Highly Cytotoxic Metabolites from the Culture Supernatant of the Temperate Dinoflagellate *Protoceratium* cf. *reticulatum*†

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In the course of our search for antitumor compounds in dinoflagellates, the culture broths of two strains of *Protoceratium* cf. *reticulatum* showed extremely potent cytotoxicity against human tumor cell lines. The four equally active principles, named protoceratins I (**1**), II (**2**), III (**3**), and IV (**4**), were purified and their structures were studied. The major principle, protoceratin I (**1**), proved to be identical with 2-homoyessotoxin, a well-known shellfish toxin. Protoceratins II (**2**), III (**3**), and IV (**4**) were determined to be di-, mono-, and triarabinoside of **1**, respectively. They are the first examples of glycosides of dinoflagellate polyethers.

Microalga have been shown to be a very rich source of novel bioactive metabolites including antitumor compounds.[1−8] As a part of a concerted effort to discover anticancer therapeutic agents in marine organisms,[9] the authors' group has been screening microalgae from temperate and tropical waters. The screening work involved the establishment of single-cell cultures, the extraction of the culture broths and cells, and evaluation of their bioactivity in the multiple assay systems. The efforts have been focused on the lower eukaryotes, protoctista, in particular dinoflagellates, that showed a remarkably high hit rate in the screening.[9] In this paper, we report the isolation and characterization of four active principles (named protoceratins) isolated from the two strains of *Protoceratium* cf. *reticulatum* (Gonyaulacaceae), which are rather ubiquitous oceanic dinoflagellates in temperate waters.

Results and Discussion

Of about 500 dinoflagellate strains examined, 10 isolates collected from the New England, Californian, and Japanese coasts possessed morphological features fitting the description of *P. reticulatum*. Two of them, URI strain numbers S1-83-6 and S2-191-1, passed the top oncological test criteria set in our program and were chosen for further study.

The two strains, which were isolated from the seawater collected by one of the authors (Y.S.) at different times at Sanriku, Iwate, Japan, were grown in enriched seawater medium, Guillard f/2[10] at 20 °C, and produced more or less the same pharmacological and chemical profiles. The activity was largely found in the culture medium rather than in the cells, which had been the primary source of active metabolites in previous studies of dinoflagellate. The active fraction was extracted from the culture medium using the nonionic resin Diaion HP-20 and separated into four highly cytotoxic compounds, which we called collectively protoceratins. The most abundant of them was protoceratin I (**1**), which was followed by protoceratin II (**2**) and protoceratins III (**3**) and IV (**4**). Chromatographic behavior indicated a polarity ranking of **1** < **3** < **2** < **4** from least polar to most polar. Later this series was shown to be due to the number of sugar moieties attached.

Protoceratin I (**1**) has a disodium salt molecular formula of $C_{56}H_{82}O_{21}S_2Na_2$ as stipulated from the mass spectrometry ($[M - Na]^-$, $C_{56}H_{82}O_{21}S_2Na$; $[M + Na]^+$, $C_{56}H_{82}O_{21}S_2Na_3$). Hydrolysis of **1** afforded 1.65 molar equiv of sulfate, indicating it is an *O*-disulfate salt. The high content of oxygen atoms and characteristic NMR spectra implied that it belongs to the class of linear all-*trans*-fused polycyclic ethers such as brevetoxins and ciguatoxins, which are uniquely known in dinoflagellates.[2,11−14] ^{13}C NMR and DEPT-135 spectra revealed 56 carbons, consisting of 6 methyls, 19 methylenes, 24 methines, and seven quaternary carbons. There are eight olefinic carbons, of which three are terminal methylene carbons. One terminal methylene is in conjugation with a disubstituted double bond (λ_{max} 231 nm). All these data strongly suggested that **1** is a homologous analogue of yessotoxin (**5**), $C_{55}H_{80}O_{21}S_2Na_2$, a shellfish toxin originally found in Japanese scallops (Figure 1).[15] The proton and carbon assignments and connectivities were established by $^1H-^1H$ COSY, HMQC, and HMBC (Tabe 1). The cross-peaks are in total agreement with the spin systems in yessotoxin (**5**). The location of the extra carbon or homologation was determined to be in the side chain part of the molecule by the clear connectivities from H-1 (4.01 ppm) to H-2 (1.83 ppm) and H-2a (1.65 and 1.8 ppm) seen in the COSY spectrum. As to the location of sulfate groups, they are located at 1-OH and 4-OH as in yessotoxin (**5**). The mass spectral fragmentation pattern also supported this conclusion (Figure 2). Subsequently, it was found that **1** is identical with 2-homoyessotoxin, a shellfish toxin reported in mussel and plankton samples of the Adriatic Sea.[16]

The other three more polar components, protoceratins II (**2**), III (**3**), and IV (**4**), are also equally potent cytotoxins. The structure elucidation work was mostly done with the most abundant **2**, and subsequently, the structures of **3** and **4** were deduced in comparison with the spectroscopic data of **2**.

The physicochemical properties and spectral data of protoceratin II (**2**) suggested its close resemblance to **1**, but the molecular formula of $C_{66}H_{98}O_{29}S_2Na_2$ assigned from FAB mass spectra ($[M - Na]^-$, $C_{66}H_{98}O_{29}S_2Na$; $[M + Na]^+$,

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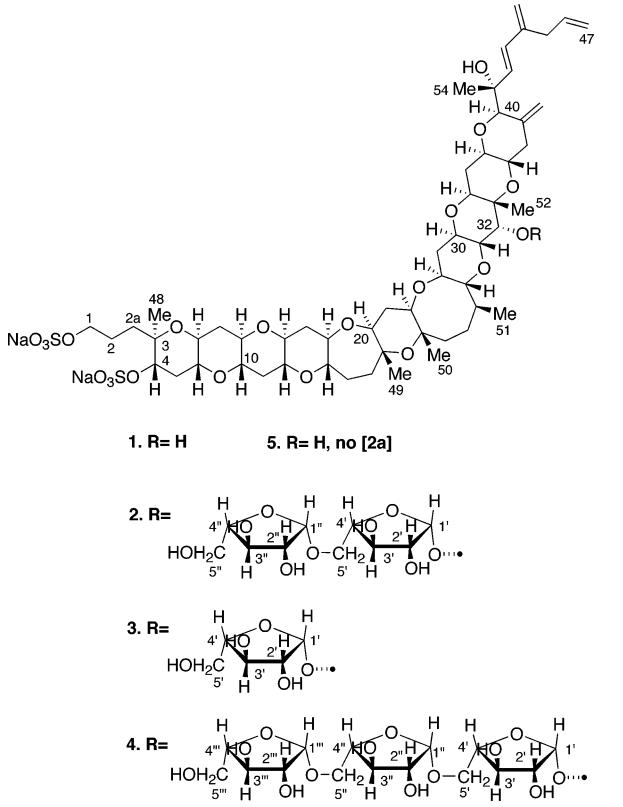

Figure 1. Structures of **1**, **2**, **3**, **4**, and **5**.

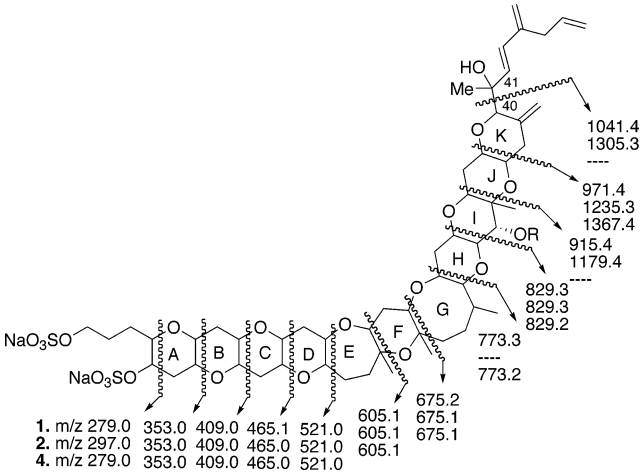

Figure 2. Negative ion FAB mass fragmentation patterns of **1**, **2**, and **4**.

$C_{66}H_{98}O_{29}S_2Na_3$) indicated the presence of an additional $C_{10}H_{16}O_8$ unit in the molecule. The ^{1}H NMR of **2** was compared with that of **1**, and with assistance of ^{1}H−^{1}HCOSY, HOHAHA, and HMBC, we were able to assign all signals and connectivities, as summarized in Table 1. The results support that **1** and **2** have the same base skeleton. The proton NMR spectrum of **2** was very similar to that of **1** except for the two anomeric proton signals at 5.42 ppm (d, J = 3.7 Hz) and 4.98 ppm (d, J = 4.2 Hz) and additional proton signals in the region of 3.0−4.3 ppm. These spectral data, the molecular formula, and the polarity of the compound strongly suggested that **2** is a glycoside of **1** with two pentose units. After acid hydrolysis, both pentose units were determined to be arabinose by gas chromatographic analysis.[17] The chirality of arabinose was assumed to be the L-form, as in the case of most natural products.

Theoretically, a great number of regioisomers are possible in attaching two sulfate and two sugar moieties to the eight hydroxyl groups available in the molecule. The FAB mass spectrum of **2** showed the characteristic fragment ions formed by cleavage of the ether rings reported for the polycyclic ethers.[18] As shown in Figure 2, the fragment ions of **1** and **2** are identical up to the cleavage of ring I at the C-30−C-31 bond, but, after that ring, **2** gave fragments 264 mass units higher than those from **1**. This indicates that two sugar units are linked to 32-O and/or 41-OH, and the two sulfate groups are in the same positions as in **1**. However, the fragment formed by the cleavage of the side chain at C-40−C-41 appeared still 264 mass units higher, suggesting the sugar moiety is linked to 32-O as a disaccharide. In fact, in the proton NMR spectrum of **2**, the H-32 signal was significantly deshielded due to the sugar linkage and appeared at 4.15 ppm, in comparison with that of **1** (3.84 ppm). Also, the shifts of H-30 (−0.17 ppm) and H-34 (−0.14 ppm) seen in the proton spectrum of **2** are considered due to the deshielding effect of the disaccharide moiety in 1,3-diaxial positions. This also confirms that the sugar-linked O-32 has the same position and relative stereochemistry as proposed for yessotoxin (**5**).[19] The ^{13}C NMR spectrum of **2** closely parallels that of **1**. Again, however, C-31, C-32, C-33, and C-34 were observed at significantly lower fields (0.6−1.7 ppm) due to the deshielding effect of the neighboring disaccharide unit.

As described earlier, the two anomeric protons observed at 5.42 and 4.97 ppm showed coupling constants of 3.7 and 4.2 Hz, respectively, which suggested β-furanoside structures for both arabinose units.[20] In the ^{13}C NMR, the anomeric carbons resonated at 103.1 and 104.1 ppm, respectively, which also supports the furanoside structures for both units.[21,22] The atom connectivities of the two arabinose units were fully assigned by ^{1}H−^{1}H COSY, HOHAHA, and HMBC. In the HMBC experiment, the anomeric proton, H-1′ (5.42 ppm), showed a cross-peak to C-32 (75.0 ppm), and conversely, H-32 (4.15 ppm) to C-1′ (103.1 ppm), confirming the linkage location of the sugar to the aglycon. The anomeric proton of the second sugar unit, H-1″ (4.98 ppm), showed a cross-peak to C-5′ (68.9 ppm), and conversely, C-1″ (104.1 ppm) to the two methylene protons, H-5′a and H-5′b (3.59 and 3.82 ppm). Also, it is very diagnostic that one of the methylene protons, H-5′b, is remarkably deshielded (3.82 ppm) as a result of sugar attachment. Similarly, C-5′ showed a significant lower field shift (68.9 ppm) due to the sugar attachment. Thus, the structure of **2** was established as 32-*O*-[β-L-arabinofuranosyl-(1→5)-β-L-arabinofuranosyl]protoceratin I, or 32-*O*-[β-L-arabinofuranosyl-(1→5)-β-L-arabinofuranosyl]2-homoyessotoxin.

The minor components, protoceratin III (**3**) and protoceratin IV (**4**), were determined to be mono- and triarabinosides of **1**, respectively, by comparing their NMR data with those of protoceratin II (**2**). Protoceratin III (**3**) was assigned a molecular formula $C_{61}H_{90}O_{25}S_2Na_2$ from the negative ion FAB data. Its ^{1}H NMR exhibited one anomeric proton at 5.4 ppm (d, J = 3.7 Hz) and five other protons in the region 3.6−4.1 ppm. The coupling patterns and chemical shifts corresponded well with those of β-arabinofuranoside (Table 1). The chemical shifts of both ^{1}H and ^{13}C NMR spectra of the skeleton part are very close to those of **2**, and the chemical shift of H-32 (4.18 ppm) indicated that the sugar is also linked to 32-OH.

The minor component protoceratin IV (**4**) was deduced to be a triarabinoside of **1** from its molecular formula, $C_{71}H_{106}O_{33}S_2$ Na_2. The negative ion FAB spectrum also

Table 1. ^{1}H and ^{13}C NMR Data for Protoceratins I (**1**), II (**2**), III (**3**), and IV (**4**)[a]

	1		**2**		**3**	**4**		**1**		**2**		**3**	**4**
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_H	position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_H
1	4.01	70.0	3.98	70.0	4.02	4.02	33		76.9		78.6		
			4.01			3.98	34	3.76	73.4	3.90	74.0	3.89	3.88
2	1.83	24.0	1.77	24.0	1.85	1.78	35	1.47	31.9	1.48	31.9	1.48	1.48
			1.85		1.98	1.85		2.08		2.13		2.12	2.12
2a	1.65	37.1	1.65	37.0	1.65	1.65	36	4.04	73.4	4.08	73.4	4.04	4.06
	1.80		1.82		1.80	1.80	37	3.39	73.1	3.37	73.1	3.38	3.36
3		77.2		77.2			38	2.43	39.1	2.41	39.3	2.41	2.42
4	4.23	77.8	4.24	77.8	4.24	4.24		2.71		2.67		2.66	2.67
5	1.70	32.9	1.71	33.2	1.70	1.70	39		143.3		143.2		
	2.56		2.58		2.58	2.58	40	3.86	85.3	3.85	85.2	3.86	3.85
6	3.03	78.6	3.03	78.8	3.04	3.03	41		78.6		78.6		
7	3.34	70.8	3.31	70.8	3.31	3.32	42	5.80	136.9	5.80	136.9	5.80	5.80
8	1.38	36.7	1.38	36.7	1.38	1.38	43	6.28	130.8	6.27	130.8	6.27	6.27
	2.17		2.16		2.16	2.17	44		145.6		145.6		
9	3.15	78.4	3.14	78.4	3.14	3.13	45	2.95	37.9	2.95	38.0	2.96	2.95
10	3.13	78.5	3.13	78.6	3.14	3.13	46	5.90	137.7	5.88	137.9	5.88	5.88
11	1.43	36.4	1.42	36.4	1.43	1.41	47	5.06	116.7	5.03	116.6	5.03	5.04
	2.26		2.27		2.26	2.26		5.09		5.09		5.09	5.09
12	3.04	77.8	3.04	77.8	3.04	3.04	48	1.20	16.9	1.21	16.9	1.21	1.21
13	3.04	78.2	3.05	78.3	3.04	3.04	49	1.25	24.1	1.25	24.1	1.25	1.25
14	1.41	38.1	1.41	38.2	1.51	1.41	50	1.15	20.9	1.15	20.8	1.15	1.15
	2.26		2.28		2.26	2.27	51	1.02	22.5	1.04	23.0	1.04	1.04
15	3.34	81.3	3.32	81.4	3.33	3.32	52	1.20	15.5	1.21	15.6	1.23	1.23
16	3.21	82.3	3.23	82.3	3.23	3.23	53	4.77	115.8	4.77	115.8	4.76	4.76
17	1.79	30.4	1.79	30.4	1.79	1.79		4.96		5.00		4.99	4.98
	1.93		1.95		1.96	1.96	54	1.38	26.3	1.38	26.3	1.38	1.38
18	1.81	41.2	1.79	41.3	1.79	1.80	55	5.04	116.7	4.96	116.6	4.97	4.99
	1.81		1.94		1.94	1.94		5.04		5.05		5.05	5.04
19		78.6		78.7			1′			5.42	103.1	5.44	5.42
20	3.39	82.6	3.40	82.7	3.39	3.40	2′			4.10	74.0	3.94	4.10
21	1.72	33.3	1.74	33.2	1.74	1.75	3′			3.94	73.3	3.86	3.95
	1.91		1.92		1.92	1.92	4′			4.26	86.1	4.14	4.27
22	3.48	87.5	3.48	87.7	3.47	3.48	5′a			3.59	68.9	3.58	3.61
23		77.2		77.1			5′b			3.82			3.84
24	1.47	47.1	1.48	47.1	1.47	1.47	1″			4.98	104.1		5.01
	1.75		1.75		1.80	1.78	2″			3.97	73.4		3.98
25	1.47	33.1	1.45	32.7	1.46	1.46	3″			3.95	71.2		4.05
	1.72		1.70		1.70	1.70	4″			4.01	87.1		4.15
26	1.69	41.0	1.68	40.7	1.68	1.69	5″a			3.62	63.4		3.63
27	2.81	89.6	2.81	89.7	2.82	2.81	5″b			3.66			3.86
28	3.31	84.2	3.28	84.2	3.28	3.30	1‴						4.99
29	1.53	40.1	1.51	40.1	1.52	1.52	2‴						4.11
	2.26		2.26		2.26	2.26	3‴						4.12
30	3.58	73.4	3.75	73.8	3.75	3.74	4‴						4.01
31	3.21	79.8	3.26	79.2	3.27	3.27	5‴a						3.62
32	3.84	74.0	4.15	75.0	4.18	4.14	5‴b						3.66

[a] ^{1}H NMR: 400 MHz in CD_3OD. ^{13}C NMR: 100 MHz in CD_3OD.

showed characteristic fragment ions of the cyclic ether ring structure (Figure 2), suggesting that the three sugar units are attached at 32-O as a trisaccharide. The assignment and connectivities of all three sugar protons were established by COSY experiments. The chemical shifts and coupling patterns were very close to those of **2** and **3**; especially, the lower field shifts of the 5b′-H and 5b″-H methylene protons (3.84 and 3.86 ppm, respectively) indicate that the two sugars are linked (1→5) in this compound as in **2**.

Dinoflagellates have been attracting attention mostly as a source of many conspicuous toxins found in marine environments, but they also have enormous potential as a source of therapeutic agents. In fact, our studies in the past 15 years showed an extremely high hit rate with dinoflagellates in anticancer screening assays.[9] Dinogflagellates take a very unique position in phylogeny, and equally they produce very unique metabolites with unique pharmacological activity. Regarding anticancer agents, previous work on dinoflagellates has been mostly focused on the macrolides produced by *Amphidinium* spp. from tropical waters and symbiotic invertebrate hosts.[4,8,23,24] In this study, however, we discovered very high cytotoxicity against human cancer cell lines with *Protoceratium* spp., which are rather common dinoflagellates found in temperate waters. There are a few more intriguing aspects worth mentioning. First, the active compounds turned out to be polycyclic ethers. The ladder-like all-*trans*-fused polycyclic ether structures were uniquely dinoflagellate metabolites. They were first recognized with brevetoxins,[11−14] the culprits of neurotoxic shellfish poisoning (NSP), and later with other important marine toxins such as maitotoxin and ciguatoxins.[2] They are well known as very specific neurotoxins that act on sodium and other ion channels, but this is the first time they have been recognized in the screening of anticancer agents. Yessotoxin, the basic skeleton of protoceratins, was first isolated by Yasumoto's group in Japan as a diarrhetic shellfish toxin from scallops,[15] but, in view of its characteristic polyether structure, its primary source had been long speculated to be a dinoflagellate; only recently the presence of yessotoxin was reported in New Zealand *Protoceratium reticulatum*.[25] It is also interesting that yessotoxin and 45,46,47-trinoryessotoxin were found in *P. reticulatum* collected at the Sanriku coast in Japan,[26] whereas our isolates produced neither of them. Homoyessotoxin, along with 45-hydroxyhomoyessotoxin, was also

found in toxic mussel and dinoflagellate samples of the Adriatic Sea.[16] It was quite coincidental that we also independently came across the compound in *Protoceratium* during our comprehensive screening of dinoflagellates for antitumor compounds. A number of polycyclic ether compounds have been isolated from the dinoflagellate genera, such as *Gymnodinium* (*Karenia*) and *Gambierdiscus*, but as far as we know, this is the first time the glycosidic forms have been discovered in dinoflagellates.[27]

Another interesting aspect is that the activity was mostly found in the culture medium and not in the cells. In the past, most studies of dinoflagellate metabolites were on the cell bodies, and the medium was neglected. This was partly due to the difficulty in dealing with small amounts of water-soluble organic compounds in an overwhelming volume of seawater medium. We now have evidence that the organisms actually release a large amount of the compounds into the medium. In fact, since this finding (in 1994), we have completely changed our screening strategy and put more emphasis on the culture medium portions. This change has resulted in a much higher hit rate and higher recovery of activity.

The pharmacology of protoceratins will be reported in separate reports, but all four protoceratins showed extremely high cytotoxicity, with mean IC_{50} values less than 0.0005 μM against human cancer cell lines, and demonstrated some cell-line selectivity. The mechanism of their extreme cytotoxicity has been a subject of our great interest. Although it still remains in question, according to the cell cycle analysis, they do not seem to be protein synthesis inhibitors as often as the cases of many other highly cytotoxic compounds including the dinoflagellate metabolites amphidinolide B and caribenolide 1.[23,24]

Experimental Section

General Experimental Procedures. The optical rotation measurement was done on a Rudolf AUTOPOL III automatic polarimeter, and UV spectra on a Milton Roy Spectronic 1201. The NMR spectra were taken on a Bruker Avance DPX-400. The mass spectrometry was done at the Mass Spectrometry Laboratory, University of Illinois at Urbana Champaign. Continuous centrifugation was done with a Sorvall RC-5B refrigerated superspeed centrifuge equipped with a titanium continuous rotor. Diaion HP-20 (Supelco) was soaked and washed with MeOH and deionized water before use. Normal-phase separation was carried out on silica gel (Baker) and reversed-phase separation on Sepralyte C8. A Whatman ODS-1 column (10 μm, 10 × 250 mm) and Adsorbosphere C18 (5 μm, 10 × 300 mm) were used for HPLC separation. All solvents used were of HPLC grade and glass-distilled for chromatography.

Organisms and Screening Procedure. All organisms used for screening were from clonal cultures, which were established by isolating single cells under a microscope. The single cells were first transferred to 0.5 mL of the enriched seawater medium, typically Gillard f medium f/4 or f/8.[10] The culture sizes were gradually scaled up to 2−15 L in Guillard f/2 medium before extraction. This process usually takes 6 months or longer. Normally, the culture was maintained at 15−25 °C under white fluorescent lights (ca. 45 μE m^{-2} s^{-1}) and harvested by continuous centrifugation after 40 days and 80 days of culture. Both 40-day and 80-day cultures produced almost identical components, but 80-day culture produced a higher amount of metabolites. The supernatant was passed through Diaion HP-20. The methanol extract of the Diaion HP-20 and extracts of the harvested cells with *n*-hexane, CH_2Cl_2, and MeOH were submitted for the primary screening assays. Only those organisms that passed the initial oncological evaluation criteria set in the program[9] were further pursued.

The organisms were isolated from various environments or habitats by different techniques, but all the *Protoceratium* isolates screened in this study were from 10 μm plankton net concentrates of seawaters. They all matched the morphological characteristics of *P. reticulatum*,[28] but they differed significantly in pharmacological and chemical profiles. Strain S1-83-6 was first isolated in 1992, and S2-191-1 from a plankton net collection from Sanriku, Iwate, Japan, by Y.S. in August 1997. Both strains produced almost identical metabolites in culture and seemed to be identical. All active strains are maintained at the culture collection at the University of Rhode Island.

Isolation of Protoceratin I (1). The cells and supernatant of the 42-day-old culture of *Protoceratium* cf. *reticulatum* S2-191-1 were separated by continuous centrifugation. The supernatant was passed through a Diaion HP-20 column (300 g), and the column was washed with deionized water (1 L) and then with MeOH (2 L). The MeOH extract was concentrated to dryness and chromatographed on a C18 silica gel column (20 × 200 mm) with 60−80% MeOH−H_2O. The 80% MeOH eluate (58 mg) was further purified by HPLC (ODS-1 10 μm, 10 × 250 mm) with 50% MeOH, monitoring at 230 nm, to give a pure active compound, protoceratin I (30 mg).

Protoceratin I (=2-homoyessotoxin) (1): colorless solid; $[\alpha]^{20}_D$ −5.5° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 231 nm (4.29); NMR (see Table 1); negative ion FABMS *m/z* 1177.50 (calcd for $C_{56}H_{82}O_{21}S_2Na$, 1177.47); HRFABMS (very small molecular ions) *m/z* 1223.4480 (calcd for $C_{56}H_{82}O_{21}S_2Na_3$, 1223.4483).

Isolation of Protoceratins II, III, and IV. The 80-day-old culture of S1-83-6 (280 L from 23 carboys) was centrifuged, and the supernatant was passed through Diaion HP-20 (1.2 L volume). The resin was washed with 6 L of deionized water and then eluted with MeOH (8 L). The MeOH eluate was concentrated to dryness, and the residue was reextracted with MeOH. The MeOH extract was chromatographed on a silica gel column (Baker, 17 × 380 mm). Elution was done with CH_2Cl_2−MeOH by stepwise increase of MeOH from 99:1 to 50:50. Protoceratin I (**1**) was first eluted with CH_2Cl_2−MeOH, 90:10−85:15, to give semipure protoceratin I (**1**) (281.7 mg). The subsequent fractions gave a mixture of more polar protoceratins (148.4 mg), which were further separated by HPLC (Sepralyte C8, 1.5 × 20.0 cm). The column was developed with 50%, 60%, and 70% MeOH sequentially. Protoceratin IV, II, and III fractions were eluted with 60% MeOH in that order, and some protoceratin I with 70% MeOH. The semipure fractions of protoceratins IV (4.1 mg), II (14.0 mg), and III (4.3 mg) were further purified by HPLC (Adsorbophere C18, 5 mm, 10 × 300 mm, solvent 62% MeOH−38% 0.01 mM pH 7.05 phosphate buffer, UV detection at 220 nm). Those fractions that contained only pure compounds were combined and desalted on C18 cartridges (Waters Sep Pak) with H_2O−85% MeOH to give pure protoceratin II (**2**) (4.7 mg) and two minor components, protoceratin III (**3**) (1.3 mg) and protoceratin IV (**4**) (1.0 mg). Compounds **2**, **3**, and **4** are all amorphous solids, readily soluble in MeOH, DMSO, and DMF and slightly soluble in H_2O, CH_2Cl_2, and $CHCl_3$. The R_f values of **1**, **2**, **3**, and **4** on TLC (silica gel, $CHCl_3$−MeOH−H_2O, 65:35:10, lower phase) were 0.40, 0.34, 0.38, and 0.32, respectively. In reversed-phase HPLC (Adsorbosphere C18 5 μm, 10 × 300 mm, 62% MeOH−38% 0.01 mM phosphate buffer pH 7.05, 2 mL/min, UV 220 nm detection), **1**, **2**, **3**, and **4** were eluted at retention times of 46.0, 38.1, 29.9, and 24.4 min, respectively.

Protoceratin II (2): $[\alpha]^{20}_D$ +12.1° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 nm (4.24); NMR (Table 1); negative ion FABMS *m/z* 1441.6 $[M - Na]^-$ (calcd for $C_{66}H_{98}O_{29}S_2Na$, 1441.55); HRFABMS *m/z* 1487.5332 $[M + Na]^+$ (calcd for $C_{66}H_{98}O_{29}S_2Na_3$, 1487.5328).

Protoceratin III (3): NMR (Table 1); negative ion FABMS *m/z* 1309.5 (calcd for $C_{61}H_{90}O_{25}S_2Na$, 1309.51).

Protoceratin IV (4): NMR (Table 1); negative ion FAB *m/z* 1573.8 (calcd for $C_{71}H_{106}O_{33}S_2Na$, 1573.60). In positive ion FABMS, both **3** and **4** did not give molecular ions suitable for peak matching.

Hydrolysis of Protoceratin I (1) and Quantitation of the Sulfate Group. Protoceratin I (**1**) (2.03 mg, 1.69 μmol) was hydrolyzed in a mixture of dioxane (0.5 mL) and 6 N HCl (0.4 mL) at 100 °C for 14 h. After removal of insoluble material by filtration, the solution was concentrated and lyophilized. The residue was dissolved in water and passed through a C18 cartridge (Waters Sep Pak). The passed solution was lyophilized again, and the residue was dissolved in distilled water (0.338 mL) and analyzed by HPLC under the following conditions: column Intersil C8, 5 μm, i.d. 4.0 × 150 mm; solvent phthalic acid (1.0 mM)−tetrabutylammonium hydroxide (1.0 mM) buffer adjusted to pH 4.60 by tetrabutylammonium hydroxide; flow rate 1 mL/min; UV detection at 295 nm; calibration standard 0.05−0.20 μM Na_2SO_4.

Typically, 10 μL of the test solution was injected, and the results were calibrated by the standard solutions. The amount of sulfate was calculated to be 0.0825 μmol or 1.65 molar equiv per molecule.

Determination of the Sugar Moiety of Protoceratin II (2). The analysis was done by the method reported by Crowell and Burnett[17] and modified for micromanipulation. A solution of **2** (1.1 mg) in 2 N HCl was heated in a reaction vial at 100 °C for 2 h. The mixture was lyophilized and redissolved in 500 μL of distilled water. The soluble part was lyophilized and added with water, and this process was repeated twice to remove HCl. The mixture was then treated with a solution of 10 mg of $NaBH_4$ in 100 μL of H_2O for 3 h at room temperature. After quenching the solution with acetic acid, the mixture was dried with N_2 at 60 °C and then in vacuo. The dried residue was dissolved in a 1:1 mixture of freshly distilled pyridine and acetic anhydride (200 μL) and heated at 120 °C for 18 h. The solution was dried with N_2 and then in vacuo. The residue was extracted with ethyl acetate (200 μL) and analyzed by GLC (Varian 1400; carrier gas N_2; column OV-17 1/8 in., 5 ft; temperature 180 °C). The sample gave a single peak (retention time 12.5 min), which was identical with that of a sample prepared from authentic arabinose. Coinjection also gave a single peak. A comparison sample prepared from xylose gave a retention time of 14.5 min, and the yield of arabinose calculated from the GC peak heights was about 60%.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, DEPT-135, ^{1}H−^{1}H COSY, and HMQC spectra of **1**; ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, and HMQC spectra of **2**; ^{1}H NMR and ^{1}H−^{1}H COSY spectra of **3**, and ^{1}H NMR and ^{1}H−^{1}H COSY spectra of **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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