Anasterocerebroside A, a New Glucosylceramide from the Patagonian Starfish *Anasterias minuta*

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Eight glucosylceramides (**1–8**) were isolated from the water-insoluble lipid fraction of a methylene chloride/methanol/water extract of the Patagonian starfish *Anasterias minuta*. One of the constituents was identified as a new glucosylceramide, anasterocerebroside A (**1**), while the known glucosylceramide **7** was isolated and characterized for the first time as a pure compound. The structures of **1** and **7** were established by spectroscopic and chemical methods.

Key words: Anasterias minuta, Starfish, Glycosphingolipids

Introduction

Glycosphingolipids are a large group of biomolecules that consist of a ceramide linked at position 1 by a β-glycosidic bond to a hydrophilic carbohydrate moiety. The hydrophobic ceramide portion involves a sphingoid base and an amide-linked fatty acyl chain. Glycosphingolipids have been isolated from several marine invertebrates. Among echinoderms, glucosylceramides, gangliosides and sphingosine derivatives have been isolated from sea urchin (Babu *et al*., 1997), starfish and sea cucumber (Chludil *et al.*, 2002a). As part of our investigation on polar metabolites from cold-water echinoderms of the South Atlantic, we have previously reported the isolation and structural elucidation of glucosylceramides from the starfishes *Cosmasterias lurida* (Maier *et al*., 1998) and *Allostichaster inaequalis* (Díaz de Vivar *et al*., 2002).

As for the constituents of *Anasterias minuta*, a very common starfish collected in cold waters off the Patagonian coast, we have recently described the isolation and structural elucidation of two new sulfated steroidal hexaglycosides which showed antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum* (Chludil *et al*., 2002b). In this paper we report on the isolation and characterization of a new glucosylceramide, Anasterocerebroside A (**1**) together with seven known related compounds from the starfish *A. minuta.*

Results and Discussion

A water-insoluble lipid fraction, which was obtained from the CH_2Cl_2–MeOH (2:1 v/v) extract of the starfish *A. minuta* was treated with organic solvents followed by vacuum-dry CC and CC over silica gel to render a glycosylceramide mixture AM. This fraction exhibited the characteristic signals of a sphingosine-type glucosylceramide, possessing 2-hydroxy fatty acid and β-glucopyranose moieties in the ^{1}H NMR spectrum (Díaz de Vivar *et al*., 2002). FABMS (negative ion mode) of the mixture showed several $[M-H]^-$ peaks, revealing the presence of a complex mixture of glucosylceramides. Before separation of the mixture AM into individual glucosylceramides, the fatty acid constituents and long-chain base moieties of these molecular species were investigated. AM was methanolized (Gaver and Sweeley, 1965) with 0.9 N HCl in 82% aqueous MeOH to yield a mixture of fatty acid methyl esters (FAME) and a mixture of long chain bases (LCB) together with methyl-D-glucopyranoside. GC analysis of the peracetylated alditol derivative confirmed glucose as the hexosyl moiety.

Analysis of the FAME mixture of AM by CG-MS showed the presence of thirteen compounds (FAME 1–13), which were characterized by comparing their spectral data with those reported previously (Higuchi *et al*., 1991 and 1994). The 2-hydroxy-substituted saturated FAME, compris-

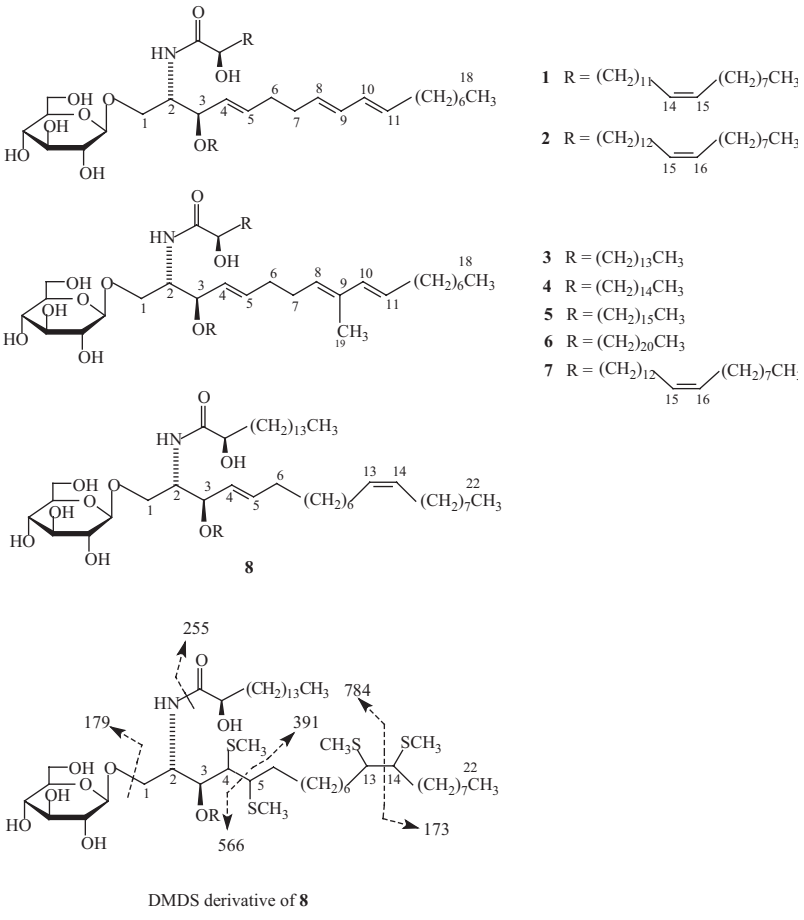
ing *ca*. 94% of the mixture, were characterized as methyl-(2*R*)-hydroxy-tetradecanoate (FAME-2), -pentadecanoate (FAME-3), -hexadecanoate (FAME-4), -heptadecanoate (FAME-5), -octadecanoate (FAME-6), -nonadecanoate (FAME-8), -eicosanoate (FAME-9), -docosanoate (FAME-10), and -tricosanoate (FAME-12). Two unsaturated and two nonsubstituted FAME were identified as methyl-(2*R*)-hydroxy-tricosenoate (FAME-11), methyl-(2*R*)-hydroxy-tetracosenoate (FAME-13), methyl-pentadecanoate (FAME-1), and methyl-eicosanoate (FAME-7) (Table I). The position of the double bond in the unsaturated methyl-(2*R*)-hydroxyacids (FAME-11 and 13) was determined by GC-MS analysis of the DMDS derivatives (Vincenti *et al*., 1987). Unsaturated FAME comprised 38.5% of the mixture and were identified as methyl-(2*R*)-hydroxy-14-tricosenoate (FAME-11) and methyl-(2*R*)-hydroxy-15-tetracosenoate (FAME-13). Optical rotation of the FAME mixture ($[\alpha]_D = -4.8°$ (c = 1.0, $CHCl_3$)) is in good agreement with the data reported in the literature (Pretorius and Horn, 1954); therefore, the absolute stereochemistry at C-2′ is suggested to be *R*.

The LCB mixture derived from methanolysis of AM was acetylated and analyzed by GC-MS, showing the presence of 2-acetamido-1,3-diacetoxy-4,8,10-octadecatriene (acetylated LCB-1) (*m/z* 421 $[M]^+$, 361 $[M-CH_3COOH]^+$, 302 $[M-CH_3COOH-CH_3COO]^+$, 234, 196, 144, 85), 2-acetamido-1,3-diacetoxy-9-methyl-4,8,10-octadecatriene (acetylated LCB-2) (*m/z* 435 $[M]^+$, 375 $[M-CH_3COOH]^+$, 316 $[M-CH_3COOH-CH_3COO]^+$, 273, 256, 179, 85), and 2-acetamido-1,3-diacetoxy-4,13-docosadiene (acetylated LCB-3) (*m/z* 479 $[M]^+$, 419 $[M-CH_3COOH]^+$, 360 $[M-CH_3COOH-CH_3COO]^+$, 335, 275, 266, 123). The relative stereochemistry at C-2 and C-3 was proposed as 2*S*,3*R* from optical rotation data of the acetylated LCB mixture ($[\alpha]_D = -13.7°$ (c = 0.8, $CHCl_3$)), in good agreement with data for synthetic 1-*O*-2-*N*-3-*O*-triacetyl-D-erythro-sphingosine (Julina *et al*., 1986).

Fraction AM was separated repeatedly by RP-HPLC into eight major fractions each displaying a single peak on RP-HPLC and single molecular ion peaks in their negative FAB mass spectra. Glucosylceramide **1** (Fig. 1) was obtained as a white amorphous powder. The FABMS (negative ion mode) showed a $[M-H]^-$ peak at *m/z* 806 and the corresponding $[M-H-162]^-$ ion at *m/z* 644 due to the loss of the hexose unit. The ^1H and ^{13}C NMR spectra (Table II) showed characteristic signals due to C-1–C-11, C-1′, C-2′, C-1″–C-6″ of a 1-*O*-β-glucopyranoside containing a sphingosine-type LCB moiety and a 2-hydroxy fatty acid. The ^{13}C NMR spectrum confirmed the ceramide nature of compound **1** (δ 176.8 ppm, C=O) with normal-chain base and fatty acid (terminal methyl at δ 14.4 ppm) and a (2*S*,3*R*,4*E*)-sphingosine-type base indicated by C-2 and C-3 chemical shifts at δ 54.2 and 72.6 ppm, respectively (Chludil *et al*., 2002a). The unsaturation pattern in the ^1H NMR spectrum showed a double doublet at 5.48 ppm and a double triplet at 5.72 ppm, characteristic of a Δ^4 sphingosine with *trans* configuration (J = 15.3 Hz) and multiplets at δ 5.34 (2H), 5.54 (2H) and 5.98 (2H) ppm. The $^1H.^1H$ COSY experiment

Table I. FAME composition of glucosylceramide mixture of *A. minuta* (in % of the total fatty acid methyl esters mixture) and MS data.

Fatty acid methyl ester	%	$[M]^+$	$[M-32]^+$	$[M-59]^+$
Methyl-pentadecanoate (FAME-1)	1.8	256	224	197
Methyl-(2*R*)-hydroxy-tetradecanoate (FAME-2)	1.7	258	226	199
Methyl-(2*R*)-hydroxy-pentadecanoate (FAME-3)	2.2	272	240	213
Methyl-(2*R*)-hydroxy-hexadecanoate (FAME-4)	16.9	286	254	227
Methyl-(2*R*)-hydroxy-heptadecanoate (FAME-5)	5.8	300	268	241
Methyl-(2*R*)-hydroxy-octadecanoate (FAME-6)	6.4	314	282	255
Methyl-eicosanoate (FAME-7)	2.7	326	294	227
Methyl-(2*R*)-hydroxy-nonadecanoate (FAME-8)	2.0	328	296	269
Methyl-(2*R*)-hydroxy-eicosanoate (FAME-9)	2.0	342	310	283
Methyl-(2*R*)-hydroxy-docosanoate (FAME-10)	1.8	370	338	311
Methyl-(2*R*)-hydroxy-tricosenoate (FAME-11)	8.6	382	350	323
Methyl-(2*R*)-hydroxy-tricosanoate (FAME-12)	2.3	384	352	325
Methyl-(2*R*)-hydroxy-tetracosenoate (FAME-13)	19.9	396	364	337



DMDS derivative of **8**

Fig. 1. Chemical structures of glucosylceramides **1–8** and FABMS (negative ion mode) fragmentation peaks of DMDS derivative of **8**.

showed correlations between the H-4 signal (δ 5.48), the broad triplet at 4.12 ppm (H-3) and the double triplet at 5.72 ppm (H-5). The latter signal also correlated with a multiplet at δ 2.10 ppm (H-6). Further correlations between olefinic signals at δ 5.54 and 5.98 ppm and vicinal methylene groups at positions 7 and 12 allowed us to establish the olefinic pattern of the LCB unit as a 4,8,10-triene (Díaz de Vivar *et al.*, 2002). The same LCB was identified in a mixture of LCB obtained from hydrolysis of the glucosylceramide mixture from spermatozoa of the starfish *Asterias amurensis* (Irie *et al.*, 1990) and in a glucosylceramide isolated from gonads and body walls of the starfish *Allostichaster inaequalis* (Díaz de Vivar *et al.*, 2002). The isolated olefinic triplet at 5.34 ppm which correlated only with a methylene signal at 2.03 ppm, was therefore assigned to the acid moiety. *Z* geometry was deduced from the *J* value (5.0 Hz) and the triplet shape of the signal (Higuchi *et al.*, 1991) in the ^{1}H NMR spectrum and was confirmed by the chemical upfield shift of allylic methylene carbon signals at 27.9 ppm (Kim *et al.*, 1997).

Acidic methanolysis of **1** yielded a FAME, a methylglycoside and a LCB. GC-MS analysis of the FAME showed a single peak. The molecular ion peak at *m/z* 382 $[M]^+$ and fragment ion peaks at *m/z* 350 $[M\text{-}32]^+$ and 323 $[M-CH_3COO]^+$ confirmed the presence of methyl-2-hydroxytricosenoate (FAME-11, Table I). The presence of a double bond was confirmed by the triplet at δ 5.34 ppm in the ^{1}H NMR spectrum of the FAME and its position was determined by GC-MS analysis of the DMDS derivative (Vincenti *et al.*, 1987) according to peaks at *m/z* 476 $[M]^+$, 429 $[M-SCH_3]^+$, 417 $[M-CH_3COO]^+$, 303 $[M\text{-}173]^+$ and 173. Acetylation of LCB derived from methano-

Table II. ^{1}H and ^{13}C NMR chemical shifts for **1** and **7** (500 MHz, CD_3OD). δ in ppm, *J* in Hz[a].

Position	**1** δ_H, *J* (Hz)	**1** δ_C	**7** δ_H, *J* (Hz)	**7** δ_C	**8** δ_H, *J* (Hz)	**8** δ_C
1a	3.70 *dd* (10.5, 3.7)	69.3 t	3.71 dd (10.3, 3.7)	69.7 t	3.71 *dd* (10.3, 3.4)	69.8 t
1b	4.09 *dd* (10.5, 5.5)		4.09 *dd* (10.3, 5.5)		4.08 *dd* (10.3, 5.5)	
2	3.98 *m*	54.2 d	3.98 *m*	54.6 d	3.98 *m*	54.6 d
3	4.12 *bt* (7.3)	72.6 d	4.14 *bt* (7.3)	73.1 d	4.12 *bt* (7.3)	73.1 d
4	5.48 *dd* (15.3, 7.3)	130.8 δ	5.50 *dd* (15.2, 7.3)	130.3 d	5.46 *dd* (15.3, 7.3)	130.8 d
5	5.72 *dt* (15.3, 5.4)	134.1 d	5.74 *dt* (15.2, 6.4)	134.3 d	5.72 *dt* (15.3, 6.4)	135.0 d
6	2.10 *m*	33.0 t	2.07 *m*	33.9 t	2.03 *m*	33.4 t
7	2.12 *m*	32.9 t	2.20 *m*	35.9 t	2.00 *m*	35.1 t
9	5.98 *m*	131.3 d[c]		135.2 s		
10	5.98 m	131.9 d[c]	6.02 *d* (15.7)	136.1 d		
11	5.54 *dt* (14.5, 6.6)	131.6 d[b]	5.56 *dt* (15.7, 6.8)	131.3 d		
$(CH_2)_n$	1.27–1.30 *bs*	30.3–30.9	1.27–1.30 *bs*	30.3–30.9	1.27 – 1.30 *bs*	30.3–30.9
CH_2CH_3	0.87 *t* (7.0)	14.4 q	0.89 *t* (6.8)	14.4 q	0.88 *t* (6.9)	14.5 q
12	2.04 *m*	33.4 t	2.06 *m*	33.5 t	2.03 *m*	28.1 t
13					5.34 *bt* (5.9)	130.9 d
14					5.34 *bt* (5.9)	130.9 d
15					2.03 *m*	28.1 t
19			1.70 *s*	12.8 q		
1′		176.8 s		177.2		177.2 s
2′	3.98 *m*	72.8 d	3.98 *m*	72.8 d	3.98 *m*	72.9 d
3′a	1.56 *m*	35.8 t	1.56 *m*	35.8 t	1.54 *m*	35.9 t
3′b	1.73 *m*		1.73 *m*		1.72 *m*	
4′	1.39 *m*	26.2 t	1.39 *m*	26.2 t	1.42 *m*	26.2 t
C=CH	5.34 *t* (5.0)	130.6 d	5.34 *t* (5.0)	130.6 d		
1″	4.25 *d* (7.8)	104.2 d	4.26 *d* (7.8)	104.7 d	4.25 *d* (8.0)	104.7 d
2″	3.19 *dd* (9.1,7.8)	74.6 d	3.18 *dd* (9.0, 7.8)	75.0 d	3.19 *dd* (8.9, 7.8)	75.0 d
3″	3.34 *m*	77.5 d	3.34 *m*	77.9 d	3.34 *m*	77.9 d
4″	3.30 *m*	71.2 d	3.30 *m*	71.6 d	3.30 *m*	71.6 d
5″	3.28 *m*	77.5 d	3.30 *m*	77.9 d	3.30 *m*	77.9 d
6″a	3.65 *dd* (12.0, 5.2)	62.4 t	3.65 *dd* (12.0, 3.8)	62.7 t	3.66 *dd* (12.0, 5.5)	62.7 t
6″b	3.85 *dd* (12.0, 2.5)		3.85 *dd* (12.0, 2.0)		3.86 *dd* (12.0, 2.0)	

[a] Assignments were based on ^{1}H-^{1}H COSY and HETCOR experiments and comparison with spectroscopy data reported in the literature (Chludil *et al*., 2002a).
[b,c] Assignments could be reversed. Carbon multiplicities were determined by DEPT experiments.

lysis of **1** and GC-MS analysis showed the presence of a C-18 sphingosine-type base (acetylated LCB-1) on the basis of peaks at *m/z* 421 $[M]^+$, 196 (allylic cleavage to Δ^5) and 85 (allylic cleavage to Δ^{10}).

The absolute stereochemistry of glucose was determined as D by hydrolysis of **1** with 2 N trifluoroacetic acid, reaction of the monosaccharide residue with L-cysteine methyl ester hydrochloride, acetylation and GC analysis and comparison with D- and L-glucose derivatives (Hara *et al*., 1986).

All these data allowed us to determine the structure of the new glucosylceramide **1** as (2*S*,3*R*,4*E*,8*E*,10*E*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[(*R*)-2-hydroxy-14-tricosenoyl]amino-4,8,10-octadecatriene.

Glucosylceramide **2** showed the same ^{1}H and ^{13}C NMR signals as **1**. The FABMS (negative ion mode) showed a $[M-H]^-$ peak at *m/z* 820 and the corresponding $[M-H\text{-}162]^-$ ion at *m/z* 658 due to the loss of the hexose unit. Glucosylceramide **2** was methanolized and the FAME analyzed by GC-MS. A single FAME was detected for **2** and identified as FAME-13 (Table I) on the basis of peaks at *m/z* 396 $[M]^+$, 364 $[M\text{-}32]^+$, and 337 $[M-CH_3COO]^+$. The double bond position of FAME-13 was determined by GC-MS analysis of the DMDS derivative according to peaks at *m/z* 490 $[M]^+$, 443 $[M-SCH_3]^+$, 417 $[M-CH_3COO]^+$, 317 $[M\text{-}173]^+$ and 173. LCB obtained from methanolysis of **2** was acetylated and analyzed by GC-MS showing the presence of acetylated LCB-1. Taking into account the FABMS and NMR data and the methanolysis products, glucosylceramide **2** was identified as (2*S*,3*R*,4*E*,8*E*,10*E*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[(*R*)-2-hydroxy-15-tetra-

cosenoyl]amino-4,8,10-octadecatriene, previously isolated from the starfish *Allostichaster inaequalis* (Díaz de Vivar *et al*., 2002).

Glucosylceramides **3–7** (Fig. 1) showed the same characteristic ^{1}H and ^{13}C NMR signals of a 1-*O*-β-glucopyranoside of a (2*S*,3*R*,4*E*,8*E*,10*E*)-2-amino-1,3-dihydroxy-9-methyl-4,8,10-octadecatriene ceramide possessing a (2*R*)-hydroxy-fatty acid. (Table II). The presence of a singlet at δ 1.70 ppm in the ^{1}H NMR spectrum and of the corresponding signal at 12.8 ppm in the ^{13}C NMR spectrum was diagnostic for the presence of a methyl group (C-19) attached to an olefinic carbon. These spectra were almost superimposable with those of glucosylceramides isolated from the ascidian *Phallusia fumigata* (Durán *et al*., 1998) and the starfishes *Ophidiaster ophidiamus* (Jin *et al*., 1994), *Cosmasterias lurida* (Maier *et al*., 1998) and *Allostichaster inaequalis* (Díaz de Vivar *et al*., 2002). Glucosylceramides **3–7** were methanolized and the FAME analyzed by GC-MS; a single FAME was detected for glucosylceramide **3** (FAME-3), **4** (FAME-4), **5** (FAME-5), **6** (FAME-9), and **7** (FAME-13). Double bond position in FAME-13 was determined from GC-MS analysis of the DMDS derivative as described previously in the analysis of glucosylceramide **2**. LCB obtained from methanolysis of **3–7** were acetylated and analyzed by GC-MS, showing the presence of 2-acetamido-1,3-diacetoxy-9-methyl-4,8,10-octadecatriene (acetylated LCB-2). Taking into account the FABMS spectra and the methanolysis products of each fraction, glucosylceramide **3** was identified as phalluside-1, previously isolated from the ascidian *Phallusia fumigata* (Durán *et al*., 1998), whereas **4** and **5** are also known compounds previously isolated from *C. lurida* (Maier *et al*., 1998) and **6** was identified as ophidiacerebroside C, previously isolated from *O. ophidiamus* (Jin *et al.,* 1994).

This is the first time that glucosylceramide **7** has been isolated and characterized as a pure compound. It has been isolated previously as a mixture of related glucosylceramides from the patagonian starfishes *Cosmasterias lurida* (Maier *et al*., 1998) and *Allostichaster inaequalis* (Díaz de Vivar *et al*., 2002). The FABMS (negative ion mode) of **7** showed a $[M-H]^-$ peak at *m/z* 834 and the corresponding $[M-H-162]^-$ ion at *m/z* 672 due to the loss of the hexose unit. The ^{1}H and ^{13}C NMR spectra (Table II) showed characteristic signals for a glucosylceramide containing sphingosine-type LCB-2. The ^{13}C NMR spectrum with DEPT displayed signals due to one methyl (δ 12.8) attached to a quaternary olefinic carbon (δ 135.2) and five olefinic methine carbons (δ 128.6, 130.3, 131.3, 134.3 and 136.1). The ^{1}H NMR signals together with the ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR cross-peaks indicated the presence of a Δ^4 sphingosine with *trans* configuration and a *trans* conjugated olefinic system which differed from the olefinic pattern observed for glucosylceramides **1** and **2**, which lack the methyl group at C-9. The singlet at δ 1.70 ppm (H-19) and the doublet at δ 6.02 (J = 15.7, H-10) were diagnostic signals for LCB-2 in glucosylceramide **7**. Taking into account the FABMS spectrum, the NMR data and the methanolysis products of **7**, it was identified as (2*S*,3*R*,4*E*,8*E*,10*E*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[(*R*)-2-hydroxy-15-tetracosenoyl]-amino-9-methyl-4,8,10-octadecatriene.

Glucosylceramide **8** (Fig. 1) was obtained as a white amorphous powder. The ^{1}H NMR spectrum of **8** (Table II) showed characteristic signals at δ 3.71 (H-1), 4.08 (H-1), 3.98 (H-2 and H-2′), 4.12 (H-3), 5.46 (H-4) and 5.72 (H-5) for a glucosylceramide containing a sphingosine-type LCB moiety and a 2-hydroxy fatty acid. The monosaccharide signals in the ^{1}H and ^{13}C NMR spectra (Table II) indicated the presence of glucose as the hexose unit. This was confirmed by GC analysis of the peracetylated alditol derivative of the monosaccharide obtained by methanolysis of **8**. The ^{1}H NMR spectra of **8** showed the presence of an additional *cis* disubstituted double bond on the basis of a triplet at δ 5.34 ppm (J = 5.9 Hz, 2H) which correlated with a signal at δ 130.9 ppm in the ^{1}H-^{13}C HETCOR spectrum. The J value and the chemical upfield shift of allylic methylene carbon signals at 28.1 ppm (Kim *et al*., 1997) allowed us to assign the *Z* geometry for this double bond. GC-MS analysis of the FAME obtained by methanolysis of **8** allowed identification of methyl-2-hydroxyhexadecanoate (FAME-4) on the basis of ions at *m/z* 286 $[M]^+$, 254 $[M-32]^+$, and 227 $[M-CH_3COO]^+$. Acetylation and GC-MS of the acetylated LCB showed the presence of 2-acetamido-1,3-diacetoxy-4,13-docosadiene (acetylated LCB-3). The position of the additional double bond in the LCB moiety of **8** was determined to be Δ^{13} on

the basis of peaks at *m/z* 785 $[M\text{-}173+H]^+$ and 174 $[M\text{-}784+H]^+$ in the FABMS (positive ion mode) spectrum of the DMDS derivative of intact glucosylceramide **8** (Fig. 1). Taking into account all these data and the $[M-H]^-$ and $[M-H\text{-}162]^-$ peaks at *m/z* 768 and 606, respectively, in the FABMS (negative ion mode) spectrum, **8** was characterized as (2*S*,3*R*,4*E*,13*Z*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[(*R*)-2-hydroxyhexadecanoyl]amino-4,13-docosadiene, previously isolated from the starfish *Asterias amurensis versicolor* (Higuchi *et al*., 1991). Recently, we have isolated a related glucosylceramide with a Δ^{15} LCB moiety from the starfish *Allostichaster inaequalis* (Díaz de Vivar *et al*., 2002).

Experimental

General methods

^1H and ^{13}C NMR and 2D spectra were recorded in CD_3OD or $CDCl_3$ in a Bruker AM 500 spectrometer. FABMS were registered on a VG-ZAB BEQ spectrometer. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. Preparative HPLC was carried out on an SP liquid chromatograph equipped with a Spectra Series P100 solvent delivery system, a Rheodyne manual injector and a refractive index detector, using a Phenomenex Ultracarb ODS 20 column (250 × 10 mm i.d., 5 μ) at a flow rate of 2 ml/min, eluting with MeOH–H_2O (99:1 v/v). TLC was performed on precoated silica gel 60 F_{254} (CH_2Cl_2/MeOH/AcOEt/H_2O (8.5:1.5:2:0.1 v/v/v/v). Reversed-phase TLC was performed on C_{18} silica gel 60 F_{254} (MeOH–H_2O (99:1 v/v)).

GC (for peracetylated alditols): Hewlett Packard 5890A gas chromatograph equipped with FID and a capillary column SP2330 (25 m × 0.2 mm i.d.). Carrier gas: Nitrogen. Temperature program: 160–280 °C at 10 °C·min^{-1}.

GC-MS (for FAME, LCB and DMDS derivatives): Hewlett Packard 5890A + VG Trio-2 with a capillary column Ultra 2 (25 m × 0.2 mm i.d.). Carrier gas: Helium. Temperature program: 100–280 °C at 10 °C·min^{-1}.

Animal material

Specimens of *A. minuta,* Perrier 1885 (family Asteriidae, order Forcipulatida) were collected in January 1999 off the Golfo San Jorge near Comodoro Rivadavia (Chubut province), on the Argentine Patagonian coast. The organisms were identified by Dr. Alejandro Tablado of the Museo de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires, Argentina, where a voucher specimen is preserved (MACN N° 34118).

Extraction and isolation

The starfish (2.7 kg wet weight) were kept frozen until worked up. The frozen animals were then cut into small pieces, homogenized and extracted three times with CH_2Cl_2–MeOH (1:2 v/v) (6 l) and centrifuged. The combined CH_2Cl_2–MeOH extracts were evaporated to dryness *in vacuo* and the residue was partitioned between H_2O and AcOEt–*n*-BuOH (2:1 v/v). The organic extract was concentrated to give a residue (31 g), which was subjected to vacuum-dry column chromatography on SiO_2 using CH_2Cl_2 and CH_2Cl_2–MeOH mixtures with increasing amounts of MeOH. The fraction eluted with CH_2Cl_2–MeOH (85:15 v/v) (1.1 g) contained the crude glycosphingolipid mixture, which was purified by silica gel column chromatography with CH_2Cl_2/MeOH/H_2O (8:1.3:0.1 v/v/v) to give fraction AM (0.7 g), showing a single spot on normal-phase and three spots on reversed-phase TLC. A part of AM (0.5 g) was repeatedly submitted to HPLC to give the pure glucosylceramides **1** (14.4 mg), **2** (18.1 mg), **3** (11.1 mg), **4** (17.5 mg), **5** (14.2 mg), **6** (22.3 mg), **7** (17.1 mg) and **8** (18.4 mg).

Methanolysis of AM

A solution of AM (140 mg) in 0.9 N HCl in 82% aqueous MeOH (40 ml) was heated at reflux for 18 h. The reaction mixture was extracted with cyclohexane (3 × 10 ml). The combined cyclohexane extracts were concentrated *in vacuo* and the residue was purified by silica gel column chromatography (cyclohexane–AcOEt (7:3 v/v)) to yield a mixture of fatty acid methyl esters (FAME) (18 mg). The aqueous MeOH layer was neutralized with Dowex 1 resin, concentrated *in vacuo* and the residue partitioned between H_2O and AcOEt. The organic phase contained the mixture of long chain bases (LCB) (33.4 mg) and the aqueous phase the methyl-D-glucopyranoside (14.7 mg).

Derivatization of methyl-D-glucopyranoside

Methyl-D-glucopyranoside (5 mg) was heated in a screwcap vial with 2 N trifluoroacetic acid (2 ml) at 120 °C for 2 h. After extraction with CH_2Cl_2, the aqueous residue was evaporated under reduced pressure. The sugar was treated with 0.5 M NH_3 (0.7 ml) and $NaBH_4$ (5 mg) at room temperature for 18 h. After acidification with 1 M AcOH, the reaction mixture was treated with MeOH (0.7 ml) and evaporated under reduced pressure. The alditol was peracetylated with Ac_2O (0.35 ml) and pyridine (0.35 ml) at 100 °C for 45 min. The reaction mixture was cooled and poured into $CHCl_3$–H_2O (1:1 v/v), and the aqueous phase extracted with $CHCl_3$. The combined $CHCl_3$ extracts were washed with H_2O (0.5 ml), saturated $NaHCO_3$ solution (0.5 ml), and H_2O (0.5 ml) and evaporated to dryness under nitrogen. The peracetylated alditol was identified by GC using standard peracetylated alditols as reference samples.

Acetylation of the LCB mixture of AM

LCB mixture (15 mg) was heated in a screwcap vial with Ac_2O (0.7 ml) and pyridine (0.7 ml) at 70 °C for 120 min. The reaction mixture was cooled and poured into $CHCl_3$–H_2O (1:1 v/v) (1.5 ml) and the aqueous phase extracted with $CHCl_3$ (3 × 1.0 ml). The combined $CHCl_3$ extracts were washed with H_2O (0.6 ml), saturated $NaHCO_3$ solution (0.6 ml) and H_2O (2 × 0.6 ml), and evaporated to dryness under nitrogen. The mixture of acetylated LCB was purified by silica gel column chromatography (Cl_2CH_2) and analyzed by GC-MS.

(2S,3R,4E,8E,10E)-1-(β-D-Glucopyranosyloxy)-3-hydroxy-2-[(R)-2-hydroxy-14-tetracosenoyl]-amino-4,8,10-octadecatriene (**1**): White solid. $[\alpha]_D$ −6.7° (*c* 0.62, $CHCl_3$); ^1H and ^{13}C NMR see Table II; FABMS (negative ion mode) *m/z* 806 $[M-H]^-$, 644 $[M-H-162]^-$.

(2S,3R,4E,8E,10E)-1-(β-D-Glucopyranosyloxy)-3-hydroxy-2-[(R)-2-hydroxy-15-tetracosenoyl]-amino-9-methyl-4,8,10-octadecatriene (**7**): White solid. $[\alpha]_D$ −6.1° (*c* 0.51, $CHCl_3$); ^1H and ^{13}C NMR see Table II; FABMS (negative ion mode) *m/z* 834 $[M-H]^-$, 672 $[M-H-162]^-$.

(2S,3R,4E,13Z)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[(R)-2-hydroxy-hexadecanoyl]amino-4,13-docosadiene (**8**): White solid. ^1H and ^{13}C NMR see Table II; FABMS (negative ion mode) *m/z* 768 $[M-H]^-$, 606 $[M-H-162]^-$.

Methanolysis of glucosylceramide 1

A solution of glucosylceramide **1** (6.0 mg) in 0.9 N HCl in 82% aqueous MeOH (1.8 ml) was heated at reflux for 18 h. The reaction mixture was extracted with cyclohexane (3 × 1.0 ml). The combined cyclohexane extracts were concentrated *in-vacuo* and the residue (1.5 mg) was analyzed by GC-MS and ^1H NMR.

Methyl 2-hydroxy-14-tricosenoate. ^1H NMR (500 MHz, $CDCl_3$): δ 0.87 (t, *J* = 6 Hz, $-CH_3$), 1.27 (s, $-CH_2-$), 2.2 (m, $-C\mathbf{H}_2-CH=CH-$), 3.77 (s, CH_3O), 4.18 (dd, *J* = 4.4 and 7 Hz, H-2), 5.34 (t, *J* = 4.5 Hz, $-C\mathbf{H}=C\mathbf{H}-$). GC-MS *m/z*: 382 $[M]^+$, 350 $[M-32]^+$, 323 $[M-59]^+$.

The aqueous MeOH layer was neutralized with Dowex 1 resin, concentrated *in vacuo* and the residue partitioned between H_2O and AcOEt. The organic phase contained the long chain base (1.4 mg) and the aqueous phase the methyl-D-glucopyranoside (0.9 mg).

Acetylation of LCB of glucosylceramide 1

LCB (1.4 mg) was heated in a screwcap vial with Ac_2O (0.12 ml) and pyridine (0.12 ml) at 70 °C for 2 h. The reaction mixture was cooled and poured into $CHCl_3$–H_2O (1:1 v/v) (0.4 ml) and the aqueous phase extracted with $CHCl_3$ (3 × 0.5 ml). The combined $CHCl_3$ extracts were washed with H_2O (0.3 ml), saturated $NaHCO_3$ solution (0.3 ml) and H_2O (2 × 0.3 ml), and evaporated to dryness under nitrogen. The LCB was analyzed by GC-MS.

2-acetamido-1,3-diacetoxy-4,8,10-octadecatriene: $[\alpha]_D^{20}$ −13.7° ($CHCl_3$, *c* 0.10), GC-MS *m/z* (rel. int.): 421 $[M]^+$ (5); 378 $[M-43]^+$ (2); 361 $[M-60]^+$ (1); 302 $[M-59-60]^+$ (1.2); 318 $[M-43-60]^+$ (5) 336 (3), 234 (2), 207 (6), 196 (3), 144 (13), 133 (4), 112 (6), 85 (70).

Synthesis of DMDS derivatives of FAME

The FAME (0.9 mg) was dissolved in carbon disulfide (0.12 ml), and dimethyl disulfide (DMDS) (0.12 ml) and iodine (0.5 mg) were added to the solution. The mixture was kept at 60 °C for 40 h in a small-volume sealed vial. The reaction was quenched with aqueous $Na_2S_2O_3$ (5%), and the reaction mixture was extracted with cyclohexane (0.3 ml). The extract was evaporated to dryness *in-*

vacuo and the residue (DMDS Derivative) was redissolved in CH_2Cl_2 and analyzed by GC-MS.

DMDS of methyl-2-hydroxy-14-tricosenoate: GC-MS *m/z*: 476 $[M]^+$, 429, 417, 303, 173.

DMDS of methyl-2-hydroxy-15-tetracosenoate: GC-MS *m/z*: 490 $[M]^+$, 431, 317, 173.

Determination of the absolute configuration of glucose

A solution of Anasterocerebroside A (**1**) (3.0 mg) in 2 N trifluoroacetic acid (1.0 ml) was heated at 120 °C for 1 h in a screwcap vial. After extracting with AcOEt (1.0 ml), the aqueous layer was concentrated to furnish the monosaccharide. The sugar and L-cysteine methyl ester hydrochloride (1 mg) were dissolved in pyridine (0.8 ml) and heated in a screwcap vial at 60 °C for 1 h. Then, Ac_2O (0.7 ml) was added and the mixture heated at 100 °C for 0.75 h. The reaction mixture was cooled and poured into $CHCl_3-H_2O$ (1:1, v/v)., and the aqueous phase was extracted with $CHCl_3$. The combined chloroform extracts were washed with H_2O (0.5 ml), saturated $NaHCO_3$ solution (0.5 ml) and H_2O (0.5 ml) and evaporated to dryness under nitrogen. The monosaccharide derivative was analysed by GC (R_t = 24.7 min) and compared to the corresponding derivatives of D-glucose (R_t = 24.7 min) and L-glucose (R_t = 25.9 min) as reference samples.

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Babu U. V., Bhandari S. P. S., and Garg, H. S. (1997), Temnosides A and B, two new glycosphingolipids from the sea urchin *Temnopleurus toreumaticus* of the Indian coast. J. Nat. Prod. **60**, 732–734.

Chludil H. D., Seldes A. M., and Maier M. S. (2002a), Glycosphingolipids from starfishes and sea cucumbers. Res. Adv. in Lipids **2**, 9–18.

Chludil H. D., Seldes A. M., and Maier M. S. (2002b), Antifungal steroidal glycosides from the Patagonian starfish *Anasterias minuta*: structure-activity correlations. J. Nat. Prod. **65**, 153–157.

Díaz de Vivar M. E., Seldes A. M., and Maier M. S. (2002), Two novel glucosylceramides from gonads and body walls of the Patagonian starfish *Allostichaster inaequalis*. Lipids **37**, 597–603.

Durán R., Zubía E., Ortega M. J., Naranjo S., and Salvá J. (1998), Phallusides, new glucosphingolipids from the ascidian *Phallusia fumigata*. Tetrahedron **54**, 14597–14602.

Gaver R. C., and Sweeley C. C. (1965), Methods for methanolysis of sphingolipids and direct determination of long-chain bases by gas chromatography. J. Am. Oil Chem. Soc. **42**, 294–298.

Hara Sh., Okabe H., and Mihashi K. (1987), Gas-liquid chromatography separation of aldose enantiomers as trimethylsilyl ethers of methyl 2-(polyhydroxyalkyl)-thiazolidine-4(*R*)-carboxylates. Chem. Pharm. Bull. **35**, 501–506.

Higuchi R., Jhou J. X., Inukai K., and Komori T. (1991), Isolation and structure of six new cerebrosides, Asteriacerebrosides A–F, and two known cerebrosides, astrocerebroside A and acanthacerebroside C. Liebigs Ann. Chem. 745–752.

Higuchi R., Inagaki M., Togawa K., Miyamoto T., and Komori T. (1994), Isolation and structure of cerebrosides from the sea cucumber *Pentacta australis*. Liebigs Ann. Chem. 653–658.

Irie A., Kubo H., and Hoshi M. (1990), Glucosylceramide having a novel tri-unsaturated long-chain base from the spermatozoa of the starfish, *Asterias amurensis*. J. Biochem. **107**, 578–586.

Jin W., Rinehart K. L., and Jares-Erijman E. A. (1994), Ophidiacerebrosides: cytotoxic glycosphingolipids containing a novel sphingosine from a sea star. J. Org. Chem. **59**, 144–147.

Julina R., Herzig T., Bernet B., and Vasella A. (1986), Enantioselective synthesis of D-*erythro*-sphingosine and of ceramide. Helv. Chim. Acta **69**, 368–373.

Kim S. Y., Choi Y., Huh H., Kim J., Kim Y., and Lee H. S. (1997), Antihepatotoxic cerebrosides from *Lycium chinense* fruits. J. Nat. Prod. **60**, 274–276.

Maier M. S., Kuriss A., and Seldes A. M. (1998), Isolation and structure of glucosylceramides from the starfish *Cosmasterias lurida*. Lipids **33**, 825–827.

Pretorius Y. Y., and Horn D. (1954), The synthesis and stereochemistry of the straight-chain α-hydroxy-acids. J. Chem. Soc. 1460–1464.

Vincenti M., Guglielminetti G., Cassani G., and Tonini C. (1987), Determination of double bond position in diunsaturated compounds by mass spectrometry of dimethyl disulfide derivatives. Anal. Chem. **59**, 694–699.