Rhizochalin A, a Novel Two-Headed Sphingolipid from the Sponge *Rhizochalina incrustata*

Tatyana N. Makarieva,*,† Alla G. Guzii,† Vladimir A. Denisenko,† Pavel S. Dmitrenok,† Elena A. Santalova,† Evgenii V. Pokanevich,† Tadeusz F. Molinski,‡ and Valentin A. Stonik†

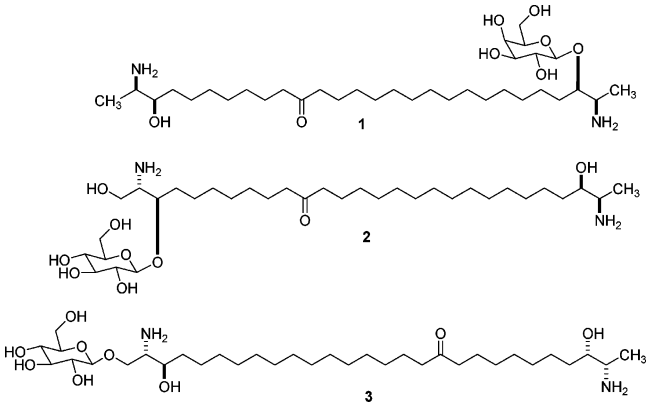
Laboratory of the MaNaPro Chemistry, Pacific Institute of Bioorganic Chemistry of the Russian Academy of Sciences, 690022 Vladivostok-22, Russia, and Department of Chemistry, University of California, One Shields Avenue, Davis, California 95616

Received August 31, 2004

Rhizochalin A (**4**), the fourth representative of two-headed glycosphingolipids, was isolated as its peracetate from the sponge *Rhizochalina incrustata*. Its structure has been established as the 2-ethyl carbamoate of rhizochalin on the basis of spectroscopic data and chemical transformations.

Sphingolipids appear to play an important role in cellular regulation. Unusual sphingolipid analogues may act on the metabolism of normal sphingolipids, or as agonists or antagonists interacting with sphingolipid recognition sites in regulatory processes.[1]

Two-headed sphingolipids from marine sponges are striking because of their rare α,ω-bifunctionalized structures and high biological activity. Since the discovery, in 1989, of rhizochalin (**1**), the first member of this series,[2] only two additional members of this group have been described. One of these compounds, oceanapiside (**2**), exhibits significant antifungal activity against the pathogenic fluconazole-resistant yeast *Candida glabrata*.[3] Rhizochalin (**1**) shows antibacterial activity against *Staphylococcus aureus* and cytotoxic activity against mouse Ehrlich carcinoma cells (IC_{50} 10 μg/mL).[2] Another analogue of **1**, calyxoside (**3**), is a selective DNA-damaging agent, but lacks inhibitory activity against topoisomerase I or II.[4]



In the course of our continuing studies on marine natural products,[5] we have found additional two-headed sphingolipids, including the new compound **4**, which we have named rhizochalin A. In this report we describe the isolation and structure elucidation of **4** containing a rare *N*-substituted carbamoyl group from the sponge *Rhizochalina incrustata* (order Haplosclerida, family Phloeodictydiae).

The EtOH extract of the lyophilized sponge was concentrated and sequentially partitioned with hexane and chloroform. Chloroform-soluble materials were further separated by column chromatography on Polychrome-1 (powdered Teflon) eluting with EtOH−H_2O, 1:1, then on silica gel ($CHCl_3$−EtOH−H_2O, 3:2:0.2) to obtain a crude mixture containing **1** and **4**. To obtain structural information on the new compound, the mixture was acetylated (Ac_2O/pyr), and products were purified by preparative TLC followed by HPLC (YMC-Pack ODS-A column) to provide rhizochalin A peracetate (**4a**, 0.0006%, based on dry weight of sponge).

Table 1. ^1H and ^{13}C NMR Data for Compounds **4a** and **5** ($CDCl_3$, TMS)[a]

	4a		**5**	
atom no.	δ_H (m, Hz)	δ_C	δ_H (m, Hz)	δ_C
1	1.11, d, 6.8	18.8	1.11, d, 6.8	18.8
2	3.92, m	48.8	3.90, m	49.0
3	4.84 td, 3.5, 6.7	76.4	4.85 m	76.4
4	1.55, m	31.5		31.7
5		25.2		25.3
6−8, 14−23	1.25, bs	29.1−29.8		29.1−29.8
9	1.55, m	23.9	1.55, m	24.0
10	2.37, t, 7.5	42.8	2.37, t, 7.5	42.9
11		211.6		211.6
12	2.36, t, 7.5	42.7	2.36, t, 7.5	42.8
13	1.55, m		1.55, m	
24		25.3		
25	1.42	30.7		
26	3.50, dt, 2.7, 6.2, 6.2	82.5	4.85, m	76.6
27	4.10, m	46.7	4.20, m	47.3
28	1.17, d, 6.8	18.6	1.10, d, 6.8	18.5
2-NH	4.71, d, 9.7		4.71, d, 8.5	
27-NH	5.81, d, 8.5		5.51, d, 8.7	
1′		156.2		
2′	4.10, m	60.8	4.11, q, 7.0	60.9
3′	1.25, t, 6.8	14.6	1.25, t, 6.8	14.7
1″	4.48, d, 7.8	100.4		
2″	5.16, dd, 7.8 10.5	69.2		
3″	5.04, dd, 3.4, 10.5	70.7		
4″	5.39, dd, 1.1, 3.4	67.0		
5″	3.91, dt, 1.11, 6.6, 6.6	70.7		
6″	4.10, dd, 6.6, 11.2; 4.19, dd, 6.6, 11.2	61.3		

[a] All ^1H NMR experiments were performed at 300 and 500 MHz; ^{13}C NMR experiments were performed at 75 and 125 MHz in $CDCl_3$.

The molecular formula $C_{49}H_{84}N_2O_{16}$ of **4a** was obtained from a high-resolution mass measurement of the $[M + Na^+]$ ion in HRMALDI-TOF-MS and consideration of FABMS and EIMS data. The ^1H and ^{13}C NMR data of **4a** (Table 1) showed signals typical of a pseudo-dimeric amino alcohol

* To whom correspondence should be addressed. Tel: 7 (4232) 31-11-68. Fax: 7 (4232) 31-40-50. E-mail: makarieva@piboc.dvo.ru.
† Laboratory of the MaNaPro Chemistry.
‡ University of California.

10.1021/np049710z CCC: $30.25
Published on Web 02/03/2005

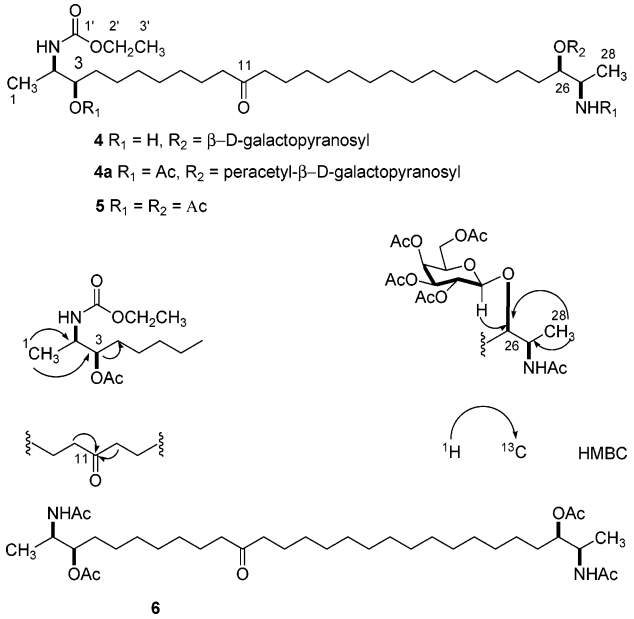


Figure 1. Rhizochalin A (**4**) and substructures from HMBC spectra.

glycoside, reminiscent of **1**. These included signals for two secondary methyl groups (δ_H 1.11, 1.17; δ_C 18.8, 18.6), two *N*-substituted CH carbons (δ_H 3.92, 4.10; δ_C 48.8, 46.7), two oxymethines (δ_H 4.84, 3.50; δ_C 76.4, 82.5), and one ketone carbonyl group (δ_C 211.6), flanked by two α-CH_2 groups (δ_H 2.37, 2.36; δ_C 42.8 and 42.7). The remainder of the signals was assigned to long CH_2 chains (δ_H 1.25; δ_C 29.1−29.8). ^{1}H NMR data of **4a** were similar to those of rhizochalin peracetate except that the amide doublet (C_2−NH) was shifted upfield from δ 5.63 to δ 4.71.[2] Consequently, the structure of **4** was formulated as an analogue of rhizochalin with a modification at C-2 that was subsequently revealed by analysis of ^{13}C NMR, COSY, and HMBC data. The ^{13}C NMR spectrum of **4a** revealed signals due to an ethoxyl group (OCH_2CH_3: δ_H 1.25, t, J = 6.8 Hz, 3H; 4.10 m, 2H; δ_C 14.6, q; 60.8, t). The balance of the formula indicated a C=O group whose ^{13}C chemical shift (δ 156.2, s) was consistent only with a carbamoyl group. HMBC correlations (Figure 1) placed this $NH(CO)OCH_2$-CH_3 group at C-2 in **4**. The galactopyranosyl group in **4** has the β-configuration at the anomeric carbon, as revealed by the H1″ coupling constant (δ 4.48, d, J = 7.8 Hz). The cross-peak with C-26 (δ 82.5) in the HMBC spectrum established the attachment of monosaccharide to this position. Hydrolysis of **4a** (6 N HCl, 100 °C, 2.5 h) liberated D-galactose and two aglycone-derived compounds, which were peracetylated (Ac_2O/pyr, 1:1) and separated by silica chromatography. The earlier-eluting compound was identified as the peracetate **5**, and the second product proved to be peracetylaglycone, **6**, identical to that derived from rhizochalin (**1**) by NMR, EIMS, and $[\alpha]_D$ data.[2] Therefore, the keto groups in **1** and **4** are located at the same position (C-11), and the absolute configuration of **4** is the same as that of **1** (2*R*,3*R*,26*R*,27*R*).[6]

Rhizochalin A is the first example of a natural product among known sphingolipids, including the family of two-headed sphingolipids that contains the rare *N*-alkyl carbamoyl group. *N*-Carbamates have also been detected in other marine alkaloids,[7−9] while the polyketides discodermolide A[10] and kabiramide C[11] contain *O*-alkyl carbamoyl groups. Since the latter compounds were obtained from MeOH extracts and the specimen of *R. incrustata* used in this study was stored in ethanol, it is possible that **4** and naturally derived *O*-Me carbamates originate from as-yet-unidentified biosynthetic intermediates of divergent carbamoyl or carbonate transferase reactions, followed by solvolytic interception.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Perkin-Elmer 343 polarimeter. The NMR experiments were performed with Bruker DPX-300 and Bruker DRX-500 spectrometers. FAB and EI mass spectra were obtained on an AMD-604S mass spectrometer (AMD-Intectra, Germany). MALDI-TOF mass spectra were obtained on a Bruker Biflex III laser desorption mass spectrometer coupled with delayed extraction using an N_2 laser (337 nm) on α-cyano-4-hydroxycinnamic acid as matrix.

Low-pressure column liquid chromatography was performed using Polichrom-1 (powder Teflon, Biolar, Latvia), Sephadex LH-20 (Sigma, Chemical Co.), and silica gel L (40/100 μm, Chemapol, Praha, Czech Republic); silica gel plates of 4.5 × 6.0 cm (5−17 μm, Sorbfil, Russia) were used for thin-layer chromatography.

Animal Material. The sponge *Rhizochalina incrustata* (Porifera, class Demospongiae, subclass Ceratinomorpha, order Haplosclerida, family Phloeodictydiae) was collected using scuba (depth 3−12 m) during the third scientific cruise of *R/V Akademik Oparin* (November 1986, Seychelles Islands, 4°26′45″ N, 54°54′75″ E) and identified by Prof. V. M. Koltun (Zoological Institute, St. Petersburg, Russia). A voucher specimen (03-297) was deposited in the collection at the Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia.

Extraction and Isolation. The fresh collection of the sponge *R. incrustasta* was immediately lyophilized and kept at −20 °C until required. The lyophilized material (400 g) was extracted with EtOH (1 L × 3). The ethanolic extract after evaporation in vacuo was redissolved with EtOH−H_2O (9:1). The *n*-hexane-soluble fraction was extracted three times by partitioning with equal volumes of hexane. The water content of the aqueous EtOH extract was adjusted to 7:1 EtOH−H_2O. The $CHCl_3$-soluble fraction was extracted three times by partitioning with equal volumes of $CHCl_3$. The $CHCl_3$ extracts were evaporated in vacuo at 50 °C to give a brown oil, which was separated over a Polichrome I (powder Teflon, Biolar, Latvia) by elution with a gradient of H_2O → 50% EtOH → EtOH. The two-headed sphingolipid fraction (ninhydrin positive) eluted with 50% EtOH. The latter was further separated over a SiO_2 column using $CHCl_3$ → $CHCl_3$−EtOH (10:1 → 3:2) and $CHCl_3$−EtOH−H_2O (3:2:0.2) mixtures as eluents to give a mixture (50 mg) of the known rhizochalin (**1**)[2] and crude **4**.

Peracetate Derivatives, **1a and 4a.** A sample of the mixture of **1** and **4** (50 mg) was dissolved in pyridine (1.0 mL) and acetic anhydride (1.0 mL) and allowed to stand at 25 °C for 18 h. Removal of the volatile material gave a residue (55 mg) containing **1a** and **4a**. The latter was separated on a SiO_2 column using EtOAc to give **1a** (30 mg) and a fraction containing less polar compounds (10.7 mg), which was purified by preparative TLC (SiO_2, EtOAc) to give **4a** (5.3 mg). Preparative HPLC (YMC-Pack ODS-A, 80:20 EtOH−H_2O) gave rhizochalin A peracetate (**4a**) (2.3 mg; 0.0006%, based on dry weight of sponge).

Rhizochalin A peracetate (4a): amorphous solid, $[\alpha]^{18}_D$ +15° (*c* 0.22 EtOH); HRMALDI *m/z* (M + Na^+) 979.5759 (cald for $C_{49}H_{84}N_2O_{16}Na$ 979.5719); EIMS *m/z* 956 (M^+), 911, 897, 841, 798, 782, 609, 563; FABMS *m/z* 957 (M + H)$^+$; FABMS *m/z* 955 (M^+ − H)$^-$; ^{1}H NMR ($CDCl_3$, see Table 1); ^{13}C NMR ($CDCl_3$, see Table 1).

Hydrolysis of Rhizochalin A Peracetate (4a). A solution of **4a** (2.2 mg) in 6 N HCl (1 mL) was heated at 100 °C for 2.5 h. The mixture was cooled and treated with Dowex ion-exchange resin (HCO_3^- form) and extracted with *n*-BuOH. The aqueous solution was separated and concentrated to afford D-galactose (0.4 mg). The *n*-BuOH layer was concentrated, and

a residue was dissolved in pyridine (0.5 mL) and acetic anhydride (0.5 mL) and allowed to stand at 25 °C for 18 h. Removal of the volatile material gave a residue (1.6 mg) containing a mixture of **5** and **6**. The latter was separated on a SiO_2 column with SiO_2, EtOAc → EtOAc−EtOH (100:3) to give pure **5** (0.3 mg) and **6** (1.1 mg).

Compound 5: amorphous solid; $[\alpha]^{18}_D$ +30° (*c* 0.03 $CHCl_3$); MALDI *m/z* 691 ($M + Na^+$); 707 ($M + K^+$); EIMS *m/z* 623 ($M^+ - 45$), 535, 519, 450, 434, 350, 340, 294, 280; FABMS *m/z* 669 ($M^+ + H$); ^1H NMR ($CDCl_3$, see Table 1).

Compound 6: amorphous solid; $[\alpha]^{18}_D$ +35° (*c* 0.11 $CHCl_3$); EIMS *m/z* 638 (M^+); 595, 578, 553, 535, 519, 493, 450, 434, 350, 340, 294, 280; ^1H NMR ($CDCl_3$, see Table 1).

Acknowledgment. This investigation was supported by the Fogarty International Center and NIH (TWOO6301-01). The research described in this publication was made possible in part by the Program of Presidium of RAS "Molecular and Cell Biology", Grant N SS-725.2003.4 from the President of RF.

References and Notes

(1) Shier, W. T.; Shier, A. C. *J. Toxicol*. **2000**, *19*, 189−246.
(2) Makarieva, T. N.; Denisenko, V. A.; Stonik, V. A.; Milgrom, Yu. N.; Rashkes, Ya. W. *Tetrahedron Lett*. **1989**, *30*, 6581−6584.
(3) Nicholas, G. M.; Hong, T. W.; Molinski, T. F.; Lerch, M. L.; Cancilla, M. T.; Lebrilla, C. B. *J. Nat. Prod*. **1999**, *62*, 1678−1681.
(4) Zhou, B. N.; Mattern, M. P.; Johnson, R. K.; Kingston, D. G. I. *Tetrahedron* **2001**, *57*, 9549−9554.
(5) Makarieva, T. N.; Rho, J.-R.; Lee, H.-S.; Santalova, E. A.; Stonik, V. A.; Shin, J. *J. Nat. Prod*. **2003**, *66*, 1010−1012.
(6) Molinski, T. F.; Makarieva, T. N.; Stonik, V. A. *Angew. Chem., Int. Ed*. **2000**, *39*, 4076−4079.
(7) Pina, I. C.; Gautschi, J. T.; Wang, G. Y. S.; Sanders, M. L.; Schmitz, F. J.; France, D.; Cornell-Kennon, S.; Sambucetti, L. C.; Remiszewski, S. W.; Perez, L. B.; Bair, K. W.; Crews, P. *J. Org. Chem*. **2003**, *68*, 3866−3873.
(8) Uy, M. M.; Ohta, S.; Yanai, M.; Ohta, E.; Hirata, T.; Ikegami, S. *Tetrahedron* **2003**, *59*, 731−736.
(9) Ovenden, S. P. B.; Capon, R. J. *J. Nat. Prod*. **1999**, *62*, 1246−1249.
(10) Matsunaga, S.; Fusetani, N.; Hashimoto, K.; Koseki, K.; Noma, M. *J. Am. Chem. Soc.* **1986**, *108*, 847−849.
(11) Gunasekera, S. P.; Gunasekera, M.; Longley, R. E.; Schulte, G. K. *J. Org. Chem.* **1990**, *55*, 4912−4915.

NP049710Z