A New Ceramide with a Novel Branched-Chain Fatty Acid Isolated from the Epiphytic Dinoflagellate *Coolia monotis*

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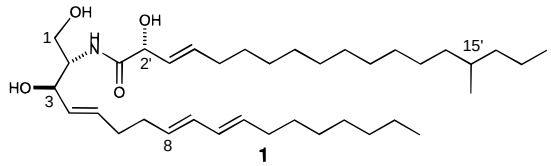
From the dinoflagellate *Coolia monotis*, a new ceramide (**1**) bearing a 2-hydroxy-15-methyl-3-octadecenoyl moiety was isolated as a cellular constituent. The structure, including the partial absolute configuration, was elucidated as **1** on the basis of the 2D NMR of **1**, and chiral HPLC and CD examinations with benzoyl derivatives of its degradation products. To our knowledge, this is the first example, from natural sources, of a C_{18} fatty acid with a methyl group substituted at a C15 carbon.

Dinoflagellates are known to be a rich source of structurally and biologically intriguing natural products.[1] In particular, epiphytic species, which dwell on the surface of algae and corals, have been shown to produce unique metabolites[2] with powerful bioactivities, such as ciguatoxins,[3] maitotoxin,[4] and palytoxin derivatives.[5] During our search for bioactive compounds from microalgae, we have encountered potent toxins at a much higher incidence in epiphytic species than in planktonic species.[6] Among the former, *Coolia monotis* Meunier (Ostreopsidaceae) attracted our attention because of its production of interesting metabolites, including cytotoxins.[7] Separation of the cytotoxic constituents from cultured cells led to the isolation of diacylgalactosylglycerolipid (**2**), which is a rather common metabolite of marine algae, as one of major active components.[8] Further HPLC fractionation resulted in the isolation of an unknown ceramide. In this report, we describe the isolation and structure of a new ceramide bearing a novel branched-chain fatty acid.



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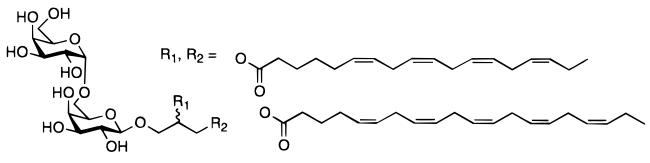

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From *C. monotis* cells harvested from 180 L of media, ceramide (**1**) (2.4 mg) was obtained as an amorphous solid; no prominent bioactivity has been found so far.[9] Structure elucidation was carried out mainly by spectroscopic methods including 2D NMR, chiral HPLC, and CD spectra. The HRFABMS revealed $[M + Na]^+$ at *m/z* 612.4968. By considering this along with ^{13}C NMR and $^{13}C-^1H$ COSY data, the molecular formula of **1** was deduced to be $C_{37}H_{67}O_4N$ (calcd 612.4946). The NMR spectra of **1** implied a ceramide-like structure because the ^1H NMR spectrum of **1** revealed signals at δ 4.08, 3.83, 3.80, and 3.68, which agreed well with those for an usual *N*-acyl-D-erythrosphingosine.[10] On the basis of $^1H-^1H$ COSY data, the sphingosine moiety of **1** was elucidated to be an 8,9,10,11-tetradehydrosphingosine derivative, although the number of carbons on an alkyl chain remained unknown. NMR data also suggested that the fatty acid part had a 2-hydroxyl-3,4-dehydroacyl structure. Methanolysis of **1** yielded sphingosine **3** and fatty acid methyl ester **4**; their molecular weights were shown to be *m/z* 295 and 326, respectively, which indicated that there were 18 carbon atoms in **3** and 19 in **4**. A notable characteristic of the ^1H NMR spectra was the presence of one doublet methyl signal together with two triplet methyl groups corresponding to the two termini for sphingosine and fatty acyl moieties. The position of the doublet methyl substitution was elucidated by NMR examination of **1** and **4**. The doublet signal at δ 0.85 was observed only for **4**, and its location was determined unambiguously by HMBC experiments with **1**; a methylene signal at δ 39.1 gave rise to HMBC crosspeaks against both doublet methyl and terminal methyl signals, while the methyl-substituted methine carbon (C15′) gave an HMBC correlation only to the doublet methyl signal. The methyl substitution at C15 was further supported by the reported values by Higuchi et al.[11] and chemical shift calculations.[12] These spectral interpretations led to the elucidation of the planar structure of **1**. The 8,9,10,11-tetradehydrosphingosine moiety was previously reported by Irie et al. as a cerebroside from starfish.[13]

The stereochemistry of **1** was elucidated on the basis of chiral HPLC and CD spectra of benzoate derivatives derived from degradation products. The fatty acid methyl ester **4** was degraded by O_3, and then reduced with $NaBH_4$, to furnish methyl glycerate. Bisbenzoyl ester **5** derived from the methyl glycerate was subjected to chiral HPLC analysis together with authentic samples of *R*- and *S*-bis-benzoylmethylglycerate. The HPLC retention time of **5** agreed well with that of the authentic *R*-derivative, which was clearly separated from the *S*-enantiomer.

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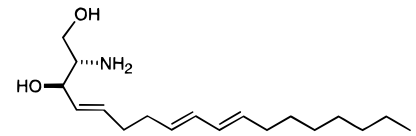
The relative configuration between C2 and C3 was shown to be erythro because the ^{1}H chemical shift and coupling constants for H_2-1, H-2, and H-3 of **3** agreed well with those of authentic D-erythrosphingosine. To convert **3** to a stereochemically defined structure for CD measurements, the sphingosine was subjected to hydrogenation followed by benzoylation with *p*-bromobenzoyl chloride to afford a trisbromobenzoyl derivative **6**. The corresponding authentic sample was prepared from D-erythrosphingosine by the same procedure. Their CD spectra agreed very well; **6** gave typical split cotton effects with the first negative peak at 253 nm ($\Delta\epsilon$ −11.6) and the second positive peak at 232 ($\Delta\epsilon$ 5.5) nm. These data unambiguously supported the absolute configuration of **1** (2*S*,3*R*,2′*R*).



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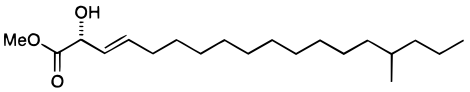

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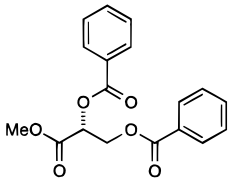

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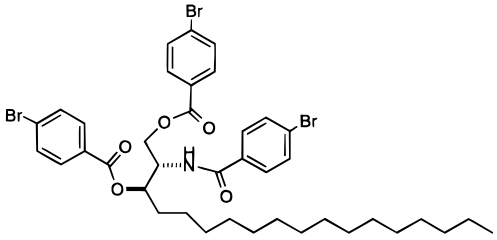

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The stereochemistry of **1** is consistent with that of ceramides reported so far,[14] including the C2′ position of 2-hydroxylacyl moieties. Regarding the remaining chiral center at C15′, attempts at chiral resolution, including gas chromatography, for synthetic 11-methylpentadecanol have thus far been unsuccessful. To our knowledge, this is the first example of a C_{18} fatty acid with a methyl group substituted on a C15 carbon, although similar branched-chain fatty acids with shorter carbon chains have been suggested to be present in marine fishes.[15] Hydrocarbon chains with a methyl group at this position have been reported from several organisms, including a sphingosine part of cerebroside isolated from starfish.[16] From a biosynthetic point of view, it is less plausible that the fourth carbon from the terminus will be methylated compared with the second or third positions, where a methyl group has frequently been introduced to fatty acids.[17,18] The biosynthesis of the latter could be explained by the incorporation of 2- or 3-methylbutyric acid as a starter unit, which was derived from the biosynthetic pathway for isoleucine and leucine, respectively. The methyl branching at the even number of carbon was reported from mammalian sources, such as secretions from Harderian gland of guinea pig,[19] in which methylmalonyl-CoA is involved in lieu of malonyl-CoA.[20] However, the C15 position of **1** should fall on a carbonyl carbon in fatty acid synthesis, hence ruling out the possibility of the involvement of methylmalonyl-CoA. These observations suggest that an unknown pathway of the fatty acid biosynthesis that is unique to the dinoflagellate(s) should be implicated in the introduction of a methyl group; for example, methyl transfer to a C=C bond at C14−C15 from methionine[18] or to a ketone at C15 from acetate as seen for the mevalonate biosynthesis.

In addition to its biosynthetic interest, this unique fatty acid could possibly be used as a chemical marker inasmuch as methyl-branching at the odd number of carbons seems to be very rare and possibly characteristic of *C. monotis* and other related dinoflagellates. This fatty acid could be used for investigating the transportation and accumulation of substances among marine creatures that are part of complicated food-webs.

Experimental Section

General Experimental Procedures. NMR spectra were measured with a JNM A-500 spectrometer (500 MHz, JEOL). Chemical shifts (δ) are referred to internal CD_2HOD at 3.35 ppm for ^{1}H NMR and $^{13}CD_3OD$ at 49.0 ppm for ^{13}C NMR. HRFABMS were recorded on a JMS SX-102 mass spectrometer (JEOL). CD spectra were obtained with a J-720 spectrometer (JASCO) in a quartz cuvette with a cell-pass of 0.2 mm. IR spectra were measured with an FT/IR-300E spectrometer (JASCO). Sphingosine (D-erythro) and *R*- and *S*-glyceric acid were purchased from Sigma.

Culture of *Coolia monotis*. *C. monotis* was collected on the coast of Motobu, Okinawa, Japan, in 1993, and identified by Prof. Y. Fukuyo, the University of Tokyo. This species has been deposited at the Microbial Culture Collection of the National Institute for Environmental Studies, Japan (NIES 615). Unialgal culture of *C. monotis* was carried out in seawater media enriched with ES-1 supplements; in 1 L of seawater were dissolved 820 μM $NaNO_3$, 46 μM α-glycerophosphate Na^+, 825 μM tris[(hydroxylmenthyl)amino]-methane, 16 μM H_3BO_3, 13 μM Na_2−EDTA−$2H_2O$, 87 nM $ZnSO_4$−$7H_2O$, and 3.6 nM $CoSO_4$−$7H_2O$ adjusted to pH 7.8 by HCl. Seawater was passed through a charcoal column and then filtered through a membrane filter (pore size 0.45 μm, nitrocellulose, Advantech). The supplemented media (7 L) in a 10-L cylindrical glass flask were autoclaved at 121 °C for 20 min. After cooling to room temperature, the medium was supplemented with vitamins (300 nM thiamine hydrochloride, 4.1 nM biotin, 1.5 nM vitamin B12) through a membrane filter (pore size 0.2 μm). Seed culture of *C. monotis* (ca. 15 000 cells/mL) was inoculated to a density of 400−800 cells/mL. The culture was maintained at 23−28 °C with a 18-light/6-dark photocycle (1500−3000 Lx) for 4−6 weeks.

Isolation of Ceramide (1). Cells of *C. monotis* were harvested by filtering a culture medium through a glass fiber filter (Advantech, GP-100) and stored at −30 °C until extraction. The algal cells obtained from 180 L of the culture were extracted with MeOH four times and then with Me_2CO three times. The combined extracts were evaporated to dryness and suspended in MeOH−H_2O (1:1). The suspension was washed with *n*-hexane and extracted with CH_2Cl_2. The hexane solution was again extracted with MeOH−H_2O (98:2), and the MeOH phase was combined with the CH_2Cl_2 extracts. After removal of the solvents, the extracts were dissolved in MeOH and passed through a column of HP-20 to remove nonpolar constituents. The eluate was then loaded onto a DIOL column (Lobar Pre-packed, Merck, Size B) and eluted with MeOH. The fraction of 70−110 mL was subjected to further purification with HPLC. Reversed-phase chromatography was carried out with an ODS column (YMC, AM-323−7, ϕ 10 × 250 mm, flow rate, 2.0 mL/min) and linear gradient elutions from MeOH−H_2O (4:1) to 100% MeOH (0.8% per min). A series of ceramides were eluted at 128 min−156 min. Final purification was carried out on the same column with the following elution program; CH_3CN−H_2O−AcOH (17:3:0.04) for 30 min and then a linear gradient of the same mobile phase to 100% CH_3CN at 0.6%/min at a flow rate of 2.0 mL/min. Several ceramides were obtained, among which **1** (2.4 mg) was eluted at 120−128 min as a major constituent.

Methanolysis of Ceramide (1). Ceramide (**1**) (0.5 mg) was subjected to methanolysis in 0.8 mL of 0.75 N HCl in MeOH at 80 °C for 18 h. Methyl ester **4** was extracted from the MeOH solution with hexane. After removal of MeOH, the residue was suspended in H_2O, and sphingosine **3** was extracted with EtOAc.

Preparation of Bis-benzoylmethylglycerate 5. Methyl ester **4** (200 μg) was dissolved in MeOH (800 μL) and oxidized with O_3 at −78 °C followed by the addition of $NaBH_4$ in MeOH until the ozone color disappeared. After removal of the solvent, the product was treated with benzoyl chloride in pyridine for 48 h. The products were subjected to HPLC using a Si gel column (YMC-pack A-024 SIL; ϕ 10 × 250 mm; flow rate, 2.0 mL/min) and eluted with hexane−EtOAc (3:1). The UV-positive fraction at 15 min was separated and used for chiral HPLC. Authentic *R*-glyceric acid (2 mg) was dissolved in H_2O and added along with trimethylsilyldiazomethane (10% hexane solution). After being allowed to stand for 1 h, the excess reagent was quenched with AcOH, and the solvents were removed to afford methylglycerate. The ester was derivatized and purified by the same procedure as described above. Authentic *S*-ester was prepared from *S*-glyceric acid in the same manner.

Chiral HPLC Analysis. Comparison of the benzoyl derivative **5** obtained from the natrual ceramide **1** with authentic bis-benzoylmethylglycerate with *R*- and *S*-configurations was carried out by chiral HPLC analysis; column, Chiral Pak AD (∅ 4.6 × 250 mm, Daicel Chemical Industory); mobile phase, hexane−iPrOH, 500:4; flow rate, 0.5 mL/min; monitor, UV at 254 nm. Bis-benzoylmethylglycerate derived from **1** was eluted at 12′53″ and authentic *R*-ester at 12′56″, while *S*-derivative was detected at 20′34″.

Preparation of Tris(bromo)benzoyl sphingosine 6. Sphingosine **3** (200 μg) obtained by methanolysis was hydrogenated with H_2 on Rh−Al_2O_3 in MeOH for 12 h. After removal of the catalyst and solvent, the product was dissolved in pyridine (100 μL) containing *p*-bromobenzoyl chloride (3 mg) and (dimethylamino)pyridine (2 mg), and left to stand for 12 h at room temperature. After the addition of H_2O, the product was extracted with EtOAc and subjected to HPLC on a Si gel column (YMC-pack A-024 SIL; ϕ 10 × 250 mm; flow rate, 2.0 mL/min) and eluted with hexane−EtOAc (8:1). The UV-positive fraction at 17 min was separated and used for CD measurements. Authentic sphingosine (D-erythro) was derivatized to the corresponding bromobenzoate and purified by the same procedure.

Ceramide (1): IR bands (liquid film) ν_{max} 3360, 2852, 1650, 1538, 1463, 1050 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 7.48 (1H, d, J = 8.5 Hz, N−H), 6.00 (2H, m, H-9, H-10), 5.84, (1H, dtd, J = 1.6, 6.8, 15.5 Hz, H-4′), 5.71 (1H, dt, J = 5.9, 15.3 Hz, H-5), 5.55 (2H, m, H-8, H-11), 5.52 (1H, m, H-3′), 5.49 (1H, m, H-4), 4.44 (1H, d, J = 7.1 Hz, H-2′), 4.08 (1H, t, J = 4.4 Hz, H-3), 3.83 (1H, m, H-2), 3.80 (1H, dd, J = 3.5, 11.1 Hz, H-1), 3.68 (1H, dd, J = 3.1, 11.1, H-1), 2.12 (2H, m, H_2-7), 2.08 (2H, m, H_2-6), 2.05 (4H, m, H_2-12, H_2-5′), 1.38 (5H, m, H_2-13, H_2-6′, H-15′), 1.25−1.35 (24H, m, H_2-14, H_2-15, H_2-16, H_2-17, H_2-7′, H_2-8′, H_2-9′, H_2-10′, H_2-11′, H_2-12′, H_2-13′, H_2-14′), 1.10 (2H, m, H_2-16′), 0.88 (6H, t, J = 6.7 Hz, H_3-18, H_3-18′), 0.85 (3H, d, J = 6.8 Hz, H_3−C15′); ^{13}C NMR (CD_3OD, 125 MHz) δ 174.7 (s, C1′), 134.3 (d, C4′), 133.3 (d, C5), 132.8 (d, C11[a]), 131.6 (d, C9), 131.6 (d, C10), 131.1 (d, C8[a]), 130.8 (d, C4), 128.5 (d, C3′), 73.3 (d, C2′), 72.7 (d, C3), 61.1 (t, C1), 55.5 (d, C2), 40.1 (t, C16′[b]), 37.5 (t, C14′[b]), 32.5−33.2, 29.2−30.5, 27.6 (t, C13′), 23.2 (t, C17), 20.8 (t, C17′), 19.6 (q, *C*H3−C15′), 14.2 (q, C18′), 13.8 (q, C18). [a,b]Assignments with the same letter may be interchanged.

Sphingosine 3: ^1H NMR (CD_3OD, 500 MHz, CD_2HOD taken as δ 3.35) 6.00 (2H, m, H-9, H-10), 5.77 (1H, dt, J = 5.9, 15.3 Hz, H-5), 5.55 (2H, m, H-8, H-11), 5.53 (1H, m, H-4), 4.03 (1H, t, J = 6.4 Hz, H-3), 3.72 (1H, dd, J = 3.5, 11.1 Hz, H-1), 3.54 (1H, dd, J = 3.5, 11.1 Hz, H-1), 2.82 (1H, m, H-2), 2.12 (2H, m, H_2-7), 2.08 (2H, m, H_2-6), 2.05 (2H, m, H_2-12), 1.38 (2H, m, H_2-13), 1.25−1.35 (8H, m, H_2-14, H_2-15, H_2-16, H_2-17), 0.88 (3H, t, J = 6.7 Hz, H_3-18).

Fatty acid methyl ester 4: ^1H NMR ($CDCl_3$, 500 MHz, $CHCl_3$ taken as δ 7.24) 5.86 (1H, dtd, J = 1.6, 6.5, 15.5 Hz, H-4′), 5.48 (1H, m, H-3′), 4.58 (1H, dd, J = 6.5, 1.6 Hz, H-2′), 2.04 (2H, m, H_2-5′), 1.25−1.38 (19H, m, H_2-6′, H_2-7′, H_2-8′, H_2-9′, H_2-10′, H_2-11′, H_2-12′, H_2-13′, H_2-14′, H-15′), 1.05 (2H, m, H_2-16′), 0.86 (3H, t, J = 6.7 Hz, H_3-18′), 0.81 (3H, d, J = 6.8 Hz, H_3−C15′).

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Japan Society for the Promotion of Science (JSPS-RFTF96I00301).

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(9) Ceramide (**1**) showed weak hemolytic activity against rabbit blood cells at 25 μg/mL and no cytotoxicity against mouse leukemia cell P-388 at 40 μg/mL.

(10) 2*S*,3*R*-sphingosine (D-erythro, Sigma) was *N*-acetylated by Ac_2O in MeOH.; ^{1}H NMR in CD_3OD, δ 3.82 (1H, dd, J = 3.1, 11.6 Hz, H-1); 3.68 (1H, dd, J = 3.5, 11.6 Hz, H-1); 3.85 (1H, dt, J = 4.3, 3.4 Hz, H-2); 4.10 (1H, t, J = 4.3 Hz, H-3).

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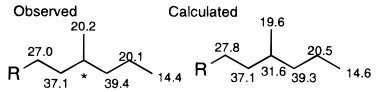
(12) The chemical shifts of 4-methyl-alkane were calculated as follows using the method by Lindemann, L. P. and Adams, J. Q. (*Anal. Chem.* **1971**, *43*, 1245; and Higuchi et al.[11]). δc values were



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