Pitipeptolides A and B, New Cyclodepsipeptides from the Marine Cyanobacterium *Lyngbya majuscula*

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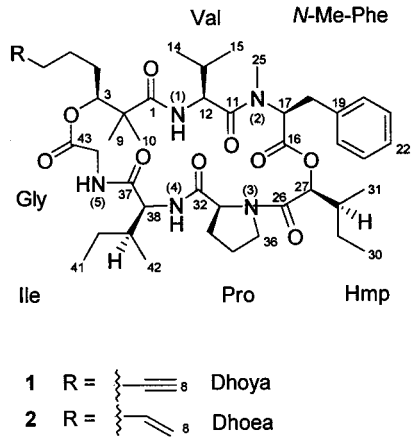
Two new cyclodepsipeptides have been isolated from a population of the marine cyanobacterium *Lyngbya majuscula* collected at Piti Bomb Holes, Guam. They appear to be unique to this particular Guamanian collection and have been named pitipeptolides A (**1**) and B (**2**). Their structures have been elucidated by spectroscopic techniques and by characterization of degradation products. Distinctive features include the presence of a 2,2-dimethyl-3-hydroxy-7-octynoic acid residue in **1** and a 2,2-dimethyl-3-hydroxy-7-octenoic acid residue in **2**, previously shown to be biosynthetic signatures of cyanobacterial metabolites. Pitipeptolides A (**1**) and B (**2**) exhibit weak cytotoxicity against LoVo cancer cells, but possess moderate antimycobacterial activity and stimulate elastase activity.

Cyanobacteria produce a wide array of bioactive secondary metabolites, and therefore, are valuable as a potential source of novel pharmaceuticals.[1] Nevertheless, cyanobacteria may represent a health threat if potent toxins are being produced, especially under bloom conditions.[2] Increasing attention has focused on both the biomedical and ecological aspects of these organisms.[3] We recently initiated chemical investigations of Guamanian varieties of marine cyanobacteria, predominantly strains of *Lyngbya majuscula* Harvey ex Gomont, affording several new cytotoxins whose anticancer activities have been assessed.[4] *L. majuscula* occurs abundantly in many habitats around the island of Guam. Strains that differ morphologically based on width of cells and trichomes and in secondary metabolite production are sometimes quite similar based on 16S rDNA analysis. For example, a population of *L. majuscula* in Cocos Lagoon produces majusculamides A and B[5] and malyngamides A and B,[6] while a population at Piti Bomb Holes produces the compounds reported here (<1.5% sequence divergence).[7] A large collection of *L. majuscula* was made in January 2000 at Piti Bomb Holes, Guam. Pitipeptolides A (**1**) and B (**2**) were the major secondary metabolites produced by this organism (UOG strain VP627).

Results and Discussion

The organic extract of the cyanobacterial collection afforded a mixture of compounds **1** and **2** after solvent partition and chromatography on silica. Their separation was achieved by normal-phase HPLC, yielding both compounds as colorless amorphous solids.

HRFABMS data for the major compound, pitipeptolide A (**1**), suggested a molecular formula of $C_{44}H_{65}N_5O_9$. The ^{1}H NMR spectrum indicated the peptide nature of **1** by showing signals for exchangeable protons at δ 6.09, 6.38, and 7.92 and an *N*-Me singlet at δ 2.78, arising from secondary and tertiary amide functionalities, respectively, in the molecule. It also revealed the presence of a monosubstituted phenyl group as well as a terminal alkyne moiety (t at δ 1.96, $J \approx 2.5$ Hz) consistent with the



diagnostic ^{13}C NMR signals at δ 83.6 for a quaternary carbon and at δ 69.1 for a methine carbon. Seven ester/amide carbonyl resonances were observed in the ^{13}C NMR spectrum. The presence of both functionalities could be deduced from the IR spectrum displaying strong absorption bands centered at 1725 and 1645 cm^{-1}. Oxygenated sp^3 carbons were suspected because of signals at δ 78.1 and 77.2 in the ^{13}C NMR spectrum, suggesting the presence of two hydroxy acids in addition to amino acids. Seven partial structures could be assembled by analysis of 1D and 2D NMR data (Table 1), i.e., *N*-methylphenylalanine (*N*-Me-Phe), glycine (Gly), proline (Pro), valine (Val), isoleucine (Ile), 2-hydroxy-3-methylpentanoic acid (Hmp), and a 2,2-dimethyl-3-hydroxy-7-octynoic acid (Dhoya) unit that has been identified previously in cyanobacterial metabolites.[8] The sequential relationship of the different spin systems could be established from HMBC experiments (Table 1), leading to the gross structure as depicted for **1**, except for the linkage between Hmp and Pro cyclizing the molecule, which at this point had only been assessed to account for the molecular formula and the last degree of unsaturation. Evidence for this linkage was found by evaluating NOE data. The α-H of the Hmp residue (H-27) did not show a ROESY correlation to the δ-H's of Pro (H-36), but to a signal at δ 4.62. However, this could not be unambiguously assigned to the α-H of Pro (H-33) since its doublet overlaps with the doublet of doublets of one of the glycine methylene protons (H-44) (Table 1). 1D NOE experiments were more

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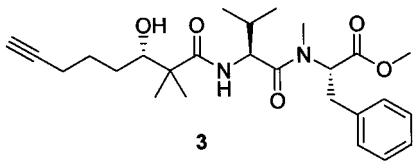
Table 1. NMR Spectral Data for Pitipeptolide A (**1**) and B (**2**) at 400 MHz (^{1}H) and 100 MHz (^{13}C) in $CDCl_3$

		pitipeptolide A (**1**)			pitipeptolide B (**2**)	
unit	C/H no.	δ_H (*J* in Hz)	δ_C	HMBC[a,b]	δ_H (*J* in Hz)	δ_C
Dhoya[c]/Dhoea[d]	1		175.4, s	H-3, H-9, H-10, NH-(1), H-12[e]		175.5, s
	2		45.5, s	H-9, H-10		45.5, s
	3	4.95, dd (9.6, 2.9)	77.2, d	H-9, H-10	4.94, dd (10.4, 3.3)	77.3, d
	4	1.58, m, 1.83, m	28.8, t		1.47, m, 1.59, m	29.4, t
	5	1.43, m	24.4, t		1.32, m	25.0, t
	6	2.22, m	18.0, t	H-4, H-8	2.05, m	33.4, t
	7		83.6, s		5.75, ddt (17.1, 10.2, 6.9)	138.1, d
	8	1.96, ~t (~2.5)	69.1, d		4.96, br dd (10.2, 1.7), 5.00, br dd (17.1, 1.7)	115.2, t
	9	1.31, s	19.4, q	H-3, H-10	1.28, s	19.4, q
	10	1.16, s	22.9, q	H-9	1.12, s	22.9, q
Val	11		171.9, s	H-12, H-25		171.9, s
	12	4.70, dd (9.2, 2.1)	53.3, d	H-14	4.70, dd (9.2, 2.3)	53.2, d
	13	1.76, m	29.5, d	H-14	1.76, m	29.6, d
	14	0.90, d (7.1)	16.0, q	H-12	0.90, d (7.1)	16.0, q
	15	0.88, d (6.9)	20.3, q		0.89, d (6.9)	20.3, q
	NH-(1)	6.09, d (9.2)			6.09, d (9.2)	
N-Me-Phe	16		172.5, s	H-17, H-18, H-27		172.5, s
	17	3.85, dd (11.2, 3.8)	65.7, d	H-18, H-25	3.84, dd (11.2, 3.9)	65.8, d
	18	3.11, dd (14.2, 11.2), 3.21, dd (14.2, 3.8)	33.9, t	H-17	3.11, dd (14.3, 11.2), 3.21, dd (14.3, 3.9)	33.9, t
	19		137.5, s	H-17, H-18, H-21/23		137.6, s
	20/24	7.11, d (6.9)	128.9, d	H-18, H-24/20	7.11, d (6.9)	128.9, d
	21/23	7.28, m	128.6, d	H-23/21	7.28, m	128.6, d
	22	7.24, m	126.9, d	H-20/24	7.24, m	126.9, d
	25	2.78, s	39.2, q	H-17	2.78, s	39.2, q
Hmp	26		169.5, s	H-27,[e] H-28[e]		169.5, s
	27	4.93, d (7.1)	78.1, d	H-29, H-31	4.94, d (7.1)	78.2, d
	28	1.81, m	37.1, d	H-27, H-30, H-31	1.81, m	37.2, d
	29	1.14, m, 1.58, m	24.9, t	H-27, H-30, H-31	1.15, m, 1.58, m	24.9, t
	30	0.89, t (7.4)	11.6, q		0.89, t (7.4)	11.6, q
	31	0.93, d (7.1)	14.5, q	H-27, H-29	0.93, d (6.9)	14.5, q
Pro	32		170.3, s	H-33, H-34, H-38, NH-(4)		170.2, s
	33	4.62, d (7.1)	61.1, d		4.62, d (7.3)	61.2, d
	34	1.97, m, 2.64, m	31.2, t	H-33	1.96, m, 2.66, m	31.2, t
	35	1.76, m, 1.97, m	21.7, t	H-33	1.77, m, 1.96, m	21.7, t
	36	3.55, dd (11.9, 9.6), 3.70, m	46.3, t		3.55, dd (11.7, 9.4), 3.70, m	46.3, t
Ile	37		171.8, s	H-38, NH-(5), H-44		171.6, s
	38	4.19, dd (8.9, 8.1)	61.0, d		4.21, dd (8.9, 8.3)	60.9, d
	39	2.03, m	35.1, d	H-38, H-41	2.05, m	35.1, d
	40	1.24, m, 1.58, m	25.8, t	H-38, H-41, H-42	1.25, m, 1.59, m	25.8, t
	41	0.875, t (7.4)	10.8, q	H-40	0.88, t (7.4)	10.8, q
	42	1.00, d (6.9)	15.8, q	H-38, H-40	1.00, d (6.9)	15.8, q
	NH-(4)	7.92, d (8.1)			7.95, d (8.3)	
Gly	43		170.2, s	H-44, H-3		170.1, s
	44	3.98, d (17.5), 4.62, dd (17.5, 8.9)	41.0, t		3.98, d (17.5), 4.61, dd (17.5, 8.9)	41.0, t
	NH-(5)	6.38, d (8.9)			6.38, d (8.9)	

[a] Protons showing long-range correlation with indicated carbon. [b] If not indicated otherwise, correlations were observed after optimization for $^nJ_{CH} = 7$ Hz. [c] Refers to compound **1**. [d] Refers to compound **2**. [e] Correlation observed after optimization for $^nJ_{CH} = 4$ Hz.

conclusive. Irradiation at δ 4.93 for H-27 resulted in enhancement of the doublet at δ 4.62 corresponding to H-33. The NOE between H-27 and H-33 not only supports the Hmp-Pro linkage, confirming the gross structure for **1**, but also discloses the cis geometry of the propyl amide bond between these two units.[9] Upon methanolysis of **1**, methyl esters **3** and **4** were formed, providing additional support for the gross structure.

The HRMS for pitipeptolide B (**2**) indicated that the molecular weight was two mass units higher than that of pitipeptolide A (**1**). The NMR spectra revealed that both compounds are closely related. The ^{1}H NMR spectrum of **2** lacked the triplet at δ 1.96 for H-8 and showed additional resonances in the olefinic region (δ 4.96, 5.00, and 5.75). Signals in the ^{13}C NMR spectrum of **2** for sp^2 carbons at δ 115.2 and 138.1 instead of acetylenic signals supported that the Dhoya unit had been substituted by a 2,2-dimethyl-3-hydroxy-7-octenoic acid (Dhoea) unit in **2**, accounting for the molecular formula of $C_{44}H_{67}N_5O_9$ derived from the HRMS. The presence of all other units was confirmed by TOCSY experiments and the sequence by HMBC and ROESY experiments.



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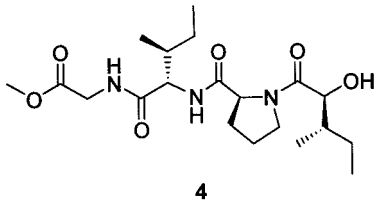

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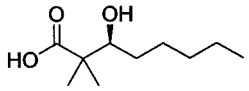
Table 2. Antimycobacterial Disk Diffusion Susceptibility Assay Results for Compounds **1** and **2**

sample	amount[a] (μg)	Diameter (mm) of zone of growth inhibition for *M. tuberculosis* strains	
		ATTC 25177	ATTC 35818
streptomycin	25	50	55
	5	15	33
	1	0	10
pitipeptolide A (**1**)	100	25	15
	25	10	9
pitipeptolide B (**2**)	100	30	15
	25	15	10

[a] Sample was dissolved in ethanol, applied to each 6 mm disk, and air-dried prior to placement on the plate.

Chiral HPLC analysis of the acid hydrolyzates of **1** and **2** revealed the L-configuration of all amino acids and detected the presence of (2*S*,3*S*)-Hmp in both cases. Since conversion of **3** to the corresponding Mosher esters failed,[10] the stereocenter in **1** at C-3 was assigned after hydrogenation of **1** on Pd/C, acid hydrolysis of the resulting product, tetrahydropitipeptolide A, and isolation of 2,2-dimethyl-3-hydroxyoctanoic acid (**5**, Dhoaa). Its negative optical rotation revealed 3*S* configuration by comparison with data for the known (*S*)-Dhoaa (**5**).[11] Closely matching NMR data suggested the same relative stereochemistry of **1** and **2**, and their identical optical rotations indicated identical absolute configuration. Furthermore, since the hydrogenation products of **1** and **2** (tetrahydropitipeptolide A and dihydropitipeptolide B, respectively) were the same, the configuration of the Dhoea unit in **2** was *S* as well.



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Pitipeptolides A (**1**) and B (**2**) exhibit weak cytotoxicity against LoVo cells with IC_{50} values of 2.25 and 1.95 μg/mL, respectively. Both compounds show activity in the antimycobacterial diffusion susceptibility assay. Magnitudes of growth inhibition for *Mycobacterium tuberculosis* strains ATCC 25177 and ATCC 35818 are listed in Table 2 in comparison with a standard drug, streptomycin, which possesses superior activity. No other antibacterial, antifungal, or protease-inhibiting activity (elastase, papain, thrombin, trypsin, plasmin, chymotrypsin) was detected, but both compounds increased elastase activity significantly (2.76-fold and 2.55-fold, respectively, at 50 μg/mL). We had made a similar observation for the lipopeptide apramide A,[12] indicating a possible correlation between the stimulation of elastase activity and the presence of hydrophobic portions in the molecule. This result is not surprising; other studies have shown that certain unsaturated long-chain alcohols markedly enhance elastase activity.[13]

The most distinctive features of **1** and **2**, the Dhoya and Dhoea units, are found in some other marine natural products such as the kulolides[11,14] (Dhoya and Dhoea), isolated from marine mollusks known for sequestering cyanobacterial metabolites via their diet, and yanucamides A and B[8] (Dhoya), directly obtained from a cyanobacterial source. The isolation of compounds **1** and **2** provides more evidence that Dhoya and Dhoea units are diagnostic for metabolites of cyanobacterial origin and that they can be considered biosynthetic signatures. Pitipeptolides A (**1**) and B (**2**) represent the second group of novel secondary cyanobacterial metabolites from this collection site. The isolation of the chlorinated lipids pitiamide A and B from cyanobacteria tufts overgrowing coral tips has been reported earlier.[15]

Experimental Section

General Experimental Procedures. Unless otherwise stated, ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 400 and 100 MHz, respectively. The HSQC experiments were optimized for $^1J_{CH} = 140$ Hz, and the HMBC experiments for $^nJ_{CH} = 7$ or 4 Hz. HRMS were obtained by FAB, MALDI, or CI as indicated.

Biological Material. Cyanobacterium VP627 was a *L. majuscula* collected at Piti Bomb Holes, Guam, in January 2000. A specimen preserved in formalin has been deposited at the University of Guam Marine Laboratory.

Extraction and Isolation. The freeze-dried organism VP627 (~35 g) was extracted with EtOAc−MeOH (1:1) three times to afford the crude organic extract VP627L (3.69 g). VP627L was partitioned between EtOAc and H_2O and the concentrated organic phase applied to a Si Bond-Elut column. After washing with hexane, compounds **1** and **2** were eluted with hexane−EtOAc (1:3). The fraction was dried and the mixture separated by normal-phase HPLC (Econosil Silica, 10 μm, 250 × 10 mm, 3.0 mL/min; detection at 250 nm) using an isocratic system of hexane−EtOAc (3:7) to afford pitipeptolide B (**2**) (9.5 mg, t_R 23.5 min) and pitipeptolide A (**1**) (46.9 mg, t_R 25.0 min).

Pitipeptolide A (1): colorless amorphous solid; $[\alpha]^{25}_D$ −109° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (4.48) nm; IR (film) ν_{max} 3401, 2965, 2872, 1725, 1643 (br), 1514, 1448, 1407, 1373, 1284, 1185, 1026 cm^{-1}; ^1H NMR, ^{13}C NMR, and HMBC data, see Table 1; HRFABMS *m/z* $[M + H]^+$ 808.4867 (calcd for $C_{44}H_{66}N_5O_9$, 808.4861).

Pitipeptolide B (2): colorless amorphous solid; $[\alpha]^{25}_D$ −109° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (4.48) nm; IR (film) ν_{max} 3401, 2966, 2870, 1731, 1643 (br), 1514, 1455, 1414, 1367, 1279, 1185, 1026 cm^{-1}; ^1H NMR, ^{13}C NMR data, see Table 1; HRMS (MALDI) *m/z* $[M + H]^+$ 810.5005 (calcd for $C_{44}H_{68}N_5O_9$, 810.5012).

Methanolysis of 1. Compound **1** (10.0 mg) was dissolved in 1 mL of MeOH. A 0.1 M solution of NaOMe in MeOH (100 μL) was added to the mixture, which was then stirred at room temperature for 72 h. The solvent was evaporated and the residue partitioned between diluted HCl (1 mL, pH 4) and EtOAc (3 × 2 mL). The organic phase was concentrated and applied to a SiO_2 Sep Pak. Elution was initiated with hexane followed by hexane solutions containing progressively increasing amounts of EtOAc and final washing with MeOH. Methyl ester **3** (2.7 mg) eluted first (hexane−EtOAc, 2:1 to 1:1) followed by unreacted **1** (2.3 mg) (hexane−EtOAc, 1:2 to 1:3), and methyl ester **4** (3.3 mg) was encountered in the MeOH fraction.

3: colorless oil; $[\alpha]^{25}_D$ −85° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (4.33) nm; IR (film) ν_{max} 3413 (br), 2955, 2872, 1737, 1631, 1525, 1455, 1261, 1214, 1167, 1085, 1008 cm^{-1}; HRMS (MALDI) *m/z* $[M + Na]^+$ 481.2674 (calcd for $C_{26}H_{38}N_2O_5$ + Na, 481.2673).

4: colorless oil; $[\alpha]^{25}_D$ −75° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (4.25) nm; IR (film) ν_{max} 3410 (br), 2955, 2870, 1743, 1637 (br), 1537, 1455, 1385, 1202, 1132, 1038 cm^{-1}; HRMS (MALDI) *m/z* $[M + Na]^+$ 436.2424 (calcd for $C_{20}H_{35}N_3O_6$ + Na, 436.2418).

Acid Hydrolysis of 1 and 2. Compounds **1** and **2** (0.7 mg each) were treated with 0.5 mL of 6 N HCl and the suspensions heated at 110 °C for 12 h. The product mixtures were dried and analyzed by chiral HPLC for their amino acid contents [column, Chirex phase 3126 (D) (4.6 × 250 mm), Phenomenex; solvent, 2 mM $CuSO_4$−MeCN (95:5), except for *N*-Me-Phe, 2 mM $CuSO_4$−MeCN (85:15); flow rate, 0.8 mL/min; detection at 254 nm]. The retention times (t_R, min) of the authentic amino acids were Gly (7.1), L-Pro (11.5), D-Pro (22.2), L-Val (19.3), D-Val (26.1), L-Ile (47.6), D-Ile, (63.0), L-*allo*-Ile (40.1), D-*allo*-Ile (52.0), *N*-Me-L-Phe (34.0), and *N*-Me-D-Phe (37.2). The retention times of the amino acids in the hydrolyzates

were 7.1, 11.5, 19.3, 47.6 (solvent mixture 95:5), and 34.0 (solvent mixture 85:15), indicating the presence of Gly, L-Pro, L-Val, L-Ile, and *N*-Me-L-Phe. The HPLC traces of both hydrolyzates were identical.

The stereochemistry of the Hmp unit in **1** and **2** was determined in a similar manner by chiral HPLC analysis of the acid hydrolyzates, but using a different stationary phase [column, CHIRALPAK MA(+) (4.6 × 50 mm), Daicel Chemical Industries, Ltd.; solvent, 2 mM $CuSO_4$−MeCN (90:10); flow rate, 1.0 mL/min; detection at 254 nm]. Authentic standards of the hydroxy acid eluted as follows (t_R, min): (2*R*,3*S*)-Hmp (38.2), (2*R*,3*R*)-Hmp (46.0), (2*S*,3*R*)-Hmp (60.0), and (2*S*,3*S*)-Hmp (75.0). The HPLC profiles of both hydrolyzates showed a peak at 75.0 min, corresponding to (2*S*,3*S*)-Hmp. The amino acids eluted within the first 15 min under these conditions.

Hydrogenation of 1 and 2. Compounds **1** (10.0 mg) and **2** (2.0 mg) were hydrogenated on 10% Pd/C (catalytic amount) in MeOH at room temperature for 6 and 3 h, respectively, by bubbling hydrogen through the solution. Each solution was filtered through a pad of Celite and concentrated to dryness, and the residues were applied to a SiO_2 Sep Pak. Elution with hexane−EtOAc (1:1) afforded tetrahydropitipeptolide A (9.5 mg) and dihydropitipeptolide B (1.8 mg), respectively.

Tetrahydropitipeptolide A: colorless amorphous solid; R_f 0.30 [hexanes−EtOAc (1:1)]; $[\alpha]^{25}_D$ −104° (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (4.48) nm; IR (film) ν_{max} 3401, 2955, 2872, 1731, 1648 (br), 1514, 1455, 1367, 1190, 1026 cm^{-1}; HRMS (MALDI) *m*/*z* $[M + H]^+$ 812.5169 (calcd for $C_{44}H_{70}N_5O_9$, 812.5168).

Dihydropitipeptolide B: colorless amorphous solid; R_f 0.30 [hexane−EtOAc (1:1)]; $[\alpha]^{25}_D$ −106° (*c* 0.65, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (4.48) nm; IR (film) ν_{max} 3408, 2961, 2870, 1729, 1644 (br), 1504, 1455, 1371, 1190, 1030 cm^{-1}; HRMS (MALDI) *m*/*z* $[M + H]^+$ 812.5161 (calcd for $C_{44}H_{70}N_5O_9$, 812.5168).

The ^1H NMR spectra of tetrahydropitipeptolide A and dihydropitipeptolide B were identical.

Acid Hydrolysis of Tetrahydropitipeptolide A. The hydrogenation product of **1** (9.0 mg) was subjected to acid hydrolysis (6 N HCl, 110 °C, 12 h). The hydrolyzate was dried under N_2 and the residue partitioned between 1 N HCl and EtOAc. The organic phase was concentrated and the remainder purified by reversed-phase HPLC (YMC-Pack ODS-AQ-323, 5 μm, 250 × 10 mm, 2.0 mL/min; PDA detection from 190 to 540 nm) using a MeCN−0.02 N TFA linear gradient (0−100% over 55 min after a 5 min period at 0% MeCN) for elution. This afforded (2*S*,3*S*)-Hmp (1.1 mg) at t_R 28.1 min and (*S*)-Dhoaa (**5**) (1.0 mg) at t_R 40.8 min.

(*S*)-2,2-Dimethyl-3-hydroxyoctanoic acid (5): colorless oil; $[\alpha]^{25}_D$ −24° (*c* 0.33, MeOH); ^1H NMR (300 MHz, $CDCl_3$) δ 0.90 (3H, t, J = 6.7 Hz), 1.20 (3H, s), 1.25 (3H, s), 1.26−1.42 (6H, m), 1.51 (1H, m), 1.58 (1H, m), 3.64 (1H, dd, J = 10, 2 Hz); CIMS (NH_3) *m*/*z* 206 $[M + NH_4]^+$ 206 (96), 188 $[M + NH_4 - H_2O]^+$ (40); HRCIMS (NH_3) *m*/*z* $[M + NH_4 - H_2O]^+$ 188.1645 (calcd for $C_{10}H_{20}O_3 + NH_4 - H_2O$, 188.1651).

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References and Notes

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