Penasins A−E, Long-Chain Cytotoxic Sphingoid Bases, from a Marine Sponge *Penares* sp

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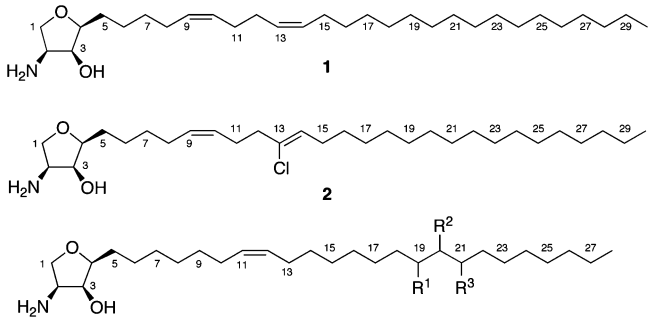
Five sphingoid bases, penasin A (**1**), penasin B (**2**), and a mixture of penasins C−E (**3**−**5**), were identified from a marine sponge *Penares* sp. as cytotoxic constituents. The structure of the common polar head part was assigned by analysis of the NMR data, whereas the structures of the long aliphatic chains including the locations of double bond(s) and a branched methyl group were determined by analysis of tandem FABMS and ^{13}C NMR data together with the GC-MS analysis of ozonolysis products. The absolute configuration of the headgroup was defined for the mixture of **3**−**5** by the modified Mosher method. Penasins exhibit moderate cytotoxicity against HeLa and P388 cells.

Sphingolipids play important structural roles in the plasma membrane by comprising an integral part of lipid rafts.[1] They also function as signaling molecules to regulate cellular processes.[2] In spite of the extreme structural diversity of sphingolipids in higher organisms, the structures of their sphingosine bases fall into only a few classes.[3] On the other hand, a wide variety of differentially modified sphingosine bases have been isolated from marine sponges and tunicates.[4] They exhibit a variety of biological activities. One such compound, pachastrissamine, is the C_{18}-anhydrophytosphingosine (1,4-anhydro-D-*lyxo*-phytosphingosine) obtained from a marine sponge *Pachastrissa* sp.[5] The same compound and the 4-hydroxybutyl aldehyde adduct were reported under the name jaspines.[6] In our study to discover antitumor lead compounds from marine invertebrates, the extract of a marine sponge *Penares* sp. exhibited cytotoxity against P388 murine leukemia cells. Bioassay-guided fractionation afforded C_{29}- or C_{30}-anhydrophytosphingosine bases termed penasins (**1**−**5**). Marine sponges of the genus *Penares* were the source of modified sphingosine bases penaresidins and penazetidine A.[7]

The organic extract of the sponge was subjected to a solvent partitioning process. The $CHCl_3$ and *n*-hexane layers were combined and fractionated by silica gel column chromatography, ODS flash chromatography, and ODS-HPLC to afford penasin A (**1**), penasin B (**2**), and an inseparable mixture of penasins C−E (**3**−**5**).



Penasin A (**1**) had a molecular formula of $C_{30}H_{57}NO_2$, which was established by the HRESIMS data. The ^1H NMR spectrum exhibited a cluster of four olefinic protons, five deshielded protons (δ 3.7−4.3), a methylene envelope, and a terminal methyl group (Table 1). Interpretation of the COSY and HSQC data (Table 2) revealed that compound **1** contained a 2-amino-3-hydroxy-4-alkyltetrahydrofuran ring, as found in pachastrissamine.[5] The molecular formula and the HSQC data suggested that penasin A had an unbranched alkyl chain with two unsaturations. The double bonds were located at C-9/C-10 and C-13/C-14 on the basis of the tandem FABMS data (Figure 1). The geometries of the double bonds were determined as 9*Z*,13*Z* on the basis of the chemical shifts of 27.9 ppm for the allylic methylene carbons (C-8, C-11, C-12, and C-15).[8] The relative configuration of the tetrahydrofuran ring was assigned to be identical with that of pachastrissamine by the intense NOESY cross-peaks between H-2 and H-3 and between

Table 1. ^1H NMR Data of **1**, **2**, the Mixture of **3**−**5**, and the Mixture of **6**−**8**[a]

position	**1**[b]	**2**[b]	**3**−**5**[b]	**6**−**8**[c]
1a	3.81	3.81	3.81	3.61
1b	3.91	3.91	3.92	4.09
2	3.88	3.88	3.88	4.62
3	4.25	4.26	4.26	4.20
4	3.72	3.71	3.72	3.79
5	1.67	1.65	1.65	1.65
6	1.42	1.47	1.43	1.35, 1.47
7	1.43	1.42	1.20−1.40	1.20−1.40
8	2.06	2.10	1.20−1.40	1.20−1.40
9	5.36	5.42	1.36	1.35
10	5.36	5.33	2.04	2.03
11	2.06	2.3	5.35	5.36
12	2.06	2.36	5.35	5.36
13	5.36		2.04	2.03
14	5.36	5.52	1.36	1.36
15	2.06	2.16		1.20−1.40
16	1.44	1.39		
17−28	1.20−1.40	1.20−1.40		
28				
29	1.32	1.32		
30	0.89	0.91		
27			1.32	1.28
28			0.90	0.89
29			0.86	0.86
δ[d]			1.20−1.40	1.20−1.40
γ			1.30	1.37
β			1.10, 1.30	1.09, 1.26
α			1.39	1.28
β			1.10, 1.30	1.09, 1.26
γ			1.30	1.37
δ			1.20−1.40	1.20−1.40

[a] $J_{H,H}$ in Hz: (1) tetrahydrofuran ring: 1a,1b = 8.9; 1a,2 = 4.8; 2,3 = 4.9; 3,4 = 3.5; 4,5ab =7.5, 6.2: (2) methyl signals were split by 7 Hz. [b] Chemical shift values in methanol-d_4. [c] Chemical shift values in $CDCl_3$. [d] For the designations of the Greek letters, see Figure 2, unit b.

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Table 2. ^{13}C NMR Data of **1**, **2**, the Mixture of **3**−**5**, and the Mixture of **6**−**8**[a]

	1[b]	**2**[b]	**3**−**5**[b]	**6**−**8**[c]
1	68.8	68.7	68.9	70.2
2	54.1	54.1	54.2	53.0
3	70.7	70.7	70.8	72.0
4	84.0	84.1	84.1	82.2
5	29.4	29.2	29.6	28.8
6	27.0	27.0	27.0	26.2
7	29−31	29.0−31.0	29.0−31.0	29.0−30.0
8	27.9	27.9	29.0−31.0	29.2[d]
9	130.0−131.0	131.5	29.0−31.0	29.6[e]
10	130.0−131.0	128.7	27.9	27.3
11	27.9	26.9	130.6	130.0
12	27.9	40.2	130.6	130.0
13	130.0−131.0	135.1	27.9	27.3
14	130.0−131.0	126.9	29−31	29.8[e]
15	27.9	29.2		29.4[d]
16−26	29.0−31.0	29.0−31.0		
25			29.0−31.0	29.6
26	29.0−31.0	29.0−31.0	31.8	32.0
27	29.0−31.0	29.0−31.0	23.6	22.7
28	31.8	31.8	14.3	14.2
29	23.6	23.6	20.0	19.8
30	14.3	14.3		
δ[f]			29.0−31.0	29.6
γ			28.0	27.1
β			38.0	37.2
α			33.6	32.8
β			38.0	37.2
γ			28.0	27.1
δ			29−31	29.6

[a] Chemical shifts were determined on the basis of the HSQC or HSQC-TOCSY data. [b] Chemical shift values in methanol-d_4. [c] Chemical shift values in $CDCl_3$. [d] Assignments may be interchanged. [e] Assignments may be interchanged. [f] For the designations of the Greek letters, see Figure 2, unit b.

H-3 and H-4, together with the coincidence of the ^1H and ^{13}C chemical shifts.

Penasin B (**2**) exhibited $(M + Na)^+$ ions at *m*/*z* 498 and 500 in a ratio of 3:1, suggesting the presence of a chlorine atom. The molecular formula was determined to be $C_{30}H_{56}NO_2Cl$ by the HRESIMS data. The NMR data showed the presence of one disubstituted and one trisubstituted double bond each, a terminal methyl group, and a methylene envelop in addition to the 2-amino-3-hydroxytetrahydrofuran headgroup (Tables 1 and 2). Analysis of the 2D NMR data demonstrated that the two double bonds (C-9, C-10 and C-13, C-14) were separated by two methylene carbons (C-11 and C-12). The position of the non-hydrogenated sp^2 carbon atom was assigned to be adjacent to one of the central methylene carbons by analysis of the COSY spectrum. The HMBC data showed the chemical shift of the non-hydrogenated carbon to be

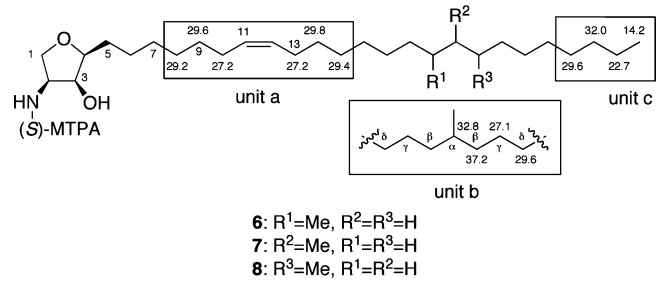

Figure 2. ^{13}C NMR assignments for **6**−**8** on the basis of the HSQC-TOCSY data.

135.1 ppm, which was in agreement with an sp^2 carbon substituted by a chlorine atom.[9,10] The *Z*-geometry of the disubstituted olefinic functionality was assigned via the chemical shifts of allylic carbons C-8 and C-11, whereas the geometry of the trisubstituted double bond was assigned as *Z* by the NOESY cross-peak between H-14 and H_2-12. The latter assignment was supported by comparing the carbon chemical shifts with those of model compounds.[9,10] The relative configuration of the tetrahydrofuran ring was assigned to be identical with that of pachastrissamine by comparing the NMR data. The positions of the double bonds were determined by the tandem FABMS data (Figure 1).

Penasins C−E (**3**−**5**), isomeric compounds differing in the position of a methyl branch, were obtained as an inseparable mixture. The molecular formula of this fraction was determined as $C_{29}H_{58}NO_2$ by HRESIMS. The ^1H NMR spectrum differed from that of penasin A (**1**) in the intensity of the olefinic and allylic proton signals (integrated to be 2H and 4H, respectively) and the presence of a methyl doublet. The NMR data suggested that this fraction also contained the common tetrahydrofuran unit (Tables 1 and 2). The *Z*-geometry of the double bond was established on the basis of the chemical shifts of allylic carbons C-8 and C-13.[8] The position of the double bond in the side chain was assigned by analysis of the tandem FABMS data (Figures S18 and S19). Even though the ^1H NMR spectrum is clean, the tandem FABMS of this fraction did not give a pair of intense ions separated by 28 Da, which was expected for a methyl branch. Instead, all the ions larger than 262 Da were separated by 14 Da (Figure S18). By expecting to get a better resolution of NMR spectra and to determine the absolute configuration of the stereogenic centers of the tetrahydrofuran ring, the mixture of **3**−**5** was converted to the (*S*)-MTPA amides (**6**−**8**, respectively), whose ^1H NMR data of the tetrahydrofuran ring portion matched well with the corresponding data of the (S)-MTPA amide of pachastrissamine,[5] indicating the identical absolute configuration for the headgroups. By taking advantage of

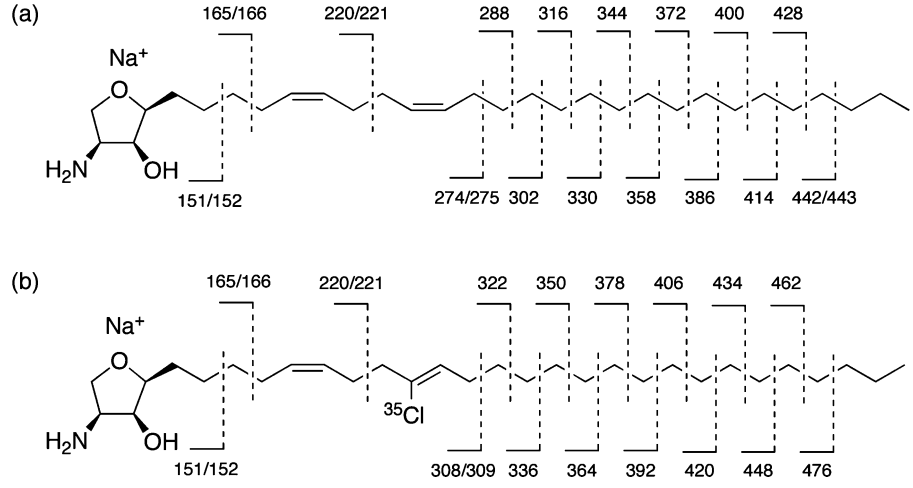

Figure 1. Tandem FABMS data for **1** and **2**.

the sharper ^{1}H NMR signals of this derivative, the HSQC-TOCSY spectrum was analyzed (Figure 2 and Table 2), which showed that the chemical environment of the branched methyl group was homogeneous: the C-10 and C-13 allylic carbons were both connected to two or more methylene carbons (unit a); the methine at the methyl branch was in the center of a C_7 or longer methylene chain (unit b), and the terminal methyl was located at one end of an *n*-butyl or longer chain (unit c). Although it was not possible to observe correlations among units a−c, the position of the methyl branch was confined within the positions between C-19 and C-21. The location of the methyl branch was determined by GC-MS analysis of the ozonolysis products. The mixture of **3**−**5** was subjected to ozonolysis followed by reduction and silylation with TMSCl to afford the 1-OTMS ethers. The mixture was subjected to GC-MS, which gave a broad peak with an intense $(M - CH_3)^+$ ion at *m*/*z* 327 as expected. Even though scant fragmentations were observed, when the mass chromatograms of potential fragment ions (*m*/*z* 171, 185, and 199) were generated, there appeared three peaks within the broad peak (Figure S24). If we hypothesize that these ions were generated due to the cleavage of the bond between the branched methine and the methylene carbon at the side of the polar group (Figure S24), it was possible to locate the methyl branch at C-19, C-20, and C-21, consistent with the ^{13}C NMR analysis. The compounds within this group were named penasins C (**3**), D (**4**), and E (**5**), respectively.

Penasin A (**1**), pensin B (**2**), and the mixture of penasins C−E (**3**−**5**) exhibit moderate cytotoxic activity against HeLa cells (with IC_{50} values of 10, 10, and 2 μg/mL, respectively) and weak cytotoxic activity against P388 cells (IC_{50} value of 50 μg/mL each). It is interesting to note that another class of modified sphingosine bases, termed penaresidines, have been isolated from a marine sponge *Penares* sp.[11]

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. NMR spectra were recorded on a JEOL alpha 600 NMR spectrometer at 300 K. Chemical shifts were referenced to solvent peaks: δ_H 3.31 and δ_C 49.15 for methanol-d_4. ESI mass spectra were measured on a JEOL JMS-T100LC. GC-MS was conducted with a Shimadzu GC-2010/Parvum 2 gas chromatograph mass spectrometer.

Animal Material. A marine sponge *Penares* sp. was collected by hand using scuba off Shikine Island, Tokyo Prefecture, Japan. A voucher specimen was deposited at the Zoological Museum of the University of Amsterdam (ZMAPOR19855).

Extraction and Isolation. The sponge was frozen after collection and kept frozen until extraction. The sponge (400 g, wet weight) was homogenized and extracted with MeOH (2 L × 2) and $CHCl_3$/MeOH (1:1) (2 L × 1). The combined extracts were concentrated, and the residue was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ layer was concentrated and partitioned between 90% MeOH and *n*-hexane. The 90% MeOH layer was diluted with H_2O to afford a solution of 60% MeOH and extracted with $CHCl_3$. The $CHCl_3$ and *n*-hexane layers were combined and subjected to silica gel column chromatography eluting with $CHCl_3$, $CHCl_3$/MeOH (98:2), $CHCl_3$/MeOH (95:5), $CHCl_3$/MeOH (9:1), and $CHCl_3$/MeOH/H_2O (8:2:0.1). The $CHCl_3$/MeOH (95:5 and 9:1) eluates were concentrated and fractioned by ODS flash chromatography with 50% MeOH, 70% MeOH, 90% MeOH, 100% MeOH, and $CHCl_3$/MeOH/H_2O (7:3:0.5). The fractions eluted with 90% MeOH, 100% MeOH, and $CHCl_3$/MeOH/H_2O (7:3:0.5) were combined and purified by ODS HPLC with a gradient elution of 50−80% 1-PrOH containing 1% HOAc followed by ODS-HPLC with either 80% MeCN containing 0.05% TFA or 55% 1-PrOH containing 0.05% TFA to afford penasin A (**1**, 2.3 mg), penasin B (**2**, 0.7 mg), and a mixture of penasins C−E (**3**−**5**, 2.7 mg).

Penasin A (1): colorless oil; $[\alpha]^{24}_D$ +11 (*c* 0.12, MeOH); ^{1}H NMR (600 MHz, methanol-d_4) and ^{13}C NMR (150 MHz, methanol-d_4) data, see Tables 1 and 2, respectively; HRESIMS *m*/*z* 464.4460 $[(M + H)^+$, calcd for $C_{30}H_{58}NO_2$, 446.4462].

Penasin B (2): colorless oil; ^{1}H NMR (600 MHz, methanol-d_4) and ^{13}C NMR (150 MHz, methanol-d_4) data, see Tables 1 and 2, respectively; HRESIMS *m*/*z* 498.4069 $[(M + H)^+$, calcd for $C_{30}H_{57}NO_2{}^{35}Cl$, 448.4072].[11]

Penasins C−E (3−5): colorless oil; ^{1}H NMR (600 MHz, methanol-d_4) and ^{13}C NMR (150 MHz, methanol-d_4) data, see Tables 1 and 2, respectively; HRESIMS *m*/*z* 452.4460 $[(M + H)^+$, calcd for $C_{29}H_{58}NO_2$, 452.4462].[12]

GCMS Analysis. O_3 was bubbled into a solution of the mixture of **3**−**5** (0.2 mg) in MeOH (0.5 mL) at −78 °C for 5 min. The solution was flashed with N_2 gas. To the solution was added 1 mg of $NaBH_4$ in 0.5 mL of MeOH, and the reaction mixture was left at rt for 5 min. The reaction mixture was concentrated and partitioned between H_2O and EtOAc. The organic phase was concentrated, dissolved in *N,O*-bis(trimethysilyl)trifluoroacetamide (BSTFA) (0.1 mL), and left at rt for 1 h. The reaction mixture was subjected to GC-MS analysis: column TC-5 (GL Science), 0.32 mm × 60 m; 50 °C for 1 min; raised to 210 at 10 °C/min; raised to 240 at 5 °C/min; and held at 240 °C for 10 min.

MTPA Amides (6−8). To a solution of a mixture of **3**−**5** (0.3 mg) and DMAP (1 mg) in CH_2Cl_2 was added (−)-MTPACl (5 μL), and the reaction mixture was left at rt for 5 min. The solution was diluted with $CHCl_3$ (2 mL) and washed with 0.1 M $NaHCO_3$ (2 mL × 3), 0.1 N HCl (2 mL × 2), and H_2O (2 mL × 2). The organic layer was concentrated and purified by preparative silica gel TLC developed with *n*-hexane/EtOAc (8:2) to afford the mixture of **6**−**8**.

6−8: ^{1}H NMR ($CDCl_3$) and ^{13}C NMR data, see Tables 1 and 2, respectively; ESIMS *m*/*z* 690 $(M + Na)^+$, 668 $(M + H)^+$.

Cytotoxicity Assay. Cytotoxicity of compounds **1**, **2**, and the mixture of **3**−**5** against P388 and HeLa cells was determined as previously described.[13] P388 murine leukemia cells (JCRB17) were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Ltd.) containing 10% fetal bovine serum, 2 μg/mL of antibiotic−antimicotic (a mixture of 10 000 units of penicillin, 10 mg of streptomycin, and 25 μg of amphotericin B per mL), and 10 μg/mL of 2-hydroxyethyldisulfide at 37 °C under an atmosphere of 5% CO_2. To each well of the 96-well microplate containing 100 μL of tumor cell suspension (1 × 10^4 cells/mL) was added 100 μL of test solution dissolved in RPMI-1640 medium, and the plate was incubated in a TABAI BNA-111 CO_2 incubator (Espec Co., Tokyo, Japan) at 37 °C for 96 h. After addition of 50 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) saline solution (1 mg/mL) to each well, the plate was incubated for 3 h under the same conditions to stain live cells. After the incubation, the plate was centrifuged, the supernatants were removed, and the cells were dissolved in 150 μL of DMSO to determine the IC_{50} values. HeLa human cervical cells were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Ltd.) containing 10% fetal bovine serum, 2 μg/mL gentamycin, and 2 μg/mL antibiotic−antimicotic at 37 °C under an atmosphere of 5% CO_2. To each well of a 96-well microplate containing 200 μL of tumor cell suspension (1 × 10^4 cells/mL) was added test solution after the 24 h preincubation, and the plate was incubated for 72 h. To determine the IC_{50} values, the plate was processed as described for P388 cells. The experiments were duplicated, but statistical analyses for the IC_{50} values have not been conducted.

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Supporting Information Available: NMR and tandem FABMS data for penasins, HSQC-TOCSY spectrum of MTPA amides, and GC-MS of ozonolysis products. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Piike, L. J. *J. Lipid Res.* **2009**, *50*, S323–S328.
(2) Modrak, D. E.; Gold, D. V.; Godenberg, D. M. *Mol. Cancer Ther.* **2006**, *5*, 200–208.
(3) Pruett, S. T.; Bushnev, A.; Hagedorn, K.; Adiga, M.; Haynes, C. A.; Sullards, M. C.; Liotta, D. C.; Merrill, A. H., Jr. *J. Lipid Res.* **2008**, *49*, 1621–1639.
(4) Shier, W. T.; Shier, A. C. *J. Toxicol. Toxin Rev.* **2000**, *19*, 189–246.
(5) Kuroda, I.; Musman, M.; Ohtani, I. I.; Ichiba, T.; Tanaka, J.; Gravalos, D. G.; Higa, T. *J. Nat. Prod.* **2002**, *65*, 1505–1506.
(6) Ledroit, V.; Debitus, C.; Lavaud, C.; Massiot, G. *Tetrahedron* **2003**, *44*, 225–228.

(7) Pruett, S. T.; Bushnev, A.; Hagedorn, K.; Agida, M.; Haynes, C. A.; Sullards, M. C.; Liotta, D. C.; Merrill, A. H., Jr. *J. Lipid Res.* **2008**, *49*, 1621–1639.
(8) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*; VCH: Weinheim, 1990; pp 192−199.
(9) Coleman, M. M.; Tabb, D. L.; Brame, E. G. *Rubber Chem. Technol.* **1977**, *50*, 49–62.
(10) For natural products with the chlorinated olefinic group, see: Ueda, M.; Yamaura, M.; Ikeda, Y.; Suzuki, Y.; Yoshizato, K.; Hayakawa, I.; Kigoshi, H. *J. Org. Chem.* **2009**, *74*, 3370–3377.
(11) Kobayashi, J.; Tsuda, M.; Cheng, J. F.; Ishibashi, M.; Takikawa, H.; Mori, K. *Tetrahedron Lett.* **1996**, *37*, 6775–6776.
(12) Optical rotation was not determined due to the paucity of the material.
(13) Ueoka, R.; Ito, A.; Izumikawa, M.; Maeda, S.; Takagi, M.; Shin-ya, K.; Yoshida, M.; van Soest, R. W. M.; Matsunaga, S. *Toxicon* **2009**, *53*, 680–684.

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