CHARACTERIZATION OF A COMPLEX MIXTURE OF PHYTOSPHINGOSINE-TYPE CERAMIDES FROM THE TUNISIAN *Reaumuria vermiculata*

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ABSTRACT: The aerial parts of *Reaumuria vermiculata* afforded a complex mixture of eight ceramides ($C_{36}H_{71}O_5$ + n CH_2 (n=0; 2 to 8)), previously identified in other natural sources and isolated for the first time from *Reaumuria vermiculata* (Tamaricaceae) : (2*S*, 3*S*, 4*R*, 2'*R*, 8*E*)-2-(2'-hydroxyoctadecanoylamino)-8-octadecen-1,3,4-triol **(1)**, (2*S*, 3*S*, 4*R*, 2'*R*, 8*E*)-2-(2'-hydroxyeicosanoylamino)-8-octadecen-1,3,4-triol **(2)**, (2*S*, 3*S*, 4*R*, 2'*R*, 8*E*)-2-(2'-hydroxyuneicosanoylamino)-8-octadecen-1,3,4-triol **(3)**, (2*S*, 3*S*, 4*R*, 2'*R*, 8*E*)-2-(2'-hydroxydocosanoylamino)-8-octadecen-1,3,4-triol **(4)**, (2*S*, 3*S*, 4*R*, 2'*R*, 8*E*)-2-(2'-hydroxytricosanoylamino)-8-octadecen-1,3,4-triol **(5)**, (2*S*, 3*S*, 4*R*, 2'*R*, 8*E*)-2-(2'-hydroxytétracosanoylamino)-8-octadecen-1,3,4-triol **(6),** (2*S*, 3*S*, 4*R*, 2'*R*, 8*E*)-2-(2'-hydroxy-pentacosanoylamino)-8-octadecen-1,3,4-triol **(7)**, (2*S*, 3*S*, 4*R*, 2'*R*, 8*E*)-2-(2'-hydroxyhexacosanoylamino)-8-octadecen-1,3,4-triol **(8).** The ceramides were characterized on the bases of spectroscopic and chemical evidences. The resulting mixture of hydroxy-fatty acid methyl esters of the acid-catalyzed methanolysis of the ceramides was analyzed and quantified by GC. Fatty acid composition was characterized by the predominance of acids with 21, 19 and 20 carbon atoms representing 38.5, 35.3 and 14.9 % of the total fatty acids, respectively. The location of the double bond in the common long chain base (LCB) was evidenced by the IE mass spectrum of the derivation product showing significant fragmentations which were strongly influenced by the sulfurated groups introduced.

Key words*: Reaumuria vermiculata*, ceramides, fatty acids, long-chain base, RMN, GC/MS.

1. Introduction

In the course of our biological and chemical studies of Tunisian medicinal plants [1-3], we have continued our investigation of the aerial part constituents of *Reaumuria vermiculata* (Tamaricaceae) [3]. Pottier Alapetite indicated in the Tunisian flora [4] that *vermiculata* was the unique specie of the *Reaumuria* genus. It is a plant which can reach a height ranging between 0.5 and 1 meter, with leaves of a glaucous green, a stem of white colour and solitary flowers. It grows essentially in the salted areas and it is available in several regions of Tunisia. The same reference adds that this plant also grows in Algeria, in Saudi Arabia, in Palestine and in Sicily.

Earlier chemical investigations have led to the isolation of three fatty alcohols (eicosan-9-ol, heptacosan-1-ol and nonacosan-1-ol), two aromatic acids (gallic acid and 3,4-dihydroxybenzoic acid), an *O*-heteroside (β-Sitosterol-3-*O*-β-D-glucoside) as well as a phytosterol (3-Oxostigmast-4-en-6-β-ol) [3].

We wish to report here the isolation and the structure determination of eight ceramides from the methanolic extract of *R. vermiculata* aerial parts, previously identified in other natural sources [5-6], and isolated for the first time from *Reaumuria vermiculata*. It is known that ceramides are key compounds in the metabolism of Sphingolipids and are emerging as important second messengers for various cellular processes including cell cycle arrest, differentiation, senescence, apoptosis and others [7-8].

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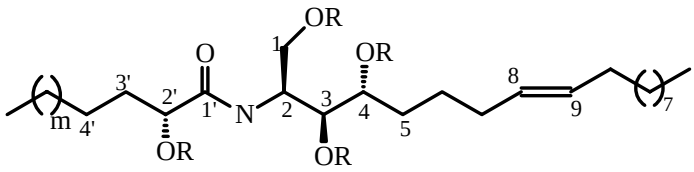
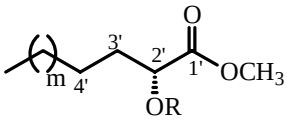
2. Results and discussion

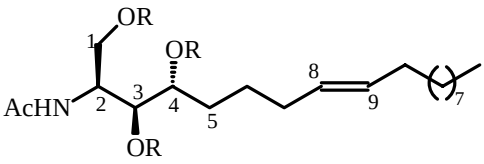


1-8 R = H m = 13 and m=15 to 21
1a-8a R = Ac

Figure 1: Structures of the natural ceramides mixture and the acetylated derivatives.



9 FAME, R = H



10 Acetylated LCB

Figure 2: Structures of fatty acid methyl esters mixture and acetylated long-chain base (LCB).

The ^{1}H NMR spectrum of **1-8** identified particularly in the down field shift, ranging from 4.00 to 7.00 ppm, a significant number of large signals poorly resolved, may correspond to protons worn by oxygenated carbon or oxygen atom, as well as ethylenic protons.
The same spectrum shows the presence of huge methylenes envelope at δ 1.22-1.30 ppm and a triplet at 0.88 ppm (t, J = 6.4 Hz) due to the terminal methyl protons. In order to facilitate the analysis of the spectra, an acetylation of the mixture **1-8** was carried out.

The FAB mass spectrum of the acetylated derivatives **1a-8a** of the mixture **1-8** displayed a cluster of eight quasimolecular ion peaks $[M+Na]^{+}$ at *m/z* 788, 816, 830, 844, 858, 872, 886 and 900, indicative of a mixture of homologous compounds (**1-8**) (Figure 1) difficult to be separated. These mass spectroscopic data are in accordance with the molecular formula $C_{44}H_{79}NO_9$ + nCH_2 (n=0; 2 to 8). Examination of the IR spectrum exhibited bands attributable to hydroxyl (υ_{O-H} = 3336 cm^{-1}) and amide ($\upsilon_{NC=O}$ = 1622 and υ_{N-H} = 3220 cm^{-1}) functionalities, and to a long aliphatic chain at 722 cm^{-1}.
The examination of the ^{1}H NMR spectrum of **1a-8a** showed a triplet at 0.86 ppm (t, J = 6.4 Hz), indicating the presence of two terminal methyls. A huge methylenes envelope at δ 1.25-1.40 ppm and four singlets at 2.0-2.2 ppm attributable to the methyles of the acetyloxy groups were also observed. The same spectrum showed signals at 4.01 (dd; *J*= 11.6 and 2.8 Hz), 4.34 (dd, *J*= 11.6 and 6.4), 4.45 (m), 5.05 (m), 4.95 (m) and 5.10 (m) attributable to protons attached to carbons bearing acetyloxy groups and also showed two olefinic protons at δ 5.36 (m) and an amide proton signal at 6.60 (d, J = 8.9 Hz). The ^{13}C-NMR spectrum of the mixture **1-8** confirmed the above spectral data by the observation of one quaternary carbon at δ 176.1 ppm (CONH, C-1'), four methyne at δ 51.9 ppm (CHNH, C-2), δ 72.2 ppm (CHOH, C-3), δ 72.3 ppm (CHOH, C-4), and δ 75.8 ppm (CHOH, C-2'), and a methylene at δ 61.3 ppm (CH_2OH, C-1). A detailed analysis of the COSY and HMQC spectra allowed us to delineate the spin system from 1-$H_{a/b}$ to 5-$H_{a/b}$ including the amidic proton at C-2 as a doublet at δ = 6.60 (d, J = 8.0 Hz) and the partial structure (C-2' to C-3'), both were connected to long aliphatic chains deduced by the correlation spots (see Table 1). The aforementioned spectral data were in good agreement with those reported for phytosphingosine-type ceramides possessing a 2-hydroxyl fatty acid moiety [10-14].

Table1: NMR spectral data of Mixtures 1-8 [a] and 1a-8a [b]

	Mixture 1-8	Mixture 1a-8a		
Atom no.	^{13}C	^{13}C	^{1}H	COSY
1	61.3	62.3	4.01 (dd , 11.6, 2.8) 4.34 (dd, 11.6, 6.4)	H-2
2	51.9	47,9	4.45 (m)	NH, H-1[a/b], H-3
3	72.5	72.6	5.08 (m)	H-2, H-4
4	72.3	72.3	4.95 (m)	H-3, H-5
5[a/b]	34.7	32.2	1.65 (m)	H-4, H-6
6 [a/b]	24.9	24.9	1.24 (m)	-
7 [a/b]	32.8	32.6	1.95 (m)	H-6, H-8
8	130.9	129.3	5.37 (m)	H-7, H-10
9	132.2	131.2	5.37 (m)	H-7, H-10
10 [a/b]	32.3	32.2	1.95 (m)	H-9, H-11
11 [a/b] -17 [a/b]	29.6-32.3	28.1-31.9	1.2-1.3 (m)	-
CH_3-18	14.2	14.1	0.87 (t, 6.6)	H-17
1’	176.1	170.8	-	-
2’	75.8	74.0	5.10 (m)	H-3’
3’ [a/b]	32.8	31.9	1.82 (m)	H-2’, H-4’
4’ [a/b]	22.9	22.7	1.29 (m)	-
5’ [a/b] -17’ [a/b] *	28.0-32.1	28.1-31.9	1.2-1.3 (m)	-
CH_3-18’**	14.2	14.1	0.87 (t, 6.6)	-
NH	-	-	6.60 (d, 8.0)	H-2’
5 x ($\underline{CH_3}$-CO-)	-	20.7-21.0	2.02-2.18 (s)	-
5 x (CH3-$\underline{CO}$-)	-	170.0-171.3		

[a] ^{13}C NMR (100 MHz) in $CDCl_3$/ CD_3OD.
[b] ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in $CDCl_3$.
17’* for **1**, 19’ for **2,** 20’ for **3,** 21’ for **4,** 22’ for **5,** 23’ for **6,** 24’ for **7** and 25’ for **8;**
18’** for **1**, 20’ for **2,** 21’ for **3,** 22’ for **4,** 23’ for **5**, 24’ for **6,** 25’ for **7** and 26’ for **8**

The completion of the structure elucidation of the mixture **1-8** required a determination of the nature of the alkyl chains at C-5 and C-4’ in the ceramide. This was accomplished by acidic methanolysis of the mixture with 1N HCl in 90 % MeOH (Fig. 3) [9]. The resulting reaction mixture was separated in two fractions **A** and **B**.

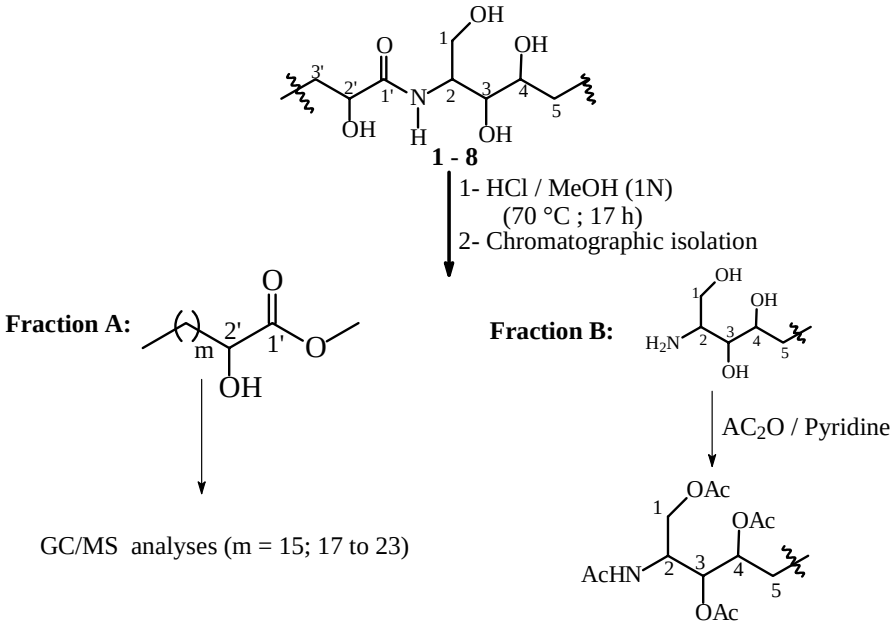


Figure 3: Acidic methanolysis of the ceramides mixture.

Fraction **B** was a long-chain base (LCB) (Figure 2). The ^{1}H NMR spectrum of its acetylated derivative with acetic anhydride in pyridine, the triacetate monoacetamide (Figure 2) showed four singlets at δ 2.0 - 2.4 ppm attributable to the methyls of the acetyloxy groups (4× $COCH_3$) in addition to characteristic signals at δ 5.96 ppm (NHCO, *d*, *J* = 6.9 Hz), δ 5.37 ppm (H-8 and H-9, *m*), 5.09 ppm (H-3, *d*, *J* = 7.2 Hz), 4,93 (H-4, *d*, *J* = 9.2 Hz), 4.46 (H-2, *m*), 4.0 (H-1a, *d*, *J* = 11.1 Hz), 4.29 (H-1b, *dd*, *J* = 6.2 and 11.1 Hz), a large $(CH_2)_n$ signal at 1.25 ppm, methylene groups at 1.95 and 1.61 ppm and a methyl group at 0.87 ppm (H-18, *t*, *J* = 6.4 Hz). The absolute stereochemistry was determined by the comparison of the optical rotation $[\alpha]_D$ = + 23 (1.4, $CHCl_3$) with literature data [15-16] of natural and synthetic sphingamines supporting the 2*S*, 3*S*, 4*R* configurations.

On the other hand, its EI mass spectrum shows several ion peaks, in particular, the molecular ion at m/z 483 $[M]^{+\cdot}$, and the ion fragments at *m/z* 423 $[M\text{-}AcOH]^{+\cdot}$, *m/z* 364 $[M\text{-}AcOH\text{-}AcO^{\cdot}]^{+}$ and *m/z* 304 $[M\text{-}2AcOH\text{-}AcO^{\cdot}]^{+}$, corresponding to the triacetate monoacetamide. The E stereochemistry of the double bond was determined on the basis of the ^{13}C NMR chemical shift of the methylene carbons adjacent to the olefinic carbons, which was observed at δ ≈ 27 in *Z* isomers and at δ ≈ 32 in *E* isomers [17].

To determine the double bond position, we investigated the reaction of dimethyl disulfide with the acetylated derivative of the long-chain base. The diene system reacted with DMDS and a stoichiometric amount of iodine in CS_2 (solvent) [18-19].

The derivation led to thioethers (Figure 4). The EI mass spectrum of the derived obtained product showed fragmentation which is strongly influenced by the sulfurated groups introduced, so that the original position of the double bond could be easily deduced (Figure 4 and 5).

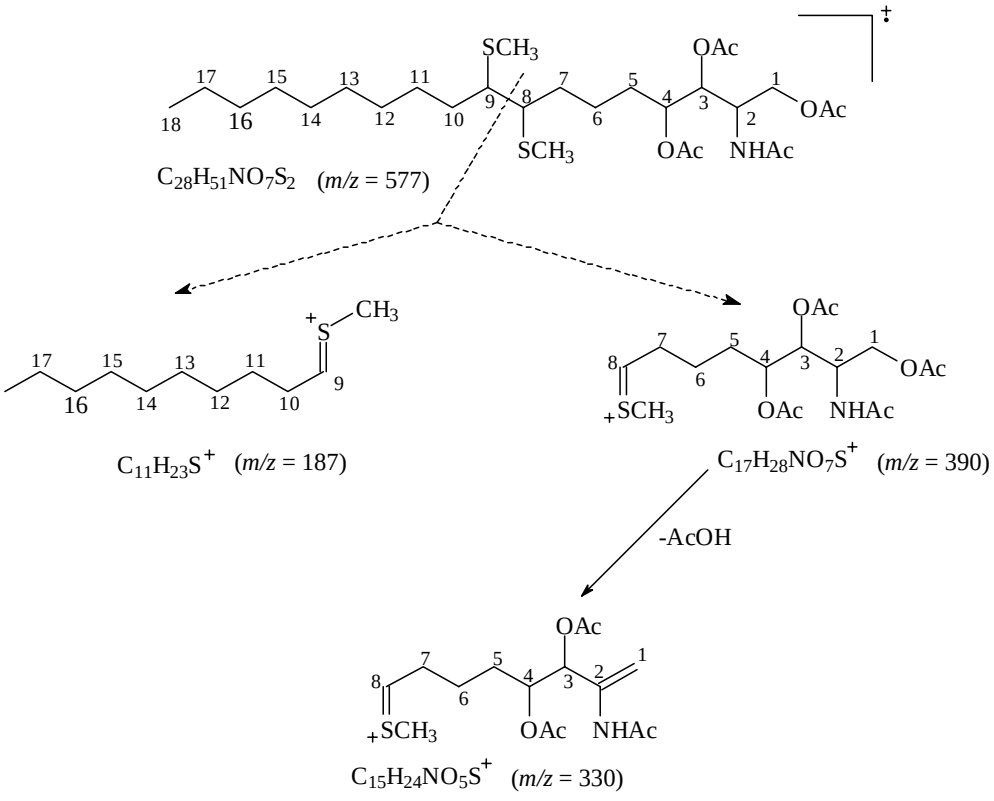


Figure 4: Characteristic ion fragments of DMDS derivative of the acetylated LCB.

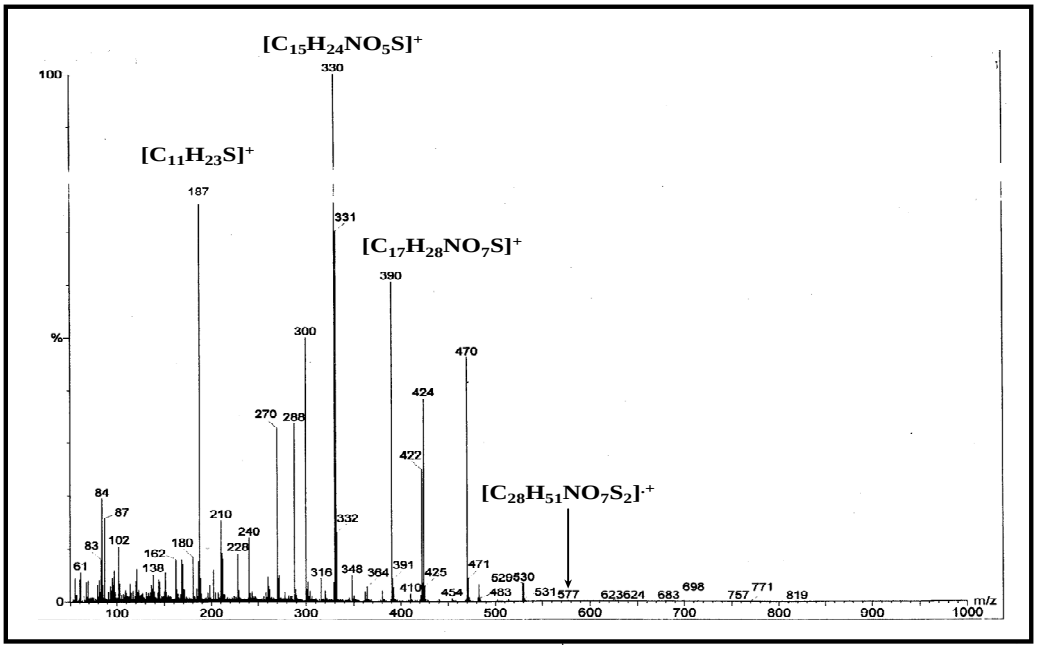


Figure 5: IE Mass spectrum of DMDS derivative of acetylated derivative LCB.

The ^{1}H NMR spectrum of the less polar fraction A containing a mixture of fatty acid methyl esters exhibits signals at δ 4.17 (m, H-2'), 3.77 (s, OCH_3), large CH_2 signal at 1.25 ppm and a methyl group at 0.87 ppm (*t*, -CH_3). Their nature was ascertained by analysis of their trimethylsilyl (TMS) derivatives, by GC-MS showing the existence of eight components which were identified as the trimethylsillylether of a methylated α-hydroxy octadecanoate (m/z = 386), α-hydroxyeicosanoate (m/z = 414), α-hydroxyuneicosanoate (m/z = 428), α-hydroxydocosanoate (m/z = 442), α-hydroxytricosanoate (m/z = 456), α-hydroxytetracosanoate (m/z = 470), α-hydroxypentacosanoate (m/z = 484) and α-hydroxy hexacosanoate (m/z = 498). The results of the analysis are summarized in Table 2.

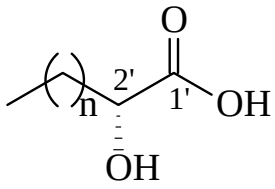


Table 2 Saturated fatty acid methyl esters composition by GC/MS EI

Retention time	% total fatty acids	Molecular ion (*m/z*)	Fatty acids (nC)
19.07	0.125	386	15
20.64	2.866	414	17
21.38	0.720	428	18
22.18	35.314	442	19
22.85	14.936	456	20
23.65	38.483	470	21
24.34	0.472	484	22
25.28	2.839	498	23

3. EXPERIMENTAL SECTION

3.1. Chromatographic and instrument methods. Optical rotations were obtained using a PerkinElmer 241-MC polarimeter. FTIR spectra were measured on PerkinElmer 157G IR spectrophotometer in KBr pellets. The NMR spectra were recorded on Bruker ARX 400 NMR spectrometer (^{1}H at 400 MHz; ^{13}C at 100 MHz), using $CDCl_3$ and CD_3OD as solvents. MALDIMS was carried out on a Voyager spectrometer DE-STR. GC-MS performed on an HP5890 gas chromatograph-mass spectrometer in EI mode at 70eV with an HP5-MS capillary column (30 m x 0.25 mm) packed with 5% diphenyl-polysiloxane/95 % dimethyl-polysiloxane. Helium was used as carrier gas, and the column temperature was rose from 80 to 250 °C at 5°C/min.

3.2. Material. Aerial parts of *R. vermiculata* were collected from Sidi le-ghdemssi, (Monastir, Tunisia) in July 2001, and identified by Dr. F. HARZALLAH- SKHIRI from the Laboratoire de Biologie végétale et Botanique, Institut Supérieur Agronomique, Université de Sousse (Sousse, Tunisia). A voucher specimen (RV-01) is deposited at the above Laboratory.

3.3. Extraction and isolation. The air-dried aerial parts of *Reaumuria vermiculata* (2.450 kg) were powdered and extracted with MeOH by soxhlet apparatus. The residue obtained after the removal of the solvent *in vacuo* (500 g) was further extracted with EtOAc, and the resulting extract (35 g) was then submitted to gradient CC on silica gel by elution with petroleum ether/EtOAc and EtOAc/MeOH mixtures with increasing polarity to give 19 fractions. The fraction 18 (1.131 g) afforded a white precipitate which was recuperated by filtration, then purified by recristallisation from acetone and subjected to silica gel CC ($CHCl_3$ / MeOH 9 : 1, Vol. / Vol.), afforded the mixture of compounds **1-8** (27 mg), which showed a single spot on TLC plates.
Mixture 1-8: White amorphous powder $[\alpha]_D^{22}$ = + 23 (c = 1.2, $CHCl_3$ / MeOH, 9.5: 0.5 Vol. / Vol.). IR (KBr) ν_{max} 3336 (OH), 3220 (N-H),2918 (N-CO), 1622 (NC=O), 1545, 1467, 1277, 1022, 963, 722 cm^{-1}; ^{13}C NMR data see Table 1.

3.4. Acetylation of 1-8: A solution of the mixture **1-8** (12 mg) in pyridine (1 mL) was treated with acetic anhydride (1mL) and was left overnight at room temperature. The reaction mixture was then diluted with water (5 mL) and extracted with ($CHCl_3$, 3 × 10mL). The chloroform extract was dried over anhydrous $MgSO_4$ and the obtained residue was subjected to silica gel CC (n-Hexane / EtOAc 7:3, Vol. / Vol.) to give 13.2 mg of the peracetate mixture **1a–8a** as white powder; ^{1}H and ^{13}C NMR data see Table 1; MALDIMS *m/z* (relative intensity, %): 788 $[M_1+Na]^+$, 816 $[M_2+Na]^+$, 830 $[M_3+Na]^+$, 844 $[M_4+Na]^+$, 858 $[M_5+Na]^+$, 872 $[M_6+Na]^+$, 886 $[M_7+Na]^+$, 900 $[M_8+Na]^+$.

3.5. Methanolysis of 1-8: The mixture **1-8** (10 mg) was dissolved in 1 mL MeOH containing 4mL of 1 N HCl and refluxed for 18 h [9]. The reaction mixture was extracted with n-hexane (3 × 10mL). The resulting n-hexane fraction after drying anhydrous ($MgSO_4$) was purified on Silica gel CC using Hexane/EtOAc (9:1, Vol./Vol.) as eluent, to yield a 6 mg mixture of fatty acid methyl esters (FAME) **(9)**, which were identified by GC-MS as methyl-2-hydroxyoctadecanoate {*m/z* 314 $[M]^{+\cdot}$ (0.1 %)}, methyl-2-hydroxyeicosanoate {*m/z* 342 $[M]^{+\cdot}$ (2.9 %)}, methyl-2-hydroxyuneicosanoate {*m/z* 356 $[M]^{+\cdot}$ (0.7 %)}, methyl-2-hydroxydocosanoate {*m/z* 360 $[M]^{+\cdot}$ (35.3 %)}, methyl-2-hydroxytricosanoate {*m/z* 374 $[M]^{+\cdot}$ (14.9 %)}, methyl-2-hydroxytetracosanoate {*m/z* 388 $[M]^{+\cdot}$ (38.5 %)}, methyl-2-hydroxypentacosanoate {*m/z* 412 $[M]^{+\cdot}$ (0.5 %)} and methyl-2-hydroxyhexcosanoate {*m/z* 498 $[M]^{+\cdot}$ (2.839 %)}. White amorphous powder; $[\alpha]_D^{22}$ = + 5.3 (c = 0.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87 (*t*, *J*=7.0 Hz, CH_3), 1.25 (*brs*, CH_2), 3.77 (*s*, OCH_3), 4.17 (*m*, H-2'); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.7 (CH_2), 24.7 (CH_2), 29.3 (CH_2), 29.5 (CH_2), 31.8 (CH_2), 34.4 (CH_2), 52.4 (OCH_3), 70.5 (CHOH), 175.9 (CO). The MeOH/H_2O

phase was evaporated and the residue acetylated as previously described. Purification by silica gel CC eluted with (Hexane/EtOAc 6:4, Vol./Vol.) yielded 4,4 mg of the tetraacetylated long-chain base (LCB) (2*S*, 3*S*, 4*R*, 8*E*)-2-acetamido-1,3,4-triacetoxy-7-octadecene **(10)**. White amorphous powder; $[\alpha]_D^{22}$ = + 27 (c = 1.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87 (*t*, *J*=6.4 Hz, CH_3), 1.20-130 [*m*, $(CH_2)_n$], 2.0 (*s*, COH_3), 2.2 (*s*, COH_3), 2.4 (*s*, COH_3), 4.0 (*d*, *J*=11.1 Hz, H-1a), 4.29 (*dd*, *J*=11.1, 6.2 Hz, H-1b), 4.46 (*m*, H-2), 4.93 (*d*, *J*=9.2 Hz, H-4), 5.09 (*d*, *J*=7.2 Hz, H-3), 5.37 (*m*, H-8, H-9), 5.96 (*d*, *J*=6.9 Hz, NH); EI-MS (70 ev) m/z (relative intensity, %) 483 $[M]^{+\cdot}$ (2), 423 $[M- AcOH]^{+\cdot}$ (24), 364 $[M - AcOH - AcO^{\cdot}]^+$ (52), 304 $[M – 2 AcOH - AcO^{\cdot}]^+$ (29), 84 (94), 184 (100).

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