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Amphimelibiosides A−F, Six New Ceramide Dihexosides Isolated from a Japanese Marine Sponge *Amphimedon* sp.

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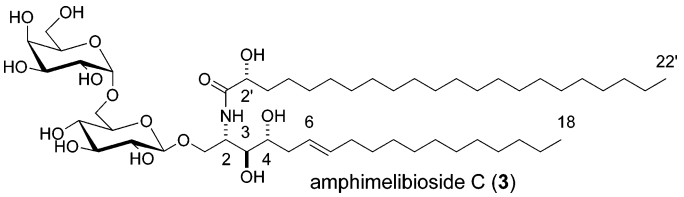

amphimelibioside C (**3**)

Six new ceramide dihexosides, amphimelibiosides A−F (**1**−**6**), were isolated from a Japanese marine sponge *Amphimedon* sp. The structure of amphimelibioside C (**3**), which is a major component of amphimelibiosides, was determined by 2D NMR techniques, chemical degradation, and a semisynthetic method to be 1-*O*-[β-D-glucopyranosyl-(1→6)-α-D-galactopyranosyl]-(2*S*,3*S*,4*R*,6*E*)-2-[(2′*R*)-2-hydroxydocosanoyl]-2-amino-6-octadecene-1,3,4-triol. The structures of the other constituents were elucidated by a combination of mass spectra, ^{1}H NMR, and GC−MS analysis.

Introduction

A variety of glycosphingolipids (GSLs) have been reported from marine invertebrates. Most of the GSLs from marine invertebrates have been found in echinoderms and marine sponges. GSLs from echinoderms (e.g., starfish,[1] sea cucumber,[2] sea urchin,[3] and feather star[4]) characteristically contain a sialic acid in the carbohydrate moiety. Some GSLs exhibit neuritogenic activity, while GSLs from marine sponges (e.g., genus *Amphimedon*,[5] *Agelas*,[6] *Axinella*,[7] and *Aplysinella*[8]) contain an *N*-acetylglucosamine, α-galactose, and α-fucose, which exhibit antifungal, antitumor, immunomodulating, and nitric oxide release inhibiting activities. Generally, GSLs consist of a sphingosine, a fatty acid, and one or more carbohydrates. Each sphingosine and fatty acid is a mixture of various long alkyl chains, since applying the reversed-phase HPLC for final purification is effective.[9]

In our continuing research on biologically active constituents from marine invertebrate,[10] six new ceramide dihexosides, one of which comprises an inseparable mixture of C18:C21′/C17:C22′ ceramides, amphimelibioside A−F (**1**−**6**), have been isolated. In this paper, we report on the isolation, structure determination, and semisynthesis of amphimelibiosides from a Japanese marine sponge *Amphimedon* sp.

Results and Discussion

The Et_2O soluble fraction obtained from the EtOH extract of a Japanese marine sponge, *Amphimedon* sp. collected near Fukuoka, was subjected to a silica gel and

(1) Kawatake, S.; Inagaki, M.; Isobe, R.; Miyamoto, T.; Higuchi, R. *Chem. Pharm. Bull*. **2002**, *50*, 1386-1389.
(2) Kaneko, M.; Kisa, F.; Yamada, K.; Miyamoto, T.; Higuchi, R. *Eur. J. Org. Chem*. **2003**, 1004−1008.
(3) Kubo, H.; Irie, A.; Inagaki, F.; Hoshi, M. *J. Biochem*. **1990**, *108*, 185−192.
(4) Arao, K.; Inagaki, M.; Higuchi, R. *Chem. Pharm. Bull*. **2001**, *49*, 695−698.
(5) Hirsch, S.; Kashman, Y. *Tetrahedron* **1989**, *45*, 3897−3906.
(6) (a) Natori, T.; Morita, M.; Akimoto, K.; Koezuka, Y. *Tetrahedron* **1994**, *50*, 2771−2784. (b) Costantino, V.; Fattorusso, E.; Mangoni, A. *Tetrahedron* **1996**, *52*, 1573−1578. (c) Costantino, V.; Fattorusso, E.; Imperatore, C.; Mangoni, A. *J. Org. Chem*. **2004**, *69*, 1174−1179.
(7) Costantino, V.; Fattorusso, E.; Mangoni, A.; Aknin, M.; Gaydou, E. M. *Liebigs Ann. Chem*. **1994**, 1181−1185.
(8) Borbone, N.; De Marino, S.; Iorizzi, M.; Zollo, F.; Debitus, C.; Ianaro, A.; Pisano, B. *Eur. J. Org. Chem*. **2001**, 4651−4656.
(9) Miyamoto, T.; Inagaki, M.; Isobe, R.; Tanaka, Y.; Higuchi, R.; Iha, M.; Teruya, K. *Liebigs Ann./Recueil* **1997**, 931−936.
(10) Jeong, S.-J.; Inagaki, M.; Higuchi, R.; Miyamoto, T.; Ono, M.; Kuwano, M.; Van Soest, W. M. *Chem. Pharm. Bull*. **2003**, *51*, 731−733.

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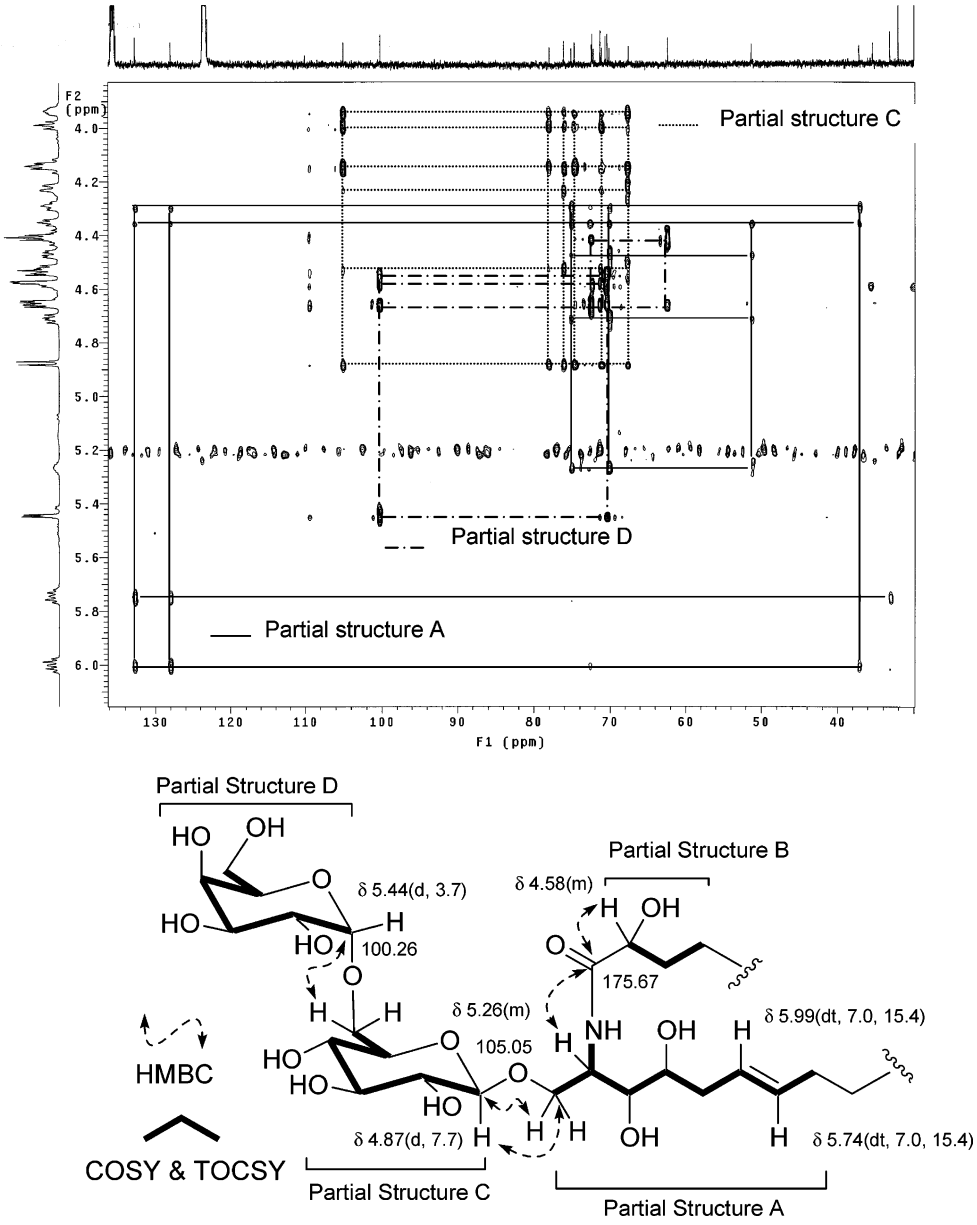


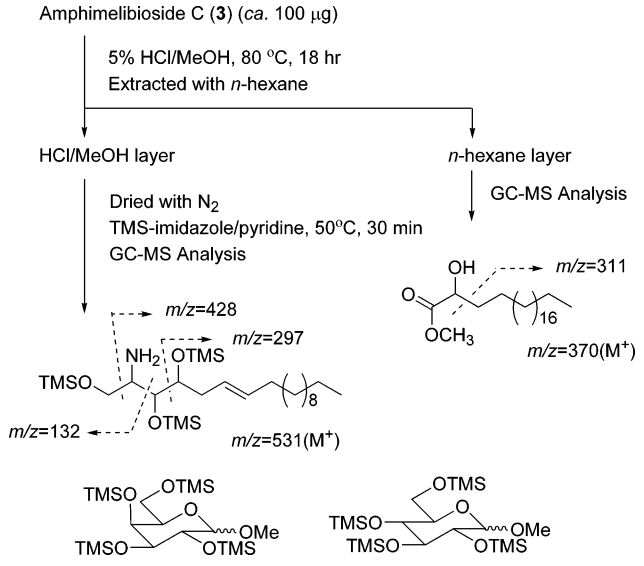
FIGURE 1. HSQC-TOCSY spectrum and the core structure of amphimelibioside C (**3**)

reversed phase chromatography to give an amphimelibiosides mixture (Fr. 3-3). The positive ion FABMS of Fr. 3-3 showed some pseudomolecular ion peaks at *m/z* = 1000 $[M + Na]^+$ as a center because this fraction was successively subjected to reversed-phase HPLC to give amphimelibioside A (**1**, 0.0017% of wet weight), B (**2**, 0.0028%), C (**3**, 0.0042%), D (**4**, 0.0023%), E (**5**, 0.0018%), and F (**6**, 0.00096%).

Structure determination was achieved with amphimelibioside C (**3**). The positive ion FABMS of **3** showed pseudo-molecular ion peaks at *m/z* = 978 $[M + H]^+$ and 1000 $[M + Na]^+$, together with a fragment ion peak at *m/z* = 654. In addition, the negative FABMS of **3** also showed a pseudo-molecular ion peak at *m/z* = 976 $[M - H]^-$, together with fragment ion peaks at *m/z* = 814 $[M - H - 162]^-$, 652 $[M - H - 162 - 162]^-$ due to the cleavage of the glycosidic linkage. These FABMS data suggested the molecular weight of **3** for 977, and the molecular formula of **3**, $C_{52}H_{99}O_{15}N$, was determined by positive-ion HRFABMS measurement [*m/z* = 1000.6878 $[M + Na]^+$, Δ −3.4 mmu]. The ^1H and ^{13}C NMR spectra of **3** showed the presence of two terminal methyls, a number of aliphatic methylenes, three oxygenated methylenes, 11 oxygenated methines, a nitrogenous methine, two acetals, a disubstituted olefin, and an amide carbon and an amide proton.

These spectral features were almost the same as those of Fr. 3-3, and these features suggested that amphimelibiosides are glycosphingolipids that can be classified as ceramide dihexosides. The $^1H-^1H$ COSY, TOCSY, and HSQC-TOCSY spectra of **3** afforded four partial structures A, B, C, and D. The correlations from an oxygenated methylene [δ_H 4.70, 4.46, δ_C 70.10(C-1)] to a nitrogeneous methine [δ_H 5.26, δ_C 51.25(C-2)], two oxygenated methines [δ_H 4.34, δ_C 75.15(C-3), δ_H 4.28, δ_C 72.48(C-4)], an allylic methylene [δ_H 3.01, 2.70, δ_C 37.11 (C-5)], a trans olefin [δ_H 5.99, δ_C 128.03(C-6), δ_H 5.74(H-7), δ_C 132.74-(C-7)], and another allylic methylene [δ_H 2.06(2H), δ_C

SCHEME 1. Methanolysis and GC−MS Analysis of Amphimelibioside C (3)



33.11(C-8)] gave the partial structure of A. The correlations from an oxygenated methine [δ_H 4.58(H′-2), δ_C 72.22(C′-2)] to two methylenes [δ_H 2.18, 1.98(H′-3), δ_C 35.32] and [δ_H 1.75, 1.67(H′-4), δ_C 25.78] gave the partial structure of B. The partial structures of C and D were assigned to β-linked glucopyranose, and α-linked galactopyranose from their chemical shifts and coupling constants, respectively. These four partial structures were merged by the aid of the HMBC experiment. The HMBC correlation between an amide carbon [δ_C 175.67(C′-1)] and the oxygenated methine proton of C′-2, and the nitrogenous methine proton of C-2 gave the connectivity of partial structures A and B. The connectivity of partial structures A and C was determined based on the HMBC correlations between the anomeric proton of β-Glc*p* [δ_H 4.87] and the oxygenated methylene carbon of C″-1 [δ_C 70.10]. Furthermore, the HMBC correlation between the anomeric carbon of α-Gal*p* [δ_C 100.26(C‴-1)] and the H″-6 of β-Glc*p*[δ_H 4.22] gave the connectivity of β-Glc*p* and α-Gal*p*. Accordingly, the core structure of **3** was determined as shown in Figure 1.

Identification of α-hydroxy fatty acid, sphingosine, and hexopyranoses was conducted using GC−MS analysis following the methanolysis of **3**. The *n*-hexane layer which contains a fatty acid methylester was subjected to GC−MS and gave one peak, which was assigned to be methyl 2-hydroxydocosanoate (C22). The TMS-ether of sphingosine and hexopyranoses were also identified to be 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-octadecene (C18), methyl 2,3,4,6-tetra-*O*-trimethylsilylglucoside (Glc), and methyl 2,3,4,6-tetra-*O*-trimethylsilylgalactoside(Gal) using GC−MS analysis as shown in Scheme 1. Accordingly, the planar structure of **3** was assigned to be a melibiosyl ceramide.

The relative stereochemistry of the ceramide moiety was presumed to be 2*S**,3*S**,4*R**,2′*R** from the ^{1}H and ^{13}C chemical shifts.[11] To confirm the stereochemistry of amphimelibiosides, a dihydroamphimelibioside (**11**) was prepared by the semisynthetic method. The glycosyl acceptor, (2*S*,3*S*,4*R*)-2-[(2′*R*)-2-benzoyloxydocosanoyl]-2-amino-3,4-dibenzoyloxyhexadecan-1-ol (**8**) was prepared in accordance with our previous report.[12] Glycosyl donor (1-bromoheptabenzoyl melibiose, **9**) was prepared from commercially available melibiose. The coupling of donor (**9**) with acceptor (**8**) in the presence of $AgClO_4$, Ag_2CO_3, and molecular sieves 4A in CH_2Cl_2 made the glycosylated product (**10**) available in a 58% yield. Perbenzoylated **10** was de-protected with NaOMe to give the dihydroamphimelibioside (**11**). On the other hand, amphimelibioside C (**3**) was hydrogenated using Pd−C as catalyst to give dihydroamphimelibioside C (**7**) in a 53% yield. Comparison of the ^{1}H NMR spectrum of **7** and **11** gave excellent agreement; furthermore, the specific rotation of **7** and **11** was in good agreement (**7**: $[\alpha]_D$ + 18.7, **11**: + 18.9) as shown in Scheme 2. Consequently, the structure of **3** was determined to be 1-*O*-[β-D-glucopyranosyl-(1→6)-α-D-galactopyranosyl]-(2*S*,3*S*,4*R*,6*E*)-2-[(2′*R*)-2-hydroxydocosanoyl]-2-amino-6-octadecene-1,3,4-triol.

The negative-ion FABMS of amphimelibioside A (**1**) showed pseudo-molecular ion peaks at *m/z* = 962 [M − H]$^-$ as well as fragment ion peaks due to the cleavage of glycosidic linkage at *m/z* = 800 [M − 162]$^-$ and 638 (ceramide). The ^{1}H NMR spectrum was nearly identical to that of **3**. GC−MS analysis following methanolysis identified methyl 2-hydroxyhenicosanoate (C21) and methyl 2-hydroxydocosanoate (C22) as its fatty acid component in a ratio of 64:36. In addition, 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-heptadecene (C17) and 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-octadecene (C18) were identified as the sphingosine in a ratio of 24:76. Thus, **1** was determined to be a mixture of the corresponding analogues shown in Chart 1.

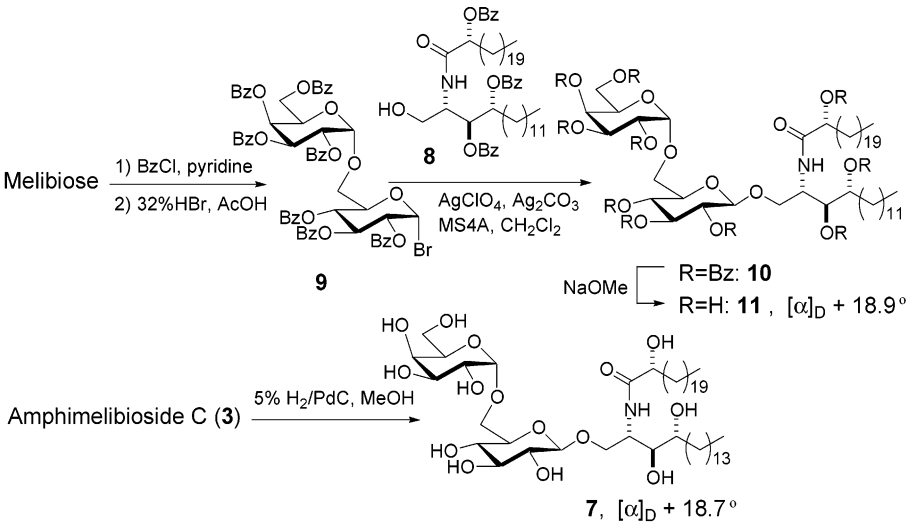
Amphimelibioside B (**2**) revealed a pseudo-molecular ion peak at *m/z* = 976 [M − H]$^-$, the same as that of **3**. Methylation analysis of **2** gave a fatty acid methyl ester, which was identified by GC-MS as methyl 2-hydroxydocosanoate (C22). A trimethylsilylated sphingosine base gave a similar mass spectrum of C18 sphingosine as that of **3**. However, the retention times of them were different from each other [t_R = 26.9 min (**2**), and t_R = 27.5 min (**3**)]. The ^{1}H NMR spectrum of **2** showed terminal methyl signals at δ_H 0.83 (6H, m) and a multiplet methine proton at δ_H 1.45. The 1D TOCSY experiment was conducted from this methine proton, and extracted terminal secondary methyl signals (see the Supporting Information). Therefore, the sphingosine of **2** was revealed to 1,3,4-trihydroxy-2-amino-16-methyl-6-heptadecene (C18), and the structure of **2** was assigned as shown in Chart 1.

The negative-ion FABMS of amphimelibioside D (**4**) showed a pseudo-molecular ion peak at *m/z* = 990 [M − H]$^-$ together with the fragment ion peaks at *m/z* = 828 and 666. GC−MS analysis of fatty acid and sphingosine revealed the methyl 2-hydroxydocosanoate (C22) and 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-nonadecene (C19). Furthermore, the ^{1}H NMR spectrum of **4** showed a signal due to terminal primary methyls [δ_H 0.89 (6H)]. Therefore, the structure of **4** was assigned as shown in Chart 1.

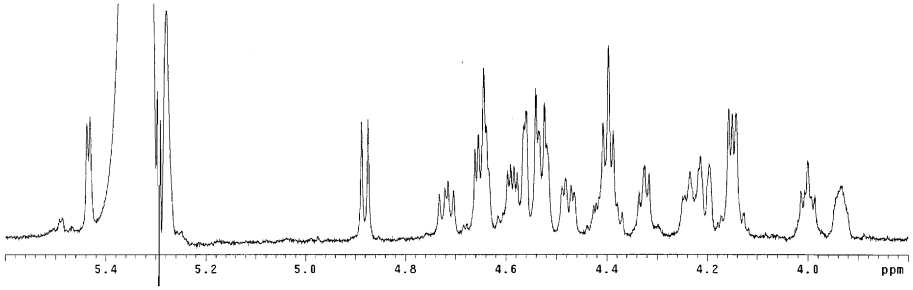
(11) (a) Sugiyama, S.; Honda, M.; Komori, T. *Liebigs Ann Chem*. **1988**, 619−625. (b) Kawano, Y.; Higuchi, R.; Isobe, R.; Komori, T. *Liebigs Ann. Chem*. **1988**, 19−24.

(12) Sugata, T.; Kan, Y.; Nagaregawa, Y.; Miyamoto, T.; Higuchi, R. *J. Carbohydr. Chem*. **1997**, *16*, 917−925.

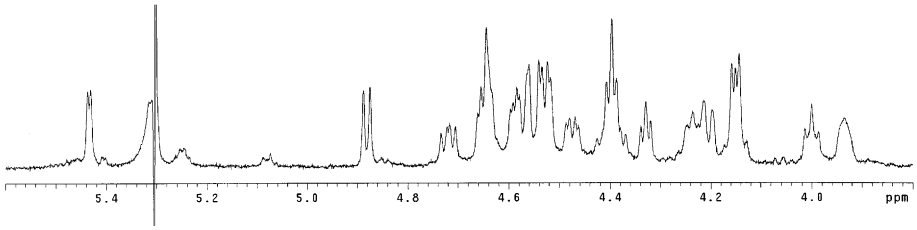
SCHEME 2. Synthesis of Dihydroamphimelibiosides and Their ^{1}H NMR Spectra



^{1}H NMR Spectrum of **11** (synthetic)



^{1}H NMR Spectrum of **7** (derived from **3**)



Amphimelibioside E (**5**) gave a similar negative ion FABMS and a ^{1}H NMR spectrum as that of **4**. Methyl 2-hydroxytricosanoate (C23) and 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-octadecene (C18) were identified as a fatty acid and a sphingosine of **5**, respectively. Thus, the structure of **5** was deduced to be that shown in Chart 1.

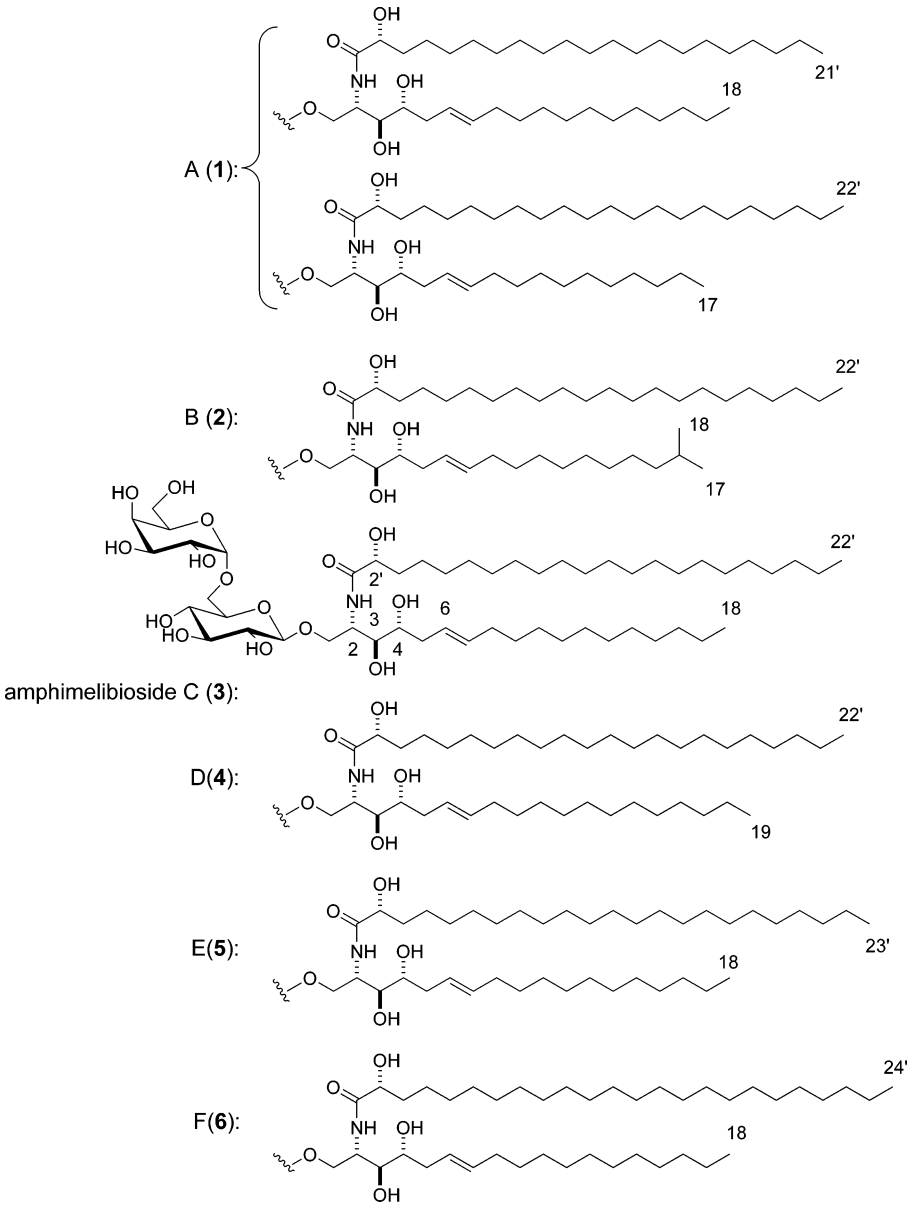
The negative-ion FABMS of amphimelibioside F (**6**) featured a pseudo-molecular ion peak at $m/z = 1004$ [M $-$ H]$^-$, and the ^{1}H NMR spectrum was quite similar to those of other amphimelibiosides except for **2**. GC$-$MS analysis revealed methyl 2-hydroxytetracosanoate (C24) and the same sphingosine (C18) as that of **3**. Accordingly, the structure of **6** was assigned as shown in Chart 1.

Conclusion

Five new β-melibiosylceramides (**2**$-$**6**) were individually isolated by reversed-phase HPLC, along with amphimelibioside A (**1**), which consists of an inseparable mixture of two combinations of alkyl chains in the ceramide moiety which could not be isolated. Agelasphines were the first isolated α-galactosyl glycosphingolipid, and they showed potent antitumor activity.[6a] Next, a variety of α-galactosyl glycosphingolipids were reported from marine sponges, the genuses *Agelas*[6b] and *Axinella*.[7] Amphimelibiosides have an α-galactopyranose as the second sugar, and this carbohydrate moiety is well-known as melibiose. Melibiose is commonly found in honey and soybean in trace amounts or produced by *Escherichia coli*, although the existence of melibiose, which is in a glycosidic form, has been reported only from sea urchin eggs.[13] Amphimelibiosides are the first naturally occurring melibiosyl ceramides isolated from a marine sponge. On the other hand, the ceramide moiety of amphimelibiosides has already been reported in halicerebroside A and amphicerebrosides.[5] Halicerebroside A possesses the normal terminus in the sphingosine, and β-Glc*p* in the carbohydrate moiety, and exhibited mild antitumor activity. In contrast, amphicerebrosides possesses the isopropyl terminus and α- or β-glucosamines, and their heptaacetyl derivatives exhibited low antifun-

(13) Kubo, H.; Irie, A.; Inagaki, F.; Hoshi, M. *J. Biochem.* **1988**, *104*, 755$-$760.

CHART 1. Structures of Amphimelibiosides A−F (1−6)



gal activity. The elucidation of the structure of amphimelibiosides has been conducted using a combination of 2D NMR analysis and GC−MS analysis for planar structure, and the semisynthetic method has been used to determine the stereostructure. Almost all phytosphingosines possessed 2*S*,3*S*,4*R* configurations because their biosynthetic origin is derived from L-serine.[14] In this study, we used 1-hydroxytribenzoylceramide as the glycosyl acceptor. This acceptor was easily prepared from the cerebroside of a starfish, *Acanthaster planci*, and might be used for the rapid and facile synthesis of the other biologically active glycosphingolipids. Most GSLs in marine invertebrates were isolated in peracetate form, and the structures were determined with them. We succeeded in the isolation of intact GSLs, and in determination of the structures. In this study, we have not surveyed any biological activities for amphimelibiosides yet. However, we can easily speculate that amphimelibiosides are moistening agents used as the source of some cosmetics, because ceramides have potent moistening activity[15] and melibiose improves skin conditions.[16]

Experimental Section

Collection, Extraction, and Isolation. *Amphimedon* sp. (wet weight 145.8 g) was collected by hand at depths of 20 m off Orono Island, Fukuoka Prefecture, Japan, in July of 2001. A voucher specimen was deposited in the Zoological Museum in University of Amsterdam (ZMA POR.16790). The sponge was homogenized and extracted with EtOH (3 × 1 L) and filtered. The extract was evaporated in vacuo, and the resulting aqueous suspension (350 mL) was diluted with H_2O (650 mL) and extracted with Et_2O (0.8 L, 0.5 L, 0.3 L) and *n*-BuOH (0.5 L, 0.5 L, 0.3 L). These organic layers were evaporated to give Et_2O extract (842.4 mg) and *n*-BuOH extract (189.2 mg),

(14) (a) Sprinson, B. D.; Coulon, A. *J. Biol. Chem*. **1954**, *207*, 585−591. (b) Weiss, B. *J. Biol. Chem*. **1963**, *238*, 1953−1959.

(15) Loden, M. *Am. J. Clin. Dermatol.* **2003**, *4*, 771−788.

(16) Shigematsu, T.; Kaneko, I.; Kajiwara, N.; Sayama, K.; Nagura, T.; Kikuchi, H. Jpn. Kokai Tokkyo Koho 2003, JP2003160494.

TABLE 1. ^{1}H and ^{13}C Chemical Shifts (ppm) for Amphimelibioside C (3) and Fr. 3-3

	3		fr. 3-3	
	^{1}H	^{13}C	^{1}H [b]	^{13}C
C-1 (sphingosine)	4.70 (dd, 6.6, 10.6)[a]	70.10 (t)	4.67[c]	70.29 (t)
	4.46 (dd, 4.0, 10.6)[a]		4.46[c]	
C-2	5.26 (m)	51.25 (d)	5.24 (m)	51.56 (d)
C-3	4.34 (t, 6.6)	75.15 (d)	4.31 (t, 7.0)	75.45 (d)
C-4	4.28 (m)	72.48 (d)	4.26[c]	72.67 (d)
C-5	3.01 (dd, 7.0, 12.8)	37.11 (t)	2.99 (m)	37.35 (t)
	2.70 (quintet, 7.0)		2.67 (m)	
C-6	5.99 (dt, 7.0, 15.4)	128.03 (d)	5.97 (dt, 7.0, 15.0)	128.16 (d)
C-7	5.74 (dt, 7.0, 15.4)	132.74 (d)	5.70 (dt, 7.0, 15.0)	132.79 (d)
C-8	2.06 (2H, q, 7.0)	33.11 (t)	2.03 (2H, m)	33.20 (t)
term. CH_3	0.88 (3H, t, 7.2)	14.17 (q)	0.85 (m)	14.25 (q), 22.90 (q)
NH	8.53 (d, 9.2)		8.52 (d, 9.2)	
C′-1 (fatty acid)		175.67 (s)		175.70 (s)
C′-2	4.58 (m)	72.22 (d)	4.54[c]	72.37 (d)
C′-3	2.18 (m)	35.32 (t)	2.16 (m)	35.54 (t)
	1.98 (m)		1.95 (m)	
C′-4	1.75 (m)	25.78 (t)		25.88 (t)
	1.67 (m)			
term. CH_3	0.88 (3H, t, 7.2)	14.17 (q)	0.85 (m)	14.25 (q)
C″-1 (*β*-Glc*p*)	4.87 (d, 8.1)	105.05 (d)	4.86 (d, 7.7)	105.29 (d)
C″-2	3.98 (dd, 8.1, 8.4)	74.71 (d)	3.95[c]	74.96 (d)
C″-3	4.14 (dd, 8.4, 8.8)[a]	77.99 (d)	4.11 (m)	78.33 (d)
C″-4	4.12 (dd, 6.6, 8.8)[a]	71.17 (d)	4.07 (m)	71.55 (d)
C″-5	3.93 (m)	76.11 (d)	3.91 (m)	76.28 (d)
C″-6	4.22 (dd, 2.0, 10.6)[a]	67.54 (t)	4.23[c]	67.99 (t)
	4.51 (dd, 5.1, 10.6)[a]		4.48[c]	
C‴-1 (α-Gal*p*)	5.44 (d, 3.7)	100.26 (d)	5.43 (d, 3.7)	100.53 (d)
C‴-2	4.65 (dd, 3.7, 9.9)[a]	70.38 (d)	4.62[c]	70.67 (d)
C‴-3	4.53 (dd, 3.3, 9.9)[a]	71.33 (d)	4.52[c]	71.63 (d)
C‴-4	4.57 (br d, 3.3)[a]	70.67 (d)	4.57[c]	70.94 (d)
C‴-5	4.65 (m)	72.40 (d)	4.64[c]	72.57 (d)
C‴-6	4.40 (2H, m)	62.34 (t)	4.39 (2H, m)	62.59 (t)

[a] Coupling constants were measured from the 1D-TOCSY experiment. [b] Chemical shifts were measured from the HSQC experiment. [c] Submerged by other signals.

respectively. The Et_2O extract was subjected to silica gel column chromatography with $CHCl_3$/MeOH/H_2O (8/2/0.1 → 6/4/0.2) to give six fractions [Fr. 1 (309.2 mg), Fr. 2 (53.2 mg), Fr. 3 (180.4 mg), Fr. 4 (68.2 mg), Fr. 5 (112.9 mg), and Fr. 6 (62.6 mg)]. Fraction 3 was chromatographed on Lobar RP-8 with 95% MeOH to give six fractions [Fr. 3-1 (47.1 mg), Fr. 3-2 (26.9 mg), Fr. 3-3 (29.6 mg), Fr. 3-4 (30.4 mg), Fr. 3-5 (15.2 mg), and Fr. 3-6 (17.1 mg)]. Fraction 3-3 was dissolved in 300 μL of pyridine. Aliquots of 30 μL of this solution were each subjected to reversed-phase HPLC with 100% MeOH to give amphimelibioside A (**1**, 2.5 mg), B (**2**, 4.1 mg), C (**3**, 6.1 mg), D (**4**, 3.3 mg), E (**5**, 2.6 mg), and F (**6**, 1.4 mg), respectively.

Fr. 3-3 (Amphimelibioside Mixture): white powder; FABMS positive ion *m/z* = 1028, 1015, 1014, 1000, 998, 987, 986 $[M + Na]^+$, 654, 636; negative ion *m/z* = 1004, 990, 976, 962 $[M - H]^-$; ^{1}H NMR and ^{13}C NMR (5% D_2O in pyridine d_5) Table 1.

Amphimelibioside C (3): white powder; $[\alpha]_D = +3.5$ (*c* = 0.26, MeOH); HR-FABMS found $[M + Na]^+$ *m/z* = 1000.6878 ($C_{52}H_{99}O_{15}NNa$, requires 1000.6912); FABMS positive ion *m/z* = 1016 $[M + K]^+$, 1000 $[M + Na]^+$, 654, 636; negative ion *m/z* = 976 $[M - H]^-$, 814 $[M - 162]^-$, 653, 652, 339; ^{1}H NMR and ^{13}C NMR (5% D_2O in pyridine d_5) Table 1.

Methanolysis and GC−MS Analysis of 3. Approximately 100 μg of **3** was dissolved in 100 μL of 5% HCl/MeOH, and solution was heated at 80 °C in a sealed tube for 18 h. The reaction mixture was diluted with 400 μL of MeOH, neutralized with Ag_2CO_3, and then filtered. The filtrate was extracted with *n*-hexane (3 × 1 mL), and the remaining layer was dried under nitrogen. The product was reacted with 1-(trimethylsilyl)imidazole (50 μL) and pyridine (50 μL) at 50 °C for 30 min. The reaction mixture was quenched with MeOH (1 mL) and extracted with *n*-hexane (2 mL) to give a mixture of TMS ether of sphingosine and 1-*O*-methyl sugars. The fatty acid methyl ester and TMS derivatives were subjected to GC−MS, respectively. The results were as follows: methyl 2-hydroxydocosanoate, t_R = 28.5 min, *m/z* = 370 (M^+), 311 ($M^+ - 59$), 57 (base peak), 43; methyl 2,3,4,6-tetra-*O*-trimethylsilylglucoside, t_R = 15.1, 15.4 min (standard, 15.1, 15.4 min), methyl 2,3,4,6-tetra-*O*-trimethylsilylgalactoside, t_R = 13.1, 13.8, 14.5 min (standard, 13.1, 13.8, 14.5 min); 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-octadecene, t_R = 27.5 min, *m/z* = 531 (M^+), 428 ($M^+ - 103$), 297, 132 (base peak), 116, 73.

Hydrogenation of 3. A solution of **3** (1.9 mg, 1.9 μmol) in MeOH (1 mL) was added to catalytic amounts of 5% Pd−C and stirred for 2 h at room temperature in an atmosphere of hydrogen gas. The reaction mixture was filtered through a bed of Celite and evaporated to dryness. The residue was subjected on silica gel short column with $CHCl_3$/MeOH/H_2O (8/2/0.1) to give dihydroamphimelibioside C (**7**, 1.0 mg): white powder; $[\alpha]_D = +18.7$ (*c* = 0.075, MeOH); HR-FABMS found $[M + Na]^+$ *m/z* = 1002.7111 ($C_{52}H_{101}O_{15}NNa$, requires 1002.7069), FABMS positive ion *m/z* = 1002 $[M + Na]^+$, 656, 638, negative ion *m/z* = 978 $[M - H]^-$, 816 $[M - Gal]^-$, 654; ^{1}H NMR (5% D_2O in pyridine-d_5) δ 0.88 (6H, t, *J* = 7.1 Hz, *term*. CH_3), 1.2−1.4 (ca. 55H, m, CH_2), 1.44 (1H, m), 1.69 (2H, m), 1.76 (1H, m),1.90 (2H, m, H_2-5), 2.00 (1H, m, H′-3), 2.21 (2H, m), 3.95 (1H, m, Glc-5), 4.00 (1H, m, Glc-2), 4.15 (2H, m, Glc-3 and Glc-4), 4.21 (1H, m, Glc-6), 4.24 (1H, m, H-4), 4.33 (1H, t, *J* = 6 Hz, H-3), 4.40 (2H, m, Gal-6), 4.48 (1H, dd, *J* = 4.0, 10.6 Hz, H-1), 4.53 (1H, m, Glc-6), 4.55 (1H, m, Gal-3), 4.59 (1H, m, H′-2), 4.65 (3H, m, Gal-2,Gal-4, Gal5), 4.72 (1H, dd, *J* = 6.6, 10.6 Hz, -1), 4.88 (1H, d, *J* = 7.7 Hz, Glc-1), 5.25 (1H, m, H-2), 5.43 (1H, d, *J* = 3.7 Hz, Gal-1), 8.55 (1H, d, *J* = 9.0 Hz, NH).

Glycosyl Acceptor (8). (2*S*,3*S*,4*R*,6*E*)-2-[(2′*R*)-2-Benzoyloxydocosanoyl]-2-amino-3,4-dibenzoyloxyhexadecane-1-ol (**8**) was prepared in accordance with our previous report.[12]

Glycosyl Donor (9). A solution of melibiose (1.27 g, 3.7 mmol) in pyridine (8 mL) was added to benzoyl chloride (5 mL) and stirred for 3 h at 60 °C. The reaction mixture was poured into saturated aqueous $NaHCO_3$ and extracted with Et_2O. The Et_2O layer was washed with 1 N HCl and then saturated aqueous NaCl. The extract was dried with Na_2SO_4 and evaporated in vacuo. The residue was dissolved in CH_2Cl_2 (4 mL), and 32% HBr/AcOH (4 mL) was added. After being stirred for 2 h at room temperature in the dark, the reaction mixture was poured into saturated aqueous $NaHCO_3$ and extracted with Et_2O. The Et_2O layer was dried with Na_2SO_4 and evaporated in vacuo. The residue was chromatographed on silica gel with *n*-hexane/ EtOAc (3/1) to afford 1-bromo-heptabenzoylmelibiose (**9**, 2.52 g, 60%): white powder; FABMS positive ion *m/z* = 1134, 1132 $[M]^+$, 1053 $[M - Br]^+$, 1052 $[M - HBr]^+$, 579, 105 (base peak); ^1H NMR ($CDCl_3$) δ 3.77 (1H, dd, J = 1.8, 11.9 Hz, Glc-6), 4.03 (1H, dd, J = 3.7, 11.9 Hz, Glc-6), 4.32 (1H, dd, J = 5.5, 11.4 Hz, Gal-6), 4.45 (1H, dd, J = 7.2, 11.4 Hz, Gal-6), 4.50 (1H, br dd, J = 1.8, 10.1 Hz, Glc-5), 4.64 (1H, t, J = 6.4 Hz, Gal-5), 4.95 (1H, dd, J = 3.9, 9.9 Hz, Glc-2), 5.51 (1H, d, J = 3.7 Hz, Gal-1), 5.71 (1H, dd, J = 3.7, 10.1 Hz, Gal-2), 5.77 (1H, t, J = 9.9 Hz, Glc-4), 6.09−6.12 (2H, m, Gal-3 and Gal-4), 6.16 (1H, t, J = 9.9 Hz, Glc-3), 6.61 (1H, d, J = 3.9 Hz, Glc-1), 7.20−8.15 (35H, benzoyl). Anal. Calcd for $C_{61}H_{49}BrO_{17}$: C, 64.61; H, 4.36. Found: C, 64.38; H, 4.43.

Synthesis of Amphimelibioside Analogue (11). In a typical procedure,[17] a solution of **8** (15.0 mg, 16 μmol) and **9** (34 mg, 30 μmol) in dry CH_2Cl_2 (100 μL) with molecular sieves 4A was stirred for 3 h and successively treated with $AgClO_4$ (3.3 mg, 16 mmol) and Ag_2CO_3 (4.4 mg, 16 mmol) under rigorously anhydrous conditions. The suspension was in the dark for 18 h at room temperature and filtered through a bed of Celite. The filtrate was evaporated to dryness, and the residue was chromatographed on silica gel with *n*-hexane/ EtOAc (2/1) to give perbenzoyl derivative **10** (18.4 mg, 58% yield): colorless amorphous; FABMS positive ion *m/z* = 1872 $[M - OBz]^+$, 1053, 1052, 818, 579, 105 (base peak); ^1H NMR ($CDCl_3$) δ 0.87 (6H, m, *term*. CH_3), 1.10−1.50 (CH_2), 1.86 (2H, m), 2.00 (2H, m), 3.16 (1H, dd, J = 2.5, 9.5 Hz, H-1), 3.47 (1H, dd, J = 2.2, 9.8 Hz, H-1), 3.58 (1H, dd, J = 2.0, 11.0 Hz, Glc-3), 3.77 (1H, t, J = 8.5 Hz, Glc-2), 3.81 (1H, dd, J = 5.6, 10.7 Hz, Glc-4), 4.26 (1H, dd, J = 5.6, 11.2 Hz, Gal-6), 4.47 (1H, m, Gal-6), 4.50 (1H, m, H-2), 4.52 (2H, m, Glc-6), 4.61 (1H, t, J = 6.1 Hz, Gal-5), 5.27 (1H, d, J = 8.4 Hz, Glc-1), 5.28 (1H, m, Gal-4), 5.32 (1H, dt, J = 2.9, 10.0 Hz, H-4), 5.44 (2H, m, H′-2 and Glc-5), 5.64 (1H, dd, J = 2.9, 9.0 Hz, H-3), 5.72 (1H, dd, J = 3.4, 10.7 Hz, Gal-3), 5.89 (1H, dd, J = 3.4, 10.7 Hz, Gal-2), 5.99 (1H, d, J = 3.2 Hz, Gal-1), 6.93 (1H, d, J = 9.8 Hz, NH), 7.00−8.15 (50H, benzoyl).

Part of **10** (14.4 mg, 7.2 μmol) was dissolved in dry CH_2Cl_2 (1 mL) and added 0.1 mol NaOMe/MeOH(1 mL), and the mixture was stirred for 1 h at room temperature. The reaction mixture was neutralized with Dowex 50WX ion-exchange resin and filtered. The filtrate was evaporated, and the resulting residue was chromatographed on silica gel with $CHCl_3$/MeOH/H_2O (8/2/0.1) to give an amphimelibioside analogue (**11**, 1.8 mg, 26% yield): white powder; $[\alpha]_D$ = +18.9 (c = 0.058, MeOH); HR-FABMS found $[M + Na]^+$ *m/z* = 974.6693 ($C_{50}H_{97}O_{15}NNa$, requires 974.6756); FABMS positive ion *m/z* = 974 $[M + Na]^+$, 628, 610; ^1H NMR (5% D_2O in pyridine-d_5) δ 0.88 (6H, t, J = 7.1 Hz, *term*. CH_3), 1.2−1.4 (ca. 55H, m, CH_2), 1.44 (1H, m), 1.69 (2H, m), 1.76 (1H, m), 1.90 (2H, m, H_2-5), 2.00 (1H, m, H′-3), 2.21 (2H, m), 3.95 (1H, m, Glc-5), 4.00 (1H, m, Glc-2), 4.15 (2H, m, Glc-3 and Glc-4), 4.21 (1H, m, Glc-6), 4.24 (1H, m, H-4), 4.33 (1H, t, J = 6 Hz, H-3), 4.40 (2H, m, Gal-6), 4.48 (1H, dd, J = 4.0, 10.6 Hz, H-1), 4.53 (1H, m, Glc-6), 4.55 (1H, m, Gal-3), 4.59 (1H, dd, J = 3.7, 7.7 Hz, H′-2), 4.65 (3H, m, Gal-2, Gal-4, Gal5), 4.72 (1H, dd, J = 6.6, 10.6 Hz, -1), 4.88 (1H, d, J = 7.7 Hz, Glc-1), 5.26 (m, H-2, overlapped in H_2O), 5.43 (1H, d, J = 3.7 Hz, Gal-1), 8.55 (1H, d, J = 9.0 Hz, NH).

Amphimelibioside A (1): white powder; HR-FABMS found $[M + Na]^+$ *m/z* = 986.6719 ($C_{51}H_{97}O_{15}NNa$, requires 986.6756); FABMS positive ion *m/z* = 986 $[M + Na]^+$; negative ion *m/z* = 962 $[M - H]^-$, 800, 638, 619; ^1H NMR (5% D_2O in pyridine-d_5) δ 0.89 (6H, m, term. CH_3), 1.2−1.4 (m, $(CH_2)_n$), 1.68 (1H, m), 1.75 (1H, m), 1.98 (1H, m), 2.05 (2H, m), 2.18 (1H, m), 2.70 (1H, m), 3.02 (1H, m), 3.93 (1H, m), 3.98 (1H, t, J = 8.4 Hz), 4.12 (1H, t, J = 8.8 Hz), 4.14 (1H, t, J = 8.8 Hz), 4.22 (1H, br d, J = 10.3 Hz), 4.28 (1H, m), 4.34 (1H, br t, J = 5.1 Hz), 4.40 (2H, m), 4.46 (1H, dd, J = 4.0, 10.6 Hz), 4.52 (2H, m), 4.57 (2H, m), 4.64 (2H, m), 4.70 (1H, m), 4.87 (1H, d, J = 7.7 Hz), 5.26 (1H, m), 5.44 (1H, d, J = 3.7 Hz), 5.74 (1H, m), 5.99 (1H, m), 8.53 (1H, d, J = 9.2 Hz).

Methanolysis and GC−MS Analysis of 1. Approximately 100 μg of **1** was methanolyzed in a similar manner as **3**, and the results were as follows: methyl 2-hydroxyhenicosanoate (63%), t_R = 26.7 min, *m/z* = 356 (M^+), 297 $(M^+ - 59)$, 57 (base peak), 43; methyl 2-hydroxydocosanoate (37%), t_R = 28.3 min, *m/z* = 370 (M^+), 311$(M^+ - 59)$, 57 (base peak), 43; methyl 2,3,4,6-tetra-*O*-trimethylsilylglucoside, t_R = 15.1, 15.4 min (standard, 15.1, 15.4 min); methyl 2,3,4,6-tetra-*O*-trimethylsilylgalactoside, t_R = 13.1, 13.8, 14.5 min (standard, 13.1, 13.8, 14.5 min); 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-heptadecene (24%), t_R =25.9 min, *m/z* = 517 (M^+), 503 $(M^+ - 14)$, 414 $(M^+ - 103)$, 297, 132 (base peak), 116, 73, 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-octadecene (76%), t_R =27.5 min, *m/z* = 517 $(M^+ - 14)$, 428 $(M^+ - 103)$, 297, 132 (base peak), 116, 73.

Amphimelibioside B (2): white powder; HR-FABMS found $[M + Na]^+$ *m/z* = 1000.6931 ($C_{52}H_{99}O_{15}NNa$, requires 1000.6912); FABMS negative ion *m/z* = 976 $[M - H]^-$, 814, 652, 619; ^1H NMR (5% D_2O in pyridine-d_5) δ 0.88 (9H, m, *term*. CH_3), 1.2−1.4 (m, $(CH_2)_n$), 1.49 (1H, m), 1.68 (1H, m), 1.75 (1H, m), 1.98 (1H, m), 2.06 (2H, m), 2.18 (1H, m), 2.70 (1H, m), 3.02 (1H, m), 3.93 (1H, m), 3.99 (1H, t, J = 8.4 Hz), 4.14 (2H, m), 4.22 (1H, br d, J = 10.3 Hz), 4.28 (1H, m), 4.35 (1H, br t, J = 5.0 Hz), 4.40 (2H, m), 4.46 (1H, dd, J = 4.0, 10.3 Hz), 4.52 (2H, m), 4.58 (2H, m), 4.65 (2H, m), 4.71 (1H, dd, J = 6.6, 10.3 Hz), 4.87 (1H, d, J = 7.7 Hz), 5.26 (m), 5.44 (1H, d, J = 3.7 Hz), 5.74 (1H, m), 6.00 (1H, m), 8.54 (1H, d, J = 9.5 Hz).

Methanolysis and GC−MS Analysis of 2. Approximately 100 μg of **2** was methanolyzed in a similar manner as **3**, and the results were as follows: methyl 2-hydroxydocosanoate, t_R = 28.3 min, *m/z* = 370 (M^+), 311 $(M^+ - 59)$, 57 (base peak), 43; methyl 2,3,4,6-tetra-*O*-trimethylsilylglucoside, t_R = 15.1, 15.4 min (standard, 15.1, 15.4 min), methyl 2,3,4,6-tetra-*O*-trimethylsilylgalactoside, t_R = 13.1, 13.8, 14.5 min (standard, 13.1, 13.8, 14.5 min); 1,3,4-tri-*O*-trimethylsilyl-2-amino-16-methyl-6-heptadecene, t_R =26.9 min, *m/z* = 517 $(M^+ - 14)$, 429 $(M^+ - 102)$, 297, 132 (base peak), 116, 73.

Amphimelibioside D (4): white powder; HR-FABMS found $[M + Na]^+$ *m/z* = 1014.7072 ($C_{53}H_{101}O_{15}NNa$, requires 1014.7069); FABMS positive ion *m/z* = 992 $[M + H]^+$, 830, 668, 650, negative ion *m/z* = 990 $[M - H]^-$, 828, 666, 619; ^1H NMR (5% D_2O in pyridine d_5) δ 0.88 (6H, m, *term*. CH_3), 1.2−1.4 (m, $(CH_2)_n$), 1.68 (1H, m), 1.75 (1H, m), 1.98 (1H, m), 2.06 (2H, m), 2.18 (1H, m), 2.70 (1H, m), 3.02 (1H, m), 3.93 (1H, m), 3.99 (1H, t, J = 8.4 Hz), 4.14 (2H, m), 4.22 (1H, br d, J = 10.3 Hz), 4.28 (1H, m), 4.35 (1H, br t, J = 5.0 Hz), 4.40 (2H, m), 4.46 (1H, dd, J = 4.0, 10.3 Hz), 4.52 (2H, m), 4.58 (2H, m), 4.65 (2H, m), 4.71 (1H, dd, J = 6.6, 10.3 Hz), 4.87 (1H, d, J = 7.7 Hz), 5.26 (m), 5.44 (1H, d, J = 3.7 Hz), 5.74 (1H, m), 6.00 (1H, m), 8.54 (1H, d, J = 9.5 Hz).

Methanolysis and GC−MS Analysis of 4. Approximately 100 μg of **4** was methanolyzed in a similar manner as **3**, and the results were as follows: methyl 2-hydroxydocosanoate, t_R = 28.3 min, *m/z* = 370 (M^+), 311 $(M^+ - 59)$, 57 (base peak), 43; methyl 2,3,4,6-tetra-*O*-trimethylsilylglucoside, t_R = 15.1, 15.4 min (standard, 15.1, 15.4 min); methyl 2,3,4,6-tetra-*O*-trimethylsilylgalactoside, t_R = 13.1, 13.8, 14.5 min (standard, 13.1, 13.8, 14.5 min); 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-

(17) Paulsen, H.; Paal, M. *Carbohydr. Res*. **1984**, *135*, 71−84.

nonadecene, t_R = 28.6 min, *m/z* = 545 (M^+), 531 ($M^+ - 14$), 443 ($M^+ - 102$), 311, 132 (base peak), 116, 73.

Amphimelibioside E (5): white powder; HR-FABMS found $[M + Na]^+$ *m/z* = 1014.7050 ($C_{53}H_{101}O_{15}NNa$, requires 1014.7069); FABMS positive ion *m/z* = 1014 $[M + Na]^+$, negative ion *m/z* = 990 $[M - H]^-$, 666; ^{1}H NMR (5% D_2O in pyridine-d_5) δ 0.89 (6H, m, term. CH_3), 1.2−1.4 (m, $(CH_2)_n$), 1.68 (1H, m), 1.75 (1H, m), 1.98 (1H, m), 2.05 (2H, m), 2.18 (1H, m), 2.70 (1H, m), 3.02 (1H, m), 3.93 (1H, m), 3.98 (1H, t, J = 8.4 Hz), 4.12 (1H, t, J = 8.8 Hz), 4.14 (1H, t, J = 8.8 Hz), 4.22 (1H, br d, J = 10.3 Hz), 4.28 (1H, m), 4.34 (1H, br t, J = 5.1 Hz), 4.40 (2H, m), 4.46 (1H, dd, J = 4.0, 10.6 Hz), 4.52 (2H, m), 4.57 (2H, m), 4.64 (2H, m), 4.70 (1H, m), 4.87 (1H, d, J = 7.7 Hz), 5.26 (1H, m), 5.44 (1H, d, J = 3.7 Hz), 5.74 (1H, m), 5.99 (1H, m), 8.53 (1H, d, J = 9.2 Hz).

Methanolysis and GC−MS Analysis of 5. Approximately 100 μg of **5** was methanolyzed in a similar manner as **3**, and the results were as follows: methyl 2-hydroxytricosanoate, t_R = 30.1 min, *m/z* = 384 (M^+), 325 ($M^+ - 59$), 57 (base peak), 43; methyl 2,3,4,6-tetra-*O*-trimethylsilylglucoside, t_R = 15.1, 15.4 min (standard, 15.1, 15.4 min); methyl 2,3,4,6-tetra-*O*-trimethylsilylgalactoside, t_R = 13.1, 13.8, 14.5 min (standard, 13.1, 13.8, 14.5 min); 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-octadecene, t_R = 27.5 min, *m/z* = 517 ($M^+ - 14$), 428 ($M^+ - 103$), 297, 132 (base peak), 116, 73.

Amphimelibioside F (6): white powder; HR−FABMS found $[M + Na]^+$ *m/z* = 1028.7186 ($C_{54}H_{103}O_{15}NNa$, requires 1028.7225); FABMS positive ion *m/z* = 1028 $[M + Na]^+$, 1027, 682, 664; negative ion *m/z* 1004 $[M - H]^-$, 680; ^{1}H NMR (5% D_2O in pyridine-d_5) δ 0.89 (6H, m, *term*. CH_3), 1.2−1.4 (m, $(CH_2)_n$), 1.68 (1H, m), 1.75 (1H, m), 1.98 (1H, m), 2.05 (2H, m), 2.18 (1H, m), 2.70 (1H, m), 3.02 (1H, m), 3.93 (1H, m), 3.98 (1H, t, J = 8.4 Hz), 4.12 (1H, t, J = 8.8 Hz), 4.14 (1H, t, J = 8.8 Hz), 4.22 (1H, br d, J = 10.3 Hz), 4.28 (1H, m), 4.34 (1H, br t, J = 5.1 Hz), 4.40 (2H, m), 4.46 (1H, dd, J = 4.0, 10.6 Hz), 4.52 (2H, m), 4.57 (2H, m), 4.64 (2H, m), 4.70 (1H, m), 4.87 (1H, d, J = 7.7 Hz), 5.26 (1H, m), 5.44 (1H, d, J = 3.7 Hz), 5.74 (1H, m), 5.99 (1H, m), 8.53 (1H, d, J = 9.2 Hz).

Methanolysis and GC−MS Analysis of 6. Approximately 100 μg of **6** was methanolyzed in a similar manner as **3**, and the results were as follows: methyl 2-hydroxytetracosanoate, t_R = 32.2 min, *m/z* = 398 (M^+), 339 ($M^+ - 59$), 57 (base peak), 43; methyl 2,3,4,6-tetra-*O*-trimethylsilylglucoside, t_R = 15.1, 15.4 min (standard, 15.1, 15.4 min); methyl 2,3,4,6-tetra-*O*-trimethylsilylgalactoside, t_R = 13.1, 13.8, 14.5 min (standard, 13.1, 13.8, 14.5 min); 1,3,4-tri-*O*-trimethylsilyl-2-amino-6-octadecene, t_R = 27.5 min, *m/z* = 517 ($M^+ - 14$), 428 ($M^+ - 103$), 297, 132 (base peak), 116, 73.

Supporting Information Available: ^{1}H, ^{13}C NMR, COSY, TOCSY, ROESY, HSQC, HSQC-TOCSY, HMBC, and FABMS spectra and GC−MS data of **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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