Rhizochalins C and D from the Sponge *Rhizochalina incrustata.* A Rare *threo*-Sphingolipid and a Facile Method for Determination of the Carbonyl Position in α,ω-Bifunctionalized Ketosphingolipids

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Received September 10, 2007

Rhizochalins C and D (**1**, **2**), new representatives of two-headed glycosphingolipids, were isolated from the sponge *Rhizochalina incrustata*. Rhizochalin D is an unexpected C_{29} homologue of the canonical C_{28} dimeric sphingolipid structures. Their structures including absolute configurations were established using spectroscopic data, micromolar-scale Baeyer–Villiger oxidation, and LCMS interpretation of the products. Application of the latter method leads to a revision of the structure of oceanapiside and placement of the keto group at C-18 rather than C-11.

Two-headed sphingolipids represent a group of rare bis-α,ω-amino alcohols from the marine sponges *Rhizochalina incrustata,*[1,2] *Oceanapia phillipensis*,[3] *Oceanapia* sp.,[4] *Calyx* sp.,[5] *Leucetta leptorhapsis,*[6] and an unidentified sponge.[7] Early findings on biological properties of two-headed sphingolipids included antibacterial activity against *Staphylococcus aureus*, cytotoxic activity against mouse Ehrlich carcinoma cells,[1] antifungal activity against the pathogenic fluconazole-resistant yeast *Candida glabrata*,[3,4] selective DNA-damaging activity,[5] and inhibition of protein kinase C.[7]

In the course of our continuing studies on marine natural products,[8,9] we have found several additional two-headed sphingolipids, including two new compounds we have named rhizochalins C (**1**) and D (**2**). In this report we describe the isolation and structure elucidation of both **1** and **2** including absolute configurations. In contrast to the regular (2*S*,3*R*)-configuration found in normal sphingoid bases, rhizochalin C contains the rare (2*R*,3*R*) *threo* stereochemistry, while **2** possesses an odd-numbered C_{29} hydrocarbon chain instead of C_{28} found in canonical members of this series.[1–5]

Results and Discussion

The EtOH extract of lyophilized *R. incrustata*, collected in Madagascar,[1] was concentrated and partitioned between *n*-hexane and aqueous EtOH. The aqueous EtOH-soluble materials were further separated by column chromatography on Polychrome-1 (powdered Teflon) by eluting with EtOH−H_2O, 1:1, followed by Si gel chromatography ($CHCl_3$−EtOH−H_2O, 3:2:0.2) to obtain a crude mixture, which was purified by preparative HPLC (Dynamax C_{18} column) to provide pure rhizochalin (**3**)[1] and rhizochalins C (**1**) and D (**2**) (0.12%, 0.008%, and 0.011%, respectively, based on dry weight of the sponge).

The molecular formula $C_{34}H_{68}N_2O_9$ of **1** was obtained from a high-resolution mass measurement of the $[M + H]^+$ ion peak in the HRFABMS and interpretation of MALDI-TOF-MS and ESIMS data, as well as ESIMS data for the corresponding octaacetate (**1a**). Interestingly, the base peak at *m/z* 325 in the ESIMS of **1** corresponded to a doubly charged ion $[M + H_2]^{2+}$ as previously seen for oceanapiside.[3] The ^1H and ^{13}C NMR data of **1** (Table 1) revealed signals typical of a pseudo-dimeric amino alcohol glycoside, reminiscent of **3** and its known analogues.[1–5] These included signals for one secondary methyl group (δ_H 1.25, δ_C 16.0), one oxymethylene group (O-CH_2, δ_H 3.76, 3.64; δ_C 59.6), two *N*-substituted CH carbons (δ_H 3.12, 3.05; δ_C 57.5, 53.3), two oxymethines (δ_H 3.86, 3.40; δ_C 77.0, 73.0), and one ketone carbonyl group (δ_C 215.0), flanked by two CH_2 groups (2 × CH_2, δ_H 2.43; 2.42; δ_C 43.4; 43.5). The remainder of the signals were assigned to $(CH_2)_n$ chains (δ_H 1.27−1.29; δ_C 30.8–31.6) and a sugar moiety. In contrast with **3**, the carbon backbone of sphingolipid **1** contains two different end-groups, the α-terminus formally derived from serine and the ω-terminus from alanine. The structures of these moieties were confirmed by 2D NMR data including HMBC experiments. Collectively, the NMR data of **1** were found to be similar to those previously seen in oceanpiside except signals due to the sugar group.[3] A galactose unit was suggested by signals at δ_C 104.6 and δ_H 4.32 (d, J = 7.2 Hz), δ_C 73.3 and δ_H 3.51 (dd, 7.2, 9.8), δ_C 75.1 and δ_H 3.47 (dd, 3.4, 9.8), δ_C 71.1 and δ_H 3.78 (dd, 3.4), δ_C 77.6 and δ_H 3.54 (dd, 4.6, 6.5), and δ_C 63.6 and δ_H 3.72 and 3.74 (m). The ^3J-coupling constant of the anomeric proton H-1′ (d, J = 7.2 Hz) indicated that rhizocalin C is a β-galactoside. The HMBC spectrum also showed a cross-peak from H-1′ (δ_H 4.32) to C-3 (δ 77.0), confirming the attachment of the galactopyranosyl fragment to O-3. Hydrolysis of **1** gave an aglycone named rhizochalinin C (**1b**). The NMR spectra of **1b** were congruent with those of **1**. Therefore, rhizochalin C (**1**) was formulated as a C-1-hydroxy analogue of **3**.

The molecular formula $C_{35}H_{71}N_2O_8$ of rhizochalin D (**2**) was obtained from HRFABMS measurement of the $[M + H]^+$ ion, together with interpretation of MALDI-TOF-MS and ESIMS data, and ESIMS data of the corresponding peracetate (**2a**). As for compound **1**, the base peak in the ESIMS of **2** was the doubly charged ion at *m/z* 324 corresponding to $[M + H_2]^{2+}$. The ^1H and ^{13}C NMR data of **2** (Table 1) showed signals typical of a pseudo-dimeric amino alcohol glycoside similar to **3**,[1–5] but the MS data showed a molecular mass for **2** that was 14 amu higher than those reported for **3**.[1] The ^{13}C NMR, COSY, and HMBC data of **2** (Table 1) were essentially the same as those of **3**, suggesting that the carbon chain of **2** is extended by one additional CH_2 group with respect to **3**. Hydrolysis of **2** gave an aglycone designated as rhizochalinin D (**2b**).

The assignment of the D-configuration of the sugar was based on comparison of NMR data for H-2 and H-3 adjoining the sugar

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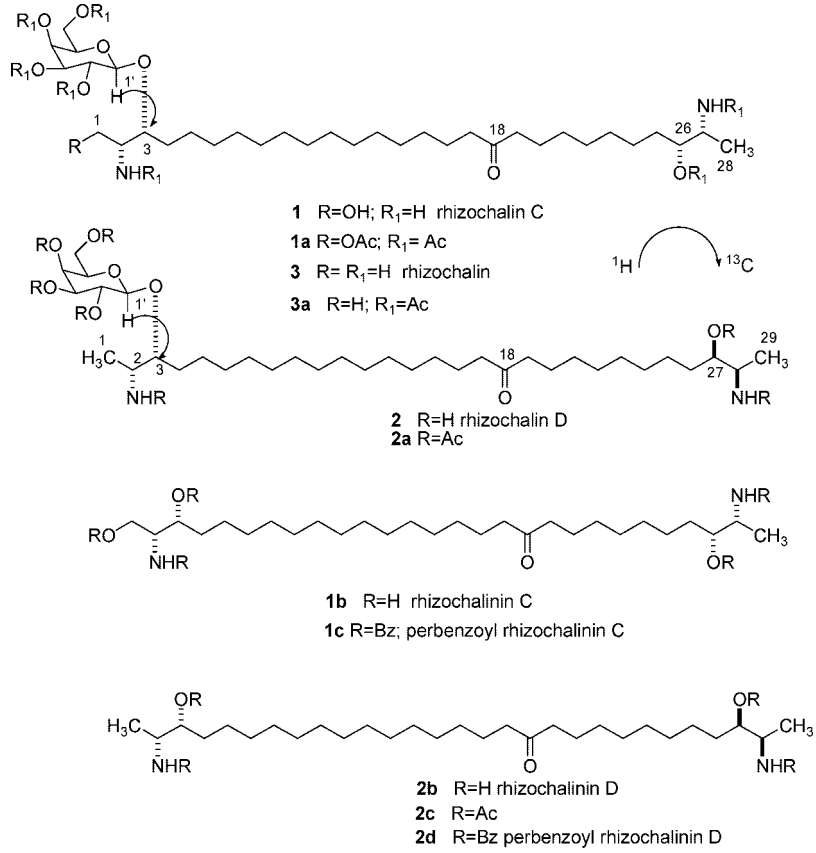
10.1021/np0704811 CCC: $37.00
Published on Web 12/06/2007



in **2** and **2a** with those of related[1,2,4] and model compounds, as it is found that the latter signals are sensitive to the sugar absolute configuration.[10] The D-galactose is the most common sugar encountered among the rhizochalinins from the sponge *R. incrustata*[1,2] and is also found here in rhizochalin C (**1**).

The foregoing evidence established the structures of rhizochalins C and D as unusual glycosylated two-headed sphingolipids, having a ketone group embedded in a hydrocarbon chains. However, interpretation of spectroscopic data could not establish the position of the carbonyl group or the configuration of these natural products. In agreement with previous studies on ketosphingolipids, direct attempts to determine the position of the carbonyl group in the compounds by EI mass spectrometry were unsuccessful due to uncertainty in the interpretation of low-intensity ions from McLafferty-type rearrangements. In our earlier work, the position of the C═O group in **3** was established by Baeyer–Villiger oxidation followed by alkaline hydrolysis and full characterization of the liberated alcohol and carboxylic moieties.[1] The C═O position in calyxoside was assigned by reductive amination to afford a primary amine that facilitated α-cleavage in EIMS and location of the carbonyl group.[5] However, the applicability of these degradative methods is limited in the case of minor metabolites that are available in submilligram amounts.

In order to secure the location of the C═O group, we have developed a procedure based on *in situ* MS analysis of Baeyer–Villiger oxidation products obtained from the ketone. Preliminary analyses were carried out using an available sample of rhizochalin peracetate (**3a**). In order to ascertain limits of detection (LOD), variable amounts of **3a** (0.13, 0.26, 0.5, 1.0, and 2.0 mg) in methanol solution were subjected to Baeyer–Villiger conditions (TFA−H_2O_2) to effect three successive chemical transformations: oxidation, hydrolysis of the derived esters (**4** and **4a**), and methylation of the resultant carboxylic acids. Removal of the volatiles under vacuum followed by acetylation (Ac_2O−pyr) in one pot and subsequent analysis of the degradation products by MALDI-TOF-MS without purification permitted confirmation of the C-18 position of the carbonyl in **3**. As little as about 0.13 mg of **3a** is sufficient for identification of principal ions at *m/z* 724 $[M + Na]^+$ in the MALDI-TOF-MS of **9** and **12** and at *m/z* 338 $[M + Na]^+$ in the MS of **14** and **16**. Furthermore, preparative isolation of **9**, **12**, **14**, and **16** obtained from a larger amount of **3a** (2.0 mg) using HPLC gave sufficient amounts of target compounds to record ^1H NMR spectra (Table 2) in addition to MALDI-TOF-MS.

The foregoing methodology was also applied to rhizochalin C peracetate (**1a**), rhizochalin D peracetate (**2a**), and rhizochalinin D peracetate (**2c**) (∼0.2 mg each) to determine the C═O location in **1** and **2**. Baeyer–Villiger oxidation of rhizochalin C peracetate (**1a**) and subsequent MALDI-TOF analysis (Scheme 1) gave ions at *m/z* 782 $[M + Na]^+$ and *m/z* 338 $[M + Na]^+$, consistent with the presence of fragments **8**, **11**, **14**, and **16**. Interpretation of these results placed the C═O group at C-18 in **1** and flanked by $(CH_2)_{14}$ and $(CH_2)_7$ chain fragments. Thus, the C═O group in **1** is placed at the same position as those of previously described α,ω-bifunctionalized ketosphingolipids[1–5] (see reassignment of oceanapiside, below). The same analysis applied to rhizochalin D peracetate (**2a**) gave ions at *m/z* 724 $[M + Na]^+$ and *m/z* 352 $[M + Na]^+$, consistent with the presence of fragments **9**, **12**, **15**, and **17**. Rhizochalinin D peracetate gave ions at *m/z* 436 $[M + Na]^+$ and *m/z* 352 $[M + Na]^+$, consistent with the presence of fragments **10**, **13**, **15**, and **17**. These combined results confirmed that the carbonyl group in **2** is flanked by $(CH_2)_{14}$ and $(CH_2)_8$, in contrast to all known α,ω-bifunctionalized ketosphingolipids from sponges.

Having validated the Baeyer−Villager/MS method, we turned our attention to oceanapiside (**18**), in which the keto group was assigned to C-11 using charge-localized fragmentation of the Li^+ adduct of **18** and d_3-**18**,[3] which relied upon an assumption of the site of Li^+ coordination in the molecule. Since the interpretation of these results led to an unorthodox structure—the one exception to the C-18 keto-substitution pattern in the family of dimeric sphingolipids—we applied a modification of the Baeyer–Villiger oxidation−MS analysis (Scheme 2)[11] in order to resolve the question of location of the keto group. Oxidation of oceanapiside

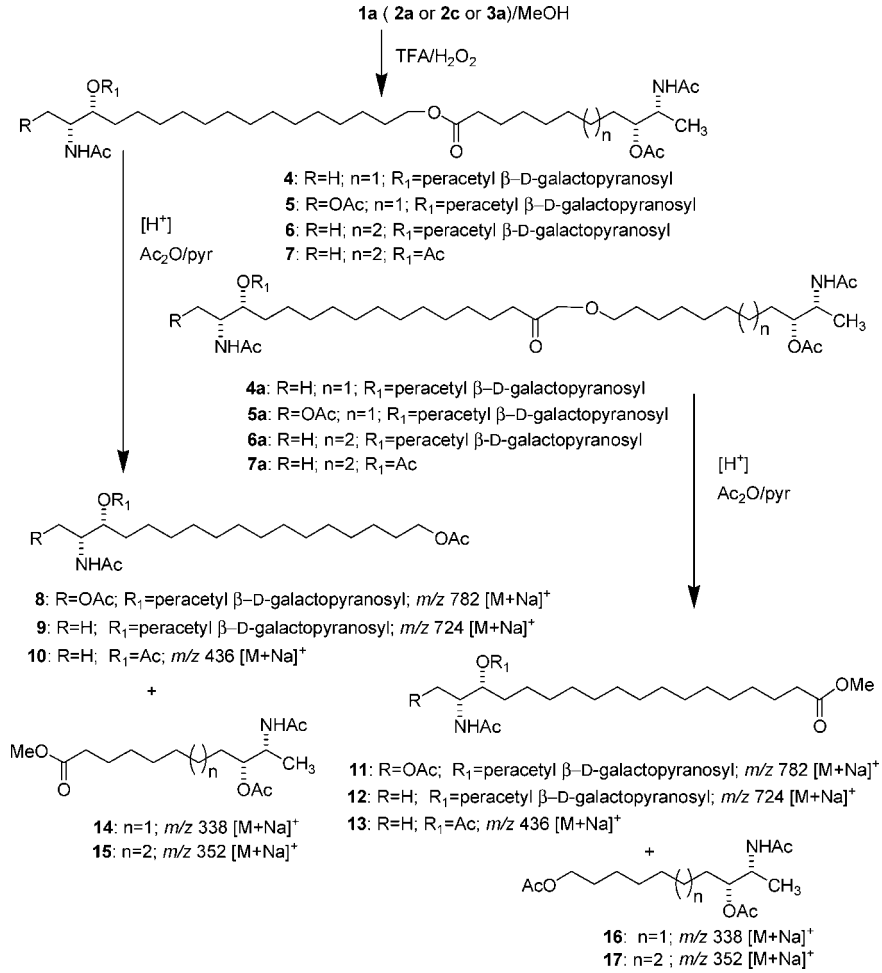
Table 1. ^{1}H and ^{13}C NMR Data for Rhizochalins C (**1**) and D (**2**) (CD_3OD, TMS)

	rhizochalin C				rhizochalin D			
atom no.	δ_C	δ_H (mult, *J* Hz)	COSY	HMBC (10 Hz)	δ_C	δ_H (mult, *J*Hz)	COSY	HMBC
1a	59.6	3.76 (dd, 3.8, 11.8)	H2		16.0	1.27 (d, 6.7)	H2	C2, C3
1b		3.64 (dd, 9.2, 11.8)						
2	57.5	3.12 (m)	H1, H3		52.7	3.17 (m)	H1, H3	
3	77.0	3.86 (m)	H2, H4a		81.0	3.67 (ddd, 3.2, 7.2, 9.7)	H2, H4a	
4a	33.7	1.56 (m)	H3		33.2	1.56 (m)	H3	
4b		1.70 (m)				1.70 (m)		
5–14	30.8–31.6	1.27–1.29 (bs)			30.8–31.6	1.27–1.29 (bs)		
15	29.2	1.30 (m)	H16		31.4	1.30 (m)	H16	
16	24.8	1.56 (m)	H17	C18, C15	25.5	1.56 (m)	H17	C18, C15
17	43.4	2.43 (t, 7.0)	H16	C15, C16, C18	44.1	2.43 (t, 7.0)	H16	C15, C16, C18
18	215.0				215.0			
19	43.5	2.42 (t, 7.0)	H20	C18, C20, C21	44.2	2.42 (t, 7.0)	H20	C18, C20, C21
20	24.0	1.51 (m)	H19	C18, C21	24.0	1.51 (m)	H19	C18, C21
21	29.2	1.28 (m)	H20		29.2	1.28 (m)	H20	
22–24	30.8–31.6	1.27–1.29			30.8–31.6	1.27–1.29		
25a					35.2	1.39 (m)	H25b	
25b						1.56 (m)	H25a	
25	30.8–31.6	1.27–1.29						
26a	34.5	1.39 (m)	H25b					
26b		1.56 (m)	H25a					
26	73.0	3.40 (m)	H25a, H25b, H227					
27	53.3	3.05 (m)	H26, H27	C26, C28	73.3	3.42 (m)	H26a, H26b, H28	
28	16.0	1.25 (d, 6.7)	H27	C26, C27	54.0	3.07 (m)	H29, H27	C29, C27
29					16.6	1.25 (d, 6.7)	H28	C28, C27
1′	104.6	4.32 (d, 7.2)	H2′	C3	104.6	4.32 (d, 7.2)	H2′	C3
2′	73.3	3.51 (dd, 7.2, 9.8)	H3′, H1′		73.3	3.51 (dd, 7.2, 9.8)	H3′, H1′	
3′	75.1	3.47 (dd, 3.4, 9.8)			75.1	3.47 (dd, 3.4, 9.8)		
4′	71.1	3.78 (d, 3.4)			71.1	3.78 (d, 3.4)		
5′	77.6	3.54 (dd, 4.6, 6.5)			77.6	3.54 (dd, 4.6, 6.5)		
6′	63.6	3.72 (m) 3.74 (m)			63.6	3.72 (m) 3.74 (m)		

Table 2. ^{1}H NMR Data for Peracetates **9**, **12**, **14**, and **16** ($CDCl_3$, TMS)

	14	**16**	**9**	**12**
atom no.	δ_H (mult, *J* Hz)	δ_H (mult, *J* Hz)	δ_H (mult, *J* Hz)	δ_H (mult, *J* Hz)
1			1.165 (d, 6.8)	1.165 (d, 6.8)
2			4.09 (m)	4.09 (m)
3			3.49 (dt, 2.7, 6.3)	3.49 (dt, 2.7, 6.3)
4a,b			1.42 (m	1.42 (m)
5–16			1.27–1.29 (bs)	1.27–1.29 (bs)
17			4.05 (t, 7.0)	2.30 (t, 7.0)
17-$COOCH_3$				3.70 (s)
19-$COOCH_3$	3.70 (s)			
19	2.29 (t, 7.0)	4.05 (t, 7.0)		
20–25	1.27–1.29 (bs)	1.27–1.29 (bs)		
26	4.85 (m)	4.85 (m)		
27	4.20 (m)	4.20 (m)		
28	1.101 (d, 6.8)	1.101 (d, 6.8)		
2-NHAc			5.89 (d, 9.0)	5.89 (d, 9.0)
27-NHAc	5.50 (d, 9.0)	5.50 (d, 9.0)		
1′			4.48 (d, 8.0)	4.48 (d, 8.0)
2′			5.16 (dd, 8.0, 10,6)	5.16 (dd, 8.0, 10,6)
3′			5.04 (dd, 3.3, 10.6)	5.04 (dd, 3.3, 10.6)
4′			5.39 (dd, 0.8, 3.3)	5.39 (dd, 0.8, 3.3)
5′			3.91 (dt, 0.8, 6.6)	3.91 (dt, 0.8, 6.6)
6′			4.10 (dd, 6.6, 11.3) 4.19 (dd, 6.6, 11.3)	4.10 (dd, 6.6, 11.3) 4.19 (dd, 6.6, 11.3)

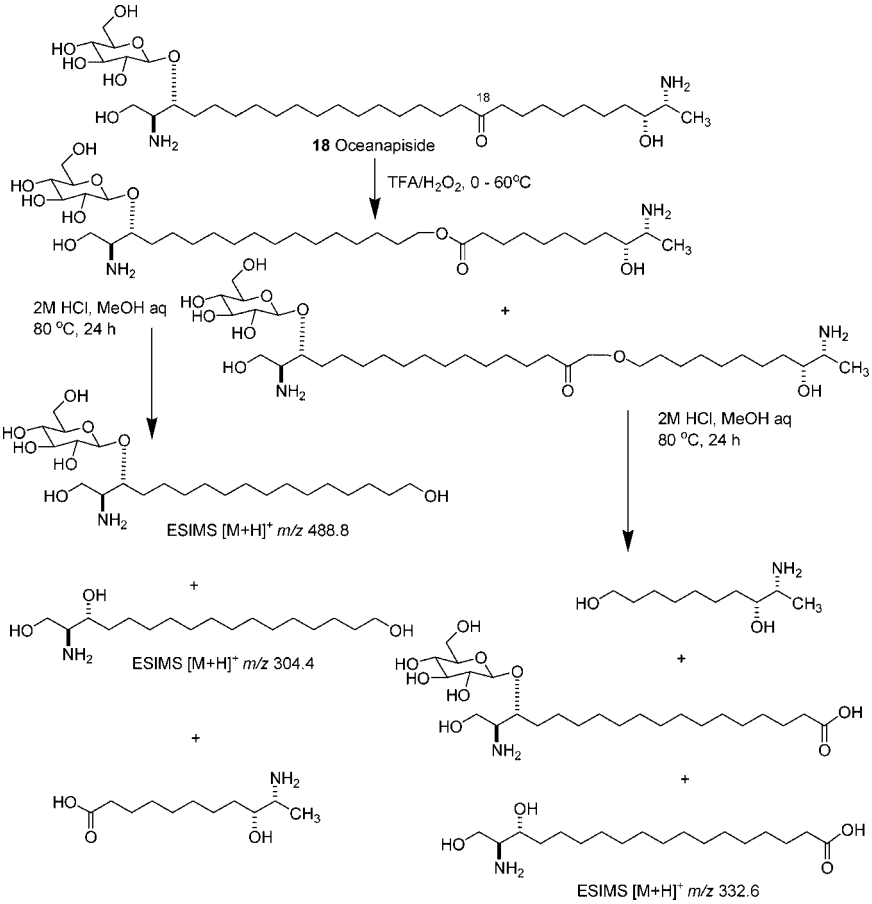
Scheme 1. Degradation of Rhizochalin C, Rhizochalin D, Rhizochalinin D, and Rhizochalin Peracetates (**1a**, **2a**,**c**, and **3a**) by Baeyer–Villiger Oxidation

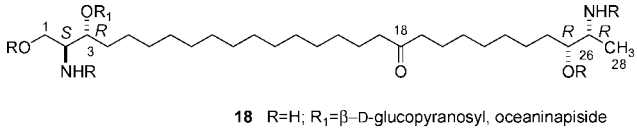


18 ($TFA-H_2O_2$) gave a mixture of esters, which was hydrolyzed and directly analyzed by ESIMS without separation. The *m*/*z* pattern of carboxylic acid−alcohol pairs (Scheme 2) was consistent only with a keto group at C-18, not C-11. The reassignment was confirmed by reductive amination of oceanapiside and analysis of EIMS fragmentation patterns as previously described,[5] and the results are entirely self-consistent. Thus, the revised structure of oceanapiside is depicted as shown.

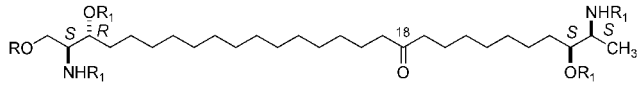
The absolute configurations of rhizochalins C and D were assigned from analysis of the ^{1}H NMR and CD spectra of the corresponding perbenzoyl aglycones following a procedure we developed specifically for rhizochalin (**3**) and oceanapiside (**18**)[12,13]

Scheme 2. Degradation of Oceanpiside (**18**) by Baeyer–Villiger Oxidation

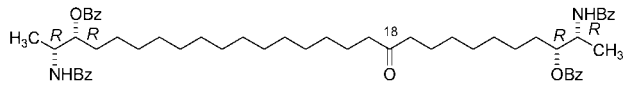




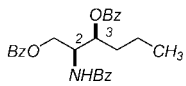
18 R=H; R_1=β–D-glucopyranosyl, oceaninapiside
18a R=R_1=Bz, perbenzoyl oceanin



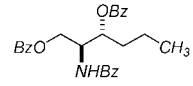
19 R=β–D-glucopyranosyl, calyxoside; R_1=H
19a R=R_1=Bz, perbenzoyl calyxinin



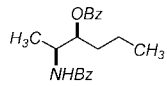
3b perbenzoyl rhizochalin



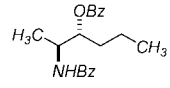
20a *threo-2S,3S*



20b *erythro-2S,3R*



20c *threo-2S,3S*



20d *erythro-2S,3R*

and applied, later, by Kingston and co-workers to calyxoside (**19**; here, for convenience we refer to the corresponding aglycone as "calyxinin").[5] By comparing CD spectra of the natural product-derived perbenzoyl aglycones with "hybrid CD spectra" generated from 16 linear combinations of all possible terminal group configurations,[12] a unique solution to the absolute configurations

Table 3. Partial ^{1}H NMR Data of the Compounds **18a**, **19a**, **20a**, Rhizochalinin C (**1c**), and Rhizochalinin D (**2d**) Perbenzoates

	chemical shifts (ppm)			*J*-values (Hz)		
α-terminus	$\delta_{C2\text{-}NH}$	$\delta_{3\text{-}H}$	$\delta_{2\text{-}H}$	$\delta_{1\text{-}H}$	$J_{NH\text{-}H\text{-}2}$	$J_{H\text{-}2\text{-}H\text{-}3}$
erythro-**18a**	7.08	5.38	4.88	4.61, 4.64	8.6	3.8
erythro-**19a**[a]	7.10	5.38	4.88	4.62, 4.66	8.7	4.1
threo-**20a**[b]	6.63	5.56	4.89	4.48, 4.57	9.2	4.8
threo-**1c**	6.63	5.54	4.89	4.48, 4.55	9.1	4.9
threo-**2d**	6.39	5.22	4.54	1.29	9.0	5.3

[a] See ref 5. [b] See ref 12.

Table 4. Partial ^{1}H NMR Data of the Compounds **3b**, **18a**, **19a**, Rhizochalinin C (**1c**), and Rhizochalinin D (**2d**) Perbenzoates

	chemical shifts (ppm)			*J*-values (Hz)		
α-terminus	$\delta_{C27\text{-}NH}$	$\delta_{26\text{-}H}$	$\delta_{27\text{-}H}$	$\delta_{28\text{-}H}$	$J_{NH\text{-}H\text{-}27}$	$J_{H\text{-}26\text{-}H\text{-}27}$
erythro-**18a**	6.38	5.21	4.53	1.28	8.9	5.2
erythro-**19a**	6.38	5.21	4.53	1.29	9.0	5.3
threo-**3b**	6.38	5.21	4.53	1.29	8.9	5.3
threo-**1c**	6.38	5.21	4.53	1.29	8.9	5.3
threo-**2d**	6.39	5.22	4.54	1.29	9.0	5.3

of all four stereocenters emerges. Perbenzoyl compounds **1c** and **2d** were prepared using a conventional procedure (benzoic acid, EDC, CH_2Cl_2), purified by HPLC, and their ^{1}H NMR spectra were compared with those of the perbenzoyl oceanin (**18a**), perbenzoyl calyxinin (**19a**), and **1c** as shown in Tables 3 and 4. The similarities of the chemical shifts and coupling constants for the α-terminus of rhizochalinin C perbenzoate (**1c**) with those of the *N*,*O*,*O*′-tribenzoyl model compound (**20a**)[12] indicated that rhizochalinin C perbenzoate (**1c**) and its natural product parent **1** share the *threo* configuration at C-2,C-3 of the α-terminus (Table 3). Rhizochalin C (**1**), with a rare *threo* configuration, differs in relative configuration from oceanapiside[12] and calyxoside,[5] which possess an

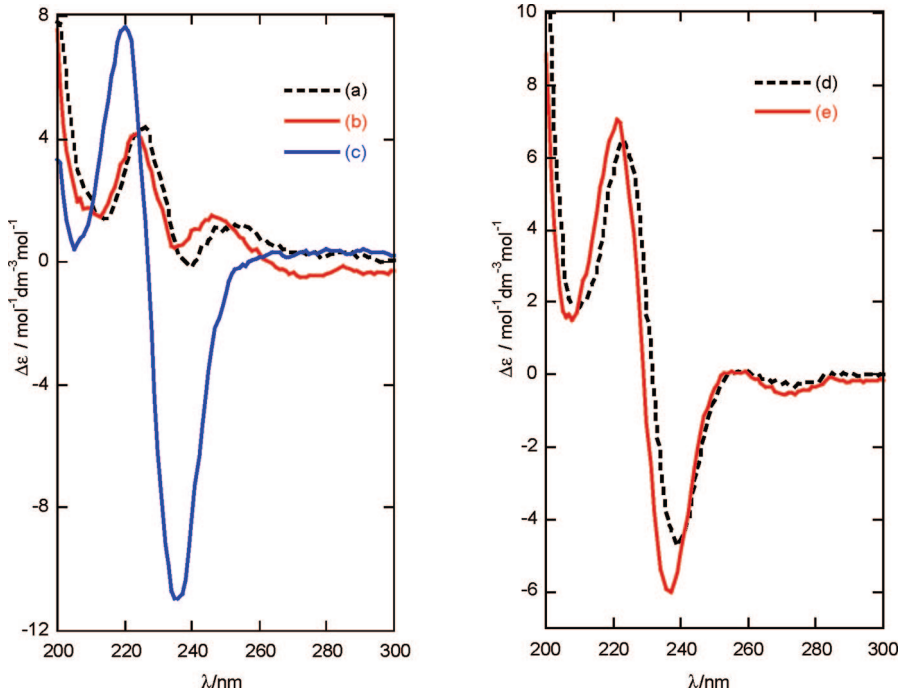


Figure 1. Measured CD and "hybrid CD" spectra (see ref 12) (MeOH, 25 °C, $c = 0.13-0.016$ mM): (a) perbenzoyl rhizochalinin C (**1c**); (b) hybrid CD of (2*R*,3*R*,26*R*,27*R*)- *ent*-**20a** + *ent*-**20c**; (c) hybrid CD of (2*S*,3*R*,26*R*,27*R*)-**20b** + *ent*-**20c**; (d) perbenzoyl rhizochalinin D (**2d**); (e) hybrid CD of (2*R*,3*R*,26*R*, 27*R*)- *ent*-**20c** + *ent*-**20c**.

erythro configuration at the α-termini, as found in "normal" sphingoid bases.[14] The ^{1}H NMR chemical shifts and coupling constants for α-and ω-termini of rhizochalinin D perbenzoate (**2d**) and rhizochalin perbenzoate (**3b**) and ω-termini of rhizochalinin C perbenzoate (**1c**), oceanin perbenzoate (**18a**), and calyxinin perbenzoate (**19a**) are identical (Tables 3 and 4). Rhizochalin D (**2**) thus has the same *threo* relative configuration at both the α-and ω-termini as rhizochalin (**3**),[13] and rhizochalin C (**1**) has the *threo* relative configuration at the ω-terminus shared by oceanapiside[12] and calyxoside. The foregoing ^{1}H NMR analysis does not distinguish *S,S* or *R,R* stereoisomers from their antipodes at either terminus; for this purpose we analyzed the CD spectra of the perbenzoyl compounds.

Measurement of the CD spectra of rhizochlinin C perbenzoate (**1c**) and comparison with "hybrid CD" spectra generated from linear combinations of CD spectra of model compounds **20a**−**d**, with all possible terminal group configurational combinations (Figure 1), clearly showed that **1c** bears the (2*R*,3*R*,26*R*,27*R*) configuration. The amplitude of the split Cotton effect in the CD spectrum of **1c** (Figure 1a) was considerably smaller in magnitude ($A = 4.5$) than the hybrid CD spectrum (Figure 1c), corresponding to perbenzoyl oceanin (**18a**, Figure 1c, $A = 18.6$), but matched perfectly in sign and magnitude the split Cotton effects of the (2*R*,3*R*,26*R*,27*R*) hybrid CD spectrum (Figure 1b, rhizochalin numbering), including the presence of a weak positive shoulder at λ 245 nm. This surprising finding reveals a rare example of a *threo* sphingolipid with inverted 2*R* configuration at C-2 that is ostensibly derived from C-2 of the amino acid L-serine.

The CD spectra of rhizochalinin D perbenzoate (**2d**) (Figure 1d) showed a CD spectrum that is essentially superimposible upon that of the (2*R*,3*R*,26*R*,27*R*)-pseudo-C_2 "hybrid CD" spectrum (Figure 1e). Thus, the configuration of the C_{29} carbon chain sphingolipids **2d** and **2** is each (2*R*,3*R*,27*R*,28*R*). It is worth noting that hybrid CD spectral comparisons,[10] based on deconvolution of CD Cotton effects by comparison of "virtual" CD spectra constructed from appropriate amino alcohol and amino diol models of known configuration, are accurate, sensitive (limits of detection ~nM), and responsive to changes in configuration at a *single* stereogenic center at either terminus of a dimeric sphingolipid.

Rhizochalin and oceanapiside exhibited antifungal activity against the fluconazole-resistant yeast *Candida glabrata* (ED_{50} ~40 μM).[3,4] However, rhizochalins C and D were not active ($ED_{50} > 150$ μM) under the same conditions.

Rhizochalin D is the first representative of two-headed sphingolipids from sponges containing an odd-numbered carbon chain. Reassignment of oceanapiside reveals that the position of the keto group is conserved in all known two-headed ketosphingolipids, including the new compounds **1** and **2**. It is possible that a unified biosynthetic pathway links these natural compounds to a common, as yet unidentified biosynthetic lipid intermediate; however the details of two-headed sphingolipid biosynthesis remain to be elucidated.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Perkin-Elmer 343 polarimeter and a Jasco DIP-370. CD spectra were recorded on a Jasco J800 spectrometer using quartz cells (0.2 mm path length) at a scan rate of 50 nm/s. The NMR spectra were recorded on Bruker DPX-400, DRX-500, and DRX-600 spectrometers at 400, 500, and 600 for ^{1}H and 100, 125, and 150 MHz for ^{13}C, respectively, with $(CH_3)_4Si$ as an internal standard. MALDI-TOF mass spectra were obtained on a Bruker Biflex III laser desorption spectrometer coupled with delayed extraction using N_2 laser (λ 337 nm) on 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CCA) as matrix. ESI mass spectra were obtained on an LCMS system comprising an MSQ Thermo Finnigan quadrupole spectrometer, coupled to an Agilent 1100 series HPLC, or by direct infusion in MeOH containing HCOOH (0.1%). FAB and EI mass spectra were obtained on an AMD-604S spectrometer (AMD-Intectra, Germany). HRFAB mass spectra were provided by the University of California, Riverside mass spectrometry facility.

Low-pressure column liquid chromatography was performed using Si gel L (50/160 μm, Sorbpolymer, Russia). Si gel plates (4.5 × 6.0 cm, 5–17 μm, Sorbfil, Russia) were used for thin-layer chromatography. Preparative HPLC for isolation and separation of sphingolipids was carried out using a Rainin binary HPLC system (Dynamax C_{18} column 10 × 250 mm, 5 μm, 3 mL/min) in 80:20:0.1% MeOH−H_2O−TFA coupled to a refractive index detector (Waters R401). Preparative HPLC separation of Baeyer–Villiger oxidation products was performed on a YMC Pack-ODS-A column (10 × 250 mm, 5 μm, 1.0 mL/min) in

80:20 EtOH−H_2O using an Agilent Series 1100 instrument equipped with the differential refractometer RID-DE14901810. Preparative HPLC separation of perbenzoate products was performed on the same instrument and column with slightly different solvent conditions (1.4 mL/min in 93% EtOH−H_2O).

Animal Material. The sponge *Rhizochalina incrustata* (phylum Porifera, class Demospongiae, subclass Ceratinomorpha, order Haplosclerida, family Phloeodictyidiae) was collected using scuba (depth 3–5 m) during the 3-day scientific cruise on board *R/V "Akademik Oparin"* (November 1986, Madagascar) and identified by Prof. V. M. Koltun (Zoological Institute, St-Petersburg, Russia). A voucher specimen is kept under registration number PIBOC #03-98 in the marine invertebrate collection of 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. The lyophilized material (18 g) was extracted with EtOH (200 mL × 3). The combined EtOH extract was concentrated under reduced pressure and redissolved in EtOH−H_2O (9:1). This solution was partitioned three times with equal volumes of *n*-hexane. The aqueous EtOH layer was evaporated *in vacuo* at 50 °C to give a brown oil, which was separated over Polichrome I (powder Teflon, Biolar, Latvia) by elution with gradient of H_2O → 1:1 H_2O−EtOH → EtOH. The two-headed sphingolipid fraction (ninhydrin positive) was eluted with 50% EtOH. The latter was further separated on a Si gel column (3:2:0.2, $CHCl_3$−EtOH−H_2O) to give a mixture of the known rhizochalin (**3**)[1] and new compounds **1** and **2**.

Preparative HPLC of the mixture (Dynamax C_{18} 80:20:0.1% MeOH−H_2O−TFA) gave **3** (20.0 mg, 0.12% based on dry weight of sponge) and rhizochalins C (**1**, 1.4 mg, 0.008% based on dry weight of sponge) and D (**2**, 1.9 mg, 0.011% based on dry weight of sponge).

Rhizochalin C (1): colorless solid; $[\alpha]^{25}_D$ −14.0 (*c* 0.093 MeOH); MALDI-TOF-MS *m/z* 671 $[M + Na]^+$; ESIMS *m/z* $[M + H]^+$ 649 (25%), $[M + H_2]^{2+}$ 325 (100%); HRFABMS *m/z* 649.5000 $[M + H]^+$ calcd, $C_{34}H_{68}N_2O_9$ 649.5003; NMR (CD_3OD, see Table 1).

Rhizochalin C Peracetate (1a). A sample of **1** (0.2 mg) was dissolved in a mixture of pyridine (50 μL) and Ac_2O (50 μL) and allowed to stand at 25 °C for 18 h. Removal of the volatile materials gave a residue (0.2 mg) of **1a**: MALDI-TOF-MS *m/z* 1007 $[M + Na]^+$; ESIMS *m/z* 1007 $[M + Na]^+$.

Baeyer–Villiger Oxidation of Rhizochalin C Peracetate (1a). A mixture of TFA (100%) and H_2O_2 (30%) [TFA−H_2O_2, 3:1 (60 μL)] was added to a solution of **1a** (0.2 mg) in MeOH (50 μL). The mixture was heated at 60 °C for 2 h, then left for 200 h at room temperature. The volatiles were removed under reduced pressure and the residue was acetylated with Ac_2O−pyridine (1:1, 100 μL). The resulting mixture of peracetates was analyzed by MALDI-TOF-MS: *m/z* 782 $[M + Na]^+$, *m/z* 338 $[M + Na]^+$.

Baeyer–Villiger Oxidation of Oceanapiside (18). A modification of the Bayer−Villager oxidation was applied to oceanapiside as follows. H_2O_2 (0.0965 g, 2.8 mmol, 30% aqueous solution) was added dropwise into TFA (0.294 g, 2.6 mmol) at 0 °C. The cold bath was removed and the solution of oceanapiside (3.0 mg) in MeOH (0.5 mL) was added dropwise. After addition, the mixture was refluxed for 2 h and then left stirring overnight at room temperature. $Na_2S_2O_4$ solution (aq) was added until the mixture turned basic (pH ∼8). The aqueous mixture was concentrated and extracted with MeOH to remove most of the salts. The crude product was redissolved in 2 M HCl in aqueous MeOH (obtained by adding 1.64 mL of 37% aqueous HCl into 8.36 mL of MeOH) and heated at 80 °C for 24 h. The reaction mixture was concentrated and redissolved in MeOH and analyzed by ESIMS. See Scheme 2.

Rhizochalinin C (1b). Rhizochalin C (0.5 mg) in 2 N HCl in MeOH (100 μL) was heated at 80 °C for 24 h in a sealed vial. Then the solution was cooled and concentrated under a stream of N_2. The residue was subjected to microcolumn Si gel chromatography with successive elution by (a) 1:4 MeOH−$CHCl_3$ and (b) 9:4:1 $CHCl_3$−MeOH−NH_4OH to provide the ninhydrin-positive product (**1b**, 0.5 mg): ^1H NMR (CD_3OD) 3.76 (1 H, dd, *J* = 4.0, 11.6, 1-H); 3.64 (1 H, dd, *J* = 6.7, 11.6, 1-H); 3.04 (1 H, m, 2-H); 3.66 (1 H, m, 3-H); 2.435 (2 H, t, *J* = 7.5, 17-H); 2.427 (2 H, t, *J* = 7.5, 19-H); 3.43 (1 H, m, 26-H); 3.08 (1 H, m, 27-H); 1.26 (3 H, d, *J* = 6.5, 28-H); ^{13}C NMR (CD_3OD) 59.7 (C-1); 61.1 (C-2); 69.8 (C-3); 44.1 (C-17); 44.0 (C-18); 73.7 (C-26); 54.1 (C-27); 16.6 (C-28); MALDI-TOF-MS *m/z* 487 $[M + H]^+$.

Rhizochalinin C Perbenzoatate (1c). A mixture of rhizochalinin C (0.5 mg, 1.0 μmol), DMAP (1 mg), benzoic acid (9.2 mg, 0.075 mmol), and EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, 12.2 mg, 0.067 mmol) in CH_2Cl_2 (1 mL) was stirred at room temperature for 6 days. The product was purified by Si gel column chromatography (20:1 $CHCl_3$−EtOAc) followed by preparative HPLC (93:7 EtOH−H_2O) to obtain rhizochalin C perbenzoate (**1d**, 0.3 mg): MALDI-TOF-MS *m/z* 1029 $[M + Na]^+$; ^1H NMR (see Tables 3 and 4).

Rhizochalin D (2): colorless solid; $[\alpha]^{25}_D$ −12.0 (*c* 0.10 MeOH); MALDI-TOF-MS *m/z* 669 [M + Na]; ESIMS *m/z* $[M + Na]^+$ 647 (17%), $[M + H_2]^{2+}$ 323 (100%); HRMSFAB *m/z* 647.5234 $[M + H]^+$ calcd, $C_{35}H_{71}N_2O_8$; 647.5210; NMR (CD_3OD, see Table 1).

Rhizochalin D Peracetate (2a). A sample of **2** (0.2 mg) was dissolved in a mixture of pyridine (50 μL) and Ac_2O (50 μL) and allowed to stand at 25 °C for 18 h. Removal of the volatile material under reduced pressure gave a residue of **2a** (0.2 mg): ^1H NMR ($CDCl_3$): 1.18 (3 H, d, *J* = 6.7, 3-H); 4.09 (1 H, m, 2-H); 3.49 (1 H, td, *J* = 2.2, 6.6, 3-H); 2.37 (2 H, t, *J* = 7.5, 17-H); 2.38 (2 H, t, *J* = 7.5, 19-H); 4.85 (1 H, m, 26-H); 4.20 (1 H, m, 27-H); 1.10 (3 H, d, *J* = 6.5, 28-H); 4.48 (1 H, d, *J* = 8.0, 1′-H); 5.16 (1 H, dd, *J* = 8.0, 10.6, 2′-H); 5.04 (1 H, dd, *J* = 3.3, 10.6, 3′-H); 5.39 (1 H, *J* = dd, 0.8, 3.3, 4′-H); 3.91 (1 H, *J* = dt, 0.8, 6.6, 5′-H); 4.10 (1-H, *J* = dd, 6.6, 11.3, 6′-H); 4.19 (1-H, *J* = dd, 6.6, 11.3, 6′-H); MALDI-TOF-MS *m/z* 963 $[M + Na]^+$.

Baeyer–Villiger Oxidation of Rhizochalin D Peracetate (2a). Compound **2a** (0.2 mg) was subjected to Baeyer–Villiger oxidation followed by acetylation under essentially the same conditions used for **1a**, and the mixture of peracetates was analyzed by MALDI-TOF-MS: *m/z* 724 $[M + Na]^+$; *m/z* 352 $[M + Na]^+$.

Rhizochalinin D (2b). Rhizochalin D (0.5 mg) was hydrolyzed in 2 N HCl in MeOH and separated, as described above for rhizochalin C, to provide the ninhydrin-positive product rhizochalinin D (**2b**, 0.5 mg): MALDI-TOF-MS *m/z* 485 $[M + H]^+$.

Rhizochalinin D Peracetate (2c). A sample of **2b** (0.2 mg) was acetylated and purified, as described above for rhizochalinin C peracetate, to give a residue of rhizochalinin D peracetate (**2c**, 0.2 mg): MALDI-TOF-MS *m/z* 675 $[M + H]^+$.

Baeyer–Villiger Oxidation of Rhizochalinin D Peracetate (2c). Compound **2c** (0.2 mg) was subjected to Baeyer–Villiger oxidation, followed by acetylation, as described above for **1c** to provide a mixture of peracetates that was analyzed by MALDI-TOF-MS: *m/z* 436, *m/z* 352.

Rhizochalinin D Perbenzoate (2d). Rhizochalinin D (**2**, 0.3 mg) was perbenzoylated according to the procedure described above for **1** and purified by preparative HPLC to give rhizochalin C perbenzoate (**1d**, 0.1 mg): MALDI-TOF-MS *m/z* 923 $[M + Na]^+$; ^1H NMR (see Tables 3 and 4).

Preparative Isolation of Baeyer–Villiger Oxidation Products of Rhizochalin Peracetate (3a). A mixture of TFA (100%) and H_2O_2 (30%) [TFA−H_2O_2, 3:1 (180 μL)] was added to a solution of rhizochalin peracetate (2.0 mg) in methanol (200 μL). The mixture was heated at 60 °C for 2 h and then left for about 200 h at room temperature. The volatiles were removed under reduced pressure, and the residue was acetylated with Ac_2O−pyr (1:1, 200 μL) to obtain a mixture of peracetates. Preparative separation of the mixture by HPLC (YMC-Pack ODS-A, 80:20 EtOH−H_2O) gave peracetates **9**, **12**, **14**, and **16**.

Peracetate 9: MALDI-TOF-MS *m/z* 724 $(M + Na^+)$; ^1H NMR ($CDCl_3$, see Table 2).

Peracetate 12: MALDI-TOF-MS *m/z* 724 $(M + Na^+)$; ^1H NMR ($CDCl_3$, see Table 2).

Peracetate 14: MALDI-TOF-MS *m/z* 338 $(M + Na^+)$; ^1H NMR ($CDCl_3$, see Table 2).

Peracetate 16: MALDI-TOF-MS *m/z* 338 $(M + Na^+)$; ^1H NMR ($CDCl_3$, see Table 2).

Acknowledgment. This investigation was supported by the Fogarty International Center and NIH (TWOO6301-01) and the National Institutes of Allergies and Infectious Diseases (AI039987, to T.F.M.). The research described here was made possible in part by Program of Presidium of RAS "Molecular and Cell Biology", Grant No. SS-6491.2006.4 from the President of RF. The authors kindly acknowledge R. Kondrat (UC Riverside) for HRFABMS measurements.

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

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NP0704811