Structure of Curacin A, a Novel Antimitotic, Antiproliferative, and Brine Shrimp Toxic Natural Product from the Marine Cyanobacterium *Lyngbya majuscula*

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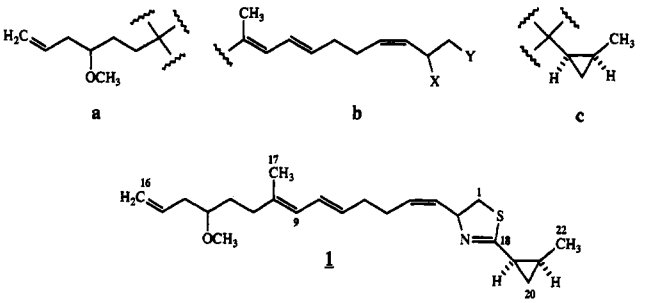
Received January 7, 1994•

Summary: Bioassay-guided fractionation of the organic extract of a Curaçao collection of *Lyngbya majuscula* led to the isolation of a new lipid, curacin A, with exceptional brine shrimp toxic and antiproliferative activities. Its unique thiazoline-containing structure has been deduced from spectroscopic information. Pure curacin A is an antimitotic agent (IC_{50} values in three cell lines ranging from 7 to 200 nM) that inhibits microtubule assembly and the binding of colchicine to tubulin.

The cyanobacteria, or blue-green algae, are a well-recognized source of structurally unique and biologically active natural products.[1,2] Consequently, our anticancer drug discovery efforts have focused on a Caribbean collection of the cyanobacterium *Lyngbya majuscula* which was strongly cytotoxic against a Vero cell line (ATCC CCL81).[3] Subsequent evaluation in the National Cancer Institute 60 cell line assay uncovered a potent antiproliferative and cytotoxic activity showing some selectivity for colon, renal, and breast cancer-derived cell lines. The extract was also found to be highly toxic to brine shrimp (LC_{50} = 25 ng/mL).[4] Using the brine shrimp assay to guide fractionation, a unique metabolite, isolated as 8–10% of the crude extract, was found to be responsible for the potent brine shrimp toxicity (LC_{50} = 3 ng/mL) and mammalian cell antiproliferative activity (IC_{50} = 6.8 ng/mL in the Chinese hamster Aux B1 cell line).[5] Herein, we report the isolation, structural elucidation, and mechanism of antiproliferative activity of this new bioactive marine natural product, which we have named curacin A (**1**).[3]

Processing of a 543-mg batch of the *L. majuscula* crude $CHCl_3$/MeOH (2:1) extract by two successive tiers of vacuum chromatography gave **1** contaminated by fatty acids. After confirming (^1H NMR, GC–MS, brine shrimp toxicity) that the bioactive component was unreactive to CH_2N_2, this fraction was methylated and further purified by HPLC to yield 44.2 mg of **1** showing $[\alpha]_D$ = +86° (*c* = 0.64, $CHCl_3$).[6] High-resolution FAB MS (positive ion, 3-nitrobenzyl alcohol) gave a major $[M + H]^+$ ion at *m/z* 374.2520 analyzing for $C_{23}H_{36}NOS$ (0.3 mmu deviation). Hence, curacin A, of $C_{23}H_{35}NOS$ composition, possessed seven degrees of unsaturation, five of which were due to double bonds (Figure 1) and two due to rings.

Data from ^1H–^1H COSY and ^1H–^{13}C HETCOR were used to generate three partial structures (**a**-**c**) for **1**. Partial



structure **a** contained a monosubstituted terminal olefin adjacent to a methylene group (Figure 1).[7] Neighboring this methylene was a deshielded methine which was shown by HMBC to bear an OMe group. Sequential ^1H–^1H correlations placed two consecutive methylene groups contiguous to this deshielded methine, the latter of which was adjacent to a quaternary carbon, concluding partial structure **a**. Partial structure **b** possessed a terminal methylene group bearing a heteroatom which was adjacent to a methine carbon also bearing a heteroatom. This latter resonance coupled to an olefin proton at δ 5.69, which was in turn coupled by 10.7 Hz to its olefinic partner. Two methylene groups spanned between this olefin and a conjugated diene (λ_{max} = 242 nm, hex). The first of these two latter olefins was disubstituted and *trans*, while the second was trisubstituted and possessed methyl and alkyl substituents at its distal position. The geometry of this latter olefin was shown to be *E* by virtue of the high-field ^{13}C NMR chemical shift of the methyl group (δ 16.56) and observing NOE between the C-17 methyl group (δ 1.74) and H-8 (δ 6.28) in $CDCl_3$. Partial structure **c** was readily formulated by spin coupling information as a *cis*-disubstituted cyclopropyl ring with methyl and quaternary carbon substituents (Figure 1).

HMBC data were used to connect these three partial structures as well as confirm the above structural assignments. Critically, the protons α to the two heteroatoms in partial structure **b** were both correlated to the quaternary carbon of partial structure **c**. Similarly, partial structures **a** and **b** were readily connected by observing long-range coupling between the olefinic proton at δ 6.02

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• Abstract published in *Advance ACS Abstracts*, March 1, 1994.

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(3) One liter preserved in isopropyl alcohol was collected from CARMABI beach, Curaçao, Netherland Antilles, –0.3 m, 13 Dec 1991. Compound **1** is named to reflect this site of collection.
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(6) HPLC was peformed using 5% EtOAc in hexanes, 2 × 30 cm Versapack Silica 10 μm, collected elution volume 57–66 mL.
(7) Coupling constants for curacin A (1, 300 MHz, benzene-d_6): J_{1a-1b} = 10.7 Hz, J_{1a-2} = 10.0 Hz, J_{2-3} = 8.7 Hz, J_{2-4} = 1.1 Hz, J_{3-4} = 10.7 Hz, J_{4-5} = 7.1 Hz, J_{6-7} = 7.0 Hz, J_{7-8} = 15.0 Hz, J_{8-9} = 10.8 Hz, J_{9-17} = 1.0 Hz, J_{19-20a} = 8.2 Hz, $J_{20a-20b}$ = 4.2 Hz, J_{20a-21} = 8.2 Hz, J_{21-22} = 6.2 Hz.

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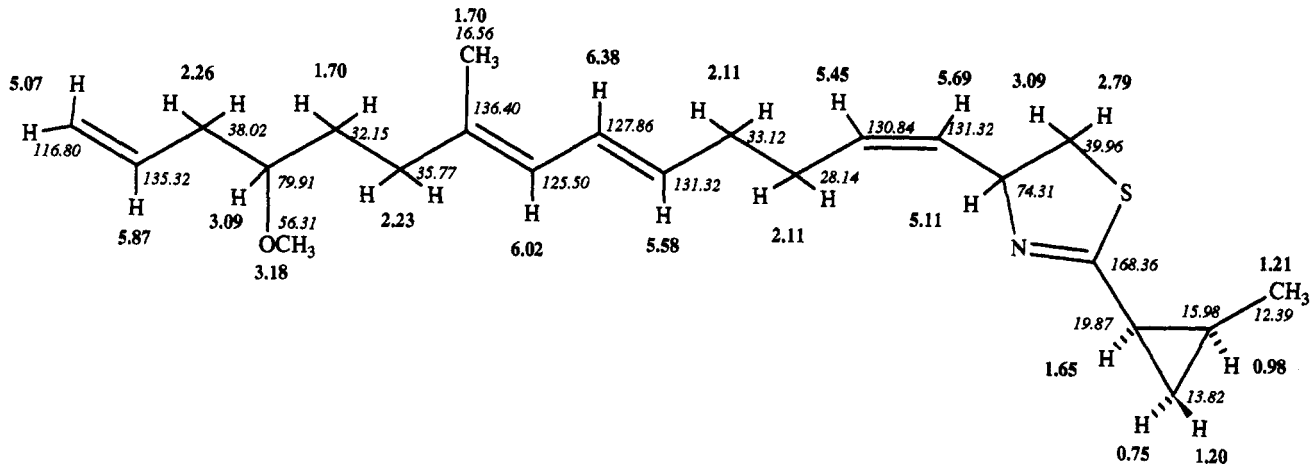

Figure 1. ^1H (boldface) and ^{13}C (italics) NMR data assignments for curacin A (1). Coupling constants in ref 7 (300-MHz, benzene-d_6).

(H-9) and methylene carbon at δ 35.77 (C-11). Placement of sulfur at C1 and nitrogen at C2 was based on comparisons of ^{13}C NMR chemical shifts with model compounds,[8] hence defining a thiazoline ring and yielding the overall planar structure of curacin A (1).

Curacin A was examined in the NCI cell line screen, and its differential cytotoxicity pattern was evaluated by the COMPARE algorithm.[9] As this study indicated that 1 was an antitubulin agent,[10] it was subsequently evaluated for its effect on tubulin polymerization. Curacin A was found to inhibit the polymerization of purified tubulin induced either by glutamate or microtubule-associated protein-dependent microtubule assembly.[11] Figure 2 presents a study of the latter reaction condition, comparing curacin A to podophyllotoxin, a well-studied inhibitor of the reaction.[12] The two drugs were highly similar in their inhibitory effects, inhibiting the extent of assembly about 50% when present at a concentration of 4 μM and nearly completely at 6 μM.

Cytotoxic agents that inhibit tubulin polymerization routinely cause the accumulation of cells arrested in mitosis at cytotoxic drug concentrations.[12,13] This is also the case with curacin A. We compared it with colchicine in two cell lines (Table 1). The number of mitotic cells increased with both drugs as the cell number decreased and was maximal at 1 μM, the highest concentration examined. Furthermore, flow cytometric analysis of Chinese hamster cells treated with 1 (100 ng/mL) clearly demonstrated that the drug causes cells to accumulate in the G_2/M phase of the cell cycle; G_2/M arrest was also observed with vincristine (300 ng/mL) while hydroxyurea, a ribonucleotide reductase inhibitor (100 ng/mL), produced a distinctly different profile (supplementary material).

Most inhibitors of tubulin polymerization appear to interact at one of two independent drug binding domains on tubulin and inhibit the binding of either colchicine or vinblastine, but not both. We examined curacin A for

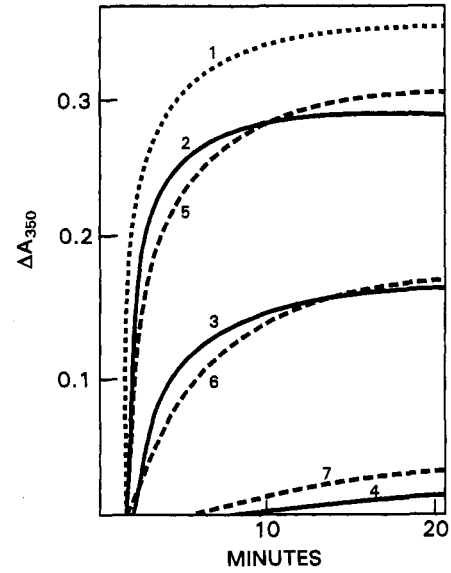

Figure 2. Inhibition of microtubule assembly by curacin A and podophyllotoxin. Each 0.25-mL reaction mixture contained 1.5 mg/mL of tubulin (15 μM), 0.5 mg/mL of heat-treated microtubule-associated proteins (prepared as in ref 10), 0.2 mM GTP, 0.2 mM $MgCl_2$, 1% (v/v) dimethyl sulfoxide (the drug solvent), 0.1 M 4-morpholinoethanesulfonate (Mes) (pH 6.9 with NaOH), and drug as follows: curve 1 (dots), none; curves 2–4 (solid), 2, 4, and 6 μM curacin A, respectively; curves 5–7 (dashes), 2, 4, and 6 μM podophyllotoxin, respectively. Baselines were established with the cuvette contents held at 0 °C; at t_0 the reaction was initiated by a 75-s temperature jump to 37 °C (absorbance measured at 350 nm).

such inhibitory activity, comparing it to podophyllotixin, a known inhibitor of colchicine binding,[12] and to maytansine, a known inhibitor of vinblastine binding[13] (Table 1). The data demonstrate that 1 belongs to the colchicine class of inhibitors, as it inhibits the binding of radiolabeled colchicine, but not radiolabeled vinblastine, to tubulin.

From chemical and biochemical perspectives, the alkylated thiazoline clearly represents the structurally unique portion of curacin A. In this respect, it bears some relationship to the potent cytotoxins patellazoles A–C from the tunicate *Lissoclinum patella*.[14] Similar to curacin A, these latter metabolites possess four-carbon and long polyketide chain substituents on a thiazole ring. The

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Table 1. Effects of Curacin A on Growth of Leukemia Cells and Ligand Binding to Tubulin

	L1210 leukemia cells[a]		CA46 Burkitt lymphoma cells[a]		ligand binding (% inhibition)	
drug	IC_{50} (M)	Mitotic cells[b] (%)	IC_{50} (M)	Mitotic cells[b] (%)	colchicine[c]	vinblastine[d]
curacin A (1)	9×10^{-9}	46	2×10^{-7}	51	76	4.6
colchicine	4×10^{-9}	41	1×10^{-8}	67		7.5
podophyllotoxin					94	
maytansine					0	90

[a] Cells were grown for 15 h (L1210 murine leukemia cells) or 24 h (CA46 human Burkitt lymphoma cells) in RPMI-1640 medium supplemented with 16% fetal bovine serum and 0.03% L-glutamine at 37 °C in 5% CO_2 atmosphere. [b] Mitotic cells are defined as those with condensed chromosomes. Drug concentration was 1.0 μM. L1210 cells were harvested, fixed, and stained with Giemsa. The mitotic index of control cells was 2–3% for both cell lines. [c] The binding of [^{3}H]colchicine to tubulin was measured by the DEAE–cellulose filter technique. Each 0.1-mL reaction mixture contained 0.1 mg/mL (1.0 μM) of tubulin, 2.0 μM [^{3}H]colchicine (DuPont), potential inhibitor at 10 μM (curacin A, podophyllotoxin) or 50 μM (maytansine), 1.0 M monosodium glutamate (pH 6.6 with HCl), 1 mM $MgCl_2$, 1 mM GTP, 0.1 M glucose 1-phosphate, 0.5 mg/mL of bovine serum albumin, and 5% (v/v) DMSO. Incubation was for 30 min at 37 °C. In the control reaction mixture, 0.20 mol of colchicine was bound per mol tubulin. [d] The binding of [^{3}H]vinblastine to tubulin was measured by centrifugal gel filtration. Each 0.32-mL reaction mixture contained 0.5 mg/mL (5.0 μM) of tubulin, 10 μM [^{3}H]vinblastine (Amersham), potential inhibitor at 100 μM, 0.1 M Mes (pH 6.9 with NaOH), 0.5 mM $MgCl_2$, and 1% (v/v) DMSO. Incubation was for 10 min at 22 °C. Triplicate 100-μL aliquots were placed on syringe columns containing G-50 (superfine) equilibrated with a solution containing 0.1 M Mes (pH 6.9) and 0.5 mM $MgCl_2$. In the control reaction mixture 0.21 mol of vinblastine was bound per mol of tubulin.

relationship of heterocyclic ring to diene chromophore in curacin A is similar to that seen in the patellazoles as well as two other antimitotic agents, maytansine and rhizoxin. However, these latter two metabolites inhibit the binding of vinblastine, but not colchicine, to tubulin.[13] Biosynthetically, curacin A may derive from two polyketides joined together through a decarboxylated cysteine residue.

Acknowledgment. We gratefully acknowledge the permission and assistance in collection activities of the CARMABI Tropical Research Center in Curaçao, Mary Roberts for taxonomic identification, Patrick Varga for performing the brine shrimp toxicity assays, and Mark Zabriskie for critically reading the manuscript. We further appreciate the initial cytotoxicity measurements on the crude extract which were performed by Randy Schatzman, Syntex Discovery Research. We also thank Nixy Zutshi and Laura Chiu for flow cytometric evaluation. This work was supported by the National Cancer Institute under Grant CA 52955.

Note Added in Proof. Curacin A has been found to be unstable when stored neat. Preliminary repetition of several of the biological assays reported herein, including inhibition of colchicine binding, with curacin A continuously maintained under solvent indicate that it is approximately 5-fold more potent than reported.

Supplementary Material Available: Results and experimental details of flow cytometric analysis of Chinese hamster cells treated with curacin A, vincristine, or hydroxyurea and ^{1}H and ^{13}C NMR spectra of curacin A in benzene-d_6 (4 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.