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Thraustochytrosides A–C: new glycosphingolipids from a unique marine protist, *Thraustochytrium globosum*

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Abstract

Three new glycosphingolipids, thraustochytrosides A–C (**1–3**) were produced, in culture, by a marine protist, identified as *Thraustochytrium globosum*, collected in the Bahamas. Two-dimensional NMR methods, coupled with tandem mass spectrometry (MS–MS), were used to establish the structures of the new compounds.

Marine microorganisms have been clearly demonstrated as a new source of novel natural products.[1] The search for bioactive compounds often leads to specialized biological niches in search of unusual and unstudied microorganisms. Examining diverse and cryptic microbes is an effective method for the discovery of new natural products. As part of a biological expansion of our program, we examined a group of marine protists, the thraustochytrids, which proved to be readily amenable to chemical study, and we isolated one strain that produced a series of new glycosphingolipids, the thraustochytrosides A–C (**1–3**). While glycosphingolipids are well known constituents of marine invertebrates, molecules of this class have not been isolated from microorganisms.[2,3]

The marine protist, designated as strain CNK-018, was isolated from the surface of the tropical seagrass *Thalassia testudinum*, collected in the Bahamas Islands. The thraustochytrid, identified as *Thraustochytrium globosum* (Kingdom Stremophila, Phylum Labyrinthulomycota, Family Thraustochytriaceae) was cultured in a marine nutrient medium.[4] The cells were separated from the culture broth, freeze-dried, and extracted with 1:1 methylene chloride:methanol. The concentrated cellular extract was then subjected to a series of solvent partitions followed by flash chromatography on a RP C-18 column.[5] Purification by reversed-phase C-18 HPLC (100% MeOH) yielded 35 mg of an inseparable mixture of thraustochytrosides A and C (**1**, **3**) and a complex fraction which, upon repeated HPLC separation, afforded 1.4 mg of thraustochytroside B (**2**).[6] Thraustochytrosides A (**1**) and C (**3**) were isolated as a mixture of two homologous compounds which could be distinguished by mass spectrometry. The com-

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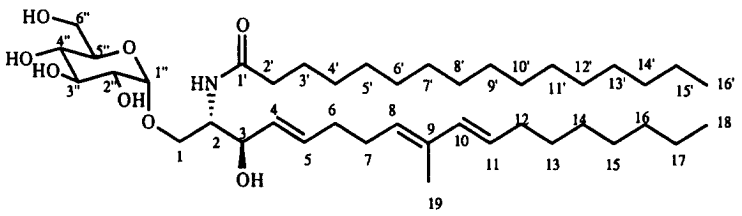

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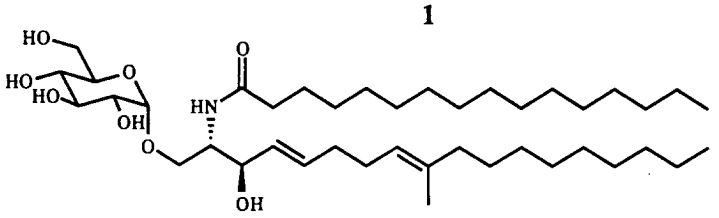

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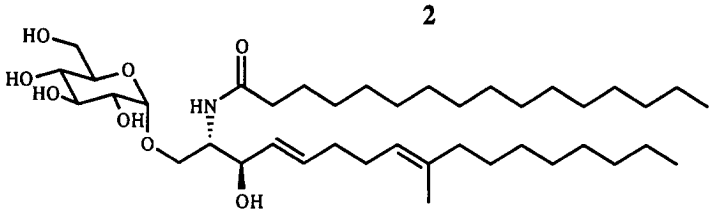

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pounds were considered inseparable since repeated chromatographic experiments failed to provide adequate separation of the metabolites. The NMR signals for the two compounds in the mixture were nearly identical. Significant differences occurred only in the relevant portion of the molecule where a single degree of unsaturation was absent in **3**. The inability to separate the metabolites is probably due to their chromatographic properties being overshadowed by the polar nature of the glycoside, thus making these metabolites difficult to separate to analytical purity. The 3:1 ratio of thraustochytroside A (**1**) to C (**3**) was determined by integration of the C-19 and C-18 methyl proton signals in the NMR spectrum.

Thraustochytroside A (**1**), readily observed in the mixture, analyzed for $C_{41}H_{75}NO_8 \cdot Na$ by HR-FABMS ($[M+Na]^+$ *m/z* 732.5368; calcd 732.5390), and through interpretation of NMR spectral data. The ^1H and ^{13}C NMR spectra for **1** displayed resonances typical of an aliphatic chain (δ 1.23) and a glycoside (Table 1). Integration of the olefinic region of the ^1H NMR spectrum clearly indicated that it was composed of a mixture of closely related metabolites, a complication not uncommon for metabolites incorporating long chain aliphatic moieties which are often composed of varied chain lengths and degrees of unsaturation.[7] The structure of **1** was assigned by analysis of mass spectral and 2D NMR data, including COSY, HMQC and HMBC experimental methods.

The double bonds in **1** were assigned in the sphingoid long chain base (LCB) through extensive analysis of 2D NMR data. The geometry of the C-10/C-11 double bond was determined to be *trans* by the large vicinal coupling constant (J=15.6 Hz) displayed between H-10 and H-11. The C-8/C-9 double bond was assigned as *E* based on the typical upfield shift of the C-19 methyl group.[8] The assignment of the stereochemistry for the remaining C-4/C-5 double bond was complicated by overlapping protons and was assigned a *trans* configuration based on the non-shielded chemical shift of the adjacent methylene (δ 32.0).[9] The relative stereochemistry of **1** at C-2 and C-3 was assigned as *erythro* (2*S**, 3*R**), identical to that of D-sphingosine, based on the similarity of the chemical shifts to those of glucosyl-*erythro*-ceramide. The sugar was assigned as an α-glucopyranoside based on the chemical shift of the anomeric proton (δ 4.66), its small coupling constant (J=3.6 Hz), and on the basis of extensive analysis of ring

Table 1
Spectral data for thraustochytrosides

	Thraustochytroside A				Thraustochytroside B			Thraustochytroside C	
C#	^{1}H δ [m, *J* (Hz)][a]	^{13}C δ[b]	COSY[a]	C#	^{1}H δ [m, *J* (Hz)][a]	^{13}C δ[b]	C#	^{1}H δ [m, *J* (Hz)][a]	^{13}C δ[b]
1	3.56 (m)	66.7	H2	1	3.56 (m)	66.7	1	3.56 (m)	66.7
2	3.78 (m)	53.3	H1, H3, NH	2	3.78 (m)	53.3	2	3.78 (m)	53.3
3	3.95 (m)	70.5	H2, H4	3	3.95 (m)	70.5	3	3.95 (m)	70.5
4	5.40 (m)	131.4	H3, H5	4	5.39 (dd; 6.9, 15.9)	131.2	4	5.40 (m)	131.5
5	5.60	130.6	H4	5	5.57	130.8	5	5.60 (m)	130.2
6	2.05	32.0		6	1.95	32.1	6	1.95 (m)	32.0
7	2.10	35.6	H8	7	2.02	36.0	7	2.10 (m)	35.6
8	5.35	129.4	H7	8	5.10	123.4	8	5.35 (m)	127.2
9		133.1		9		134.8	9		134.5
10	6.00 (d, 15.6)	134.5	H11	10	1.88	39.5			
11	5.50	127.2	H10						
12	2.05	32.3							
13-17	1.23 (br s)	22.1-32.0		11-17	1.23 (br s)	22.0-31.2	10-16	1.23 (br s)	22.1-32.0
18	0.85 (t, 5.7)	13.9		18	0.85 (t, 6.3)	13.9	17	0.85 (t, 5.7)	13.9
19	1.65 (s)	12.9		19	1.54 (s)	15.7	18	1.54 (s)	15.6
NH	7.50 (d, 8.7)			NH	7.47 (d, 9.0)		NH	7.50 (d, 8.7)	
1'		171.7		1'		171.7	1'		171.7
2'	2.11	29.0		2'-15'	1.23 (br s)	22.0-31.2	2'-15'	1.23 (br s)	22.1-32.0
3'-15'	1.23 (br s)	22.1-32.0							
16'	0.85 (t, 5.7)	13.9		16'	0.85 (t, 6.3)	13.9	16'	0.85 (t, 5.7)	13.9
1''	4.66 (m)	99.4	H2''	1''	4.66 (d, 3.9)	99.4	1''	4.66 (m)	99.4
2''	3.15 (m)	72.2	H1'', H3''	2''	3.13 (m)	72.1	2''	3.15 (m)	72.2
3''	3.36 (m)	72.7	H2'', H4''	3''	3.42 (m)	72.7	3''	3.36 (m)	72.7
4''	3.07 (m)	70.1	H3'', H5''	4''	3.07 (m)	70.1	4''	3.07 (m)	70.1
5''	3.42	73.4	H4'', H6''	5''	3.42	73.4	5''	3.42 (m)	73.4
6''	3.45 (m)	60.8	H5''	6''	3.45 (m)	60.8	6''	3.45 (m)	60.8
	3.59 (m)				3.59 (m)			3.59 (m)	

a) Spectra recorded in DMSO-d_6 at 300 MHz. b) Spectra recorded in DMSO-d_6 at 100 MHz. Assignments on the basis of DEPT and HMQC experiments.

coupling constants, derived from an E-COSY experiment on the per-acetate derivative.[10] Methanolysis under standard methods failed to yield the methyl glucoside in sufficient quantities for analysis. Thus, the absolute configuration of the sugar remains unresolved. The presence of the glycoside moiety, and assignment of the remaining carbinol at position C-3, led to the proposal that the *N*-acyl moiety was composed of a non-hydroxyl-substituted fatty acid. The composition of the two acyl chains was assigned by electrospray tandem mass spectral analysis (ESI MS–MS) for the protonated molecular ion (*m/z* 710). The MS–MS spectrum displayed an intense fragment ion at *m/z* 290 which is characteristic of the protonated methyl branched sphingosine base, thus establishing the identity of the fatty acid acyl chain as an hexadecanoic acid.

Thraustochytroside B (**2**) analyzed for $C_{41}H_{77}NO_8$ by HR-FABMS data, a formula that indicated one less degree of unsaturation. Extensive analysis of the HMBC NMR data for **2**, and comparison with the data derived for **1**, led to the assignment of the double bonds in the LCB fragment (Table 1), clearly indicating the loss of the C-10/C-11 double bond. This conclusion is further supported by an HMBC correlation from H-19 to a methylene carbon at δ 39.5 (C-10), in agreement with similar spin systems.[11] The configuration of the C-4/C-5 double bond was assigned as *trans* based on the large coupling constant (*J*=15.0 Hz) displayed between H-4 and H-5. The stereochemistry of the C-8/C-9 double bond was assigned the *E* configuration by comparison of the chemical shift for the C-19 methyl group with published data.[11] Here again, the length of the acyl chain was assigned by interpretation of the MS–MS data which displayed a fragment at *m/z* 298 ($[M+Na]^+$) which the presence of hexadecanoic acid.

Thraustochytroside C (**3**) exhibited nearly identical ^{1}H and ^{13}C NMR data to that of **2** (Table 1), but the molecular formula,[6] derived from HR-FABMS data, indicated one less methylene group in comparison

to **2**. The complete structure of **3** was assigned by comparison of NMR spectral data for **2** and by comprehensive analysis of the data acquired from 2D NMR and MS–MS experiments.

This is the first report of a secondary metabolite from a thraustochytrid marine protist, a chemically poorly known group of obligate marine microorganisms. In the few cases where thraustochytrids have been studied, they have been shown to produce a number of sterols, including brassicasterol[12] and cholesterol, and polyunsaturated fatty acids (PUFA's).[13] GSLs containing α-glycosides are typically found only in marine sponges. Thraustochytroside A (**1**) is particularly interesting since it contains a fairly unusual tri-unsaturated sphingosine LCB with a methyl branch at C-9, a structural moiety previously reported only from the ophidiacerebrosides[2] and the agelasphins[3], marine GSLs which have been shown to exhibit impressive bioactivities. While not the main thrust of this study, we are currently evaluating the bioactivity of the thraustochytrosides to determine their bioactivity profile.

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4. Strain CNK-018 was cultured in a liquid marine medium (B1 medium composed of 2.5 g peptone, 1.5 g yeast extract, 3.0 ml 50% glycerol per 1 L autoclaved filtered seawater) for 3 days while shaking at 150 rpm.
5. The crude extract was first adsorbed onto Celite and eluted with isooctane, EtOAc and MeOH. The isooctane fraction was further partitioned between hexane and MeOH. The MeOH soluble portion was dried and dissolved in EtOAc before being partitioned with water, to yield a white precipitate. The EtOAc fraction and ppt. were recombined and separated by flash chromatography on a RP C-18 column (30×2.5 cm) eluting with 100% MeOH.
6. The mixture of thraustochytroside A (**1**) and thraustochytroside C (**3**) was obtained as a white solid that exhibited the following spectral data: (**1**) HR-FABMS (*m/z*): $[M+Na]^+$ calcd for $C_{41}H_{75}NO_8 \cdot Na$, 732.5390; found, 732.5368 (Δ 3.0 ppm), (**3**) HR-FABMS (*m/z*): $[M+Na]^+$ calcd for $C_{40}H_{75}NO_8 \bullet Na$, 720.5390; found, 720.5370 (Δ 2.8 ppm). Thraustochytroside B (**2**) was obtained as a white amorphous solid which showed: HR-FABMS (*m/z*): $[M+Na]^+$ calcd for $C_{41}H_{77}NO_8 \cdot Na$, 734.5547; found, 734.5527 (Δ 2.7 ppm).
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10. Compound **1** (5.0 mg) was dissolved in 250 μl of pyridine, 250 μl of acetic anhydride was then added, and the solution was allowed to stir at 25°C for 48 hours. Typical work-up gave primarily one pure product with the following spectral data for the sugar moiety: ^{1}H NMR (300 MHz, DMSO): δ 5.02 (H-1″, d, *J*=3.6), 4.92 (H-2″, dd, *J*=3.6, 10.1), 5.50 (H-3″, dd, *J*=10.4, 8.4), 5.02 (H-4″, dd, *J*=8.4, 11.1), 4.00 (H-5″, m), 4.26 (H-6″, dd, *J*=4.6, 12.4), 4.12 (H-6″, dd, *J*=1.81, 12.4).
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