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Absolute Configuration of a Ceramide with a Novel Branched-chain Fatty Acid Isolated from the Epiphytic Dinoflagellate, *Coolia monotis*

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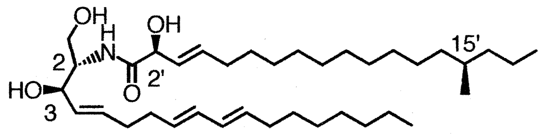
The absolute configuration of the chiral center at the C15 position of a novel branched-chain fatty acid derived from a new ceramide isolated from the epiphytic dinoflagellate *Coolia monotis* was determined to be of *R* from by reversed-phase HPLC after cleavage to 12-methylpentadecanoic acid and subsequent conversion with the chiral fluorescent reagent, (1*R*,2*R*)-2-(2,3-anthracenedicarboximido)cyclohexanol.

Key words: absolute configuration; chiral discrimination; branched-chain fatty acid; chiral conversion reagent

A new ceramide (**1**) with a novel branched-chain fatty acid has been isolated from the epiphytic dinoflagellate, *Coolia monotis* (Fig. 1).[1)] The fatty acid moiety was the first example of a C18 fatty acid with a methyl branch at C15. The structure of the ceramide including, the absolute configurations of the chiral centers at C2, C3 and C2′, was determined mainly by 2D NMR, chiral HPLC and circular dichroism spectra. However, the absolute configuration of the remaining chiral center at C15′ was not determined due to the difficulty of discriminating the chirality of the branched methyl group located far from such functional groups as the carboxylic, hydroxy and amino groups. The prediction of the absolute configuration of optically active methyl branched pheromones by Mori *et al.* has been the only one reported to our knowledge.[2)] Their method made it possible to predict the absolute configuration of some branched-chain alkyl alcohols by combining them with mesogens to give liquid crystalline properties and then measuring the helical sense of the chiral nematic phases. However, the method is not sensitive enough to determine the absolute configuration with a μg order of sample.

We have recently reported some chiral fluorescent conversion reagents with a very strong fluorescent 2,3-anthracenedicarboximide group.[3–6)] These reagents have made it possible to discriminate the chirality of methyl branched fatty acids having a chiral center from the C2 to C12 position of the carbon chain by reversed-phase HPLC. Their chiral discrimination ability was partly attributable to the chiral bent conformation of the labelled derivative. In that conformation, the methyl groups of (*S*) and (*R*)-branched fatty acid derivatives had different spatial directions toward the 2,3-anthracenedicarboximide group of the reagent. Among these reagents, (1*R*,2*R*)-2-(2,3-anthracenedicarboximido)cyclohexanol (**2**) has successfully discriminated the chirality of 12-methyltetradecanoic acid by reversed-



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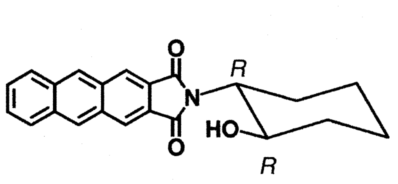

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Fig. 1. Structures of the Ceramide (**1**) and Chiral Conversion Reagent (**2**).

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Abbreviations: HRMS, high-resolution mass spectrum; NBA, 3-nitrobenzyalcohol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimido hydrochloride phase HPLC.[6)] Moreover, the derivative could be detected with high sensitivity (10^{-15} mole level) by fluorometric detection. In this present study, the determination of the absolute configuration of the fatty acid moiety of ceramide **1** with **2** is reported.

Materials and Methods

The sample conversion and clean-up were performed by the method described previously.[6)]

The HPLC pump used was a Jasco PU-980 (Japan Spectroscopic Co., Tokyo, Japan) equipped with a Rheodyne 7125 sample injector with a 20-μl sample loop, and fluorescence was detected with a Jasco FP-920 with a 3-μl flow cell. The integrator was a Chromatocorder 12 (System Instrument, Tokyo, Japan), and a Cryocool CC100-II was used to control the column temperature. HPLC separation was performed in a Develosil ODS-3 column (3 μm particle size, 4.6 mm I.D. × 150 mm, Nomura Chemical Co., Japan) eluted with methanol/acetonitrile/*n*-hexane (300:200:22.5, v/v/v) at 0.6 ml min^{-1} and -40°C. Diastereomeric derivatives were detected by fluorometry at 462 nm (excitation at 298 nm).

Measurement of the ^{1}H-NMR and mass spectra were carried out by Varian Unity INOVA 500 and Jeol JMS HX-105 instruments, respectively, and the products were isolated by silica gel (70–230 mesh) column chromatography.

(R)-2,6-Dimethyl-2-nonen (**4**). A mixture of (*S*)-(−)-citroneroll (**3**, 10 g, 64 mmol, $[\alpha]^{20}_{\mathrm{D}} = -3.12°$, $\mathrm{CHCl_3}$, $c = 0.995$; 59.8% e.e., from Kanto Chemical Co.) and *p*-toluenesulfonyl chloride (22 g, 115 mmol) was stirred in pyridine (80 ml) for 3 h at *r.t.* to give a tosylate which was purified by silica gel column chromatography developed with toluene/ethyl acetate (80:1; 18.1 g yield, 91%). This tosylate (15 g, 48 mmol) was reacted with 30 ml of 3.0 M methylmagnesium chloride (90 mmol) overnight in dry THF in a nitrogen atmosphere in the presence of CuI (1.9 g, 10 mmol) to give (*R*)-2,6-dimethyl-2-nonen (**4**, colorless oil, 5.8 g yield, 78.5%). $[\alpha]^{20}_{\mathrm{D}} = -0.91°$ ($c = 2.09$, $\mathrm{CHCl_3}$); IR (NaCl disk): 2700–3000 cm^{-1}; RSMS (Fab, triethanolamine) $[\text{M-1}]^-$: calcd. for $\mathrm{C_{11}H_{21}}$; 153.1635; found, 153.1629; ^{1}H-NMR (δ ppm, $\mathrm{CDCl_3}$, at 20°C): 0.86 (3H, t, 7.3 Hz), 0.88 (3H, t, 7.3 Hz), 1.05–1.16 (2H, m), 1.22–1.36 (4H, m), 1.60 (3H, s), 1.68 (3H, d, 1.0 Hz), 5.10 (1H, tq, 7.1 Hz and 1.0 Hz).

(R)-4-Methylheptanoic acid (**6**). A mixture of $\mathrm{KMnO_4}$ (15.2 g, 96 mmol) and tetrabutylammonium bromide (3.0 g, 10 mol) in $\mathrm{H_2O/CH_2Cl_2}$ (1:1, 100 ml) was stirred for 1 h at 0°C, before **4** (5 g, 32 mmol) in $\mathrm{CH_2Cl_2}$ (15 ml) was added dropwise to it. After stirring for 20 h at *r.t.*, the excess $\mathrm{KMnO_4}$ was quenched with sodium hydrogen sulfite. The reaction mixture was extracted with chloroform after filtration with Celite 545, before a diol (**5**) and carboxylic acid (**6**) were separated in a silica gel column developed with *n*-hexane/ethyl acetate (10:1) to give 3.7 g (62.2%) and 0.55 g (12%), respectively, of the diol and carboxylic acid. Diol **5** (2.3 g, 12 mmol) was oxidized by a mixture of 0.9 g of $\mathrm{KMnO_4}$ (5.7 mmol) and 11.8 g of $\mathrm{NaIO_4}$ (55 mmol) in acetone/water (2:1, 150 ml) for 20 h at *r.t.* to give **6**. Combined acid **6** was purified by passing through a silica gel column developed with *n*-hexane/ethyl acetate (10:1) as a colorless oil in a yield of 1.24 g (72.0%). $[\alpha]^{20}_{\mathrm{D}} = -1.09°$ ($c = 1.40$, $\mathrm{CHCl_3}$); IR (NaCl disk), 3500–3000 cm^{-1} (broad, -OH), 1710 cm^{-1} (carbonyl); HRMS (Fab, triethanolamine) $[\text{M-1}]^-$: calcd. for $\mathrm{C_8H_{15}O_2}$, 143.1072; found, 143.1069; ^{1}H-NMR (δ ppm, $\mathrm{CDCl_3}$, at 20°C): 0.88 (6H, t, 5.6 Hz), 1.08–1.16 (1H, m), 1.24–1.38 (3H, m), 1.40–1.50 (2H, m), 1.68 (1H, m), 2.30–2.44 (2H, m), 11.00 (1H, s).

*(R)-12-Methylpentadecanoic acid (R-**8**)*. Carboxylic acid **6** (4 g, 28 mmol in 20 ml of dry THF) was added dropwise to a suspension of $\mathrm{LiAlH_4}$ (1.6 g, 42 mmol) in 60 ml of dry THF below 10°C, and the mixture was refluxed for 16 h. After cooling it to below 10°C, 50 ml of diethylether and then 7 ml of water were carefully added. After stirring for 30 min and filtering through Celite 545, the alcohol was extracted with diethylether and purified by silica gel column chromatography developed with *n*-hexane/ethyl acetate (6:1; 2.0 g, 63%). A mixture of this alcohol (0.9 g, 6.9 mmol) and *p*-toluenesulfonyl chloride (2.7 g, 14 mmol) was stirred in dry pyridine (20 ml) at 0°C for 3 h to give a tosylate. This tosylate was purified by silica gel column chromatography developed with toluene/ethyl acetate (80:1; 1.78 g, 90.5%). The tosylate (1.8 g, 6.3 mmol) and CuI (3.86 g, 20 mmol) were added at -10°C to an 8-benzyloxyoctylmagnesium bromide (**7**) solution (in dry THF, 50 ml), and the resulting mixture was reacted at -10°C for 1 h and then at *r.t.* for 14 h. The sample of **7** was prepared from magnesium (0.6 g, 25 mmol) and 1-benzyloxyoctyl-8-bromooctane (7.6 g, 25 mmol), which had been synthesized in a 42.8% yield by the monobenzylation of 1,8-octanediol with equimolar benzylbromde in DMF and then by bromination with $\mathrm{CBr_4}$ in the presence of triphenylphosphine in $\mathrm{CH_2Cl_2}$. (*R*)-1-Benzyloxy-12-methylpentadecane was extracted with ethyl acetate and purified by silica gel column chromatography developed with *n*-hexane/ethyl acetate (50:1; 1.1 g, 52.4%), and then 0.81 g (24 mmol) was reductively debenzylated with $\mathrm{H_2}$ in the presence of 10% palladium carbon in ethanol (50 ml) to give (*R*)-12-methyl-1-pentadecanol; this was purified by silica gel column chromatography developed with *n*-hexane/ethyl acetate (6:1; 0.50 g, 84.7%; HRMS (EI) $[\text{M}]^+$, Calcd. for $\mathrm{C_{16}H_{34}O}$, 242.2608; Found, 242.2610). The alcohol (2.7 mmol) was ad-

ded dropwise to a $KMnO_4$ solution (1.27 g, 8 mmol in 20 ml of water/pyridine = 1:1), and the mixture stirred for 14 h at 55°C. After filtration, the filtrate was acidified with a 1N HCl solution, and acid *R*-**8** was extracted with ethyl acetate and purified by silica gel column chromatography developed with *n*-hexane/ethyl acetate (4:1) and obtained as a colorless crystaline mass (0.23 g, 33%). Mp 32–33°C, $[\alpha]^{20}_{D} = -1.38°$ ($c = 1.16$, $CHCl_3$); IR (NaCl disk): 3112 cm^{-1} (broad, -OH), 1706 cm^{-1}(carbonyl); HRMS (EI)$[M]^+$: calcd. for $C_{16}H_{32}O_2$, 256.2401; found, 256.2401; ^{1}H-NMR (δ ppm, $CDCl_3$, at 20°C): 0.84 (3H, d, 6.0 Hz), 0.88 (3H, t, 7.2 Hz), 1.04–1.11 (2H, m), 1.22–1.39 (19H, m), 1.63 (2H, m), 2.35 (2H, t, 7.8 Hz), 11.00 (1H, s).

*(rac)-12-Methylpentadecanoic acid (rac-*__8__*).* Sodium hydride (0.73 g, 30 mmol) was added to dry DMSO (50 ml) at 75°C while vigorously stirring. After cooling, 10-carboxydecyltriphenylphosphonium bromide (**9**, 10 g, 20 mmol, in 30 ml of dry DMSO) was added dropwise while keeping the temperature between 20 and 25°C, and the mixture was stirred for 25 min at *r.t.* The sample of **9** was prepared from 11-bromoundecanoic acid and equimolar triphenylphosphine by refluxing for 16 h in dry acetonitrile, and was obtained as a white powder after evaporating the acetnitrile under reduced pressure, before adding toluene (76.0%). 2-Pentanone was added, the mixture was stirred for 2 h at *r.t.*, and the reaction mixture was then acidified with 1N HCl and extracted with *n*-hexane. The acid was purified by silica gel column chromatography developed with *n*-hexane/ethyl acetate (4:1; 1.1 g, 20.7%). This pure acid (0.9 g) was hydrogenated under H_2 in ethanol in the presence of 10% palladium-activated carbon. *rac*-**8** was purified by silica gel column chromatography developed with *n*-hexane/ethyl acetate (4:1) and obtained as a colorless crystalline mass (0.66 g, 70.4%). Mp 34–35°C; IR (NaCl disk): 3362 cm^{-1} (broad, -OH), 1712 cm^{-1} (carbonyl), HRMS [Fab, 3-nitrobenzylalcohol (NBA)] $[M-1]^-$: calcd. for $C_{16}H_{31}O_2$, 255.2333; found, 255.2333; ^{1}H-NMR (δ ppm, $CDCl_3$, at 20°C): 0.84 (3H, d, 6.0 Hz), 0.88 (3H, t, 7.2 Hz), 1.04–1.11 (2H, m), 1.22–1.39 (19H, m), 1.63 (2H, m), 2.35 (2H, t, 7.8 Hz), 11.00 (1H, s).

(1R,2R)-2-(2,3-Anthracenedicarboximido)cyclohexyl 12-methylpentadecanoate. *R*-**8** (100 mg) was esterified with (1*R*,2*R*)-2 (30 mg) by 50 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in the presence of 32 mg of 4-(dimethylamino)pyridine in 40 ml of toluene/acetonitrile (1:1, v/v). The mixture was reacted at 40°C for more than 10 h. The *R*-**8** derivative was purified by silica gel column chromatography developed with *n*-hexane/ethyl acetate (40:1) and by crystallization with acetone-methanol. The *R*-**8** derivative was obtained as yellow crystals, mp 98–99°C. Elemental analysis: Calcd. for $C_{38}H_{49}NO_4$: C, 78.17; H, 8.47; N, 2.40. Found: C, 78.06; H, 8.34; N, 2.40. HRMS (Fab, NBA) $[M]^-$: calcd for $C_{38}H_{49}NO_4$, 583.3651; found, 583.3659. ^{1}H-NMR (δ ppm, $CDCl_3$, at −20°C): 0.81 (3H, d, 7.8 Hz), 0.88 (3H, t, 7.8 Hz), 0.62–1.59 (26H, m), 1.83–1.91 (3H), 2.13 (2H, t, 8.4 Hz), 2.23 (1H, m), 2.47 (1H, m), 4.33 (1H, m), 5.58 (1H, m), 7.65 (2H, m), 8.11 (2H, m), 8.52 (2H, s), 8.66 (2H, s). Optical purity: 60% e.e. determined by HPLC.

By a similar prodecure, the authentic *rac*-**8** derivative was also obtained as yellow crystals, mp 111°C. HRMS (Fab, NBA) $[M]^-$: calcd. for $C_{38}H_{49}NO_4$, 583.3651; found, 583.3659. ^{1}H-NMR (δ ppm, $CDCl_3$, at −20°C): 0.806 (1.5H, d, 7.8 Hz, branched methyl of (*S*)-8 derivative), 0.808 (1.5H, d, 7.8 Hz, branched methyl of (*R*)-8 derivative) 0.879 (1.5H, t, 7.8Hz, terminal methyl of (*S*)-8 derivative), 0.882 (1.5H, t, 7.8 Hz, terminal methyl of (*R*)-8 derivative), 0.62–1.59 (26H, m), 1.83–1.91 (3H), 2.13 (2H, t, 8.4 Hz), 2.23 (1H, m), 2.47 (1H, m), 4.33 (1H, m), 5.58 (1H, m), 7.65 (2H, m), 8.11 (2H, m), 8.52 (2H, s), 8.66 (2H, s).

Preparation of 12-methylpentadecaoic acid from the ceramide **1**. Ceramide **1** (80 μg), which had been prepared by the reported method from the epiphytic dinoflagellate, *Coolia monotis*,[1)] was acetylated by acetic anhydride in pyridine. Ozonolysis then gave 12-methylpentadecanal in methanol at −78°C. Hydrogen peroxide (31%) was added to the mixture at −78°C, which was reacted for a further 1 h at *r.t.* After extracting with *n*-hexane, **8** was purified by normal-phase HPLC (YMS SIL 06, 6.0 mm I.D. × 150 mm, 1.0 ml min^{-1} flow rate) eluted with *n*-hexane-isopropanol (150:1). This procedure enabled about 5 μg of 12-methylpentadecanoic acid (**8**) to be obtained. After methylation, its presence and purity were confirmed by GC-MS by comparing with an authentic sample of methyl *rac*-12-methylpentadecanoate.

Results and Discussion

To determine the absolute configuration at C15′ of new ceramide **1**, the chiral discrimination of 12-methylpentadecanoic acid was tested because this acid could be easily obtained from the ceramide by oxidative degradation of the double bond between C3′ and C4′. Since chiral discrimination of the 12-methyltetradecanoate of **2** was possible by reversed-phase HPLC,[6)] it was expected that this would be also possible for the 12-methylpentadecanoate of **2**, because discrimination of the chiral center formed by a methyl and propyl group was easier than that formed by a methyl and ethyl group.[6)] It was necessary for the determination of the absolute configuration to prepare authentic optically active and racemic 12-

methylpentadecanoic acids as references.

Preparation of authentic (R) and (rac) 12-methylpentadecanoic acid

Authentic (*R*)-12-methylpentadecanoic acid was prepared from (*S*)-(−)-citronellol (**3**; 59.8% e.e.) as shown in Scheme 1. After tosylation of **3**, the tosylate was converted to (*R*)-2,6-dimethyl-2-nonen (**4**) by reacting with methylmagnesium chloride. The double bond of **4** was oxidized with $KMnO_4$ in the presence of a phase-transfer reagent to give a diol (**5**) and carboxylic acid (**6**) in 62.2% and 12% yields, respectively. After separating **5** and **6**, **5** was oxidized to **6** with $NaIO_4/KMnO_4$ (72.0%). After the reduction of carboxylic acid **6** and subsequent tosylation, the tosylate was reacted with 8-benzyloxyoctylmagnesium bromide (**7**) to give (*R*)-1-benzyloxy-12-methylpentadecane in a 52.4% yield. After debenzylation, the resulting alcohol was oxidized to (*R*)-12-methylpentadecanoic acid (*R*-**8**). *Racemic* 12-methylpentadecanoic acid (*rac*-**8**) was prepared from **9** and 2-pentanone by the Wittig reaction and subsequent catalytic hydrogenation (Scheme 2).

HPLC separation of the 12-methylpentadecanoate of 2

Branched-chain fatty acid *R*- and *rac*-**8** were esterified with **2** in the presence of EDC and 4-dimethylaminopyridine in acetonitrile/toluene.[6] The resulting esters were purified by silica gel TLC before their separation by reversed-phase HPLC. The HPLC separation conditions used were slightly modified from those for 12-methyltetradecanoate (methanol:acetonitrile:*n*-hexane = 300:200:20 to 300:200:22.5) because their retention times were too long (more than 250 min) under the original conditions. The *rac*-**8** ester of **2** was separated into two peaks at 187 min and 195 min (Fig. 2-a). The *R*-**8** (60% e.e.) derivative also gave the same two peaks but the latter peak was 4 times larger than the former peak (Fig. 2-b). This unequivocally showed that the latter peak was the one derived from *R*-**8**. This elution order was the same as that of 12-methyltetradecanoate. It should be noted for all branched-chain fatty acids tested until now that their elution order was dependent on the position of the branched methyl group and independent of the chain length.

This method made it possible to separate the 12-methylpentadecanoate of **2** and it could detected at the 10^{-14} mole level by fluorescence detection since the 2,3-anthracenedicarboximide group was a very

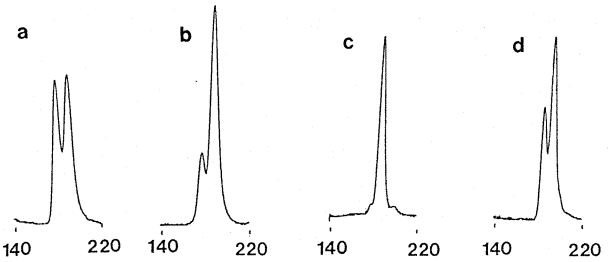
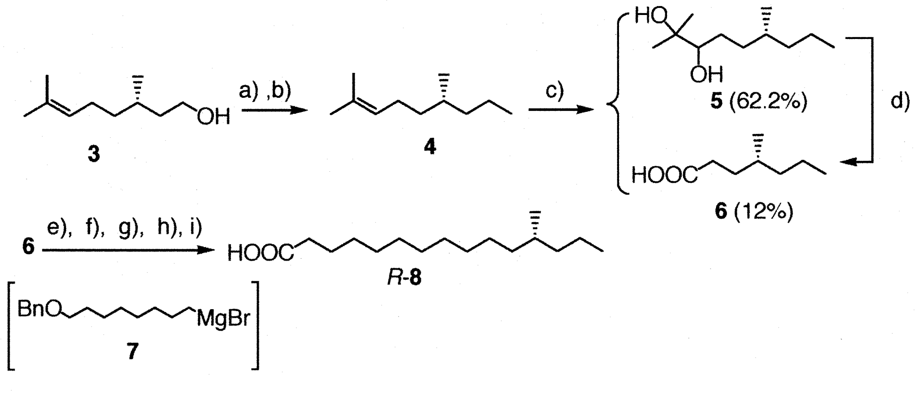
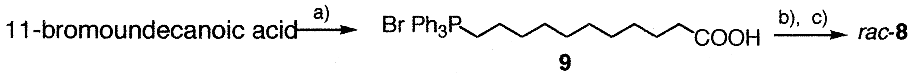


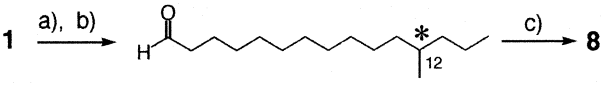
Fig. 2. Chromatograms of the12-Methylpentadecanoate of **2**.
a) **2** derivative of *rac*-**8**, b) **2** derivative of (*R*)-**8**, c) **2** derivative of 12-methylpentadecanoic acid prepared from ceramide **1**, d) a mixture of a) and c).



Scheme 1. Synthesis of (*R*)-12-Methylpentadecanoic Acid (*R*-**8**).
a) tosyl chloride/pyridine, 91.2%; b) MeMgBr, CuI/THF, 0°C→*r.t.*, 78.5%; c) $KMnO_4$, $Et_4NBr/H_2O + CH_2Cl_2$; d) $KMnO_4$, $NaIO_4/H_2O$ + acetone, 72.0%; e) $LiAlH_4$/THF, 63.0%; f) tosyl chloride/pyridine, 90.5%; g) **7**, CuI/THF, 0°C→*r.t.*, 52.4%; h) H_2, Pd-C/EtOH, 84.7%; i) $KMnO_4/H_2O$ + pyridine (1:1) at 50°C, 33.0%.



Scheme 2. Synthesis of (*rac*)-12-Methylpentadecanoic Acid (*rac*-**8**).
a) triphenylphosphine/dry acetinitrile, reflux for 16 h, 76.0%; b) ① NaH/DMSO, 75°C ② 2-pentanone, 20–25°C, 20.7%; c) H_2, Pd-C/EtOH, 70.4%.



Scheme 3. Preparation of the 12-Methylpentadecanoic Acid (**8**) from Ceramide **1**.
a) Ac_2O/pyridine; b) O_3/MeOH, at −78°C; c) 31% H_2O_2, −78°C→*r.t.* (1 h).

strong fluorophore.[7] This method is therefore very useful for the enatiomeric discrimination of methyl-branched fatty acids on a μg to ng scale.

Determination of the absolute configuration of ceramide **1**

To discriminate the chiral center at C15′ of ceramide **1**, **1** (80 μg) was decomposed to 12-methylpentadecanoic acid by the procedure shown in Scheme 3, about 5 μg of **8** being obtained. A 0.6-μg aliquot of **8** converted with reagent **2** and purified by silica gel TLC. Since the retention time of the derivative was agreed with that of the authentic (*R*)-12-methylpentadecanoate of **2**, the absolute configuration at C15′ of the branched-chain fatty acid moiety of ceramide **1** was determined to be *R* (Figs. 2-c and d).

It was possible by this method to determine absolute configuration of less than 80 μg of the ceramide by a comparison with an authentic sample. Branched-chain fatty acids have also been found in some mammalian tissues,[8] fish[9] and sponge.[10] The highly sensitive remote chiral discrimination method will be one of the most powerful tools for determing the absolute configuration of many natural products.

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