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Structure Determination of Metabolites Isolated from Urine and Bile after Administration of AY4166, a Novel D-Phenylalanine-Derivative Hypoglycemic Agent

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Abstract—Molecular structures of 10 metabolites, which were isolated from urine (M1–M8) or bile (M9 and M10) after administration of AY4166 (*N*-(*trans*-4-isopropylcyclohexanecarbonyl)-D-phenylalanine), a novel amino acid derivative with hypoglycemic activity, have been elucidated by mass spectrometry and nuclear magnetic resonance. Four of these (M1, M2, M3 and M8) were determined to be hydroxyl derivatives of AY4166, two (M9 and M10) were carboxylate derivatives via oxidization of M2 and M3, three (M4, M5 and M6) were glucronic acid conjugates and the other (M7) was a dehydro derivative. The estimated structures for M1, M2, M3, M7, M8, M9 and M10 were confirmed by the coincidence of the retention time of HPLC, MS and ^{1}H NMR spectra between the isolated metabolites and authentic synthesized substances. For three glucronic acid conjugates, M4, M5 and M6, structural confirmation was performed by a selective enzymatic digestion with β-glucronidase. M1 and M2/3 were about 5–6 and 3 times less potent than AY4166, respectively, and M7 was almost as potent as AY4166. Copyright © 1996 Elsevier Science Ltd

Introduction

N-(*trans*-4-Isopropylcyclohexanecarbonyl)-D-phenylalanine (AY4166) is a novel amino acid derivative which is expected to be a favorable insulinotropic agent for reducing postprandial hyperglycemia.[1–3] It lowers blood glucose levels in nondiabetic and diabetic animals, showing quicker on-set and shorter duration time than sulfonylureas, which are also insulinotropic agents.[4] It has recently been shown that in murine pancreatic β-cells AY4166 increases cytosolic free Ca^{2+} by stimulating Ca^{2+} influx[5] through the regulation of ATP-sensitive potassium channels.[6]

In general, structure determination of metabolites after administration of an agent has a key importance for better understanding of its functioning mechanism and the safety assurance of an agent. Accordingly, 10 metabolites of AY4166 were isolated from urine or bile of rat, dog or human and the molecular structures elucidated by mass spectrometry and nuclear magnetic resonance and confirmed with chemical synthesis or enzymatic digestion. The determined structures are reported in this paper along with the biological activity.

Results and Discussion

The molecular structures of M1–M10 were elucidated by means of one- and two-dimensional NMR and MS experiments and shown in Figure 1, together with the structure of AY4166. A summary of ^{1}H and ^{13}C NMR spectroscopic data of 10 metabolites are given in Tables 1 and 2.

Structure estimation of metabolites

M1, M2, M3, M7 and M8. M1, M2 and M3 showed similar retention time in HPLC. They have the same molecular weight, $[M+H]^+=334$ m.u., and they were estimated to be hydroxyl derivatives of AY4166 ($M_r=317.43$). Comparing the ^{1}H spectrum of M1 with that of AY4166, a large doublet signal at 0.86 ppm (6H) in AY4166 was replaced by a new singlet signal at 1.10 ppm (6H) in M1. In addition, the number of unexchangable protons was less than that of AY4166 by one proton (1H). These results indicate that although two methyl groups remained in M1, the coupling with a proton was lost. This suggests that the methyne proton of the isopropyl group was converted into a hydroxyl group, and the structure of M1 shown in Figure 1 has been proposed. In the ^{13}C spectrum, the methyne carbon signal C-7' of AY4166 at 34.1 ppm was replaced by the quaternary carbon signal at 73.1 ppm in M1.

In the ^{1}H spectrum of M2, the methyl signal at 0.85 ppm was a doublet, but the integral value was reduced to 3H. Two double doublet signals were observed at 3.35 and 3.49 ppm and these two proton signals were correlated to the carbon signal at 66.3 ppm in the C, H-COSY spectrum. This indicates one of the two methyl groups of AY4166 was converted into the methoxyl group. Thus, the structure of M2 in Figure 1 has been proposed.

M3 showed a very similar proton spectrum to that of M2, though the retention time of M3 was slightly slower than that of M2. We considered that M3 was a

diastereomer of M2. One methyl group of the isopropyl group in AY4166 changed into methoxyl in M2 and M3. Accordingly, C-7' became the new chiral center and two diastereomers were produced.

M8 was estimated to be a hydroxyl derivative in the same way as M1, M2 and M3 from the mass spectrometry results. The ^{1}H NMR spectrum of M8 showed that the symmetrical two doublet signals in the aromatic region and the integral of aromatic protons was reduced to 4H from 5H, which is common to AY4166 and other metabolites. This suggests that in M8, the phenylalanyl residue was replaced by the tyrosyl residue.

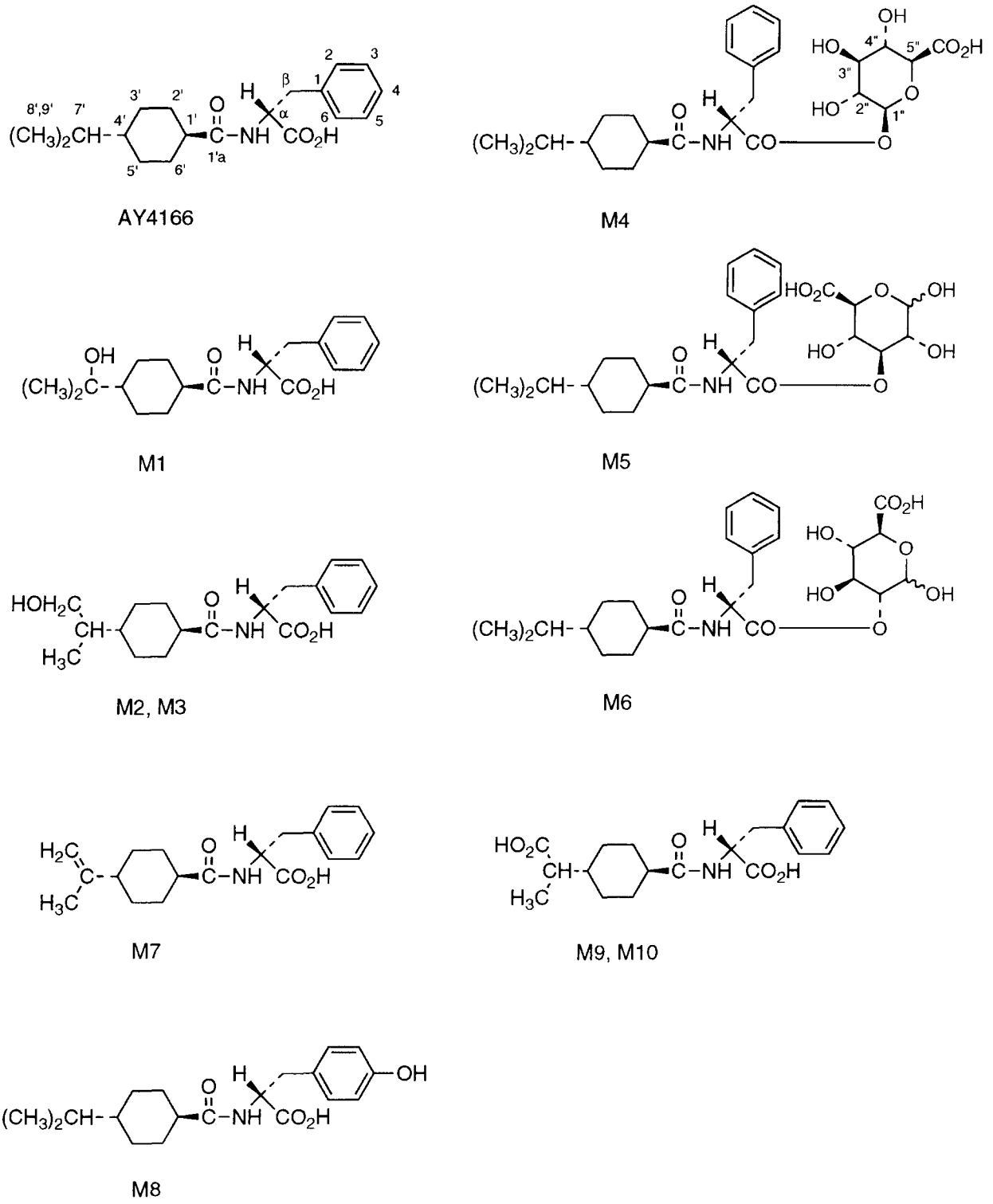


Figure 1. Molecular structure of AY4166 and its 10 metabolites. The numbering of the atoms in the chemical formulas refers to the position numbers of Tables 1 and 2.

The spectra of M7 shows the following differences, compared to the ^1H and ^{13}C spectra of AY4166 itself: (i) a large doublet signal at 0.86 ppm (6H) was replaced by a singlet signal at 1.69 ppm (3H), which indicates one of the two methyl groups in AY4166 disappeared. (ii) In the ^{13}C spectrum of M7, a new CH_2 signal was observed at 108.9 ppm, which showed correlation with the 4.66 ppm proton signal (2H) in the C,H-COSY spectrum. Taking into account the mass spectral information that one double bond may have been formed, the structure of M7 was proposed. The C7' signal, the quaternary carbon, was observed at 151.5 ppm in M7.

M4, M5 and M6. M4, M5 and M6 were estimated to be three isomers of glucronic acid (M_r 194.14) conjugates, from the results of mass measurements which gave $[M+H]^+$, $[M+Na]^+$, and $[M+2Na]^+$ as 494, 516, and 538 m.u., respectively. ^1H NMR spectra of these metabolites showed glucronic acid signals in the region between 3.4 and 5.5 ppm, which were not observed in AY4166. However, for the residual signals, there were little changes in comparison with AY4166 spectrum. Figure 2 shows a comparison between the spectra of metabolites M4, M5 and M6 and that of glucronic acid (CD_3OD solution) in the region between 3.0 and 6.0 ppm. The signal assignments based on H,H-COSY and TOCSY(total COSY) measurements were shown at the top of each signal. Both α- and β-anomeric signals were observed for the authentic glucronic acid and metabolites M5 and M6. But, for M4, only β-anomeric signals were observed. Compared to the standard glucronic acid, β1″ proton signal of M4, α3″ and β3″-H signals of M5, and α2″-H and β2″-H signals of M6 were shifted down field more than 1.0 ppm. Figure 3 shows the chemical shift differences of each glucronic acid signal of three metabolites. A large shift of more than 1.0 ppm was thought to be caused by the dehydro-condensation between AY4166 and glucronic acid.

The functional groups which can cause dehydro-condensation between AY4166 and glucronic acid are the COOH group of the phenylalanyl residue in AY4166, four OH groups and the COOH group of glucronic acid. A large shift of specific protons of glucronic acid observed in M4, M5 and M6 was thought to indicate the dehydro-condensation sites in each metabolite. Thus, we thought that the phenylalanyl COOH group of AY4166 will be ester bonded with the 1″-OH of glucronic acid in M4, the 3″-OH in M5, and the 2″-OH in M6, as a result, β1″-H of M4, α3″-H and β3″-H of M5, and α2″-H and β2″-H of M6

Table 1. ^1H NMR data of M1–M10 in ppm relative to tetramethylsilane

Position	M1	M2, M3	M4	M5	M6	M7	M8	M9, M10
1′*[a]	2.10	2.07	2.08	2.08	2.10	2.14	2.10	2.10
2′/6′*[b]	1.37, 1.83	1.36, 1.78	1.36, 1.78	1.37, 1.78	1.32, 1.77	1.44, 1.83	1.37, 1.80	1.38, 1.80
	1.28, 1.67	1.31, 1.68	1.30, 1.62	1.30, 1.60	1.27, 1.60	1.35, 1.66	1.30, 1.68	1.29, 1.62
3′/5′*[b]	1.06, 1.87	1.00, 1.70	1.00, 1.73 1.00, 1.73	1.00, 1.73	0.98, 1.73	1.22, 1.80	1.00, 1.77	1.01, 1.80
	1.06, 1.87	1.10, 1.70	1.00, 1.73	1.00, 1.73	0.98, 1.73	1.22, 1.73	1.00, 1.77	1.07, 1.71
4′	1.25	1.30	1.00	1.00	1.00	1.85	1.00	1.48
7′	—	1.43	1.40	1.40	1.37	—	1.40	2.18
8′*[c]	1.10 s	0.85 d	0.86 d	0.86 d	0.86 d	1.69 s	0.86 d	1.08 d
9′*[c]	1.10 s	3.35, 3.49 dd	0.86 d	0.86 d	0.86 d	4.66 s	0.86 d	—
α*[d]	4.66	4.49	4.79	4.72	4.78	4.66	4.58	4.64
β*[d]	2.94, 3.21	2.96, 3.22	2.97, 3.30	2.95, 3.25	2.94, 3.25	2.95, 3.22	2.85, 3.10	2.92, 3.21
2, 6	7.21	7.20	7.18	7.23	7.25	7.22	7.02 d*[e]	7.20
3, 5	7.25	7.22	7.25	7.23	7.25	7.27	6.68 d*[e]	7.25
4	7.19	7.15	7.20	7.18	7.20	7.20	—	7.19
1″*[f]	—	—	5.54	5.18, 4.58	5.28, 4.65	—	—	—
2″*[f]	—	—	3.40	3.58, 3.34	4.65, 4.74	—	—	—
3″*[f]	—	—	3.47	5.29, 5.00	3.97, 3.62	—	—	—
4″*[f]	—	—	3.46	3.60, 3.66	3.52, 3.53	—	—	—
5″*[f]	—	—	3.68	4.17, 3.68	4.12, 3.60	—	—	—

NMR data of compounds M1–M3, M7–M10 were obtained from synthetic materials in CD_3OD. For M4–M6, data were obtained from isolated materials.

[a] 1′ signals apparently look like triple triplet, though they should be all double double double doublet (dddd). The values of $J_{ax,ax}$ and $J_{ax,eq}$ (Hz) for M1–M10 are in turn, (12.1, 3.4), (12.1, 3.2), (12.0, 3.4), n. d., n. d., (12.0, 3.4), (12.2, 3.4) and (12.2, 3.1).

[b] Positions 2′/6′ or 3′/5′ could not be distinguished. ^1H chemical shift values of 2′/6′ and 3′/5′ were paired data of axial and equatorial protons. In the block of 2′/6′ and 3′/5′ data, the upper pair of proton data refers to the first value of the corresponding carbon data in Table 2. The lower pair correlates to the second value of carbon data; e.g. 30.5 ppm signal of 2′/6′ carbon of M1 correlates to the proton pairs of 1.37 and 1.83, and the 30.8 ppm signal correlates to the proton pairs of 1.28 and 1.67.

[c] The coupling constant of 8′ and 9′ methyl signals ($J_{7'H,8'CH_3}=J_{7'H,9'CH_3}$) is 6.8 Hz for M4, M5, M6 and M8. In M9 (M10) $J_{7'H,8'CH_3}$ is 7.3 Hz. In M2, $J_{7'H,8'CH_3}$ is 7.1, $J_{9'CH_2\text{-}1,9'CH_2\text{-}2}$ is 10.7, and $J_{7'H,9'CH_2\text{-}1}$ and $J_{7'H,9'CH_2\text{-}2}$ are 6.7 and 5.9 Hz, respectively.

[d] α and β signals are all double doublet (dd). The values of $J_{\alpha,\beta\text{-}1}$, $J_{\alpha,\beta\text{-}2}$ and $J_{\beta\text{-}1,\beta\text{-}2}$ are (9.3, 4.9, 13.9) for M1, (8.3, 5.1, 13.9) for M2, (9.3, 4.9, 13.7) for M4, (9.3, 5.1, 13.9) for M7, (9.0, 5.1, 14) for M8, (9.8 Hz, 4.9 Hz, 14.0 Hz) for M9. For M5 and M6, these values were not determined.

[e] J = 8.5 Hz.

[f] For the chemical shift values of 1″–6″ in M5 and M6, the first value corresponds to α anomers, and the second one to β anomers (see text). The proton signals of 1″ and 5″ are doublet (d) and those for 2″, 3″ and 4″ are triplet (t). In M4, $J_{1'',2''}=8.3$, $J_{2'',3''}=8.8$, $J_{3'',4''}$ = n.d., $J_{4'',5''}=9.3$ Hz. In M5 the α anomer, $J_{1'',2''}=3.4$, $J_{2'',3''}=J_{3'',4''}=9.8$, $J_{4'',5''}=9.8$ Hz, and for the β anomer $J_{1'',2''}=7.8$, $J_{3'',4''}=9.3$ Hz, $J_{2'',3''}$ and $J_{4'',5''}$ were n.d. In M6 the α anomer, $J_{1'',2''}=3.4$, $J_{2'',3''}=J_{3'',4''}=9.3$, $J_{4'',5''}=10.3$ Hz, and for the β anomer $J_{1'',2''}=9.3$ Hz, the other J's were not determined.

Table 2. ^{13}C NMR data of M1–M10 in ppm relative to tetramethylsilane

Position	M1	M2, M3	M4	M5	M6	M7	M8	M9, M10
1′	46.1	46.5	46.3	46.3	46.3	45.9	46.3	45.8
2′/6′	30.5, 30.8	30.4, 31.2	30.5, 30.9	30.6, 30.9	30.6, 30.9	30.3, 30.7	30.5, 30.9	30.2,30.5
3′/5′	27.7, 27.8	28.7, 31.2	30.1, 30.2	30.1, 30.2	30.1, 30.2	32.0, 32.1	30.1, 30.2	29.7,31.2
4′	49.7	39.9	44.8	44.8	44.8	46.0	44.8	41.2
7′	73.1	41.8	34.1	34.1	34.1	151.5	34.1	46.3
8′	26.8	13.8	20.2	20.2	20.1	21.0	20.1	14.4
9′	26.9	66.3	20.2	20.2	20.1	108.9	20.1	180.0
1′a	178.9	178.5	179.1	179.1,179.2	179.2	178.9	179.1	178.7
α	54.6	56.9	54.6	54.9, 54.9	54.8, 54.7	54.7	54.9	54.7
αCOOH	174.9	179.7	171.9	173.0,172.8	172.7,172.5	175.0	175.1	174.9
β	38.4	38.8	37.9	38.4	38.35,38.41	38.4	37.7	38.4
1	138.6	139.4	138.4	138.7, 138.3	138.3,138.5	138.5	129.2	138.6
2,6	130.3	130.6	130.6	130.6,130.3	130.5,130.6	130.3	131.3	130.3
3,5	129.4	129.2	129.4	129.4,129.5	129.4,129.4	129.4	116.2	129.4
4	127.8	127.4	127.8	127.7,127.9	127.8,127.8	127.8	157.3	127.8
1″[a]	—	—	96.1	93.9, 98.2	91.1, 96.2	—	—	—
2″[a]	—	—	73.7	71.8, 74.3	76.1, 77.7	—	—	—
3″[a]	—	—	77.8	77.9, 80.1	72.0, 76.2	—	—	—
4″[a]	—	—	73.4	72.3, 72.0	74.2, 73.7	—	—	—
5″[a]	—	—	76.7	71.7, 76.4	71.2, 75.8	—	—	—
6″[a]	—	—	175.8	177.7, 176.3	177.9, 176.6	—	—	—

NMR data of compounds M1–M3, M7–M10 were obtained from synthetic materials in CD_3OD. For M4–M6, data were obtained from isolated materials.

[a]For the chemical shift values of 1″–6″ in M5 and M6, the first value corresponds to α anomers, and the second one to β anomers (see text).

shifted downfield by more than 1.0 ppm. Thus, the molecular structures of M4, M5 and M6 in Figure 1 are proposed. Since the linkage site in M4 is at position 1″-OH (equatorial), only the β anomer is present. On the other hand, the linkage sites in M5 and M6 are at positions 3″-OH and 2″-OH, respectively, as position 1″-OH may be both axial and equatorial, both α and β anomers can be present.

HMBC measurements, which can observe long range couplings between proton and carbon, support the above estimation. In the HMBC spectrum of M4 (Fig. 4), a cross-peak was observed between β1″-H of glucronic acid (5.54 ppm) and the carbonyl carbon of the phenylalanyl COOH group (171.9 ppm), they were three bonds apart from each other. This fact strongly supports the estimated structure of M4. Similarly, in the HMBC spectrum of M5, a cross-peak was observed between α3″-H (5.29 ppm) of glucronic acid and the carbonyl carbon of the phenylalanyl COOH group (173.0 ppm), and this finding also supports the estimated structure of M5. In the case of M6, no HMBC measurement was made because of unstability and sample amount deficiency, however a large shift in position of the 2″-H signal indicates that there is a similar ester bond between position 2″-OH of glucronic acid and the phenylalanyl COOH.

From the strength of carbon signals, α anomers are suggested to be present in greater quantities than β anomers in M5 and M6.

M9 and M10. Comparing the proton and carbon spectra of AY4166, the spectra of M9 shows the following differences: (i) methyl groups at 0.86 ppm (doublet, 6H) changed into a doublet signal at 1.08 ppm (3H), which indicates one methyl group of isopropyl group in AY4166 was converted to the other group. (ii) In the carbon spectrum of M9, a new quaternary carbon signal was observed at 180.0 ppm. This signal showed long range correlation with the methyl proton at 1.08 ppm and the methyne proton at 2.18 ppm in the HMBC spectrum. Considering the MS results, we thought one methyl group changed into a carboxyl group. Thus, the structure of M9 was proposed.

M10 showed quite a similar proton spectrum to that of M9, though the retention time of M10 was slightly longer than that of M9. We thought that M10 was a diastereomer of M9. C-7′ became the new chiral center in M9 and M10, as shown in the relation of M2 to M3.

Confirmation of estimated structures

M1, M2, M3, M7, M8, M9 and M10 by synthetic materials. The estimated structures of M1, M2, M3, M7, M8, M9 and M10 were confirmed by the coincidence of the retention time of HPLC, the mass spectrum and ^1H NMR spectrum between the isolated metabolites and authentic substances which were prepared by the method shown in Schemes 1–3.

trans-1,4-Cyclohexanedicarboxylic acid methyl ester (**1**)[7] was methylated with methylmagnesium bromide to give **2**, which was converted to **3** by the coupling with D-phenylalanine benzyl ester (D-Phe-OBn) using DCC-HOBt. Catalytic hydrogenolysis of **3** removed the

benzyl group to give the required product M1 (Scheme 1).

Treating the acyl chloride of **1** with lithium dimethylcuprate[8] followed by Lombardo's methylenation[9] gave the olefin **5**. Hydroboration of **5** in THF and hydrolysis gave the racemic alcohol **6** quantitatively, this was hydrolysed to **7**. The coupling of D-Phe-OBn with **7** was followed by hydrogenolysis to give M2 and M3 as a 1:1 diastereomeric mixture (Scheme 1). The mixture was separated by HPLC with the first eluate being taken as M2 and the later eluate as M3.

The methyl ester **5** was hydrolysed to **9**, which was coupled with D-phenylalanine methyl ester (D-Phe-OMe) and hydrolysed to give M7 (Scheme 1). Metabolite M8 was derived from **11** which was coupled with D-Phe-OBn as a *N*-hydroxysuccinimide ester **12**[2] (Scheme 2). A mixture of M2 and M3 was easily oxidized with Jones reagent to give a mixture of M9 and M10 (Scheme 3).[10] The mixture was separated by HPLC with the first eluate being taken as M9 and the later eluate as M10.

M4, M5 and M6 by β-glucronidase digestion. Since compounds corresponding to M4, M5 and M6 are very unstable, structural confirmation was made by enzymatic digestion using β-glucronidase. This enzyme is known to catalyse the reaction in which β-D-glucronide is hydrolysed and glucronic acid freed. This requires the carboxyl group of glucronic acid and acts selectively on the β-1″ position linkage, but not on the position 2″ or the position 3″ linkages. When M4, M5 and M6 were treated with β-glucronidase, only M4 was decomposed to produce AY4166 and glucronic acid, but M5 and M6 were not decomposed and remained unmodified. These findings are not inconsistent with the estimated structures of M4, M5 and M6.

The 2″-, 3″- and 4″-*O*-acyl glucronide isomers of zomepirac were reported by Smith and Bent,[11] based on the proton NMR data which showed a large downfield shift of the proton geminal to the acyloxy

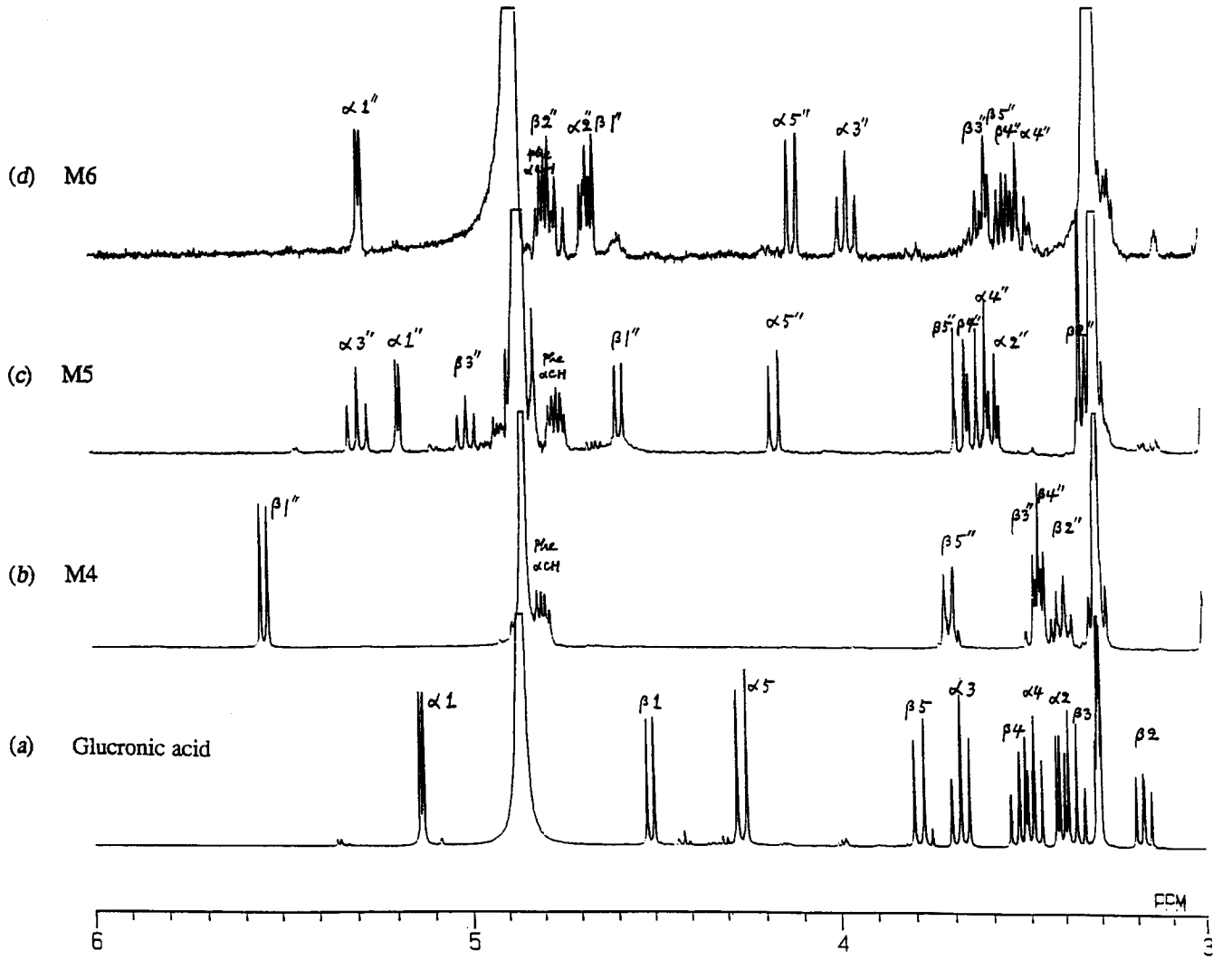


Figure 2. Comparison of ^{1}H NMR (3.0–6.0 ppm) of M4, M5 and M6 and that of glucronic acid. (a) Glucronic acid (b) M4 (c) M5 (d) M6. At the top of each signal, the assignment of glucronic acid protons is shown (see text). α, β Indicate α and β anomers, and the number shows the position in glucronic acid. In M4, M5 and M6, double dashed numbers are used in accord with Figure 1.

group. They suggested that these positional isomers of glucronides were formed by sequential acyl migration of β1″-*O*-glucronide of zomepirac. Although we have no evidence to suggest the acyl migration of glucronides of AY4166, the positional isomers were clearly characterized by ^{13}C NMR and 2D NMR measurement including HMBC.

Biological activities of metabolites

Fasted beagle dogs were intravenously injected with AY4166 or its metabolites. Some of these metabolites produced a quick and short-lasting hypoglycemic action like AY4166 did. Based on the extent of maximal decrease in blood glucose, the potency of the hypoglycemic effect of these metabolites was calculated. It was observed, M1 was about 5–6 times less potent, and M2 and M3 were 3 times less potent than AY4166, while M7 was almost as potent as AY4166 (unpublished observation).

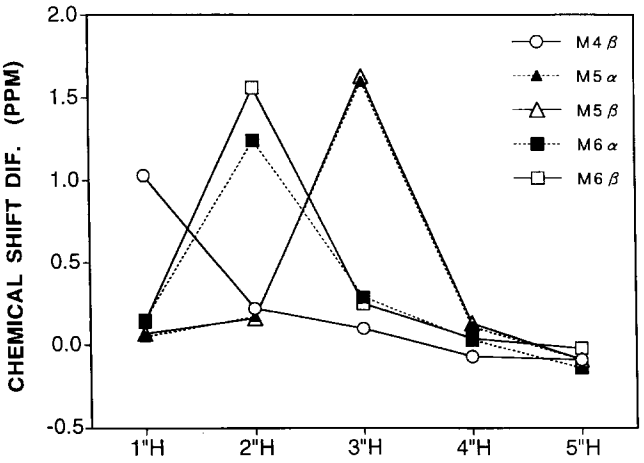


Figure 3. The chemical shift difference (ppm) of each glucronic acid signal of M4, M5 and M6, compared to the standard glucronic acid. β1″-H of M4, α3″-H and β3″-H of M5, and α2″-H and β2″-H of M6 shift downfield more than 1 ppm.

Conclusion

Molecular structures of AY4166 metabolites have been determined. M1, M2, M3 and M8 were found to be hydroxyl derivatives of AY4166, M9 and M10 were carboxylate derivatives, M4, M5 and M6 were glucronic acid conjugates, and M7 was a dehydro derivative. Three glucronic acid conjugates were distinguished from each other in the position of ester linkage between AY4166 and glucronic acid. M7 showed almost the same potency of hypoglycemic effect as AY4166 did, and the precise biological activity results will be published elsewhere.

Experimental

Materials and reagents

AY4166 was prepared by the method previously reported.[3] Deuterium oxide (Kanto Chemical Co., Inc., Japan, NMR spectroscopy use grade), methyl alcohol-d_4 (Isotec Inc., U.S.A.), $CDCl_3$ (Merck, U.S.A.), tetramethylsilane (Isotec Inc., U.S.A.) and β-glucronidase (Sigma, Japan) were used.

Separation of the metabolites

M1, M2 and M3 were obtained from the urine of rats to which AY4166 (500 mg/kg) was administered. The pooled urine of rats (0–6 h) was adjusted to pH 3.5 with 1 M HCl and extracted with ethyl acetate. The ethyl acetate layer was evapd to dryness in vacuo and applied into a prep. HPLC (Inertsil PREP 30×250 mm, GLScience, Tokyo, Japan). The stepwise elution of the prep. HPLC was carried out with the mixed solution of acetonitrile at 20, 30 and 40% in 0.02 M sodium phosphate buffer (pH 6.6) as eluent.

M4, M5, M6 and M7 were obtained from human urine after administration of AY4166 (60 mg/kg). The pooled urine of human (0–4 h) was adjusted to pH 2.0 with 1 M HCl and extracted with ethyl acetate. The ethylacetate layer was evapd to dryness in vacuo and

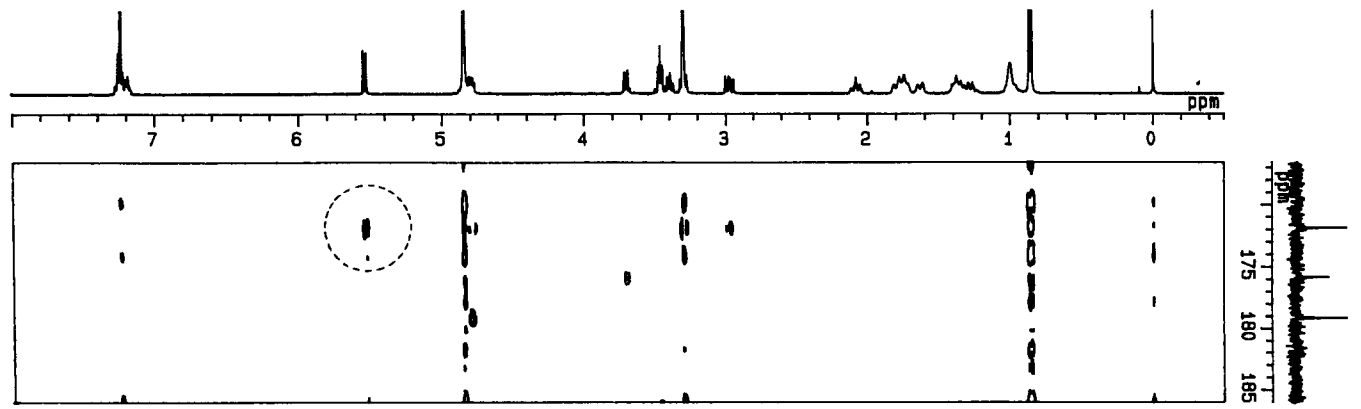
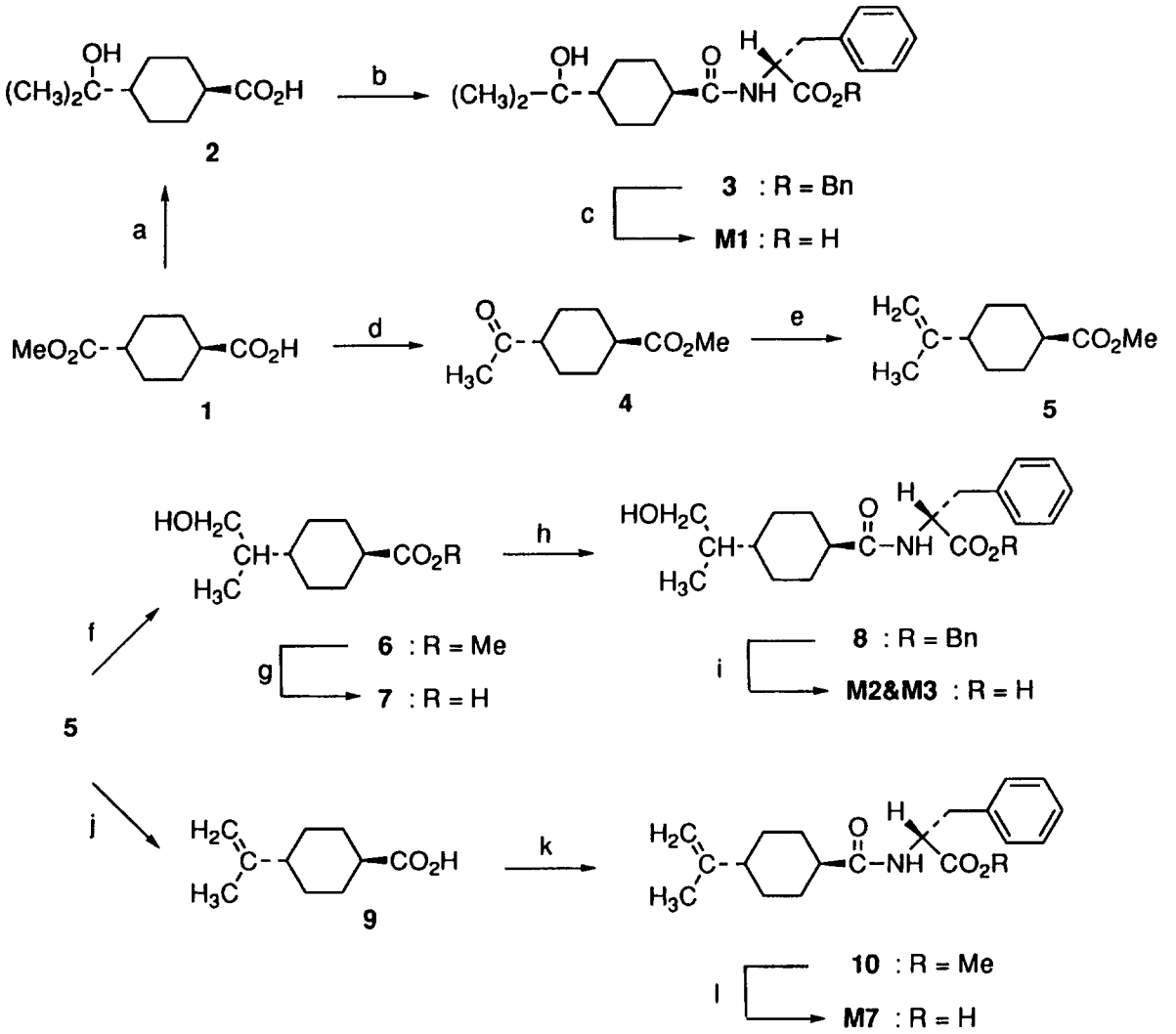
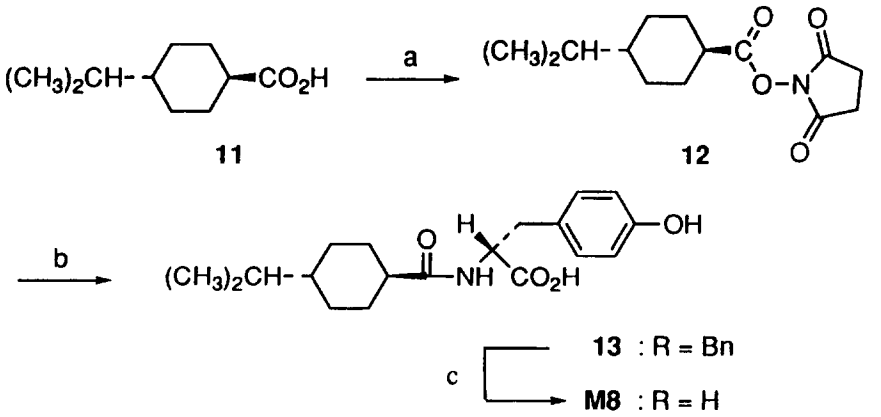


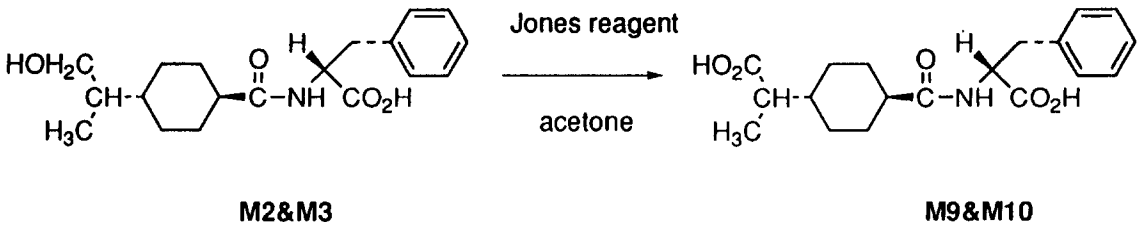
Figure 4. HMBC spectrum of M4 (part). The cross peak, circled with a dotted line, is observed between β1″-H of glucronic acid at 5.54 ppm and the carbonyl carbon of the phenylalanyl COOH group at 171.9 ppm.



Scheme 1. Conditions: (a) MeMgBr/Et_2O-THF; (b) D-Phe-OBn/DCC/HOBt/CH_2Cl_2-THF; (c) 10%Pd-C/MeOH, (d) (1) $SOCl_2$, (2) $LiCuMe_2$/Et_2O/THF; (e) $Zn/TiCl_4/CH_2Br_2$/THF; (f) (1) BH_3/THF, (2) 30% H_2O_2/NaOH; (g) 1M NaOH/acetone/MeOH; (h) D-Phe-OBz/DCC/HOBt/CH_2Cl_2-THF; (i) 10% Pd-C/MeOH; (j) 1M NaOH/MeOH; (k) D-Phe-OMe/DCC/HOBt/CH_2Cl_2-THF; (l) 1M NaOH/MeOH.



Scheme 2. Conditions: (a) HOSu/WSC·HCl/CH_2Cl_2; (b) D-Tyr-OBn/CH_2Cl_2/THF; (c) 10% Pd-C/MeOH. HOSu: *N*-Hydroxysuccinimide; WSC·HCl: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride.



Scheme 3.

the residue dissolved in methanol. After dilution with water, the solution was injected to solid-phase extraction cartridge (SEP-PAK C18, Waters, Milford, U.S.A.) and crude metabolites were eluted with methanol. The crude metabolites solution was applied to prep medium pressure liquid chromatography (YMC S-30, 20 × 100 mm, YMC, Kyoto, Japan) and fractionated with the mixed solution of acetonitrile at 10, 20, 30, 33 and 36% in 0.02 M sodium phosphate buffer (pH 6.6) as eluent. Each fraction was then applied to prep. HPLC (Inertsil PREP 30 × 250 mm, GLScience, Tokyo, Japan) and fractionated with the mixed solution of acetonitrile at 33 and 40% in 0.02 M sodium phosphate buffer (pH 6.6) as eluent.

M8 was obtained from the urine of dog by way of the same procedure as M7 from human urine.

M9 and M10 were obtained from the bile of rats to which AY4166 was administered perorally (30 mg/kg). One tenth volume of 1 M HCl was added to the pooled bile (0–8 h) and extracted with ethyl acetate. The ethylacetate layer evaporated in vacuo was applied onto a prep. TLC (silica gel, cica-Merck, Tokyo, Japan). The solvent for TLC was chloroform: *n*-butanol: acetic acid:water, 66:26:2:6. After extraction from TLC, the crude M9 and M10 were applied to prep. medium pressure liquid chromatography (C18, 20 × 300 mm, GLScience, Tokyo, Japan) and fractionated with the mixture of 0.05 M phosphate buffer (pH 6.6) and acetonitrile (90:10) as mobile phase. The collected fraction was then applied to a prep. HPLC (Inertsil ODS, 20 × 250 mm, GLScience, Tokyo, Japan) and fractionated with the same mobile phase as the above medium pressure liquid chromatography.

Spectral methods

The NMR spectra of M1–10 and AY4166 were obtained by using samples of approximately 0.5–5 mg substances dissolved in 0.6 mL of CD_3OD. ^1H and ^{13}C NMR spectra of M1–10 and AY4166 were recorded on a JEOL EX400 spectrometer and a JEOL α400 spectrometer operating at 399.78 MHz (EX400) or 400.18 MHz (α400) for ^1H, and 100.53 MHz (EX400) or 100.63 MHz (α400) for ^{13}C. The NMR experiments were performed using microprograms from the standard JEOL library. The measurement temperature was 25 °C. The other ^1H and ^{13}C NMR spectra were observed on a Varian Gemini at 300 and 75 MHz, respectively. FAB-MS were recorded on a JEOL DX300 instrument. High resolution-mass spectra (HR-MS) of synthetic compounds were performed by Toray Research Center and Ajinomoto Co., Inc. IR spectra were obtained on a Perkin–Elmer 1600 series FT-IR spectrometer. Optical rotations were measured on a JASCO DIP-370 Digital Polarimeter. Melting points were measured on a Yamato Model MP-21 apparatus and uncorrected.

Enzymatic digestion

β-Glucronidase reaction was performed as follows. M4, M5 and M6 (1 mg/1.7 mL phosphate buffer, pH 5.0) was treated with the enzyme (1000 unit/mL, 10, 100, 200, 1000 × dilution) at 35 °C, for 2 h. Reaction products were analysed by HPLC.

Synthesis

***trans*-4- (1-Hydroxy-1-methylethyl) cyclohexanecarboxylic acid (2).** Methyl magnesium bromide (250 mL of a 1 M solution in THF, 250 mmol) was added to a soln of methyl ester **1** (15.13 g, 81.25 mmol) in THF (340 mL) under cooling in an ice bath. The mixture was stirred at room temperature for 3 h, then at 40 °C overnight, and finally 1 M hydrochloric acid (85 mL) was added. After sepn, the aq phase was extracted with ethyl acetate (300 mL) and the combined organic layers dried over $MgSO_4$. Evapn gave a yellow oil which was column chromatographed on silica gel eluting with dichloromethane:methanol (20:1) to give **2** (12.69 g, 84%) as a colorless solid. Mp 129.6–130.0 °C; IR (KBr, cm^{-1}) 1715; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.08 (dq, J=3.0, 12.9 Hz, 2H), 1.18 (s, 6H), 1.30 (tt, J=3.0, 12.3 Hz, 1H), 1.44 (dq, J=3.0, 12.9 Hz, 2H), 1.85–1.99 (m, 2 H), 2.03–2.16 (m, 2 H), 2.26 (tt, J=3.6, 12.3 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 26.44, 27.01, 28.86, 42.90, 48.15, 72.70, 181.23; HRMS(EI) calcd for $C_{10}H_{18}O_3$ (M^+) 186.1256, found 186.1247.

***N*-[*trans*-4-(1-Hydroxy-1-methylethyl) cyclohexanecarbonyl]-D-phenylalanine (M1).** 0.52 M sodium carbonate (170 mL) was added to a soln of D-Phe-OBn *p*-toluenesulfonate (33.33 g, 80.99 mmol) in ethyl acetate (250 mL), then stirred at room temperature for 30 min. The organic layer was sepd, and the aq layer extracted with ethyl acetate (2 × 100 mL). The combined extracts were dried over $MgSO_4$, filtered and evapd to give an oil. This was dissolved in a mixture of dichloromethane (330 mL) and THF (240 mL), and **2** (12.69 g, 68.13 mmol) was added. The mixture was cooled in an ice

bath, HOBt (9.94 g, 73.56 mmol) and DCC (15.12 g, 73.28 mmol) were added and, then stirred overnight. The precipitate was filtered off, washed with ethyl acetate, and the combined filtrate and washings evapd. The residue was dissolved in ethyl acetate (300 mL), and washed, successively with 0.1 M hydrochloric acid (200 mL), water (200 mL), 5% sodium bicarbonate (200 mL), and water (200 mL). The organic layer was dried over $MgSO_4$, filtered and evapd to give an oil. The crude product was column chromatographed on silica gel eluting with ethyl acetate:hexane (3:1) to give crude **3** as a colorless amorphous solid (27.0 g).

The benzyl ester **3** (22.05 g, 52.06 mmol) was dissolved in methanol (500 mL), treated with 10% Pd/C (5.81 g), and stirred at room temperature under a hydrogen atmosphere (1 atm) for 22 h. The mixture was purged thoroughly with Ar gas, filtered through Millipore filter, and concd in vacuo. The residue was recrystallized from ethyl acetate to give M1 (8.08 g, 47%) as a colorless solid. Mp 175.4–175.8 °C; $[\alpha]_D^{22}$ −34.4° (*c* 1.0, 0.1 M; NaOH); IR (KBr, cm^{-1}) 1726, 1651, 1531, 1212, 701;^{1}H NMR (CD_3OD, 400 MHz): δ 0.98–1.15 (m, 2H), 1.10 (s, 6H), 1.20–1.44 (m, 3H), 1.63–1.71 (m, 1H), 1.80–1.92 (m, 3H), 2.10 (tt, J=3.4, 12.1 Hz, 1H), 2.94 (dd, J=9.3, 13.9 Hz, 1H), 3.21 (dd, J=4.9, 13.9 Hz, 1H), 4.66 (dd, J=4.9, 9.3 Hz, 1H), 7.16–7.28 (m, 5H);^{13}C NMR (CD_3OD, 100 MHz): δ 26.85, 26.89, 27.70, 27.77, 30.47, 30.81, 38.43, 46.08, 49.67, 54.65, 73.18, 127.75, 129.38, 130.33, 138.57, 174.90, 178.95; HRMS (FAB) calcd for $C_{19}H_{28}NO_4$ (MH^+) 334.2019, found 334.1987.

***trans*-4-Acetylcyclohexanecarboxylic acid methyl ester (4).** Monoester **1** (15.06 g, purity 85%, 68.74 mmol) was added to thionyl chloride (27 mL) and stirred with heated in an oil bath (50 °C) for 3 h, then evapd in vacuo to give the acid chloride. Methyllitium (225 mL of 1.4 M solution in diethyl ether, 0.315 mol) was added dropwise to cuprous iodide (27.01 g, 0.142 mol) in THF (30 mL) at −40 °C in 20 min, then the mixture was stirred for 2 h. The mixture was cooled to −60 °C, and a soln of the acid chloride in THF (30 mL) was added dropwise. After 30 min, methanol (35 mL) was added and allowed to reach room temperature, and ethyl acetate (100 mL), then satd ammonium chloride solution (150 mL) added. The organic phase was sepd and the aq layer extracted with ethyl acetate (3 × 100 mL). The combined extracts were dried over $MgSO_4$, filtered and evapd. The crude product was column chromatographed on silica gel eluting with ethyl acetate:hexane (1:2) to give **4** (6.56 g, 52%) as a colorless oil.: IR (neat, cm^{-1}) 1734, 1707; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.27–1.56 (m, 4H), 1.94–2.13 (m, 4H), 2.15 (s, 3H), 2.22–2.40 (m, 2H), 3.67 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 27.36, 28.06, 28.11, 42.47, 50.38, 51.58, 175.90, 211.33; HRMS (EI) calcd for $C_{10}H_{16}O_3$ (M^+) 184.1099, found 184.1101.

***trans*-4-Isopropenylcyclohexanecarboxylic acid methyl ester (5).** To a suspension of zinc powder (21.62 g, 0.330 mol) in dibromomethane (7.6 mL) and THF (250 mL) at −40 °C was added dropwise $TiCl_4$ (8.5 mL, 77.5 mmol). The mixture was allowed to warm to 5 °C and stirred at this temperature for 4 days under an Ar atmosphere. The slurry was cooled in an ice-water bath and dichloromethane (40 mL) was added. To the stirred mixture was added **4** (13.82 g, 75.0 mmol) in dichloromethane (40 mL) over 20 min. The cooling bath was removed and the mixture was stirred at room temperature for 3.5 h. A slurry of sodium bicarbonate (120 g) in water (200 mL) was added to the mixture over 45 min and stirred for 1.5 h, then extracted with hexane (2 × 200 mL). The residue was filtered, washed with hexane (4 × 100 mL), and the filtrate further extracted with hexane (100 mL). The combined extracts and washings were dried over $MgSO_4$, filtered and evapd. The crude product was column chromatographed on silica gel eluting with ethyl acetate:hexane to give **5** (6.95 g, 51%) as a colorless oil. IR (neat, cm^{-1}) 1738, 1643; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.12–1.30 (m, 2H), 1.47 (dq, J=3.0, 12.9 Hz, 2H), 1.71 (s, 3H), 1.78–1.95 (m, 3H), 1.97–2.10 (m, 2H), 2.25 (tt, J=3.6, 12.3 Hz, 1H), 3.66 (s, 3H), 4.67 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 20.79, 29.00, 30.72, 43.01, 44.39, 51.35, 108.35, 149.92, 176.31; HRMS (EI) calcd for $C_{11}H_{18}O_2$ (M^+)182.1307, found 182.1283.

***trans*-4-(2-Hydroxy-1-methylethyl)cyclohexanecarboxylic acid methyl ester (6).** A soln of **5** (8.04 g, 44.1 mmol) in THF (70 mL) was cooled in an ice-water bath, 1 M BH_3–THF (33.5 mL, 33.5 mmol) was added dropwise, and the mixture stirred at room temperature under Ar for 30 min. To the mixture cooling in an ice-water bath was added water (34 mL), 1 M sodium hydroxide (34 mL), and 30% hydrogen peroxide (17 mL). After stirring at room temperature for 1 h, the mixture was acidified to pH 2 with 1 M hydrochloric acid and extracted with ethyl acetate (3 × 100 mL). The combined extracts were dried over $MgSO_4$, filtered, and evapd to give an oil. The crude product was column-chromatographed on silica gel eluting with ethyl acetate:hexane to give **6** (7.62 g, 86%) as a colorless oil. IR (neat, cm^{-1}) 3408, 1735; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (d, J=6.9 Hz, 3H), 0.94–1.20 (m, 2H), 1.30–1.59 (m, 4H), 1.68–1.82 (m, 2H), 1.94–2.08 (m, 2H), 2.23 (tt, J=3.6, 12.3 Hz, 1H), 3.48 (dd, J= 6.6, 10.5 Hz, 1H), 3.61 (dd, J= 6.0, 10.5 Hz, 1H), 3.66 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.32, 27.70, 29.03, 29.16, 29.83, 38.45, 40.53, 43.35, 51.46, 66.07, 176.54; HRMS (EI) calcd for $C_{11}H_{20}O_3$ (M^+) 200.1412, found 200.1409.

***trans*-4-(2-Hydroxy-1-methylethyl)cyclohexanecarboxylic acid (7).** To a stirred soln of **6** (7.12 g, 35.6 mmol) in a mixture of methanol (70 mL) and acetone (70 mL) was added 1 M sodium hydroxide (90 mL). After stirring at room temperature overnight, the mixture was acidified to pH 2.5 with 6 M hydrochloric acid and extracted with ethyl acetate (3 × 100 mL). The combined extracts were dried over $MgSO_4$, filtered and dried in vacuo to give **7** (6.19 g, 93%) as a colorless solid. Mp 111.6–112.2 °C; IR (KBr, cm^{-1}) 3301, 1681; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.90 (d, J= 6.9 Hz, 3H), 0.96–1.22 (m, 2H), 1.29–1.59 (m, 4H), 1.68–1.83

(m, 2H), 1.98–2.12 (m, 2H), 2.25 (tt, J=3.6, 12.3 Hz, 1H), 3.50 (dd, J=6.6, 10.5 Hz, 1H), 3.62 (dd, J=5.7, 10.5 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.32, 27.63, 28.84, 28.98, 29.77, 38.40, 40.48, 43.13, 66.10, 181.65; HRMS (EI) calcd for $C_{10}H_{18}O_3$ (M^+) 186.1256, found 186.1244.

***N*-[*trans*-4-(2-Hydroxy-1-methylethyl)cyclohexanecarbonyl]-D-phenylalanine (M2 and M3)**. By a procedure similar to that for **2**, **7** (5.21 g) was coupled with D-Phe-OBn (14.87 g) to give **8** (11.43 g, 90%). The crude **8** (10.50 g) was hydrogenolysed by treating with 10%Pd/C (1.75 g) and recrystallized from ethanol:water to give a mixture of diastereomers M2 and M3 as a colorless solid (4.03 g, 49%). IR (KBr cm^{-1}) 3316, 1714, 1639, 1539, 1012, 696; ^{1}H NMR (CD_3OD, 400 MHz): δ 0.85 (d, J=7.1 Hz, 3H), 0.93–1.16 (m, 2H), 1.25–1.38 (m, 3H), 1.43 (m, 1H), 1.63–1.73 (m, 3H), 1.74–1.82 (m, 1H), 2.07 (tt, J=3.2, 12.1 Hz, 1H), 2.96 (dd, J=8.3, 13.9 Hz, 1H), 3.22 (dd, J=5.1, 13.9 Hz, 1H), 3.35 (dd, J=6.7, 10.7 Hz, 1H), 3.49 (dd, J=5.9, 10.7 Hz, 1H), 4.49 (dd, J=5.1, 8.3 Hz, 1H), 7.12–7.25 (m, 5H); ^{13}C NMR (CD_3OD, 100 MHz): δ 13.78, 28.71, 30.37, 31.18, 31.25, 38.83, 39.86, 41.79, 46.49, 56.91, 66.25, 127.43, 129.21, 130.58, 139.43, 178.54, 179.67; HRMS (FAB) calcd for $C_{19}H_{28}NO_4$ (MH^+) 334.2019, found 334.1986.

***trans*-4-Isopropenylcyclohexanecarboxylic acid (9)**. To the soln of **5** (12.5 g, 68.6 mmol) in methanol (140 mL) under cooling in an ice-water bath was added 1 M sodium hydroxide (140 mL). After being stirred for 4 h at room temperature, methanol was removed by rotary evaporation. The residue was washed with diethyl ether (2×100 mL), acidified to pH 1 with 2 M HCl, and extracted with dichloromethane (2×200 mL). The combined extracts were dried over $MgSO_4$, filtered and dried in vacuo to give **9** (10.5 g, 91%) as a colorless solid. Mp 107.2–107.6 °C; IR (KBr, cm^{-1}) 1692, 1643; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.14–1.32 (m, 2H), 1.49 (dq, J=3.0, 12.9 Hz, 2H), 1.72 (s, 3H), 1.81–1.95 (m, 3H), 2.02–2.14 (m, 2H), 2.29 (tt, J=3.6, 12.3 Hz, 1H), 4.69 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.90, 28.85, 30.69, 42.94, 44.40, 108.52, 149.98, 182.44; HRMS (EI) calcd for $C_{10}H_{16}O_2$ (M^+) 168.1150, found 168.1145.

***N*-(*trans*-4-Isopropenylcyclohexanecarbonyl)-D-phenylalanine (M7)**. By a procedure similar to that for **2**, **9** (10.42 g, 61.94 mmol) was coupled with D-Phe-OMe to give crude **10** (20.7 g). To a soln of **10** in methanol (400 mL) was added 1 M sodium hydroxide (140 mL) and this mixture was stirred at room temperature for 1 h. The mixture was cooled in an ice-water bath and 2 M hydrochloric acid was added to pH 11.5. After evapn of methanol, the residue was filtered and washed with water. The filtrate and washing were combined and acidified to pH 1.5 with 2 M hydrochloric acid, then stirred for 2 h at 0 °C. The precipitate was filtered and washed with water to give a crude product which was repeatedly recrystallized from methanol–water to give M7 (11.09 g, 57%) as a colorless solid. Mp 130.2–130.8 °C; $[\alpha]_D^{22}$ −35.4° (*c* 1.0, 0.1 M NaOH); IR (KBr, cm^{-1}) 3311, 1735, 1646, 1540, 699; ^{1}H NMR (CD_3OD, 400 MHz): δ 1.15–1.49 (m, 4H), 1.62–1.70 (m, 1H), 1.69 (s, 3H), 1.70–1.90 (m, 4H), 2.14 (tt, J=3.4, 12.0 Hz, 1H), 2.95 (dd, J=9.3, 13.9 Hz, 1H), 3.22 (dd, J=5.1, 13.9 Hz, 1H), 4.66 (s, 2H), 4.66 (dd, J=5.1, 9.3 Hz, 1H), 7.17–7.30 (m, 5H); ^{13}C NMR (CD_3OD, 100 MHz): δ 21.04, 30.35, 30.70, 32.03, 32.10, 38.42, 45.87, 46.00, 54.69, 108.94, 127.78, 129.40, 130.32, 138.53, 151.46, 175.03, 178.90; HRMS (FAB) calcd for $C_{19}H_{26}NO_3$ (MH^+) 316.1912, found 316.1934.

***N*-(*trans*-4-Isopropylcyclohexanecarbonyl)-D-tyrosine (M8)**. A *N*-hydroxysuccinimide ester (**12**) was prepared in the manner described in the literature.[2] To a suspension of D-Tyr-OBn *p*-toluenesulfonate (10.0 g, 22.5 mmol) in a mixture of THF (70 mL) and dichloromethane (70 mL) was added triethylamine (3.1 mL, 22.2 mmol) and **12** (6.0 g, 22.4 mmol). After being stirred for 19 h at room temperature, dichloromethane was removed by rotary evaporation and the residue extracted with ethyl acetate (200 mL). The extract was washed, successively with 0.1 M hydrochloric acid, water, satd sodium bicarbonate, and brine, then dried over Na_2SO_4. After evapn, the residue was column chromatographed on silica gel eluting with ethyl acetate:hexane (1:2) to give **13** (7.81 g) which contained unreacted **12** (0.9 g) from ^{1}H NMR observation.

In a similar manner for **3**, crude **8** (7.49 g, 17.7 mmol) was hydrogenolysed with 10% Pd/C (2 g) in methanol (130 mL). The crude product was washed with dichloromethane (100 mL), then recrystallized from acetone–water to give M8 (4.2 g, 71%) as colorless crystals. Mp 164.8–165.4 °C; IR (KBr, cm^{-1}) 1723, 1615, 1538, 1516, 828; $[\alpha]_D^{22}$ −60.0° (*c* 1.0, 0.1 M NaOH); ^{1}H NMR (CD_3OD, 400MHz): δ 0.86 (d, J=6.8 Hz, 6H), 0.95–1.16 (m, 3H), 1.24–1.44 (m, 3H), 1.64–1.71 (m, 1H), 1.71–1.85 (m, 3H), 2.10 (tt, J=3.4, 12.2 Hz, 1H), 2.85 (dd, J=9.0, 14.0 Hz, 1H), 3.10 (dd, J=5.1, 14.0 Hz, 1H), 4.58 (dd, J=5.1, 9.0 Hz, 1H), 6.68 (d, J=8.5 Hz, 2H), 7.02 (d, J=8.5 Hz, 2H); ^{13}C NMR (CD_3OD, 100 MHz): δ 20.15, 30.09, 30.18, 30.55, 30.90, 34.12, 37.68, 44.83, 46.30, 54.95, 116.16, 129.20, 131.32, 157.32, 175.08, 179.05; HRMS (FAB) calcd for $C_{19}H_{28}NO_4$ (MH^+) 334.2018, found 334.2026.

***N*-[*trans*-4-(1-Carboxyethyl)cyclohexanecarbonyl]-D-phenylalanine (M9 and M10)**. To an acetone (1 mL)-solution of Jones reagent (0.4 mL) which was prepared by dissolving chromium trioxide (2.67 g) in concd sulfuric acid (2.3 mL) and diluting to 10 mL with water, was added portionwise a suspension of a mixture of M2 and M3 (0.2 g, 0.60 mmol) in acetone (4 mL), while stirring and cooling in an ice-water bath. The mixture was stirred for 3 h while cooling, then 2-propanol (0.2 mL) was added. After being further stirred for 1.5 h, the resultant mixture was poured into water (10 mL), and extracted with ethyl acetate (3×50 mL). The combined extracts were washed with brine, dried over Na_2SO_4, filtered and evapd. The residue was washed with dichloromethane to give a mixture of diastereomers M9 and M10 (0.16 g, 77%) as a colorless

solid. IR (KBr, cm^{-1}) 1712, 1624, 1543, 698;^{1}H NMR (CD_3OD, 400 MHz): δ 0.96–1.04 (m, 1H), 1.04–1.12 (m, 1H), 1.08 (d, J=7.3 Hz, 3H), 1.25–1.33 (m, 1H), 1.33–1.43 (m, 1H), 1.44–1.53 (m, 1H), 1.57–1.67 (m, 1H), 1.67–1.76 (m, 1H), 1.75–1.85 (m, 2H), 2.10 (tt, J=3.1, 12.2 Hz, 1H), 2.14–2.22 (m, 1H), 2.92 (dd, J=9.8, 14.0 Hz, 1H), 3.21 (dd, J=4.9, 14.0 Hz, 1H), 4.64 (dd, J=4.9, 9.8 Hz, 1H), 7.17–7.28 (m, 5H);^{13}C NMR (CD_3OD, 100 MHz): δ 14.44, 29.73, 30.15, 30.50, 31.21, 38.41, 41.21, 45.84, 46.34, 54.65, 127.75, 129.37, 130.31, 138.57, 174.86, 178.69, 180.03; HRMS (FAB) calcd for $C_{19}H_{26}NO_5$ (MH^+) 348.1810, found 348.1814.

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