** and the new *cis-p*-coumaroyl myo-inositol (**4**) from *T. mairei* seeds.

Results and Discussion

The EtOH extract of the seeds of *T. mairei* was partitioned between *n*-hexane, MeOH and H_2O (4 : 3 : 1) to give an *n*-hexane soluble and MeOH–H_2O soluble fractions. The MeOH–H_2O soluble fraction was extracted with *n*-butanol to afford an *n*-butanol-soluble fraction. Extensive chromatography of the *n*-hexane-soluble residue by combination of Si gel and Sephadex LH-20 columns as well as normal (Si gel) and reverse-phase (RP-C_{18}) HPLC yielded **1** (0.00036%), **2** (0.0018%), taxuspine X (0.00018%), decinnamoyltaxinine E (0.0018%), taxinine A (0.00036%), and a mixture of 5α-cinnamoyloxy-9α,10β,13α-triacetoxy-taxa-4(20)11-diene and 5α-cinnamoyloxy-2α,9α,10β,13α-tetraacetoxy-taxa-4(20)11-diene (0.057%). The *n*-butanol-soluble residue was chromatographed on Sephadex LH-20 and reverse-phase RP-C_{18} columns to yield *trans*- and *cis-p*-coumarate esters of myo-inositol. The structures of the known taxoids and *p*-coumarate ester of myo-inositol were identified on the basis of spectral evidence and comparison with authentic samples. The structural elucidation of compounds **1** and **4** is discussed below.

Compound **1**, [α] −306° ($CHCl_3$), had the composition $C_{26}H_{32}O_8$ as deduced by a combination of low resolution EI



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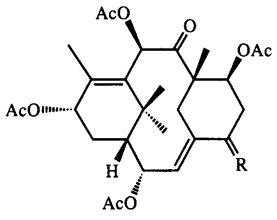

2 R = α-OH, β-H
3 R = O

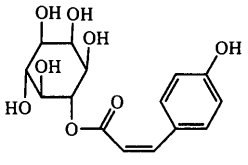

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Table 1. ^{1}H- and ^{13}C-NMR ($CDCl_3$) Spectral Data of Taxumairone A (**1**)

Position	^{13}C (ppm)[a]	^{1}H[b]	COSY	HMBC
1	47.8 d	1.80 m		H2, Me16, 17
2	69.4 d	5.76 (dd, 10.5)	H20	
3	35.5 t	2.60 (d, 14)	H3	H20, Me19
		3.08 (dd, 14, 2)		H3
4	134.1 s			H3
5	189.1 s			H3, 7, 20
6	131.4 d	6.29 (d, 10.2)	H7	
7	150.1 d	6.76 (dd, 10.2, 1.8)	H6	H3, Me19
8	52.5 s			H6, Me19
9	202.8 s			H10, Me19
10	80.0 d	6.08 s		
11	130.1 s			H10, Me16
				Me17, 18
12	139.2 s			H10, Me16
13	67.6 d	5.26 (d, 10)	H14	H1, Me18
14	26.8 t	2.67 m (β)	H13, 14	H2
		2.02 m (α)	H13, 14	
15	37.6 s			H10, Me16, Me17
16	25.0 q	1.26 s		Me17
17	33.7 q	1.11 s		Me16
18	17.3 q	1.51 s		
19	28.2 q	1.45 s		H3, 7
20	134.0 d	6.35 (dd, 10.5, 2)	H2	H3
OAc	170.6 s	2.19 s[c]		
OAc	170.0 s	2.17 s[c]		
OAc	169.7 s	2.04 s[c]		

a) Multiplicities and assignments made by the HMQC and HMBC techniques. *b*) Multiplicities and coupling constants in Hz in parentheses. *c*) Data interchangeable.

mass (*m/z* 472), FAB-MS (*m/z* 495, M+Na), ^{13}C-NMR spectroscopy and further confirmed by HR-FAB-MS. Its UV and IR bands indicated the presence of conjugated ketone (1674 cm^{-1}, 240 nm) and acetyl (1736 cm^{-1}) groups. This was also supported by fragment ions at *m/z* 430 $(M-Ac)^+$ and *m/z* 413 $(M-OAc)^+$ in the EIMS spectrum. The ^{1}H-NMR spectrum of **1** exhibited proton signals due to four methyls (δ 1.11, 1.26, 1.45 and 1.51), three acetyl methyls (δ 2.04, 2.17 and 2.19) and three olefinic (δ 6.76, 6.29 and 6.35) as well as three oxygenated methine (δ 6.08, 5.76, and 5.26) protons. The ^{13}C-NMR spectrum showed the presence of a ketone carbonyl (δ 202.8), a conjugated carbonyl (δ 189.1) and three olefinic double bonds (δ 130.1, 131.4, 134.0, 134.1, 139.2 and 150.1) in addition to three oxygenated methines (δ 69.4, 80.0 and 67.6). The connectivities of each proton and carbon were established by detailed analysis of ^{1}H–^{1}H COSY and HMBC spectra (Table 1) of **1**. A multiplet at δ 2.67, assigned to H-14β, correlated with H-14α (δ 2.02) and a doublet at δ 5.26 (H-13). The H-2 (δ 5.76) showed correlation with a doublet of doublets at δ 6.35 (H-20). Two isolated spin systems were also found. One set was a doublet of doublets at δ 6.76 (H-7) and a doublet at δ 6.29 (H-6). Another characteristic AB spin pattern was located at 3.08 ppm and 2.60 ppm, which were assigned to the C-3 methylene protons. The remaining singlet at δ 6.08 was therefore assigned to H-10. On the basis of COSY experiments, the structure of **1** was similar to that of **2**, which belongs to 2(3→20) abeotaxanes.[3,8] However, the C ring in compound **1** was an α,β-unsaturated cyclohexenone rather than a cyclohexane as in **2**. In the HMBC spectrum, correlations between Me19 and C-3 (δ 35.5), C-7 (δ 150.1), C-8 (δ 52.5), C-9 (δ 202.8) and between H-3, H-7, H-20 and C-5 (δ 189.1) revealed that **1** possessed an α,β-unsaturated cyclohexenone (ring C). This moiety was corroborated by an UV absorption at 240 nm.[15] HMBC correlations of H-2/Me16/Me17/C-1 (δ 47.8), H-10/Me19/C-9 and H-10/Me16/Me17/Me18/C-11 (δ 130.1) as well as H-2/C-14 (δ 26.8) agreed with a ten membered ring (ring B). Correlations of H-1/ Me18/C-13 (δ 67.6), H-10/Me16/C-12 (δ 139.2) and H-10/Me16/Me17/C-15 (δ 37.6) indicated that **1** contained a geminal dimethylcyclohexene moiety (ring A).

The relative stereochemistry of **1** was determined by analysis of NOESY spectrum. Correlations between H-1/H-2, H-13/H-14β, H-1/H-14β, H-1/Me17 and Me16/Me17 agreed with a β-configuration for H-2 and H-13. NOESY correlations among H-10/H-7/Me-18 in **1** suggested H-10 was in α-orientation. These findings were consistent with an unusual cage conformation previously reported for taxuspine B and other taxin B derivatives.[3—5] Thus the configurations in **1** are the same as those of 2(3→20) abeo-taxanes and its possible conformation is indicated in Fig. 1. To confirm the assigned structure **1**, **2** was oxidized with CrO_3/pridine to yield 5-dehydrotaxin B (**3**) and a compound identical with **1**.

Compound **4** was obtained as an amorphous solid. The ^{1}H-NMR spectrum of **4** resembled that of *trans-p*-coumaroyl myo-inositol, suggesting that **4** was an isomer. An AX spin system at δ 6.00 and δ 7.12 (d, J=12.6 Hz) in **4**, rather than the two doublets at δ 6.37 and δ 7.62 (d, J=16 Hz) in *trans-p*-coumaroyl myo-inositol, indicated the presence of a *cis-p*-coumaroyl moiety in **4**. Compound **4** seems to be unstable and it decomposed after ^{1}H-NMR measurement.

Taxumairone A is a new 2(3→20) abeo-taxane having an α,β-unsaturated carbonyl system at C-5, C-6 and C-7 plus an exocyclic double bond at C-4/C-20. This compound was believed to be a natural product because it was also obtained from another batch of ripe seeds of *T. mairei* under a normal

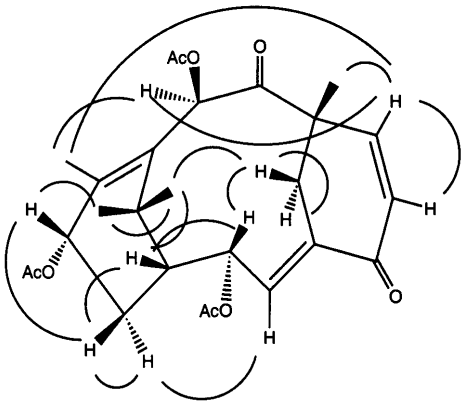

Fig. 1. NOESY and Proposed Comformation of **1**

fractionated procedure. Furthermore, taxumairone A exhibited significant cytotoxicity against human colon (COLO-205) and epidermoid carcinoma (KB) cells with an ED_{50} of 0.1 and 1.84 μg/ml, respectively.

Experimental

Optical rotations were measured with a JASCO DIP-1000 polarimeter. IR and UV spectra were recorded with a HORIBA FT-720 and a HITACHI U-3210 spectrophotometer, respectively. EI and FAB-MS spectra were measured with a VG Quattro 5022 mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded using a Varian FT-300 or a Bruker AM-400 NMR instrument.

Plant material The seeds of *Taxus mairei* were collected in December, 1997, in Tien-shiung, Hua-lein County, Taiwan and were identified by one of the authors (Y. C. S.). A voucher specimen of seeds (TPG8-2) is deposited in the Institute of Marine Resources, National Sun Yat-sen University.

Extraction and Isolation Unripe fresh seeds (1.1 kg) of *T. mairei* were ground and extracted with EtOH to afford a crude extract (117 g), which was partitioned between *n*-hexane (600 ml) and 25% aq. MeOH (600 ml) to yield an *n*-hexane-soluble fraction (12 g). The 25% aq. MeOH was extracted with *n*-butanol to give an *n*-butanol-soluble fraction (65 g). The *n*-hexane-soluble fraction was chromatographed on a Si gel column (150 g) and eluted with *n*-hexane–EtOAc of increasing polarity to give five fractions, A (mixture of 5α-cinnamoyloxy-9α,10β,13α-triacetoxy-taxa-4(20)11-diene and 5α-cinnamoyloxy-2α,9α,10β,13α-tetraacetoxy-taxa-4(20)11-diene, 625 mg), B (taxinine, 890 mg), C (0.61 g), D (1 g) and E (0.46 g). Fraction C was rechromatographed on a Si gel column and eluted with *n*-hexane–$CHCl_3$–MeOH (100 : 100 : 1) to yield decinnamoyl-taxinine E and a residue (0.18 g). Further separation of the residue by HPLC column (Si gel, *n*-hexane–$CHCl_3$–MeOH, 10 : 10 : 1) gave taxinine A (4 mg), taxin B (9 mg) and a less polar fraction (74 mg). The latter was separated by PTLC (Si gel) developed with *n*-hexane–$CHCl_3$–MeOH (10 : 10 : 1) to afford two fractions a (16 mg) and b (39 mg). Fraction a was separated by ODS HPLC (LiChrosorb RP-18 column, 90% aq. MeOH) to yield taxumairone A (**1**, 4 mg) taxinine A and taxuspine X (2 mg). Fraction b was also separated by ODS HPLC (90 % aq. MeOH) to provide taxin B (**2**, 11 mg), taxuspine X and taxezopidine G (16 mg). Part of the *n*-butanol-soluble fraction (5.3 g) was applied to a Sephadex LH-20 and eluted with MeOH to give a residue, which was chromatographed by a reverse-phase C_{18} column (H_2O/MeOH, 20 : 1 and 10 : 1) to yield myo-inositols of *trans-p*-coumarate ester (100 mg) of and *cis-p*-coumarate ester (**4**, 20 mg).

Taxumairone A (**1**): Isolated as an amorphous solid, $[\alpha]_D^{26}$ −306° (c= 0.26, $CHCl_3$); FAB-MS: *m/z* 495 $[M+Na]^+$; UV λ_{max} (log ε) (MeOH) nm: 208 (3.71), 240 (3.28); IR (KBr) cm^{-1}: 2929, 1736, 1674, 1666, 1635, 1612, 1439, 1234, 1022, 968, 840, 737, 663; ^{1}H- and ^{13}C-NMR: Table 1. EIMS *m/z* (rel. int.): 472 (0.02, M^+), 430 (0.2), 413 (0.2), 371 (0.3), 325 (1), 311 (1), 295 (0.8), 282 (3.6), 264 (10), 249 (9), 239 (2.8), 217 (3.7), 197 (3.5), 175 (1), 160 (17), 145 (18), 133 (17), 121 (25), 105 (17), 91 (15), 83 (21), 77 (13), 69 (11), 55 (19); HR-FAB-MS *m/z*: 473.2158 $[M+H]^+$ (Calcd for $C_{26}H_{33}O_8$, 473.2175), *m/z*: 495.1976 $[M+Na]^+$ (Calcd for $C_{26}H_{32}O_8Na$, 495.1995).

Oxidation of Taxin B (**2**): To a solution of **2** (8 mg) in $CHCl_3$ was added CrO_3 (40 mg) and pyridine (2 drops), and the reaction mixture was stirred for 30 min at room temperature. After filtration to remove the CrO_3, the filtrate was concentrated to give a residue. Purification of the residue by HPLC (LiChrosorb RP-18 column, 80% aq. MeOH) yielded 5-dehydrotaxin B (**3**, 5 mg): an amorphous solid, $[\alpha]_D^{26}$ −224° (c=0.9, $CHCl_3$); FABMS: *m/z* 555 $[M+Na]^+$; HR-EI-MS *m/z*: 532.2309 (Calcd for $C_{28}H_{36}O_{10}$, 532.2308); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.78 (1H, m, H-1), 5.70 (1H, dd, J=2.2, 10.2 Hz, H-2), 2.40 (1H, d, J=14, 1.5 Hz, H-3α), 2.91 (1H, d, J=14 Hz, H-3β), 2.80 (1H, d, J=10 Hz, H-6β), 5.42 (1H, dd, J=3.9, 12.6 Hz, H-7), 6.50 (1H, s, H-10), 5.28 (1H, d, J=10.2 Hz, H-13), 2.70 (1H, m, H-14β), 1.32 (3H, s, H-16), 1.14 (3H, s, H-17), 1.83 (3H, s, H-18), 1.39 (3H, s, H-19), 5.26 (1H, dd, J=1.6, 10.2 Hz, H-20), 2.04, 2.09, 2.17, 2.20 (3H×4, s, $OCOCH_3$); EI-MS *m/z* (rel. int.): 532 (0.1, M^+), 504 (0.3), 473 (1.7), 430 (1.2), 413 (0.9), 384 (1.1), 371 (2.6), 342 (2.8), 324 (3.1), 300 (2.2), 282 (10), 249 (6), 239 (4), 217 (6.3), 175 (9), 160 (9), 145 (14), 133 (15), 121 (22), 105 (12), 91 (9.4), 79 (5.6), 55 (8.1). Compound **2** (5 mg) in pyridine (1 ml) was reacted with CrO_3 (20 mg)/pyridine (0.033 ml)/$CHCl_3$ (0.25 ml) and the reaction mixture was stirred for 30 min. Work-up and separation by HPLC as mentioned above (LiChrosorb RP-18 column, 80% aq. MeOH) yielded 5-dehydrotaxin B (**3**, 1 mg, t_R 11.92 min) and a compound (0.5 mg, t_R 9.96 min), which showed identical spectral data with those of **1**.

Cis-p-coumaroyl myo-inositol (**4**): Isolated as an amorphous powder, ^{1}H-NMR δ (300 MHz, CD_3OD): 3.3—3.8 (5H, m, H-2, 3, 4, 5, 6), 5.50 (1H, m, H-1), 6.00 (1H, d, J=12.6 Hz, H-2′), 7.12 (1H, d, J=12.6 Hz, H-3′), 6.90 (2H, H-6′, 8′), 7.54 (2H, H-5′, 9′).

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A New 3,4-*seco*-Ursane Triterpenoid from *Glyptopetalum sclerocarpum*

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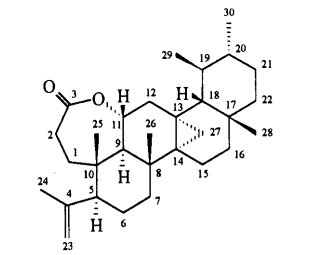
A new ursane-type triterpene, glyptopetolide, was isolated along with two known triterpenoids, isoarborinol and cangoronine, from the stem bark of *Glyptopetalum sclerocarpum* LAWS. (Celastraceae). The structure of glyptopetolide was elucidated as 3,4-*seco*-14α,27-cyclo-urs-4(23)-en-3,11α-olide by spectroscopic analysis.

Key words *Glyptopetalum sclerocarpum*; Celastraceae; triterpenes; glyptopetolide

In the northeastern part of Thailand, folk herbalists use the bark of *Glyptopetalum sclerocarpum* LAWS., a tree belonging to the family Celastraceae, to treat skin infection and malaria. Our previous investigations have led to the isolation of a number of new quinone-methide triterpenes and a new sesquiterpene from the stem bark of this plant.[1,2] Terpenoids from this plant were shown to possess cytotoxic,[3] antiviral[2] and antimicrobial activities.[4] In this paper, we report the isolation and structure elucidation of a novel *seco*-ursane triterpenoid named glyptopetolide (**1**), along with two known triterpenes, isoarborinol (**2**) and cangoronine (**3**), from the *n*-hexane extract of the bark of this plant.

Gompound (**1**) was obtained as colorless needles, mp 288—290 °C, $[\alpha]_D$ +58° (c=0.08, $CHCl_3$) and its molecular formula was determined as $C_{30}H_{46}O_2$ based on elemental analysis, EI-MS, ^{1}H- and ^{13}C-NMR, and distortionless enhancement by polarization transfer (DEPT) experiments. The ^{1}H-NMR spectrum displayed the presence of four methyl singlets at δ_H 0.89, 1.10, 1.13 and 1.74, two methyl doublets at δ_H 0.88 and 1.03, and two doublets of the geminally-coupled protons of a cyclopropane ring at δ_H 0.09 and 1.09. The chemical shifts of the two methyl doublets (H_3-29, d, J= 6.4 Hz and H_3-30, d, J=5.8 Hz) were indicative of the attachment of these groups at C-19 and C-20 of the ursane basic structure.[5,6] The broad methyl singlet at δ_H 1.74, together with a doublet at δ_H 4.69 (1H, J=1.8 Hz) and a quintet at δ_H 4.86 (1H, J=1.8 Hz), were characteristic of an isopropenyl group, which was also supported by IR bands at 1634 and 909 cm^{-1}. In the heteronuclear multiple bond correlation (HMBC) spectrum, long-range correlations could be observed between the downfield methyl signal (δ_H 1.74, H_3-24) and C-23 methylene carbon (δ_C 114.3), as well as between C-5 (δ_C 57.5) and each proton signal of the isopropenyl moiety, suggesting the 3,4-*seco* nature of ring A of this triterpenoid.

The ^{13}C-NMR signal of a carbonyl carbon (C-3) at δ_C 176.0 and the IR absorption bands at 1736 and 1296 cm^{-1} indicated the presence of a lactone ring as part of the structure of **1**. Three-bond correlation between this carbon signal and the deshielded H-11 methine proton at δ_H 4.44 suggested the formation of an ester linkage between the positions 3 and 11, creating a seven-membered lactone ring. The orientation of the lactonyloxy proton could be assigned as α according to the nuclear Overhauser effect spectroscopy (NOESY) cross peak observed between this proton and H_3-25. Another cross peak between H-12β and H_3-26 indicated the C-ring as adopting the boat conformation, as shown in Fig. 2. This re-



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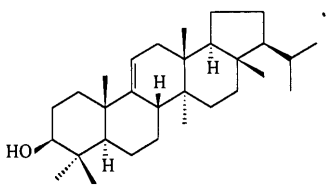

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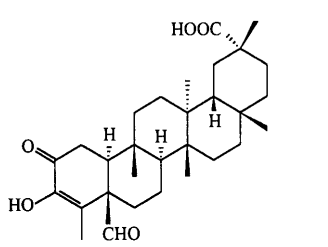

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Fig. 1

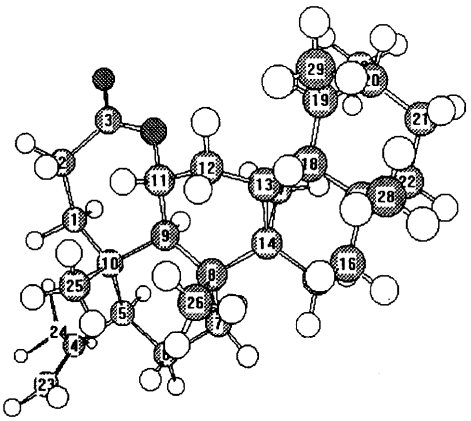

Fig. 2. Conformational Drawing of **1**

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Table 1. NMR Data of **1**

	^{1}H	^{13}C	HMBC	NOESY
1α	1.39 t (J=14.4 Hz)	40.9 t	C-3, C-10, C-25	H-5, H-9
1β	1.82 ddd (J=14.4, 7.8, 1.5 Hz)		C-3, C-9, C-10, C-25	H-2α, H$_3$-25
2α	2.38 ddd (J=14.4, 7.8, 1.1 Hz)	24.3 t	C-1, C-3, C-10	H-1β
2β	2.66 brt (J=14.4 Hz)		C-1, C-3, C-10	H-11, H$_3$-25
3		176.0 s		
4		146.3 s		
5	1.80 m	57.5 d	C-6, C-9, C-10, C-25	H-1α, H-6α, H-9, H-23b
6α	1.43 m	29.6 t		H-5
6β	1.27 m			H-7β
7α	1.30 m	36.5 t		
7β	1.68 dt (J=13.1, 3.1 Hz)			H-6β, H$_3$-26
8		37.0 s		
9	1.63 d (J=7.2 Hz)	53.9 d	C-5, C-7, C-8, C-10, C-11, C-14, C-25, C-26	H-1α, H-5, H-27a
10		38.8 s		
11	4.44 t (J=7.2 Hz)	74.1 d	C-3, C-9, C-10, C-13	H-2β, H-12α, H$_3$-25
12α	2.19 d (J=16.2 Hz)	39.6 t	C-9, C-11, C-13, C-14	H-11, H-18, H$_3$-29
12β	2.30 dd (J=16.2, 6.7 Hz)		C-13, C-27	H-18, H$_3$-26, H$_3$-28
13		23.9 s		
14		30.0 s		
15α	1.49 dd (J=13.4, 5.4 Hz)	21.0 t	C-13, C-14, C-17	H-16α
15β	1.73 m		C-27	
16α	1.37 m	27.3 t	C-17, C-28	H-15α
16β	0.79 ddt (J=13.4, 6.4, 1.5 Hz)		C-14, C-17, C-18	
17		31.6 s		
18	0.74 dd (J=10.5, 1.4 Hz)	50.3 d	C-12, C-13, C-16, C-17	H-12α, H-12β, H$_3$-28, H$_3$-29
19	0.88 m	41.3 d		
20	0.99 m	38.3 d	C-30	H-22β, H$_3$-29, H$_3$-30
21α	1.31 m	31.0 t		
21β	1.78 m			
22α	1.02 m	42.0 t	C-28	
22β	1.32 m			H-20, H$_3$-28
23a	4.86 quintet (J=1.8 Hz)	114.3 t	C-5, C-24	H$_3$-24
23b	4.69 d (J=1.8 Hz)		C-5, C-24	H-5, H$_3$-24
24	1.74 brs	23.4 q	C-4, C-5, C-23	H-23a, H$_3$-25
25	1.10 s	17.5 q	C-1, C-5, C-9, C-10	H-1β, H-2β, H-11, H-23b, H$_3$-24, H$_3$-26
26	1.13 s	17.5 q	C-7, C-8, C-9, C-14	H-12β, H$_3$-25, H$_3$-28
27a	1.09 d (J=6.1 Hz)	10.5 t	C-13, C-18	H-9
27b	0.09 d (J=6.1 Hz)		C-8, C-12, C-13, C-15, C-18	
28	0.89 s	28.2 q	C-16, C-17, C-18, C-22	H-18, H-22β, H$_3$-26, H$_3$-29
29	1.03 d (J=6.4 Hz)	17.9 q	C-18, C-19, C-20	H-12α, H-18, H-20, H$_3$-28, H$_3$-30
30	0.88 d (J=5.8 Hz)	20.6 q	C-19, C-20, C-21	H-20, H$_3$-29

sulted in the absence of the coupling between H-11 and H-12α, due to their dihedral angle of nearly 90°, and a dihedral angle of about 140° between H-9 and H-11, producing a smaller J value for these *trans* di-axial protons than might be expected.

The location of the cyclopropane ring at the ring C/D junction could be confirmed through long-range correlations observed of the most upfield proton signal at δ_H 0.09 (1H, d, J=6.1 Hz, H-27b) with the carbon signals at δ_C 37.0 (C-8), 39.6 (C-12), 23.9 (C-13), 21.0 (C-15) and 50.3 (C-18), and of both proton signals at δ_H 2.30 (1H, dd, J=16.2, 6.7 Hz, H-12β) and 1.73 (1H, m, H-15β) with the C-27 carbon signal at δ_C 10.5. A NOESY experiment showed a cross peak between H-9 and H-27a, indicating the orientation of the cyclopropane ring as α. Therefore, the structure of **1** was elucidated as the novel triterpenoid 3,4-*seco*-14α,27-cyclo-urs-4(23)-en-3,11α-olide, and named as glyptopetolide. A 3,4-*seco*-ursane-type triterpenoid possessing a cyclopropane ring at the ring C/D junction is very rare in natural products.

Compounds **2** and **3** were identified as the triterpenoids isoarborinol (**2**)[7,8] and cangoronine (**3**),[9,10] respectively, by comparison of their physical and spectroscopic data with the literature. Compound **2** has been reported as a constituent of several different plant families, whereas **3** is found mainly in the family Celastraceae.

Experimental

All melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin Elmer 341 polarimeter. UV spectra were determined in methanol solution using a Hitachi U-2000 spectrophotometer. IR spectra were taken on a Nicolet Magna-IR Spectrometer 750. Mass spectra (EI-MS) were measured on a JEOL DX300 mass spectrometer. NMR spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a JEOL JNM-A500 (Alpha series) spectrometer, and chemical shifts are given on a δ (ppm) scale with tetramethylsilane as an internal standard. Column chromatography was carried out on Kieselgel 60 (230—400 mesh, Merck) and Sephadex LH-20 (Pharmacia).

Extraction and Isolation The stem bark of *G. sclerocarpum* was collected in May 1993 in Chaiyaphum province, Thailand, and identified by Dr. Thawatchai Santisuk (Royal Forest Department, Bangkok, Thailand). The voucher specimen (RB-93051) is deposited with the Herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The *n*-hexane extract (49.4 g) of the dried stem bark (700 g) was dissolved in a small amount of $CHCl_3$. Addition of acetone produced a gum, which was then removed. Crystallization of the

filtrate in acetone yielded the quinone-methide triterpenes, as described in a previous paper.[1] The dried mother liquor (39.6 g) was subjected to a Sephadex LH-20 column eluted with $CHCl_3$–MeOH (2 : 1) to give 4 fractions (F1—F4). Fraction F3 (12.2 g) was dissolved in MeOH and partitioned with *n*-hexane. The *n*-hexane soluble part (9.2 g) was chromatographed over a silica gel column, eluted with $CHCl_3$–MeOH (30 : 1), to provide 2 fractions. The first fraction (600 mg) was further separated with silica gel [$CHCl_3$, *n*-hexane–$CHCl_3$–acetone (2 : 1 : 0.1)] to yield glyptopetolide (**1**) (50 mg) and isoarborinol (**2**) (90 mg). On the other hand, the *n*-hexane insoluble part (2.3 g) was combined with fraction F2 (7.4 g) and subjected to silica gel column chromatography using a *n*-hexane–acetone gradient system to give 4 fractions (A—D). Elution of fraction D (1.1 g) with *n*-hexane–EtOAc (5 : 1→1 : 2) gave a residue (800 mg), which was purified by two successive silica gel columns, using *n*-hexane–EtOAc–acetone (8 : 1 : 1) and *n*-hexane–$CHCl_3$–MeOH (2 : 8 : 0.3), respectively, as eluents to afford cangoronine (**3**) (12 mg).

Glyptopetolide (**1**): Colorless needles ($CHCl_3$), mp 288—290 °C, $[\alpha]_D$ +58° (*c*=0.08, $CHCl_3$). IR (thin film) cm^{-1}: 3200—3700, 2924, 1736, 1634, 1296. EI-MS *m/z*: 438 (M^+), 423, 355, 337, 281, 255, 171, 157, 145, 119, 105. *Anal.* Calcd for $C_{30}H_{46}O_2$: C, 82.14; H, 10.57. Found: C, 81.76; H, 10.48. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: Table 1 and ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: Table 1.

Isoarborinol (**2**): Colorless needles (acetone), mp 280—284 °C, $[\alpha]_D$ +35° (*c*=0.08, $CHCl_3$). The identification was made by comparison of the ^{1}H- and ^{13}C-NMR data with those reported in the literature.[7,8]

Cangoronine (**3**): Colorless needles (acetone), mp 310—314 °C. The identification was made by comparison of the ^{1}H- and ^{13}C-NMR data with those reported in the literature.[9,10]

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Spirostanol Sapogenins from the Underground Parts of *Tupistra chinensis*

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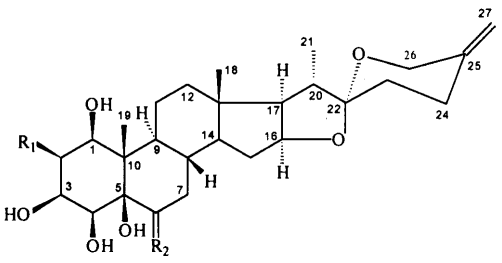
Chemical examination of the underground parts of *Tupistra chinensis* led to the isolation of two new 5β-spirostane type steroidal sapogenins, tupichigenin B (1) and C (2), together with two known steroidal sapogenins, ranmogenin A (3) and $\Delta^{25(27)}$-pentrogenin (4). The structures of 1 and 2 were established as spirost-25(27)-ene-1β,3β,4β,5β,6β-pentaol and 1β,2β,3β,4β,5β-pentahydroxyspirost-25(27)-en-6-one, respectively, on the basis of detailed analysis of their physical and spectral data.

Key words *Tupistra chinensis*; Liliaceae; steroidal sapogenin; spirostanol sapogenin; tupichigenin B, C

Tupistra chinensis BAKER (Liliaceae) is mainly distributed in southwestern China.[1] As a folkloric Chinese medicine, this species has usually been used to treat rheumatic diseases and snake-bite.[1] This species is used as a substitute for *Euphorbia helioscopia* L. (Euphorbiaceae) in Taiwan. However, to our knowledge, these two species show different chemical constituents. Therefore, the biological activities of these two species need to be further investigated. In a previous paper, we have reported the isolation and structural elucidation of two steroidal sapogenins, tupichigenin A and 1β,2β,3β,4β,5β,7β-hexahydroxyspirost-25(27)-en-6-one from *T. chinensis*.[2] In a continuation of our investigation of the constituents of *T. chinensis*, we describe here the isolation and structural elucidation of two new 5β-spirostane type steroidal sapogenins, spirost-25(27)-ene-1β,3β,4β,5β,6β-pentaol (**1**) and 1β,2β,3β,4β,5β-pentahydroxyspirost-25(27)-en-6-one (**2**), as well as two known steroidal sapogenins, spirost-25(27)-ene-1β,3β,4β,5β-tetraol (ranmogenin A) (**3**)[3,4] and spirost-25(27)-ene-1β,2β,3β,4β,5β-pentaol ($\Delta^{25(27)}$-pentrogenin) (**4**).[3,4] Steroidal sapogenins are of great commercial utility as starting materials in the synthesis of a variety of steroid hormones.[5] The 5β-spirostanol sapogenins and saponins (AB *cis* ring junction) which are widely distributed in a large number of higher plants, especially in Liliaceae, have attracted great research interests not only for their chemistry but also for such biological activities as inhibitory effects on human spermatozoa,[6] inhibition of platelet aggregation,[7] reduction of blood glucose level,[8] molluscicidal activities,[9,10] spasmolytic activity in rat duodenum,[11] and *in vitro* inhibitory activity on cAMP phosphodiesterase.[12]

Compound **1**, obtained as white microneedles, $[\alpha]_D^{24}$ −0.1° (*c*=0.002, pyridine), showed in the HR-FAB-MS (positive mode) a *pseudo*molecular $[M+Na]^+$ peak at *m/z* 501.2832 (Calcd 501.2829), consistent with the molecular formula $C_{27}H_{42}O_7$. The IR spectrum showed a strong absorption at 3374 cm^{-1} due to hydroxyl groups, but lacked the characteristic bands of the spirostane ring.

Unambiguous complete assignments for the ^1H- and ^{13}C-NMR signals were made by combination of distortionless enhancement by polarization transfer (DEPT), ^1H–^1H correlated spectroscopy (^1H–^1H COSY), heteronuclear chemical shift correlation (HETCOR) and nuclear Overhauser and exchange spectroscopy (NOESY) spectra. The ^1H-NMR spectrum (Table 1) in pyridine-d_5 of **1** showed signals for two tertiary methyl groups at δ 1.90 (3H, s, Me-19) and 0.89 (3H, s, Me-18), and a secondary methyl group at δ 1.10 (3H, d, *J*=6.8 Hz, Me-21). The ^{13}C-NMR spectrum (Table 1) showed a total of 27 carbon signals, which were assigned by DEPT as three methyls, nine methylenes, ten methines (including five oxygenated methines), and five quaternary carbons. The carbonyl resonance at δ 109.4 (C) was assigned to C-22 of the spirostanol skeleton. Two signals at δ 144.4 (C) and 108.7 (CH_2) were assigned to the C-25 and C-27 positions,[13] respectively. Three diagnostic signals at δ 81.4 (CH), 65.0 (CH_2) and 63.1 (CH) were assigned to the C-16, C-26, and C-17 positions, respectively.[14] These ^1H-NMR data and ^{13}C-NMR signals suggested that **1** is a C-25(27) unsaturated spirostane type steroidal sapogenin. The oxygenated methine protons at δ 4.17 (1H, br s), 4.56 (1H, dd, *J*=3.6, 2.8 Hz), 4.21 (1H, d, *J*=3.6 Hz) and 4.90 (1H, br s) were assigned to H-1, H-3, H-4, and H-6, respectively. The methylene protons at δ 2.13 (1H, m, H-2$_\alpha$) and δ 2.53 (1H, dt, *J*=14.8, 2.8 Hz, H-2$_\beta$) were determined, and were shown to be coupled to both of the two oxygenated methine protons at δ 4.17 (H-1) and δ 4.56 (H-3) in the ^1H–^1H COSY spectrum. The oxygenated methine proton at δ 4.21 (H-4) was in turn coupled with the oxygenated methine proton at δ 4.56 (H-3). The oxygenated methine proton at δ 4.90 was assigned to H-6, which was coupled with two methylene protons at δ 1.56 (1H, td, *J*=14.0, 3.6 Hz, H-7$_\alpha$) and δ 2.09 (1H, m, H-7$_\beta$). These findings supported the placement of four hydroxyl groups on C-1, C-3, C-4, and C-6 positions. Furthermore, four signals at δ 75.0 (CH), 71.1 (CH), 69.5 (CH), and 69.5 (CH) were assigned to the C-1, C-3, C-4, and C-6 positions, respectively, by HETCOR spectrum. The coupling patterns of H-1 at δ 4.17 (br s), H-3 at δ 4.56 (dd, $J_{3\alpha,4\alpha}$=3.6,



1: R_1=H, R_2=β-OH, H **3**: R_1=H, R_2=H, H
2: R_1=β-OH, R_2=O **4**: R_1=β-OH, R_2=H, H

Chart 1

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Table 1. ^{13}C-NMR and ^{1}H-NMR Data for **1** and **2** (100 and 400 MHz in Pyridine-d_5)

Position	**1**		**2**	
	δ_C	δ_H, J (Hz)	δ_C	δ_H, J (Hz)
1	75.0, d	4.17, br s	75.8, d	4.28, br s
2	33.1, t	2.53, dt (14.8, 2.8), H_α 2.13, m, H_β	67.3, d	4.33, br s
3	71.1, d	4.56, dd (3.6, 2.8)	74.9, d	4.79, br s
4	69.5, d	4.21, d (3.6)	70.8, d	4.83, d (3.6)
5	79.2, s		85.5, s	
6	69.5, d	4.90, br s, H_α	210.7, s	
7	35.4, t	1.56, td (14.0, 3.6), H_α 2.09, m, H_β	42.1, t	2.50, dd (13.6, 4.0)
8	30.3, d	2.40, qd (11.2, 3.6)	37.3, d	2.01, m
9	45.7, d	1.30, td (11.2, 4.0)	44.4, d	1.89, m
10	45.5, s		49.6, s	
11	21.3, t	1.40, d (14.0), H_α 1.50, m, H_β	21.6, t	1.58, m, H_α 1.50, m, H_β
12	40.0, t	1.14, m, H_α 1.71, m, H_β	39.0, t	1.12, m, H_α 1.67, m, H_β
13	40.7, s		40.6, s	
14	56.1, d	1.18, m	55.6, d	1.46, m
15	32.2, t	2.07—2.10, m, H_α 1.45, m, H_β	31.5, t	1.97, m, H_α 1.39, m, H_β
16	81.4, d	4.61, m	80.8, d	4.52, m
17	63.1, d	1.88, m	62.2, d	1.81, t (8.0)
18	16.5, q	0.89, s	16.0, q	0.72, s
19	16.3, q	1.90, s	12.8, q	1.31, s
20	42.0, d	1.98, quin. (6.8)	41.5, d	1.91, m
21	15.0, q	1.10, d (6.8)	14.5, q	1.02, d (6.8)
22	109.4, s		109.4, s	
23	33.2, t	1.74, m	32.7, t	1.74, m
24	28.9, t	2.70, td (13.2, 5.6), H_{ax} 2.23, td (13.2, 2.4), H_{eq}	28.5, t	2.61, d (12.8), H_{ax} 2.22, d (12.8), H_{eq}
25	144.4, s		143.7, s	
26	65.0, t	4.47, d (12.0), H_{ax} 4.04, d (12.0), H_{eq}	64.8, t	4.38, d (12.0), H_{ax} 3.99, d (12.0), H_{eq}
27	108.7, t	4.78, s, H_A 4.82, s, H_B	108.8, t	4.75, s, H_A 4.79, s, H_B

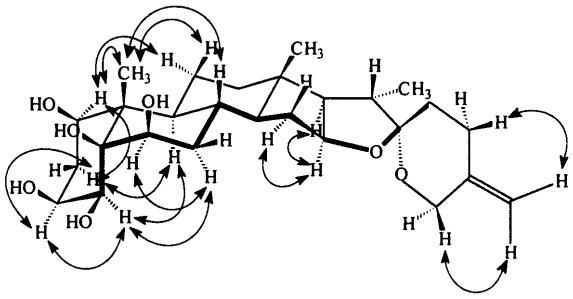

Fig. 1. NOESY Correlations of **1**

$J_{3\alpha,2\alpha}$=2.8 Hz) and H-6 at δ 4.90 (br s) indicated that H-1, H-3 and H-6 are α-equatorial.

The relative stereochemistry of **1** was also established by NOESY correlation, as shown in Fig. 1. NOESY correlations between H-4$_\alpha$ and H-7$_\alpha$/H-9$_\alpha$, between H-1$_\alpha$ and Me-19, and between H-2$_\alpha$ and H-9$_\alpha$ supported the A/B *cis* ring junction pattern and also indicated α-axial configurations of H-2, H-4, H-7, and H-9. Thus, the hydroxyl group at C-5 has a β-orientation and the signal at δ 79.2 (C) was assignable to the C-5 position. The proton at δ 4.61 (1H, m) was assigned to the H-16 position.[15] NOESY correlations between H-16 and H-15$_\alpha$/H-17 indicated that H-16, H-15$_\alpha$ and H-17 were *cis* to each other and oriented α. This fact also supported the D/E *cis* ring junction pattern. The protons at δ 4.04 (1H, d, J=12 Hz) and δ 4.47 (1H, d, J=12 Hz) were assigned to H-26$_{eq}$ and H-26$_{ax}$,[15] respectively. The geminal protons at C-27 were observed at δ 4.78 and δ 4.82 as two singlets, and coupling constants of approximately 0 Hz were characteristic of an exocyclic methylene.[15] The methylene group at C-26 appeared as two doublets. In the NOESY spectrum, cross peaks were observed between δ 4.78 (H-27$_A$) and δ 2.23 (H-24$_{eq}$), and between δ 4.82 (H-27$_B$) and δ 4.04 (H-26$_{eq}$). These properties further confirmed the presence of an exocyclic methylene group at C-25. On the basis of the above spectroscopic evidence, the structure of compound **1** was deduced to be spirost-25(27)-ene-1β,3β,4β,5β,6β-pentaol, which we have named Tupichigenin B.

Compound **2** was obtained as white microneedles, $[\alpha]_D^{24}$ −10.3° (c=0.02, $CHCl_3$). The HR-FAB-MS (positive mode) gave a *pseudo*molecular $[M+H]^+$ ion at m/z 493.2810 (Calcd 493.2801), consistent with the molecular formula $C_{27}H_{40}O_8$. The ^{1}H- and ^{13}C-NMR spectral data of **2** are shown in Table 1. All signals were assigned unequivocally according to DEPT, ^{1}H–^{1}H COSY, ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC), heteronuclear multiple-bond connectivity (HMBC) and NOESY analysis. The ^{1}H-NMR spectrum in pyridine-d_5 of **2** was similar to that of $\Delta^{25(27)}$-pentrogenin (**4**). Two methylene proton signals (δ 1.47 and

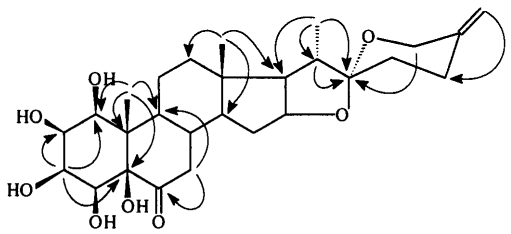

Fig. 2. HMBC Correlations (H to C) of **2**

1.11), assigned to the methylene at C-6 in **4**, disappeared in **2**. The ^{13}C-NMR spectrum of **2** revealed 27 carbon signals, which were assigned by DEPT as three methyls, eight methylenes, ten methines (including five oxygenated methines), and six quaternary carbons. The ^{13}C-NMR spectrum of **2** showed good similarity with that of **4** except the signals of C-5 to C-10. The presence of a carbonyl group in **2** was recognized by the IR (1711 cm^{-1}) and ^{13}C-NMR spectra (δ 210.7). The ketone functionality at C-6 was confirmed by its HMBC correlation to resonance at δ 2.50 (H_2-7). In turn, H_2-7 showed an additional correlation to the methine carbon at δ 44.4 (C-9), as shown in Fig. 2. The downfield shift of the quaternary carbon at C-5 from **4** (δ 77.7) to **2** (δ 85.5) and the downfield shift of the methylene carbon at C-7 from **4** (δ 30.1) to **2** (δ 42.1) also confirmed the ketone functionality at C-6. On the basis of the above spectroscopic evidence, the structure of compound **2** was confirmed to be 1β,2β,3β,4β,5β-pentahydroxyspirost-25(27)-en-6-one, which we have named Tupichigenin C.

Compound **3** and $\Delta^{25(27)}$-pentrogenin (**4**) were known steroidal sapogenins and identified by FAB-MS, IR, ^{1}H- and ^{13}C-NMR spectra, and by two-dimensional NMR spectral data as spirost-25(27)-ene-1β,3β,4β,5β-tetraol and spirost-25(27)-ene-1β,2β,3β,4β,5β-pentaol, respectively.[3,4] The ^{1}H-NMR data of the compounds **3** and **4** were not revealed in the previous report,[3,4] and their chemical shift assignments of C-23 and C-24 in the ^{13}C-NMR data needed to be revised. We reported herein the complete spectral data of **3** and **4** in the experimental section.

Experimental

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were obtained in MeOH using a JASCO V-530 spectrophotometer. Melting points were determined using a Yanagimoto micro-melting point apparatus and are uncorrected. The IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra (all in pyridine-d_5) were recorded with Varian NMR spectrometers, using TMS as an internal standard. LR-FAB-MS and LR-EI-MS spectra were obtained with a JEOL JMS-SX/SX 102A mass spectrometer or a Quattro GC/MS spectrometer with a direct inlet system. HR-FAB-MS spectra were measured on a JEOL JMS-HX 110 mass spectrometer. Silica gel 60 (Macherey-Nagel, 230—400 mesh) was used for column chromatography, precoated silica gel plates (Macherey-Nagel, SIL G-25 UV_{254}, 0.25 mm) were used for analytical TLC, and precoated silica gel plates (Macherey-Nagel, SIL G/UV_{254}, 0.25 mm) were used for preparative TLC. The spots were detected by spraying with 50% H_2SO_4 followed by heating on a hot plate.

Plant Material *Tupistra chinensis* was purchased in Kaohsiung, Taiwan, in August 1997, and identified by Professor Yueh-Cherng Li, Sichuan Provincial Laboratory of Drugs, People's Republic of China. A voucher specimen (No. 970808) is deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Separation The air-dried underground parts of *T. chinensis* (17 kg) were extracted repeatedly with MeOH at room temperature. The combined MeOH extracts were evaporated and partitioned to yield *n*-hexane (140 g), $CHCl_3$ (60 g), EtOAc (100 g), *n*-BuOH (130 g), and aqueous (280 g) extracts. A portion of the $CHCl_3$ extract was concentrated and chromatographed over silica gel and eluted with *n*-hexane–EtOAc mixtures of increasing polarity to yield eleven fractions. Fraction 6, eluted from *n*-hexane–EtOAc (1 : 7), was further chromatographed on silica gel elution with $CHCl_3$–MeOH (15 : 1) and recrystallized with $CHCl_3$–MeOH (15 : 1) to afford compound **3** (30 mg, 0.05% dry weight), then eluted with $CHCl_3$–MeOH (10 : 1) and recrystallized with $CHCl_3$–MeOH (15 : 1) to afford compound **1** (150 mg, 0.25% dry weight). Fraction 6 was further chromatographed on silica gel elution with $CHCl_3$–MeOH (8 : 1) and recrystallized with $CHCl_3$–MeOH (15 : 1) to afford compound **2** (290 mg, 0.48% dry weight). Fraction 7, eluted from *n*-hexane–EtOAc (1 : 10), was further chromatographed on silica gel elution with $CHCl_3$–MeOH (16 : 1) and recrystallized with $CHCl_3$–MeOH (20 : 1) to afford compound **4** (75 mg, 0.13% dry weight).

Tupichigenin B (**1**): White microneedles, mp 247—248 °C, $[\alpha]_D^{24}$ −0.1° (c=0.002, pyridine). Positive FAB-MS (positive mode) *m/z*: 501 $[M+Na]^+$. HR-FAB-MS *m/z*: Found 501.2832 $[M+Na]^+$ (Calcd for $C_{27}H_{42}O_7Na$ 501.2829). IR ($CHCl_3$) ν_{max} cm^{-1}: 3374 (OH), 3020, 2936, 2400, 1522, 1422, 1216, 1047, 928. ^{1}H-NMR (400 MHz, pyridine-d_5) and ^{13}C-NMR (100 MHz, pyridine-d_5) data see Table 1.

Tupichigenin C (**2**): White microneedles, mp 252—253 °C, $[\alpha]_D^{24}$ −10.3° (c=0.02, $CHCl_3$). FAB-MS (positive mode) *m/z*: 515 $[M+Na]^+$. HR-FAB-MS *m/z*: Found 493.28109 $[M+H]^+$ (Calcd 493.28014). IR ($CHCl_3$) ν_{max} cm^{-1}: 3414 (OH), 2945, 2832, 2586, 2517, 2149, 2048, 1711, 1422, 1365, 1224, 1031, 928. ^{1}H-NMR (400 MHz, pyridine-d_5) and ^{13}C-NMR (100 MHz, pyridine-d_5) data see Table 1.

Ranmogenin A (**3**): White microneedles, $[\alpha]_D^{24}$ −24.4° (c=0.06, pyridine). FAB-MS (positive mode) *m/z*: 485 $[M+Na]^+$, 463 $[M+H]^+$. HR-FAB-MS *m/z*: 463.3052 (Calcd for $C_{27}H_{43}O_6$ 463.3060). IR ($CHCl_3$) ν_{max} cm^{-1}: 3363 (OH), 3019, 2946, 1523, 1424, 1365, 1027, 929. ^{1}H-NMR (400 MHz, pyridine-d_5) δ: 5.50 (1H, br s, OH-5), 4.83, 4.79 (each 1H, s, H_2-27), 4.61 (1H, m, H-16), 4.60 (1H, s, H-3$_\alpha$), 4.49 (1H, d, J=12 Hz, H-26$_{ax}$), 4.28 (1H, d, J=2.8 Hz, H-4), 4.22 (1H, br s, H-1), 4.05 (1H, d, J=12 Hz, H-26$_{eq}$), 2.72 (1H, td, J=12.8, 5.6 Hz, H-24$_{ax}$), 2.54, 2.10 (each 1H, dt, J=15.2, 2.8 Hz, H_2-2), 2.49, 1.70 (each 1H, dt, J=13.2, 3.2 Hz, H_2-6), 2.25 (1H, d, J=12.8 Hz, H-24$_{eq}$), 1.61 (3H, s, H_3-19), 1.09 (3H, d, J=7.2 Hz, H_3-21), 0.89 (3H, s, H_3-18). ^{13}C-NMR (100 MHz, pyridine-d_5) δ: 73.8 (C-1), 33.5 (C-2), 71.2 (C-3), 68.1 (C-4), 78.4 (C-5), 30.4 (C-6), 28.5 (C-7), 35.0 (C-8), 45.7 (C-9), 45.4 (C-10), 21.5 (C-11), 40.1 (C-12), 40.7 (C-13), 56.3 (C-14), 32.2 (C-15), 81.4 (C-16), 63.0 (C-17), 16.6 (C-18), 13.9 (C-19), 41.9 (C-20), 15.0 (C-21), 109.4 (C-22), 33.2 (C-23), 29.0 (C-24), 144.4 (C-25), 65.0 (C-26), 108.7 (C-27).

$\Delta^{25(27)}$-Pentrogenin (**4**): White microneedles, $[\alpha]_D^{24}$ −6.1° (c=0.001, pyridine). FAB-MS (positive mode) *m/z*: 501 $[M+Na]^+$. HR-FAB-MS *m/z*: 501.2839 (Calcd for $C_{27}H_{42}O_7Na$ 501.2828). IR ($CHCl_3$) ν_{max} cm^{-1}: 3419 (OH), 3020, 2949, 2400, 1522, 1434, 1216, 1056, 928. ^{1}H-NMR (400 MHz, pyridine-d_5) δ: 4.82 (1H, dd, J=4.0, 3.2 Hz, H-3), 4.80, 4.77 (each 1H, s, H_2-27), 4.56 (1H, dd, J=14.6, 8.0 Hz, H-16), 4.44 (1H, d, J=12.0 Hz, H-26$_{ax}$), 4.33 (1H, d, J=3.2 Hz, H-1), 4.32 (1H, d, J=4.0 Hz, H-4), 4.18 (1H, t, J=3.2 Hz, H-2), 4.02 (1H, d, J=12.0 Hz, H-26$_{eq}$), 2.69 (1H, td, J=12.4, 6.8 Hz, H-24$_{ax}$), 2.45 (1H, m, H-7$_\alpha$), 2.23 (1H, d, J=13.6 Hz, H-24$_{eq}$), 1.98 (1H, m, H-15$_\alpha$), 1.95 (1H, m, H-20), 1.81 (1H, m, H-17), 1.69 (1H, m, H-8), 1.65 (1H, m, H-7$_\beta$), 1.59 (3H, s, H_3-19), 1.56, 0.99 (each 1H, m, H_2-12), 1.47 (1H, m, H-6$_\alpha$), 1.40 (1H, m, H-15$_\beta$), 1.18 (1H, td, J=11.2, 4.4 Hz, H-9), 1.11 (1H, m, H-6$_\beta$), 1.06 (3H, d, J=7.2 Hz, H_3-21), 1.02 (1H, m, H-14), 0.82 (3H, s, H_3-18). ^{13}C-NMR (100 MHz, pyridine-d_5) δ: 77.9 (C-1), 67.1 (C-2), 75.3 (C-3), 68.0 (C-4), 77.7 (C-5), 28.2 (C-6), 30.1 (C-7), 34.7 (C-8), 45.2 (C-9), 44.9 (C-10), 21.5 (C-11), 39.8 (C-12), 40.5 (C-13), 56.0 (C-14), 31.9 (C-15), 81.3 (C-16), 62.8 (C-17), 16.4 (C-18), 13.6 (C-19), 41.7 (C-20), 14.8 (C-21), 109.4 (C-22), 33.0 (C-23), 28.7 (C-24), 144.1 (C-25), 64.0 (C-26), 108.7 (C-27).

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Benzophenone *C*-Glucosides from *Polygala telephioides*

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Three novel benzophenone *C*-glucosides, 3′-*C*-β-D-glucopyranosyl-2′,4′,6′,3-tetrahydroxy-4-methoxybenzophenone, 3′-*C*-β-D-glucopyranosyl-2′,4′,6′-trihydroxy-3,4-dimethoxybenzophenone and 4′-*C*-β-D-glucopyranosyl-2′,3′,5′,6′,3-pentahydroxybenzophenone, named telephenones A, B, and C were isolated from the whole plant of *Polygala telephioides* Willd and their structures determined by analysis of spectroscopic data.

Key words *Polygala telephioides*; Polygalaceae; benzophenone; *C*-glucoside; telephenone

The root of *Polygala tenuifolia* Willd. is a famous crude drug used as an expectorant and as a tonic in China and Japan, and the saponins,[1] xanthones,[2] and oligosaccharide multi-esters[3] of this plant have been studied previously. A plant of the same genera, *Polygala telephioides* Willd. is widely distributed in southern China and has gradually come to be used as a detoxification agent for heroin poisoning in China. In order to characterize the active substance responsible for the above activity, we examined the whole plant and discovered three new oligoglycoside esters, a flavonoid and three new benzophenone *C*-glucosides. In a previous paper, we reported the isolation and structural determination of the three oligosaccharide esters.[4] This paper deals with the structures of the benzophenone *C*-glucosides, named telephenones A, B, and C.

The dried plant was extracted with 95% EtOH by refluxing to give extract that was treated with hexane. The insoluble part was chromatographed on Diaion, Chromatorex ODS, Sephadex LH-20 and silica gel to give telephenones A (**1**), B (**2**), and C (**3**).

The molecular formula of **1**, obtained as a pale yellow powder, $[\alpha]_D$ −10.4° (MeOH), was estimated as $C_{20}H_{22}O_{11}$ based on the negative FAB-MS. Its IR spectrum showed absorptions due to hydroxyl (3300 cm^{-1}) and carbonyl (1616 cm^{-1}) functions. The ^{13}C-NMR spectrum (Table 1) displayed twenty signals in total, among which six were assigned to a *C*-β-D-glucopyranosyl moiety.[5] The remaining signals were reminiscent of a benzophenone or a xanthone framework.

Table 1. ^{13}C-NMR Data for Telephenones A—C (**1**—**3**) in Pyridine-d_5

	1	2	3
C-1	134.8	134.1	142.8
C-2	117.6	113.0	120.9
C-3	147.6	149.2	119.8
C-4	152.2	153.3	129.4
C-5	111.1	110.9	159.9
C-6	122.7	124.8	116.0
C-1′	108.2	108.0	109.1
C-2′	160.8	162.7	158.7
C-3′	105.6	106.9	158.8
C-4′	162.6	160.7	106.2
C-5′	96.7	96.6	158.8
C-6′	160.4	160.4	158.7
C=O	197.4	197.0	198.3
CH_3 (3)		55.8	
CH_3 (4)	55.8	55.8	
glc-1	77.0	77.0	77.3
glc-2	74.2	74.2	74.8
glc-3	80.3	80.3	79.8
glc-4	71.4	71.4	70.8
glc-5	82.9	82.9	82.8
glc-6	62.1	62.1	61.6

The ^{1}H-NMR spectrum (in pyridine-d_5) showed signals due to an isolated aromatic proton at δ 6.49 (1H, s) and ABX-system aromatic protons at δ 6.90 (1H, d, J=8.6 Hz), 7.70 (1H, dd, J=2.4, 8.6 Hz) and 8.04 (1H, d, J=2.4 Hz) together with one methoxyl group. The heteronuclear multiple bond correlation experiment (HMBC) revealed the benzophenone framework of **1** and the locations of the hydroxyl and methoxyl groups and glucosyl C–C linkage, as shown in Fig. 1. Consequently, the structure of **1** was characterized as 5′-*C*-β-D-glucopyranosyl-2′,4′,6′,3-tetrahydroxy-4-methoxybenzophenone.

The molecular formula of **2**, obtained as a pale yellow powder, $[\alpha]_D$ −14.5° (MeOH), was assigned as $C_{21}H_{24}O_{11}$, which corresponds to a methyl derivative of **1**, based on the negative FAB-MS. Its IR spectrum also showed absorptions due to hydroxyl (3300 cm^{-1}) and carbonyl functions (1615 cm^{-1}). The total of twenty-one carbon signals in the ^{13}C-NMR spectrum (Table 1) were comprised of six carbon signals assignable to a *C*-β-D-glucopyranosyl moiety, two methoxyl signals and a benzophenone moiety. The ^{1}H-NMR spectrum (in pyridine-d_5) showed signals due to an isolated aromatic proton at δ 6.55 (1H, s) and ABX-system aromatic protons at δ 6.84 (1H, d, J=8.5 Hz), 7.74 (1H, dd, J=2.4, 8.5 Hz), and 7.78 (1H, d, J=2.4 Hz) along with two methoxyl groups. A comparative study of the ^{13}C-chemical shifts of **2** with those of **1** showed shifts at C-2 (−4.6 ppm), C-3 (+1.6 ppm), and C-4 (+1.1 ppm), suggesting attachment of the methyl group at C-3 OH. The HMBC experiment revealed the structure of **2**, as shown in the Formulae. Therefore, the structure of **2** was characterized as 3′-*C*-β-D-glucopyranosyl-2′,4′,6′-trihydroxy-3,4-dimethoxybenzophenone.

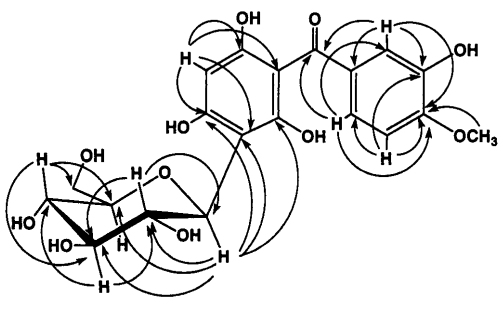

Fig. 1. HMBC Correlations for Telephenone A (in Pyridine-d_5)

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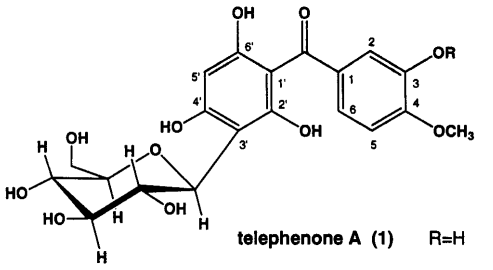
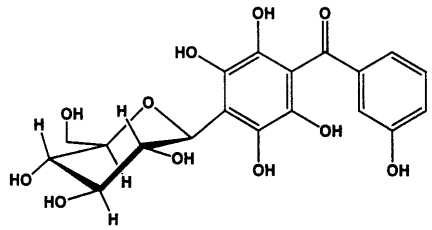


telephenone A (1) R=H

telephenone B (2) R=CH_3



telephenone C (3)

The molecular formula of **3**, obtained as a pale yellow powder, $[\alpha]_D$ +22.8° (MeOH), was estimated as $C_{19}H_{20}O_{11}$ based on the negative FAB-MS. Its IR spectrum showed absorptions due to hydroxyl (3300 cm^{-1}) and carbonyl functions (1615 cm^{-1}). Six signals among the total of nineteen signals in the ^{13}C-NMR spectrum could be assigned to a *C*-*β*-D-glucopyranosyl moiety. The remaining signals were attributable to a xanthone or benzophenone framework for **3**. The ^{1}H-NMR spectrum (in DMSO-d_6) showed signals due to ABCX-system aromatic protons at δ 6.95 (1H, ddd, J=2.4, 2.4, 7.9 Hz), 7.24 (1H, dd, J=7.9, 7.9 Hz), 7.15 (1H, ddd, J=2.4, 2.4, 7.9 Hz) and 7.13 (1H, dd, J=2.4, 2.4 Hz). The duplicate signals at δ 158.7 and 158.8 and the proton coupling system suggested the structure for **3** as shown in the Formulae. Consequently, the structure of **3** was characterized as 4′-*C*-*β*-D-glucopyranosyl-2′,3′,5′,6′,3-pentahydroxybenzophenone.

To the best of our knowledge, this is the first report of these *C*-glucosyl benzophenone derivatives which are worthy of note as novel natural products.

Experimental

General procedures are the same as those used in previous work.[4] ^{1}H- and ^{13}C-NMR were taken in pyridine-d_5.

Isolation and Identification Dried plants (1.5 kg) of *Polygala telephioides* WILLD. were extracted by refluxing with 95% EtOH to give an extract (198 g), which was divided into hexane-soluble and insoluble parts (49.5 g) by refluxing with hexane. The insoluble part was absorbed on a Diaion HP-20 column and eluted successively with H_2O, 30% MeOH, 50% MeOH, 80% MeOH and MeOH, the respective eluates of which were evaporated to give extracts (6.45 g, 10.8 g, 18.4 g, 11.2 g, and 3.7 g, respectively). A part (1.05 g) of the 50% MeOH eluate was chromatographed on a Chromatorex ODS column with 30—50% MeOH to give fractions 1—11. Fraction 3 was further chromatographed on a Sephadex LH-20 column with MeOH and a silica gel column with $CHCl_3$: MeOH : water = 8 : 2 : 0.1—7 : 3 : 0.5 to give telephenone A (**1**, 7 mg) and telephenone C (**3**, 6 mg). Fraction 4 was subjected to silica gel chromatography to provide telephenone B (**2**, 6 mg).

Telephenone A (**1**) Pale yellow powder, $[\alpha]_D$ −10.4° (c=0.35, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1616 (C=O), UV λ_{max}^{MeOH} 211.0, 313.5 nm, negative FAB-MS (*m/z*): 437 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 5.84 (1H, d, J=9.8 Hz, glc H-1), 6.49 (1H, s, H-5′), 6.90 (1H, d, J=8.6 Hz, H-5), 7.70 (1H, dd, J=2.4, 8.6 Hz, H-6), 8.04 (1H, d, J=2.4 Hz, H-2), 3.69 (3H, s, C_4–OCH_3); (DMSO-d_6) δ: 4.60 (1H, d, J=9.8 Hz, glc H-1), 5.95 (1H, s, H-5′), 6.94 (1H, d, J=8.6 Hz, H-5), 7.14 (1H, overlapped, H-6), 7.16 (1H, overlapped, H-2), 3.82 (3H, s, C_4–OCH_3). ^{13}C-NMR (pyridine-d_5): Table 1.

Telephenone B (**2**): Pale yellow powder, $[\alpha]_D$ −14.5° (c=0.30, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1615 (C=O), UV λ_{max}^{MeOH} 210.0, 313.5 nm, negative FAB-MS (*m/z*): 451 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 5.87 (1H, d, J=9.8 Hz, glc H-1), 6.55 (1H, s, H-5′), 6.84 (1H, d, J=8.5 Hz, H-5), 7.74 (1H, dd, J=2.4, 8.5 Hz, H-6), 7.78 (1H, d, J=2.4 Hz, H-2), 3.70 (3H, s, C_3–OCH_3), 3.68 (3H, s, C_4–OCH_3). (DMSO-d_6) δ: 4.60 (1H, d, J=9.8 Hz, glc-1), 5.97 (1H, s, H-5′), 6.98 (1H, d, J=8.6 Hz, H-5), 7.25 (1H, dd, J=1.8, 8.6 Hz, H-6), 7.30 (1H, d, J=1.8 Hz, H-2), 3.82 (3H, s, C_3–OCH_3), 3.78 (3H, s, C_4–OCH_3). ^{13}C-NMR (pyridine-d_5): Table 1.

Telephenone C (**3**): Pale yellow powder, $[\alpha]_D$ +22.8° (c=0.29, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1615 (C=O), negative FAB-MS (*m/z*): 423 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 5.66 (1H, d, J=9.8 Hz, glc H-1), 7.10 (1H, dd, J=7.3, 7.9 Hz, H-5), 7.19 (1H, overlapped, H-6), 7.24 (1H, overlapped, H-4), 7.81 (1H, s, H-2); (DMSO-d_6) δ: 6.95 (1H, ddd, J=2.4, 2.4, 7.9 Hz, H-2), 7.13 (1H, dd, J=2.4, 2.4 Hz, H-6), 7.15 (1H, ddd, J=2.4, 2.4, 7.9 Hz, H-4), 7.24 (1H, dd, J=7.9, 7.9 Hz, H-3). ^{13}C-NMR (pyridine-d_5): Table 1.

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Structures of New Ceramides from the Fruit Bodies of *Grifola frondosa*[1)]

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Four new phytosphingosine-type ceramides, (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxydocosanoylamino]-1,3,4-octadecanetriol (1), (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxytricosanoylamino]-1,3,4-octadecanetriol (2), (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxypentacosanoylamino]-1,3,4-octadecanetriol (3) and (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxyhexacosanoylamino]-1,3,4-octadecanetriol (4), have been isolated from the fruit bodies of *Grifola frondosa*. The structures of the new compounds were elucidated on the basis of their spectral data.

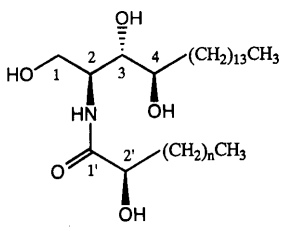
Key words phytosphingosine-type ceramide; *Grifola frondosa*; Polyporaceae; mushroom

The fruit bodies of *Grifola* (*G.*) *frondosa* (FR.) S. F. GRAY (Maitake in Japanese, Polyporaceae) are known as an edible mushroom. The anti-hypertensive, anti-diabetic and anti-hyperliposis effects of this mushroom have been reported.[2—4)] In previous papers, we reported the isolation and structural elucidation of twenty three ergostane-type sterols from the mushroom.[5)] In a continuation of our investigation of the chemical constituents in the fruit bodies of *G. frondosa*, we describe herein the isolation and structural elucidation of four new phytosphingosine-type ceramides, (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxydocosanoylamino]-1,3,4-octadecanetriol (**1**), (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxytricosanoylamino]-1,3,4-octadecanetriol (**2**), (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxypentacosanoylamino]-1,3,4-octadecanetriol (**3**), and (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxyhexacosanoylamino]-1,3,4-octadecanetriol (**4**). Extraction and isolation were carried out as described in the Experimental section.

Compound **1** was isolated as an amorphous powder. The molecular formula was determined to be $C_{40}H_{81}NO_5$ by negative ion high-resolution (HR)-FAB-MS. The IR spectrum showed absorption bands at 3340 cm^{-1} (hydroxyl), 1621, 1546 cm^{-1} (amide), 2918, 2849 and 1469 cm^{-1} (aliphatic). The ^{1}H-NMR spectrum of **1** (*vide* Experimental), obtained with the aid of ^{1}H –^{1}H shift correlation spectroscopy (^{1}H –^{1}H COSY) spectrum, showed signals due to two terminal methyl groups [δ_H 0.85 (3H, t, J=7.0 Hz), 0.86 (3H, t, J=7.0 Hz)], aliphatic methylenes [δ_H 1.25 (56H, br s)], an oxygenated methylene group [δ_H 4.44 (1H, m), 4.52 (1H, m)], three oxygenated methine groups [δ_H 4.30 (1H, m), 4.37 (1H, ddd, J=6.6, 6.2, 4.8 Hz), 4.63 (1H, ddd, J=7.7, 5.1, 3.7 Hz)], a methine group [δ_H 5.12 (1H, m)] and an amide proton [δ_H 8.60 (1H, d, J=9.2 Hz)]. The ^{13}C-NMR spectrum (*vide* Experimental) showed the characteristic signals appearing due to an amide carbonyl at δ_C 175.3 and a methine carbon linked to amide nitrogen at δ_C 53.0.[6)] These spectral data were virtually identical with those of (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxytricosanoylamino]-1,3,4-hexadecanetriol (**5**), which was isolated from the starfish *Acanthaster planci*,[7)] except for the lengths of the long chain base and the fatty acid. The lengths of the long chain base and the fatty acid were determined by the electron ionization (EI)-MS, which showed significant fragment ion peaks at *m/z* 225 (a)[8)] and 356 (b)[9)] (Fig. 1). Thus, the long chain base and fatty acid of **1** must be 2-amino-1,3,4-octadecanetriol and 2-hydroxydocosanoic acid, respectively. The stereochemistry at C-2, C-3, C-4 and C-2′ was determined as the 2*S*, 3*S*, 4*R*, 2′*R* configuration by comparing the optical rotation values of **1** ($[\alpha]_D$+12.9°) and **5** ($[\alpha]_D$+10.0°).[7)] On the basis of this evidence, the structure of **1** was determined to be (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxydocosanoylamino]-1,3,4-octadecanetriol.

Compound **2** was isolated as an amorphous powder. The molecular formula was determined to be $C_{41}H_{83}NO_5$ by negative ion HR-FAB-MS. The ^{1}H-NMR spectrum was virtually identical with that of **1** except for the integration of the aliphatic methylene protons at δ_H 1.25 (58 H). The EI-MS gave fragment ion peaks at *m/z* 225 (a) and 370 (b), indicating that the long chain base was same as that of **1** and fatty acid must be 2-hydroxytricosanoic acid. The optical rotation values of **2** ($[\alpha]_D$+14.9°) and **1** ($[\alpha]_D$+12.9°) suggested that **2** has the same absolute configuration as that of **1** for the C-2, C-3, C-4, and C-2′ parts. Therefore, the structure of **2** was determined to be (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxytricosanoylamino]-1,3,4-octadecanetriol.

Compound **3** was isolated as an amorphous powder. The



1 n = 19
2 n = 20
3 n = 22
4 n = 23

Chart 1

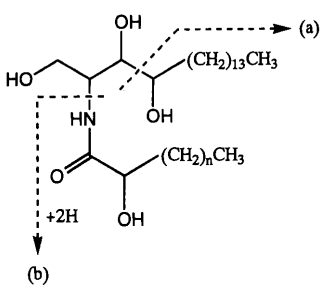


Fig. 1. EI-MS Fragmentation of **1**—**4**

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molecular formula was determined to be $C_{43}H_{87}NO_5$ by negative ion HR-FAB-MS. Inspection of the ^{1}H-NMR spectrum revealed that **3** was identical to **1** except for the integration of the aliphatic methylene protons at δ_H 1.25 (62 H). The EI-MS gave fragment ion peaks at *m/z* 225 (a) and 398 (b), indicating that the long chain base was the same as that of **1** and the fatty acid must be 2-hydroxypentacosanoic acid. Comparison of the optical rotation values of **3** ($[\alpha]_D$+13.8°) and **1** ($[\alpha]_D$+12.9°) allowed the assignment of the 2*S*, 3*S*, 4*R* and 2′*R* stereochemistry. Accordingly, the structure of **3** was determined to be (2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-hydroxypentacosanoylamino]-1,3,4-octadecanetriol.

Compound **4** was isolated as an amorphous powder. The molecular formula was determined to be $C_{44}H_{89}NO_5$ by negative ion HR-FAB-MS. The ^{1}H-NMR spectrum was similar to that of **1** except for the integration of the signal of the aliphatic methylene protons at δ_H 1.25 (64 H). The EI-MS of **4** gave fragment ion peaks at *m/z* 225 (a) and 412 (b), indicating that the long chain base was the same as that of **1** and the fatty acid must be 2-hydroxyhexacosanoic acid. The optical rotation values of **4** ($[\alpha]_D$+11.1°) and **1** ($[\alpha]_D$+12.9°) suggested that **4** has the same absolute configuration as that of **1** for the C-2, C-3, C-4, and C-2′ carbons. Based on this evidence, the structure of **4** was determined to be (2*S*, 3*S*,4*R*)-2-[(2′*R*)-2′-hydroxyhexacosanoylamino]-1,3,4-octadecanetriol.

Compounds **1**—**4** have the same long chain base, (2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecanetriol, and differ in the chain length of the (2*R*)-2-hydroxy fatty acids. Recently, phytosphingosine-type ceramides similar to **1**—**4** were reported from the soft coral *Sinularia leptoclados*[6] and the starfish *Acanthaster planci*.[7] Furthermore, **1** and **2** were the ceramide parts of the glycosphingolipids named regulosides A and B, respectively, which were recently isolated from the starfish *Pentaceraster regulus*.[10] Although the compositions of sphingolipids of *G. frondosa* have been reported by Ohnishi *et al.*,[11] this is the first report of the isolation and structural elucidation of each ceramide from *G. frondosa*.

Experimental

General Procedures Optical rotations were determined with a JASCO DIP-360 digital polarimeter. IR spectra were recorded with a Perkin–Elmer FT-IR 1725X IR spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded with JEOL JNM-LA 600 (600 and 150 MHz, respectively) spectrometer. Chemical shifts are given on a δ (ppm) scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; ddd, double double doublet; t, triplet; q, quartet; br, broad; m, multiplet). The EI- and FAB-MS were recorded on a JEOL JMS-DX 303 mass spectrometer. Column chromatography was carried out on Kieselgel 60 (Merck; 230—400 mesh). Preparative HPLC was carried out on a Tosoh HPLC system (pump, CCPD; detector, RI-8010) using a TSK gel ODS-120T (7.8 mm i.d.×30 cm) column (Tosoh).

Material The fresh fruit bodies of *Grifola frondosa* were purchased from Mogamimachi Maitake Seisan Kumiai (Yamagata Prefecture, Japan).

Extraction and Isolation The fresh fruit bodies of *G. frondosa* (20 kg) were extracted three times with Et_2O (20 l) at room temperature for 10 d. The Et_2O extract (21 g) was chromatographed on a silica gel column using *n*-hexane–EtOAc (7 : 3—1 : 7), EtOAc, and MeOH, to afford 9 fractions (frs. 1—9). Fraction 9 was purified by preparative HPLC (mobile phase, MeOH; flow rate, 1.5 ml/min; column temperature, 40 °C) to give **1** (1.2 mg), **2** (0.7 mg), **3** (1.1 mg) and **4** (1.2 mg).

(2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-Hydroxydocosanoylamino]-1,3,4-octadecanetriol (**1**): Amorphous powder. $[\alpha]_D^{22}$ +12.9° (*c*=0.1, C_5H_5N). IR ν_{max} (KBr) cm^{-1}: 3340, 2918, 2849, 1621, 1546, 1469. FAB-MS (negative ion mode; matrix, triethanolamine) *m/z*: 654 [M−H]$^-$. HR-FAB-MS (negative ion mode; matrix, triethanolamine) *m/z*: 654.5985 ([M−H]$^-$, Calcd for $C_{40}H_{80}NO_5$: 654.6036). EI-MS [the sample was dissolved in $CHCl_3$–MeOH (2 : 1)] *m/z*: 356 (b), 225 (a). ^{1}H-NMR (600 MHz, C_5D_5N) δ: 0.85 (3H, t, *J*=7.0 Hz, H_3-18 or H_3-22′), 0.86 (3H, t, *J*=7.0 Hz, H_3-18 or H_3-22′), 1.25 (56H, br s, H-7—H-17, H-5′—H-21′), 1.70 (2H, m, Ha-6, Ha-4′), 1.79 (1H, m, Hb-6), 1.94 (2H, m, Ha-5, Hb-4′), 2.05 (1H, m, Ha-3′), 2.25 (2H, m, Hb-5, Hb-3′), 4.30 (1H, m, H-4), 4.37 (1H, ddd, *J*=6.6, 6.2, 4.8 Hz, H-3), 4.44 (1H, m, Ha-1), 4.52 (1H, m, Hb-1), 4.63 (1H, ddd, *J*=7.7, 5.1, 3.7 Hz, H-2′), 5.12 (1H, m, H-2), 6.25 (1H, d, *J*=6.6 Hz, OH-4), 6.73 (2H, d, *J*=6.2 Hz, OH-1, OH-3), 7.66 (1H, d, *J*=5.1 Hz, OH-2′), 8.60 (1H, d, *J*=9.2 Hz, NH). ^{13}C-NMR (150 MHz, C_5D_5N) δ: 14.3 (q, C-18, C-22′), 23.0—32.2 (t, C-7—C-17, C-5′—C-21′), 25.9 (t, C-6), 26.7 (t, C-4′), 34.2 (t, C-5), 35.7 (t, C-3′), 53.0 (d, C-2), 62.1 (t, C-1), 72.5 (d, C-2′), 73.0 (d, C-4), 76.8 (d, C-3), 175.3 (s, C-1′).

(2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-Hydroxytricosanoylamino]-1,3,4-octadecanetriol (**2**): Amorphous powder. $[\alpha]_D^{20}$ +14.9° (*c*=0.07, C_5H_5N). IR ν_{max} (KBr) cm^{-1}: 3338, 2919, 2850, 1621, 1545, 1468. FAB-MS (negative ion mode; matrix, triethanolamine) *m/z*: 668 [M−H]$^-$. HR-FAB-MS (negative ion mode; matrix, triethanolamine) *m/z*: 668.6243 ([M−H]$^-$, Calcd for $C_{41}H_{82}NO_5$: 668.6193). EI-MS [the sample was dissolved in $CHCl_3$–MeOH (2 : 1)] *m/z*: 370 (b), 225 (a). ^{1}H-NMR (600 MHz, C_5D_5N) δ: 0.85 (3H, t, *J*=7.0 Hz, H_3-18 or H_3-23′), 0.86 (3H, t, *J*=7.0 Hz, H_3-18 or H_3-23′), 1.25 (58H, br s, H-7 H-17, H-5′—H-22′), 1.70 (2H, m, Ha-6, Ha-4′), 1.79 (1H, m, Hb-6), 1.94 (2H, m, Ha-5, Hb-4′), 2.05 (1H, m, Ha-3′), 2.26 (2H, m, Hb-5, Hb-3′), 4.30 (1H, m, H-4), 4.37 (1H, ddd, *J*=7.0, 6.2, 4.8 Hz, H-3), 4.44 (1H, m, Ha-1), 4.52 (1H, m, Hb-1), 4.63 (1H, ddd, *J*=7.7, 5.1, 3.7 Hz, H-2′), 5.12 (1H, m, H-2), 6.25 (1H, d, *J*=6.2 Hz, OH-4), 6.73 (2H, d, *J*=6.2 Hz, OH-1, OH-3), 7.66 (1H, d, *J*=5.1 Hz, OH-2′), 8.60 (1H, d, *J*=8.8 Hz, NH). ^{13}C-NMR (150 MHz, C_5D_5N) δ: 14.3 (q, C-18, C-23′), 23.0—32.2 (t, C-7—C-17, C-5′—C-22′), 25.9 (t, C-6), 26.7 (t, C-4′), 34.2 (t, C-5), 35.7 (t, C-3′), 53.0 (d, C-2), 61.9 (t, C-1), 72.5 (d, C-2′), 73.0 (d, C-4), 76.8 (d, C-3), 175.3 (s, C-1′).

(2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-Hydroxypentacosanoylamino]-1,3,4-octadecanetriol (**3**): Amorphous powder. $[\alpha]_D^{21}$ +13.8° (*c*=0.1, C_5H_5N). IR ν_{max} (KBr) cm^{-1}: 3333, 2919, 2850, 1621, 1545, 1468. FAB-MS (negative ion mode; matrix, triethanolamine) *m/z*: 696 [M−H]$^-$. HR-FAB-MS (negative ion mode; matrix, triethanolamine) *m/z*: 696.6446 ([M−H]$^-$, Calcd for $C_{43}H_{86}NO_5$: 696.6506). EI-MS [the sample was dissolved in $CHCl_3$–MeOH (2 : 1)] *m/z*: 398 (b), 225 (a). ^{1}H-NMR (600 MHz, C_5D_5N) δ: 0.85 (3H, t, *J*=7.0 Hz, H_3-18 or H_3-25′), 0.86 (3H, t, *J*=7.0 Hz, H_3-18 or H_3-25′), 1.25 (62H, br s, H-7 H-17, H-5′—H-24′), 1.70 (2H, m, Ha-6, Ha-4′), 1.79 (1H, m, Hb-6), 1.94 (2H, m, Ha-5, Hb-4′), 2.05 (1H, m, Ha-3′), 2.24 (2H, m, Hb-5, Hb-3′), 4.30 (1H, m, H-4), 4.38 (1H, ddd, *J*=6.6, 6.2, 4.8 Hz, H-3), 4.44 (1H, m, Ha-1), 4.52 (1H, m, Hb-1), 4.63 (1H, ddd, *J*=7.7, 5.1, 3.7 Hz, H-2′), 5.12 (1H, m, H-2), 6.25 (1H, d, *J*=6.2 Hz, OH-4), 6.73 (2 H, d, *J*=6.2 Hz, OH-1, OH-3), 7.66 (1H, d, *J*=5.1 Hz, OH-2′), 8.60 (1H, d, *J*=9.2 Hz, NH). ^{13}C-NMR (150 MHz, C_5D_5N) δ: 14.3 (q, C-18, C-25′), 23.0—32.2 (t, C-7—C-17, C-5′—C-24′), 25.9 (t, C-6), 26.7 (t, C-4′), 34.2 (t, C-5), 35.7 (t, C-3′), 53.0 (d, C-2), 62.1 (t, C-1), 72.5 (d, C-2′), 73.1 (d, C-4), 76.8 (d, C-3), 175.3 (s, C-1′).

(2*S*,3*S*,4*R*)-2-[(2′*R*)-2′-Hydroxyhexacosanoylamino]-1,3,4-octadecanetriol (**4**): Amorphous powder. $[\alpha]_D^{22}$ +11.1° (*c*=0.1, C_5H_5N). IR ν_{max} (KBr) cm^{-1}: 3334, 2919, 2850, 1621, 1545, 1469. FAB-MS (negative ion mode; matrix, triethanolamine) *m/z*: 710 [M−H]$^-$. HR-FAB-MS (negative ion mode; matrix, triethanolamine) *m/z*: 710.6598 ([M−H]$^-$, Calcd for $C_{44}H_{88}NO_5$: 710.6663). EI-MS [the sample was dissolved in $CHCl_3$–MeOH (2 : 1)] *m/z*: 412 (b), 225 (a). ^{1}H-NMR (600 MHz, C_5D_5N) δ: 0.86 (6H, t, *J*=7.0 Hz, H_3-18, H_3-26′), 1.25 (64H, br s, H-7—H-17, H-5′—H-25′), 1.70 (2H, m, Ha-6, Ha-4′), 1.79 (1H, m, Hb-6), 1.94 (2H, m, Ha-5, Hb-4′), 2.05 (1H, m, Ha-3′), 2.25 (2H, m, Hb-5, Hb-3′), 4.30 (1H, m, H-4), 4.38 (1H, ddd, *J*=6.6, 6.2, 4.8 Hz, H-3), 4.43 (1H, m, Ha-1), 4.52 (1H, m, Hb-1), 4.63 (1H, ddd, *J*=7.7, 5.1, 3.7 Hz, H-2′), 5.12 (1H, m, H-2), 6.25 (1H, d, *J*=6.2 Hz, OH-4), 6.73 (2H, d, *J*=6.2 Hz, OH-1, OH-3), 7.66 (1H, d, *J*=5.1 Hz, OH-2′), 8.60 (1H, d, *J*=9.2 Hz, NH). ^{13}C-NMR (150 MHz, C_5D_5N) δ: 14.3 (q, C-18, C-26′), 23.0—32.2 (t, C-7—C-17, C-5′—C-25′), 25.9 (t, C-6), 26.7 (t, C-4′), 34.2 (t, C-5), 35.7 (t, C-3′), 53.0 (d, C-2), 62.1 (t, C-1), 72.5 (d, C-2′), 73.1 (d, C-4), 76.8 (d, C-3), 175.3 (s, C-1′).

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Synthesis of 19-Oxygenated 4β,5β-Epoxy Derivatives of 16α-Hydroxyandrostenedione as Mechanistic and Catalytic Probes for Aromatase Reaction

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4β,5β-Epoxy derivatives of 16α-hydroxyandrostenedione (2), one of the natural substrates for aromatase, and its 19-oxygenated compounds 4 and 5 were synthesized as mechanistic and catalytic probes for the enzyme reaction. Treatment of 16α-bromoandrostenedione (13) or its 19-hydroxy analog 19 which was prepared from 3β-hydroxy-19-(*tert*-butyldimethylsiloxy)androst-5-en-17-one (16) in three steps, with H_2O_2 and NaOH followed by controlled alkaline hydrolysis with NaOH in aqueous pyridine stereospecifically yielded 4β,5β-epoxy-16α-ol 15 or 4β,5β-epoxy-16α,19-diol 22, respectively. Oxidation of 16β-bromo-4β,5β-epoxy-19-ol 21 with pyridinium dichromate followed by controlled alkaline hydrolysis produced 4β,5β-epoxy-16α-hydroxy-19-al 24.

Key words aromatase reaction; 16α-hydroxyandrostenedione; 4β,5β-epoxy steroid; 19-oxygenated steroid; controlled alkaline hydrolysis

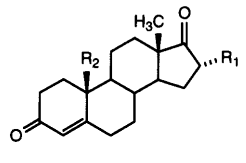
Aromatase is the enzyme responsible for catalyzing the conversion of androgens, androst-4-ene-3,17-dione (androstenedione, **1**) and its 16α-hydroxy androgen (**2**), into estrogens, estrone and estriol[1] (Chart 1). Aromatization of the androgens appears to involve three sequential oxygenations of the C-19 methyl group: the first two are sequential hydroxylations to produce 19-hydroxy and 19-oxo intermediates **4** and **5**, respectively, in the case of the substrate **1**.[2] In the third step, the angular methyl at C-19 and the 1β,2β-hydrogens are eliminated to result in the aromatization of the A-ring of the androgen molecule to form estrogen. The chemical nature of the third step remains elusive. A leading theory for the last step proposes nucleophilic attack of the heme ferric peroxide species on the 19-aldehyde intermediate **5** to produce a 19-hydroxy-19-ferric peroxide intermediate.[3] Several theories with alternate routes including 4,5-epoxidation[4] and 2β-hydroxylation[5] have also been proposed.

Chemical model reaction studies carried out by Robinson's group have shown that hydrogen peroxide reacted with 19-oxo steroid **5** in the absence of strong base to generate the 4β,5β-epoxy **8**,[6] in contrast, a 2,4-diene isomer of steroid **5** is well aromatized on treatment with H_2O_2 under the above conditions.[7] Recently, we reported that 6-oxoandrostenedione, a typical suicide substrate of aromatase, and its 5-ene-4,7-dione isomer inactivate the enzyme through the 4β,5β-epoxy-19-oxo intermediate **9**[8] and its 5β,6β-epoxy isomer **11**,[9] respectively, as reactive electrophiles. Moreover, epoxyketones **10** and **12** having 19-oxo and 19-methyl functions, respectively, irreversibly inactivate aromatase in a time-dependent manner.[10] It has also been reported that androstenedione 4β,5β-epoxide **6** is a fair competitive inhibitor of aromatase[11] and its 19-hydroxy analog **7** is converted into estrone by incubation with human placental microsomes in the presence of NADPH probably through the 19-oxo intermediate **8**.[4a] On the other hand, 16α-hydroxyandrostenedione (**2**), an alternate substrate of aromatase, is known to bind to a different binding site of aromatase from the androstenedione binding site, causing a non-competitive type inhibition of androstenedione aromatization.[12]

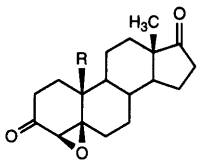
Taken together, we had interest in 4β,5β-epoxy derivatives of compound **2** as mechanistic and catalytic probes for the aromatase reaction. Thus, we report here synthesis of a series of 16α-hydroxy-4β,5β-epoxyandrosta-3,17-diones, **15**, **22**, and **24**.

Results and Discussion

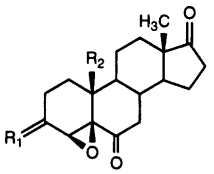
We initially focused on epoxidation of the 16α-hydroxy steroid **2** with H_2O_2 and NaOH in MeOH. It is well known that a 16α-ketol is easily isomerized into the most thermodynamically stable 17β-hydroxy-16-ketone under basic conditions,[13] thus, to avoid the ketol rearrangement during the alkaline epoxidation, this less stable steroid **2** was converted into *tert*-butyldimethylsilyl (TBDMS) ether **3** by treatment with TBDMS chloride and imidazole. However, treatment of compound **3** with H_2O_2 and NaOH yielded a complex mix-



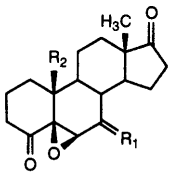
1; R_1= H, R_2= CH_3
2; R_1= OH, R_2= CH_3
3; R_1= OTBDMS, R_2= CH_3
4; R_1= H, R_2= CH_2OH
5; R_1= H, R_2= CHO



6; R= CH_3
7; R= CH_2OH
8; R= CHO



9; R_1= O, R_2= CHO
10; R_1= H (α), R_2= CHO



11; R_1= O, R_2= CHO
12; R_1= H (α), R_2= CH_3

TBDMS: *tert*-butyldimethylsilyl

Chart 1

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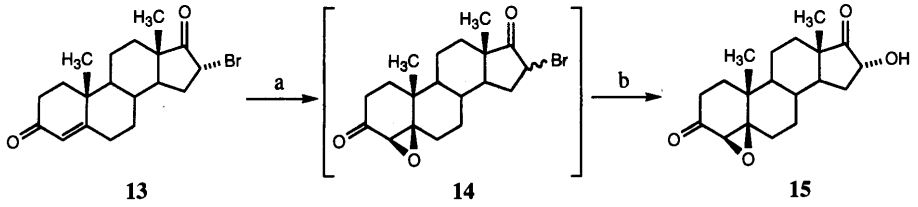
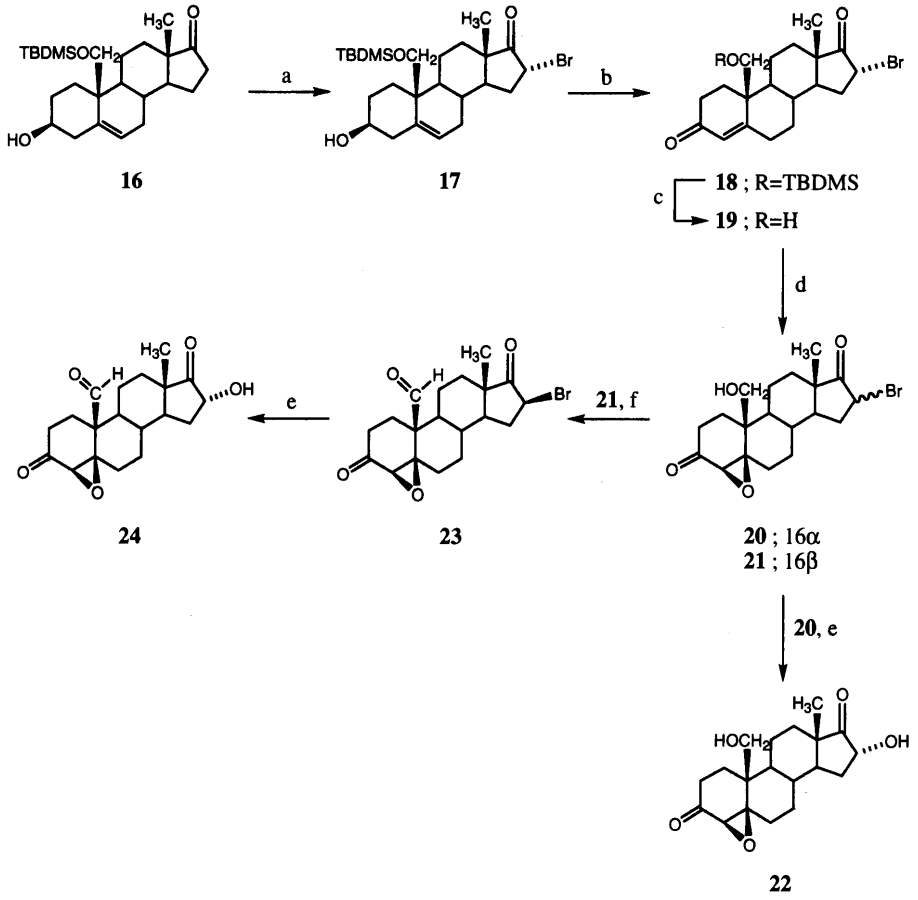


Reagents: a) H_2O_2, NaOH, MeOH, 4 °C; b) NaOH, pyridine, H_2O, r.t.

Chart 2



Reagents: a) $CuBr_2$, MeOH, r.t.; b) i Jones reagent, acetone, 0 °C. ii *p*-TsOH, acetone, r.t.; c) 3mol/l HCl, THF, 2-propanol, r.t.; d) H_2O_2, NaOH, MeOH, 4 °C; e) NaOH, pyridine, H_2O, r.t.; f) pyridinium dichromate, CH_2Cl_2, r.t.

Chart 3

ture of products and the desired 4β,5β-epoxy derivative could not be isolated in a pure form. The 16α-siloxy-17-ketone structure might be unstable towards the alkaline epoxidation conditions.

Then, we employed a different synthetic path in which the alkaline epoxidation of a 4-en-3-one steroid is followed by a stereospecific introduction of a 16α-hydroxyl moiety to a 17-keto steroid through controlled alkaline hydrolysis of a 16-bromo-17-ketone with 1.2 mol eq of NaOH in aqueous pyridine.[14] Treatment of 16α-bromoandrostenedione (**13**) with H_2O_2 in the presence of NaOH produced a mixture of 4β,5β-epoxy-16α- and 16β-bromides **14**, based on the ^{1}H-NMR analysis of the product (Chart 2). This mixture, without further purification, was subjected to controlled alkaline hydrolysis giving 16α-hydroxy-4β,5β-epoxy-17-keto steroid **15** (34% from **13**). This hydrolysis proceeds through an initial equilibration between a 16α-bromo-17-ketone and its 16β-isomer followed by a stereoselective hydrolysis of the 16β-bromide, yielding a 16α-hydroxy-17-ketone without an isomerization.[14] It is known that the alkaline epoxidation of 4-en-3-one steroids usually gives the β-epoxide as the major product.[15] Moreover, the β-configuration of the epoxy ring of compound **15** was consistent with a lower shift of the 19-methyl proton signals (δ=1.18) and a higher field shift

of the epoxy proton at C-4 (δ=3.00) compared to the α-epoxide (δ=1.09 for the 19-methyl and 3.06 for the epoxy proton).[9b,16]

Then, we employed a similar sequence for synthesis of 19-oxygenated 4β,5β-epoxy compounds **22** and **24** to that described above (Chart 3). We chose the known compound 3β,19-dihydroxy-5-en-17-one 19-TBDMS ether **16**[17] as the starting material. Bromination of this with 3 mol eq of $CuBr_2$ in MeOH[8b] at room temperature gave 16α-bromide **17** (75%) of which Jones oxidation followed by treatment with *p*-toluenesulfonic acid yielded 4-en-3-one steroid **18** (58%). Deprotection of the 19-TBDMS group with dil. HCl and a subsequent epoxidation with H_2O_2 and NaOH in MeOH produced a 1 : 1.35 mixture of 16α- and 16β-bromo-4β,5β-epoxy-19-ols **20** and **21**. The latter, the major product, was an alkaline-catalyzed isomerization product of the former and its structure was assigned on the basis of the ^{1}H- NMR spectrum [δ: 1.11 (18-Me), 2.93 (4α-H), 3.83 and 4.11 (19-CH_2), 4.11 (16α-H)]; the β-configuration of the 16-bromo function was revealed by a lower field shift of the 18-Me signal and a higher field shift of the 16α-proton, compared to the 16α-bromo isomer **20** [δ: 0.94 (18-Me), 4.53 (16β-H)].[14] After separation of the mixture by silica gel column chromatography, the 16α-bromide **20** was converted stereoselectively into the 16α-ketol **22** through controlled alkaline hydrolysis in good yield. On the other hand, the major product **21** was converted into 4β,5β-epoxy-16α-hydroxy-17,19-dione **24** (66%) in two steps involving pyridinium dichromate oxidation followed by controlled alkaline hydrolysis.

The spectroscopic and elemental analysis data of these 4β,5β-epoxy steroids were consistent with the assigned structures, respectively.

Experimental

Melting points were measured on a Yanagimoto melting point apparatus (Kyoto, Japan) and are uncorrected. IR spectra were recorded in KBr pellets for the solid products or in neat forms for the oily products on a Perkin-Elmer FT-IR 1725X spectrophotometer (Norwalk, CT, U.S.A.), and UV spectra were determined in 95% ethanol on a Hitachi 150-20 UV spectrophotometer (Tokyo, Japan). ^{1}H-NMR spectra were obtained in $CDCl_3$ solutions with JEOL EX 270 (270 MHz) spectrometer (Tokyo, Japan) using tetramethylsilane as an internal standard. High-resolution mass spectra (HRMS) were determined with a JEOL JMS-DX 303 spectrometer. Thin-layer chromatography (TLC) was performed on E. Merck precoated TLC silica gel plates (silica gel 60F-254, layer thickness 0.25 and 0.5 mm for the analytical and preparative use, respectively; Darmstadt, Germany). Column chromatography was conducted with silica gel 60, 70—230 mesh (E. Merck, 70—230 mesh).

16α-Hydroxyandrost-4-ene-3,17-dione (**2**),[14] 16α-bromoandrost-4-ene-3,17-dione (**13**),[14] and 3β-hydroxy-19-(*tert*-butyldimethylsiloxy)androst-5-en-17-one (**16**)[17] were synthesized according to the known methods.

16α-(*tert*-Butyldimethylsiloxy)androst-4-ene-3,17-dione (3) 16α-Hydroxy-17-ketone **2** (443 mg, 1.47 mmol) was treated with TBDMS chloride (665 mg, 4.41 mmol) and imidazole (300 mg, 4.41 mmol) in DMF (14 ml) at room temperature for 3 h. The reaction mixture was diluted with EtOAc (200 ml), washed with 5% HCl, sat. $NaHCO_3$ solution, and water, sequentially, and dried with Na_2SO_4. Evaporation of the solvent afforded a solid which was purified by column chromatography (hexane–EtOAc) to give compound **3** (529 mg, 86.7%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.10 (6H, s, 16α-$OSiMe_2$), 0.89 (9H, s, 16α-$OSiCMe_3$), 0.94 (3H, s, 18-Me), 1.21 (3H, s, 19-Me), 4.33 (1H, d, J=6.8 Hz, 16β-H), 5.75 (1H, d, J=1.0 Hz, 4-H). FT-IR (neat) cm^{-1}: 1753 and 1677 (C=O). UV λ_{max} (EtOH) nm (ε): 240 (15400). HRMS Calcd for $C_{25}H_{40}O_3Si$: 416.2747. Found: 416.2741.

Reaction of the 16α-Silyl Ether 2 with H_2O_2 Aqueous 30% H_2O_2 (0.24 ml, 2.1 mmol) was added to a solution of compound **2** in MeOH (2.7 ml) containing 10% NaOH solution (0.1 ml, 0.25 mmol) and the reaction mixture was allowed to stand at 4 °C for 2 h. After this time, the mixture was diluted with EtOAc (50 ml), washed with $Na_2S_2O_3$ solution, 5% $NaHCO_3$ solution, and water, sequentially, and dried with Na_2SO_4. Evaporation of the solvent gave an oil. TLC and ^{1}H-NMR spectroscopic analysis of the oil revealed it to be a complex mixture of products, thus, isolation of the desired 4β,5β-epoxy steroid was not carried out.

Reaction of 16α-Bromoandrost-4-ene-3,17-dione (13) with H_2O_2 and NaOH The 16α-bromide **13** (200 mg, 0.55 mmol) was treated with aqueous H_2O_2 (1.9 ml, 16.4 mmol) (MeOH, 20 ml; 10% NaOH solution, 0.48 ml; 4 °C; 4 h) similarly as described above. Based on ^{1}H-NMR spectroscopy of the crude solid **14** (102 mg) obtained revealed that this consists of a *ca.* 1 : 2 mixture of 16α-bromo-4β,5β-epoxyandrosta-3,17-dione and its 16β-isomer. ^{1}H-NMR ($CDCl_3$) δ: the 16α-bromide 0.94 (s, 18-Me), 1.19 (s, 19-Me), 3.00 (s, 4α-H), 4.53 (m, 16β-H) and 16β-bromide 1.11 (s, 18-Me), 1.19 (s, 19-Me), 3.00 (s, 4α-H), 4.11 (t, J=8.7 Hz, 16α-H). The mixture was subjected to the next step without further purification.

16α-Hydroxy-4β,5β-epoxyandrosta-3,17-dione (15) A mixture of the 16-bromo-4,5-epoxides **14** (100 mg, 0.26 mmol) obtained above was dissolved in 75% pyridine (4.0 ml). NaOH (12 mg, 0.30 mmol) was added to this solution. The mixture was stirred at room temperature for 3 h, poured into 5% HCl and extracted with EtOAc. The organic layer was washed with 5% $NaHCO_3$ solution and water, and dried with Na_2SO_4. Evaporation of the solvent gave a crude solid which was recrystallized from EtOAc–hexane to give compound **15** (70 mg, 34% from **13**) as colorless needles, mp 141—142 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.99 (3H, s, 18-Me), 1.18 (3H, s, 19-Me), 3.00 (1H, s, 4α-H), 4.38 (1H, d, J=7.3 Hz, 16β-H). FT-IR (KBr) cm^{-1}: 3467 (OH), 1749 and 1702 (C=O). *Anal.* Calcd for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23. Found: C, 71.38; H, 8.47.

16α-Bromo-3β-hydroxy-19-(*tert*-butyldimethylsiloxy)androst-5-en-17-one (17) A solution of 3β-hydroxy-19-(*tert*-butyldimethylsiloxy)androst-5-en-17-one (**16**) (1.55 g, 3.7 mmol) and $CuBr_2$ (2.48 g, 11.1 mmol) in 40 ml of dry MeOH was stirred at room temperature for 15 h and then poured into water. The precipitates were collected by filtration, dissolved in EtOAc, and dried with Na_2SO_4. Evaporation of the solvent yielded a solid which was recrystallized from acetone to afford the 16α-bromide **17** (1.38 g, 75%) as colorless needles; mp 136—137 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.02 and 0.03 (3H each, s, 19-$OSiMe_2$), 0.86 (9H, s, 19-$OSiCMe_3$), 0.95 (3H, s, 18-Me), 3.53 (1H, m, 3α-H), 3.60 and 3.79 (1H each, d, J=10.7 Hz, 19-H_2), 4.53 (1H, m, 16β-H), 5.59 (1H, m, 6-H). FT-IR (KBr) cm^{-1}: 3336 (OH), 1746 (C=O). *Anal.* Calcd for $C_{25}H_{42}O_3SiBr$: C, 60.34; H, 8.31. Found: C, 60.06; H, 8.54.

16α-Bromo-19-(*tert*-butyldimethylsiloxy)androst-4-ene-3,17-dione (18) Jones reagent (0.46 ml) was added to a solution of the 3β-ol **17** (625 mg, 1.26 mmol) in acetone (61 ml) and the mixture was stirred at 0 °C for 5 min. After this time, the mixture was poured into water. The precipitates were collected by filtration, washed with water, dried under reduced pressure, and then dissolved in acetone (13 ml). *p*-Toluenesulfonic acid monohydrate (44 mg, 0.24 mmol) was added to this solution. The mixture was allowed to stand at room temperature for 1.5 h and then poured into saturated NaCl solution. The precipitates were collected by filtration, dried under reduced pressure, and recrystallized from acetone–hexane to give compound **18** (360 mg, 58%) as colorless needles, mp 145—146 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.04 and 0.05 (3H each, s, 19-$OSiMe_2$), 0.86 (9H, s, 19-$OSiCMe_3$), 0.96 (3H, s, 18-Me), 3.89 (2H, s, 19-CH_2), 4.54 (1H, m, 16β-H), 5.88 (1H, s, 4-H). FT-IR (KBr) cm^{-1}: 1752 and 1671 (C=O). UV λ_{max} (EtOH) nm (ε): 241.5 (15900). *Anal.* Calcd for $C_{25}H_{40}O_3SiBr$: C, 60.59; H, 7.93. Found: C, 60.58; H, 8.15.

16α-Bromo-19-hydroxyandrost-4-ene-3,17-dione (19) 3 mol/l HCl (5.1 ml) was added to a solution of the 19-silyl ether **18** (315 mg, 0.64 mmol) in THF (5.5 ml) and 2-propanol (8.3 ml). The resulting mixture was allowed to stand at room temperature for 1 d, diluted with EtOAc (200 ml), washed with 5% $NaHCO_3$ solution and water, and dried with Na_2SO_4. Evaporation of the solvent yielded a solid which after recrystallization from acetone gave compound **19** (211 mg, 87%) as colorless prisms, mp 196—198 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.96 (3H, s, 18-Me), 3.94 and 4.06 (1H each, d, J=10.8 Hz, 19-H_2), 4.55 (1H, m, 16β-H), 5.96 (1H, s, 4-H). FT-IR (KBr) cm^{-1}: 3343 (OH), 1745 and 1666 (C=O). UV λ_{max} (EtOH) nm (ε): 242.0 (14900). *Anal.* Calcd for $C_{19}H_{25}O_3Br$: C, 59.85; H, 6.61. Found: C, 59.58; H, 6.85.

Epoxidation of the 16α-Bromo-4-en-3-one Steroid 19 with H_2O_2 and NaOH Compound **19** (223 mg, 0.59 mmol) was subjected to the reaction with H_2O_2 under the similar conditions to those described for the reaction of compound **2** [21.6 ml of MeOH, 0.52 ml (1.3 mmol) of 10% NaOH solution, 1.95 ml (17.2 mmol) of 30% H_2O_2, 4 °C, 30 min]. After the same work-up as above, an oily product was obtained and was purified by column chromatography (hexane–EtOAc).

16α-Bromo-19-hydroxy-4β,5β-epoxyandrosta-3,17-dione (20) The more polar product obtained by the above epoxidation was recrystallized from EtOAc to give compound **20** (71 mg, 31%) as colorless prisms, mp 217—218 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.94 (3H, s, 18-Me), 2.94 (1H, s, 4α-H), 3.83 and 4.13 (1H each, d, J=11.0 Hz, 19-H_2), 4.53 (1H, m, 16β-H). FT-IR (KBr) cm^{-1}: 3506 (OH), 1733 and 1709 (C=O). *Anal.* Calcd for $C_{19}H_{25}O_4Br$: C, 57.44; H, 6.34. Found: C, 57.37; H, 6.39.

16β-Bromo-19-hydroxy-4β,5β-epoxyandrosta-3,17-dione (21) The less polar product obtained by the above epoxidation was recrystallized from acetone–hexane to afford compound **21** (98 mg, 42%) as colorless needles, mp 199—201 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.11 (3H, s, 18-Me), 2.93 (1H, s, 4α-H), 3.83 (1H, d, J=11.1 Hz, 19-H), 4.11 (2H, m, 16α-H and 19-H). FT-IR (KBr) cm^{-1}: 3509 (OH), 1753 and 1704 (C=O). *Anal.* Calcd for $C_{19}H_{25}O_4Br$: C, 57.44; H, 6.34. Found: C, 57.17; H, 6.36.

16α,19-Dihydroxy-4β,5β-epoxyandrosta-3,17-dione (22) The 16α-bromide **20** (44 mg, 0.11 mmol) was subjected to controlled alkaline hydrolysis with NaOH under similar conditions to those described for the synthesis of the 16α-ol **15** (75% pyridine, 2.3 ml; NaOH, 5.2 mg 0.13 mmol; 1 h). After the same work-up as above, the solid product obtained was recrystallized from EtOAc to yield compound **22** (30 mg, 81%) as colorless prisms, mp 188—190 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.99 (3H, s, 18-Me), 2.93 (1H, s, 4α-H), 3.82 and 4.15 (1H each, d, J=10.9 Hz, 19-H_2), 4.37 (1H, d, J=7.8 Hz, 16β-H). *Anal.* Calcd for $C_{19}H_{26}O_5$: C, 68.24; H, 7.84. Found: C, 67.96; H, 8.04.

16β-Bromo-4β,5β-epoxyandrosta-3,17,19-trione (23) Pyridinium dichromate (95 mg, 0.25 mmol) was added to a solution of the 16β-bromide **21** (84 mg, 0.21 mmol) in CH_2Cl_2 (3.3 ml). The mixture was stirred at room temperature for 2 h, diluted with EtOAc (80 ml), washed with 5% $NaHCO_3$ solution and water, and dried with Na_2SO_4. After evaporation of the solvent, the solid product obtained was subjected to column chromatography (EtOAc–hexane) to give compound **23** (63 mg, 75%) as colorless plates, mp 154—156 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.19 (3H, s, 18-Me), 3.06 (1H, s, 4α-H), 4.11 (1H, t, J=8.8 Hz, 16α-H), 9.77 (1H, d, J=2.1 Hz, 19-H). FT-IR (KBr) cm^{-1}: 1744 and 1717 (C=O). *Anal.* Calcd for $C_{19}H_{23}O_4Br$: C, 57.73; H, 5.86. Found: C, 57.58 H, 5.98.

16α-Hydroxy-4β,5β-epoxyandrosta-3,17,19-trione (24) The 16α-bromide **23** (109 mg, 0.28 mmol) was subjected to controlled alkaline hydrolysis with NaOH under similar conditions to those described for the synthesis of the 16α-ol **15** (75% pyridine, 4.6 ml; NaOH, 13.5 mg, 0.34 mmol; 1 h). After the same work-up as above, the crude solid obtained was recrystallized from EtOAc to afford compound **24** (60 mg, 66%) as colorless plates, 170—171 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.06 (3H, s, 18-Me), 3.05 (1H, s, 4α-H), 4.38 (1H, d, J=7.6 Hz, 16β-H), 9.79 (1H, d, J=2.1 Hz, 19-H). FT-IR (KBr) cm^{-1}: 3504 (OH), 1747 and 1717 (C=O). *Anal.* Calcd for $C_{19}H_{24}O_5$: C, 68.65; H, 7.28. Found: C, 68.36; H, 7.35.

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Preparation of *N'*-[2-(5,6-Dimethylbenzothiazolyl)]-*N*-furfuryloxamide with Plant Growth Regulatory Activity

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The reaction of the *N*-furfuryloxamic acid sodium salt (12) with 1,1'-oxalyldiimidazole (ODI) yielded the imidazolide (13) as an intermediate, and this directly reacted with 2-aminothiazole derivatives (14) or 2-aminobenzothiazole derivatives (15) under essentially neutral conditions to afford the *N'*-[2-(substituted thiazolyl)]- or *N'*-[2-(substituted benzothiazolyl)]-*N*-furfuryloxamides (6 or 7).

The prepared compounds (6 and 7) were examined for plant growth regulatory activity in a seed germination assay. The examination resulted in the discovery of some new revelations that *N'*-[2-(5,6-dimethylbenzothiazlyl)]-*N*-furfuryloxamide (7c) at the concentration of 1.0×10^{-3} M completely inhibited the radicle growth of both rape and leek seedlings.

Key words furan derivative; oxamide derivative; plant growth regulator; seedling; benzothiazole derivative; herbicide

Thiazole and benzothiazole derivatives have attracted much attention from both botanical and plant physiological viewpoints.[1] For example, the 5-thiazolecarboxamide derivative (**1**) at 50 ppm showed 75% control of the fungus *Pseudoperonospora cubenis*.[2] Compound (**2**) totally protected wood against *Coniophora puteana*.[3] Compound (**3**) gave ≧90% control of *Amaranthus viridis* at 0.05 kg/ha and caused no damage to beets. The herbicidal activity of **3** is greater than that of triflusulfuron-methyl.[4] The benzothiazole derivative (**4**) showed 90—100% control against *Sesbania exaltata*, *Abutilon theophrasti*, *Solanum* sp. and *Viola* sp. at 0.1 kg/ha.[5] The 2-(2-benzothiazolyloxy)acetamide derivative (**5**) inhibited the growth of barnyard grass and *Cyperaceae* weeds.[6]

In previous papers, we showed that *N'*-(2-thiazolyl)-*N*-furfuryloxamide (**6a**) induced about a 23% promotion in rooting in a seed germination assay using rape and leek.[7] As part of our efforts to identify more effective plant growth regulators among the derivatives of **6a**, we were prompted to examine the effect of the structural changes of the thiazole ring on plant physiology. Namely, our attention focused on the expectation that the modest promotion property mentioned above may vary with the structure of thiazole ring either to be reduced or to be strengthened. Here we describe the preparation of the thiazole and benzothiazole derivatives (**6b—f** and **7a—c**) and the examination of their activity as plant growth regulators in a seed germination assay.

We synthesized the thiazole derivatives (**6a—f**) and benzothiazole derivatives (**7a—c**) as follows. Furfurylamine (**9**) was condensed with the potassium salt (**10**) of the oxalic acid monomethyl ester using 1,1'-oxalyldiimidazole (ODI) to afford methyl *N*-furfuryloxamate (**11**), which was hydrolyzed in sodium hydroxide solution at room temperature to give the sodium salt (**12**) of *N*-furfuryloxamic acid.

The carboxyl group of **12** was activated with ODI to form the imidazolide intermediate (**13**), which was subjected to amidation with the 2-aminothiazole derivatives (**14**) or 2-aminobenzothiazole derivatives (**15**) to afford the corresponding *N'*-substituted *N*-furfuryloxamides (**6** or **7**) in yields of 40% to 83%.

The plant growth regulatory property was assayed according to the method reported by Inamori *et al.*[8] using seeds of rape, *Brassica campestris* L. (Brassicaceae), as a dicotyledon and leek, *Allium tuberosum* ROTTLER (Liliaceae), as a monocotyledon. 2,4-Dichlorophenoxyacetic acid (2,4-D) was used as the positive control. The results are summarized in Tables 1 and 2.

First, we chose a bromine group as the substituent on the thiazole ring because agrochemicals containing this halogen atom, such as bromacil or diquat, often cause a negative mode of action. The 5-bromothiazole derivative (**6b**) at high concentrations of 1.0×10^{-3} M then exhibited a marked inhibitory effect, whereas the parent 2-thiazole derivative (**6a**) showed about 23% promotion of root growth for the rape and leek seedlings. On the other hand, the 4,5-dimethylthiazole derivative (**6e**) showed about 45% inhibition, which suggest that a small alkyl group such as a methyl group did not have a great effect on the inhibition activity.

We are continuously interested in the more bulky substituent such as the phenyl ring. For example, the thiazole de-

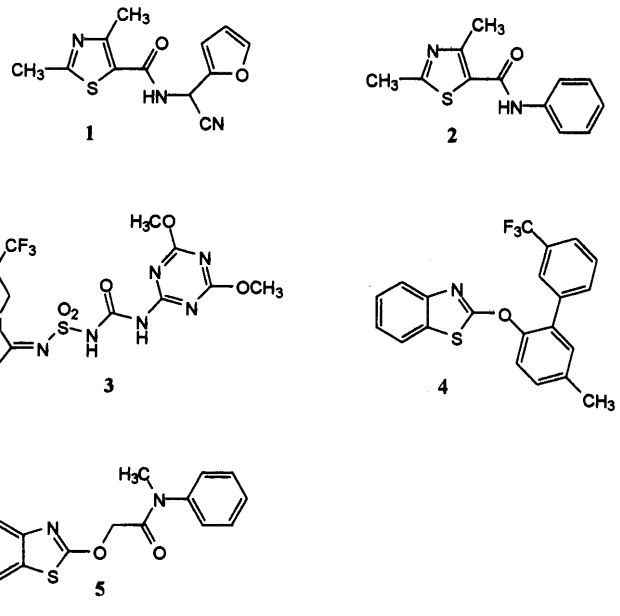

Chart 1

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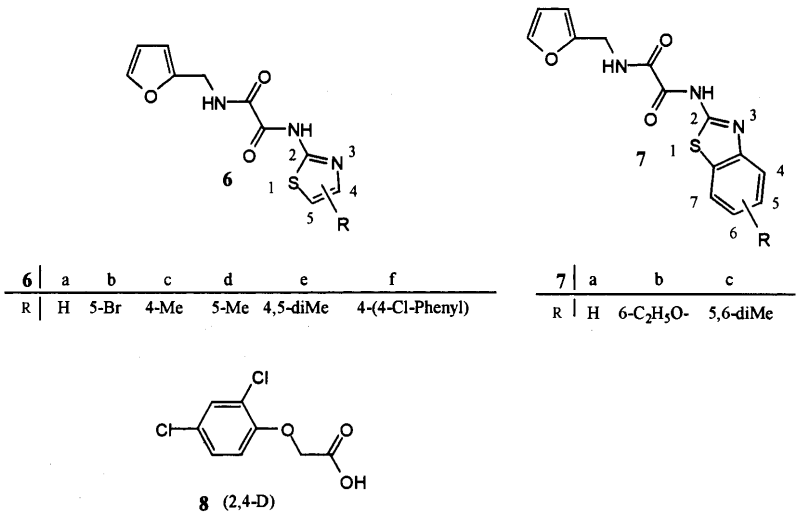
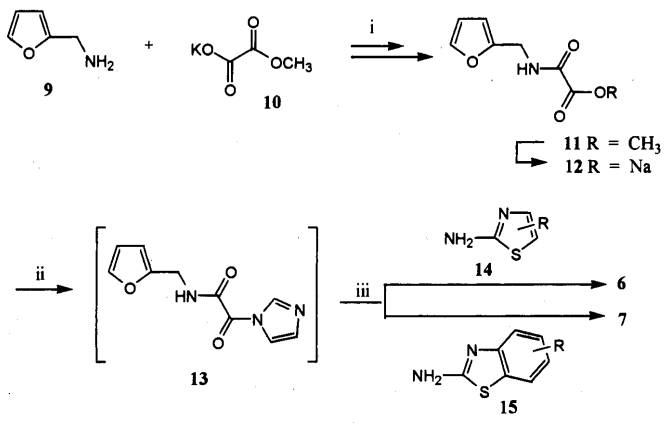


6	a	b	c	d	e	f
R	H	5-Br	4-Me	5-Me	4,5-diMe	4-(4-Cl-Phenyl)

7	a	b	c
R	H	6-C_2H_5O-	5,6-diMe

Chart 2



i, condensation using 1,1′-oxalyldiimidazole (ODI) to give an amide-ester, and the hydrolysis using NaOH. ii, activation using ODI. iii, amidation.

Chart 3

rivative (**6f**), which has a 4-chlorophenyl group at the 4-position of the thiazole ring, exhibited a 98% inhibition for rape and 95% for leek at the concentration of 1.0×10^{-3} M.

It would appear quite reasonable to us that increasing the steric crowding on the *b*-side of the thiazole ring enhances the inhibitory effect on root growth. Therefore, by postulating that all the oxamide groups, thiazole rings and 4-chlorophenyl rings lie on the same plane, it may be useful to speculate that the more the coplanarity is increased, the more clearly the inhibition property will appear. This idea is supported by the finding that the 5,6-dimethylbenzothiazole derivative (**7c**) showed complete inhibition at the concentration of 1.0×10^{-3} M for both rape and leek seedlings, while the 6-ethoxybenzothiazole derivatives (**7b**) showed only about a 70% inhibition at the same concentration because the whole coplanarity formed by oxamide-benzothiazole groups is slightly broken by bonding of the 6-ethoxy group with the benzene ring. As shown in Table 2, the activity of **7c** is similar to that of 2,4-D (**8**) as the positive control. Therefore, **7c** appears to be a new seed compound available as a growth retardant.

In summary, we synthesized the *N*′-[2-(substituted thiazolyl)] and *N*′-[2-(substituted benzothiazolyl)]-*N*-furfuryloxamides (**6** and **7**). The *N*′-[2-(5,6-dimethylbenzothiazolyl)]-*N*-furfuryloxamide (**7c**) at the concentration of 1.0×10^{-3} M completely inhibited the rooting of the rape and leek seedlings.

Experimental

Methyl oxalate, oxalyl chloride, imidazole, dimethyl sulfoxide (DMSO), furfurylamine, the 2-aminothiazole derivatives (**14b**—**f**), the 2-aminobenzothiazole derivatives (**15a**—**c**), and 2,4-D were purchased from commercial sources and used as received. *N*′-Thiazolyl-*N*-furfuryloxamide (**6a**) and the *N*-furfuryloxamic acid sodium salt (**12**) were prepared according to the reported procedures.[7a] *N*-Furfuryloxamic acid is very hygroscopic; therefore, for convenience, it was converted into the corresponding sodium salt. When compounds **14c**, **14d**, **14f**, **15a**—**15c** were chosen as the free amine components, no triethylamine was used as a scavenger for hydrogen halide. Melting points were taken on a Yanagimoto melting point apparatus. All melting points are uncorrected. The IR spectra were measured using a Hitachi model 270-30 IR spectrophotometer. The NMR spectra were measured on a Bruker DPX-400 spectrometer (400 MHz) with tetramethylsilane as the internal reference, and chemical shifts were recorded as δ-values.

***N*′-[2-(5-Bromothiazolyl)]-*N*-furfuryloxamide (6b)** A solution of oxalyl chloride (1.28 g, 10 mmol) in acetonitrile (10 ml) was added dropwise to an ice-cold, stirred solution of imidazole (2.7 g, 40 mmol) in acetonitrile (150 ml). The mixture was stirred at room temperature for 5 min, then a suspension of the *N*-furfuryloxamic acid sodium salt (**12**) (1.8 g, 10 mmol) and methanesulfonic acid (1 g, 10 mmol) in acetonitrile (10 ml) was rapidly added as a single portion. The mixture was stirred at room temperature for 20 min, and then a suspension of 2-amino-5-bromothiazole mono hydrobromide (**14b**) (1.14 g, 10 mmol) and triethylamine (1.0 g, 10 mmol) in acetonitrile (10 ml) was added in a single portion. The resultant mixture was stirred for 3 h at 40 °C. The solvent was removed *in vacuo*, and the remaining residue was poured onto ice and extracted with ethyl acetate. Washing of the ethyl acetate extract with 5% hydrochloric acid and water, followed by drying and evaporation of the solvent left 2.3 g (69%) of the crude product (**6b**). Recrystallization from toluene gave **6b** with a mp of 210—212 °C. IR (KBr) cm^{-1}: 1662 (CO). ^{1}H-NMR (DMSO-d_6) δ: 4.3 (d, 2H, J=6.1 Hz, CH_2), 6.2—7.5 (m×3, 1H×3, furan-4H, -3H and -5H), 7.6 (s, 1H, thiazole-4H), 9.5—9.6 (t, 1H, $CH_2\underline{NH}$), 13.4 (s, 1H, CONH). *Anal.* Calcd for $C_{10}H_8BrN_3O_3S$: C, 36.38; H, 2.44; N, 12.73. Found: C, 36.12; H, 2.31; N, 12.72.

***N*′-2-(4-Methylthiazolyl)-*N*-furfuryloxamide (6c)** **6c** was prepared as described for **6b**, through the reaction of **12** with 2-amino-4-methylthiazole (**14c**) in 68% yield. Recrystallization from ethanol gave **6c**, mp 172—173 °C. IR (KBr) cm^{-1}: 1665 (CO). ^{1}H-NMR (DMSO-d_6) δ: 2.2 (s, 3H, CH_3), 4.3—4.4 (d, 2H, J=6.1 Hz, CH_2), 6.2—7.5 (m×3, 1H×3, furan-4H, -3H and -5H), 6.9 (s, 1H, thiazole-5H), 9.5 (t, 1H, $CH_2\underline{NH}$), 12.4 (s, 1H, CONH). *Anal.* Calcd for $C_{11}H_{11}N_3O_3S$: C,49.8; H, 4.18; N, 15.84. Found: C, 49.97; H, 4.24; N, 15.86.

***N*′-2-(5-Methylthiazolyl)-*N*-furfuryloxamide (6d)** **6d** was prepared as described for **6b**, through the reaction of **12** with 2-amino-5-methylthiazole (**14d**) in 66% yield. Recrystallization from ethanol gave **6d**, mp 195—

Table 1. Plant Growth-Modulating Activities of *N'*-Substituted *N*-(2-Furfuryl)oxamides (**6** and **7**)

Compound	*N'*-Subs. groups	Dicotyledoneae Rape; *Brassica campestris* L.				Monocotyledoneae Leek; *Allium tuberosum* Rottler			
		Growth (mm)[a] Control	Growth (mm)[a] 1.0×10^{-3} (M)	Promotion (%)[b]	Inhibition (%)[b]	Growth (mm)[a] Control	Growth (mm)[a] 1.0×10^{-3} (M)	Promotion (%)	Inhibition %)
6a	2-Thiazolyl	57±17.5	70±10.5**	21	—	8±5.0	10±5.7*	25	—
6b	2-(5-Bromothiazolyl)	57±15.4	1±0.9**	—	99	7±3.9	0**	—	100
6c	2-(4-Methylthiazolyl)	52±16.3	31±19.1**	—	41	7±3.7	2±1.9**	—	71
6d	2-(5-Methylthiazolyl)	59±16.9	28±19.2**	—	5	7±3.9	3±2.2**	—	57
6e	2-(4,5-Dimethylthiazolyl)	60±16.1	32±18.9**	—	47	7±5.1	4±3.3*	—	42
6f	2-[4-(4-Chlorophenyl)thiazolyl]	58±17.2	0.8±0.9**	—	98	7±4.9	0.3±0.4**	—	95
7a	2-Benzthiazolyl	57±15.2	4±4.7**	—	92	6±3.5	0.9±0.5**	—	85
7b	2-(6-Ethoxybenzthiazolyl)	62±15.7	21±9.7**	—	67	6±3.6	1±1.1**	—	83
7c	2-(5,6-Dimethylbenzthiazolyl)	53±15.6	0**	—	100	7±3.4	0**	—	100
	2,4-D[c]	64±25.7	0**	—	100	10±2.2	0**	—	100

a) The values represent mean±S.D. of 40 seeds after seven days (*A. tuberosum*: 10 d). Significant differences from the corresponding control level are indicated, * and ** show $p<0.05$ and $p<0.01$, respectively. Quantity of light: 127 μmol·m^{-2}·s^{-1}. Temperature 25 °C. Relative humidity of 60%. Experimental size: 20 seeds/group, 2 groups. *b*) [(The mean value of the control−the mean value at the concentration (M) of 1.0×10^{-3})/the mean value of control]×100=*P* (%); when *P* shows a positive value, the absolute value of *P* corresponds to the promotion effect, and in the case of *P* showing a negative value, the absolute value of that corresponds to the inhibition effect. *c*) 2,4-Dichlorophenoxyacetic acid (2,4-D) was used as a positive control.

Table 2. Plant Growth Activities of Compounds **6b**, **6f**, **7a**, **7c** and **8**

Compound	*N'*-Subs. groups	Dicotyledoneae Rape; *Brassica campestris* L.				Monocotyledoneae Leek; *Allium tuberosum* Rottler			
		Inhibition (%)[a] 1.0×10^{-3} (M)	5.0×10^{-4} (M)	1.0×10^{-4} (M)	5.0×10^{-5} (M)	Inhibition (%)[a] 1.0×10^{-3} (M)	5.0×10^{-4} (M)	1.0×10^{-4} (M)	5.0×10^{-5} (M)
6b	2-(5-Bromothiazolyl)	99	79	20	2	100	85	27	5
6f	2-[4-(4-Chlorophenyl)thiazolyl]	98	90	85	72	96	96	68	57
7a	2-Benzthiazolyl	92	70	41	3	85	64	43	19
7c	2-(5,6-Dimethyl)benzthiazolyl	100	100	95	62	100	98	95	53
8	2,4-D[b]	100	99	98	98	100	100	99	97

a) See the footnotes *a*) and *b*) of Table 1. *b*) 2,4-Dichlorophenoxyacetic acid (2,4-D) was used as a positive control.

198 °C. IR (KBr) cm^{-1}: 1663 (CO). ^{1}H-NMR (DMSO-d_6) δ: 2.1(s, 3H, CH_3), 4.2—4.3 (d, 2H, J=6.1 Hz, CH_2), 6.1—7.4 (m×3, 1H×3, furan-4H, -3H and -5H), 7.1 (s, 1H, thiazole-5H), 9.3 (t, 1H, $CH_2\underline{NH}$), 12.1 (s, 1H, CONH). *Anal.* Calcd for $C_{11}H_{11}N_3O_3S$: C, 49.8; H, 4.18; N, 15.84. Found: C, 49.65; H, 4.23; N, 15.80.

***N'*-[2-(4,5-Dimethylthiazolyl)]-*N*-furfuryloxamide (6e)** **6e** was prepared as described for **6b**, through the reaction of **12** with 2-amino-4,5-dimethylthiazole hydrochloride (**14e**) in 83% yield. Recrystallization from toluene gave **6e**, mp 168—169 °C. IR (KBr) cm^{-1}: 1659 (CO). ^{1}H-NMR (DMSO-d_6) δ: 2.1 (s, 3H, CH_3), 2.2 (s, 3H, CH_3), 4.3 (d, 2H, J=6.1 Hz, CH_2), 6.2—7.5 (m×3, 1H×3, furan-4H, -3H and -5H), 9.4 (t, 1H, $CH_2\underline{NH}$), 12.4 (br s, 1H, CONH). *Anal.* Calcd for $C_{12}H_{13}N_3O_3S$: C, 51.61; H, 4.69; N, 15.04. Found: C, 51.59; H, 4.69; N, 15.09.

***N'*-[2-[4-(4-Chlorophenyl)thiazolyl]]-*N*-furfuryloxamide (6f)** **6f** was prepared as described for **6b**, through the reaction of **12** with 2-amino-4-(4-chlorophenyl)thiazole (**14f**) in 46% yield. Recrystallization from methanol gave **6f**, mp 205—207 °C. IR (KBr) cm^{-1}: 1662 (CO). ^{1}H-NMR (DMSO-d_6) δ: 4.4 (d, 2H, J=6.1 Hz, CH_2), 6.3—7.6 (m×3, 1H×3, furan-4H, -3H and -5H), 7.5—7.9 (two d, each 2H, J=8.0 Hz, phenyl -2H, -6H and phenyl-3H, -5H), 7.8 (s, 1H, thiazole-5H), 9.5 (t, 1H, $CH_2\underline{NH}$), 12.6 (s, 1H, CONH). *Anal.* Calcd for $C_{16}H_{12}ClN_3O_3S$: C, 53.12; H, 3.34; N,11.61. Found: C,53.33; H,3.43; N,11.51.

***N'*-(2-Benzothiazolyl)-*N*-furfuryloxamide (7a)** **7a** was prepared as described for **6b**, through the reaction of **12** with 2-aminobenzothiazole (**15a**) in 40% yield. Recrystallization from toluene gave **7a**, mp 161—162 °C. IR (KBr) cm^{-1}: 1660 (CO). ^{1}H-NMR (DMSO-d_6) δ: 4.2 (d, 2H, J=6.1 Hz, CH_2), 6.3—7.7 (three m, each 1H, furan-3H, 4H and 5H), 7.3—7.5 (m, 4H, benzothiazole-4H, 5H, 6H and -7H), 9.6 (t, 1H, $CH_2\underline{NH}$), 12.3 (br s, 1H, CONH). *Anal.* Calcd for $C_{14}H_{11}N_3O_3S$: C, 55.81; H, 3.68; N, 13.95. Found: C, 56.14; H, 3.89; N, 14.18.

***N'*-[2-(6-Ethoxybenzothiazole)]-*N*-furfuryloxamide (7b)** **7b** was prepared as described for **6b**, through the reaction of **12** with 2-amino-6-ethoxybenzothiazole (**15b**) in 83% yield. Recrystallization from toluene gave **7b**, mp 172—173 °C. IR (KBr) cm^{-1}: 1668 (CO). ^{1}H-NMR (DMSO-d_6) δ: 1.3 (s, 3H, CH_3), 4.0 (q, 2H, $\underline{CH_2}CH_3$), 4.4 (d, 2H, J=6.1 Hz, $\underline{CH_2}NH$), 6.3—7.0 (three m, each 1H, furan-3H, 4H and 5H), 7.5—7.9 (m, 3H, benzothiazol-4H, 5H and 7H), 9.5 and 9.6 (t, 1H, $\underline{CH_2}NH$), 12.6 (br s, 1H, CONH). *Anal.* Calcd for $C_{16}H_{15}N_3O_4S$: C, 55.65; H, 4.34; N, 12.17. Found: C, 55.57; H, 4.27; N, 11.81.

***N'*-[2-(5,6-Dimethylbenzothiazolyl)]-*N*-furfuryloxamide (7c)** **7c** was prepared as described for **6b**, through the reaction of **12** with 2-amino-5,6-dimethylbenzothiazole (**15c**) in 81% yield. Recrystallization from toluene gave **7c**, mp 250—251 °C. IR (KBr) cm^{-1}: 1707 and 1617 (CO). ^{1}H-NMR (DMSO-d_6) δ: 2.3 (two s, each 3H, each CH_3), 4.3 (d, 2H, J=6.1 Hz, CH_2), 6.2—7.7 (three m, each 1H, furan-3H, 4H and 5H), 7.2—7.3 (m, 2H, benzothiazole-4H and 7H), 9.6 (t, 1H, each $CH_2\underline{NH}$), 12.6 (br s, 1H, CONH). *Anal.* Calcd for $C_{16}H_{15}N_3O_3S$: C, 58.35; H, 4.59; N, 12.76. Found: C, 58.22; H, 4.55; N, 12.80.

Plant Growth-Inhibitory Activity Test This test was carried out according to the method reported by Inamori *et al.*[8] The DMSO solution (1.0 ml) containing a test oxamide derivative (**6b—f** or **7a—c**), 2,4-D as the positive control, or DMSO alone (1.0 ml) as the control, was diluted in 100 ml of sterilized agar (0.8%, Nacalai Tesque, Inc.) to give concentrations of 5×10^{-5} M, 1.0×10^{-4} M, 5×10^{-4} M, and 1.0×10^{-3} M. Agar containing the test compounds (**6**, **7** and 2,4-D) or DMSO as the control was poured into a 500 ml sterilized culture jar. Twenty seeds of each plant species, sterilized with 70% ethanol and 1% NaClO, were placed on the agar and left for seven days (*A. tuberosum*; ten days) at 25 °C under a relative humidity of 60% and a light intensity of 127 μmol·m^{-2}·s^{-1}. The results are summarized in Tables 1 and 2.

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A New Anti-HIV Triterpene from *Geum japonicum*

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Geumonoid (1), a new triterpene, was isolated from *Geum japonicum*. Its structure was elucidated on the basis of 1D, 2D NMR and MS spectroscopic analysis. Compound 1 showed inhibitory activity against HIV-1 protease.

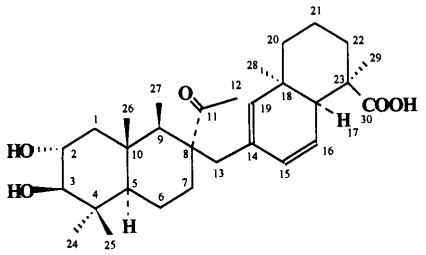
Key words *Geum japonicum*; triterpene; geumonoid; anti-HIV protease; Rosaceae

Previous phytochemical studies on the constituents of *Geum japonicum* Thunb. (Rosaceae) led to the discovery of many compounds, including triterpenoids and tannins.[1—6] As part of our continuous searching for novel bioactive agents from medicinal plants, a methanol extract from the whole plant of *G. japonicum* was found to show significant inhibitory activity against HIV-1 protease. Bioactivity-guided chromatographic fractionation of the active extract led to the isolation and characterization of a new compound, named geumonoid (**1**). Herein, we report the isolation and structural elucidation of the new compound and its inhibitory activity against HIV-1 protease.

Geumonoid (**1**), obtained as an amorphous powder, showed a molecular peak at *m/z* 486 $[M]^+$ in its EIMS. A molecular formula $C_{30}H_{46}O_5$ was determined from comprehensive examination of the spectral data (EI-MS and NMR) and elemental analysis. It gave a positive response to the Liebermann–Burchard reagent. Its IR spectrum showed the presence of a carbonyl (1714 cm^{-1}), a carboxyl (1700 cm^{-1}), and hydroxyl (3424 cm^{-1}) groups. The ^{1}H-NMR spectrum of **1** showed signals for seven methyl groups at δ 0.89, 0.99, 1.05, 1.06, 1.07, 2.11 (each 3H, s), and 1.26 (d, J=6.8 Hz); two oxymethine groups at δ 4.15 (ddd, J=11.2, 9.3, 4.4 Hz) and 3.40 (d, J=9.3 Hz); three olefinic protons at δ 5.77 (1H, d, J=10.1 Hz), 6.16 (1H, dd, J=10.1, 2.8 Hz), and 5.85 (1H, s). The coupling constant of proton signals at δ 5.77 and 6.16 is 10.1, suggesting the presence of two *cis*-olefinic protons. The olefinic proton at δ 5.85 occurred as a singlet, indicating that no proton was affixed to the adjacent carbon.

The ^{13}C-NMR spectrum and DEPT experiments showed 30 carbon signals: seven methyl, seven methylene, eight methine (including three olefinic methine and two oxymethine), and eight quaternary (including an olefinic, a carboxylic, and a carbonyl) carbons. The carbonyl, carboxyl and olefinic groups account for four of the eight units of unsaturation required by the molecular formula, and thus, compound **1** was determined to be tetracyclic, which suggested that this compound might be a triterpene.

The HMQC spectrum established the one bond correlation between carbons and hydrogens in compound **1**. The connections of the groups were derived from ^{1}H–^{1}H COSY NMR and HMBC spectra. In the ^{1}H–^{1}H COSY NMR spectrum, the doublet at δ 3.40 (H-3) showed a cross peak with δ 4.15 (ddd) attributed to the H-2 proton. The latter was coupled with two multiplets at δ 2.50 (1H) and 1.36 (1H), assigned as the two protons (H-1) of a methylene. The cross peaks between δ 1.10 (H-5) and 1.44 (H-6a), 1.44 (H-6a) and 1.63 (H-6b), 1.63 (H-6b) and 2.18 (H-7a), 2.18 (H-7a) and 1.25 (H-7b) were also observed in the ^{1}H–^{1}H COSY NMR spectrum. The above correlations revealed the connectivities of CH (3)–CH (2)–CH_2 (1) and CH (5)–CH_2 (6)–CH_2 (7). The HMBC spectrum of **1** (see Fig. 1) revealed the long-range correlations from δ 55.43 (C-5) to δ 1.07 (H-24), and 0.99 (H-25); from δ 83.77 (C-3) to δ 2.50, 1.36 (H-1); from δ 47.42 (C-9) to δ 2.18, 1.25 (H-7), and 1.10 (H-5); and from δ 211.40 (C-11) to δ 2.11 (H-12), and δ 2.52 (H-9). This information, together with the EI-MS fragments (see Fig. 2) at *m/z* 281, 279, 263, 261, 169, 167, 149, and 43, suggest the presence of partial structure A in compound **1**.



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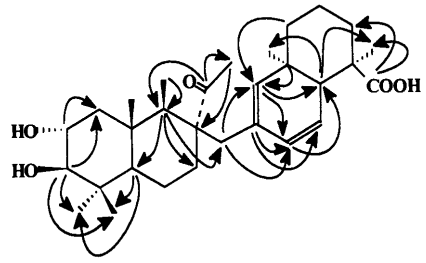

Fig. 1. Selective HMBC Observations for Geumonoid
Arrows denote HMBC correlation from C to H.

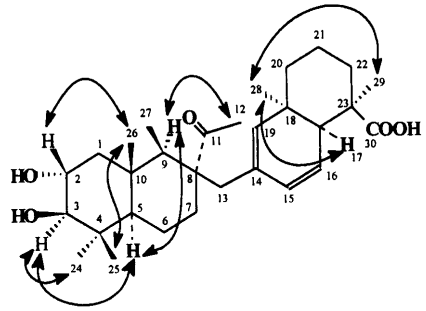

Fig. 2. NOE Interactions Obtained in the NOESY Spectrum of **1**

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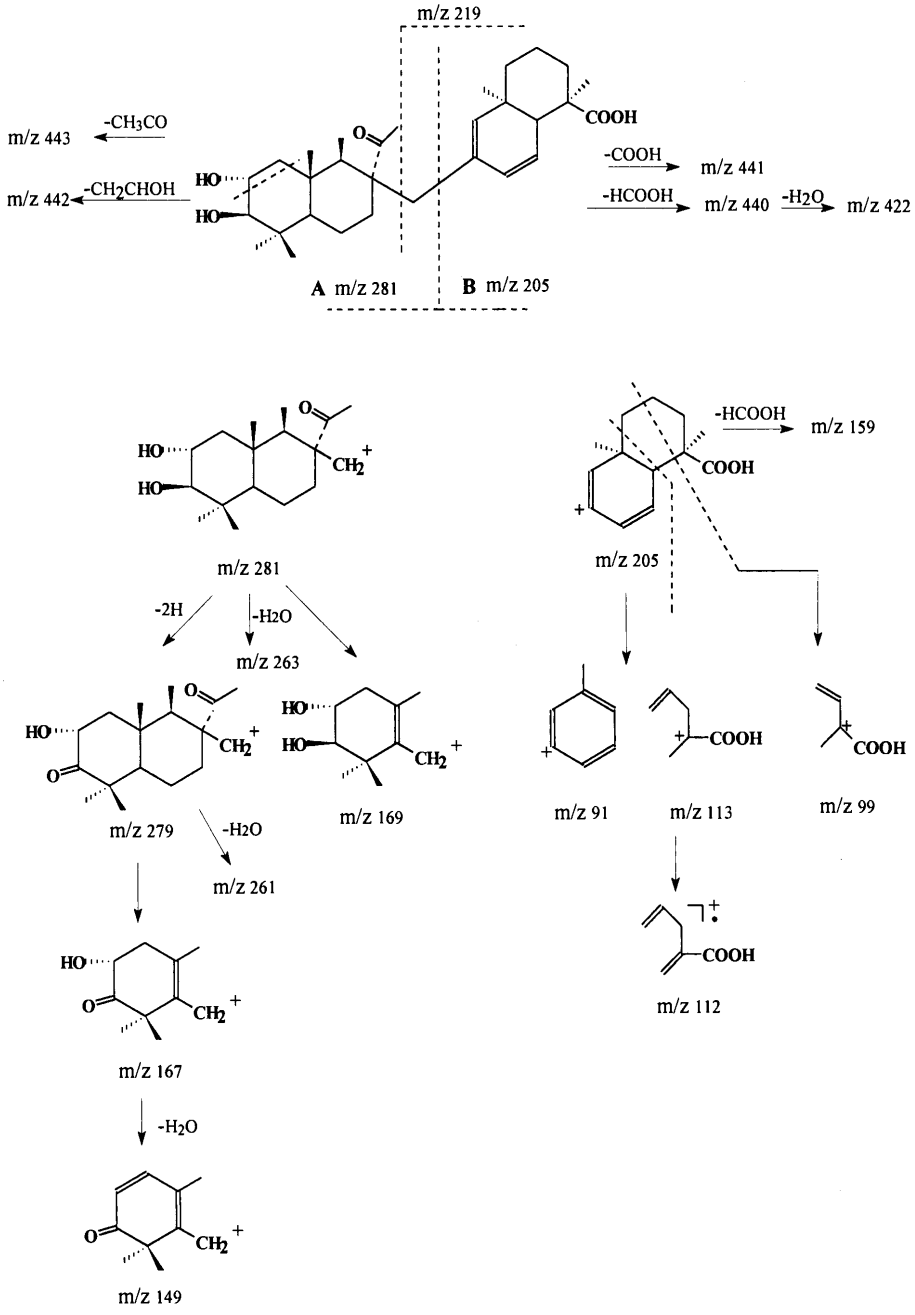

Fig. 3. The EIMS Fragments of **1**

In addition to the above data, the correlations between δ 5.77 (d, J=10.1 Hz, H-15) and 6.16 (dd, J=10.1, 2.8 Hz, H-16) and 2.17 (d, J=2.8 Hz, H-17), δ 2.57 (m, H-20a) and 1.64 (m, H-20b), δ 1.64 (m, H-20b) and 2.01 (m, H-21a), δ 2.01 (m, H-21a) and 1.65 (m, H-21b), 1.65 (m, H-21b) and 1.38 (m, H-22a) and 1.34 (m, H-22b) were also observed in the ^{1}H–^{1}H COSY NMR spectrum, and the long-range correlations from H-16 to C-14, C-17 and C-23; from H-15 to C-13, C-17, and C-19; from H-19 to C-13, C-18, and C-20; from C-17 to H-28, H-29; from C-30 to H-22, H-29; and from C-13 to H-15 were observed in the HMBC spectrum of **1** (see Fig. 1). This information, together with the EI-MS fragments (see Fig. 3) at *m/z* 219, 205, 113, 112, 99, implied partial structure B existing in the molecule of compound **1** and determined the linkage position of the partial structures of A and B.

The C-3 proton appeared to be a doublet with a coupling constant of 9.3 Hz, which suggested axial–axial couplings between C-2H and C-3H. Comparing the chemical shift values of carbon signals at C-2 and C-3 (δ 68.56, 83.77) with those of the known compound tormentic acid[7] suggested that they have the same configurations at C-2 and C-3. Thus, the configurations of protons at C-2 and C-3 were deduced to be β and α, respectively, which were further confirmed by the NOE interactions in the NOESY spectrum of **1** (see Fig. 2). The cross peaks observed in the NOESY spectrum between H-3 and H-5, H-3 and H-24, H-5 and H-9, H-2 and H-26, H-26 and H-27 implied that the relative configurations of H-2, H-5, H-9, 9-methyl and 10-methyl were α, α, α, β, and β, respectively. The NOESY spectrum also showed cross peaks between H-17 and H-28, and between H-28 and H-29. Thus, the orientation between H-17, 18-methyl, and 23-methyl are all *cis* in the opposite direction to the carboxylic group. The above information enabled us to determine the structure and

Table 1. NMR Data for Geumonoid (in Pyridine-d_5)

Position	δ_H	δ_C
1	2.50 m, 1.36 m	47.34 (t)
2	4.15 ddd (11.2, 9.3, 4.4)	68.56 (d)
3	3.40 d (9.3)	83.77 (d)
4		39.85 (s)
5	1.10 m	55.43 (d)
6	1.44 m, 1.63 m	18.65 (t)
7	2.18 m, 1.25 m	26.60 (t)
8		40.88 (s)
9	2.52 s	47.42 (d)
10		38.19 (s)
11		211.40 (s)
12	2.11 s	28.04 (q)
13	2.01 m, 1.70 m	38.97 (t)
14		142.45 (s)
15	5.77 d (10.1)	127.41 (d)
16	6.16 dd (10.1, 2.8)	130.55 (d)
17	2.17 d (2.8)	54.65 (d)
18		41.53 (s)
19	5.85 s	128.98 (d)
20	2.57 m, 1.64 m	27.42 (t)
21	1.38 m, 1.34 m	32.50 (t)
22	2.01 m, 1.65 m	28.16 (t)
23		47.53 (s)
24	1.07 s	16.33 (q)
25	0.99 s	19.39 (q)
26	1.05 s	17.08 (q)
27	1.26 d (6.8)	28.99 (q)
28	1.06 s	20.16 (q)
29	0.89 s	16.82 (q)
30		177.87 (s)

relative stereochemistry of this molecule, as shown in **1**.

Compound **1** was tested for HIV-1 protease inhibitory effects with a recombinant enzyme, which exhibited strong activity with 89% inhibition at the concentration of 17.9 μg/ml. The studies of the structure–activity relationships and action model of the compound are currently in progress in our laboratory.

Experimental

General Melting points were measured with an XT4-100x micro-melting point apparatus and are uncorrected. An optical rotation was obtained on a Perkin-Elmer 241 polarimeter. The IR spectrum was recorded on a Perkin-Elmer 1600 series FT-IR spectrophotometer. NMR spectra were run on a Bruker AM-500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). EIMS was recorded on a Micro-Mass 7035E Mass spectrometer at 70 eV. Elemental analysis was performed on a MoD1106 elemental analyzer. Silica gel 60 (Merck 100—200 mesh) was used for column chromatography. Precoated silica gel Kieselgel 60 F_{254} plates (0.25 mm thickness) were used for TLC, and the spots were detected by spraying with 25% phosphomolybdic acid, followed by heating. Merck silica gel 60 F_{254} was used for preparative thin layer chromatography (PTLC, 0.5 mm thickness).

Plant Material The whole plant of *G. japonicum* used in this experiment was collected from China in Aug. 1994. The material was identified as *G. japonicum* Thunb. by Dr. Dao-Feng Chen, Department of Pharmacognosy, Shanghai Medical University, China. A voucher specimen was deposited in the Department of Pharmacognosy, Shanghai University of Traditional Chinese Medicine, China.

Extraction and Isolation Dried whole plants (2.6 kg) were chopped into small pieces and extracted three times with MeOH (20 l) at room temperature for 7 d; then the extract was evaporated *in vacuo* to yield MeOH extract (350 g). The MeOH extract was suspended in distilled water (1 l) and successively extracted with hexane (2 l×5) and EtOAc (2 l×5). The EtOAc soluble fraction was filtered, and the filtrate was evaporated under reduced pressure to give a brown oil. The EtOAc extract (100 g) was subjected to silica gel column chromatography using a linear gradient with a $CHCl_3$–MeOH system. The fractions with $CHCl_3$: MeOH (8 : 2) were further purified by repeated silica gel column chromatography and preparative TLC to give geumonoid (**1**, 10 mg).

Geumonoid (**1**): An amorphous powder, $[\alpha]_D^{25}$ +10.1° (c=1.0, EtOH); IR (KBr) ν_{max} cm^{-1}: 3424, 2934, 1714, 1700, 1654, 1636, 1560, 1458, 1384, 1048; ^{13}C- and ^{1}H-NMR data, see Table 1; EI-MS *m/z*: 486 (20), 468 (60), 443 (25), 442 (70), 441 (15), 440 (30), 422 (20), 281 (5), 279 (20), 263 (15), 261 (20), 219 (10), 205 (40), 169 (20), 167 (65), 159 (28), 149 (90), 113 (25), 112 (20), 99 (55), 91 (40), 43 (100). *Anal.* C, 74.12; H, 9.45, Calcd for $C_{30}H_{46}O_5$: C, 74.07; H, 9.47.

Biological Assay Recombinant HIV-1 protease was obtained from the expression vector, PGEX-PR 107, in *Escherichia coli* DH5a and was purified according to the method in literature.[8] The proteolytic activity of HIV-1 PR was measured using the synthetic heptapeptide Ser–Gln–Asn–Tyr–Pro–Ile–Val (SANYPIV), corresponding to the p24–p17 cleavage site in the natural gag precursor, as a substrate. The products of cleavage were analyzed by HPLC (Ultrasphere ODS, 5 μm, 4.6 mm×15 cm, Beckman, CA, U.S.A.) with a 7—33% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA). Product peak areas were integrated by a data system (Chromatopac C-R3A, Shimadzu, Japan) and compared with the standard peptide SQNY, which is expected to be generated from heptapeptide SQNYPIV after HIV-1 protease digestion. The extinction coefficient of peptide SQNY was used to estimate the rate of proteolysis of the heptapeptide substrate. The assay was performed in a volume of 140 μl containing 90 pmol of substrate, 2 U of HIV-1 protease, and compound solution in 0.1 M NaOAc buffer, pH 5.5, with 1 M $(NH_4)_2SO_4$, at 37 °C. The reaction, processed for 2 h, was terminated by the addition of 20 μl of aqueous 10% trifluoroacetic acid. The supernatant obtained by centrifugation at 12000 rpm for 3 min was then analyzed by the HPLC method. The unit of enzyme activity was defined as the amount of enzyme which yields 1 nmol of tetrapeptide SQNY under the above conditions in 1 min.

The proeolytic activity of HIV-1 protease inhibited by the compound was calculated as the activity of the control minus that of the sample and then divided by itself.

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Synthetic Studies of Zoanthamine/Norzoanthamine: Biogenetic-like One-Step Construction of the Heterocyclic Aminal Core (CDEFG Ring) from a Monocyclic Precursor

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Biogenetic-like one-step construction of the pentacyclic aminal core of zoanthamine/norzoanthamine alkaloids was accomplished in high yield from the suitably protected monocyclic aminohydroxy diketocarboxylic acid 7 by heating in aqueous acetic acid.

Key words aminal; alkaloid; norzoanthamine; polycycle; tandem reaction

Zoanthamine[3] (**1**) and norzoanthamine[4] (**2**) are marine alkaloids having significant biological activities. For example, norzoanthamine and its hydrochloride strongly suppress the decrease in bone weight and strength in ovariectomized mice without showing serious side effects such as are observed in the case of 17β-estradiol, and has been considered to be a promising osteoporotic drug.[4c] In addition to their interesting biological activities, their unique and complicated heptacyclic structure, including a bisaminal skeleton, has resulted in stimulatimg increasing synthetic efforts.[5] Uemura proposed a biosynthetic pathway for norzoanthamine which involves the cyclization of an assumed acyclic precursor **3**.[4c] Although the precise pathway, particularly for the ABC ring, is unclear, it seems plausible that a DEFG ring moiety is constructed from the tricyclic precursor **4** either in an enzymatic or a non-enzymatic way.

During the course of our investigation towards the total synthesis of norzoanthamine, we became interested in the construction of the CDEFG ring moiety by a biogenetic-like cyclization which is most attractive from a synthetic point of view as well. Furthermore, we also envisaged that the construction of this moiety could be achieved at the final stage of the total synthesis. These considerations led us to investigate the development of an efficient methodology for the synthesis of fully functionalized aminal moiety **5**, and we recently reported the first synthesis of the pentacyclic aminal core **5**.[6] The synthesis of **5** was accomplished in two steps from the protected aminohydroxy diketocarboxylic acid **6** as shown in Chart 1. Thus, **6** was initially converted to the monoaminal **8** by treating with hydrochloric acid in THF in 78% yield, and hydrogenolysis of the Cbz group in **8** resulted in simultaneous cyclization affording the desired pentacyclic **5** in 71% yield after MS3A treatment. Although this is the first entry to the aminal core, it would be more desirable if pentacyclic aminal core **5** could be constructed in one-step from a suitably protected precursor in a similar manner to a plausible biogenetic-like tandem cyclization. Here we wish to describe the successful transformation of Boc derivative **7** to **5**.

The preparation of the cyclization precursor **7** followed the same strategy developed for the preparation of Cbz-derivative **6**.[6] (Chart 2) Thus, the aldehyde **9**[6] was coupled with the sulfone **11**, prepared from the corresponding Cbz-derivative **10** by 4 steps[7] in 99% yield. The resulting hydroxysulfone was further transformed to the cyclization precursor **7** by conventional functional group manipulation in 38% overall yield from **9**.[8]

We then investigated the cyclization of **7**. At first, **7** was treated with 2 N HCl–THF (1 : 3), the same conditions for the cyclization of Cbz-derivative **6** to monoaminal **8**, to obtain the corresponding monoaminal **12** and spiroketal **13** in 89% and 7% yields, respectively. The structure of **12** was determined by comparison of its ^{1}H-NMR spectrum with that of the corresponding Cbz-derivative **8**. This result indicated that the Boc group remained untouched under these conditions. Deprotection of the Boc group was next examined under various standard conditions[9] such as HCl–THF, HCl (g)–AcOEt, CF_3CO_2H–CH_2Cl_2, TMSI–CH_3CN, *etc.* However, only decomposition of **12** occurred under these forcing conditions and the formation of **5** could not be detected by TLC. Among a number of unsuccessful efforts, we observed that deprotection of the Boc group was accelerated by adding a small amount of H_2O in CH_3CO_2H, and we focused on an H_2O–CH_3CO_2H system for deprotection of the Boc group.

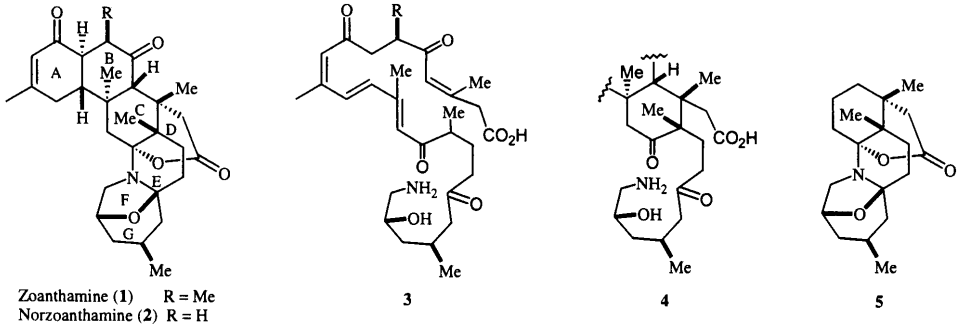

Fig. 1

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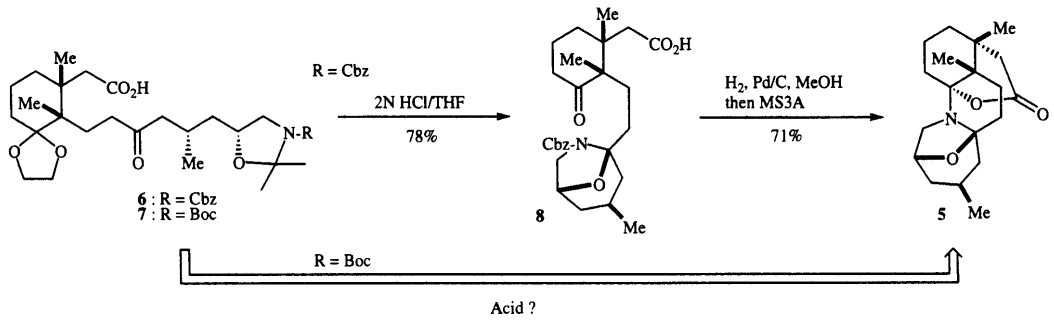

Chart 1

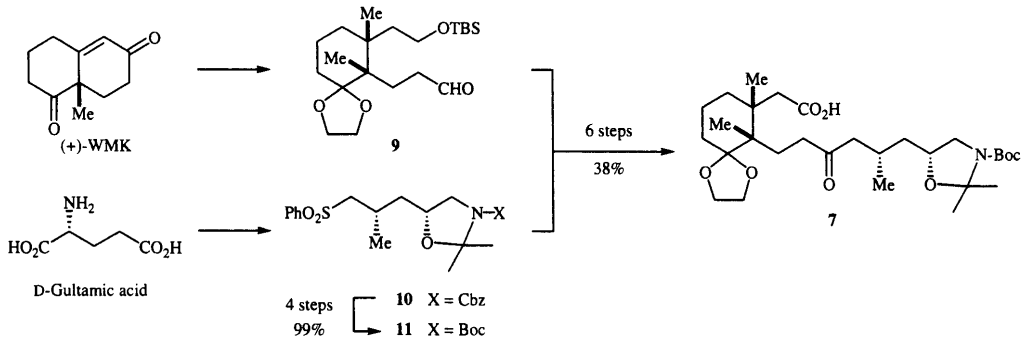

Chart 2

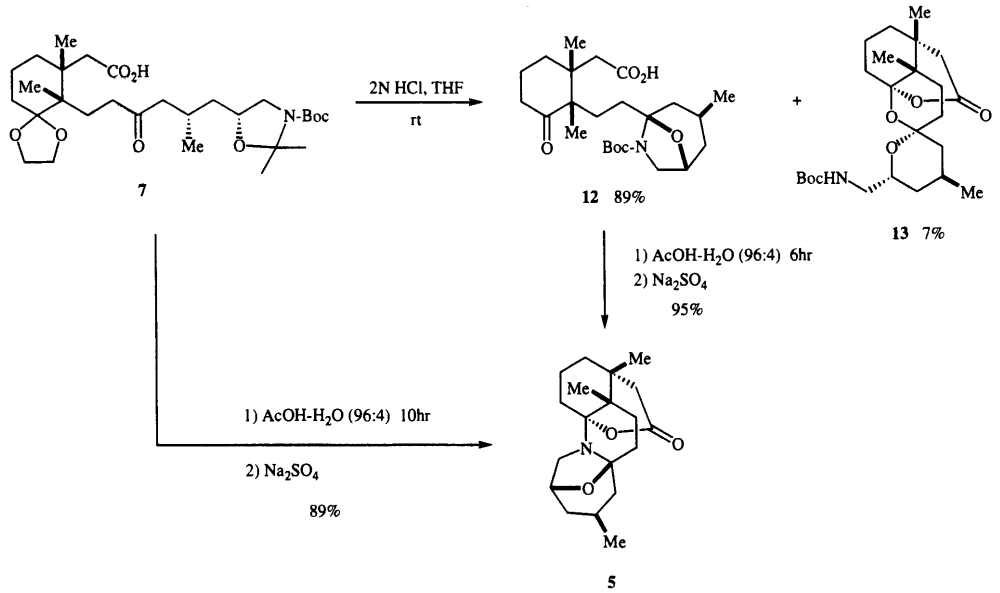

Chart 3

After a systematic survey of varying ratios ($CH_3CO_2H : H_2O$) and reaction temperatures, we were finally able to obtain the pentacyclic aminal **5** in 95% yield by heating at 100 °C in CH_3CO_2H–H_2O (96 : 4) for 6 h, followed by the addition of anhydrous Na_2SO_4. Without Na_2SO_4 treatment, aminal **5** could not be isolated. The obtained **5** was unambiguously identified by comparing its NMR spectrum with that of the authentic sample prepared by the previous method.[6] Further, the present conditions, CH_3CO_2H–H_2O (96 : 4) at 100 °C, were found to be effective for the one-step and biogenetic-like conversion of **7** to **5**. Thus, pentacyclic **5** was produced in 89% yield from **7**.

In summary, we were able to establish an efficient methodology for the construction of the fully functionalized CDEFG ring moiety **5** of the zoanthamine and norzoanthamine family. It should be emphasized that a pentacyclic aminal system could be constructed from the monocyclic Boc-derivative **7** in one-step by finding the appropriate acidic conditions. We think that the present conditions might be applicable to the final step of the total synthesis of norzoanthamine, and studies along this line are now in progress.

Experimental

General NMR spectra were recorded on a JEOL JNM-LA-400, JNM AL-300 or JNM-EX-270 spectrometer. Chemical shifts are shown in ppm downfield internal tetramethylsilane. The abbreviations used in ^{1}H-NMR data are as follows: s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet. IR were recorded on a Horiba FT-210 spectrometer. Optical rotations were measured on JASCO DIP-360 or JASCO P-1030 spectrometer. Mass spectra were obtained on a JEOL JMS-BU20, JMS-SX102A or JMS-700 spectrometer.

2-[(1*R*,2*S*)-2-{2-[(1*S*,3*S*,5*S*)-6-Aza-6-*tert*-butoxycarbonyl-3-methyl-8-oxa-bicyclo[3.2.1]oct-5-yl]ethyl}-1,2-dimethyl-3-oxo-cyclohexyl] Acetic Acid (12) and (1*R*,3*S*,6*S*,7*R*)-6,7-Dimethyl-2,10-dioxa-9-oxo-tricyclo-[4.4.3^{1,7}]decane-3-spiro-1′-[(3′*S*,5′*S*)-2′-oxa-5′-methyl-3′-(*tert*-butoxycarbonylaminomethyl)]Cyclohexane (13) To a stirred solution of **7** (24.1 mg, 44.7 μmol) in THF (3 ml) was added 2 M HCl (1 ml) at room temperature. After stirring for 21 h, the reaction mixture was poured into saturated $NaHCO_3$ (5 ml), and the mixture was extracted with AcOEt (20 ml). The organic layer was washed with saturated NaCl (5 ml), dried and concentrated. The residual oil was subjected to chromatography (1 : 1 hexane/AcOEt) to give monoaminal **12** (17.4 mg, 89%) and spiroketal **13** (1.3 mg, 7%).

Monoaminal (**12**): $[\alpha]_D^{27}$ +59.2° (c=0.53, $CHCl_3$). IR (film) cm^{-1}: 3440, 2981, 1674, 1394. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.89 (3H, d, J=6.4 Hz), 0.97 (3H, s), 0.98 (3H, s), 1.07—1.30 (3H, m), 1.45 (9H, s), 1.37—1.45 (1H, m), 1.57 (1H, dt, J=5.2, 12.0 Hz), 1.69—1.82 (3H, m), 1.89—2.17 (5H, m), 2.21—2.54 (4H, m), 3.35—3.55 (2H, m), 4.39 (1H, br s). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 14.5, 21.2, 21.5, 21.8, 24.1, 26.5, 28.4, 31.0, 31.9, 37.6, 38.3, 41.5, 42.2, 42.8, 51.2, 55.5, 72.4, 80.5, 93.9, 152.8, 176.7, 215.2. FAB-MS m/z: 438 (M+H), 338, 198. HRMS: Calcd for $C_{24}H_{40}NO_6$ (M+H), 438.2856. Found 438.2846.

Spiroketal (**13**): $[\alpha]_D^{27}$ −53.7° (c=0.12, $CHCl_3$). IR (film) cm^{-1}: 2934, 1701, 1510, 1367, 1290. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.88 (3H, d, J=5.4 Hz), 0.88 (3H, s), 0.87—0.94 (2H, m), 0.99 (1H, t, J=12.7 Hz), 1.15 (3H, s), 1.18—1.35 (3H, m), 1.46 (9H, s), 1.51—1.98 (8H, m), 2.00—2.12 (1H, m), 2.31 (1H, d, J=19.0 Hz), 2.58 (1H, d, J=19.0 Hz), 3.11 (1H, ddd, J=4.6, 7.8, 13.5 Hz), 3.26 (1H, ddd, J=2.7, 5.2, 13.5 Hz), 4.24 (1H, tdd, J=2.7, 4.6, 11.7 Hz), 5.79 (1H, br s). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 16.6, 18.1, 20.6, 21.7, 22.0, 25.0, 29.7, 31.9, 35.3, 36.9, 37.3, 40.9, 44.6, 46.2, 69.0, 78.4, 98.5, 107.8, 156.9, 171.1. FAB-MS m/z: 438 (M+H), 338, 198. HRMS: Calcd for $C_{24}H_{40}NO_6$ (M+H), 438.2856. Found 438.2847.

Bisaminal (5) A solution of **12** (7.9 mg, 18.1 μmol) in AcOH–H_2O (96 : 4, 2 ml) was stirred at 100 °C for 6 h. After the reaction mixture was cooled to room temperature, anhydrous Na_2SO_4 was added to the mixture. The mixture was stirred for 1 h, and filtered and washed with MeOH. The filtrate was concentrated. The residual oil was subjected to silica gel chromatography (4 : 1 $CHCl_3$/MeOH) and passed through activated alumina column (1 : 1 $CHCl_3$/MeOH) to give **5** (5.2 mg, 95%) as a colorless oil.

$[\alpha]_D^{27}$ +3.7° (c=0.82, $CHCl_3$). IR (film) cm^{-1}: 2941, 1709, 1458, 1250, 1159. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.90 (3H, d, J=6.6 Hz), 0.90 (3H, s), 1.08 (1H, t, J=12.4 Hz), 1.20 (3H, s), 1.23—1.28 (1H, m), 1.44 (1H, dt, J=2.9, 12.9 Hz), 1.53—1.66 (3H, m), 1.67—1.94 (6H, m), 2.02 (1H, dd, J=4.7, 14.0 Hz), 2.10 (1H, dd, J=5.1, 12.4 Hz), 2.23—2.31 (1H, m), 2.25 (1H, d, J=19.0 Hz), 2.55 (1H, dd, J=2.2, 19.0 Hz), 3.20 (1H, t, J=6.7 Hz), 3.25 (1H, d, J=6.7 Hz), 4.52—4.53 (1H, m). ^{13}C-NMR (68 MHz, $CDCl_3$) δ: 18.2, 18.4, 21.7, 22.9, 23.2, 24.9, 29.7, 30.1, 35.5, 38.8, 40.8, 44.4, 47.4, 89.8, 105.6, 173.3. EI-MS m/z: 319 (M^+), 279. HRMS: Calcd for $C_{19}H_{29}NO_3$ 319.2147. Found 319.2148.

Bisaminal (5) (One-Step Cyclization) A solution of **7** (16.7 mg, 30.9 μmol) in AcOH–H_2O (96 : 4, 2 ml) was stirred at 100 °C for 10 h. After the reaction mixture was cooled to room temperature, anhydrous Na_2SO_4 was added to the mixture. The mixture was stirred for 1 h, and filtered and washed with MeOH. The filtrate was concentrated. The residual oil was subjected to chromatography (3 : 1 $CHCl_3$/MeOH) and passed through activated alumina column (1 : 1 $CHCl_3$/MeOH) to give **5** (8.8 mg, 89%) as a colorless oil.

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7) (i) 4 N HCl/THF, (ii) H_2, Pd–C/MeOH, (iii) Boc_2O, 1 N NaOH–dioxane, (iv) 2,2-dimethoxypropane, *p*-TsOH/benzene.
8) (i) *t*-BuLi/THF, −78 °C, (ii) Dess–Martin periodinane, pyridine/CH_2Cl_2, r.t., (iii) 5% Na-Hg, Na_2HPO_4/MeOH, r.t., (iv) TBAF/THF, r.t., (v) PCC, MS4A, CH_2Cl_2, r.t., (vi) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene/*t*-BuOH–H_2O, r.t.
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A New Acylated Isoflavone Glucoside from *Pterocarpus santalinus*

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Phytochemical investigation on the constituents of heartwood of *Pterocarpus santalinus* resulted in the isolation of a new acylated isoflavone glucoside. The structure of the new compound was elucidated on the basis of spectral studies as 4′,5-dihydroxy-7-*O*-methyl isoflavone 3′-*O*-β-D-(3″-*E*-cinnamoyl)glucoside.

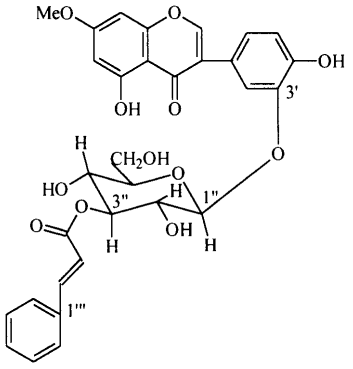
Key words *Pterocarpus santalinus*; Fabaceae; acylated isoflavone glucoside

The genus *Pterocarpus* is known to be a rich source of flavonoids and related phenolic compounds. Extracts of a few species of this genus are known for their hypoglycemic[2] and hypolipidemic activities.[3] The extracts of *Pterocarpus santalinus* L. (Fabaceae) have been used medicinally in the treatment of inflammations, mental aberrations and ulcers, while an infusion of the wood has long been regarded as useful in the treatment of diabetes.[4] As a part of our ongoing phytochemical investigation on *P. santalinus*[5—7] we report herein the isolation and characterization of a new acylated isoflavone glucoside (**1**) from the heartwood of *P. santalinus*.

Compound **1** was isolated as yellow amorphous powder whose molecular formula $C_{31}H_{28}O_{12}$ was determined by FAB-MS (*m/z* 615 $[M+Na]^+$). It gave positive visualization with ferric chloride and Molish reagent indicating that **1** is a glycoside. The IR spectrum suggested the hydroxyl group (br 3410 cm^{-1}), carbonyl groups (1690 and 1630 cm^{-1}) and ether function (1060 cm^{-1}). The broad band decoupled ^{13}C-NMR spectrum showed 29 signals corresponding to all 31 carbons (two carbons have the same chemical shift) of the molecule. Multiplicities of carbon signals were determined by DEPT spectrum: 11 quaternary carbons which includes two carbonyl carbons (181.9 and 165.2 ppm), 18 CH carbons, one CH_2 and CH_3 carbon. The ^1H-NMR spectrum of **1** exhibited a flavonoid pattern and showed a signal at δ 8.14 (1H, s) typical of a proton of C-2 of an isoflavone skeleton.[8,9] This was supported by UV spectrum, which exhibited absorptions at 265 nm and 322 (sh) nm[10,11] and this was confirmed by ^{13}C-NMR resonances at 154.8, 124.5 and 181.9 ppm, respectively for 2, 3 and 4 carbons.[12] Two *meta* coupled doublets at δ 6.54 and 6.38 (J=2.0 Hz each 1H) represented H-8 and H-6, respectively. The downfield signal at δ 12.48, exchangeable with D_2O, indicated the presence of free hydroxyl at C-5, which is in chelation with carbonyl function.[8] The signals at δ 6.96 (d, J=2.0 Hz, 1H), 6.72 (d, J=8.0 Hz, 1H) and 7.10 (dd, J=8.0 and 2.0 Hz, 1H) established the presence of three aromatic protons in ring B. The signal at δ 3.84, integrating for 3 protons, indicated the presence of a methoxy group and was assigned to C-7 on the basis of HMBC correlations (Fig. 1).

The ^1H-NMR resonances at δ 3.8—3.5 and signals in the ^{13}C-NMR spectrum just below δ 70 and a signal at 61.30 ppm showed by DEPT to represent CH_2 group, indicated the presence of sugar moiety. The signal for an anomeric proton at δ 5.35 (d, J=7.9 Hz) suggested β-configuration for sugar.[8] The other signals belonging to *trans*-cinnamoyl moiety at δ 6.65 (d, J=16.0 Hz, 1H), 7.67 (d, J=16.0 Hz, 1H), 7.40 (m, 3H) and 7.71 (m, 2H) were observed. A loss of 130 and 162 mass units from the molecular ion in the FAB-MS indicated the presence of cinnamic acid and glucose moieties in **1**. This was confirmed by the formation of *trans*-cinnamic acid and D-glucose upon hydrolysis. Alkaline hydrolysis of **1** gave *trans*-cinnamic acid and 4′,5-dihydroxy-7-*O*-methyl isoflavone 3′-*O*-β-D-glucoside,[7] indicating that the cinnamoyl moiety was attached to the glucosyl residue. The cross peak between H-3″ (δ 5.10) of the glucose and the carbonyl ester carbon (165.2 ppm) of the cinnamoyl residue (HMBC spectrum, Fig. 1), confirmed the attachment of cinnamoyl moiety at C-3″ hydroxyl of glucose.

The (proton carbon chemical shift) correlations for all the carbons directly bonded to protons were established by HETCOR experiment and ^1H–^1H COSY and HMBC (Fig. 1) confirmed the assignment of the proton and carbon frequencies. Thus, compound **1** was characterized as 4′,5-dihydroxy-7-*O*-



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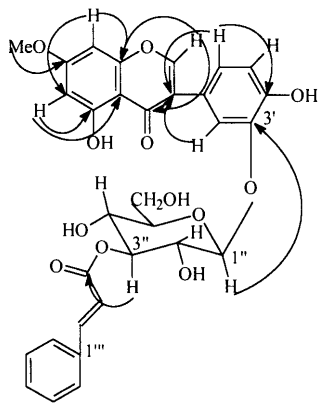

Fig. 1. HMBC Correlations of **1**

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methyl isoflavone 3′-*O*-β-D-[3″-*E*-cinnamoyl]glucoside

Experimental

General Procedures Melting points are uncorrected. UV spectra were taken in MeOH on a Beckman 25 spectrophotometer. IR spectra were run in KBr. FAB-MS was obtained using a glycerol matrix on a VG Micro Mass Zab-HF mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured on a Brucker AC 300 spectrometer at 300 and 75 MHz, respectively. Samples were run in DMSO-d_6 TMS as internal standard.

Plant Material The heartwood of *P. santalinus* was collected from the Tirumala Hills, Tirupati and a voucher specimen has been deposited in the Herbarium of the Botany Department, Sri Venkateswara University, Tirupati.

Extraction and Isolation The air-dried and powdered heartwood (600 g) of *P. santalinus* was defatted and exhaustively extracted with methanol at room temperature. The MeOH extract was concentrated under reduced pressure and the resulting residue was suspended in water and extracted with CH_2Cl_2. The CH_2Cl_2 soluble portion was subjected to column chromatography over silica gel ,eluted with varying portions of a mixture of $CHCl_3$–EtOAc and the fractions were combined on the basis of TLC analysis leading to four series. Compound **1** (35 mg) was obtained from series C, eluted with $CHCl_3$–EtOAc (7 : 3) upon removal of solvent followed by recrystallization.

4′,5-Dihydroxy 7-*O*-methylisoflavone 3′-*O*-β-D-(3″-*E*-cinnamoyl) Glucoside (**1**): Light yellow amorphous powder from MeOH, UV (MeOH); λ_{max} (log ε) 265 (4.43), 322 (3.73) nm; IR (KBr) ν_{max} cm^{-1} 3410, 3300, 1690, 1630, 1060; ^{1}H-NMR (DMSO-d_6) δ 12.48 (1H, s, OH-5), 8.14 (1H, s, H-2), 7.71 (2H, m, H-2‴, 6‴), 7.67 (1H, d, *J*=16.0 Hz, H-7‴), 7.40 (3H, m, H-3‴, 4‴, 5‴), 7.10 (1H, dd, *J*=8.0, 2.0 Hz, H-6′), 6.96 (1H, d, *J*=2.0 Hz, H-2′), 6.72 (1H, d, *J*=8.0 Hz, H-5′), 6.65 (1H, d, *J*=16.0 Hz, H-8‴), 6.54 (1H, d, *J*=2.0 Hz, H-8), 6.38 (1H, d, *J*=2.0 Hz, H-6), 5.35 (1H, d, *J*=7.9, H-1″), 5.10 (1H, dd, *J*=9.2, 9.2 Hz, H-3″), 3.84 (3H, s, OMe-7), 3.80 (1H, m, H-6″a), 3.5 (4H, m, H-2″, 4″, 5″, 6″b); ^{13}C-NMR 181.9 (C-4), 165.2 (C-9‴), 164.6 (C-7), 159.7 (C-9), 157.3 (C-5), 154.8 (C-2), 151.9 (C-3′), 144.8 (C-7‴), 139.8 (C-4′), 133.9 (C-1‴), 130.1 (C-4‴), 129.2 (C-3‴, 5‴), 128.9 (C-6′), 128.0 (C-2‴, 6‴), 126.2 (C-1′), 124.5 (C-3), 119.2 (C-8‴), 115.8 (C-5′), 114.9 (C-2′), 112.2 (C-10), 101.9 (C-1″), 98.4 (C-6), 95.7 (C-8), 78.8 (C-3″), 77.2 (C-5″), 73.9 (C-2″), 71.0 (C-4″), 61.3 (C-6″), 58.4 (OMe); FAB-MS (*m/z*) 615 [M+Na]$^+$, 462, [M+H−cinnamoyl]$^+$ (5), 301 [M+H−cinnamoyl glucosyl]$^+$ (51).

Acid hydrolysis of 1 Compound **1** (15 mg) on acid hydrolysis with 5 N HCl at 90 °C for 3 h after usual workup gave 3′,4′,5-trihydroxy-7-*O*-methyl isoflavone,[6] cinnamic acid and glucose (identified by co-paper chromatography in BAW).

Alkaline hydrolysis of 1 Ten mg of compound **1** was refluxed in 1% KOH for 2 h. The reaction mixture was processed further in the usual way which yielded 4′,5-dihydroxy-7-*O*-methyl isoflavone-3′-*O*-β-D-glucoside and cinnamic acid.

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Chemical Studies on the Philippine Crude Drug Calumbibit (Seeds of *Caesalpinia bonduc*): The Isolation of New Cassane Diterpenes Fused with α,β-Butenolide

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New cassane diterpenes named neocaesalpins C and D were isolated from the Philippine crude drug calumbibit botanically originating from the seeds of *Caesalpinia bonduc* (Fabaceae), and their structures were elucidated on the basis of the spectroscopic evidence. These compounds are characterized by the presence of the α,β-butenolide moiety. Although a number of cassane furanoditerpenes have been known to occur in the same plant species, such constituents could not be isolated from the crude drug of Philippine origin in this study. It is presumed that the chemical difference resulted from chemical differentiation of the species.

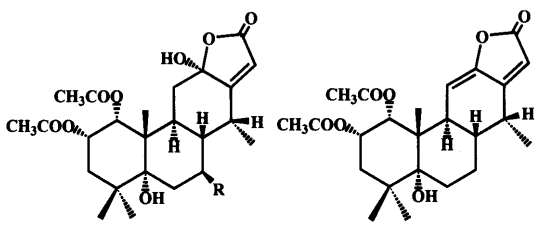
Key words *Caesalpinia bonduc*; Fabaceae; cassane diterpene; Philippine folk medicine; calumbibit

Caesalpinia bonduc (L.) ROXB. (Fabaceae) is a stout prickly climber distributed throughout the tropical and subtropical regions. This plant has been recognized as medicinal in traditional medicine of various parts of the world where it occurs. Calumbibit is one of the most esteemed crude drugs in the Philippines, and botanically originates from the seeds of this plant. It is regarded as a febrifuge and purgative and is also considered to be effective for the treatment of malaria.[1] Although the seeds of this plant are chemically predominated by oil and fats, early investigations, which were carried out under its synonymous name *C. bonducella*, revealed the presence of the characteristic cassane furanoditerpenes, α-, β-, γ-, δ-, ε-caesalpin and caesalpin F.[2—8] The author also investigated the seeds of *C. bonduc* collected in Indonesia and reported earlier on the isolation and structure elucidation of neocaesalpins A and B, which are characterized by the presence of α,β-butenolide instead of the furan in caesalpins.[9] The subsequent investigation of the Philippine crude drug calumbibit has furnished two new cassane diterpenes, and their isolation and structure elucidation are described in this paper.

The chloroform extract of crushed calumbibit was subjected to the same separation procedure described previously,[9] which led to the isolation of two new compounds for which the names neocaesalpins C and D are proposed. Neocaesalpin C (**1**) was obtained as colorless prisms and its molecular formula was calculated as $C_{24}H_{36}O_9$ based on the high-resolution fast atom bombardment (HR-FAB) mass spectral analysis. The infrared (IR) spectrum had an absorption band at 3584 cm^{-1} indicating the presence of hydroxyl groups in the molecule. The IR bands at 1736 and *ca.* 1720 cm^{-1} (overlapping with ester groups) are assignable to the α,β-butenolide, and the presence of this moiety was further substantiated by the ultraviolet (UV) absorption maximum at 214 nm (log ε=4.13). The ^{1}H-nuclear magnetic resonance (NMR) spectrum (Table 1) was very similar to that of neocaesalpin B (**2**), one of the cassane diterpenes which were obtained from the seeds of *C. bonduc* of Indonesian origin, except for proton signals at H-6, H-7, H-8, and H-14. These signals were observed more than 0.3 ppm downfield due to the presence of a hydroxyl group at the 7-position. A proton signal at the 7-position was observed at δ 4.68 ppm as ddd with the following coupling constants: $J_{7\alpha\text{-}6\beta}$=5.7, $J_{7\alpha\text{-}6\beta}$= $J_{7\alpha\text{-}8}$=10.8 Hz. This finding unequivocally assigned the configuration of the 7-hydroxyl group as β. Therefore the structure of neocaesalpin C was elucidated as **1**. Comparative analysis of the ^{13}C-NMR spectra between neocaesalpins B and C (Table 2) was also consistent with the structure **1** for neocaesalpin C, with the outstanding difference being the presence of a secondary alcoholic methine carbon (δ 66.0) in place of a methylene carbon (δ 23.8).[9]

Neocaesalpin D (**3**) was obtained in crystalline forms, mp 209—213 °C. It had the molecular formula of $C_{24}H_{32}O_7$ according to HR-FAB mass spectral analysis. It is apparent from its ^{1}H- and ^{13}C-NMR spectra (Tables 1 and 2) that this compound is a diterpene closely related to neocaesalpin B. The UV absorption maximum at 278 (log=4.23) indicated that this compound has an α,β-butenolide ring conjugated with one extra double bond. The presence of this conjugate system was also supported by the ^{1}H- and ^{13}C-NMR spectra (Tables 1 and 2), as mentioned below. Proton signals assigned to H-11α and H-11β in **2** were missing in the ^{1}H-NMR spectrum of **3**, and a new broad olefinic proton signal was observed at δ 5.92, which was assigned to H-11. The ^{13}C-NMR spectrum of **3** lacked the characteristic hemiketal sp^3 carbon found in that of **1**, and instead two quarternary sp^2 carbons were observed at δ 111.1 and 151.2 which were assigned to C-11 and C-12, respectively. The IR bands at 1788 and 1767 cm^{-1} will be assigned to this conjugate system. The spectral findings stated above assign the structure of neocaesalpin D to **2**. This structure is apparently derived from dehydration of the hemiketal hydroxyl group, but attempts at chemical dehydration from **2** to **3** failed.

All diterpenes obtained by other research groups from *C.*



R=OH neocaesalpin C (1)
R=H neocaesalpin B (2)

neocaesalpin D (3)

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Table 1. ^{1}H-NMR[a] Spectral Data for Neocaesalpins C and D

	Neocaesalpin C		Neocaesalpin D	
	δ ppm[b]	Multiplicity (J)[c]	δ ppm[b]	Multiplicity (J)[c]
H-1	5.67	d (2.9)	5.69	d (3.0)
H-2	5.55	ddd (2.9, 4.0, 13.2)	5.61	ddd (3.0, 4.7, 13.0)
H-3α	2.31	dd (13.2, 13.2)	2.35	dd (13.0, 13.0)
H-3β	1.41	dd (4.0, 13.2)	1.39	dd (4.7, 13.0)
H-6α	2.42	dd (5.7, 13.2)	1.69	ddd (2.0, 2.4, 12.8)
H-6β	1.87—1.93	m (10.8, 13.2)	1.59	ddd (4.3, 12.8, 12.8)
H-7	4.68	ddd (5.7, 10.8, 10.8)	α 2.00—2.08	m
			β 1.17	m
H-8	1.93—1.96	m (10.8, 12.8)	1.76	ddd (4.3, 10.4, 10.4)
H-9	3.29	ddd (2.8, 12.8, 12.8)	3.44	br d (10.4)
H-11	α 2.51	dd (2.6, 12.8)	5.92	br s
	β 1.46	dd (12.8, 12.8)		
H-14	3.87	dq (4.8, 7.2)	2.67	dq (4.4, 7.2)
H-15	5.83	s	5.88	d (0.9)
17-Me	1.57	d (7.2)	0.91	d (7.2)
18-Me	1.21	s	1.12	s
19-Me	1.12	s	1.03	s
20-Me	1.16	s	1.07	s
CH_3COO	2.01	s	2.03	s
	2.16	s	2.11	s

a) Spectra were measured at 400 MHz in pyridine-d_5. *b*) Chemical shifts as δ ppm with TMS as the internal standard. *c*) Multiplicity and coupling constants (J Hz) in parentheses.

Table 2. ^{13}C-NMR[a] Spectral Data for Neocaesalpins C and D

C	Neocaesalpin C	Neocaesalpin D
1	74.2	73.2
2	67.8	67.6
3	35.6	36.1
4	40.3	40.4
5	78.2	76.6
6	36.4	26.4
7	66.0	23.9
8	47.6	37.5
9	32.8	36.9
10	45.3	45.4
11	38.9	111.1
12	106.6	151.2
13	171.1	161.8
14	33.4	33.5
15	113.5	110.6
16	174.7	170.5[b]
17	13.0	14.3
18	28.4	27.8
19	25.5	24.7
20	17.6	19.5
$CH_3\underline{C}O$	170.2	170.2[b]
	170.3	170.3[b]
$\underline{C}H_3CO$	20.8	20.7
	21.0	20.9

a) Spectra were measured at 100 MHz in pyridine-d_5. *b*) Assignments in the same column may be interchanged.

bonduc (syn. *C. bonducella*) are tricarbocyclic derivatives fused with a furan ring.[2—8] However, the author's group has obtained from the seeds of the same plant species not furanoditerpenes but cassane-type diterpenes fused with the butenolide ring.[9] It is of interest to note that all of the plant sources from which furanoditerpenes were isolated are derived from the Western hemisphere, and not of Asian origin. As mentioned at the beginning of this article, the phytogeographic distribution of *C. bonduc* covers almost the entire tropics and subtropics worldwide. Although there are no data available on the morphologic diversity of this species, it can be presumed that several chemotypes exist depending upon phytogeographic distribution as a result of chemical differentiation. There is growing concern over the use of traditional medicines for either the treatment or prevention of diseases worldwide, and standardization of natural medicines has become one of the most important issues to maintain quality control. The results of this study may indicate that attention should be paid not only to the botanical origin (identification of species) but also chemical variation of natural medicines in the process of standardization.

Experimental

All melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. Spectral data were obtained using the following apparatus: ^{1}H- and ^{13}C-NMR spectra with a JEOL JNM GSX-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer with tetramethylsilane (TMS) as internal standard; mass spectra (MS) with a JEOL SX-102A mass spectrometer; IR spectra with a JASCO FT/IR-8000 infrared spectrometer; optical rotations with a JASCO DIP-370 polarimeter and UV spectra with a Shimadzu UV-240 spectrometer. Column chromatography was carried out with Wakogel C-200 (eluted with hexane–ethyl acetate).

Plant Material Calumbibit, the seeds of *C. bonduc*, was purchased at the Quiapo crude drug market in Metro Manila, the Philippines, in March 1998, when the collection of Philippine crude drugs was undertaken as part of the Monbusho International Scientific Research Program: Field Research entitled "Ethnobotanical Survey in Philippine Tropical Rain Forest," headed by the author. The sample specimen of calumbibit was deposited as a voucher at the Medicinal Plant Research Station, Faculty of Pharmaceutical Sciences, Teikyo University.

Extraction and Isolation The crushed calumbibit (502 g) was extracted three times with chloroform at room temperature, and the combined extracts were evaporated to dryness under reduced pressure to yield a brown extract (49 g). The whole extract was partitioned between methanol (200 ml) and hexane (400 ml) and the methanol layer was evaporated to dryness under reduced pressure to give the residue (13 g). The residue was subjected to silica gel column chromatography on elution with a mixed solvent system of hexane and ethyl acetate, increasing the amount of the latter gradually. Each fraction collected was evaporated to dryness under reduced pressure, and the residue was dissolved in a small amount of acetone. Certain fractions fur-

nished crystalline precipitates, which were collected and recrystallized from acetone to give neocaesalpins C (16 mg) and D (95 mg).

Neocaesalpin C (1) Colorless prisms, mp >260 °C. $[\alpha]_D^{25}$ −50° (c= 0.034). IR (KBr) cm^{-1}: 3584, 2946, 1736, 1368, 1252, 1227, 1034. UV λ_{max} (MeOH) nm (log ε): 214 (4.13). ^{1}H-NMR (pyridine-d_5) δ: Table 1. ^{13}C-NMR (pyridine-d_5) δ: Table 2. EI-MS m/z (int. %): 448 (M^+-H_2O, 1), 430 ($M^+-2\times H_2O$, 11), 406 (M^+-CH_3COOH, 25), 388 ($M^+-CH_3COOH-H_2O$, 17), 370 ($M^+-CH_3COOH-2\times H_2O$, 53), 346 ($M^+-2\times CH_3COOH$, 44), 328 ($M^+-2\times CH_3COOH-H_2O$, 89), 310 ($M^+-2\times CH_3COOH-2\times H_2O$, 100). FAB-MS m/z: 489.2116 (Calcd for $C_{24}H_{36}O_9\cdot Na^+$: 489.2100).

Neocaesalpin D (2) Colorless prisms, mp 209—213 °C. $[\alpha]_D^{25}$ +71.9° (c=0.089). IR (KBr) cm^{-1}: 2944, 1788, 1767, 1730, 1373, 1256, 1231. UV λ_{max} (MeOH) nm (log ε): 278 (4.23). ^{1}H-NMR (pyridine-d_5) δ: Table 1. ^{13}C-NMR (pyridine-d_5) δ: Table 2. EI-MS m/z (int. %): 414 (M^+-H_2O, 7), 372 (M^+-CH_3COOH, 10), 354 ($M^+-CH_3COOH-H_2O$, 42), 312 ($M^+-2\times CH_3COOH$, 59), 294 ($M^+-2\times CH_3COOH-H_2O$, 100. FAB-MS m/z: 433.2243 (Calcd for $C_{24}H_{32}O_7\cdot H^+$: 433.2226).

Acknowledgments This work was in part supported by a Grant-in Aid for Scientific Research (1997—1998; No. 09041192) from the Ministry of Education, Science, Sports and Culture of Japan.

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Re-examination of the Anodic Oxidation of *N,N*-Dimethylaniline, Using Parametric Method 3

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A semi-empirical calculation (PM3) was applied to elucidate the anodic oxidation mechanism of *N,N*-dimethylaniline (DMA) and the dimerization of a cation radical (A) derived from DMA was ruled out. The heat of reaction value of the dimerization of A was 42.43 kcal/mol. We propose the following. Cation radical A reacts with DMA to generate another cation radical (D). This reaction was exothermic and the heat of reaction value and the activation energy were −0.35 kcal/mol and 1.31 kcal/mol, respectively. Deprotonation of D by DMA gives neutral radical (E), which is oxidized to TMB by A. All these reactions were exothermic.

Key words PM3; *N,N*-dimethylaniline; oxidation; MO; radical

Much attention has been devoted to the anodic oxidation of *N,N*-dimethylaniline (DMA) and the electrolysis has been carried out in various conditions.[1] All the investigators, with the exception of Hand and Nelson, found *N,N,N′,N′*-tetramethylbenzidine (TMB) to be the predominant product.[2] It was reported that the primary electrode reaction was a one-electron transfer to form a cation radical (A) which, then, coupled to form TMB. A semi-empirical calculation (parametric method 3 (PM3)) was applied to elucidate the mechanism in more detail.

PM3 is the latest Hamiltonian contained in the MOPAC program and has been reviewed by Stewart.[3] Friant-Michel *et al.* also used PM3 effectively to elucidate the cyclization mechanism of α- and β-D-glucosyl azides.[4]

Results and Discussion

The presence of A in the primary anodic oxidation of DMA was suggested by cyclic voltammetry.[5–7] Dimerization of A, that is reaction 1) shown in Chart 1, is an established theory as the generation mechanism of TMB.

We performed the calculation based on the assumption that DMA was a proton acceptor. The total energy of reactants and products of reaction 1) were estimated using PM3, and the results are shown in Table 1.

The molecular orbital (MO) calculation was performed using HyperChem release 5.1. Before single point calculation, geometry optimization was initially performed using MM+, then with UHF (spin unrestricted Hartree–Fock) calculation using the Polak–Ribiere algorithm as the minimization algorithm until the total root-mean-square (RMS) gradient was reduced to 0.01 kcal/(Å mol).

As shown in Table 1, the total energy of reactants and products were −117665.85 kcal/mol and −117706.10 kcal/mol respectively, so the heat of reaction value ($\Delta E = E_{react}$ (sum of total energies of reactants) $- E_{prod}$ (sum of total energies of products)) was −40.25 kcal/mol. This fact suggests that the overall reaction is exothermic and reasonable. However, the reaction is a multi-step one. The coupling of A shown in Chart 2 was examined initially.

The total energy of the dication (B) was estimated at −58624.95 kcal/mol, which was more than double that of A (2×(−29333.69 kcal/mol)=−58667.38 kcal/mol) by 42.43 kcal/mol. Therefore, the reaction 2) does not proceed at room temperature.

As another mechanism, deprotonation of A prior to coupling was postulated and is shown in Chart 3. The total energy of reactants and products of reaction 3) except C are shown in Table 1 and that of C was estimated at −29103.69 kcal/mol. Therefore, the sum of total energy of products was

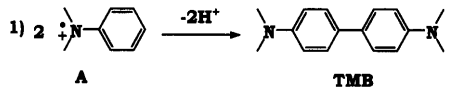

Chart 1

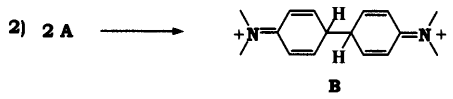

Chart 2

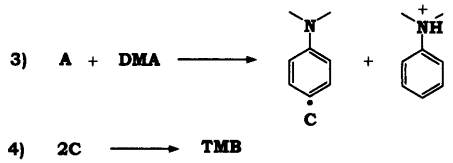

Chart 3

Table 1. Total Energy of Reactants and Products in the Reaction 1)

Reactant	Total energy (kcal/mol)	Product	Total energy (kcal/mol)
Cation radical of *N,N*-Dimethylaniline (A)	−29333.69	*N,N,N′,N′*-Tetramethylbenzidine (TMB)	−58302.10
N,N-Dimethylaniline (DMA)	−29500.11	Protonated DMA	−29702.00

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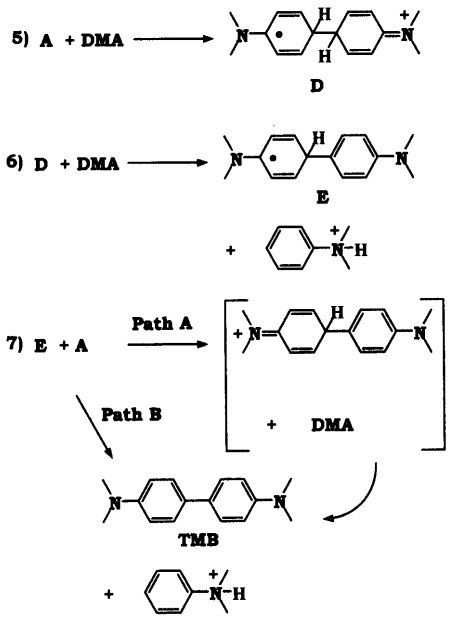

Chart 4

Table 2. Sum of Total Energy of Reactants and Products in the Reactions in Chart 4

Reaction No.	Reactants (E_{react})	Products (E_{prod})	ΔE ($E_{react}-E_{prod}$)
5)	−58833.80 kcal/mol	−58834.15 kcal/mol	−0.35 kcal/mol
6)	−88334.26 kcal/mol	−88343.32 kcal/mol	−9.06 kcal/mol
7) Path A	−87975.01 kcal/mol	−87989.44 kcal/mol	−14.43 kcal/mol
7) Path B	−87975.01 kcal/mol	−88004.10 kcal/mol	−29.09 kcal/mol

−58805.69 kcal/mol and that of reactants was −58832.94 kcal/mol. The heat of reaction value was 27.25 kcal/mol. Reaction 3) also does not proceed at room temperature.

As an alternative mechanism, an electrophilic addition of A to DMA and following reactions were postulated and are shown in Chart 4. The total energy of reactants and products are summarized in Table 2. The total energy of cation radical (D), which was a product of reaction 4), was −58834.15 kcal/mol and below −58833.80 kcal/mol, which was the sum of the total energy of reactants, that is A and DMA. The total energy of the transition state shown in Fig. 1 was −58832.49 kcal/mol, so the activation energy ($E_{act}=E_{trans}$ (sum of total energies of transition state)$-E_{react}$) was only 1.31 kcal/mol. The synchronous transit method of transition state searching was applied, then the eigenvector following was used with a RMS gradient termination criterion of 0.01 kcal/(Å/mol). DMA was abundant in the electrolyte, so the reaction 5) seems very fast. The high reactivity of reaction 5) well explains the fact that A was not detected with electron spin resonance spectrometry.[5,7]

DMA abound in the solution abstracted a proton from D to generate a neutral radical (E). Because E has one more electron than TMB, E must be oxidized to generate TMB. Using the rotating ring-disk electrode technique, Galus *et al.* reported that only DMA and TMB were oxidized on the anode.[6] Their results suggests that the oxidant in this step was not the electrode but A.

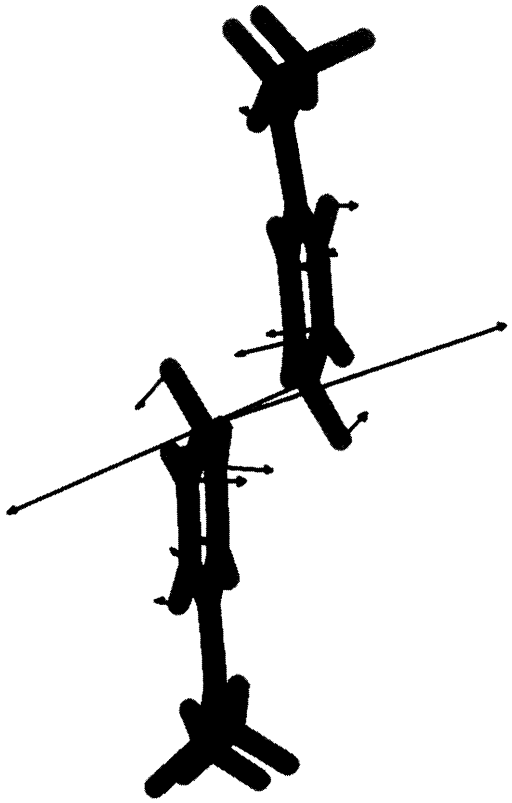

Fig. 1. Transition State of Reaction between *N*,*N*-Dimethylaniline (DMA) and Its Cation Radical

Arrows show direction of vibrations.

Two types of reactions with A were predicted: one-electron oxidation following deprotonation (Path A) and hydrogen abstraction (Path B). These reactions were exothermic as shown in Table 2. It is difficult to judge which path is followed because we failed to obtain a transition state for either one. In either case, two molecules of A were consumed to generate TMB and two molecules of DMA were protonated in the schemes shown in Chart 4. The schemes are in agreement with reaction 1) stoichiometrically.

Experimental

Apparatus Semi-empirical calculation was carried out as described previously.[8]

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Synthesis of [1]Benzopyrano[2,3,4-*kl*]acridin-3-ol and Its Analogues as Pentacyclic Compounds

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A new heterocyclic compound, [1]benzopyrano[2,3,4-*kl*]acridin-3-ol was synthesized by cyclization of xanthone derivatives. The key compound, 1-(3′-methoxyanilino)-xanthone, was prepared from 1-aminoxanthone. [1]benzopyrano[2,3,4-*kl*]acridin-3-ol analogues, [1]benzothiopyrano[2,3,4-*kl*]acridin-3-ol, pyrido[3′,2′ : 5,6]-pyrano[2,3,4-*kl*]acridin-3-ol and pyrido[3′,2′ : 5,6]thiopyrano[2,3,4-*kl*]acridin-3-ol were synthesized by the same method.

Key words heterocyclic compound; [1]benzopyrano[2,3,4-*kl*]acridin-3-ol; [1]benzothiopyrano[2,3,4-*kl*]acridin-3-ol; pyrido-[3′,2′ : 5,6]pyrano[2,3,4-*kl*]acridin-3-ol; pyrido[3′,2′ : 5,6]thiopyrano[2,3,4-*kl*]acridin-3-ol

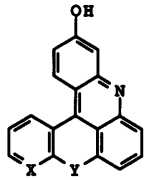
A number of polycyclic aromatic alkaloids that may have various types of biological activity have been isolated from natural marine organisms. In particular, these compounds are attractive with respect to anti-tumor activity. From this perspective, our investigation of synthesizing xanthene derivatives focused on synthesis of [1]benzopyrano[2,3,4-*kl*]-acridin-3-ol (**1**) and its analogues, because of their structural similarity to shermilamine and norse goline, an inhibitor of DNA topoisomerase type-II (Chart 1).

The [1]benzopyrano[2,3,4-*kl*]acridine derivative possesses the fused ring system which contains xanthene skeleton. In retrosynthetic analysis of **1**, a number of bond disconnections are possible, one of which is shown in Chart 2. Disconnection of carbon–carbon double bond would require the preparation of 1-(3′-methoxyanilino)xanthone (**5**) from 1-chloroxanthone or 1-aminoxanthone (**6**).

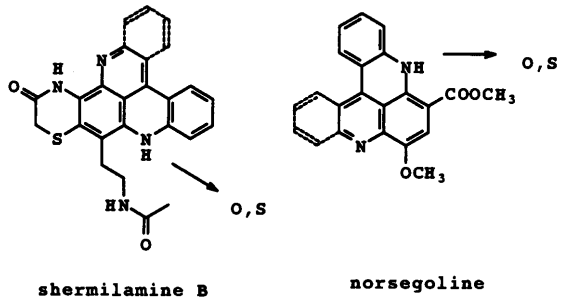
Results and Discussion

We chose to investigate two synthetic routes to 1-(3′-methoxyanilino)xanthone (**5**). The first route utilizes the reaction of 1-chloroxanthone with 3-methoxyaniline and the second (route B, in Chart 3) is the condensation of 1-aminoxanthone with 1-iodo-3-methoxybenzene.

1-Chloroxanthone was prepared according to Okabayashi's method.[1] The obtained chloroxanthone was a mixture of 1- and 3-chloroxanthone which could not be separated by recrystallization or column chromatography. For this reason, the mixture of 1- and 3-chloroxanthones was used with the following reaction. 1-Aminoxanthone was prepared from a mixture of 1- and 3-chloroxanthone by a modification of the method of 1-aminothioxanthone.[2] A mixture of 1- and 3-chloroxanthones was reacted with *p*-toluenesulfonamide to give a mixture of 1-(*p*-toluenesulfonamido)xanthone and 3-chloroxanthone. The mixture was hydrolyzed with 47% hydroboric acid to give a mixture of 1-aminoxanthone and 3-chloroxanthone. The mixture was easily separated by silica gel column chromatography to give **6**. Initially, a mixture of

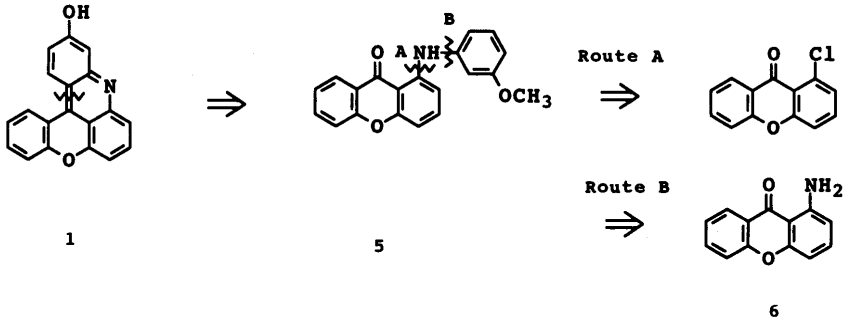


1:X=CH,Y=O:[1]benzopyrano[2,3,4-*kl*]acridin-3-ol
2:X=CH,Y=S:[1]benzothiopyrano[2,3,4-*kl*]acridin-3-ol
3:X=N, Y=O:pyrido[3',2':5,6]pyrano[2,3,4-*kl*]acridin-3-ol
4:X=N, Y=S:pyrido[3',2':5,6]thiopyrano[2,3,4-*kl*]acridin-3-ol



shermilamine B norsegoline

Chart 1



1 5 6

Chart 2

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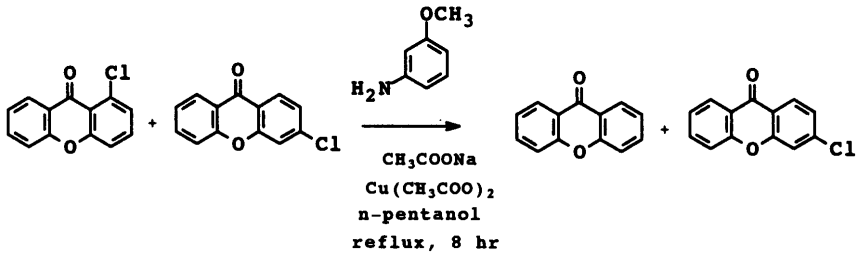


Chart 3

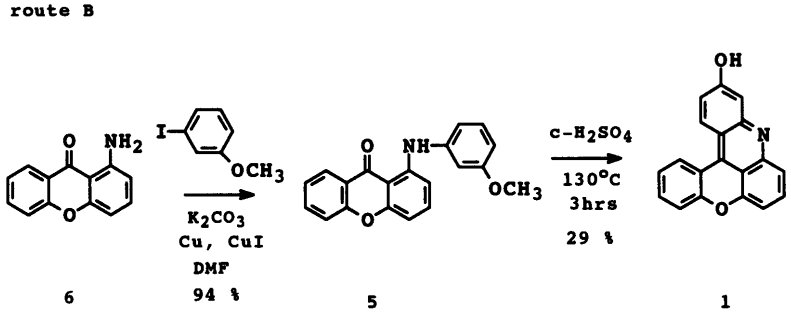


Chart 4

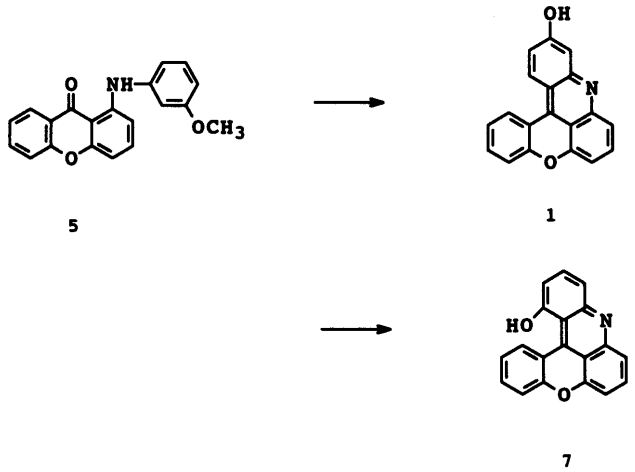


Chart 5

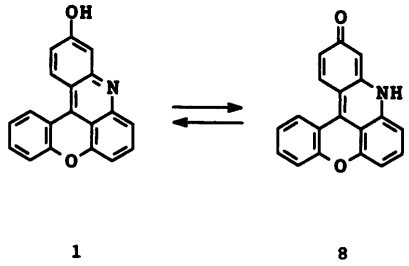


Chart 6

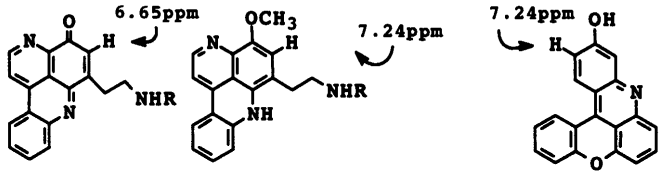


Chart 7

1- and 3-chloroxanthone was reacted with 3-methoxyaniline to give a mixture of xanthone and 3-chloroxanthone. From these results, 1-chloroxanthone gave xanthone by dechlorination. The reaction did not afford the objected compound (**5**).

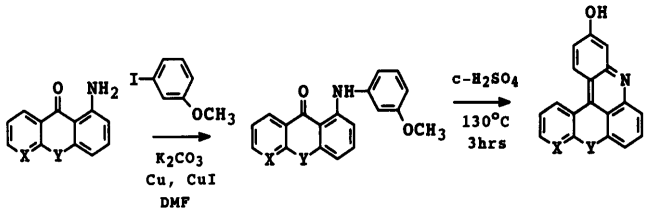
On the other hand, in route B reaction of 1-aminoxanthone with 1-iodo-3-methoxybenzene gave **5** in a 94% isolated yield, Chart 4. Analysis of the ^{1}H-NMR spectrum of **2** suggests this is the structure, due to the presence of methoxy protons at δ 3.82 ppm and aromatic protons at δ 7.0—8.5 ppm.

The obtained **5** was reacted under conc.-sulfuric acid at 130 °C for 8 h to give **1**. The reaction was considered with the possibility of two compounds, **1** and [1]benzopyrano[2,3,4-*kl*]acridin-1-ol (**7**), by condensation orientation (Chart 5). However, only compound **1** was obtained.

Furthermore, **1** had the possibility of 3*H*,5*H*-[1]benzopyrano[2,3,4-*kl*]acridin-3-one structure (**8**) which was the keto form of the relationship of keto–enol tautomerism (Chart 6). In cystodytin A,[3] the proton at the 2-position was indicated with the signal at δ 6.65 ppm and in cystodytin A methyl ether, the proton at the 2-position was indicated with the signal at δ 7.24 ppm (Chart 7). From these results, the structure of **1** was determined for the enol type in the presence of aromatic proton of δ 7.24 ppm corresponding to the proton at the 2-position of cystodytin A.

In addition, synthesis of [1]benzopyrano[2,3,4-*kl*]acridin-3-ol analogous, [1]benzothiopyrano[2,3,4-*kl*]acridin-3-ol (**2**), pyrido[3′,2′ : 5,6]pyrano[2,3,4-*kl*]acridin-3-ol (**3**) and pyrido-[3′,2′ : 5,6]thiopyrano[2,3,4-*kl*]acridin-3-ol (**4**) were examined by the procedure described above. We prepared the starting materials, 1-aminothioxanthone (**9**),[4] 1-amino-5*H*-[1]benzopyrano[2,3-*b*]pyridin-5-one (**10**)[5] and 1-amino-5*H*-[1]benzothiopyrano[2,3-*b*]pyridin-5-one (**11**)[6] by a modification of the method described by literatures. Then, **9**, **10** and

Table 1



	Yield		Yield	
6: X=CH, Y=O	94%	**5**	29%	**1**
9: X=CH, Y=S	73%	**12**	15%	**2**
10: X=N, Y=O	94%	**13**	47%	**3**
11: X=N, Y=S	50%	**14**	46%	**4**

11 were reacted with 3-iodo-1-methoxybenzene to give 1-(3′-methoxyanilino)thioxanthone (**12**), 1-(3′-methoxyanilino)-benzopyrano[2,3-*b*]pyridin-5-one (**13**) and 1-(3′-methoxyanilino)benzothiopyrano[2,3-*b*]pyridin-5-one (**14**) in good yields, respectively. The reaction of these compounds with conc.-sulfuric acid afforded **2**—**4**, as shown in Table 1. These structures were determined by a method similar to that for **1**.

Experimental

Melting points were measured on a Yanagimoto micro-melting point apparatus and are uncorrected. IR spectra were recorded with a Perkin-Elmer FT-IR 1720. ^{1}H-NMR spectra were measured on a JEOL FX-400 instrument using $CDCl_3$ as a solvent and tetramethylsilane as an internal standard. MS were taken with a Hitachi M-2500 spectrometer.

1-(3′-Methoxyanilino)xanthone (5) A mixture of 1-aminoxanthone (2.11 g, 0.01 mol), 3-iodo-1-methoxybenzene (2.34 g, 0.01 mol), copper (0.1 g), copper iodine (0.1 g), K_2CO_3 (2.76 g, 0.02 mol), and DMF (50 ml) was stirred under reflux for 5 h. The reaction mixture was filtered. The filtrate was concentrated. The residue was mixed with hot water, filtered, and dried. The obtained solid was purified by silica gel column chromatography with benzene to give **5** (3.00 g, 94%).

Compound **5**: Yellow crystal (MeOH), mp 100—101 °C, IR (KBr) cm^{-1}: 1640, 1597, ^{1}H-NMR ($CDCl_3$) δ: 3.83 (3H, s, OCH_3), 6.69 (1H, dd, J=2.44, 8.29 Hz, 4′-H), 6.71 (1H, dd, J=0.98, 8.29 Hz, 4-H), 6.90 (1H, d, J=2.44 Hz, 2′-H), 6.96 (1H, dd, J=1.46, 7.81 Hz, 6′-H), 7.07 (1H, dd, J=0.98, 8.29 Hz, 2-H), 7.28 (1H, t, J=7.81 Hz, 5′-H), 7.34 (1H, t, J=7.81 Hz, 7-H), 7.40 (1H, dd, J=1.95, 8.29 Hz, 5-H), 7.42 (1H, t, J=8.29 Hz, 3-H), 7.68 (1H, t, J=7.81 Hz, 6-H), 8.26 (1H, dd, J=1.46, 7.81 Hz, 8-H), 11.30 (1H, s, NH). *Anal.* Calcd for $C_{20}H_{15}NO_3$: C, 75.69; H, 4.76; N, 4.41. Found: C, 75.66; H, 4.75; N, 4.19. MS: *m/z* 317 [M$^+$].

1-(3′-Methoxyanilino)thioxanthone (12) A mixture of 1-aminothioxanthone (2.27 g, 0.01 mol), 3-iodo-1-methoxybenzene (2.34 g, 0.01 mol), copper (0.1 g), copper iodine (0.1 g), K_2CO_3 (2.76 g, 0.02 mol), and DMF (50 ml) was stirred under reflux for 5 h. The reaction mixture was filtered. The filtrate was concentrated. The residue was mixed with hot water, filtered, and dried. The obtained solid was purified by silica gel column chromatography with benzene to give **12** (2.43 g, 73%).

Compound **12**: Yellow needles (MeOH), mp 98—99 °C. IR (KBr) cm^{-1}: 1603, 1590, 1559. ^{1}H-NMR ($CDCl_3$) δ: 3.82 (3H, s, OCH_3), 6.70 (1H, dd, J=2.44, 8.29 Hz, 4′-H), 6.83 (1H, dd, J=0.98, 7.81 Hz, 4-H), 6.90 (1H, d, J=2.44 Hz, 2′-H), 6.94 (1H, d, J=7.81 Hz, 6′-H), 7.18 (1H, dd, J=0.98, 8.29 Hz, 2-H), 7.28 (1H, t, J=7.81 Hz, 5′-H) 7.28 (1H, t, J=7.81 Hz, 3-H), 7.43 (1H, t, J=8.29 Hz, 7-H), 7.47 (1H, dd, J=0.98, 8.29 Hz, 5-H), 7.56 (1H, t, J=8.29 Hz, 6-H), 8.52 (1H, dd, J=0.98, 7.81 Hz, 8-H), 11.83 (1H, S, NH). *Anal.* Calcd for $C_{20}H_{15}NO_2S$: C, 72.06; H, 4.54; N, 4.20. Found: C, 71.88; H, 4.60; N, 4.14. MS: *m/z* 333 [M$^+$].

1-(3′-Methoxyanilino)-5*H*-[1]benzopyrano[2,3-*b*]pyridin-5-one (13) A mixture of 1-amino-5*H*-[1]benzopyrano[2,3-*b*]pyridin-5-one (2.12 g, 0.01 mol), 3-iodo-1-methoxybenzene (2.34 g, 0.01 mol), copper (0.1 g), copper iodine (0.1 g), K_2CO_3 (2.76 g, 0.02 mol), and DMF (50 ml) was stirred under reflux for 5 h. The reaction mixture was filtered. The filtrate was concentrated. The residue was mixed with hot water, filtered, and dried. The obtained solid was purified by silica gel column chromatography with benzene to give **13** (2.98 g, 94%).

Compound **13**: Yellow needles (MeOH), mp 139—140 °C. IR (KBr) cm^{-1}: 1641, 1595, 1572. ^{1}H-NMR ($CDCl_3$) δ: 3.82 (3H, s, OCH_3), 6.72 (1H, d, J=1.95, 8.29 Hz, 4′-H), 6.82 (1H, d, J=8.3 Hz, 4-H), 6.89 (1H, s, 2′-H), 6.93 (1H, d, J=8.29 Hz, 6′-H), 7.08 (1H, d, J=8.30 Hz, 2-H), 7.28 (1H, t, J=7.8 Hz, 5′-H), 7.37 (1H, dd, J=4.88, 7.81 Hz, 7-H), 7.43 (1H, t, J=8.29 Hz, 3-H), 8.62 (1H, dd, J=1.95, 7.81 Hz, 8-H), 8.68 (1H, dd, J=1.95, 4.88 Hz, 6-H), 11.11 (1H, s, NH). *Anal.* Calcd for $C_{19}H_{14}N_2O_2$: C, 71.69; H, 4.43; N, 8.80. Found: C, 71.46; H, 4.35; N, 8.81. MS: *m/z* 318 [M$^+$].

1-(3′-Methoxyanilino)-5*H*-[1]benzothiopyrano[2,3-*b*]pyridin-5-one (14) A mixture of 1-amino-5*H*-[1]benzothiopyrano[2,3-*b*]pyridin-5-one (2.28 g, 0.01 mol), 3-iodo-1-methoxybenzene (2.34 g, 0.01 mol), copper (0.1 g), copper iodine (0.1 g), K_2CO_3 (2.76 g, 0.02 mol), and DMF (50 ml) was stirred under reflux for 5 h. The reaction mixture was filtered. The filtrate was concentrated. The residue was mixed with hot water, filtered, and dried. The obtained solid was purified by silica gel column chromatography with benzene to give **14** (1.68 g, 50%).

Compound **14**: Yellow needles (MeOH), mp 147—148 °C. IR (KBr) cm^{-1}: 1603, 1594, 1573. ^{1}H-NMR ($CDCl_3$) δ: 3.82 (3H, s, OCH_3), 6.72 (1H, dd, J=1.95, 8.30 Hz, 4′-H), 6.86 (1H, dd, J=0.98, 7.81 Hz, 4-H), 6.88 (1H, s, 2′-H), 6.93 (1H, d, J=7.81 Hz, 6′-H), 7.17 (1H, dd, J=0.98, 8.30 Hz, 2-H), 7.28 (1H, t, J=7.81 Hz, 5′-H), 7.30 (1H, t, J=8.30 Hz, 3-H), 7.36 (1H, dd, J=4.39, 7.81 Hz, 7-H), 8.69 (1H, dd, J=1.95, 4.39 Hz, 6-H), 8.71 (1H, dd, J=1.95, 7.81 Hz, 8-H), 11.71 (1H, s, NH). *Anal.* Calcd for $C_{19}H_{14}N_2OS$: C, 68.25; H, 4.22; N, 8.38. Found: C, 68.00; H, 4.26; N, 8.15. MS: *m/z* 334 [M$^+$].

[1]Benzopyrano[2,3,4-*kl*]acridin-3-ol (1) A solution of **5** (3.17 g, 0.01 mol) in 94% H_2SO_4 was heated at 130 °C for 3 h. The cooled solution was poured ice-water and filtered, and the filter cake was washed with water and dried. The residue was purified by silica gel column chromatography using $CHCl_3$–MeOH as eluent to give **1** (0.83 g, 29%).

Compound **1**: Dark red powder, mp 283—284 °C, IR (KBr) cm^{-1}: 1642, 1620, 1595. ^{1}H-NMR (DMSO-d_6) δ: 7.04 (1H, dd, J=0.98, 7.56 Hz, 8-H), 7.23 (1H, d, J=2.44 Hz, 4-H), 7.24 (1H, dd, J=2.44, 9.27 Hz, 2-H), 7.39 (1H, t, J=8.55 Hz, 12-H), 7.42 (1H, dd, J=1.47, 8.30 Hz, 10-H), 7.58 (1H, dd, J=0.98, 8.79 Hz, 6-H), 7.62 (1H, t, J=8.55 Hz, 11-H), 7.71 (1H, t, J=8.79 Hz, 7-H), 8.42 (1H, dd, J=1.47, 8.30 Hz, 13-H), 8.59 (1H, d, J=9.27 Hz, 1-H). *Anal.* Calcd for $C_{19}H_{11}NO_2$: C, 79.99; H, 3.89; N, 4.91. Found: C 79.73; H, 4.02; N, 4.71. MS: *m/z* 285 [M$^+$]. HR-EI-MS Calcd for $C_{19}H_{11}NO_2$: 285.0789. Found: 285.0808.

[1]Benzothiopyrano[2,3,4-*kl*]acridin-3-ol (2) A solution of **12** (3.33 g, 0.01 mol) in 94% H_2SO_4 was heated at 130 °C for 3 h. The cooled solution was poured ice-water and filtered, and the filter cake was washed with water and dried. The residue was purified by silica gel column chromatography using $CHCl_3$–MeOH as eluent to give **2** (0.45 g, 15%).

Compound **2**: Dark red powder, mp 273—274 °C. IR (KBr) cm^{-1}: 1636, 1596. ^{1}H-NMR (DMSO-d_6) δ: 7.23 (1H, dd, J=2.44, 9.27 Hz, 2-H), 7.28 (1H, d, J=2.44 Hz, 4-H), 7.43 (1H, dd, J=0.98, 8.78 Hz, 8-H), 7.47 (1H, t, J=7.81 Hz, 12-H), 7.53 (1H, t, J=7.81 Hz, 11-H), 7.63 (1H, t, J=8.78 Hz, 7-H), 7.67 (1H, dd, J=1.47, 7.81 Hz, 10-H), 7.75 (1H, dd, J=1.98, 8.78 Hz, 6-H), 7.97 (1H, dd, J=1.47, 7.81 Hz, 13-H), 8.30 (1H, d, J=9.27 Hz, 1-H). *Anal.* Calcd for $C_{19}H_{11}NOS$: C, 75.72; H, 3.68; N, 4.65. Found: C, 75.83; H, 3.79; N, 4.44. MS: *m/z* 301 [M$^+$]. HR-EI-MS Calcd for $C_{19}H_{11}NOS$: 301.0561. Found: 301.0590.

Pyrido[3′,2′:5,6]pyrano[2,3,4-*kl*]acridin-3-ol (3) A solution of **13** (3.18 g, 0.01 mol) in 94% H_2SO_4 (40 ml) was heated at 130 °C for 3 h. The cooled solution was poured ice-water and filtered, and the filter cake was washed with water and dried. The residue was purified by silica gel column chromatography using $CHCl_3$–MeOH as eluent to give **3** (1.34 g, 47%).

Compound **3**: Red powder, mp 310—311 °C. IR (KBr) cm^{-1}: 1640, 1595. ^{1}H-NMR (DMSO-d_6) δ: 7.10 (1H, dd, J=8.78 Hz, 8-H), 7.27 (1H, d, J=2.44 Hz, 4-H), 7.28 (1H, d, J=2.44, 9.27 Hz, 2-H), 7.45 (1H, dd, 4.39, 7.80, 12-H), 7.63 (1H, d, J=8.78 Hz, 6-H), 7.74 (1H, t, J=8.78 Hz, 7-H), 8.46 (1H, d, J=4.39 Hz, 11-H), 8.57 (1H, d, J=9.27 Hz, 1-H), 8.87 (1H, d, J=7.80 Hz, 13-H). *Anal.* Calcd for $C_{18}H_{10}N_2O_2$: C, 75.51; H, 3.52; N, 9.79. Found: C, 75.37; H, 3.56; N, 9.84. MS: *m/z* 286 [M$^+$]. HR–EI–MS Calcd for $C_{18}H_{10}N_2O_2$: 286.0741. Found: 286.0715.

Pyrido[3′,2′:5,6]thiopyrano[2,3,4-*kl*]acridin-3-ol (4) A solution of **14** (3.34 g, 0.01 mol) in 94% H_2SO_4 (40 ml) was heated at 130 °C for 3 h. The

cooled solution was poured ice-water and filtered, and the filter cake was washed with water and dried. The residue was purified by silica gel column chromatography using $CHCl_3$–MeOH as eluent to give **4** (1.39 g, 46%).

Compound **4**: Red powder, mp 297—298 °C. IR (KBr) cm^{-1}: 1636, 1597. ^{1}H-NMR (DMSO-d_6) δ: 7.24 (1H, dd, J=2.44, 9.27 Hz, 2-H), 7.28 (1H, d, J=2.44 Hz, 4-H), 7.46 (1H, dd, J=0.98, 8.78 Hz, 8-H), 7.46 (1H, dd, J=4.39, 7.80 Hz, 12-H), 7.66 (1H, t, J=8.78 Hz, 7-H), 7.78 (1H, dd, J= 0.98, 8.78 Hz, 6-H), 8.22 (1H, d, J=9.27 Hz, 1-H), 8.32 (1H, d, J=1.46, 7.80 Hz, 13-H), 8.57 (1H, d, J=1.46, 4.39 Hz, 11-H). *Anal.* Calcd for $C_{18}H_{10}NO_2S$: C, 71.52; H, 3.33; N, 9.27. Found: C, 71.78; H, 3.46; N, 9.00. MS: *m/z* 302 [M^+]. HR-EI-MS Calcd for $C_{20}H_{15}NO_3$: 302.0514. Found: 302.0554.

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A Novel Method of Preparing Amine-Modifying C-4′ Oxidized Nucleotide

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A novel method of preparing C-4′ oxidized nucleotide, a monomeric model of an alkali labile lesion (1) has been studied. The C-4′ selenated 4a and 4b were found to be effective in preparing 3, a monomeric model of 1, by the reaction with NBS (*N*-bromosuccinimide). The successive reaction of 3 with amine at room temperature afforded the α,β-unsaturated γ-methylene-γ-lactam (2) in good yield.

Key words C-4′ oxidized nucleotide; alkali labile lesion; lactam

Bleomycin-induced oxidative damage of DNA under limiting oxygen conditions results in the formation of an alkali labile lesion (**1**).[1] The pathway for the formation of **1** had been rationalized as shown in Chart 1; abstraction of the C-4′ hydrogen from deoxyribose by activated Fe-bleomycin complex and the following one electron oxidation of the resulting radical affords the C-4′ cation intermediate. Hydroxylation of the C-4′ cation intermediate leads to the formation of **1** by nucleic acid base release.[2] Recently, we found that unsaturated lactam (**2a**) and a DNA fragment were formed in the reaction of **1** with amine under mild conditions (room temperature, pH 7).[3] However, quantitative study of lactam formation from **1** has not been done.

This formation of lactam under conditions close to those in the biological systems suggested to us the possibility of using **1** as a functional (oligo)nucleotide to modify the amine-containing biomolecules. For these purposes, we needed to develop a novel method to prepare **1**. Compound **1** was too reactive to synthesize directly. We planned to prepare the C-4′ oxidized sugar unit of **1**, a 2′-deoxy-4′-pentulose derivative, by conversion of C-4′ substituted nucleotide. In this paper, we describe quantitative study of lactam formation from 3′,5′-*O*-diphosphorylated 2′-deoxy-4′-pentulose (**3**), which was prepared by NBS-mediated hydrolysis of 4′-selenated **4a** and **4b**[4] (Chart 2).

Prior to NBS-mediated hydrolysis of **4a** and **4b**, 4′-selenated **5a** and **5b**[5] were subjected to NBS-mediated methanolysis to predict the reactivity of phenyl selenyl group at the C-4′ position. Treatment of **5a** with 1.5 eq of NBS in dry MeOH at room temperature afforded 2′-deoxy-4′-methoxyadenosine derivative (**6a**) and its C-4′ epimer (**6b**) in 22% and 53% yields, respectively. The NOESY spectrum of **6a** supported its stereochemistry at the C-4′ position; the NOE correlation was observed between C-1′ H and the methoxy proton, and between C-3′ H and C-5′ H. In contrast, the reaction of diastereomer **5b** under the same conditions afforded **6a** (53%) and **6b** (28%) (Chart 3). Thus, the NBS-promoted substitution of a phenylselenyl group with a methoxy group proceeded without reaction of adenine moiety.

Next, the NBS-mediated hydrolysis of **4a, b** and successive reaction with benzylamine were studied. Compounds **4a, b** were prepared in the conventional manner (Chart 4). Compound **4a** was treated with 1.5 eq of NBS in a phosphate buffer (pH 7.8) at room temperature. After 1 h, TLC analysis of the reaction mixture showed that **4a** had disappeared.

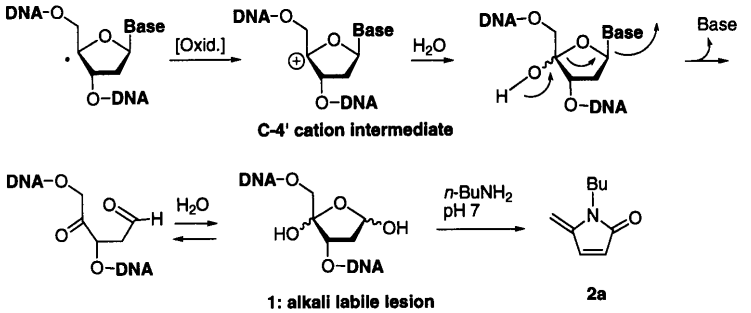

Chart 1

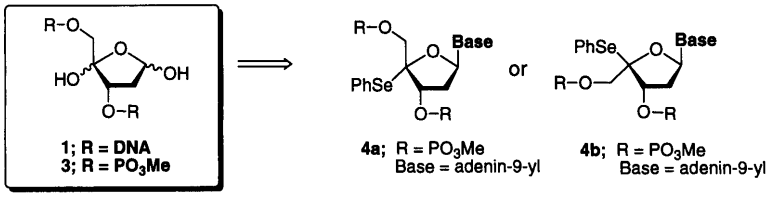

Chart 2

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HPLC analysis of the reaction mixture showed quantitative liberation of adenine from **4a**. To the reaction mixture was added 3 eq of $BnNH_2$ in a phosphate buffer (pH 7.8) containing 18% CH_3CN and the whole was stirred at room temperature. After 9 h, unsaturated lactam **2b** formed from **4a** in 74% yield (Chart 5). The same reaction of **4b** also afforded **2b** in 74% yield. Direct treatment of **4a, b** with amine only resulted in their recovery. Although the isolation of **3** was not successful, these results strongly suggest that treatment of **4a, b** with NBS effected one-step formation of **3** and its successive reaction with $BnNH_2$ gave **2b** *via* cyclic amine derivatives (i), (ii), and (iii).[3)]

Thus, it was successfully shown that the α,β-unsaturated γ-methylene-γ-lactam (**2b**) formed from **3** in good yield under mild conditions. The reactivity of **3**, a monomeric model of **1**, might reflect the behavior of **1** in the biological system and **1** might modify amine containing biomolecules with ease. As shown in methanolysis of **5a** and **5b**, the phenyl selenyl group at the C-4′ position of nucleoside (nucleotide) is possively replaced by various oxygen functional groups. The resulting C-4′ substituted (oligo)nucleotide also will be converted to **1** under conditions alternative to those with NBS. This methodology for preparing C-4′ oxidized nucleotide might provide a new route to modify a lysine residue in a protein.

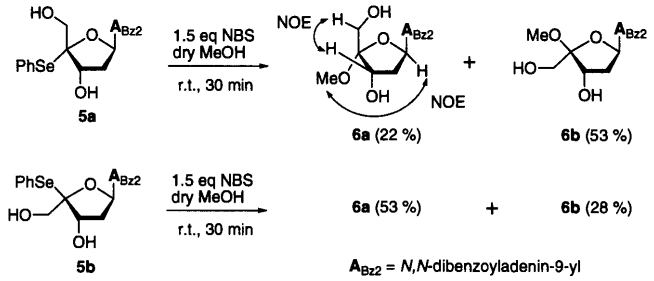

Chart 3

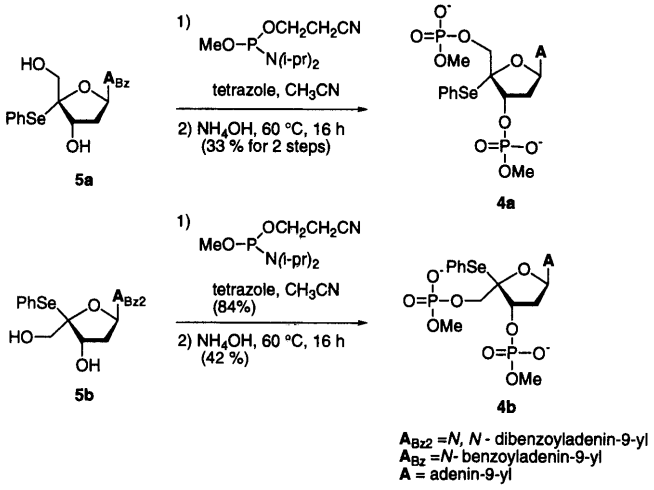

Chart 4

Experimental

Melting points were taken on a Yanagimoto melting point apparatus and are not corrected. ^{1}H-NMR spectra were obtained on a JEOL GX-270 (270 MHz). Chemical shifts are reported as δ values with respect to tetramethylsilane (TMS) as an internal standard. ^{13}C-NMR spectra were taken on a JEOL GX-270 (68 MHz) spectrometer. IR spectra were taken on a JASCO IR A-100 infrared spectrophotometer. Mass spectra and high-resolution mass spectra (HRMS) were determined on a JEOL 610 HS or SX-102 spectrometer. For thin layer chromatography, precoated TLC plates (Merck, Kieselgel 60 F_{254}) were used. Column chromatography was carried out on silica gel 70—230 mesh (Merck, Kieselgel 60).

NBS-Mediated Methanolysis of 5a To a solution of **5a** (50 mg, 81 mmol) in 3 ml of dry MeOH was added NBS (21.7 mg, 122 μmol) and the mixture was stirred for 30 min at room temperature. After addition of saturated aqueous $NaHCO_3$, the reaction mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography. Elution with $CHCl_3$: MeOH=10 : 1 gave N^6,N^6-dibenzoyl-2′-deoxy-4′-methoxyadenosine (**6a**) (8.8 mg, 22%) and 9-(2-deoxy-4-methoxy-α-L-*threo*-pento-1,4-furanosyl)-N^6,N^6-dibenzoyladenine (**6b**) (21 mg, 53%); **6a**: ^{1}H-NMR ($CDCl_3$) δ: 2.53 (m, 1H), 3.03 (m, 1H), 3.51 (s, 3H), 3.73 (d, J=11.7 Hz, 1H), 4.08 (d, J=11.7 Hz, 1H), 4.78 (dd, J=5.9, 5.3

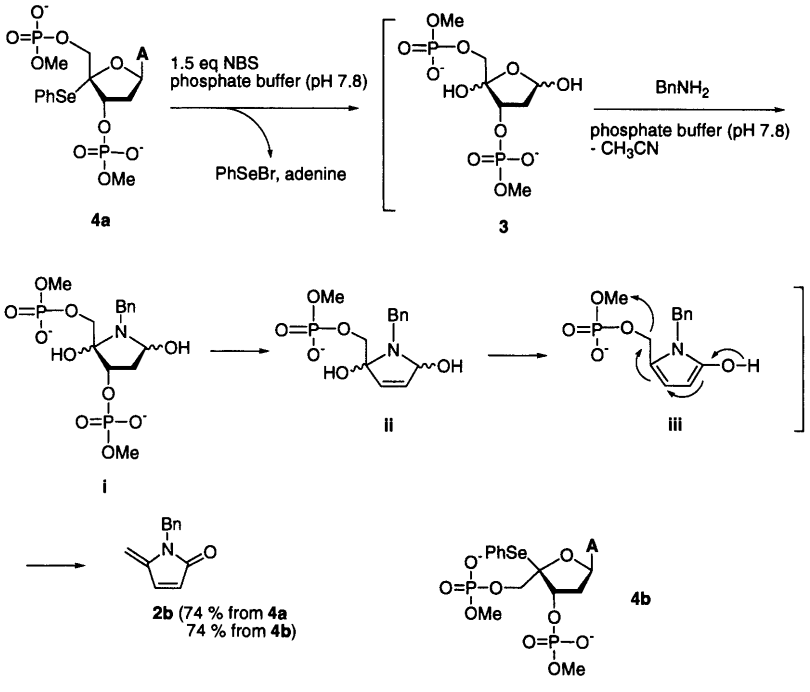

Chart 5

Hz, 1H), 6.45 (t, J=6.5 Hz, 1H), 7.34—7.48 (m, 4H), 7.49—7.53 (m, 2H), 7.84—7.87 (m, 4H), 8.20 (s, 1H), 8.64 (s, 1H). FAB-MS *m/z*: 490.1711 (Calcd for $C_{25}H_{24}N_5O_6$: 490.1727 $[M+H]^+$). **6b**: ^{1}H-NMR ($CDCl_3$) δ: 2.66 (m, 1H), 2.95 (m, 1H), 3.19 (s, 3H), 3.92 (d, J=12.4 Hz, 1H), 3.98 (d, J=12.4 Hz, 1H), 4.59 (d, J=5.3 Hz, 1H), 6.85 (t, J=7.1 Hz, 1H), 7.33—7.36 (m, 4H), 7.46—7.49 (m, 2H), 7.84—7.86 (m, 4H), 8.26 (s, 1H), 8.69 (s, 1H). FAB-MS *m/z*: 490.1737 (Calcd for $C_{25}H_{24}N_5O_6$: 490.1727 $[M+H]^+$).

NBS-Mediated Methanolysis of 5b Similar treatment of **5b** (55.4 mg, 90 mmol) with NBS (32.1 m g, 128 μmol) gave **6a** (23.3 mg, 53%) and **6b** (12.3 mg, 28%), respectively.

2′-Deoxy-3′,5′-*O*-dimethylphosphoryl-4′-phenylselenoadenosine (4a) To a mixture of N^6-benzoyl-2′-deoxy-4′-phenylselenyladenosine (270 mg, 0.53 mmol), tetrazole (382 mg, 5.45 mmol), and molecular sieves (382 mg) in dry CH_3CN (5 ml) was added cyanoethyl *N,N*-diisopropylmethyl phosphine[6] (396 mg, 1.59 mmol) at 0 °C under Ar atmosphere and the mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in 4 ml of a solution containing 0.3 g of I_2 in a mixture of THF : water : pyridine (13 ml : 1 ml : 0.2 ml). The reaction mixture was stirred at room temperature for 20 min and concentrated under reduced pressure. The residue was dissolved in 6 ml of $CHCl_3$ and washed with 1% aqueous sodium bisulfite (8 ml) and the aqueous layer was extracted with $CHCl_3$, washed with brine, and dried over $MgSO_4$. After concentration, the residue was purified by silica gel chromatography. Elution with $CHCl_3$–$CHCl_3$/MeOH (10 : 1) gave the crude mixture of N^6,N^6-dibenzoyl-2′-deoxy-3′,5′-*O*-di(2-cyanoethyl methylphosphoryl) adenosine (267 mg). A mixture of the obtained crude mixture and 10 ml of NH_4OH was heated at 55 °C in a pressure bottle for 16 h. The solvent was evaporated under reduced pressure and evaporated twice with EtOH and the residue was purified by silica gel chromatography. Elution with iso-PrOH/NH_4OH/H_2O (17 : 1 : 2—7 : 1 : 2) gave **4a** (104 mg, 33% for 2 steps) as colorless solid; ^{1}H-NMR (D_2O) δ: 2.80—2.98 (m, 1H), 3.16 (d, J=10.9 Hz, 3H), 3.58 (d, J=10 .9 Hz, 3H), 3.78 (dd, J=11.5, 5.0 Hz, 1H), 4.18 (dd, J=11.6, 3.3 Hz, 1H), 5.20 (dd, J=14.5, 7.3 Hz, 1H), 6.58 (dd, J=7.3, 5.3 Hz, 1H), 7.17—7.23 (m, 3H), 7.60—7.63 (m, 2H), 8.12 (s, 1H), 8.23 (s, 1H). FAB-MS *m/z*: 596.0233 (Calcd for $C_{18}H_{24}N_5O_9P_2Se$: 596.0214 $[M+H]^+$).

9-(2-Deoxy-3,5-*O*-dimethylphosphoryl-4-phenylseleno-α-L-*threo*-pento-1,4-furanosyl)adenine (4b) To a mixture of **5b** (204 mg, 0.33 mmol), tetrazole (277 mg, 3.96 mmol), and molecular sieves (280 mg) in dry CH_3CN (3 ml) was added cyanoethyl *N,N*-diisopropylmethyl phosphine (197 mg, 0.85 mmol) at 0 °C under Ar atmosphere and the mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in 2 ml of a solution containing 0.3 g of I_2 in a mixture of THF : water : pyridine (13 ml : 1 ml : 0.2 ml). The reaction mixture was stirred at room temperature for 20 min and concentrated under reduced pressure. The residue was dissolved in 3 ml of $CHCl_3$ and washed with 1% aqueous sodium bisulfite (5 ml) and the aqueous layer was extracted with $CHCl_3$, washed with brine, and dried over $MgSO_4$. After concentration, the residue was purified by silica gel chromatography. Elution with $CHCl_3$–$CHCl_3$/MeOH (10 : 1) gave 9-(2-deoxy-3,5-*O*-di(2-cyanoethylmethylphosphoryl)4-phenylseleno-α-L-*threo*-pento-1,4-furanosyl)-N^6,N^6-dibenzoyladenine (252 mg, 84%); ^{1}H-NMR ($CDCl_3$) δ: 2.71—2.81 (m, 4H), 3.01—3.07 (m, 1H), 3.35—3.42 (m, 1H), 3.79—3.89 (m, 6H), 4.22—4.44 (m, 6H), 5.48 (m, 1H), 6 .72 (t, J=6.6 Hz, 1H), 7.19—7.52 (m, 11H), 7.81—7.88 (m, 4H), 8.44—8.46 (m, 1H), 8.60—8.62 (m, 1H). FAB-MS *m/z*: 910.1257 (Calcd for $C_{38}H_{38}N_7O_{11}P_2Se$: 910.1270 $[M+H]^+$). A mixture of the obtained products and 10 ml of NH_4OH was heated at 55 °C in a pressure bottle for 16 h. The solvent was evaporated under reduced pressure and evaporated twice with EtOH and the residue was purified by silica gel chromatography. Elution with iso-PrOH/NH_4OH/H_2O (17 : 1 : 2—7 : 1 : 2) gave **4b** (69 mg, 42%) as colorless solid; ^{1}H-NMR (D_2O) δ: 2.80—2.90 (m, 1H), 2.99—3.08 (m, 1H), 3.53 (d, J=10.9 Hz, 3H), 3.56 (d, J=10.6 Hz, 3H), 4.03 (dd, J=10.9, 4.1 Hz, 1H), 4.15 (dd, J=11.2, 4.1 Hz, 1H), 5.06 (dd, J=14.0, 6.9 Hz, 1H), 6.25 (dd, J=6.9, 4.0 Hz, 1H), 6.79—6.85 (m, 2H), 7.00—7.10 (m, 3H), 7.79 (s, 1H), 7.95 (s, 1H); FAB-MS *m/z*: 596.0208 (Calcd for $C_{18}H_{24}N_5O_9P_2Se$: 596.0214 $[M+H]^+$).

Successive Treatment of 4a and 4b with NBS and Benzylamine To a solution of **4a** (11 mg, 17 μmol, 1.0×10^{-1} M) in a phosphate buffer (pH 7.8, 0.17 ml) was added NBS (4.5 mg, 25 μmol) and the mixture was stirred for 1 h at room temperature. To the reaction mixture was added $BnNH_2$ (6 mg, 51 μmol) in a phosphate buffer (pH 7.8; 1 ml) containing 18% CH_3CN and the whole was stirred for 16 h at room temperature. The reaction mixture was extracted with AcOEt, and dried over $MgSO_4$. After concentration, the residue was purified on silica gel eluting with hexane/AcOEt (4 : 1) to give **2b** (2.3 mg, 74%); IR (neat) cm^{-1}: 3060, 1705, 1685, 1630, 1400, 820. ^{1}H-NMR ($CDCl_3$) δ: 4.80 (d, J=2 Hz, 1H), 4.83 (d, J=2 Hz, 1H), 4.84 (s, 2H), 6.28 (ddd, J=6, 2, 1 Hz, 1H), 7.00 (d, J=6 Hz, 1H), 7.18—7.34 (m, 5H). ^{13}C-NMR ($CDCl_3$) δ: 42.6 (t), 97.6 (t), 124.9 (d), 126.9 (d), 127.3 (d), 128.6 (d), 137.0 (d), 137.5 (d), 145.2 (s), 170.4 (s). EI-MS *m/z*: 186.0922 (Calcd for $C_{12}H_{12}NO$: 186.0919 $[M+H]^+$.

The same reaction of **4b** also afforded **2b** in 74% yield.

References and Notes

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Synthesis and Anti-HIV Activity of 3-Alkylamido-3-deoxy-betulinic Acid Derivatives[1)]

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3-Alkylamido-3-deoxy-betulinic acids were synthesized and evaluated for anti-HIV activity as part of the structure–activity relationship study of the potent anti-HIV agent 3-*O*-(3′,3′-dimethyl)-succinyl-betulinic acid (DSB) (2). 3α-Diglycorylamide-3-deoxy-betulinic acid demonstrated relatively potent anti-HIV activity (EC_{50} 0.24 μM, TI 728). However, replacing the ester group at C-3 in 2 and its analogues with an amido group yielded inactive or much less potent compounds against HIV replication, indicating that the ester group at C-3 in 2—4 is essential for potent anti-HIV activity.

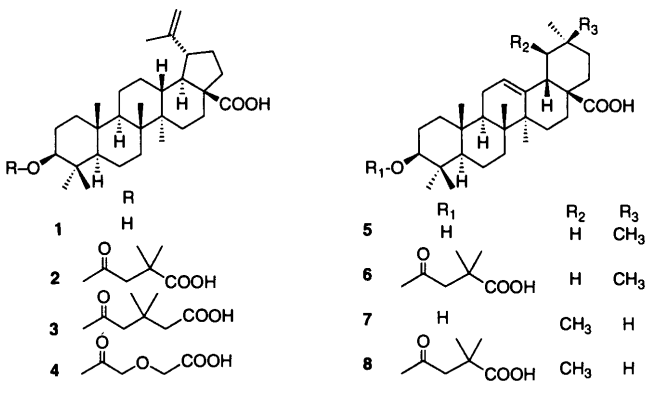
Key words Anti-HIV; betulinic acid; triterpene; 3-alkylamido-3-deoxy-betulinic acid

Betulinic acid (**1**), a pentacyclic lupan-type triterpene, was isolated from the leaves of *Syzygium claviflorum* (Myrtaceae) and identified as an anti-HIV agent with an EC_{50} value of 1.4 μM and therapeutic index (TI) of 9.3 in our search for potential anti-HIV natural products.[2)] Modification of **1** to develop more potent anti-HIV agents yielded 3-*O*-(3′,3′-dimethyl)-succinyl-betulinic acid (DSB, **2**), which demonstrated extremely potent anti-HIV activity with an EC_{50} value of <0.00035 μM and a TI of >20000. 3′,3′-Dimethylglutaryl (**3**) and diglycoryl (**4**) derivatives were also quite potent with EC_{50} values of 0.0023 and 0.01 μM, respectively, and TI values of 1974 and 1172, respectively.[3,4)] Other pentacyclic triterpenoic acids, including oleanolic acid (**5**) and ursolic acid (**7**), also were isolated from various plant sources and identified as anti-HIV agents with EC_{50} and TI values of 3.7 and 4.4 μM and 12.8 and 3.3, respectively.[5)] As part of the structure–activity relationship study of DSB, corresponding esters of oleanolic acid or ursolic acid were synthesized and evaluated. Their structures, especially the A- through D-rings, were partially correlated with that of **1**. Although 3-*O*-(3′,3′-dimethyl)-succinyl-oleanolic acid (**6**) was extremely active with an EC_{50} value of 0.00086 μM and a TI of 22326,[5)] other derivatives, including 3-*O*-(3′,3′-dimethyl)-succinyl-ursolic acid (**8**), were generally much less potent than the corresponding betulinic acid derivatives.[5,6)] This result suggested that the triterpenoic acid moiety, especially the E-ring moiety of **1**, together with the ester group at C-3, play an important role in potent anti-HIV activity. To extend the structure–activity relationship study of these betulinic acid derivatives, we now have prepared 3-alkylamido-3-deoxy-betulinic acid derivatives by replacing the ester group at C-3 in **2**—**4** with an amido group, and evaluated their anti-HIV activity.

Results and Discussion

The strategy for preparing the target compounds is shown in Chart 1. Two different pathways were attempted to introduce an amino group at C-3 in betulinic acid; reductive amination of betulonic acid (**9a**) with NH_4OAc and $NaBH_3CN$ in CH_3OH (route A)[6)] or reduction of oxime derivative (**10a**) with NH_4OAc, $NaBH_3CN$, and $TiCl_3$ in CH_3OH–THF (route B).[7)] Each reaction product was subsequently treated directly with an appropriate anhydride and pyridine. However, neither reaction gave the desired amido derivative, even in the presence of DMAP at reflux. In contrast, reductive amination of 28-*O*-methyl ester derivative (**9b**) or reduction of **10b**, followed by treatment with anhydride and pyridine, successfully yielded amido products. The amido product obtained from reductive amination was thought to be mainly the 3β-amido-betulinic acid derivative, along with a small amount of 3α-isomer.[5)] However, reduction of the oxime was expected to give only the 3β-amido derivative.[7)] HPLC examination of both reaction products showed two compounds in each case. These products were separated by semi-preparative scale HPLC, and the structures were assigned as 3β- (**13a**) and 3α-(3′,3′-dimethylglutaryl)-amido-3-deoxy-betulinic acid 28-*O*-methyl ester (**14b**) based on the coupling constant of H-3 in the ^{1}H-NMR spectra. The ratios of the 3β- and 3α isomers were estimated by HPLC examination; *ca.* 3 : 1 for route A, and *ca.* 9 : 1 for route B.

The 28-methyl ester groups in **13b** and **14b** strongly resisted subsequent alkaline hydrolysis even under drastic conditions (40% KOH–EtOH at reflux). Derivatives with other protective groups, including phenacyl (**13c**) and allyl (**13d**) ester groups, also failed to give the desired product using appropriate deprotective conditions with Zn/AcOH and $Pd(PPh_3)_4$/morpholine, respectively. In contrast, 28-(4′-mor-



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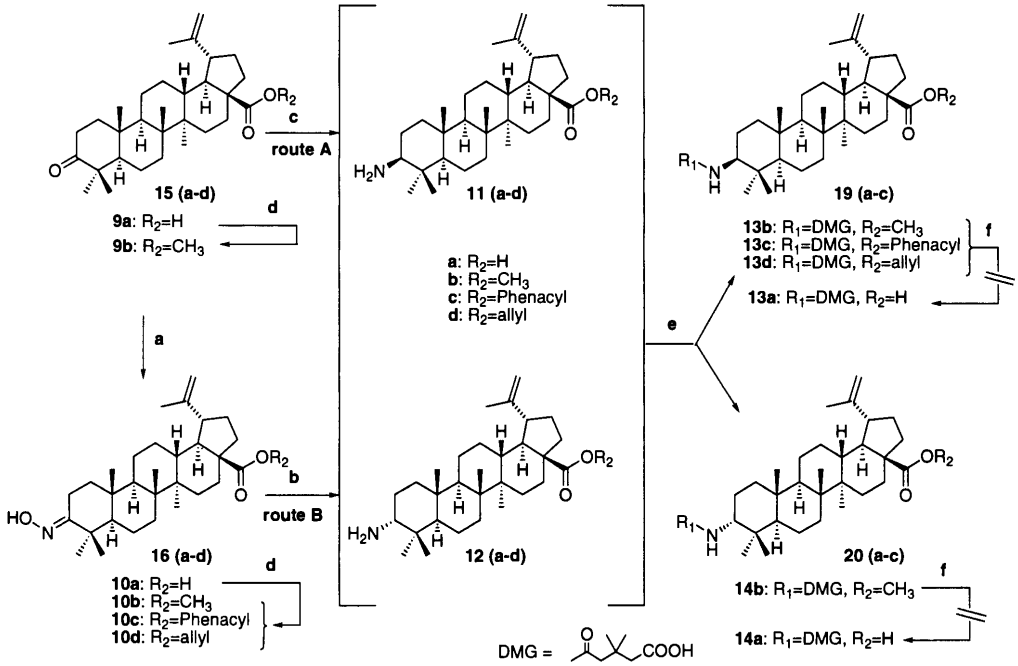
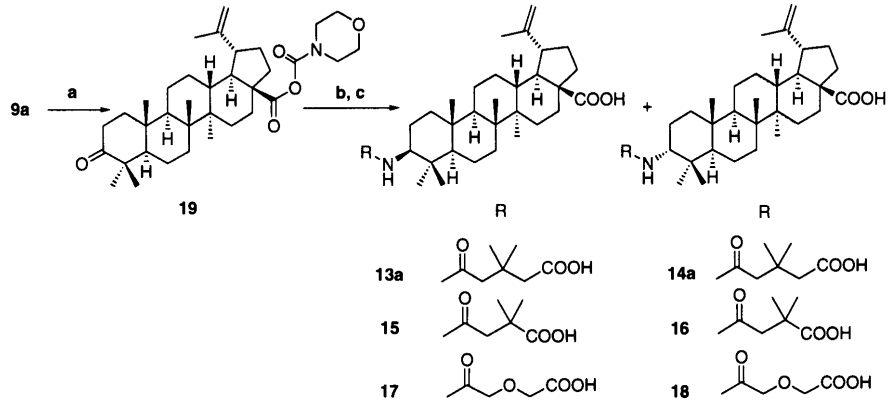


(a) NH_2OH, pyridine, (b) NH_4OAc, $NaBH_3CN$, $TiCl_3$ in CH_3OH–THF, (c) NH_4OAc, $NaBH_3CN$ in CH_3OH, (d) CH_3I, phenacyl bromide, or allylbromide/K_2CO_3/acetone, (e) anhydride, pyridine, (f) KOH–EtOH, Zn/AcOH or $Pd(PPh_3)_4$/morpholine.

Chart 1



(a) 4-Morpholine carbonyl chloride, pyridine, (b) NH_4OAc, $NaBH_3CN$, CH_3OH, (c) anhydride, pyridine, DMAP, reflux.

Chart 2

pholine)-carbonic anhydride (**19**),[4] which was prepared by the reaction of betulonic acid (**9a**) and 4-morpholine carbonyl chloride, reacted readily using reductive amination with NH_4OAc and $NaBH_3CN$ in CH_3OH. In addition, this anhydride was cleaved easily by treatment with pyridine and DMAP at reflux. Therefore, 4-morpholine-carbonic anhydride was employed as the protective group for the 28-carboxylic acid, and the target compounds (**13a**, **14a**, **15**—**18**) were finally prepared by the procedure shown in Chart 2. The resulting 3β- and 3α-isomer mixtures were separated by semi-preparative scale HPLC to afford pure samples. We expected that treatment with 2,2-dimethylsuccinic anhydride would yield a mixture of 3′,3′- and 2′,2′-dimethylsuccinylamido derivatives as was seen previously in 3-*O*-acyl betulinic acid derivatives.[3,4] However, the structures of the only two products were assigned as 3β- (**15**) and 3α-(3′,3′-dimethylsuccinyl)amido-3-deoxy-betulinic acids (**16**) by 2D-NMR spectroscopic examinations, including HSQC and HMBC; no 2′,2′-dimethyl derivatives were formed.

Anti-HIV data for 3-alkylamido-3-deoxy-betulinic acid derivatives are summarized in Table 1. Among the amido derivatives, 3α-diglycorylamido-3-deoxy-betulinic acid (**18**) demonstrated relatively potent anti-HIV activity in acutely infected H9 cells with an EC_{50} value of 0.24 μM, and inhibited uninfected H9 cell growth with an IC_{50} value of 174.9 μM. Thus, its calculated TI was 728. However, it was less potent than its corresponding ester derivative (**4**). In contrast, the 3β-amido isomer (**17**) showed only weak anti-HIV activity with an EC_{50} value of 7.9 μM and TI of 4.9. Unexpectedly, 3β- (**15**) and 3α-(3′,3′-dimethylsuccinyl)-amido-3-deoxy-betulinic acid (**16**), the amido analogs of the potent betulinic acid derivative DSB showed no anti-HIV activity up to 172 μM. Although **13a** was also inactive, the corresponding 3β-(3′,3′-dimethylglutaryl)amido-3-deoxy-betulinic acid 28-*O*-

Table 1. Anti-HIV Activities of **1**, and Its 3-*O*-Acyl- (**2**—**4**) and 3-Alkylamido Derivatives (**13a**—**d**, **14a**—**b**, **15**—**17**), and 3-*O*-Acyl-Oleanolic Acid (**6**) and -Ursolic Acid (**8**)

	IC_{50} [a]	EC_{50} [b]	TI [c]
1	12.9	1.4	9.2
2	7.0	<0.00035	>20000
3	4.5	0.0023	1974
4	11.7	0.01	1172
6	19.2	0.00086	22326
8	49.5	2.1	23.6
13a	32.4	N.S. [d]	—
13b	23.5	1.16	20.3
13c	23.6	4.77	3.54
13d	32.6	N.S. [d]	—
14a	— [e]	— [e]	—
15	35.8	N.S. [d]	—
16	32.2	N.S. [d]	—
17	38.7	7.9	4.9
18	174.9	0.24	728
AZT	1873	0.045	41622

a) Concentration (μM) which is toxic to 50% of mock-infected H9 cells. *b*) Concentration (μM) which inhibits HIV-1 replication by 50%. *c*) TI is defined by IC_{50}/EC_{50}. *d*) No suppression. *e*) Not tested due to limited solubility in DMSO.

methyl (**13b**) and phenacyl (**13c**)-ester derivatives showed weak anti-HIV activity with EC_{50} values of 1.16 and 4.77 μM, respectively. The allyl-ester derivative (**13d**) displayed no anti-HIV activity.

Overall, replacing the ester group at C-3 in DSB (**2**) and its analogues (**3** and **4**) with an amido group yielded inactive or much less potent compounds against HIV replication. The above investigation strongly indicates that the ester group at C-3 in **2**—**4** is essential for potent anti-HIV activity.

Experimental

General Methods Melting points were measured on a Yanako micro melting point apparatus and are uncorrected. Optical rotations were measured on Perkin-Elmer 241 polarimeters. Mass spectra were determined on a JEOL HX-110 spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured on JEOL A-400 spectrometers with tetramethylsilane (Me_4Si) as an internal standard. Column chromatography was performed with Kieselgel 60 PF_{254} (Merck). TLC was performed on precoated Kieselgel 60 F_{254} plates (0.2 mm, Merck), and spots were detected under UV or visualized by spraying 10% H_2SO_4 with heating. HPLC consisted of a Waters 600E solvent delivery system, a Waters 486 UV-VIS spectrometer, and a Waters R401 differential refractometer R-401 equipped with a YMC-ODS (YMC, Inc.) or Mightysil RP-18 GP (Kanto Chemical Co., Inc.) column (4 mm i.d.×250 mm and 20 mm i.d.×250 mm), for analytical and semi-preparative scale, respectively.

3-Deoxy-3-oxime-betuliniac Acid (**10a**): A solution of betulonic acid (628 mg, 1.50 mmol) and hydroxylamine hydrochloride (208 mg, 3.00 mmol) in pyridine (17 mL) was heated at 110 °C for 2.5 h. After cooling, the reaction mixture was diluted with ice-water and extracted with EtOAc three times. The combined organic layers were washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was chromatographed over silica gel with *n*-hexane–EtOAc (5 : 1) to give the corresponding oxime (580 mg, 82% yield) as a white powder, mp 230—232 °C. $[\alpha]_D^{22}$ 0° (*c*=1.0, $CHCl_3$). ^{1}H-NMR (pyridine-d_5) δ: 0.89 (3H, s, CH_3-25), 1.05 (3H, s, CH_3-24), 1.01 (3H, s, CH_3-27), 1.13 (3H, s, CH_3-23), 1.37 (3H, s, CH_3-26), 1.78 (3H, s, CH_3-29), 4.78, 4.95 (each 1H, br s, H-30), 12.25 (=N-OH).

Preparation of betulonic acid or 3-deoxy-3-oxime-betulinic acid 28-*O*-ester derivatives: A mixture of betulonic acid (70 mg) or 3-deoxy-3-oxime-betulinic acid (70—80 mg), appropriate halide (30—70 mg), and K_2CO_3 (35—300 mg) in dry acetone (8—15 ml) was refluxed for 2—4 h with stirring. After removal of the inorganic salts by filtration, the filtrate was concentrated to dryness. The corresponding 28-*O*-ester derivatives crystallized from the residue (80—90% yield).

Betulonic Acid 28-*O*-Methyl Ester (**9b**): A white powder. $[\alpha]_D^{22}$ +31.5° (*c*=1.01, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, s, CH_3-25), 0.95 (3H, s, CH_3-24), 0.97 (3H, s, CH_3-27), 1.02 (3H, s, CH_3-23), 1.07 (3H, s, CH_3-26), 1.68 (3H, s, CH_3-29), 3.68 (3H, s, OCH_3), 4.60, 4.73 (each 1H, br s, H-30). FAB MS: *m/z* 491 $(M+Na)^+$. HR-FAB MS: Calcd for $C_{31}H_{48}O_3Na$: 491.3501. Found: 491.3500.

3-Deoxy-3-oxime-betulinic Acid 28-*O*-Methyl ester (**10b**): Colorless needles. mp 239—241 °C. $[\alpha]_D^{22}$ −5.6° (*c*=0.36, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, s, CH_3-25), 0.94 (3H, s, CH_3-24), 0.95 (3H, s, CH_3-27), 1.05 (3H, s, CH_3-23), 1.15 (3H, s, CH_3-26), 1.68 (3H, s, CH_3-29), 3.67 (3H, s, OCH_3), 4.60, 4.73 (each 1H, br s, H-30). Positive FAB MS: *m/z* 506 $(M+Na)^+$. HR-FAB MS: Calcd for $C_{31}H_{49}NO_3Na$: 506.3611. Found: 506.3610.

3-Deoxy-3-oxime-betulinic Acid 28-*O*-Pheanacyl Ester (**10c**): A white powder. mp 209—211 °C. $[\alpha]_D^{22}$ −21.4° [*c*=1.02, $CHCl_3$–MeOH (1 : 1)]. ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, s, CH_3-25), 0.97 (3H, s, CH_3-24), 0.98 (3H, s, CH_3-27), 1.04 (3H, s, CH_3-26), 1.13 (3H, s, CH_3-23), 1.69 (3H, s, CH_3-29), 4.60, 4.72 (each 1H, br s, H-30), 5.28, 5.33 (each 1H, d, *J*=16.0 Hz, phenacyl CH_2), 7.48 (2H, t, *J*=7.5 Hz, phenacyl H-3′), 7.60 (1H, t, *J*=7.5 Hz, phenacyl H-4′), 7.92 (2H, d, *J*=7.5 Hz, phenacyl H-2′). Positive FAB MS: *m/z* 588 $(M+H)^+$. HR-FAB MS: Calcd for $C_{38}H_{54}NO_4$: 588.4052. Found: 588.4059.

3-Deoxy-3-oxime-betulinic Acid 28-*O*-Allylester (**10d**): A white powder. mp 177—178 °C. $[\alpha]_D^{22}$ −6.25° (*c*=0.48, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, s, CH_3-25), 0.94 (3H, s, CH_3-24), 0.95 (3H, s, CH3-27), 1.05 (3H, s, CH_3-26), 1.15 (3H, s, CH_3-23), 1.68 (3H, s, CH_3-29), 4.57, 4.73 (each 1H, br s, H-30), 4.60 (2H, br s, H-1′), 5.24 (1H, dq, *J*=1.5, 10.5 Hz, H-3′). 5.34 (1H, dq, *J*=1.5, 17.0 Hz, H-3′), 5.93 (1H, ddt, *J*=17.0, 10.0, 5.0 Hz, H-2′). Positive FAB MS: *m/z* 510 $(M+H)^+$. HR-FAB MS: Calcd for $C_{33}H_{52}NO_3$: 510.3947. Found: 510.3941.

3β and 3α-(3′,3′-Dimethyl)-glutaryl-3-deoxy-betulinic Acid 28-*O*-Methyl Ester (**13b** and **14b**): a) A solution of **9b** (40 mg, 0.085 mmol) and NH_4OAc (131 mg, 1.7 mmol) in MeOH (2 mL) was heated 60 °C for 2.5 h. After cooling to room temperarue, a solution of $NaBH_3CN$ (16 mg, 0.26 mmol) in MeOH was added, and the mixture was stirred at room temperature overnight. After addition of 10% aqueous NH_4OH solution, the resulting basic solution was extracted with $CHCl_3$ three times, and the organic layer was washed with water, dried over Na_2SO_4, and concentrated to dryness. The residue (36 mg) was subsequently treated with dimethylglutaric anhydride (92 mg, 0.65 mmol) and DMAP (19 mg, 0.16 mmol) in pyridine (2 ml) at reflux overnight. The reaction mixture was diluted with ice-water and extracted with $CHCl_3$. The organic layer was washed with water, dried over Na_2SO_4, and concentrated to dryness. The residue was separated by semi-preparative scale HPLC to afford **13b** (22 mg, 42% yield) and **14b** (9 mg, 17% yield). b) A solution of **10b** (40 mg, 0.083 mmol) and NH_4OAc (92 mg, 1.18 mmol) in MeOH (6 ml) was treated with $NaBH_3CN$ (74 mg, 1.18 mmol). The mixture was cooled to 10 °C, 15% $TiCl_3$ (0.2 ml, 0.19 mmol) was added dropwise, and the mixture was stirred at room temperature for 15 h. Work-up as for a) gave a product (37 mg) that was treated with dimethylglutaric anhydride (60 mg, 0.43 mmol) and DMAP (12 mg, 0.086 mmol) in pyridine (2 ml) at reflux. The reaction mixture was worked up as described above, and the product was separated by semi-preparative HPLC to give **13b** (32 mg, 61% yield) and **14b** (3 mg, 6% yield).

3β-(3′,3′-Dimethyl)-glutarylamido-3-deoxy-betulinic Acid 28-*O*-Methyl Ester (**13b**): Colorless needles (from *n*-hexane–EtOAc). mp 242—244 °C. $[\alpha]_D^{22}$ +6.3° (*c*=0.51, $CHCl_3$). ^{1}H-NMR (pyridine-d_5+D_2O) δ: 0.77 (3H, s, CH_3-25), 0.96 (3H, s, CH_3-24), 0.98 (3H, s, CH_3-27), 1.02 (3H, s, CH_3-23), 1.11 (3H, s, CH_3-26), 1.31, 1.32 (each 3H, s, dimethylsuccinyl-CH_3), 1.78 (3H, s, CH_3-29), 2.71, 2.78 (each 2H, br s, dimethylglutaryl-CH_2), 3.79 (3H, s, OCH_3), 4.05 (1H, dd, *J*=4.0, 12.0 Hz, H-3), 4.79, 4.90 (each 1H, br s, H-30). Positive FAB MS: *m/z* 612 $(M+H)^+$. HR-FAB MS: Calcd for $C_{38}H_{62}NO_5$: 612.4628. Found: 612.4631.

3α-(3′,3′-Dimethyl)-glutaryl-3-deoxy-betulinic Acid 28-*O*-Methyl Ester (**14b**): A white amorphous powder. $[\alpha]_D^{22}$ −5.0° (*c*=0.2, $CHCl_3$). ^{1}H-NMR (pyridine-d_5+D_2O) δ: 0.80 (3H, s, CH_3-24), 0.83 (3H, s, CH_3-25), 0.96 (3H, s, CH_3-26), 0.97 (3H, s, CH_3-27), 1.10 (3H, s, CH_3-23), 1.31 (6H, s, dimethylglutaryl-CH_3×2), 1.70 (3H, s, CH_3-29), 2.62, 2.75 (each 2H, s, dimethylglutaryl-CH_2×2), 3.75 (3H, s, OCH_3), 4.22 (1H, br s, H-3), 4.75, 4.86 (each 1H, br s, H-30). Positive FAB MS: *m/z* 634 $(M+Na)^+$. HR-FAB MS: Calcd for $C_{38}H_{61}NO_5Na$: 634.4448. Found: 634.4448.

3β-(3′,3′-Dimethyl)-glutarylamide-3-deoxy-betulinic Acid 28-*O*-Phenacyl Ester (**13c**): A white amorphous powder. $[\alpha]_D^{21}$ −6.2° (*c*=0.5, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.78 (3H, s, CH_3-25), 0.81 (3H, s, CH_3-24), 0.89 (3H, s, CH_3-27), 0.95 (3H, s, CH_3-26), 0.98 (3H, s, CH_3-23), 1.14 (dimethylglutaryl-CH_3), 1.70 (3H, s, CH_3-29), 2.45, 2.47 (each 2H, s, dimethylglutaryl-CH_2), 4.02 (1H, ddd, *J*=4.0, 10.0, 10.0 Hz, H-3), 4.60, 4.72 (each 1H, br s, H-30),

5.30 (2H, s, phenacyl CH_2),5.80 (1H, d, NH), 7.48 (2H, t, J=7.5 Hz, phenacyl H-3′), 7.60 (1H, t, J=7.5 Hz, phenacyl H-4′), 7.92 (2H, d, J=7.5 Hz, phenacyl H-2′). Positive FAB MS: *m/z* 716 $(M+H)^+$. HR-FAB MS: Calcd for $C_{45}H_{66}NO_6$: 716.4890. Found: 716.4881.

3β-(3′,3′-Dimethyl)-glutarylamide-3-deoxy-betulinic Acid 28-*O*-Allyl Ester (**13d**): A white amorphous powder, $[\alpha]_D^{21}$ +6.25° (c=0.4, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.77 (3H, s, CH_3-25), 0.82 (3H, s, CH_3-24), 0.89 (3H, s, CH_3-27), 0.91 (3H, s, CH_3-23), 0.97 (3H, s, CH3-26), 1.10, 1.12 (each 3H, s, dimethylglutaryl-CH_3), 1.69 (3H, s, CH_3-29), 2.26, 2.31 (each 1H, d, J=13.5 Hz, dimethylglutaryl-CH_2), 2.41, 2.46 (each 1H, d, J=12.5 Hz, dimethylglutaryl CH_2), 3.72 (1H, ddd, J=4.5, 11.0, 11.0 Hz, H-3), 4.58 (2H, m, H-1′), 4.58, 4.73 (each 1H, br s, H-30), 5.68 (1H, d, J=11.0 Hz, NH), 5.24 (1H, dq, J=1.5, 10.5 Hz, H-3′), 5.34 (1H, dq, J=1.5, 17.0 Hz, H-3′), 5.93 (1H, ddt, J=17.0, 10.5, 5.0 Hz, H-2′). Positive FAB MS: *m/z* 638 $(M+H)^+$. HR-FAB MS: Calcd for $C_{40}H_{64}NO_5$: 638.4784. Found: 638.4792.

3β-(3′,3′-Dimethyl)-glutarylamido-3-deoxy-betulinic Acid (**13a**): Yield 40.5% from 208 mg of **9a**. A white amorphous powder. $[\alpha]_D^{22}$ +6.3° [c=1.02, $CHCl_3$–MeOH (1 : 1)]. ^{1}H-NMR (pyridine-d_5+D_2O) δ: 0.74 (3H, s, CH_3-25), 0.95 (3H, s, CH_3-24), 1.01 (3H, s, CH_3-27), 1.07 (3H, s, CH_3-26), 1.11 (3H, s, CH_3-23), 1.38, 1.38 (each 3H, s, dimethylglugaryl-CH_3×2), 1.82 (3H, s, CH_3-29), 2.74 (2H, s, dimethylglutaryl-CH_2), 2.79, 2.76 (each 1H, d, J=14 Hz, dimethylglutaryl-CH_2), 4.06 (1H, dd, J=4.5, 12 Hz, H-3), 4.80, 4.94 (each 1H, br s, H-30). ^{13}C-NMR (pyridine-d_5+D_2O) δ: 14.9 (C-26), 16.2 (C-25), 16.4 (C-27), 17.1 (C-24), 18.9 (C-6), 19.4 (C-29), 21.1 (C-11), 26.1 (C-12), 26.1 (C-2), 28.8, 28.9 (dimethylglutaryl-CH_3), 29.0 (C-23), 30.3 (C-21), 31.2 (C-15), 32.9 (C-16), 33.5 (dimethylglutaryl C-3), 34.7 (C-7), 37.4 (C-10), 37.6 (C-22), 38.4 (C-4), 38.6 (C-13), 39.7 (C-1), 41.0 (C-8), 42.8 (C-14), 46.4, 47.8 (dimethylglutaryl-CH_2), 47.8 (C-19), 49.7 (C-18), 50.8 (C-9), 56.5 (C-5), 56.6 (C-17), 56.7 (C-3), 110.0 (C-30), 151.3 (C-20), 171.4 (NHCO), 175.0 (dimethylglutaryl-COOH), 178.9 (C-28). Positive FAB MS: *m/z* 598 $(M+H)^+$. HR-FAB MS: Calcd for $C_{37}H_{60}NO_5$: 598.4471. Found: 598.4470.

3α-(3′,3′-Dimethyl)-glutarylamido-3-deoxy-betulinic Acid (**14a**): Yield 12.6% from 208 mg of **9a**. A white amorphous powder. $[\alpha]_D^{22}$ −8.9° [c=0.52, $CHCl_3$–MeOH (1 : 1)]. ^{1}H-NMR (pyridine-d_5+D_2O) δ: 0.81 (3H, s, CH_3-24), 0.94 (3H, s, CH_3-25), 0.96 (3H, s, CH_3-26), 1.03 (3H, s, CH_3-27), 1.10 (3H, s, CH_3-23), 1.34 (6H, s, dimethylglugaryl-CH_3×2), 1.74 (3H, s, CH_3-29), 2.67, 2.75 (each 2H, s, dimethylglutaryl-CH_2×2), 4.20 (1H, br s, H-3), 4.78, 4.89 (each 1H, br s, H-30). ^{13}C-NMR (pyridine-d_5+D_2O) δ: 14.7 (C-26), 16.2 (C-25), 16.3 (C-27), 18.4 (C-6), 19.4 (C-24), 20.9 (C-11), 23.2 (C-23), 24.2 (C-2), 26.0 (C-12), 28.7, 29.0 (dimethylglutaryl-CH_3), 29.0 (C-29), 30.1 (C-21), 31.0 (C-15), 32.7 (C-16), 33.7 (dimethylglutaryl C-3), 34.4 (2C) (C-7 and 1), 36.5 (C-4), 37.5 (C-22), 37.6 (C-10), 38.5 (C-13), 41.1 (C-8), 42.7 (C-14), 46.5, 47.3 (dimethylglutaryl-CH_2), 47.7 (C-19), 49.6 (C-18), 50.1 (C-5), 50.6 (C-9), 54.7 (C-3), 56.5 (C-17),110.0 (C-30), 151.2 (C-20), 171.9 (NHCO), 175.6 (dimethylglutaryl-COOH), 179.0 (C-28). Positive FAB MS: *m/z* 598 $(M+H)^+$. HR-FAB MS: Calcd for $C_{37}H_{60}NO_5$: 598.4471. Found: 598.4476.

3β-(3′,3′-Dimethyl)-succinylamido-3-deoxy-betulinic Acid (**15**): Yield 25.6% from 253 mg of **9a**. A white amorphous powder. $[\alpha]_D^{22}$ +11.4° [c=1.0, $CHCl_3$–MeOH (1 : 1)]. ^{1}H-NMR (pyridine-d_5+D_2O) δ: 0.70 (3H, s, CH_3-25), 0.89 (3H, s, CH_3-24), 1.00 (3H, s, CH_3-27), 1.05 (3H, s, CH_3-26), 1.11 (3H, s, CH_3-23), 1.58, 1.60 (each 3H, s, dimethylsuccinyl-CH_3), 1.81 (3H, s, CH_3-29), 2.94, 2.99 (each 1H, d, J=14.0 Hz, dimethylsuccinyl-CH_2), 4.06 (1H, dd, J=6.0, 11.0 Hz, H-3), 4.79, 4.94 (each 1H, br s, H-30). ^{13}C-NMR (pyridine-d_5+D_2O) δ: 14.9 (C-26), 16.2 (C-25), 16.4 (C-27), 17.1 (C-24), 18.9 (C-6), 19.4 (C-23), 21.1 (C-11), 26.1 (C-12), 26.1 (C-2), 28.8, 28.9 (dimethylglutaryl-CH_3), 29.0 (C-29), 30.3 (C-21), 31.2 (C-15), 32.9 (C-16), 33.5 (dimethylglutaryl C-3), 34.7 (C-7), 37.4 (C-10), 37.6 (C-22), 38.4 (C-4), 38.6 (C-13), 39.7 (C-1), 41.0 (C-8), 42.8 (C-14), 46.4, 47.8 (dimethylglutaryl CH_2), 47.8 (C-19), 49.7 (C-18), 50.8 (C-9), 56.5 (C-5), 56.6 (C-17), 56.7 (C-3), 110.0 (C-30), 151.3 (C-20), 171.4 (NHCO), 175.0 (dimethylglutaryl-COOH), 178.9 (C-28). Positive FAB MS: *m/z* 598 $(M+H)^+$. HR-FAB MS: Calcd for $C_{37}H_{60}NO_5$: 598.4471. Found: 598.4470.

3α-(3′,3′-Dimethyl)-succinylamido-3-deoxy-betulinic Acid (**16**): Yield 16.0% from 253 mg of **9a**. A white amorphous powder. $[\alpha]_D^{22}$ −10.8° [c=1.0, $CHCl_3$–MeOH (1 : 1)]. ^{1}H-NMR (pyridine-d_5+D_2O) δ: 0.79 (3H, s, CH_3-24), 0.86 (3H, s, CH_3-25), 0.93 (3H, s, CH_3-26), 1.02 (3H, s, CH_3-27), 1.10 (3H, s, CH_3-23), 1.53, 1.54 (each 3H, s, dimethylsuccinyl-CH_3), 1.78 (3H, s, CH_3-29), 2.91, 2.96 (each 1H, d, J=14.0 Hz, dimethylsuccinyl-CH_2), 4.23 (1H, br t, J=3.0 Hz, H-3), 4.80, 4.91 (each 1H, br s, H-30). ^{13}C-NMR (pyridine-d_5+D_2O) δ: 14.6 (C-26), 16.2 (C-25), 16.3 (C-27), 18.5 (C-6), 19.5 (C-24), 21.0 (C-11), 23.3 (C-23), 24.3 (C-2), 26.1 (C-12), 26.2, 26.4 (dimethylsuccinyl-CH_3), 28.9 (C-29), 30.2 (C-21), 31.2 (C-15), 32.8 (C-16), 34.4 (C-1), 34.5 (C-7), 36.6 (C-4), 37.6 (C-22), 37.7 (C-10), 38.5 (C-13), 41.2 (dimethylsuccinyl C-3), 41.5 (C-8), 42.7 (C-14), 46.5 (dimethylsuccinyl CH_2), 47.7 (C-19), 49.7 (C-18), 50.3 (C-5), 51.0 (C-9), 54.2 (C-3), 56.6 (C-17),109.9 (C-30), 151.3 (C-20), 170.7 (NHCO), 178.8 (dimethylsuccinyl COOH), 180.2 (C-28). Positive FAB MS: *m/z* 598 $(M+H)^+$. HR-FAB MS: Calcd for $C_{37}H_{60}NO_5$: 598.4471. Found: 598.4476.

3β-Diglycorylamido-3-deoxy-betulinic Acid (**17**): Yield 32.5% from 221 mg of **9a**. A white amorphous powder. $[\alpha]_D^{22}$ +8.7° [c=1.03, $CHCl_3$–MeOH (1 : 1)]. ^{1}H-NMR (pyridine-d_5+D_2O) δ: 0.73 (3H, s, CH_3-25), 0.91 (3H, s, CH_3-24), 1.01 (3H, s, CH_3-27), 1.04 (3H, s, CH_3-26), 1.07 (3H, s, CH_3–23), 1.81 (3H, s, CH_3-29), 4.06 (1H, dd, J=4.0, 12.0 Hz, H-3), 4.49, 4.55 (each 1H, d, J=16.5 Hz, diglycoryl CH_2), 4.50, 4.57 (each 1H, d, J=15.0 Hz, diglycoryl CH_2), 4.79, 4.94 (each 1H, br s, H-30). ^{13}C-NMR (pyridine-d_5+D_2O) δ: 14.9 (C-26), 16.1 (C-25), 16.3 (C-27), 16.7 (C-24), 18.8 (C-6), 19.4 (C-23), 21.1 (C-11), 25.8 (C-2), 26.0 (C-12), 28.8 (C-29), 30.2 (C-21), 31.2 (C-15), 32.8 (C-16), 34.6 (C-7), 37.3 (C-10), 37.6 (C-22), 38.4 (C-4), 38.6 (C-13), 39.6 (C-1), 41.0 (C-8), 42.8 (C-14), 47.8 (C-19), 49.7 (C-18), 50.8 (C-9), 56.4 (C-5), 56.5 (C-3), 56.6 (C-17), 69.6, 72.1 (diglycoryl C-2 and 4), 110.0 (C-30), 151.3 (C-20), 169.2 (NHCO), 173.3 (diglycoryl COOH), 178.8 (C-28). Positive FAB MS: *m/z* 594 $(M+Na)^+$, 572 $(M+H)^+$. HR-FAB MS: Calcd for $C_{34}H_{54}NO_6$: 572.3951. Found: 572.3951.

3α-Diglycorylamido-3-deoxy-betulinic Acid (**18**): Yield 12.1% from 221 mg of **9a**. A white amorphous powder. $[\alpha]_D^{22}$ −3.2° [c=1.0, $CHCl_3$–MeOH (1 : 1)]. ^{1}H-NMR (pyridine-d_5+D_2O) δ: 0.80 (3H, s, CH_3-24), 0.94 (3H, s, CH_3-25), 1.02 (6H, s, CH_3-26 and 27), 1.13 (3H, s, CH_3-23), 1.75 (3H, s, CH_3-29), 4.20 (1H, br s, H-3), 4.96 (2H, s, diglycoryl CH_2), 4.51, 4.55 (each 1H, d, J=15.0 Hz, diglycoryl CH_2) 4.78, 4.89 (each 1H, br s, H-30). ^{13}C-NMR (pyridine-d_5+D_2O) δ: 14.8 (C-26), 16.2 (C-25), 16.4 (C-27), 18.5 (C-6), 19.4 (C-24), 21.0 (C-11), 23.2 (C-23), 24.0 (C-2), 26.0 (C-12), 28.6 (C-29), 30.2 (C-21), 31.2 (C-15), 32.8 (C-16), 34.4 (C-1), 34.6 (C-7), 36.9 (C-4), 37.6 (C-22), 37.6 (C-10), 38.5 (C-13), 41.8 (C-8), 42.8 (C-14), 47.8 (C-19), 49.7 (C-18), 50.5 (C-5), 51.0 (C-9), 54.0 (C-3), 56.6 (C-17), 69.8, 72.3 (diglycoryl C-2 and 4), 110.0 (C-30), 151.3 (C-20), 169.1 (NHCO), 173.5 (diglycoryl COOH), 178.4 (C-28). Positive FAB MS: *m/z* 572 $(M+H)^+$. HR-FAB MS: Calcd for $C_{34}H_{54}NO_6$: 572.3951. Found: 572.3943.

Anti-HIV Assay The anti-HIV assay was performed by the procedure described in the literature.[5)]

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Synthesis of 9Z-9-Substituted Retinoic Acids by Palladium Catalyzed Coupling Reaction of a Vinyl Triflate with Alkenyl Stannanes

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Palladium catalyzed cross coupling reactions of a vinyl triflate intermediate and various alkenyl stannanes afforded trisubstituted *Z*-olefins stereoselectively in high yields. These olefins were then converted to the corresponding 9*Z*-retinoic acids *via* Horner–Emmons reaction and subsequent basic hydrolysis in excellent yields.

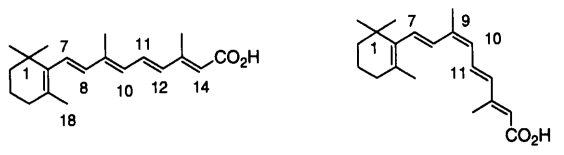
Key words retinoid X receptor; 9*Z*-retinoic acid analog; coupling reaction; vinyl triflate; alkenyl stannane

It is well known that all-*E*-retinoic acid **1** and 9*Z*-retinoic acid **2** are the ligands of retinoic acid receptors (RARα, β, γ) and retinoid X receptors (RXRα, β, γ), respectively.[1] These receptors are members of the nuclear receptor superfamily and exhibit significant biological functions including cell differentiation, cell proliferation, embryonic development, *etc.* through gene transcription.[2] Currently, much effort is being directed at the preparation of receptor-selective retinoids in order to define the functions of each receptor and to develop therapeutic agents.[3] In connection with our study on the stereoselective synthesis of retinoids and carotenoids,[4] we wish to describe here a novel synthesis of 9-substituted 9*Z*-retinoic acids using a palladium catalyzed cross-coupling reaction.[5]

Treatment of 2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde **3**,[6] prepared by Wittig reaction of β-cyclocitral with (methoxymethyl)triphenylphosphonium chloride followed by hydrolysis, with *N*-phenyltrifluoromethanesulfonimide (Tf_2NPh) in the presense of potassium *tert*-butoxide gave the vinyl triflate **4** in 58% yield as the only stereoisomer. In a preliminary study, palladium catalyzed coupling of **4** with alkenyl stannane **6a**[7] under various conditions indicated that the best results were obtained using 5 mol% tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) as catalyst at room temperature in dimethylformamide (DMF) for 2 h, to produce alcohol **9a** in 87% yield. The structure of **9a** was determined after oxidation to the aldehyde **10a** and comparison of spectral data with those in the literature.[8] However, under similar conditions the reaction of **4** with alkenyl stannanes **7** or **8** having an electron withdrawing group did not proceed and the expected coupling product was not obtained.

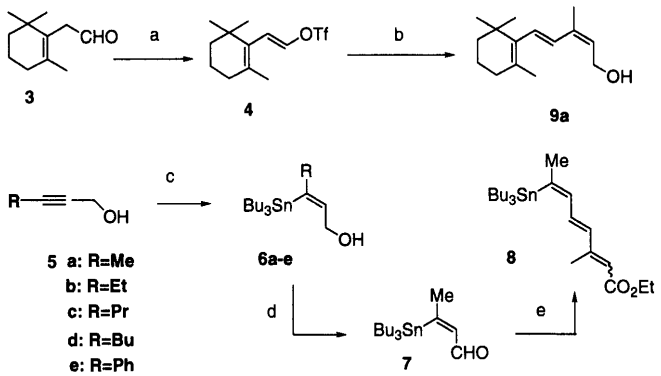
In order to determine the generality of this coupling, we examined the reaction of **4** with various alkenyl stannanes (**6b—d**), prepared from substituted propargyl alcohols **5** according to the previously reported method,[7] and the results are listed in Table 1.

In the case of **6e**, the yield was decreased dramatically (run 5), however, the yield was improved using tris(dibenzylideneacetone)dipalladium(0) (Pd_2dba_3) with triphenylarsine ($AsPh_3$) as ligand[9] at room temperature with the prolonged reaction time (overnight). Oxidation of alcohols **9** with tetra-*n*-propyl ammonium per-ruthenate (TPAP)/*N*-methyl morpholine *N*-oxide (NMO)[10] afforded the trienals **10** in good yields. The Horner–Emmons reaction of **10** with C5-phosphonate was carried out using *n*-BuLi as a base to



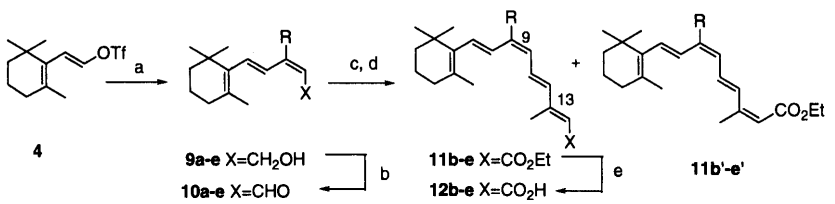
1 all-*E*-Retinoic acid 2 9*Z*-Retinoic Acid

Chart 1



Reagents: a) *t*-BuOK, Tf_2NPh / THF, 0°C; b) $Pd(PPh_3)_4$, **6a** / DMF, r.t.; c) $LiAlH_4$ then Bu_3SnOMe / THF, 0°C; d) MnO_2 / CH_2Cl_2, r.t.; e) $(EtO)_2P(O)CH_2C(Me)=CHCO_2Et$, *n*-BuLi / THF, 0°C

Chart 2



Reagents: a) $Pd(PPh_3)_4$, **6** / DMF, r.t.; b) TPAP, NMO / CH_2Cl_2, r.t.; c) $(EtO)_2P(O)CH_2C(Me)=CHCO_2Et$, *n*-BuLi / THF, 0°C; d) prep. HPLC; e) 10% KOH-EtOH, 50°C

Chart 3

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Table 1. Yields in the Synthesis of 9-Substituted 9*Z*-Retinoic Acids

Run	Substituent R	Yield of **9** (%)[a]	Yield of **10** (%)	Yield of **11** (%, 13*E*/13*Z*)	Yield of **12** (%)
1	Me	87	72	—[b]	—[b]
2	Et	88	87	80/16	95
3	Pr	87	75	53/23	Quant.
4	Bu	83	71	70/21	Quant.
5	Ph[c]	43 (Quant.)[d]	71	52/12	91

a) 10 mol% of $Pd(PPh_3)_4$ in DMF at r.t. for 2 h. *b*) Ref. 8. *c*) The stereochemistry at 9 position is *E*. *d*) Pd_2dba_3, $AsPh_3$ in DMF at r.t. for 14 h.

give esters **11** as a mixture of double bond isomers [13*E*:13*Z*=*ca.* 3:2—5:1]. After separation of the 9*Z*,13*E*-isomers by preparative high-performance liquid chromatography (HPLC), these compounds were transformed to the corresponding acids **12** by basic hydrolysis in excellent yields.

In summary, we have developed a novel method for the stereoselective synthesis of trisubstituted *Z*-olefin using the palladium catalyzed coupling reaction of a vinyl triflate with alkenyl stannanes and have achieved the synthesis of 9-substituted 9*Z*-retinoic acids **12**.[11] Biological investigations with these compounds are ongoing.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. UV spectra were recorded on a JASCO Ubest-55 instrument and IR spectra on a Perkin-Elmer FT-IR Paragon 1000 spectrometer. ^{1}H-NMR spectra were obtained on a Varian Gemini-200 or Gemini-300 NMR spectrometer. Mass spectra were determined on a Hitachi M-4100 instrument. Column chromatography (CC) under aspirator pressure (*ca.* 30 mmHg) was performed using Merck Silica gel 60. Preparative HPLC was conducted on a Shimadzu LC-6A instrument with a Shimadzu UV-VIS detector, SPD-6AV, using a LiChrosorb Si-60 column (5 μm), 1.0×30 cm. All reactions were carried out under a nitrogen atmosphere. THF and ether were purified by distillation from benzophenone–sodium ketyl under nitrogen. Materials obtained from commercial suppliers were used without further purification except when otherwise noted. Diisopropylamine was purified by distillation from CaH_2. Standard workup means that the organic layers were finally washed with brine, dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and concentrated *in vacuo* below 30 °C using a rotary evaporator.

(*E*)-2-(2,6,6-Trimethylcyclohexen-1-yl)-ethenyl (trifluoromethyl)sulfonate (4) A solution of the aldehyde (**3**, 1.01 g, 6.1 mmol)[6] in THF (12 ml) was added to a stirred solution of *tert*-BuOK (2 g, 9.0 mmol) in THF (10 ml) at 0 °C. The mixture was stirred for 15 min at 0 °C, and *N*-phenyl trifluoromethanesulfonimide (2.6 g, 4.2 mmol) was added at the same temperature. After stirring for an additional 30 min at room temperature, the reaction was quenched with saturated aqueous NH_4Cl (30 ml) and extracted with Et_2O (50 ml×3), followed by standard work up. The residue was purified by CC (ether : hexane=1 : 4 as eluent) to give the enol triflate (**4**, 1.03 g, 58%) as a colorless oil. UV λ_{max} (EtOH) nm: 235; IR ($CHCl_3$) cm^{-1}: 2934, 2868, 1645, 1423, 1215, 1110; ^{1}H-NMR (300 MHz) δ: 0.98 (6H, s), 1.43—1.66 (4H, m), 1.69 (3H, s), 2.02 (2H, br t, J=6 Hz), 6.22 (1H, d, J=12 Hz), 6.41 (1H, d, J=12 Hz); HR-MS *m/z*: 298.1944 (Calcd for $C_{20}H_{26}O_2$: 298.1944).

General Procedure for the Preparation of 3-Substituted-3-tributylstannyl-prop-2-en-1-ols (6a—e) NaOMe (0.16 g, 0.19 mmol) was added to a solution of $LiAlH_4$ (1.0 mol THF solution, 43.5 ml, 43.5 mmol) at 0 °C. The mixture was cooled to 0 °C and 3-substituted prop-2-en-1-ol (20 mmol) in THF (5 ml) was slowly added. The resulting mixture was stirred 1 h at 0 °C, then EtOAc (7.3 ml) was added and the mixture stirred for 15 min at room temperature. The reaction mixture was then cooled to 0 °C, Bu_3SnOMe (11.5 ml, 39.8 mmol) was added and the resulting mixture was left at room temperature. After 2 d, MeOH (30 ml) was added and the mixture was stirred for 1 h at room temperature and poured into H_2O (700 ml). The mixture was carefully made acidic by addition of dilute HCl and the product was extracted with Et_2O (80 ml×3), followed by standard work up. The residue was purified by CC (ether : hexane=1 : 4 as eluent) to give stannyl alcohol (**6**) as a colorless oil.

(2*Z*)-3-Tributylstannyl-2-buten-1-ol (**6a**): Prepared from 2-butyn-1-ol (**5a**, 1.68 g, 24 mmol) in 79% yield (6.58 g) as a colorless oil. The spectral data of this compound were identical with those in the literature.[7]

(2*Z*)-3-Tributylstannyl-2-penten-1-ol (**6b**): Prepared from 2-pentyn-1-ol (**5b**, 1.63 g, 19 mmol) in 62% yield (4.49 g) as a colorless oil. IR ($CHCl_3$) cm^{-1}: 3607, 3453, 2958, 2927, 1463, 1377; ^{1}H-NMR (300 MHz) δ: 0.88—1.59 (30H, m), 1.15 (1H, t, J=6 Hz), 2.24 (2H, q, J=7.5 Hz), 4.05 (2H, m), 6.25 (1H, t, J=6.5 Hz, $^3J_{Sn-H,trans}$=127 Hz). HR-MS *m/z*: 376.1767 (Calcd for $C_{17}H_{36}OSn$: 376.1786).

(2*Z*)-3-Tributylstannyl-2-hexen-1-ol (**6c**): Prepared from 2-hexyn-1-ol (**5c**, 1.91 g, 20 mmol) in 78% yield (5.92 g) as a colorless oil. IR ($CHCl_3$) cm^{-1}: 3608, 3448, 2958, 2927, 1463, 1377; ^{1}H-NMR (300 MHz) δ: 0.87—1.59 (32H, m), 1.14 (1H, t, J=5.5 Hz), 2.19 (2H, t, J=7.5 Hz), 4.05 (2H, m), 6.23 (1H, t, J=6.5 Hz, $^3J_{Sn-H,trans}$=126 Hz); HR-MS *m/z*: 390.1917 (Calcd for $C_{18}H_{38}OSn$: 390.1942).

(2*Z*)-3-Tributylstannyl-2-hepten-1-ol (**6d**): Prepared from 2-heptyn-1-ol (**5c**, 2.25 g, 20 mmol) in 74% yield (5.99 g) as a colorless oil. IR ($CHCl_3$) cm^{-1}: 3608, 3451, 2958, 2928, 1464, 1378; ^{1}H-NMR (300 MHz) δ: 0.88—1.59 (34H, m), 1.15 (1H, t, J=6.5 Hz), 2.20 (2H, t, J=7 Hz), 4.05 (2H, m), 6.22 (1H, t, J=6.5 Hz, $^3J_{Sn-H,trans}$=129 Hz); HR-MS *m/z*: 404.2074 (Calcd for $C_{19}H_{40}OSn$: 404.2099).

(2*Z*)-3-Tributylstannyl-3-phenyl-2-buten-1-ol (**6e**): Prepared from 3-phenyl-2-butyn-1-ol (**5e**, 1.42 g, 11 mmol) in 80% yield (3.65 g) as a colorless oil. The spectral data were identical with those in the literature.[7]

General Procedure for the Preparation of 9*Z*-Aldehyde (9a—e) To a stirred solution of triflate (**4**, 200 mg, 0.67 mmol) in DMF (2 ml) was added $Pd(PPh_3)_4$ (40 mg, 0.034 mmol, 0.05 eq) at room temperature under nitrogen. After 10 min, a solution of tributylstannyl olefin (**5**, 300—400 mg, *ca.* 1 mmol, 1.5 eq) in DMF (2 ml) was added and the resulting mixture was stirred for 20 min. The reaction was quenched with saturated aqueous NaCl (5 ml) and extracted with Et_2O (10 ml×3), followed by the standard work up. The residue was purified by CC (ether : hexane=1 : 4 to 3 : 7 as an eluent) to give the coupled alcohol (**9**). The alcohol (**9**, 0.5 mmol) was dissolved in CH_2Cl_2 (5 ml) containing 4 Å sieves and NMO (0.1 ml, 0.75 mmol). After stirring the mixture for 10 min, TPAP (0.025 mmol) was added and the reaction followed by TLC until complete. When complete, the mixture was diluted with CH_2Cl_2 (20 ml) and washed with sodium sulphite solution (10 ml), brine (10 ml) and finally saturated copper(II) sulphate solution (10 ml). The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by CC (ether : hexane=1 : 4 as eluent) to give the aldehyde (**9**) as a pale yellow oil.

(2*Z*)-3-Methyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4-pentadienal (**9a**): Prepared from triflate (**4**, 170 mg, 0.57 mmol) and stannylolefin (**6a**, 320 mg, 0.9 mmol) in 63% yield (79 mg, 2 steps) as a colorless oil. The spectral data were identical with those in the literature.[8]

(2*Z*)-3-Ethyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4-pentadienal (**9b**): Prepared from triflate (**4**, 98 mg, 0.33 mmol) and stannylolefin (**6b**, 188 mg, 0.5 mmol) in 76% yield (59 mg, 2 steps) as a colorless oil. UV λ_{max} (EtOH) nm: 326sh, 282; IR ($CHCl_3$) cm^{-1}: 2933, 1655, 1607; ^{1}H-NMR (300 MHz) δ: 1.05 (6H, s), 1.19 (3H, d, J=7.5 Hz), 1.46—1.69 (4H, m), 1.75 (3H, s), 2.06 (2H, br t, J=6 Hz), 2.48 (2H, q, J=7.5 Hz), 5.89 (1H, d, J=8 Hz), 6.61 (1H, d, J=16 Hz), 6.89 (1H, d, J=16 Hz), 10.15 (1H, d, J=8 Hz); HR-MS *m/z*: 232.1819 (Calcd for $C_{16}H_{24}O$: 232.1825).

(2*Z*)-3-Propyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4-pentadienal (**9c**): Prepared from triflate (**4**, 105 mg, 0.35 mmol) and stannylolefin (**6c**, 195 mg, 0.5 mmol) in 65% yield (56 mg, 2 steps) as a colorless oil. UV λ_{max} (EtOH) nm: 321sh, 279.5; IR ($CHCl_3$) cm^{-1}: 2934, 1659, 1612; ^{1}H-NMR (300 MHz) δ: 0.97 (3H, t, J=7.5 Hz), 1.04 (6H, s), 1.46—1.69 (6H, m), 1.73 (3H, s), 2.04 (2H, br t, J=6 Hz), 2.39 (2H, t, J=7 Hz), 5.86 (1H, d, J=8 Hz), 6.58 (1H, d, J=16 Hz), 6.85 (1H, d, J=16 Hz), 10.11 (1H, d, J=8 Hz); HR-MS *m/z*: 246.1987 (Calcd for $C_{17}H_{26}O$: 246.1983).

(2*Z*)-3-Butyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4-pentadienal (**9d**): Prepared from triflate (**4**, 122 mg, 0.41 mmol) and stannylolefin (**6d**, 227 mg,

0.56 mmol) in 59% yield (62 mg, 2 steps) as a colorless oil. UV λ_{max} (EtOH) nm: 319, 267; IR ($CHCl_3$) cm^{-1}: 2959, 1657, 1611; ^{1}H-NMR (300 MHz) δ: 0.95 (3H, t, J=7.5 Hz), 1.06 (6H, s), 1.48—1.65 (8H, m), 1.75 (3H, s), 2.06 (2H, br t, J=6 Hz), 2.43 (2H, t, J=7.5 Hz), 5.89 (1H, d, J=8 Hz), 6.60 (1H, d, J=16 Hz), 6.87 (1H, d, J=16 Hz), 10.11 (1H, d, J=8 Hz); HR-MS *m/z*: 260.2123 (Calcd for $C_{18}H_{28}O$: 260.2139).

(2*E*)-3-Phenyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4-pentadienal (**9e**): Prepared from triflate (**4**, 93 mg, 0.31 mmol) and stannylolefin (**6e**, 213 mg, 0.5 mmol) in 31% yield (27 mg, 2 steps) as a colorless oil.

UV λ_{max} (EtOH) nm: 299.5; IR ($CHCl_3$) cm^{-1}: 2936, 1660, 1614; ^{1}H-NMR (300 MHz) δ: 1.04 (6H, s), 1.45—1.70 (4H, m), 1.79 (3H, s), 2.06 (2H, br t, J=6.5 Hz), 6.14 (1H, d, J=8 Hz), 6.45 (2H, d, J=16 Hz), 6.93 (1H, d, J=16 Hz), 7.43 (5H, br s), 10.14 (1H, d, J=8 Hz); HR-MS *m/z*: 280.1831 (Calcd for $C_{20}H_{24}O$: 280.1826).

General Procedure for the Preparation of Ethyl 9-Substituted Retinoate (11b—e) To a solution of diethyl 3-(methoxycarbonyl)-2-methyl-2-propenylphosphonate (E:Z=4:1) (391 mg, 2 mmol) in THF (5.5 ml) was added *n*-BuLi (1.6 M hexane solution, 1.25 ml, 2 mmol) at 0 °C. After stirring for 30 min, a solution of the aldehyde (**10**, *ca.* 0.7 mmol) in THF (5 ml) was added. The resulting mixture was stirred for an additional 5 h. The reaction was quenched with saturated NH_4Cl (5 ml) and extracted with ether followed by standard workup. The residue was purified by preparative HPLC (ether : benzene : hexane=1 : 25 : 74) to give the respective pentaenyl esters (**11**) as pale yellow oils.

Ethyl (2*E*/*Z*,4*E*,6*Z*,8*E*)-7-Ethyl-3-methyl-9-(2,6,6-trimethylcyclohexen-1-yl)nona-2,4,6,8-tetraenoates (**11b**) These were prepared from the aldehyde (**10b**, 66 mg, 0.28 mmol) in 80% (**11b**, 56 mg) and 16% (**11b′**, 15 mg) yields, respectively.

2*E*,6*Z*-isomer **11b**: UV λ_{max} (EtOH) nm: 349; IR ($CHCl_3$) cm^{-1}: 2932, 1698, 1589; ^{1}H-NMR (300 MHz) δ: 1.04 (6H, s), 1.15 (3H, t, J=7 Hz), 1.29 (3H, t, J=7 Hz), 1.4—1.7 (4H, m), 1.75 (3H, s), 2.05 (2H, t, J=6.5 Hz), 2.33 (3H, s), 2.34 (2H, t, J=7 Hz), 4.17 (2H, q, J=7 Hz), 5.77 (1H, s), 6.06 (1H, d, J=11.5 Hz), 6.24 (1H, d, J=15 Hz), 6.30 (1H, d, J=16.5 Hz), 6.53 (1H, d, J=16.5 Hz), 7.08 (1H, dd, J=11.5, 15 Hz); HR-MS *m/z*: 342.2566 (Calcd for $C_{23}H_{34}O_2$: 342.2557).

2*Z*,6*Z*-isomer **11b′**: UV λ_{max} (EtOH) nm: 350.5; IR ($CHCl_3$) cm^{-1}: 2931, 1696, 1601; ^{1}H-NMR (300 MHz) δ: 1.03 (6H, s), 1.14 (3H, t, J=7 Hz), 1.30 (3H, t, J=7 Hz), 1.4—1.7 (4H, m), 1.75 (3H, s), 2.04 (2H, t, J=6.5 Hz), 2.06 (3H, s), 2.34 (2H, t, J=7 Hz), 4.16 (2H, q, J=7 Hz), 5.64 (1H, s), 6.19 (1H, d, J=12 Hz), 6.27 (1H, d, J=16 Hz), 6.53 (1H, d, J=16 Hz), 7.03 (1H, dd, J=12, 15 Hz), 7.73 (1H, d, J=15 Hz); HR-MS *m/z*: 342.2563 (Calcd for $C_{23}H_{34}O_2$: 342.2557).

Ethyl (2*E*/*Z*,4*E*,6*Z*,8*E*)-3-Methyl-9-(2,6,6-trimethylcyclohexen-1-yl)-7-propylnona-2,4,6,8-tetraenoates (**11c**): These were prepared from the aldehyde (**10c**, 160 mg, 0.65 mmol) in 53% (**11c**, 122 mg) and 22% (**11c′**, 52 mg) yields, respectively.

2*E*,6*Z*-isomer **11c**: UV λ_{max} (EtOH) nm: 349; IR ($CHCl_3$) cm^{-1}: 2930, 1696, 1601; ^{1}H-NMR (300 MHz) δ: 0.94 (3H, t, J=7 Hz), 1.03 (6H, s), 1.28 (3H, t, J=7 Hz), 1.4—1.7 (6H, m), 1.73 (3H, s), 2.04 (2H, t, J=6.5 Hz), 2.31 (2H, t, J=7 Hz), 2.33 (3H, s), 4.16 (2H, q, J=7 Hz), 5.76 (1H, s), 6.04 (1H, d, J=11 Hz), 6.23 (1H, d, J=15 Hz), 6.28 (1H, d, J=16 Hz), 6.51 (1H, d, J=16 Hz), 7.07 (1H, dd, J=11, 15 Hz); HR-MS *m/z*: 356.2698 (Calcd for $C_{24}H_{36}O_2$: 356.2713).

2*Z*,6*Z*-isomer **11c′**: UV λ_{max} (EtOH) nm: 351.5; IR ($CHCl_3$) cm^{-1}: 2932, 1698, 1601; ^{1}H-NMR (300 MHz) δ: 0.90 (3H, t, J=7 Hz), 1.04 (6H, s), 1.28 (3H, t, J=7 Hz), 1.4—1.7 (6H, m), 1.74 (3H, s), 2.04 (2H, t, J=6.5 Hz), 2.05 (3H, s), 2.31 (2H, t, J=7 Hz), 4.16 (2H, q, J=7 Hz), 5.63 (1H, s), 6.15 (1H, d, J=12 Hz), 6.28 (1H, d, J=15 Hz), 6.51 (1H, d, J=16 Hz), 7.07 (1H, dd, J=11, 15 Hz), 7.73 (1H, d, J=16 Hz); HR-MS *m/z*: 356.2712 (Calcd for $C_{24}H_{36}O_2$: 356.2713).

Ethyl (2*E*/*Z*,4*E*,6*Z*,8*E*)-7-Butyl-3-methyl-9-(2,6,6-trimethylcyclohexen-1-yl)nona-2,4,6,8-tetraenoates (**11d**): These were prepared from the aldehyde (**10d**, 98 mg, 0.38 mmol) in 70% (**11d**, 97 mg) and 21% (**11d′**, 29 mg) yields, respectively.

2*E*,6*Z*-isomer **11d**: UV λ_{max} (EtOH) nm: 350, 244.5; IR ($CHCl_3$) cm^{-1}: 2932, 1699, 1602; ^{1}H-NMR (300 MHz) δ: 0.94 (3H, t, J=7 Hz), 1.05 (6H, s), 1.30 (3H, t, J=7 Hz), 1.4—1.7 (8H, m), 1.65 (3H, s), 2.06 (2H, t, J=6 Hz), 2.34 (2H, t, J=7 Hz), 2.35 (3H, s), 4.18 (2H, q, J=7 Hz), 5.77 (1H, s), 6.05 (1H, d, J=11.5 Hz), 6.24 (1H, d, J=15 Hz), 6.29 (1H, d, J=16 Hz), 6.52 (1H, d, J=16 Hz), 7.07 (1H, dd, J=11.5, 15 Hz); HR-MS *m/z*: 370.2883 (Calcd for $C_{25}H_{38}O_2$: 370.2870).

2*Z*,6*Z*-isomer **11d′**: UV λ_{max} (EtOH) nm: 350, 244.5; IR ($CHCl_3$) cm^{-1}: 2933, 1698, 1589; ^{1}H-NMR (300 MHz) δ: 0.90 (3H, t, J=7 Hz), 1.05 (6H, s), 1.29 (3H, t, J=7 Hz), 1.4—1.7 (8H, m), 1.75 (3H, s), 2.04 (2H, t, J=6.5 Hz), 2.05 (3H, s), 2.34 (2H, t, J=7 Hz), 4.17 (2H, q, J=7 Hz), 5.64 (1H, s), 6.15 (1H, d, J=11 Hz), 6.28 (1H, d, J=16 Hz), 6.51 (1H, d, J=16 Hz), 7.07 (1H, dd, J=11, 15 Hz), 7.73 (1H, d, J=15 Hz); HR-MS *m/z*: 370.2884 (Calcd for $C_{25}H_{38}O_2$: 370.2870).

Ethyl (2*E*/*Z*,4*E*,6*E*,8*E*)-3-Methyl-9-(2,6,6-trimethylcyclohexen-1-yl)-7-phenylnona-2,4,6,8-tetraenoates (**11e**): These were prepared from the aldehyde (**10e**, 69 mg, 0.25 mmol) in 52% (**11e**, 49 mg) and 12% (**11e′**, 12 mg) yields, respectively.

2*E*,6*E*-isomer **11e**: UV λ_{max} (EtOH) nm: 360, 286, 246; IR ($CHCl_3$) cm^{-1}: 2933, 1698, 1606; ^{1}H-NMR (300 MHz) δ: 1.02 (6H, s), 1.30 (3H, t, J=7 Hz), 1.4—1.7 (4H, m), 1.81 (3H, s), 2.05 (2H, t, J=6 Hz), 2.38 (3H, s), 4.19 (2H, q, J=7 Hz), 5.81 (1H, s), 6.18 (1H, d, J=16 Hz), 6.26 (1H, d, J=11.5 Hz), 6.37 (1H, d, J=15 Hz), 6.68 (1H, d, J=16 Hz), 7.19 (1H, dd, J=11.5, 15 Hz); HR-MS *m/z*: 390.2566 (Calcd for $C_{27}H_{34}O_2$: 390.2557).

2*Z*,6*E*-isomer **11e′**: UV λ_{max} (EtOH) nm: 362, 291, 248; IR ($CHCl_3$) cm^{-1}: 2930, 1670, 1602; ^{1}H-NMR (300 MHz) δ: 1.00 (6H, s), 1.29 (3H, t, J=7 Hz), 1.4—1.7 (4H, m), 1.79 (3H, s), 2.05 (2H, t, J=6.5 Hz), 2.09 (3H, s), 4.17 (2H, q, J=7 Hz), 5.68 (1H, s), 6.18 (1H, d, J=16 Hz), 6.39 (1H, d, J=11 Hz), 6.65 (1H, d, J=16 Hz), 7.17 (1H, dd, J=11, 15 Hz), 7.73 (1H, d, J=15 Hz); HR-MS *m/z*: 390.2563 (Calcd for $C_{27}H_{34}O_2$: 390.2557).

General Procedure for the Preparation of 9*Z*-Retinoic Acid Analogs (12b—e) A mixture of the ester (**11**, *ca.* 40 mg, 0.1 mmol) and 25% NaOH solution (5 ml) in methanol (5 ml) was heated at 50 °C for 30 min. After cooling, the reaction mixture was made acidic with 5% HCl, and the organics were extracted with ethyl acetate followed by standard workup. The residue was purified by CC (ethyl acetate : hexane=3 : 1 as eluent) to give the acid (**12**, 23 mg, 98%) as a yellow solid.

(2*E*,4*E*,6*Z*,8*E*)-7-Ethyl-3-methyl-9-(2,6,6-trimethylcyclohexen-1-yl)nona-2,4,6,8-tetraenoic Acid (**12b**): Prepared from the ester (**11b**, 43 mg, 0.13 mmol) in 95% (38 mg) yield. mp 125—127 °C (ether–*n*-hexane); UV λ_{max} (EtOH) nm: 339; IR ($CHCl_3$) cm^{-1}: 3300—2600, 1681, 1586; ^{1}H-NMR (300 MHz) δ: 1.04 (6H, s), 1.15 (3H, t, J=7.5 Hz), 1.4—1.7 (4H, m), 1.75 (3H, s), 2.05 (2H, t, J=7 Hz), 2.35 (3H, s), 2.38 (2H, t, J=7.5 Hz), 5.79 (1H, s), 6.07 (1H, d, J=12 Hz), 6.27 (1H, d, J=16 Hz), 6.32 (1H, d, J=15 Hz), 6.53 (1H, d, J=16 Hz), 7.13 (1H, dd, J=12, 15 Hz); a COOH signal was not present; HR-MS *m/z*: 314.2251 (Calcd for $C_{21}H_{30}O_2$: 314.2244).

(2*E*,4*E*,6*Z*,8*E*)-3-Methyl-9-(2,6,6-trimethylcyclohexen-1-yl)-7-propylnona-2,4,6,8-tetraenoic Acid (**12c**): Prepared from the ester (**11c**, 58 mg, 0.16 mmol) in quantitative (53 mg) yield. mp 125—127 °C (ether–*n*-hexane); UV λ_{max} (EtOH) nm: 340; IR ($CHCl_3$) cm^{-1}: 3350—2600, 1679, 1586; ^{1}H-NMR (300 MHz) δ: 0.94 (3H, t, J=7 Hz), 1.03 (6H, s), 1.4—1.7 (6H, m), 1.74 (3H, s), 2.04 (2H, t, J=6.5 Hz), 2.32 (2H, t, J=7 Hz), 2.34 (3H, s), 5.79 (1H, s), 6.05 (1H, d, J=11 Hz), 6.25 (1H, d, J=15 Hz), 6.29 (1H, d, J=16 Hz), 6.51 (1H, d, J=16 Hz), 7.12 (1H, dd, J=11, 15 Hz); a COOH signal was not present; HR-MS *m/z*: 328.2416 (Calcd for $C_{22}H_{32}O_2$: 328.2401).

(2*E*,4*E*,6*Z*,8*E*)-7-Butyl-3-methyl-9-(2,6,6-trimethylcyclohexen-1-yl)nona-2,4,6,8-tetraenoic Acid (**12d**): Prepared from the ester (**10d**, 23 mg, 0.06 mmol) in quantitative (21 mg) yield. mp 135—136 °C (ether–*n*-hexane); UV λ_{max} (EtOH) nm: 345.5; IR ($CHCl_3$) cm^{-1}: 3300—2600, 1678, 1585; ^{1}H-NMR (300 MHz) δ: 0.94 (3H, t, J=7 Hz), 1.04 (6H, s), 1.4—1.7 (8H, m), 1.76 (3H, s), 2.06 (2H, t, J=6 Hz), 2.34 (2H, t, J=7 Hz), 2.35 (3H, s), 5.80 (1H, s), 6.06 (1H, d, J=11.5 Hz), 6.27 (1H, d, J=15 Hz), 6.31 (1H, d, J=16 Hz), 6.52 (1H, d, J=16 Hz), 7.14 (1H, dd, J=11.5, 15 Hz); a COOH signal was not present; HR-MS *m/z*: 342.2572 (Calcd for $C_{23}H_{34}O_2$: 342.2557).

(2*E*,4*E*,6*E*,8*E*)-3-Methyl-9-(2,6,6-trimethylcyclohexen-1-yl)-7-phenylnona-2,4,6,8-tetraenoic Acid (**12e**): Prepared from the ester (**11e**, 23 mg, 0.06 mmol) in 91% (20 mg) yield. mp 148—150 °C (ether–*n*-hexane); UV λ_{max} (EtOH) nm: 339; IR ($CHCl_3$) cm^{-1}: 3300—2600, 1681, 1603, 1583; ^{1}H-NMR (300 MHz) δ: 1.00 (6H, s), 1.4—1.7 (4H, m), 1.79 (3H, s), 2.05 (2H, t, J=6 Hz), 2.37 (3H, s), 5.70 (1H, s), 6.17 (1H, d, J=16 Hz), 6.27 (1H, d, J=11 Hz), 6.37 (1H, d, J=15 Hz), 6.67 (1H, d, J=16 Hz), 7.23 (1H, dd, J=11, 15 Hz); a COOH signal was not present; HR-MS *m/z*: 362.2260 (Calcd for $C_{25}H_{30}O_2$: 362.2244).

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11) According to nomenclature, **12e** is the 9*E*-isomer.

First Synthesis of a α-Trifluoromethyl Allenol Ether *via* the Julia-Lythgoe Process

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α-Trifluoromethyl allenol ethers 9a—e were prepared in moderate to good yields by the Julia-Lythgoe process using β-ethoxy-β-trifluoromethyl vinylic sulfone 3. Several reactions of 9c were examined to give α,β-unsaturated trifluoromethyl ketone derivatives 11 and 12.

Key words allene; fluoro enol ether; sulfone

Allenol ethers are important builiding blocks for the syntheses of a wide variety of synthetic precursors and natural products.[1] The transformation of allenol ethers using many kinds of organometalic reagents has been examined and provided propargylic,[2] allenic,[3] α,β-unsaturated carbonyl compounds,[4] vinyl stannanes[5] and various heterocyclic compounds.[6] Most synthetic approaches to allenol ethers have utilized the isomerization of propargylic ethers by appropriate bases.[7] Wittig-type olefination of carbonyl compounds is also known as a useful method for the preparation of allenol ethers.[8]

Recently, much attention has been focused on organofluorine compounds because of their interesting biological activities and chemical reactivities. In particular, the introduction of a trifluoromethyl group into organic molecules changes their properties due to its strong electron-withdrawing activity. However, no efforts have been described that uses the standard methods for the preparation of fluoroalkyl allenol ethers. Desulfonylation[9] and desulfinylation[10] has been used as an effective method for the formation of double bonds. In particular, the Julia-Lythgoe process involving β-acetoxy sulfones easily provides alkenyl componds by desulfonylation using SmI_2.[11] Sulfones such as shown in Fig. 1, should undergo elimination of the sulfonyl group and provide the expected fluoroalkyl allenol ethers, however, there are so far no reports of application of the Julia-Lythgoe process to allene formation. Here we report the synthesis of trifluoromethyl allenol ethers *via* the Julia-Lythgoe process of vinylic sulfones.

Preparation of the vinylic sulfones was examined as shown in Chart 1. The β-ethoxy vinylic sulfones **3** and **4** were easily obtained by mCPBA oxidation of the sulfides **1** and **2**. The sulfones were then treated with lithium 2,2,6,6-tetramethylpiperidide (LTMP) at −78 °C, followed by the successive reaction with the corresponding aldehydes to afford the allylic alcohols **5** and **6** in moderate to good yields. For preparation of allenes, we first performed the reaction of **5a** with SmI_2 at 0 °C, however, allene **9a** was obtained in low yield (Chart 2). Next, we examined reaction of the corresponding allylic acetate **7a** with SmI_2, and obtained the desired product **9a** in 73% yield. The structure of **9a** was determined by spectral data, which indicated on the allenic absorption at ν 1950 cm^{-1} in the IR spectrum, an allenic proton at δ 7.09 ppm in the ^{1}H-NMR spectrum, and a singlet carbon at δ 195.85 ppm in the ^{13}C-NMR spectrum. The reactions with other aldehydes were also examined, and the results are shown in Table 1. The *p*-chloro- and *p*-bromo-phenyl allenol

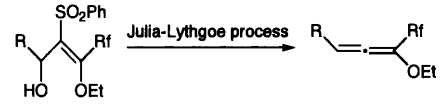


Fig. 1

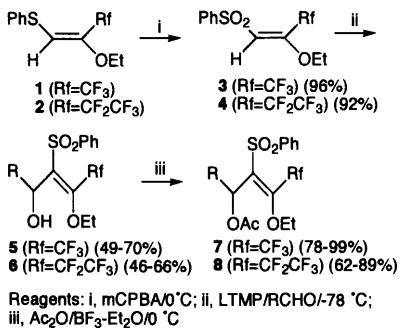


Chart 1

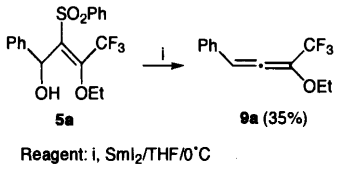
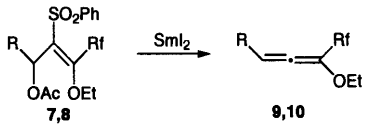


Chart 2

Table 1. Syntheses of Allenol Ethers



Entry		Vinylic sulfone		Product (% yield)
		Rf	R	
1	**7a**	CF_3	Ph	**9a** (73)
2	**7b**	CF_3	*p*-Br–Ph	**9b** (49)
3	**7c**	CF_3	*p*-Cl–Ph	**9c** (70)
4	**7d**	CF_3	Phenylethynyl	**9d** (37)
5	**7e**	CF_3	(*E*)-Styryl	**9e** (57)
6	**8a**	CF_3CF_2	Ph	**10a** (15)
7	**8b**	CF_3CF_2	(*E*)-Styryl	**10b** (7)

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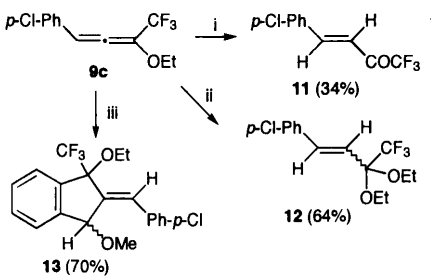

Reagent: i, HCl/EtOH-H_2O/reflux; ii, $CH(OEt)_3/BF_3$-Et_2O; iii, $PhCH(OMe)_2/BF_3$-Et_2O/$ClCH_2CH_2Cl$/0 °C

Chart 3

ethers **9b** and **9c** were readily obtained (entries 2 and 3). Phenylethynyl or cinnam-aldehydes also afforded the corresponding allenes **9d, e**, however, these compounds were found to be unstable (entries 4 and 5). The pentafluoroethyl sulfone gave low yields because they are more labile than the trifluoromethyl derivatives (entries 6 and 7).

In order to characterize the new trifluoromethyl allenol ethers, we examined some reactions of allenol ether **9c**, as shown in Chart 3. Hydrolysis of **9c** with 5% HCl gave the α,β-unsaturated trifluoromethyl ketone **11** in 34% yield. Reaction with ethyl orthoformate/BF_3–Et_2O gave the acetal **12** in good yield. The intermolecular reaction with benzaldehyde dimethyl acetal yielded the cyclized indene **13**, exclusively.

Experimental

^{1}H-, ^{13}C- and ^{19}F-NMR spectra were recorded using a Varian Inova400 spectrometer; chemical shifts are reported in ppm using $CDCl_3$ as solvent and tetramethylsilane as internal standard. ^{19}F-NMR are reported in ppm using CF_3CO_2H as an external standard. IR spectra were obtained on a JASCO IRA-100. Elemental analyses were performed at the Microanalytical Laboratory of Gifu Pharmaceutical University. Melting points were determined on a Yanaco micromelting point apparatus and are uncorrected. Electron impact (EI) mass spectra was obtained using a Shimadzu QP-1000 spectrometer with a direct insertion probe at an ionization voltage of 70 eV. The enol ethers **1** and **2** were prepared as in our previous report.[12] The allenol ethers **9** and **10** were too labile to measure the mass spectral data.

Synthesis of (*E*)-2-Ethoxy-1,1,1-trifluoro-3-(phenylsulfonyl)prop-2-ene (3) and (*E*)-3-Ethoxy-1,1,1,2,2-pentafluoro-4-(phenylsulfonyl)but-3-ene (4). Typical Procedure mCPBA (1.92 g, 8.80 mmol) was added to a $ClCH_2CH_2Cl$ (28 ml) solution of the vinyl sulfide **1** (7.45 g, 26.6 mmol) at 0 °C. The mixture was stirred for 1 h at room temperature and poured into $CHCl_3$ (100 ml). The $CHCl_3$ solution was washed with saturated $NaHCO_3$ (50×3 ml) solution. The organic layer was dried over $MgSO_4$ and the solvent removed under reduced pressure. The residue was purified by column chromatography on silica gel eluting with AcOEt–hexane (1 : 10) to give the title compound **3** (1.02 g, 96%) as a colorless oil.

3: IR (film) cm^{-1}: 1300, 1200—1100 (SO_2). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.33 (3H, t, J=7 Hz, Me), 4.21—4.26 (2H, m, OCH_2), 6.44 (1H, s, olefinic H), 7.55—7.59 (3H, m, ArH), 7.98—8.00 (2H, m, ArH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.03 (q), 71.22 (t), 116.95 (d, J=4 Hz, 3-C), 118.96 (s, J=280 Hz, CF_3), 127.98 (d×2), 129.24 (d×2), 134.07 (d), 141.06 (s), 150.61 (s, J=34 Hz, 2-C). ^{19}F-NMR δ: −8.04 (3F, s, CF_3); MS *m/z*: 280 (M^+). *Anal.* Calcd for $C_{11}H_{11}F_3O_3S$: C, 47.14; H, 3.96. Found: C, 47.26; H, 3.93.

4: IR (film) cm^{-1}: 1320, 1260—1080 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.40 (3H, t, J=7 Hz, Me), 4.46 (2H, q, J=7 Hz, OCH_2), 6.41 (1H, s, olefinic H), 7.57—7.61 (2H, m, ArH), 7.66—7.70 (1H, m, ArH), 7.95—7.98 (2H, m, ArH); ^{19}F-NMR δ: −40.47 (2F, s, CF_2), −4.91 (3F, s, CF_3). *Anal.* Calcd for $C_{12}H_{11}F_5O_3S$: C, 43.64; H, 3.36. Found: C, 43.72; H, 3.38. The molecular ion peak at *m/z* 330 (M^+) was not observed in the mass spectrum.

Preparations of Allylic Alcohols 5 and 6. Typical Procedure A THF (3.00 ml) solution of 2-ethoxy-1,1,1-trifluoro-3-(phenylsulfonyl)prop-2-ene (**3**) (0.51 g, 1.81 mmol) was added dropwise to a THF (6.00 ml) solution of lithium 2,2,6,6-tetramethylpiperidide (prepared from 2,2,6,6-tetramethylpiperidine (0.50 g, 3.54 mmol) and *n*-BuLi (1.80 ml, 2.68 mmol)) at −78 °C. The mixture was stirred for 10 min and a THF (3.00 ml) solution of benzaldehyde (0.38 g, 3.57 mmol) was added. The whole was then poured into water (100 ml) and the organic layer separated and the aqueous layer extracted with ether. The combined organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by preparative TLC on silica gel eluting with AcOEt–hexane (1 : 20) to give (*E*)-2-ethoxy-1,1,1-trifluoro-4-phenyl-3-(phenylsulfonyl)but-2-en-4-ol (0.41 g, 61%) as a yellow oil.

5a: IR (film) cm^{-1}: 3500 (OH), 1310, 1180—1120 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.45 (3H, t, J=7 Hz, Me), 4.13—4.22 (2H, m, OCH_2), 4.55 (1H, d, J=12 Hz, OH), 6.00 (1H, d, J=12 Hz, 4-H), 7.31—7.38 (7H, m, ArH), 7.49—7.56 (3H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.19 (q), 69.84 (t, J=4 Hz), 72.36 (d, 4-C), 121.02 (s, J=284 Hz, CF_3), 125.72 (d×2), 127.90 (d), 128.19 (d×2), 128.64 (d×2), 128.70 (d×2), 133.89 (d), 138.24 (s, J=2 Hz), 139.63 (s), 141.12 (s), 150.59 (s, J=34 Hz, 2-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −17.37 (3F, s, CF_3); MS *m/z* 357 (M^+−Et). *Anal.* Calcd for $C_{18}H_{17}F_3O_4S$: C, 55.95; H, 4.43. Found: C, 55.63; H, 4.39.

(*E*)-4-(*p*-Bromophenyl)-2-ethoxy-1,1,1-trifluoro-3-(phenylsulfonyl)but-2-en-4-ol (**5b**): mp 131—134 °C (dec.), IR (KBr) cm^{-1}: 3500—3400 (OH), 1270, 1200—1060 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.43 (3H, t, J=7 Hz, Me), 4.10—4.15 (1H, m, OCH_2), 4.17—4.23 (1H, m, OCH_2), 4.52 (1H, d, J=12 Hz, OH), 5.60 (1H, d, J=12 Hz, 4-H), 7.22—7.61 (9H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.23 (q), 69.49 (d, 4-C), 72.60 (t), 120.19 (s, J=284 Hz, CF_3), 122.11 (s), 127.64 (d×2), 128.10 (d×2), 128.91 (d×2), 131.79 (d×2), 134.11 (d), 137.77 (s), 138.94 (s), 141.15 (s), 151.92 (s, 2-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −17.34 (3F, s, CF_3); MS *m/z* 309 (M^+−*p*-Br–Ph). *Anal.* Calcd for $C_{18}H_{16}BrF_3O_4S$: C, 46.47; H, 3.47. Found: C, 46.42; H, 3.50.

(*E*)-4-(*p*-Chlorophenyl)-2-ethoxy-1,1,1-trifluoro-3-(phenylsulfonyl)but-2-en-4-ol (**5c**): mp 98—100 °C (dec.), IR (KBr) cm^{-1}: 3504 (OH), 1327, 1189 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.43 (3H, t, J=7 Hz, Me), 4.11—4.22 (2H, m, OCH_2), 4.52 (1H, d, J=12 Hz, OH), 5.92 (1H, d, J=12 Hz, 4-H), 7.32—7.25 (4H, m, ArH) 7.35—7.44 (2H, m, ArH), 7.60—7.55 (3H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.22 (q), 69.45 (d), 72.56 (t), 120.17 (s, J=284 Hz, CF_3), 127.28 (d×2), 128.08 (d×2), 128.82 (d×2), 128.88 (d×2), 133.96 (s), 134.08 (d), 137.82 (s), 138.39 (s), 141.17 (s), 150.93 (s, J=34 Hz, 2-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −17.81 (3F, s, CF_3); MS *m/z* 280 (M^+−$PhSO_2$). *Anal.* Calcd for $C_{18}H_{16}ClF_3O_4S$: C, 51.37; H, 3.83. Found: C, 51.15; H, 3.70.

(*E*)-2-Ethoxy-1,1,1-trifluoro-6-phenyl-3-(phenylsulfonyl)hex-2-en-5-yn-4-ol (**6d**): IR (film) cm^{-1}: 3480 (OH), 1330—1260 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.39 (3H, t, J=7 Hz, Me), 4.10 (2H, q, J=7 Hz, OCH_2), 4.52 (1H, d, J=11 Hz, OH), 5.71 (1H, d, J=11 Hz, 4-H), 7.26—7.64 (8H, m, ArH), 8.10—8.13 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.12 (q), 59.11 (d, J=5 Hz, 4-C), 72.22 (t), 86.34 (s), 87.24 (s), 122.01 (s), 122.67 (s, J=283 Hz, CF_3), 124.64 (s), 128.42 (d×2), 128.58 (d×2), 128.92 (d×2), 129.23 (d), 131.99 (d×2), 134.08 (d), 141.11 (s), 149.65 (s, J=35 Hz, 2-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −16.97 (3F, s, CF_3); MS *m/z* 410 (M^+). *Anal.* Calcd for $C_{20}H_{17}F_3O_4S$: C, 58.53; H, 4.18. Found: C, 58.64; H, 4.16.

(2*E*,5*E*)-2-Ethoxy-1,1,1-trifluoro-6-phenyl-3-(phenylsulfonyl)hex-2,5-dien-4-ol (**5f**): IR (film) cm^{-1}: 3500—3450 (OH), 1310, 1200—1100 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.36 (3H, t, J=7 Hz, Me), 4.00—4.14 (2H, m, OCH_2), 4.48 (1H, d, J=11 Hz, OH), 5.49 (1H, dd, J=6, 11 Hz, 4-H), 6.52 (1H, dd, J=6, 16 Hz, 5-H), 6.71 (1H, d, J=16 Hz, 6-H), 7.63—7.25 (8H, m, ArH), 7.93—7.98 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.09 (q), 69.60 (d, J=3 Hz, 4-C), 72.14 (t), 122.67 (s, J=284 Hz, CF_3), 127.00 (d×2), 128.08 (d), 128.17 (d×2), 128.46 (d), 128.92 (d×2), 129.23 (d), 131.99 (d×2), 134.08 (d), 141.11 (s), 149.65 (s, J=35 Hz, 2-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −16.97 (3F, s, CF_3); MS *m/z* 410 (M^+). *Anal.* Calcd for $C_{20}H_{19}F_3O_4S$: C, 58.53; H, 4.18. Found: C, 58.64; H, 4.16.

(*E*)-3-Ethoxy-1,1,1,2,2-pentafluoro-5-phenyl-4-(phenylsulfonyl)pent-3-en-5-ol (**6a**): mp 74—75 °C (dec.), IR (film) cm^{-1}: 3500—3450 (OH), 1310, 1180—1120 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.37 (3H, t, J=7 Hz, Me), 4.16—4.23 (1H, m, OCH_2), 4.29—4.36 (1H, m, OCH_2), 4.53 (1H, dd, J=2, 12 Hz, OH), 6.03 (1H, d, J=12 Hz, 5-H), 7.24—7.68 (10H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.23 (q), 69.49 (d, J=10 Hz, 5-C), 73.50 (t, J=4 Hz), 112.67 (s, J=40, 263 Hz, CF_2), 118.29 (s, J=36, 288 Hz, CF_3), 125.69 (d×2), 127.57 (d×3), 127.97 (d), 128.75 (d×3), 133.76 (d), 139.97 (s), 141.62 (s), 142.14 (s, J=4 Hz) 149.76 (s, J=25 Hz, 3-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −37.17 (1F, d, J=282 Hz, CF_2), −28.61 (1F, d, J=282 Hz, CF_2), −5.01 (3F, t, J=3 Hz, CF_3); MS *m/z* 391 (M^+−EtO). *Anal.* Calcd for $C_{19}H_{17}F_5O_4S$: C, 52.29; H, 3.93. Found: C, 51.33; H, 3.94.

(*E*)-3-Ethoxy-1,1,1,2,2-pentafluoro-7-phenyl-4-(phenylsulfonyl)hept-3-en-6-yn-5-ol (**6b**): IR (film) cm^{-1}: 3500 (OH), 1310, 1210, 1120 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.34 (3H, t, J=7 Hz, Me), 4.20 (2H, q, J=7 Hz, OCH_2), 4.52 (1H, d, J=11 Hz, OH), 5.78 (1H, d, J=10 Hz, 5-H), 7.26—7.62 (8H, m, ArH), 8.07—8.15 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.14 (q), 58.83 (d, J=9 Hz, 5-C), 73.50 (t), 86.24 (s), 87.48 (s), 111.09 (s, J=40 and 264 Hz, CF_2), 118.26 (s, J=36, 288 Hz, CF_3), 122.04 (s), 127.01 (d×2), 128.55 (d×2), 128.86 (d×2), 129.21 (d), 132.00 (d×2), 133.94 (d), 140.58 (s), 141.37 (s), 148.73 (s, J=25 Hz, 3-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −36.47 (1F, d, J=282 Hz, CF_2), −33.59 (1F, d, J=282 Hz, CF_2), −5.18 (3F, t, J=3 Hz, CF_3); MS m/z 415 (M^+−OEt). *Anal.* Calcd for $C_{21}H_{17}F_5O_4S$: C, 54.78; H, 3.72. Found: C, 52.94; H, 3.70.

Acetylation of Allylic Alcohols 5 and 6. Typical Procedure BF_3–Et_2O (0.29 g, 2.07 mmol) was added to a Ac_2O (1.80 g, 17.6 mmol) solution of (*E*)-2-ethoxy-1,1,1-trifluoro-4-phenyl-3-(phenylsulfonyl)but-2-en-4-ol (**5a**) (0.80 g, 2.07 mmol) at 0 °C. The mixture was stirred for 30 min and poured into $CHCl_3$ (50 ml). The organic layer was washed with a sat.$NaHCO_3$ (50×3 ml) and dried over $MgSO_4$. The solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel eluting with AcOEt–hexane (1 : 20) to give (*E*)-4-acetoxy-2-ethoxy-1,1,1-trifluoro-4-phenyl-3-(phenylsulfonyl)but-2-ene (**7a**) (905 mg, 99%) as a yellow oil. IR (film) cm^{-1}: 1740 (CO), 1310, 1210—1140 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.36 (3H, t, J=7 Hz, Me), 2.24 (3H, s, Me), 3.94—4.01 (1H, m, OCH_2), 4.13—4.21 (1H, m, OCH_2), 7.26—7.61 (9H, m, ArH, 4-H), 7.67—7.69 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.08 (q), 21.01 (q), 69.40 (d, J=2 Hz, 4-C), 72.36 (t), 119.78 (s, J=283 Hz, CF_3), 126.32 (d×2), 128,24 (d), 128.40 (d×2), 128.55 (d×2), 128.61 (d×2), 133.73 (d), 136.28 (s), 136.73 (s, J=2 Hz), 141.62 (s), 151.47 (s, J=34 Hz, 2-C), 169.69 (s); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −17.20 (3F, s, CF_3); MS m/z 369 (M^+−OAc). *Anal.* Calcd for $C_{20}H_{19}F_3O_5S$: C, 56.07; H, 4.47. Found: C, 55.85; H, 4.55

(*E*)-4-Acetoxy-4-(*p*-bromophenyl)-2-ethoxy-1,1,1-trifluoro-3-(phenylsulfonyl)but-2-ene (**7b**): mp 66—69 °C (dec.), IR (film) cm^{-1}: 1730 (CO), 1320—1270 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.35 (3H, t, J=7 Hz, Me), 2.23 (3H, s, Me), 3.93—3.97 (1H, m, OCH_2), 4.10—4.18 (1H, m, OCH_2), 7.23 (1H, s, 4-H), 7.25 (2H, d, J=8 Hz, ArH), 7.43—7.47 (2H, m, ArH), 7.50 (2H, d, J=8 Hz, ArH), 7.56—7.60 (1H, m, ArH), 7.73—7.75 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.02 (q), 20.90 (q), 68.84 (d, 4-C), 71.96 (t), 119.64 (s, J=283 Hz, CF_3), 122.25 (s), 127.98 (d×2), 128.26 (d×2), 128.68 (d×2), 131.61 (d×2), 133.85 (d), 135.56 (s), 136.17 (s), 141.44 (s), 151.64 (s, J=35 Hz, 2-C), 169.49 (s); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −17.08 (3F, s, CF_3); MS m/z 365 (M^+−$PhSO_2H$). *Anal.* Calcd for $C_{20}H_{18}BrF_3O_5S$: C, 47.26; H, 3.57. Found: C, 45.74; H, 3.50.

(*E*)-4-Acetoxy-4-(*p*-chlorophenyl)-2-ethoxy-1,1,1-trifluoro-3-(phenylsulfonyl)but-2-ene (**7c**): mp 61—63 °C (dec.), IR (KBr) cm^{-1}: 1740 (CO), 1310, 1240—1120 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.36 (3H, t, J=7 Hz, Me), 2.24 (3H, s, Me), 3.93—4.01 (1H, m, OCH_2), 4.09—4.20 (1H, m, OCH_2), 7.24 (1H, s, 4-H), 7.29 (2H, d, J=9 Hz, ArH), 7.35 (2H, d, J=9 Hz, ArH), 7.44—7.48 (2H, m, ArH), 7.57—7.62 (1H, m, ArH), 7.71—7.74 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.15 (q), 21.05 (q), 68.94 (d, 4-C), 72.08 (t), 119.76 (s, J=283 Hz, CF_3), 127.81 (d×2), 128.40 (d×2), 128.77 (d×2), 128.79 (d×2), 133.91 (d), 134.27 (s), 135.07 (s), 136.37 (s), 141.62 (s), 151.77 (s, J=35 Hz, 2-C), 169.49 (s); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −17.12 (3F, s, CF_3); MS m/z 321 (M^+−$PhSO_2H$). *Anal.* Calcd for $C_{20}H_{18}ClF_3O_5S$: C, 51.90; H, 3.92. Found: C, 51.84; H, 3.96.

(*E*)-4-Acetoxy-2-ethoxy-1,1,1-trifluoro-6-phenyl-3-(phenylsulfonyl)hex-2-en-5-yne (**7d**): IR (film) cm^{-1}: 1770 (CO), 1320, 1220—1140 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.24 (3H, t, J=7 Hz, Me), 2.26 (3H, s, Me), 3.77—3.82 (1H, m, OCH_2), 3.89—3.94 (1H, m, OCH_2), 7.15 (1H, s, 4-H), 7.32—7.55 (8H, m, ArH, 4-H), 8.09—8.12 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.15 (q), 21.69 (q), 61.85 (t), 84.19 (s), 110.12 (s), 121.34 (s, J=290 Hz, CF_3), 121.84 (s), 127.35 (d), 128.58 (d×2), 128.66 (d×2), 130.21 (d×2), 132.17 (d×2), 133.51 (d), 142.06 (s), 143.12 (s), 168.17 (s); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: 0.47 (3F, s, CF_3), MS m/z 393 (M^+−OAc). *Anal.* Calcd for $C_{22}H_{19}F_3O_5S$: C, 58.40; H, 4.23. Found: C, 52.72; H, 3.83.

(*Z*)-4-Acetoxy-2-ethoxy-1,1,1-trifluoro-6-phenyl-3-(phenylsulfonyl)hex-2-en-5-yne (**7d**): mp 132—133 °C (dec.), IR (KBr) cm^{-1}: 1780 (CO), 1320, 1200—1140 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.91 (3H, t, J=7 Hz, Me), 2.02 (3H, s, Me), 3.42—3.50 (1H, m, OCH_2), 3.77—3.81 (1H, m, OCH_2), 7.26—7.35 (3H, m, ArH), 7.46—7.54 (4H, m, ArH), 7.57—7.61 (1H, m, ArH), 7.74 (1H, s, 4-H), 7.90—7.93 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.74 (q), 20.67 (q), 61.17 (t), 83.59 (s), 118.51 (s), 121.73 (s), 121.88 (s, J=294 Hz, CF_3), 127.58 (d), 128.27 (d×2), 129.00 (d×2), 129.22 (d×2), 130.62 (d), 132.43 (d×2), 133.21 (d), 141.34 (s), 142.98 (s), 166.03 (s); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −0.95 (3F, s, CF_3); MS m/z 393 (M^+−OAc). *Anal.* Calcd for $C_{22}H_{19}F_3O_5S$: C, 58.40; H, 4.23. Found: C, 57.97; H, 4.18.

(2*E*,5*E*)-4-Acetoxy-2-ethoxy-1,1,1-trifluoro-6-phenyl-3-(phenylsulfonyl)hex-2,5-diene (**7e**): IR (film) cm^{-1}: 1770 (CO), 1300, 1200—1120 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.95 (3H, t, J=7 Hz, Me), 2.03 (3H, s, Me), 3.44—3.52 (1H, m, OCH_2), 3.73—3.77 (1H, m, OCH_2), 7.14 (1H, d, J=15 Hz, 6-H), 7.23—7.36 (1H, m, ArH), 7.39—7.43 (3H, m, ArH), 7.47—7.56 (4H, m, ArH), 7.56—7.58 (1H, m ArH), 7.90—7.92 (2H, m, ArH), 8.09 (1H, d, J=11 Hz, 4-H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.57 (q), 21.31 (q), 61.06 (t), 121.18 (d), 121.71 (s, J=293 Hz, CF_3), 127.95 (d×2), 128.18 (d×2), 128.61 (d×2), 129.97 (d×2), 130.50 (d), 131.60 (s), 132.76 (d), 135.33 (s), 142.10 (s), 146.32 (d), 147.74 (d), 165.48 (s); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −0.79 (3F, s, CF_3); MS m/z 395 (M^+−OAc). *Anal.* Calcd for $C_{22}H_{21}F_3O_5S$: C, 58.14; H, 4.66. Found: C, 58.21; H, 4.69.

(*E*)-5-Acetoxy-3-ethoxy-1,1,1,2,2-pentafluoro-5-phenyl-4-(phenylsulfonyl)pent-3-ene (**8a**): IR (film) cm^{-1}: 1740 (CO), 1320, 1180—1120 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.39 (3H, t, J=7 Hz, Me), 2.23 (3H, s, Me), 4.26—4.34 (2H, m, OCH_2), 7.26—7.53 (8H, m, 5H and ArH), 7.53—7.57 (1H, m, ArH) 7.71—7.74 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.18 (q), 20.84 (q), 69.22 (d, J=5 Hz, 5-C), 74.00 (t), 109.80 (s, J=40, 265 Hz, CF_2), 118.10 (s, J=36, 287 Hz, CF_3), 125.80 (d), 128.00 (d×2), 128.02 (d×2), 128.52 (d×2), 128.58 (d×2), 133.70 (d), 136.53 (s), 140.76 (s), 141.75 (s), 151.74 (s, J=25 Hz, 3-C), 169.56 (s); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −33.86 (1F, d, J=284 Hz CF_2), −30.05 (1F, d, J=284 Hz CF_2), −3.68 (3F, t, J=19 Hz, CF_3); MS m/z 337 (M^+−$PhSO_2H$). *Anal.* Calcd for $C_{21}H_{19}F_5O_5S$: C, 52.61; H, 3.99. Found: C, 50.94; H, 3.86.

(3*E*,6*E*)-5-Acetoxy-3-ethoxy-1,1,1,2,2-pentafluoro-7-phenyl-4-(phenylsulfonyl)hept-3,6-diene (**8b**): IR (film) cm^{-1}: 1780 (CO), 1310, 1240—1120 (SO_2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.04 (3H, t, J=7 Hz, Me), 2.05 (3H, s, Me), 3.41—3.49 (1H, m, OCH_2), 3.67—3.75 (1H, m, OCH_2), 7.14 (1H, d, J=15 Hz, 7-H), 7.21—7.45 (5H, m, ArH), 7.47—7.58 (3H, m, ArH), 7.85—7.92 (2H, m, ArH), 8.08 (1H, d, J=11 Hz, 5-H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.62 (q), 21.58 (q), 61.96 (t), 118.75 (s, J=35, 322 Hz, CF_3), 121.41 (d), 128.03 (d×2), 128.43 (d×2), 128.57 (d), 129.35 (d×2), 130.58 (d×2), 131.94 (s), 132.93 (d), 135.41 (s), 142.22 (s), 147.15 (d), 147.89 (d), 165.93 (s); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −40.61 (2F, s, CF_2), −0.93 (3F, s, CF_3); MS m/z 363 (M^+−$PhSO_2H$). *Anal.* Calcd for $C_{23}H_{21}F_5O_5S$: C, 54.65; H, 4.19. Found: C, 54.67; H, 4.25.

Preparations of Allenol Ethers 9 and 10 from Acetates 7 and 8 with SmI_2. Typical Procedure SmI_2 (5.00 ml, 0.20 mol/l THF solution) was added dropwise to a THF (2.0 ml) solution of (*E*)-4-acetoxy-2-ethoxy-1,1,1-trifluoro-4-phenyl-3-(phenylsulfonyl)but-2-ene (**7a**) (0.15 g, 0.35 mmol) at room temperature. The reaction mixture was stirred for 30 min and poured into water (150 ml). The organic layer was separated and the aqueous layer was extracted with ether. The combined organic layer was dried over $MgSO_4$ and the solvent removed under reduced pressure. The residue was purified by preparative TLC on silica gel eluting with hexane to afford 2-ethoxy-1,1,1-trifluoro-4-phenylbut-2,3-diene (**9a**) (58 mg, 73%) as a colorless oil.

9a: IR (film) cm^{-1}: 1950 (allene), 1200—1100 (ether); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.32 (3H, t, J=7 Hz, Me), 3.77 (2H, q, J=7 Hz, OCH_2), 7.09 (1H, q, J=3 Hz, allenic H), 7.25—7.41 (5H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.57 (q), 65.98 (t), 113.72 (d, 4-C), 120.95 (s, J=273 Hz, CF_3), 128.09 (d×2), 129.17 (d×2), 129.35 (d), 131.14 (s), 195.85 (s, 3-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −0.01 (3F, s, CF_3). *Anal.* Calcd for $C_{12}H_{11}F_3O$: C, 63.16; H, 4.89. Found: C, 62.88; H, 4.51.

4-(*p*-Bromophenyl)-2-ethoxy-1,1,1-trifluorobut-2,3-diene (**9b**): IR (film) cm^{-1}: 1950 (allene), 1200—1100 (ether); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.32 (3H, t, J=7 Hz, Me), 3.68—3.82 (2H, m, OCH_2), 7.03 (1H, q, J=3 Hz, allenic H), 7.24 (2H, brd, J=8 Hz, ArH), 7.48 (2H, brd, J=8 Hz, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.50 (q), 66.10 (t), 112.74 (d, 4-C), 120.79 (s, J=273 Hz, CF_3), 123.44 (s), 127.32 (s, J=40 Hz, 2-C), 129.47 (d×2), 132.09 (s), 132.35 (d×2), 190.84 (s, 3-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −0.01 (3F, s, CF_3). *Anal.* Calcd for $C_{12}H_{10}BrF_3O$: C, 49.63; H, 3.28. Found: C, 49.24; H, 3.19.

4-(*p*-Chlorophenyl)-2-ethoxy-1,1,1-(trifluoro)but-2,3-diene (**9c**): IR (film) cm^{-1}: 1950 (allene), 1180—1060 (ether); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.32 (3H, t, J=7 Hz, Me), 3.69—3.82 (2H, m, OCH_2), 7.05 (1H, q, J=3 Hz, allenic H), 7.31 (2H, d, J=8 Hz, ArH), 7.34 (2H, d, J=8 Hz, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.52 (q), 66.10 (t), 112.66 (d, 4-C), 120.84 (s, J=273 Hz, CF_3), 129.22 (d×2), 129.40 (d×2), 131.67 (s), 135.24 (s), 190.82 (s, 3-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −0.01 (3F, s, CF_3). *Anal.* Calcd for $C_{12}H_{10}ClF_3O$: C, 54.87; H, 3.84. Found: C, 54.50; H, 3.85.

2-Ethoxy-1,1,1-trifluoro-6-phenylhexa-2,3-dien-5-yne (**9d**): IR (film) cm^{-1}: 1940 (allene), 1200—1100 (ether); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.36 (3H, t, J=7 Hz, Me), 3.79 (2H, q, J=7 Hz, OCH_2), 6.54 (1H, q, J=2 Hz, allenic H), 7.25—7.36 (3H, m, ArH), 7.47—7.49 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.47 (q), 66.39 (t), 81.50 (s), 93.80 (s), 95.45 (d, 4-C), 120.48 (s, J=274 Hz, CF_3), 122.49 (s), 128.64 (d×2), 129.30 (d), 132.02 (d×2), 200.47 (s, 3-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −0.09 (3F, s, CF_3); MS m/z 252 (M^+). *Anal.* Calcd for $C_{14}H_{11}F_3O$: C, 66.66; H, 4.40. Found: C, 66.31; H, 4.61.

(*E*)-2-Ethoxy-1,1,1-trifluoro-6-phenylhexa-2,3,5-triene (**9e**): IR (film) cm^{-1}: 1940 (allene), 1200—1100 (ether); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.33 (3H, t, J=7 Hz, Me), 3.71—3.77 (2H, m, OCH_2), 6.63 (1H, dd, J=10, 16 Hz, olefinic H), 6.75 (1H, d, J=16 Hz, olefinic H), 6.89 (1H, dd, J=3, 10 Hz, allenic H), 7.25—7.29 (1H, m, ArH), 7.30—7.36 (2H, m, ArH), 7.43—7.44 (2H, m, ArH); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.53 (q), 65.86 (t), 113.56 (d, 4-C), 120.85 (s, J=273 Hz, CF_3), 123.73 (d), 127.07 (d×2), 128.84 (d), 128.99 (d×2), 136.33 (s), 136.78 (d), 200.47 (s, 3-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −0.01 (3F, s, CF_3); MS m/z 255 (M^++1). *Anal.* Calcd for $C_{14}H_{13}F_3O$: C, 66.14; H, 5.15. Found: C, 66.01; H, 5.17.

3-Ethoxy-1,1,1,2,2-pentafluoro-5-phenylpenta-3,4-diene (**10a**): IR (film) cm^{-1}: 1960 (allene), 1210—1160 (ether); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.31 (3H, t, J=7 Hz, Me), 3.69—3.89 (2H, m, OCH_2), 7.11 (1H, d, J=4 Hz, allenic H), 7.24—7.40 (5H, m, ArH); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −39.93 (1F, d, J=3 Hz, CF_2), −39.86 (1F, d, J=3 Hz, CF_2), −5.40 (3F, t, J=2 Hz, CF_3). *Anal.* Calcd for $C_{13}H_{11}F_5O$: C, 56.12; H, 3.99. Found: C, 56.98; H, 4.43.

3-Ethoxy-1,1,1,2,2-pentafluoro-7-phenylhepta-3,4,6-triene (**10c**): IR (film) cm^{-1}: 1940 (allene), 1220 (ether); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.32 (3H, t, J=7 Hz, Me), 3.71—3.79 (2H, m, OCH_2), 6.61 (1H, dd, J=11, 16 Hz, 6-H), 6.76 (1H, d, J=16 Hz, 5-H), 6.91 (1H, dt, J=3, 11 Hz, allenic H), 7.27—7.37 (3H, m, ArH), 7.43—7.47 (2H, m, ArH); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −39.86—−39.86 (2F, m, CF_2), −5.42 (3F, t, J=2 Hz, CF_3).

Hydrolysis of Allenol Ether 9c with HCl 5% HCl (1.0 ml) solution was added to 80% EtOH (1.0 ml) containing **9c** (0.10 g, 0.38 mmol). The mixture was refluxed for 1 h and poured into a sat. $NaHCO_3$ (50 ml). The organic layer was separated and the aqueous layer was extracted with ether. The combined organic layer was dried over $MgSO_4$ and solvent removed under reduced pressure. The residue was purified by preparative TLC on silica gel eluting with AcOEt–hexane (1 : 40) to give (*E*)-4-(*p*-chlorophenyl)-1,1,1-trifluorobut-3-en-2-one (**11**) (30 mg, 34%) as yellow prisms.

(*E*)-4-(*p*-Chlorophenyl)-1,1,1-trifluorobut-3-en-2-one (**11**): mp 43—46 °C, IR (KBr) cm^{-1}: 1718 (CO); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 6.98 (1H, dd, J=1, 16 Hz, 4-H), 7.37—7.45 (2H, m, ArH), 7.52—7.63 (2H, m, ArH), 7.91 (1H, d, J=16 Hz, 3-H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 116.19 (s, J=291 Hz, CF_3), 117.29 (d), 129.85 (d×2), 130.57 (d×2), 132.03 (s), 138.72 (s), 148.77 (d), 179.92 (s, 2-C); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: 10.34 (3F, s, CF_3); MS m/z 165 (M^+-CF_3). *Anal.* Calcd for $C_{10}H_6ClF_3O$: C, 51.20; H, 2.58. Found: C, 51.15; H, 2.84.

Reaction of 9c with Triethyl Orthoformate/BF_3–Et_2O BF_3–Et_2O (0.22 g, 1.56 mmol) was added to an EtOH (1.0 ml) solution of triethyl orthoformate (0.28 g, 1.91 mmol). The mixture was refluxed for 2 h and poured into sat. $NaHCO_3$ (150 ml) solution. The work-up procedure afforded (*E*)- and (*Z*)-2,2-diethoxy-1,1,1-trifluoro-4-(*p*-chlorophenyl)but-3-ene (**12**) (75 mg, 64%) as a yellow oil.

12: *E* : *Z*=2 : 3, IR (film) cm^{-1}: 1200—1040 (ether); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.05 (t, J=7 Hz, *Z*-Me), 1.27 (t, J=7 Hz, *E*-Me), 3.49—3.73 (m, *E* and *Z*-OCH_2), 5.49 (dd, J=1, 13 Hz, *Z*-4-H), 6.05 (dd, J=1, 16 Hz, *E*-4-H), 6.77 (d, J=13 Hz, *Z*-3-H), 7.01 (d, J=16 Hz, *E*-3-H), 7.24—7.38 (m, *E* and *Z*-ArH); ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: −0.27 (s, *E*-CF_3), 0.69 (s, *Z*-CF_3). *Anal.* Calcd for $C_{14}H_{16}ClF_3O_2$: C, 54.65; H, 5.33. Found: C, 54.96; H, 5.09.

Reaction of 9c with Benzaldehyde Dimethyl Acetal/BF_3–Et_2O BF_3–Et_2O (0.11 g, 0.76 mmol) was added to a $ClCH_2CH_2Cl$ (1.0 ml) solution of **9c** (0.10 g, 0.38 mmol) and benzaldehyde dimethyl acetal (0.12 g, 0.76 mmol) at 0 °C. The mixture was stirred for 10 min. The work-up procedure gave *cis* and *trans*-2-(*p*-chlorobenzylidene)-1-ethoxy-3-methoxy-1-(trifluoromethyl)indene (**13**) (110 mg, 70%) as a yellow oil.

13: *cis* : *trans*=1 : 1, *E* : *Z*=1 : 1, IR (film) cm^{-1}: 1280, 1150 (ether); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.85 (t, J=7 Hz, Me), 1.21 (t, J=7 Hz, Me), 3.07 (d, J=1 Hz, Me), 3.24 (d, J=1 Hz, Me), 3.35—3.50 (m, OCH_2), 3.61—3.68 (m, OCH_2), 4.65 (s, CHO), 4.69 (s, CHO), 6.89—6.92 (q, J=4 Hz, ArH), 7.06—7.36 (m, ArH), 7.41 (s, olefinic H), 7.43 (s, olefinic H). ^{19}F-NMR (376.4 MHz, $CDCl_3$) δ: 0.28 (s, CF_3), 0.60 (s, CF_3); MS m/z 382 (M^+), 353 (M^+-OEt). *Anal.* Calcd for $C_{20}H_{18}ClF_3O_3$: C, 62.75; H, 4.74. Found: C, 62.29, H, 4.69.

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