New Lipids from the Tunicate *Cystodytes* cf. *dellechiajei*, as PLA2 Inhibitors

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Cystodytes cf. *dellechiajei* collected off Djerba furnished new lipids, sphingosines **1**, as inhibitors of phospholipase A2, along with inactive homologous ceramides **2**. Structures were determined by spectroscopic methods and chemical transformations.

Sphingosines and ceramides have been isolated from a number of marine organisms including sea stars,[1−7] sea anemones,[8,9] gorgonians,[10] sponges,[11−14] tunicates,[15] dinoflagellates,[16] and green algae.[17] Some exhibit cytotoxic,[6,10,14] antitumor,[10] immunostimulatory,[12] antifungal,[14] antimicrobial,[10,15] antiviral,[15,17] or Ca^{2+}ATPase activity.[16]

Sphingolipids have received much attention in recent years, after the discovery of protein kinase C inhibition by sphingosines[18] and indirect evidence leading to the hypothesis that sphingolipid-derived products may function as second messengers.[19] Moreover, apoptotic DNA damage and cell death by ceramides have been observed in mammalian cell lines.[20]

Cystodytes cf. *dellechiajei* (Ascidiaceae) is a widespread tunicate. Previous studies of this species have led to the isolation of the aromatic alkaloids cystodytins A−I, from samples collected in the Okinawan Sea,[21] and the topoisomerase II inhibitors cystodytins J and A, kuanoniamin, dehydrokuanoniamine B, shermilamins B and C, and eilatin, from samples collected near the Fiji Islands.[22] Other samples from Tunisian coasts have furnished cystodamine[23] and a cyclotetrapeptide.[24] In this paper we report the isolation and structure elucidation of sphingosine **1a** as an inhibitor of phospholipase A2 (PLA2), as well as a mixture of homologous ceramides **2** from *Cystodytes* cf. *dellechiajei* collected in Tunisia near Djerba.[25]

Results and Discussion

Specimens were collected in the Mediterranean Sea, off the coast of Tunisia near Djerba, at 20−25 m depth, and preserved in methanol until extraction. They were successively extracted with methanol and then dichloromethane. The methanolic extract of *Cystodytes* cf. *dellechiajei* exhibited marked inhibition of PLA2, and separation of the active compounds was monitored by this bioassay. The extract was first separated on a Si gel column eluted with dichloromethane and increasing amounts of methanol. Fractions eluted with 10% and 20% MeOH were pooled and further fractionated by HPLC on a C_{18} reversed-phase column (MeOH−water 95/5) to yield compounds **1** (18 mg, 0.006% dry wt) and **2** (11 mg, 0.004% dry wt).

The FABMS (positive mode) of **1** showed a series of pseudomolecular ion peaks $[M + H]^+$ at *m*/*z* 670, 656, 642, 628, and 614, indicative of a mixture of homologous compounds. Purification by HPLC over a C_{18} reversed-phase column (MeOH) yielded pure **1a** (7 mg) as the major component. The other components were not resolved.

Compound **1a** was obtained as an amorphous powder, $[\alpha]_D$ +9.6°. HRFABMS furnished the molecular formula $C_{38}H_{78}NO_5$. The ^{1}H NMR spectrum (Table 1) indicated the presence of a D_2O-exchangeable doublet at δ 8.57 ppm (J = 9 Hz) for the NH of an amide function. Six protons were observed between δ 5.10 and 4.29 ppm and were assigned to protons attached to a carbon bearing a heteroatom. Two aliphatic chains were suggested by the presence of signals for methylene groups at δ 2.19−1.90 and 1.65 ppm, a large signal at δ 1.25 ppm, and for three methyl groups at δ 0.85 ppm, a doublet (6H), and a triplet (3H).

The ^{13}C NMR spectrum (Table 1) showed signals at δ 175.2 ppm for the carbonyl of an amide function, as well as signals corresponding to four methines, one deshielded methylene, an intense signal at δ 29−30 ppm due to methylene groups of aliphatic side-chains, signals for two nonequivalent methyl groups of an isopropyl group, and a third methyl group. Assignment of all carbon and proton signals was made possible by ^{1}H−^{1}H COSY and heteronuclear correlation (HMQC, HMBC) experiments, which led to the proposal that **1a** is a 4-hydroxy-sphingosine containing a 2-OH fatty acid (Figure 1).

The fatty acid and sphingosine chain lengths of **1a** were determined through acid methanolysis according to Gaver and Sweeley.[26] Purification by SiO_2 column chromatography afforded two compounds. The first component, which eluted with dichloromethane, corresponded to the methyl ester of the α-hydroxy acid. The ^{1}H NMR spectrum indicated that it possessed a normal aliphatic chain (one triplet at δ 0.85 ppm). GC−EIMS (*m*/*z*, % rel int) analysis, after acetylation, showed the presence of the methyl ester of acetoxy-2-heneidecanoic acid (**3a**) (*m*/*z* 398, 32.4%) (Figure 1). The optical rotation $[\alpha]_D$ −2.4° indicated the *R* configuration.[6] The second, more polar component corresponded to the sphingamine moiety. Acetylation followed by chromatography over Sephadex LH20 gave the tetraacetylsphingamine. Its ^{1}H NMR spectrum exhibited four singlets at δ 2.22−2.00 ppm, in addition to characteristic signals at δ 6.09 (d, NH), 5.10 (dd, H-3), 5.05 (1H, dd, H-1a), 4.93 (1H, dd, H-1b), 4.90 (ddd, H-4), and 4.49 (dt, H-2), a large signal at δ 1.25 ppm, methylene groups at δ 1.65 and 2.19 ppm, and signals for one methine at 1.48 ppm and two methyl groups at δ 0.85 ppm (doublet) corresponding to the isopropyl group. EIMS of the acetylated sphingamine showed one ion at *m*/*z* 398 for $[M - AcO]^+$, corresponding to the 1,3,4-triacetyl-2-acetaminoalkane (**4a**) (Figure 1). By comparison with literature data of natural[4,14] and synthetic sphingamines,[27] the optical rotation, $[\alpha]_D$ +26.7° supports the 2*S*,3*S*,4*R* configuration. The data described above and the NMR data of the mixture **1**, which is essentially

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1a** and **2**[a]

	compound **1a**		compounds **2**	
assignment	^{1}H δ ppm (m, J = Hz)	^{13}C δ ppm	^{1}H δ ppm (m, J = Hz)	^{13}C δ ppm
1	4.51 (dd, 1H, 4, 11)	62.0	4.70 (dd, 1H, 7, 10)	70.5
	4.40 (dd, 1H, 5, 11)		4.51 (dd, 1H, 4, 10)	
2	5.10 (m, 1H)	52.9	5.25 (m, 1H)	51.7
3	4.35 (dd, 1H, 4, 6)	76.8	4.30 (dd, 1H, 7, 5)	75.8
4	4.29 (m, 1H)	73.0	4.20 (m, 1H)	72.5
5	2.19 (m, 1H); 1.90 (m, 1H)	34.1	2.19 (m, 1H) 1.90 (m, 1H)	34.1
6	1.65 (m, 2H)	26.6	1.65 (m, 2H)	26.6
$(CH_2)_n$	1.20−1.35	29−30	1.20−1.35	29−30
CH *i*Pr	1.48 (m, 1H)	28.2	1.48 (m, 1H)	28.2
CH_3 *i*Pr	0.85 (d, 6H, 7)	22.8	0.85 (d, 6H, 7)	22.8
NH	8.57 (d, 1H, 9)		8.55 (d, 1H, 9)	
1′		175.2		175.7
2′	4.62 (dd, 1H, 4, 8)	72.5	4.55 (dd, 1H, 8, 4)	72.4
3′	1.98 (m, 1H), 2.18 (m, 1H)	35.6	1.98 (m, 1H), 2.18 (m, 1H)	35.6
$(CH_2)_m$	1.25	29−30	1.25	29−30
CH_2−CH_2−CH_3	1.27 (m, 2H)	32.1	1.27 (m, 2H)	32.1
CH_2−CH_2−CH_3	1.23 (m, 2H)	22.9	1.23 (m, 2H)	22.9
CH_3	0.85 (t, 3H)	14.3	0.85 (3H)	14.3
1″			4.93 (d, 1H, 8)	105.6
2″			3.90 (dd, 1H, 8, 9)	75.2
3″			4.20 (m, 1H)	78.4
4″			4.20 (m, 1H)	71.5
5″			3.80 (ddd, 1H, 2, 5, 9)	78.6
6″			4.50 (dd, 1H, 2, 12)	62.6
			4.30 (m, 1H, 5, 12)	

[a] H (300 MHz); ^{13}C (75 MHz); pyridine-d_5.

identical with that of **1a,** allowed us to assign the structures shown in Figure 1.

Acid methanolysis was performed on the mixture of the homologous compounds **1**. GC−EIMS (*m*/*z*, % rel int) analysis of the acetylated esters showed the presence of methyl esters corresponding to acetoxy-2-docosanoic acid (**3b**) (*m*/*z* 412, 15.6%), acetoxy-2-heneidecanoic acid (**3a**) (*m*/*z* 398, 28.2%), and acetoxy-2-eicosanoic acid (**3c**) (*m*/*z* 384, 15.4%). The mixture corresponding to the sphingamine moieties was acetylated to give a mixture of homologous tetraacetylsphingamines. EIMS revealed three ions for $[M - AcO]^+$ at *m*/*z* 426 for **4b**, 412 for **4c**, and 398 for **4a**, corresponding to (2*S*,3*S*,4*R*) 1,3,4-triacetyl-2-acetaminoalkanes (**4**) (Figure 1).

Mixture **2** was obtained as an amorphous powder, with its FABMS showing several $[M + H]^+$ ions at *m*/*z* 832, 818, 804, 790, and 776, indicating the presence of a homologous series of compounds. This mixture was inseparable by C_{18} reversed-phase HPLC. The ^{1}H and ^{13}C NMR spectra were similar to those of **1a**, except for the presence of additional signals for a glycopyranose moiety. The nature of the sugar unit was determined by NMR analysis and by comparison of the chemical shifts of protons and carbons with literature data.[28] The anomeric proton doublet (δ = 4.93 ppm, J = 8 Hz) was used in the analysis of the COSY spectrum as a starting point for the sequential assignment of the proton resonances in the monosaccharide unit. Five oxymethines and one oxymethylene residues were identified (Table 1). The sugar moiety was identified as a glucopyranose based on the large coupling constants of H-2″ with H-3″, H-4″ with H-5″, and H-5″ with H-6″, implying the axial position of protons H-2″, H-3″, H-4″, H-5″. The occurrence of β-glucopyranose was established through the coupling constants (J = 8 Hz) between H-1″ (δ 4.93) and H-2″ (δ 3.90).

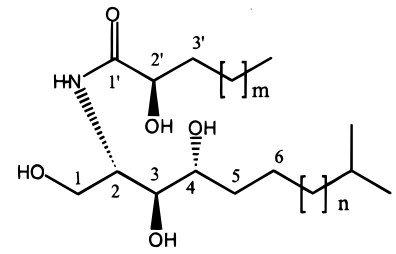
Spectral data and acid methanolysis, which led to the same series of the α-hydroxyacids methyl esters and acetylsphingamines as in **1**, as well as β-methylglucopyranose, allowed us to propose the structures shown in Figure 1 for the cerebrosides (**2**) isolated from *Cystodytes* cf. *dellechiajei*.

Compound **1a** and the mixture **1** both inhibit in vitro the activity of PLA2 (*Crotalus adamanteus*), with an ED_{50} of 100 μg/mL. This is the first report of such an activity for a sphingosine. The homologous cerebrosides (**2**) were inactive in this assay.

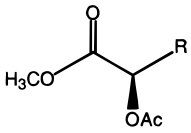
Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AC 300 spectrometer with standard pulse sequences operating at 300.13 and 75.47 MHz, respectively. The chemical shift values are reported in parts per million, units and the coupling constants are in hertz. The programs used for J_{mod}, HMQC, and HMBC (J = 7 Hz) experiments were those of the Bruker manual (1991). HR-FABMS (positive mode) was measured on a ZAB−SEQ spectrometer in a thioglycerine matrix at the "Service central d'analyses du CNRS" (Lyon) and EIMS on a Nermag R 10−10. GC−MS experiments were carried on a Hewlett-Packard 6890 chromatograph (Chrompack CP Sil 8 column 0.25 mm i.d. × 50 m, 1 mL/mn flow rate of helium, operated at 80 °C and increased 5 °C/mn to 290 °C) coupled to a Nermag R 10-10 mass spectrometer. UV spectra were obtained in MeOH, using a Kontron-type Uvikon 930 spectrophotometer, and IR spectra (KBr pellets) were recorded on a Nicolet (Impact 400D) FTIR spectrophotometer. Optical rotations were measured with a Perkin-Elmer 141 polarimeter with a sodium lamp (λ = 589 nm) in a 10-cm microcell. Si gel column chromatography was carried out using Kieselgel 60 (230−400 mesh, E. Merck), gel filtration was carried out using LH20 (Sephadex LH20 17-0090-01 Pharmacia Biotech). Fractionations were monitored by TLC using aluminum-backed sheets (Si gel 60 F_{254}, 0.25 mm thick) with visualization under UV (254 and 366 nm) and Dragendorff spray reagent. All the solvents were distilled prior to use. Semipreparative reversed-phase HPLC (Akzo Nobel RP_{18} column, 7.5 × 250 mm, MeOH/H_2O 95:5) was performed with a L-6200A pump (Merck-Hitachi) equipped with a UV−vis detector (λ = 220 nm) L-4250C (Merck-Hitachi) and a chromato-integrator D-2500 (Merck-Hitachi).

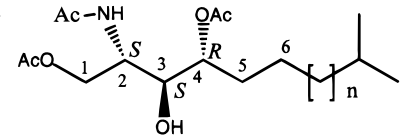
Animal Material. Specimens of the tunicate *C.* cf. *dellechiajei*, Della Valle, 1877 (Ascidiaceae) were collected in the Mediterranean Sea, off the coast of Tunisia near Djerba at 20−25 m depth and preserved in methanol until extraction. The



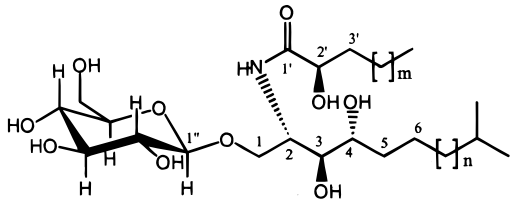
1a : m =18; n =7
1 : m =19, 18, 17; n = 9, 8, 7



3a : R= $(CH_2)_{18}$-CH_3
3b : R= $(CH_2)_{19}$-CH_3
3c : R= $(CH_2)_{17}$-CH_3



4a : n = 7; **4b** : n = 9; **4c** : n = 8



Compounds **2** m = 19, 18, 17; n = 9, 8, 7

Figure 1. Compounds **1a**, **1**, methyl esters of the acetylated α-hydroxyacids **3**, (2*S*,3*S*,4*R*) 1,3,4-triacetyl-2-acetamino-alkanes **4**, compounds **2** (m = 19, 18, 17; n = 9, 8, 7).

voucher specimens were deposited in Museum National d'Histoire Naturelle collection under the reference MNHN A3 CYS III. The animal material (2.7 kg wet wt) was ground and successively extracted with methanol and dichloromethane at room temperature. After filtration, each extract was concentrated under reduced pressure to obtain dichloromethane extract (1.8 g) and methanolic extract (3.7 g).

Extraction and Isolation. The methanolic extract of *C.* cf. *dellechiajei* was separated on a Si gel column eluting with dichloromethane with increasing amounts of methanol. Fractions eluted with 10% and 20% MeOH were pooled and fractionated by HPLC on a C_{18} reversed-phase column (MeOH/H_2O 95:5) to yield mixtures **1** and **2**. Compound **1** was fractionated by HPLC on a C_{18} reversed-phase column (MeOH) to yield compound **1a** and a mixture of homologous compounds. Compound **2** was inseparable by HPLC on C_{18} reversed phase.

Compound 1a: amorphous powder; $[\alpha]_D$ +9.6° (*c* 0.05, MeOH); IR (KBr) ν_{max} 3500, 1640 cm^{-1}; ^1H NMR, ^{13}C NMR data, see Table 1 and text; FABMS (positive mode) $[M + H]^+$ *m/z* 628; HRFABMS *m/z* 628.5897 (calcd for $C_{38}H_{78}NO_5$, 628.5860).

Compounds 2: amorphous powder; $[\alpha]_D$ +5.2° (*c* 1.2, pyridine); IR (KBr) ν_{max} 3500−3100 and 1640 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1 and text; FABMS $[M + H]^+$: *m/z* 832, 818, 804, 790, 776.

Hydrolysis of Compound 1a. A mixture of 5 mL of HCl (1N), 15 mL of MeOH, and 5 mg of **1a** was refluxed for 15 h with magnetic stirring. Water was added, and extraction with *n*-hexane yielded, after drying ($MgSO_4$) and purification on Si gel (dichloromethane/acetone, 98:2), the long-chain methyl ester. This methyl ester was acetylated as follows: a mixture of 2.5 mg of the residue, Ac_2O (1.5 mL), and pyridine (0.15 mL) was allowed to stand at 20 °C overnight, then diluted with 2 mL of H_2O and extracted with CH_2Cl_2. Purification by TLC (hexane/EtOAc, 6:4) gave pure **3a**. The MeOH/H_2O phase was evaporated and the residue acetylated as described above. Purification by filtration over a Sephadex LH20 column (CH_2Cl_2/MeOH, 1:1) gave the tetraacetylsphingamine **4a**.

Hydrolysis of Compounds 1. Hydrolysis was carried out as described for **1a** above to yield **3a**−**3c**, and **4a**−**4c**.

Hydrolysis of compounds 2. To 5 mL of HCl (1 N) and 15 mL of MeOH, 5 mg of **2** were added, and the mixture was refluxed for 15 h with magnetic stirring. Water was added, and extraction with *n*-hexane yielded, after drying ($MgSO_4$) and purification on Si gel (dichloromethane/acetone, 98:2), the long-chain methyl esters, which were acetylated in the same way as in **1a** (described above). The aqueous methanolic layer of the hydrolysate was evaporated to dryness, the residue was separated by TLC (Si gel 60, $CHCl_3$/MeOH/H_2O, 7:30:5), and the sphingamines were acetylated. The presence of methyl-β-D-glucopyranose was established by comparison of NMR data with those reported in the literature [28] and by comparison with an authentic sample (Fluka): $[\alpha]_D$ +73° (*c* 0.1 MeOH), R_f 0.46 (EtOAc/MeOH/H_2O, 5:2:0.5); authentic sample $[\alpha]_D$ +77° (*c* 0.1 MeOH), R_f 0.46).

Compound 3a: $[\alpha]_D$ −2.4° (*c* 0.5, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz) δ 4.75 (1H, dd, *J* = 8, 4 Hz, H-2), 3.52 (3H, s, CH_3O), 2.21 (3H, s, AcO), 2.18 (1H, m, H-3b), 1.98 (1H, m, H-3a), 1.25 $(CH_2)_m$, 1.27 (m, $CH_2CH_2CH_3$), 1.23 (m, CH_2−CH_3), 0.85 (3H, t); ^{13}C NMR ($CDCl_3$, 75 MHz) 170.1 (C-1), 169.8 (C=O, AcO), 77.4 (C-2), 55.2 (CH_3O), 35.6 (C-3), 32.1 ($CH_2CH_2CH_3$), 29−30 $(CH_2)_n$, 22.9 ($CH_2CH_2CH_3$), 14.3 (CH_3); GC−EIMS (*m/z*, % rel int) acetoxy-2-heneidecanoic acid (*m/z* 398, 32.4%).

Compound 4a: $[\alpha]_D$ +26.7° (*c* 0.8, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz), δ 6.09 (1H, d, *J* = 9 Hz, NH), 5.10 (1H, dd, H-3), 5.05 (1H, dd, H-1a), 4.93 (1H, dd, H-1b), 4.90 (1H, m, H-4), 4.49 (1H, ddd, H-2), 2.20−2.00 (12H, s, 4 AcO), 2.19 (1H, m, H-5a), 1.90 (1H, m, H-5b), 1.65 (2H, m, H-6), 1.48 (1H, m, CH-*i*Pr), 1.25 $(CH_2)_n$, 0.85 (6H, d, *J* = 7 Hz, CH_3-*i*Pr); ^{13}C NMR ($CDCl_3$, 75 MHz), 171.2, 170.1, 169.5 (3 C=O, AcO), 78.5 (C-3), 75.2 (C-4), 70.5 (C-1), 51.7 (C-2), 34.1 (C-5), 30−29 $(CH_2)_n$, 28.2 (CH-*i*Pr), 26.6 (C-6), 22.8 (CH_3-*i*Pr), 20.8−20.4 (CH_3, AcO); EIMS $[M - AcO]^+$ *m/z* 398.

PLA2 Assay. The enzyme was extracted from *Crotalus adamenteus* venom (EC 3.1.1.4, Sigma P-0790-270 u/mg solid). The substrate consisted of lecithine (3.5 mM) in a mixture of Triton X-100 (7 mM), NaCl (100 mM), $CaCl_2$ (10 mM), and phenol red (0.055 mM) as colorimetric indicator in 100 mL H_2O.[29] The pH of the reaction mixture was adjusted to 7.6 with NaOH (40 mM). *C. adamenteus* venom PLA2 was solubilized in HPLC-grade water at the concentration of 0.2 μg/μL; 1 μL of PLA2 was incubated with 100 μg/10 μL DMSO of each extract or pure compound for 1.5 h at room temperature. Then 1 mL of the substrate was added, and the absorbance at 588 nm was read at time 0 and 5 min. Percent inhibition of the enzyme activity was determined by comparison with a vehicle control, and manoalide at 0.25 μM was used as control for 100% inhibition.[30]

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