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Antiepileptic Ceramides from the Red Sea Sponge *Negombata corticata*

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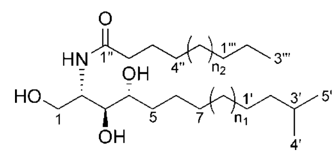
Abstract: A new antiepileptic ceramide mixture **1** was isolated from the Red Sea sponge *Negombata corticata*. The structures of the metabolites were determined by extensive spectroscopic analysis. The anticonvulsant activity of **1** was measured *in vivo* using the pentylenetetrazole-induced seizure model. This finding has important implications for biological studies with this class of compounds.

Sterols and fatty acid are potentially excellent biomarkers in marine samples due to their stability and diversity of structures. They are present in all eukaryotes and share with phospholipids a structural function in membranes due to their role in chemotaxonomic purposes and for food web tracing.[1] Sphingolipids have emerged as a new class of modulators of various cell functions. Ceramides, which are the central moiety in the biosynthesis of sphingolipids and glycosphingolipids, are involved in the regulation of different cellular events, including cell senescence, differentiation, and programmed cell death (apoptosis).[2] Ceramides also act as regulators of many biochemical and cellular responses to stress, such as exposure to heat, radiation, oxidative conditions, and chemotherapeutic agents.[3] Ceramides as well as more complex sphingolipids are required for activation of membrane fusion of Semliki Forest virus (SFV) and other alphaviruses.[4] An interest in ceramides as regulators of growth, differentiation, and cellular apoptosis has recently increased. Many marine invertebrates are rich sources of ceramides that differ in structure and biological properties from those of terrestrial organisms. Unusual ceramides have been isolated from sponges,[5–8] Coelenterata,[9–11] crabs,[12] sea stars,[13] and ascidia.[14] Marine ceramides manifest cytotoxic, antitumor, antimicrobial,[9] and antifungal activities.[7] Others are sex heromones[12] and enzyme inhibitors.[14]



$\mathbf{1_a}$ $n_1 = 13$, $n_2 = 9$
$\mathbf{1_b}$ $n_1 = 11$, $n_2 = 11$

In this work, we report on the isolation and identification of a new ceramide mixture **1** ($\mathbf{1_a}$ and $\mathbf{1_b}$) from the Red Sea sponge *Negombata corticata*. The genus *Negombata* is represented in the Red Sea by two species, namely, *Negombata magnifica* (Keller) (formerly *Latrunculia magnifica*) and *Negombata corticata* (Carter), family Podospongiidae.[15] The genus *Negombata* was shown to be a source of biologically active macrolides[16–19] and lipids.[20]

The structure elucidation of **1** began with an analysis of HRMS data. The high-resolution ESI-TOF mass spectrum of **1** displayed a pseudomolecular ion peak at *m/z* 676.6230 [M + Na]$^+$, which when combined with the detailed analysis of the ^{13}C spectrum and DEPT indicated a molecular formula of $C_{41}H_{83}O_4N$, representing one unit of unsaturation. The ^{1}H NMR spectrum in C_5D_5N showed resonances of an amide proton doublet at δ 8.44 (1H, d, J = 8.4 Hz) and protons of a long methylene chain at δ 1.28, indicating a sphingolipid skeleton. The characteristic resonances of 2-amino-1,3,4-triol of the hydrocarbon chain were observed at δ 5.10 (1H, m), 4.50 (2H, dd, J = 8.0, 4.8), 4.38 (1H, m), and 4.29 (1H, m) in the ^{1}H NMR spectrum and at δ 54.1 (CHN), 62.5 (CH_2O), 77.0 (CHOH), and 73.3 (CHOH) in the ^{13}C NMR spectrum. In addition, the ^{1}H NMR spectrum showed resonances corresponding to aliphatic hydrocarbons at δ 0.89 (6H, d, J = 7.2, H-4′ and H-5′), 0.88 (3H, t, J = 6.8, H-3‴), 1.28 (overlapped H, m), 1.84 (1H, sep, H-3′), 1.95 (2H, m, H-3″), and 2.47 (2H, t, J = 7.6, H-2″). The ^{13}C NMR spectrum showed resonances due to one terminal methyl group at δ 14.6 and two branched methyl groups in aliphatic hydrocarbon chains at δ 23.1 and an amide carbonyl at δ 173.7. Analysis of the ^{1}H−^{1}H COSY, HMQC, and HMBC spectra led to the assignment of proton and carbon signals for **1**. The positions of the hydroxyl groups were confirmed by a ^{1}H−^{1}H COSY spectrum between H_2-1/H-2, H-2/H-3, H-3/H-4, and H-4/H_2-5 and also from HMBC of H_2-1/C-2 ($^2J_{CH}$), H_2-1/C-3 ($^3J_{CH}$), H-2/C-3 ($^2J_{CH}$), H-3/C-2 ($^2J_{CH}$), H-3/C-4 ($^2J_{CH}$), H-4/C-2 ($^3J_{CH}$), and H-4/C-6 ($^3J_{CH}$), leading to the assignment of C-1/C-2/C-3/C-4. GC-MS analysis of the fatty acid methyl ester of **1** was carried out after

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Table 1. ^{13}C (100 MHz), ^{1}H (400 MHz), COSY, and HMBC NMR Spectroscopic Data of Ceramide Mixture **1**

no.[a]	δ_C in $CDCl_3$	δ_H (mult.,J_{Hz}) in $CDCl_3$	δ_C in C_5D_5N	δ_H (mult.,J_{Hz}) in C_5D_5N	COSY (H−H)	HMBC (H−C)
1	61.1	3.73 (dd, 4.8)3.90 (dd, 3.2)	62.5	4.50 (dd, 8.0, 4.8)	2	2,3
2	52.0	4.15 (m)	54.1	5.10 (m)	1	3
3	75.4	3.59 (m)	77.0	4.38 (m)	2, 4	4
4	72.4	3.62 (m)	73.3	4.29 (m)	3, 5	5, 6
5	32.7	1.25 (m)	34.2	1.28 (m)	4, 6	6, 7
6	25.7	1.25 (m)	26.8	1.28 (m)		
7	29.8	1.25 (m)	30.2	1.28 (m)		
n_1	29.6	1.25 (m)	30.2	1.28 (m)		
1′	27.3	1.25 (m)	28.0	1.28 (m)		
2′	39.0	1.25 (m)	39.6	1.28 (m)		
3′	27.8	1.50 (sep)	28.5	1.84 (sep)	2′, 4′, 5′	2′, 4′, 5′
4′	22.3	0.86 (d, 7.2)	23.1	0.89 (d, 7.2)	3′	2′, 3′, 5′
5′	22.3	0.86 (d, 7.2)	23.1	0.89 (d, 7.2)	3′	2′, 3′, 4′
1″	175.0		173.7			
2″	36.4	2.21 (t, 7.6)	37.2	2.47 (t, 7.6)	3″	1″, 3″, 4″
3″	25.7	1.61 (m)	27.0	1.95 (m)	2″, 4″	2″, 4″, n_2
4″	29.2	1.25 (m)	30.2	1.28 (m)	3″, n_2	2″, 3″, n_2
n_2	29.6	1.25 (m)	30.2	1.28 (m)		
1‴	31.8	1.25 (m)	32.4	1.28 (m)		
2‴	22.5	1.25 (m)	23.3	1.28 (m)	1‴, 3‴	n_2, 1‴, 3‴
3‴	13.7	0.84 (t, 6.8)	14.6	0.88 (t, 6.8)	2‴	1‴, 2‴
NH		6.39 (d, 7.2)		8.44 (d, 8.4)	2	1″

[a] n_1 = 13, 11, n_2 = 9, 11.

hydrolysis and yielded two peaks with molecular ions of *m/z* 270 and 298 on the chromatogram, corresponding to C_{16} and C_{18} fatty acid methyl esters with a ratio of 62.5:37.5, respectively. The characteristic ^{1}H NMR resonance in $CDCl_3$ of methyl esters at 0.87 (3H, t, J = 6.8) indicates the presence of only terminal fatty acids palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids. The ^{1}H NMR spectrum in $CDCl_3$ of the liberated sphingosine bases after hydrolysis showed a characteristic resonance at 0.81 (6H, d, J = 7.2), indicating the presence of only isopropyl terminal sphingosine bases. LRESIMS analysis of the sphingosine bases showed molecular ions at *m/z* 388.3 $[M + H]^+$ and *m/z* 416.4 $[M + H]^+$, corresponding to C_{23} and C_{25}, respectively.

The configurations of the ceramide moieties were assigned by comparison of the physical data and ^{1}H NMR and ^{13}C NMR in two different solvents (C_5D_5N and $CDCl_3$) with the analogues as reported in the literature, in which the optical rotation [+17.4 (*c* 0.08, MeOH) and +11.1 (*c* 1.00, MeOH)] and the chemical shifts of H-2 (δ 5.10), H-3 (δ 4.38), and H-4 (δ 4.29) in C_5D_5N were in good agreement with those of known synthetic ceramide (2*S*,3*S*,4*R*)-2-[(2*R*)-2-hydroxytetracosanoylamino]-1,3,4-hexadecanetriol[21] and natural ceramide gracilamide B.[22] Also the chemical shifts of H-2 (δ 4.15), H-3 (δ 3.59), and H-4 (δ 3.62) in $CDCl_3$ were similar to those of (2*S*, 3*S*, and 4*R*) ceramides from the sea sponge *Grayella cyatophora*[23] and *Oceanapia* sp.[24] This evidence indicates the absolute configurations of C-2, C-3, and C-4 to be 2*S,* 3*S*, and 4*R*, respectively.

A detailed analysis of COSY and HMBC correlations was found to be in complete agreement with the proposed structure for **1** (Table 1).

Experimental Section

General Experimental Procedures. Optical rotations were measured at ambient temperature using a Rudolph Research Analytical Autopol IV automatic polarimeter. The IR spectrum was recorded on a Bruker Tensor 27 spectrophotometer. 1D and 2D NMR spectra were recorded in $CDCl_3$ and C_5D_5N on a Bruker Avance DPX-400 spectrometer and on a Varian AS 400 spectrometer. The low-resolution ESIMS were obtained using a Finnigan Mat LCQ. The high-resolution ESIMS was measured using a Bruker Daltonic (GmbH, Germany) micro-TOF series with electrospray ionization.

Biological Material, Collection, and Identification. *Negombata corticata*, Carter (coll. no. SAA-8) was collected by hand using scuba at depths of 15–20 m from Safaga in the Egyptian Red Sea. The sponge materials were frozen immediately and kept frozen at −20 °C until processed. A voucher specimen is deposited at the Zoological Museum of the University of Amsterdam, under registration No. ZMAPOR. 18569.

Extraction and Isolation. The sponge was freeze-dried (400 g dry weight), ground, and extracted with a mixture of $MeOH/CH_2Cl_2$ (1:1) (3 × 2 L) at room temperature. The extract was evaporated under vacuum to afford 100 g of red oil. This extract was subjected to vacuum liquid chromatography on a flash silica gel column using a hexanes, EtOAc, and MeOH gradient.

Fractions eluted from 70% EtOAc in hexanes to EtOAc were concentrated to afford 7 g of reddish residue. Purification of this fraction was carried out by column chromatography using flash silica gel. The mobile phase was made from $CHCl_3$ with an increasing gradient of MeOH. Fractions eluted with 5% MeOH in $CHCl_3$ were collected and evaporated to dryness under reduced pressure to afford 250 mg of a green residue. Further purification was carried out on column chromatography over a silica gel 60/230–400 mesh (flash) column (50 × 0.5 cm) using 2% MeOH in $CHCl_3$. Final purification was carried out on Sephadex LH-20 using $MeOH/CHCl_3$ (1:1) to afford 50 mg of **1**.

Hydrolysis of Ceramide Mixture 1. The mixture (3 mg) was heated with 5 mL of 1 N HCL in 15 mL of MeOH for 4 h at 90 °C. The reaction mixture was extracted with hexanes, and the hexanes layer was concentrated under vacuum to give a mixture of fatty acid methyl esters. NaOH solution (10%) was added to the methanolic layer containing a mixture of sphingosine bases up to pH 12.0, the sphingosine bases were extracted with $CHCl_3$, and the extract was evaporated in a vacuum to dryness.

Compound 1: white solid; R_f = 0.65, 10% MeOH in $CHCl_3$; $[\alpha]^{25}_D$ +17.4 (*c* 0.08, MeOH) and $[\alpha]^{25}_D$ +11.1 (*c* 1.00, MeOH); IR (KBr) (thin film) ν_{max} 3309, 2920, 2850, 2371, 1642, 1545, 1467, 1048.2 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz and $CDCl_3$, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz and $CDCl_3$, 100 MHz), see Table 1; LRESIMS, found *m/z* 652.6 $[M - H]^-$; HRTOFMS, found *m/z* 676.6230 $[M + Na]^+$ (calcd for $C_{41}H_{83}O_4NNa$, 676.6220).

Anticonvulsant Bioassay. Animals. A total of 24 male albino rats (125–155 g) were housed separately, two to a cage, under standard laboratory conditions. They were kept at constant room temperature (27 ± 2 °C) and relative humidity of 55–65% under a 12/12 h light/dark cycle at least 10 days prior to testing. Commercial food pellets and tap H_2O were freely available. The experiments were performed during the light portion of the cycle, between 8:00 and 12:00 a.m., to avoid circadian influences. Rats were divided into 4 groups, 6 rats each: control group, reference group, drug group (dose, 0.5 mg/kg, ip), and drug group (dose, 1 mg/kg, ip).

Drugs and Dosage. The effects of **1** on seizure susceptibility were evaluated using the pentylenetetrazole (PTZ) test.[25] Thirty minutes after administration of various doses of **1** (0.5 mg/kg, ip and 1 mg/kg, ip), animals received an intraperitoneal (ip) injection of PTZ (45 mg/kg,

Table 2. Anticonvulsant Activity of Ceramide Mixture **1**

	n	X score[a]	death
control group (pentylenetetrazole)	6	4.75 ± 0.21	6
reference group (diazepam)	6	0.13 ± 2.25	2
ceramide mixture **1** (0.5 mg/kg, ip)	6	0.12 ± 2.25	no
ceramide mixture **1** (1 mg/kg, ip)	6	0.12 ± 2.25	no

[a] 0, No seizure response; 1, Immobility, eye closure, ear twitching, facial clonus; 2, Head nodding associated with more severe facial clonus; 3, Clonus of one forelimb; 3.5, Bilateral forelimb clonus without rearing; 4, Bilateral forelimb clonus with rearing; 4.5, Falling on a side (without rearing), loss of righting reflex accompanied by generalized; clonic seizures; 5, Rearing and falling on back accompanied by generalized clonic seizures.

ip) and were then observed for a 30 min period for clonic seizures. The effect of **1** was compared against a reference group, where the rats were pretreated with diazepam (DZP) (1 mg/kg, ip) (Valpam amp., Amoun Co., Egypt), 30 min before each PTZ injection, and a control group, where the rats were injected with subconvulsive doses of PTZ dissolved in saline (45 mg/kg, ip) (Sigma).[25]

Evaluation of Seizures. After each injection, the convulsive behavior was observed for 30 min. The resultant seizures were classified according to the Racine rating scale as shown in Table 2.[26] Values are expressed as mean ± SEM. For statistical analysis, one-way analysis of variances was applied. For all comparisons, differences were considered significant at $p < 0.05$.

In PTZ-induced seizure, the administration of **1** at doses of 0.5 mg/kg, ip and 1 mg/kg, ip 30 min before the injection of PTZ prolonged the latency and reduced the duration of tonic-clonic seizures and showed antiepileptic effect comparable to that of DZP in a PTZ acute model, Table 2. There was no significant difference between seizure parameters for rats in the reference group (DZP) and those treated with **1**. Furthermore, there was a significant difference between seizure parameters of rats in the control group (PTZ) and those treated with **1**, Table 2. In addition **1** was shown to prevent death significantly.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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