Identification of Sixteen New Galactocerebrosides from the Starfish *Protoreaster nodosus*

Ke Pan, Masanori Inagaki, Nagisa Ohno, Chiaki Tanaka, Ryuichi Higuchi, and Tomofumi Miyamoto*

Graduate School of Pharmaceutical Sciences, Kyushu University; 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan. Received November 2, 2009; accepted January 9, 2010; published online January 14, 2010

Twenty-one galactocerebrosides (1—21), including sixteen new compounds (3—7, 9—17, 19, 21), were identified from a cerebroside molecular species obtained from the chloroform/methanol extract of pyloric caeca dissected from the starfish *Protoreaster nodosus*. The structures of these galactocerebrosides were determined on the basis of chemical and spectroscopic evidences. Especially, one-pot GC-MS analysis following methanolysis and periodate oxidation of these galactocerebrosides gave efficient structural information of ceramide moiety rapidly in minute amounts.

Key words glycosphingolipid; galactocerebroside; starfish; *Protoreaster nodosus*

Cerebrosides, mainly glucocerebrosides and galactocerebrosides, are the simplest glycosphingolipids (GLSs) and known to be the biosynthetic precursors of most other GLSs.[1] While glucocerebrosides have been isolated from various starfishes, occurrence of galactocerebrosides seems to be limited to three species: *Stellaster equestris*,[2] *Culcita novaeguineae*,[3] and *Oreaster reticulatus*.[4]

In our continuing research on GLSs from echinoderms, a galactocerebroside molecular species (PNC-1) was purified from the starfish *Protoreaster nodosus* (kobuhitode in Japanese) collected in Okinawa. Reversed-phase HPLC separation of PNC-1 afforded sixteen new galactocerebrosides (**3**—**7**, **9**—**17**, **19**, **21**), along with five known galactocerebrosides (**1**, **2**, **8**, **18**, **20**). Further, we developed one-pot GC-MS analysis for cerebrosides which consist of phytosphingosine-type long chain base (LCB). Generally, the trimethylsilyl (TMS) derivatives of LCB are used for GC-MS analysis and give a useful structural information,[5] however, the preparation of phytosphingosine-type LCB from the parent cerebroside is in a quite low yield and not quantitative, because the glycosidic linkage between sugar and LCB is hard to cleavage against methanolysis. One-pot reaction following methanolysis and periodate oxidation, and subsequent GC-MS analysis gave efficient structural information. In this paper, the isolation and structure elucidation of galactocerebroside molecular species (PNC-1) and constituent cerebrosides (**1**—**21**) of PNC-1 from the starfish *Protoreaster nodosus* are described.

Isolation and Structure of PNC-1 The lipid fraction, obtained from $CHCl_3$/MeOH extract of pyloric caeca, which was dissected from the fresh material of the starfish *P. nodosus*, was subjected to repeated silica gel, reversed-phase and Sephadex LH-20 column chromatography to give a less polar GLS, named PNC-1, showing a single spot on silica gel TLC plate.

The positive ion FAB-MS spectrum of PNC-1 exhibited a series of homologous pseudomolecular ion peaks $[M+Na]^+$ at *m*/*z*: 798, 812, 826, 840, 854, 868 and 882. The ^{1}H- and ^{13}C-NMR spectra of PNC-1 revealed the existence of the alkyl chain (the intense methylene signal at δ_H 1.25, δ_C 29.8; two terminal methyl signals at δ_H 0.82, δ_C 14.2, and δ_H 0.82, δ_C 22.8), a secondary amide group (δ_{NH} 8.50, δ_C 175.7), and a monosaccharide moiety (the anomeric signal at δ_H 4.82, δ_C 105.6), suggesting PNC-1 to be a cerebroside molecular species (Table 1).

The ceramide moiety of PNC-1 was determined to be composed of phytospingosine-type long chain base (LCB) and α-hydroxy fatty acid on the basis of extensive 2D NMR analysis. In the correlation spectroscopy (COSY) spectrum, the nitrogenous methine signal [δ_H 5.18 (H-2)] showed correlations with an oxymethylene signal [δ_H 4.42, 4.70 (H_2-1)] and an oxymethine signal [δ_H 4.27 (H-3)]. H-3 signal also exhibited a COSY correlation with another oxymethine signal [δ_H 4.14 (H-4)], which was in the same spin system as another two oxymethylenes signals [δ_H 2.16, 1.86 (H_2-5); δ_H 1.86, 1.62 (H_2-6)] in the total correlation spectroscopy (TOCSY) spectrum. Thus, a structural fragment from H_2-1 to H_2-6 was established, suggesting the LCB of ceramide was phytosphingosine-type. Analysis of COSY and TOCSY spectra revealed another structural fragment (H-2′ to H_2-4′) in the fatty acid part of ceramide. H-2′ (δ_H 4.52) signal showed a heteronuclear multiple-bond correlation spectroscopy (HMBC) cross peak with the amide carbon signal [δ_C 175.7 (C-1′)], indicating the presence of α-hydroxy fatty acid. Further, H-2 (δ_H 5.18) signal in LCB showed a cross peak with amide carbon (C-1′), and the ceramide moiety was depicted as shown in Fig. 1.

Identification of the compositions of fatty acid and LCB of ceremide moiety was conducted by GC-MS analysis following methanolysis and periodate oxidation of PNC-1 (see Experimental). The mixture of fatty acid methyl esters (FAMEs) obtained from methanolysis and a mixture of long chain aldehydes generated by periodate oxidation were simultaneously subjected to GC-MS, which revealed five FAME components (Table 2, Fig. 2) and seven long chain

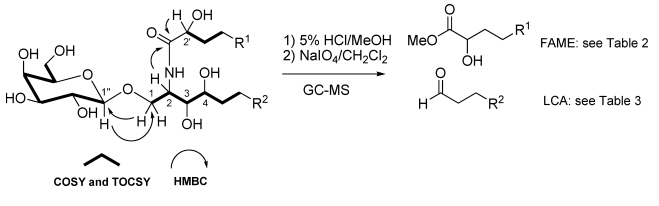


Fig. 1. Structure and Key 2D NMR Correlations of PNC-1

* To whom correspondence should be addressed. e-mail: miyamoto@phar.kyushu-u.ac.jp

Table 1. ^{1}H and ^{13}C Chemical Shifts (ppm) for PNC-1 and Compounds **3**, **7**, **21** in $C_5D_5N+D_2O$ (20 : 1)

	PNC-1		**3**		**7**		**21**	
	δ_H [a]	δ_C	δ_H [a]	δ_C	δ_H [a]	δ_C	δ_H [a]	δ_C [c]
1 (phytosphingosine)	4.70 (dd, 10.3, 6.6) 4.42[b]	70.0 (t)	4.71 (dd, 10.5, 6.4) 4.43[b]	70.0 (t)	4.70 (dd, 10.5, 6.4) 4.42[b]	70.0 (t)	4.71 (dd, 105, 6.4) 4.42[b]	70.0 (t)
2	5.18 (m)	51.2 (d)	5.19 (m)	51.2 (d)	5.17 (m)	51.2 (d)	5.19 (m)	51.2 (d)
3	4.27 (t, 6.0)	75.3 (d)	4.27 (t, 6.0)	75.3 (d)	4.27 (t, 6.0)	75.3 (d)	4.27[b]	75.3 (d)
4	4.14 (t, 7.9)	72.2 (d)	4.15 (m)	72.2 (d)	4.15 (m)	72.2 (d)	4.15 (m)	72.2 (d)
5	2.16 (m) 1.86	33.6 (t)	2.17 (m) 1.86[b]	33.6 (t)	2.15[b] 1.86[b]	33.5 (t)	2.15[b] 1.86[b]	33.5 (t)
6	1.86[b] 1.62[b]	26.4 (t)	1.86[b] 1.62[b]	26.4 (t)	1.86[b] 1.62[b]	26.3 (t)	1.86[b] 1.62[b]	26.3 (t)
Term. CH_3	0.82 (m)	14.2 (q), 22.8 (q)	0.81 (3H, t, 6.6)*	14.1 (q)	0.82 (6H, d, 6.6)	22.6 (q)	0.82 (m)	11.5 (q), 19.0 (q)
NH[d]	8.50 (d, 9.0)		8.49 (br s)		8.48 (br s)		8.50 (d, 9.5)	
1′ (fatty acid)		175.7 (s)		175.7 (s)		175.7 (s)		—
2′	4.52 (dd, 7.5, 3.2)	72.1 (d)	4.52 (dd, 7.7, 3.7)	72.1 (d)	4.52 (dd, 7.6, 3.8)	72.1 (d)	4.53 (dd, 7.1, 3.1)	72.1 (d)
3′	2.12 (m) 1.93 (m)	35.2 (t)	2.12 (m) 1.93 (m)	35.2 (t)	2.12 (m) 1.93 (m)	35.2 (t)	2.12 (m) 1.95 (m)	35.2 (t)
4′	1.70 (m) 1.64[b]	25.6 (t)	1.70 (m) 1.64[b]	25.6 (t)	1.70 (m) 1.64[b]	25.6 (t)	1.70 (m) 1.63[b]	25.6 (t)
Term. CH_3	0.82 (m)	14.2 (q)	0.82 (3H, t, 6.6)*	14.1 (q)	0.81 (3H, t, 6.6)	14.1 (q)	0.82 (m)	14.1 (q)
1″ (β-Gal*p*)	4.82 (d, 7.9)	105.6 (d)	4.82 (d, 7.9)	105.6 (d)	4.82 (d, 7.9)	105.5 (d)	4.83 (d, 7.4)	105.5 (d)
2″	4.39 (t, 8.1)	72.2 (d)	4.40 (t, 8.1)	72.2 (d)	4.39 (t, 8.1)	72.2 (d)	4.40 (t, 7.9)	72.2 (d)
3″	4.06 (dd, 9.5, 2.5)	74.8 (d)	4.06 (dd, 9.5, 3.1)	74.8 (d)	4.05 (dd, 9.5, 3.2)	74.7 (d)	4.06 (dd, 9.3, 1.7)	74.7 (d)
4″	4.44 (br s)	69.8 (d)	4.45 (br s)	69.8 (d)	4.43 (br s)	69.7 (d)	4.45 (br s)	69.7 (d)
5″	3.95 (t, 6.0)	76.7 (d)	3.97 (t, 6.0)	76.7 (d)	3.95 (t, 6.0)	76.7 (d)	3.96 (t, 6.0)	76.7 (d)
6″	4.32 (2H, m)	61.9 (t)	4.32 (2H, m)	61.9 (t)	4.32 (2H, m)	61.9 (t)	4.33 (2H, m)	61.9 (t)

a) Multiplicities and coupling constants are in parentheses. *b*) Submerged by other signals. *c*) Assignments were based on HSQC experiment. *d*) Most of amide proton are exchanged to deuterium.

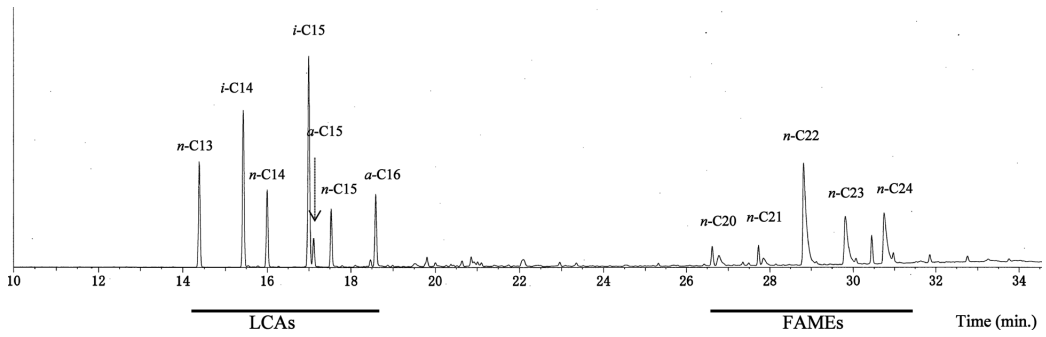


Fig. 2. Total Ion Current of LCAs and FAMEs Generated from PNC-1

n-, *i*-, and *a*-represent *normal*-, *iso*-, and *anteiso*-terminal methyl groups, respectively.

Table 2. Fatty Acid Composition of PNC-1

Fatty acid methyl ester	Composition [%]
Methyl 2-hydroxyicosanoate (*normal*-C20)	4.8
Methyl 2-hydroxyhenicosanoate (*normal*-C21)	3.1
Methyl 2-hydroxydocosanoate (*normal*-C22)	39.6
Methyl 2-hydroxytricosanoate (*normal*-C23)	25.3
Methyl 2-hydroxytetracosanoate (*normal*-C24)	27.2

Table 3. Long Chain Base Composition of PNC-1

Long chain aldehyde	Parent long chain base[a]	Composition [%]
Tridecanal (*normal*-C13)	*normal*-t16:0	14.4
12-Methyltridecanal (*iso*-C14)	*iso*-t17:0	27.6
Tetradecanal (*normal*-C14)	*normal*-t17:0	8.5
13-Methyltetradecanal (*iso*-C15)	*iso*-t18:0	29.2
12-Methyltetradecanal (*anteiso*-C15)	*anteiso*-t18:0	4.7
Pentadecanal (*normal*-C15)	*normal*-t18:0	6.8
13-Methylpentadecanal (*anteiso*-C16)	*anteiso*-t19:0	8.8

a) The chain length and number of double bonds are denoted with the prefix 't' to designate trihydroxy bases (phytosphingosine).

aldehyde (LCA) constituents (Table 3, Fig. 2). The presence of mass fragment peaks corresponding to $[M-18]^+$, $[M-44]^+$ and $[M-46]^+$ in all the LCAs confirmed their nature as aldehydes,[6—8] and the isomers with *normal*-, *iso*- and *anteiso*-terminal methyl groups could be identified by the fragmentions M-15 for *iso*-, and M-29 for *anteiso*-, neither of which was detected in the mass spectra of *normal*-LCAs.[9—11] Although the ratio of *anteiso*-type LCB was estimated to be 13.5%, the ^{13}C-NMR signals due to *anteiso*-terminal methyl groups was not confirmed.

The monosaccharide moiety of PNC-1 was determined to be β-galactopyranose according to the following evidences.

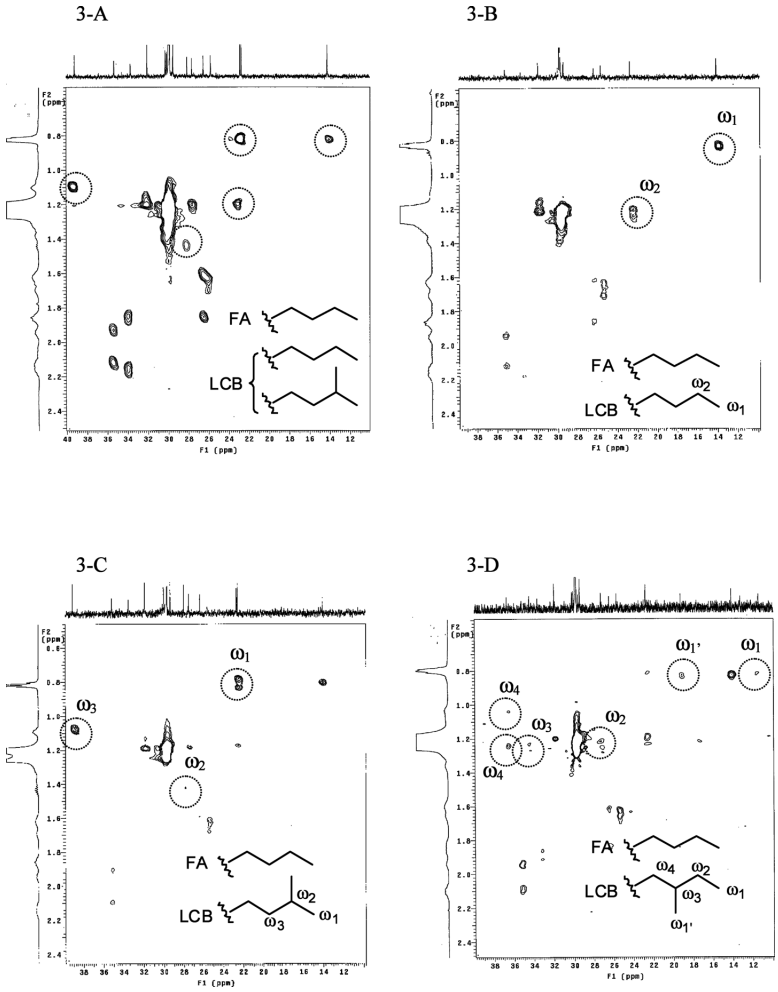


Fig. 3. Partial HSQC Spectra of PNC-1, Compounds **3**, **7**, and **21**

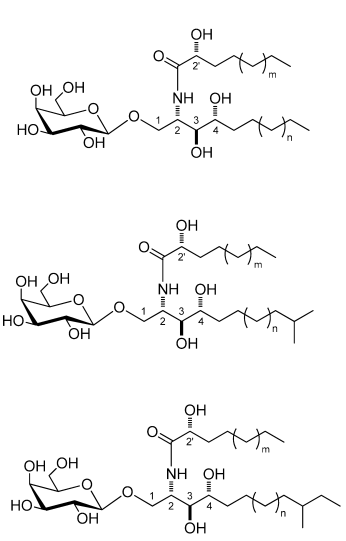
(3-A) PNC-1, (3-B) compound **3**, (3-C) compound **7**, (3-D) compound **21**. Cross peaks surround with dotted circles indicated the terminal moiety of LCB.

The anomeric proton signal [δ_H 4.82 (H-1″)] were used in the analysis of the COSY and TOCSY spectra as the starting point for the sequential assignment of all the protons in the sugar moiety. The ^{13}C-NMR signals [δ_C 105.6 (C-1″), 72.2 (C-2″), 74.8 (C-3″), 69.8 (C-4″), 76.7 (C-5″), and 61.9 (C-6″)], in combination with the coupling constant of the anomeric proton (J_{HH}=7.9 Hz) suggested the sugar residue in PNC-1 was β-galactopyranose.[2,3] The linkage of the galactopyranose unit to ceramide moiety was examined by HMBC experiment. HMBC correlations from H_2-1 to anomeric carbon C-1″ and from anomeric proton H-1″ to C-1 clearly indicated that the β-galactopyranose unit was linked to C-1 of ceramide moiety (Fig. 1).

The β-galactopyranose unit of PNC-1 was determined to be D-form according to the method of Hara *et al.* (see Experimental).[12] The absolute stereochemistry of α-hydroxy fatty acid was determined to be *R* configuration, because the optical rotator dispersion (ORD) spectra of isolated FAMEs showed the negative single cotton effect curve ($[M]_{280}$= −5254 for C22).[13] The stereochemistry of LCB was presumed to be 2*S*,3*S*,4*R*, since the ^{13}C-NMR signals assigned to C-1, C-2, C-3, and C-4 were in good agreement with those of β-D-galactocerebrosides composed of (2*S*,3*S*,4*R*)-phytosphingosines and (2*R*)-2-hydroxy fatty acid.[2,3] Therefore, the structure of PNC-1 was determined as shown in Fig. 1.

Isolation and Structures of Galactocerebrosides 1—21 from PNC-1 Reversed-phase HPLC separation of PNC-1 using a C_{18} column showed ten major peaks which were recovered to give ten fractions (PNC-1-1 to PNC-1-10). According to the results of positive ion FAB-MS and GC-MS analysis of their FAMEs and LCAs, four fractions [PNC-1-1 (**1**), PNC-1-2 (**2**), PNC-1-9 (**20**) and PNC-1-10 (**21**)] were pure compounds. The other six fractions (PNC-1-3 to PNC-1-8) each giving a mixture of FAMEs were further purified by reversed-phase HPLC employing a C_{30} column to afford eleven pure galactocerebrosides [PNC-1-3a (**3**), PNC-1-3b (**4**), PNC-1-4c (**7**), PNC-1-5a (**8**), PNC-1-5b (**9**), PNC-1-5c (**10**), PNC-1-6a (**11**), PNC-1-6d (**14**), PNC-1-7a (**15**), PNC-1-7b (**16**), and PNC-1-8a (**17**)], along with three pairs of inseparable isomers [PNC-1-4a (**5**)/PNC-1-4b (**6**), PNC-1-6b (**12**)/PNC-1-6c (**13**), and PNC-1-8b (**18**)/PNC-1-8c (**19**)].

The NMR spectra of galactocerebrosides **1**—**21** were nearly identical with parent molecular species PNC-1, except for the signals of terminal methyl groups in LCBs. According to the structure of terminal methyl in LCB, these twenty-one galactocerebrosides could be divided into three categories: *normal*-LCB (compounds **1**, **3**, **4**, **8**, **9**, **10**, **15**, **16**, **20**); *iso*-LCB (compounds **2**, **5**, **7**, **12**, **14**, **18**); *anteiso*-LCB (compounds **6**, **11**, **13**, **17**, **19**, **21**). The heteronuclear single quantum coherence (HSQC) spectrum of **3** which consists of



PNC-1-1 (**1**)	m = 16	n = 8
PNC-1-3a (**3**)	m = 16	n = 9
PNC-1-3b (**4**)	m = 17	n = 8
PNC-1-5a (**8**)	m = 16	n = 10
PNC-1-5b (**9**)	m = 17	n = 9
PNC-1-5c (**10**)	m = 18	n = 8
PNC-1-7a (**15**)	m = 17	n =10
PNC-1-7b (**16**)	m = 18	n =9
PNC-1-9 (**20**)	m = 18	n =10
PNC-1-2 (**2**)	m = 16	n = 7
PNC-1-4a (**5**)[a]	m = 16	n =8
PNC-1-4c (**7**)	m = 17	n = 7
PNC-1-6b (**12**)[b]	m = 17	n = 8
PNC-1-6d (**14**)	m = 18	n = 7
PNC-1-8b (**18**)[c]	m = 18	n = 8
PNC-1-4b (**6**)[a]	m = 16	n = 7
PNC-1-6a (**11**)	m = 16	n = 8
PNC-1-6c (**13**)[b]	m = 17	n = 7
PNC-1-8a (**17**)	m = 17	n = 8
PNC-1-8c (**19**)[c]	m = 18	n = 7
PNC-1-10 (**21**)	m = 18	n = 8

Fig. 4. Structures of Galactocerebrosides **1**—**21**

Compounds marked with the same superscripts were isolated together as an inseparable mixture.

normal-LCB showed only the correlations of normal terminal methyl [δ_H 0.81 and δ_C 14.1 (ω_1)] as shown in Fig. 3B, while the HSQC spectra of **7** and **21** which consist of *iso*-, or *anteiso*-LCB clearly showed the correlation peaks due to branched methyls [δ_H 0.82 and δ_C 22.6 (ω_1) for **7**, δ_H 0.82 and δ_C 11.5 (ω_1), 19.0 (ω_1) for **21**] as shown in Figs. 3C and D, respectively (Table 1). The HSQC experiment is efficient to discriminate the terminal methyl in LCB.

GC-MS analysis of FAME obtained from methanolysis revealed the length of fatty acid of every compound. The structure of the LCB of each compound was deduced by GC-MS analysis following periodate oxidation. Since compounds **1**—**21** were isolated from parent molecular species PNC-1, their stereochemistries were presumed to be the same as PNC-1. Therefore, the structures of compounds **1**—**21** were determined as shown in Fig. 4.

Sixteen compounds (**3**—**7**, **9**—**17**, **19**, **21**), to the best of our knowledge, are new galactocerebrosides. The structures of **1**, **2**, and **18** are the same as those of galactocerebrosides S-2b-2, S-2b-4, and S-2-16, isolated from the starfish *Stellaster equestris*.[2] Compounds **8** and **20** have been identified from bovine kidney and other mammalian tissues.[14,15] The absolute configuration of the secondary methyl in *anteiso*-LCB has not been determined in this study.

Experimental

Optical rotation was measured with a Jasco Dip-370 digital polarimeter at 25 °C. ORD spectra were measured with a Jasco J-720W spectropolarimeter at 22 °C. NMR spectra were recorded on a Varian INOVA 600 spectrometer. Positive-ion FAB-MS spectrum was acquired with a Jeol JMS-SX102 mass spectrometer (xenon atom beam; matrix, *m*-nitrobenzyl alcohol+NaCl). GC-MS was conducted with a Shimadzu QP-5050A [EI mode; ionizing potential, 70 eV; separator and ion-source temperature 250 °C; column, TC-1701 (0.25 mm×30 m, GL Science Inc.); carrier gas, He]. HPLC was performed on a JASCO PU-2089 HPLC pump and UV-2075 UV/Vis detector (210 nm) with Cadenza CD-C18 column (3 μm, 4.6×250 mm) and Develosil C30-UG-3 column (3 μm, 4.6×250 mm).

Isolation of PNC-1 Pyloric caeca (133 g) of the starfish *Protoreaster nodosus*, collected at the east coast of Okinawa in March 2008, were extracted with $CHCl_3$/MeOH (1/1, v/v) at room temperature. The extract was concentrated *in vacuo* to give a residue (15 g), which was subjected to silica gel column chromatography eluted with $CHCl_3$/MeOH/H_2O (10/1/0 to 6/4/1, v/v/v) to give four fractions. Fraction 2 (1.8 g) was further chromatographed on a RP-8 column chromatograhy (H_2O to MeOH). The MeOH elution (210 mg), containing crude less polar GLSs, was finally purified by silica gel column chromatography with $CHCl_3$/MeOH/H_2O (8/2/0.2, v/v/v) to afford PNC-1 (130 mg). PNC-1 showed a single spot on silica gel TLC ($CHC1_3$/MeOH/H_2O, 8/2/0.2, v/v/v, *Rf*=0.34).

PNC-1: White amorphous solid. $[\alpha]_D$ +3.9 (c=0.1, pyridine). Positive ion FAB-MS *m/z*: 798, 812, 826, 840, 854, 868 and 882 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR [$C_5D_5N+D_2O$ (20/1)]: see Table 1.

Preparation of FAMEs and LCAs of PNC-1 PNC-1 (2.0 mg) was heated with 5% HCl/MeOH (2.0 ml) in a sealed tube at 80 °C for 2 h. The reaction mixture was diluted with MeOH (3.0 ml) and extracted with *n*-hexane, and the extract was concentrated *in vacuo* to give a mixture of FAMEs. FAMEs: ^{1}H-NMR ($CDCl_3$) δ: 0.86 (t, J=7.1 Hz, term. CH_3), 1.2—1.8 (CH_2 chain), 3.77 (s, OCH_3), 4.17 (dd, J=4.1, 7.6 Hz); HPLC separation of FAMEs (Gadenza CD-C18 column; solvent: MeOH) gave three FAMEs, methyl 2*R*-hydroxydocosanoate ($[M]_{280}$=−5254, c=0.10, $CHCl_3$)] , methyl 2-hydroxytricosanoate ($[M]_{280}$=−37205, c=0.09, $CHCl_3$)], methyl 2-hydroxytetracosanoate ($[M]_{280}$=−37478, c=0.10, $CHCl_3$)], respectively. The remaining MeOH layer, which contained a mixture of lyso-cerebroside, free long chain base and methyl glycoside, was dried *in vacuo*. The resiude was dissolved in CH_2Cl_2 (1.0 ml), added silica gel-supported $NaIO_4$ reagent (*ca.* 2 mg), and vigorously stirred at room temperature.[16] After 30 min, the reaction mixture was filtered through a short silica gel column washing with CH_2Cl_2. Removal of solvent from the filtrate afforded a mixture of LCAs. LCAs: ^{1}H-NMR ($CDCl_3$) δ: 0.84 (m, term. CH_3), 1.13 (m, CH), 1.24 (CH_2 chain), 1.60 (m, CH_2), 1.83 (m, CH_2), 2.39 (dt, J=1.9, 7.4 Hz, CH_2–CHO), 9.74 (t, 1.9, CHO).

One-Pot Reaction of PNC-1 PNC-1 (*ca.* 0.1 mg) was heated with 5% HCl/MeOH (0.2 ml) in a sealed tube at 80 °C for 2 h. The reaction mixture was neutralized with Ag_2CO_3 and dried *in vacuo*. The resiude was dissolved in CH_2Cl_2 (0.1 ml), added silica gel-supported $NaIO_4$ reagent (*ca.* 0.2 mg), and vigorously stirred at room temperature. After 30 min, the reaction mixture was extracted with *n*-hexane, and the extract was concentrated *in vacuo* to give a mixture of FAMEs and LCAs.

GC-MS Analysis of FAMEs and LCAs from PNC-1 The mixture of FAMEs and a mixture of LCAs obtained from PNC-1 were simultaneously subjected to GC-MS [column temperature 180—320 °C (rate of temperature increase: 4 °C/min)].

Five FAMEs were detected: methyl 2-hydroxyicosanoate, t_R [min] (ratio of peak area)=26.8 (4.8), *m/z*: 342 $[M]^+$, 283 $[M-59]^+$; methyl 2-hydroxyhenicosanoate, t_R=27.8 (3.1), *m/z*: 356 $[M]^+$, 297 $[M-59]^+$; methyl 2-hydroxydocosanoate, t_R=28.9 (39.6), *m/z*: 370 $[M]^+$, 311 $[M-59]^+$; methyl 2-hydroxytricosanoate, t_R=29.9 (25.3), *m/z*: 384 $[M]^+$, 325 $[M-59]^+$; methyl 2-hydroxytetracosanoate, t_R=30.8 (27.2), *m/z*: 398 $[M]^+$, 339 $[M-59]^+$.

Seven LCAs were observed: tridecanal, t_R [min] (ratio of peak area)=14.4 (14.4), *m/z*: 198 $[M]^+$, 180 $[M-18]^+$, 154 $[M-44]^+$, 152 $[M-46]^+$, 82, 57, 43; 12-methyltridecanal, t_R=15.4 (27.6), *m/z*: 212 $[M]^+$, 197 $[M-15]^+$, 194 $[M-18]^+$, 168 $[M-44]^+$, 166 $[M-46]^+$, 82, 57, 43; tetradecanal, t_R=16.0 (8.5), *m/z*: 212 $[M]^+$, 194 $[M-18]^+$, 168 $[M-44]^+$, 166 $[M-46]^+$, 82, 57, 43; 13-methyltetradecanal, t_R=17.0 (29.2), *m/z*: 226 $[M]^+$, 211 $[M-15]^+$, 208 $[M-18]^+$, 182 $[M-44]^+$, 180 $[M-46]^+$, 82, 57, 43; 12-methyltetradecanal, t_R=17.1 (4.7), *m/z*: 226 $[M]^+$, 208 $[M-18]^+$, 197 $[M-29]^+$, 182 $[M-44]^+$, 180 $[M-46]^+$, 82, 57, 43; pentadecanal, t_R=17.5 (6.8), *m/z*: 226 $[M]^+$, 208 $[M-18]^+$, 182 $[M-44]^+$, 180 $[M-46]^+$, 82, 57, 43; 13-methylpentadecanal, t_R=18.6 (8.8), *m/z*: 240 $[M]^+$, 222 $[M-18]^+$, 211 $[M-29]^+$, 196 $[M-44]^+$, 194 $[M-46]^+$, 82, 57, 43.

Determination of Absolute Configuration of Galactose Moiety of PNC-1 (Method of Hara *et al.*) PNC-1 (*ca.* 1 mg) was heated with 2 N HCl (1 ml) at 100 °C for 24 h in a sealed vial. The reaction mixture was then extracted with *n*-hexane, and the acidic aqueous phase was concentrated by N_2 stream. The residue (sugar fraction) was heated with L-cysteine methyl ester hydrochloride (1 mg) and pyridine (0.05 ml) at 60 °C for 1 h. Then, 0.05 ml of 1-(trimethylsilyl)imidazole was added, and the mixture was heated at 60 °C for a further 20 min. To yield a trimethylsilyl ether of the methyl (4*R*)-thiazolidine-4-carboxylate derivative. The derivative was analyzed by GC-MS [column temperature: 180—250 °C (rate of temperature increase: 2.5 °C/min)]; t_R=29.2 min (derivative of D-galactose, 29.2 min; L-galactose, 30.1 min).

Isolation of Galactocerebrosides 1—21 from PNC-1 HPLC separation of PNC-1 (Cadenza CD-C18 column; flow rate: 1.0 ml/min; solvent: MeOH) showed ten major peaks (PNC-1-1 to PNC-1-10). PNC-1 (30.0 mg) was subjected to HPLC, according to the above conditions. As a result, four peaks (PNC-1-1, PNC-1-2, PNC-1-9, PNC-1-10) were collected and recov-

ered to afford four galactocerebrosides: PNC-1-1 (**1**, 2.8 mg), PNC-1-2 (**2**, 3.5 mg), PNC-1-9 (**20**, 0.4 mg) and PNC-1-10 (**21**, 0.4 mg). The other six peaks (PNC-1-3, PNC-1-4, PNC-1-5, PNC-1-6, PNC-1-7 and PNC-1-8) were recovered and each further purified on a Develosil C30-UG-3 column (flow rate: 1.0 ml/min, solvent: MeOH) to yield fourteen galactocerebrosides: PNC-1-3a (**3**, 1.6 mg), PNC-1-3b (**4**, 0.9 mg), PNC-1-4c (**7**, 1.8 mg), PNC-1-5a (**8**, 0.7 mg), PNC-1-5b (**9**, 1.5 mg), PNC-1-5c (**10**, 0.8 mg), PNC-1-6a (**11**, 0.8 mg), PNC-1-6d (**14**, 2.0 mg), PNC-1-7a (**15**, 0.4 mg), PNC-1-7b (**16**, 0.6 mg), PNC-1-8a (**17**, 0.5 mg), along with three inseparable mixtures: 2.9 mg of PNC-1-4a (**5**) and PNC-1-4b (**6**), 1.9 mg of PNC-1-6b (**12**) and PNC-1-6c (**13**), and 1.3 mg of PNC-1-8b (**18**) and PNC-1-8c (**19**).

PNC-1-1 (**1**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 812 $[M+Na]^+$. The FAME and LCA of **1** were analyzed as described for PNC-1. Methyl 2-hydroxydocosanoate and tridecanal were detected.

PNC-1-2 (**2**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 826 $[M+Na]^+$. The FAME and LCA of **2** were analyzed as described for PNC-1. Methyl 2-hydroxydocosanoate and 12-methyltridecanal were detected.

PNC-1-3a (**3**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 826 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR [$C_5D_5N+D_2O$ (20/1)]: see Table 1. The FAME and LCA of **3** were analyzed as described for PNC-1. Methyl 2-hydroxydocosanoate and tetradecanal were detected.

PNC-1-3b (**4**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 826 $[M+Na]^+$. The FAME and LCA of **4** were analyzed as described for PNC-1. Methyl 2-hydroxytricosanoate and tridecanal were detected.

PNC-1-4a (**5**) and PNC-1-4b (**6**) [**5**/**6**, 85 : 15]: White amorphous powder. Positive ion FAB-MS *m*/*z*: 840 $[M+Na]^+$. The FAME and LCA of the inseparable mixture of **5** and **6** were analyzed as described for PNC-1. The FAME was characterized as methyl 2-hydroxytricosanoate, whereas the LCA was determined to be a mixture of 13-methyltetradecanal and 12-methyltetradecanal (at a ratio of 85 : 15).

PNC-1-4c (**7**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 840 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR [$C_5D_5N+D_2O$ (20/1)]: see Table 1. The FAME and LCA of **7** were analyzed as described for PNC-1. Methyl 2-hydroxytricosanoate and 12-methyltridecanal were detected.

PNC-1-5a (**8**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 840 $[M+Na]^+$. The FAME and LCA of **8** were analyzed as described for PNC-1. Methyl 2-hydroxydocosanoate and pentadecanal were detected.

PNC-1-5b (**9**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 840 $[M+Na]^+$. The FAME and LCA of **9** were analyzed as described for PNC-1. Methyl 2-hydroxytricosanoate and tetradecanal were detected.

PNC-1-5c (**10**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 840 $[M+Na]^+$. The FAME and LCA of **10** were analyzed as described for PNC-1. Methyl 2-hydroxytetracosanoate and tridecanal were detected.

PNC-1-6a (**11**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 854 $[M+Na]^+$. The FAME and LCA of **11** were analyzed as described for PNC-1. Methyl 2-hydroxydocosanoate and 13-methylpentadecanal were detected.

PNC-1-6b (**12**) and PNC-1-6c (**13**) [**12**/**13**, 9 : 1]: White amorphous powder. Positive ion FAB-MS *m*/*z*: 854 $[M+Na]^+$. The FAME and LCA of the inseparable mixture of **5** and **6** were analyzed as described for PNC-1. The FAME was characterized as methyl 2-hydroxytricosanoate, whereas the LCA was a mixture of 13-methyltetradecanal and 12-methyltetradecanal (at a ratio of 9 : 1).

PNC-1-6d (**14**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 854 $[M+Na]^+$. The FAME and LCA of **14** were analyzed as described for PNC-1. Methyl 2-hydroxytetracosanoate and 12-methyltridecanal were detected.

PNC-1-7a (**15**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 854 $[M+Na]^+$. The FAME and LCA of **15** were analyzed as described for PNC-1. Methyl 2-hydroxytricosanoate and pentadecanal were detected.

PNC-1-7b (**16**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 854 $[M+Na]^+$. The FAME and LCA of **16** were analyzed as described for PNC-1. Methyl 2-hydroxytetracosanoate and tetradecanal were detected.

PNC-1-8a (**17**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 868 $[M+Na]^+$. The FAME and LCA of **17** were analyzed as described for PNC-1. Methyl 2-hydroxytricosanoate and 13-methylpentadecanal were detected.

PNC-1-8b (**18**) and PNC-1-8c (**19**) [**18**/**19**, 9 : 1]: White amorphous powder. Positive ion FAB-MS *m*/*z*: 868 $[M+Na]^+$. The FAME and LCA of the inseparable mixture of **18** and **19** were analyzed as described for PNC-1. The FAME was characterized as methyl 2-hydroxytetracosanoate, whereas the LCA was determined to be a mixture of 13-methyltetradecanal and 12-methyltetradecanal (at a ratio of 9 : 1).

PNC-1-9 (**20**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 868 $[M+Na]^+$. The FAME and LCA of **20** were analyzed as described for PNC-1. Methyl 2-hydroxytetracosanoate and pentadecanal were detected.

PNC-1-10 (**21**): White amorphous powder. Positive ion FAB-MS *m*/*z*: 882 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR [$C_5D_5N+D_2O$ (20/1)]: see Table 1. The FAME and LCA of **21** were analyzed as described for PNC-1. Methyl 2-hydroxytetracosanoate and 13-methylpentadecanal were detected.

References

1) Degroote S., Wolthoorn J., Van Meer G., *Semin. Cell Dev. Biol.*, **15**, 375—387 (2004).
2) Higuchi R., Harano R., Mitsuyuki M., Isobe R., Yamada K., Miyamoto T., Komori T., *Liebigs Ann.*, **1996**, 593—599 (1996).
3) Inagaki M., Nakata T., Higuchi R., *Chem. Pharm. Bull.*, **54**, 260—261 (2006).
4) Costantino V., de Rosa C., Fattorusso E., Imperatore C., Mangoni A., Irace C., Maffettone C., Capasso D., Malorni L., Palumbo R., Pedone C., *Eur. J. Org. Chem.*, **2007**, 5277—5283 (2007).
5) Yasugi E., Kasama T., Seyama Y., *J. Biochem.*, **110**, 202—206 (1991).
6) Christiansen K., Mahadevan V., Viswanathan C. V., Holman R. T., *Lipids*, **4**, 421—427 (1969).
7) Gilpin J. A., Mclafferty F. W., *Anal. Chem.*, **29**, 990—994 (1957).
8) Harrison A. G., *Org. Mass Spectrom.*, **3**, 549—555 (1970).
9) Sugawara T., Zaima N., Yamamoto A., Sakai S., Noguchi R., Hirata T., *Biosci. Biotechnol. Biochem.*, **70**, 2906—2912 (2006).
10) Perez-Camino M. C., Moreda W., Mateos R., Cert A., *J. Chromatogr. A*, **983**, 283—288 (2003).
11) Karlsson K. A., Samuelsson B. E., Steen G. O., *Biochim. Biophys. Acta*, **306**, 307—316 (1973).
12) Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **35**, 501—506 (1987).
13) Smith C. R. Jr., Wolff I. A., *Lipids*, **4**, 9—14 (1968)
14) Karlsson K. A., Samuelsson B. E., Steen G. O., *Biochim. Biophys. Acta*, **306**, 317—328 (1973).
15) Prostenik M., Gerencevic N., *Chem. Ber.*, **99**, 3480—3484 (1966).
16) Zhong Y. L., Shing T. K. M., *J. Org. Chem.*, **62**, 2622—2624 (1997).