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CURACINS B AND C, NEW ANTIMITOTIC NATURAL PRODUCTS FROM THE MARINE CYANOBACTERIUM *LYNGBYA MAJUSCULA*

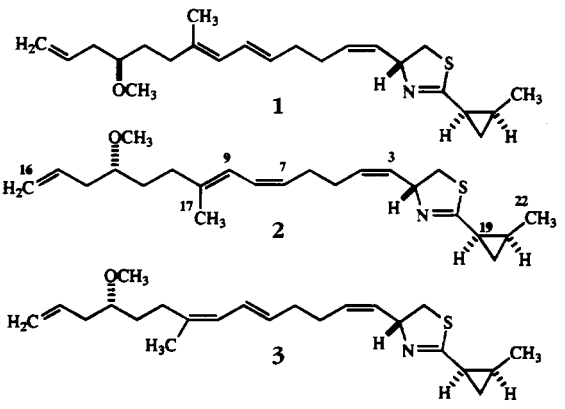
HYE-DONG YOO and WILLIAM H. GERWICK*

College of Pharmacy, Oregon State University, Corvallis, Oregon 97331

ABSTRACT.—Two new and potent antimitotic metabolites, curacins B [**2**] and C [**3**], were isolated from a Curaçao collection of *Lyngbya majuscula*. The structures of **2** and **3**, geometrical isomers of the known compound curacin A [**1**], were determined by detailed spectroscopic analysis in comparison with **1**. The absolute stereochemical configuration of **2** was deduced to be 2*R*,13*R*,19*R*,21*S* by its thermally induced interconversion with **1**.

Different isolates of the marine cyanobacterium *Lyngbya majuscula* (Oscillatoriaceae) produce an amazingly wide array of biologically active secondary metabolites, reminiscent of the rich biosynthetic capacity of some species of *Streptomyces*. The potent tumor promoters lyngbyatoxin and debromoaplysiatoxin (1,2) and the antifungal lipopeptides majusculamides A, B, and C (3,4) are examples of the range of metabolites isolated from this organism. Recently, our examination of a Curaçao (Caribbean) collection of *Lyngbya majuscula* has resulted in the isolation of an extremely potent antimitotic agent, curacin A [**1**] (5,6), which is under examination for its potential anticancer utility (7,8). In continuing investigations of this organism for minor metabolites of related structure, we have isolated and characterized two new natural products, curacins B [**2**] and C [**3**]. Both of these are toxic to brine shrimp, demonstrate strong cytotoxicity against murine L-1210 leukemia and human CA46 Burkitt lymphoma cell lines (7,8), inhibit the polymerization of purified tubulin in vitro, and in the NCI in vitro 60-cell line assay, show potent antiproliferative activity to many cancer-derived cell lines in a manner characteristic of antimitotic agents.

Isopropanol-preserved *Lyngbya majuscula* collected from Curaçao was extracted ($CHCl_3$-MeOH, 2:1) and chromatographed over Si gel to give a mixture of **1–3** contaminated by fatty acids and phytol. These contaminants were removed by methylation (CH_2N_2) and acetylation (Ac_2O/pyridine) of the curacin-containing fraction followed by normal-phase hplc to yield two peaks. The first peak was curacin A [**1**] (10.3% of extract), while the second was a mixture of the two new curacins by ^{1}H-nmr and gc-eims analysis. Repeated reversed-



phase hplc (C_8) of this latter fraction eventually gave pure curacins B [**2**] and C [**3**] as 4.7% and 0.3% of the extract, respectively.

Compounds **2** and **3**, both analyzing for $C_{23}H_{35}NOS$ by hrfabms, displayed ^{1}H- and ^{13}C-nmr bands indicative of four double bonds and one C=X functionality. Therefore, in consideration of the molecular formula, both contained two rings, similar to **1**. Comparison of the ^{1}H- and ^{13}C-nmr features of **2** and **3** with those of **1** revealed that all three metabolites were closely related (Table 1). The only nmr regions of significant difference between them were in those assigned to the C-7 to C-10 diene and the associated C-17 methyl and C-11 methylene groups. Hence, it was concluded that **2** and **3** represented geometrical isomers of **1** in this region of the molecule.

In our prior work on **1** the geometry of the C-7 to C-10 diene was established as 7(*E*),9(*E*) on the basis of a 15.0 Hz coupling between H-7 and H-8 and a strong nOe interaction between the C-17 methyl group and H-8 (see Table 1). Correspondingly, in **2** a *Z* geometry for the C-7 olefin was deduced by observing a 9.4 Hz coupling between H-7 and H-8. That **2** had the same stereochemistry as **1** for the C-9 olefin was shown by the highly comparable nature of the carbon shifts for C-11 and C-17 (Table 1), and by observing a nOe between the C-17 methyl and H-8. The combination of a 15.0 Hz coupling between H-7 and H-8 in **3** with a 7-ppm downfield shift for C-17 and a 7.5 ppm upfield shift for C-11 relative to **1** (Table 1) clearly indicated **3** to be the 7(*E*),9(*Z*) isomer.

The absolute configuration of **2** was defined through thermal interconversion with **1**, the absolute configuration of which we recently defined as 2*R*,13*R*,19*R*,21*S* (6). Refluxing a 95:5 mixture of **2** and **3** in 2,2,4-trimethylpentane (99°) for 3 h yielded a 2:1:1 mixture of all three curacins [**1–3**] by gc-eims. The **1** produced in this experiment was purified by hplc and had identical spectral properties to authentic **1** (^{1}H-nmr, gc-eims, uv) and a comparable optical rotation ($[\alpha]^{25}D$ +77°, c=0.1, $CHCl_3$; lit. $[\alpha]^{25}D$ +86°, c=0.64, $CHCl_3$) (5). Therefore, **2** possesses the same 2*R*,13*R*,19*R*,21*S* stereoconfiguration as **1**. Additionally, because hplc-purified **2** and **3** had comparable optical rotations (see Experimental), it is probable that **3** also possesses 2*R*,13*R*, 19*R*,21*S* absolute stereochemistry.

Three experiments were conducted to confirm that **2** and **3** are true natural products of this cyanobacterium and not extraction/isolation artifacts. First, a cold extraction of the preserved cyanobacterium was shown by gc-ms to contain the same proportions of **1**, **2**, and **3** as obtained from the above warm extraction. Second, direct heating of pure **1** in CH_2Cl_2-MeOH (2:1) at 35° for 10 h did not produce detectable quantities of **2** or **3**. And third, pure **1** refluxed in aqueous CH_2Cl_2-MeOH (2:1) for 90 min also did not give **2** or **3**. It should be noted that in these latter two experiments, **1** was heated at a temperature lower than that shown to effect isomerization of the **2**/**3** mixture into **1** (refluxing 2,2,4-trimethylpentane, 99°, see preceding paragraph).

In the National Cancer Institute in vitro 60 cell line screen, a 95:5 mixture of **2** and **3** showed a profile of activity similar to that of **1** as well as to other agents working by an antimitotic mechanism (Table 2). Using a purified tubulin preparation, this same mixture of **2** and **3** was only slightly less active (IC_{50} value of 1.6 μM) than **1** (IC_{50} value of 1.4 μM) at inhibiting polymerization (8). Since a 1:1 mixture of **2** and **3** had nearly the same IC_{50} value (1.7 μM) as the 95:5 mixture in the inhibition of tubulin polymerization assay, **2** and **3** must be of nearly the same potency. This same relationship was observed in the relative ability of these curacin derivatives to inhibit the binding of radiolabeled colchicine to purified tubulin (pure **1** inhibited 96%

TABLE 1. ^{1}H- and ^{13}C-Nmr Data for **1–3**.

Position	**1**		**2**		**3**	
	^{1}H (J in Hz)[a]	^{13}C[b]	^{1}H (J in Hz)[a,c]	^{13}C[b]	^{1}H (J in Hz)[a]	^{13}C[b]
1a	2.79 (dd, 10.7, 10.0)	39.96	2.79 (dd, 10.7, 10.0)	39.99	2.80 (dd, 10.5, 10.1)	39.94
1b	3.09 (dd, 10.7, 10.0)	—	3.09 (dd, 10.7, 8.4)	—	3.09 (dd, 10.5, 8.6)	—
2	5.11 (m)	74.31	5.12 (m)	74.33	5.11 (m)	74.31
3	5.69 (dd, 10.7, 8.7)	131.32	5.69 (ddt, 10.5, 8.9, 1.2)	131.40	5.69 (dd, 10.5, 8.9)	131.30
4	5.45 (br dt, 10.7, 7.1)	130.84	5.45 (br ddd, 10.5, 7.2, 1.2)	130.91	5.45 (m)	130.85
5	2.11 (m)	28.14	2.11 (m)	28.15	2.11 (m)	28.16
6	2.11 (m)	33.12	2.26 (m)	27.79	2.11 (m)	33.12
7	5.58 (br dt, 15.0 7.0)	131.32	5.40 (dt, 9.4, 7.4)	128.83	5.57 (br dt, 15.0, 6.6)	131.30
8	6.38 (dd, 15.0, 10.8)	127.86	6.35 (m)	125.84	6.51 (dd, 15.0, 10.8)	127.67
9	6.02 (d, 10.8)	125.50	6.35 (m)	120.74	5.97 (d, 10.8)	126.42
10	—	136.40	—	138.73	—	136.62
11	2.23 (m)	35.77	2.23 (m)	36.26	2.38 (m)	28.16
12	1.70 (m)	32.15	1.69 (m)	32.26	1.62 (m)	32.16
13	3.09 (m)	79.91	3.09 (m)	79.95	3.09 (m)	79.70
14	2.26 (m)	38.02	2.26 (m)	38.06	2.24 (m)	37.85
15	5.87 (m)	135.32	5.87 (m)	135.32	5.85 (m)	135.23
16	5.07 (m)	116.80	5.07 (m)	116.84	5.07 (m)	116.84
17	1.70 (s)	16.56	1.69 (s)	16.39	1.74 (s)	23.65
18	—	168.36	—	168.42	—	169.34
19	1.65 (m)	19.87	1.65 (m)	20.13	1.70 (m)	20.11
20a	0.75 (td, 8.2, 4.2)	13.82	0.75 (td, 8.1, 4.4)	14.24	0.75 (td, 8.1, 4.1)	14.21
20b	1.20 (m)	—	1.21 (m)	—	1.23 (m)	—
21	0.98 (m)	15.98	0.99 (m)	15.99	0.98 (m)	15.98
22	1.21 (d, 6.3)	12.39	1.22 (d, 6.3)	12.35	1.22 (d, 6.1)	12.31
23 (OMe)	3.18 (s)	56.31	3.19 (s)	56.37	3.20 (s)	56.23

[a]300 MHz in C_6D_6.
[b]75 MHz in C_6D_6.
[c]J values measured from 400 MHz ^{1}H-nmr spectrum in C_6D_6.

TABLE 2. In Vitro Tumor Cell Growth Inhibition and Cytotoxicity of a Mixture (95:5) of **2** and **3**.[a]

Panel/cell line	Log_{10} GTI_{50}[b]	Log_{10} TGI[b]	Log_{10} LC_{50}[b]
Non-small cell lung cancer			
NCI-H226	−7.71	−7.26	>−4.00
NCI-H460	−7.56	−7.04	−4.06
NCI-H522	<−8.00	−7.68	−7.17
Colon cancer			
COLO 205	−7.77	−7.49	−7.20
HCC-2998	−7.63	−7.25	−4.16
HCT-116	−7.85	−7.15	>−4.00
HCT-15	<−8.00	−7.52	−5.58
HT29	−7.82	−7.43	—
KM12	−7.88	−7.46	—
Melanoma			
M14	−7.56	−6.89	>−4.00
SK-MEL-2	−7.21	−6.41	>−4.00
Ovarian cancer			
OVCAR-3	−7.74	−7.42	—
SK-OV-3	−7.53	−7.07	>−4.00
Prostate cancer			
DU-145	−7.74	−7.45	—
Breast cancer			
MCF7/ADR-RES	<−8.0	−7.58	—
MDA-MB-231/ATCC	−7.75	−7.27	>−4.0
MDA-N	<−8.00	−7.61	—

[a]Data obtained from the NCI 60 cell line in vitro screen [see Boyd (9) for details].

[b]GI_{50}, concentration required for 50% growth inhibition; TGI, concentration required for total growth inhibition; LC_{50}, concentration required for 50% lethality.

while a 95:5 mixture of **2** and **3** inhibited 65% and a 1:1 mixture of **2** and **3** inhibited 58%, all at 5.0 μM) (8). In the brine shrimp assay, **2** (LD_{50} value of 38 nM) and **3** (LD_{50} value of 54 nM) were only slightly less potent than **1** (LD_{50} value of 8 nM) (5). Based on these data, it is apparent that geometrical isomerization of the C-7 and C-9 olefins in **1** has relatively little effect on the ability of these compounds to inhibit tubulin polymerization, inhibit cancer cell proliferation, or exhibit toxicity to brine shrimp.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded on Bruker AM 400 and ACP 300 spectrometers in the solvent specified. Chemical shifts were referenced to solvent (C_6D_6) signal at 7.2 ppm for ^1H and 128 ppm for ^{13}C. Mass spectra were recorded on Kratos MS 50 TC and Finnigan 4023 mass spectrometers. Gc/ms was carried out utilizing a Hewlett-Packard 5890 Series II gc connected to a Hewlett-Packard 5971 mass spectrometer. Uv spectra were obtained on a Hewlett-Packard 8452 A uv-vis spectrophotometer and ir spectra on a Nicolet 510 spectrophotometer. All solvents were distilled from glass prior to use.

PLANT MATERIAL.—*Lyngbya majuscula* was collected in December 1991 from Carmabi beach, Curaçao, and preserved in *i*-PrOH at low temperature until workup. A voucher specimen is available from W.H.G. (collection NAC-13Dec91-1).

EXTRACTION AND ISOLATION.—*i*-PrOH preserved *Lyngbya majuscula* was extracted with $CHCl_3$-MeOH (2:1). Gradient vacuum chromatography of the crude extract (543 mg) with EtOAc/hexanes gave a mixture of curacins contaminated by fatty acids and phytol. This was treated first with excess ethereal CH_2N_2 for 5 min, the solvents removed *in vacuo*, and then treated with 1 ml of Ac_2O-pyridine (1:1) for 24 h. *In vacuo* removal of excess reagents and solvents was followed by repetitive hplc on a Phenomenex Si column (10 μm, 500×10 min) with 4% EtOAc/hexanes, yielding curacin A (**1**, 44.2 mg) and a mixture of curacins B [**2**] and C [**3**] (29 mg). The latter fraction was subjected to further hplc on a Phenomenex phenosphere C_8 column (10 μm, 250×4.6 mm) with 64% CH_3CN/H_2O to afford curacins B (20 mg, 4.7%) and C (1.1 mg, 0.3%).

Curacin B [**2**].—Pure curacin B showed the following data: $[\alpha]^{25}D$ +62° (*c*=0.84, $CHCl_3$); uv

λ max (MeOH) 242 (ε 22 000) nm; ir ν max (film) 2975, 2930, 2874, 1615, 1441, 1379, 1093, 1075, 1056, 998, 967, 914 cm^{-1}; lreims *m/z* 373 (3), 342 (9), 332 (13), 274 (8), 181 (28), 180 (100), 166 (16), 140 (14), 99 (17), 91 (27), 85 (20), 79 (31), 67 (17), 55 (15); hrfabms (positive-ion) $[M+H]^+$ *m/z* 374.2526 (0.9 mmu error for $C_{23}H_{36}ONS$); ^1H- and ^{13}C-nmr data, see Table 1.

Curacin C [**3**].—Pure curacin C showed the following data: $[\alpha]^{25}D$ +56° (*c*=0.15, $CHCl_3$); uv λ max (MeOH) 242 (ε 25 000) nm; ir ν max (film) 2970, 2927, 2885, 1617, 1438, 1095, 1074, 1054, 998, 964, 914 cm^{-1}; lreims *m/z* 373 (4), 342 (9), 332 (12), 274 (8), 181 (29), 180 (100), 166 (16), 140 (14), 99 (17), 91 (26), 85 (25), 79 (30), 67 (18), 55 (16); hrfabms (positive-ion) $[M+H]^+$ *m/z* 374.2532 (1.5 mmu error for $C_{23}H_{36}ONS$); ^1H- and ^{13}C- nmr data, see Table 1.

THERMAL ISOMERIZATION OF **2** TO **1** AND **3**.—To 6 mg of a 95:5 mixture of curacins B and C was added 2.5 ml of trimethylpentane and a boiling stone at room temperature. The reaction mixture was refluxed for 3 h at which time the solvent was concentrated *in vacuo*. The concentrate was diluted with 4% EtOAc/hexanes, filtered through sintered glass, and subjected to hplc (Phenomenex Maxil 10 μm Si, 500×10 mm, eluted in 4% EtOAc/hexanes, flow rate at 9 ml/min, uv detection at 254 nm) yielding curacin A [**1**] (0.9 mg, 15% yield) and a mixture of curacins B [**2**] and C [**3**] (0.8 mg, 13%).

CONFIRMATION THAT CURACINS B [**2**] AND C [**3**] ARE NATURAL PRODUCTS.—First, preserved *Lyngbya majuscula* (15 g) was cold-extracted by grinding in a mortar with liquid N_2. Solvent (CH_2Cl_2-MeOH, 2:1, 30 ml) was added to the algal powder and stirred for 1 h. The algal material was removed by filtration and the solvent reduced *in vacuo*, dissolved in minimal 4% EtOAc/hexanes, passed through a plug of Si gel to remove polar compounds, and analyzed by gc-eims. This gave a similar profile of **1–3** to that from the hot extracted material. A second approach involved heating pure **1** (2.5 mg) at 35° in CH_2Cl_2-MeOH (2:1, 4 ml) for 10 h, followed by gc-eims analysis. Finally, **1** (2 mg) was refluxed in CH_2Cl_2-MeOH (2:1, 3 ml) containing 4 drops H_2O for 90 min, and then analyzed by gc-eims. These latter two experiments showed only the presence of **1**.

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