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CURACIN D, AN ANTIMITOTIC AGENT FROM THE MARINE CYANOBACTERIUM *LYNGBYA MAJUSCULA*

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Key Word Index—*Lyngbya majuscula*: Oscillatoriaceae; cyanobacteria; blue–green alga; thiazoline alkaloid; antiproliferative.

Abstract—Curacin D is a novel brine shrimp toxic metabolite isolated from a Virgin Islands collection of the marine cyanobacterium *Lyngbya majuscula*. Structure elucidation of curacin D was accomplished through multidimensional NMR, GC/MS, and comparisons with curacin A. Curacin D provides new insights into structure–activity relationships in this natural product class as well as some aspects of the likely biosynthetic pathway of the curacins.

INTRODUCTION

Marine cyanobacteria (blue–green algae) are rapidly proving to be an extremely important source of biologically-active metabolites with potential benefits against human disease [1, 2]. For example, we obtained curacin A (**1**), a structurally novel inhibitor of tubulin polymerization that binds to the colchicine drug binding site, from a Curaçao collection of *Lyngbya majuscula* [2]. In this paper, we report the bioassay-guided fractionation of a Virgin Islands collection of *Lyngbya majuscula* which led to a new natural product in the "curacin family", curacin D (**2**). The new carbon skeleton of curacin D is intriguing because it gives insight into the contribution of the C10 methyl group to the biological properties of the curacins, and because the absence of a C10 methyl group in curacin D implies some biosynthetic flexibility in the nature of the precursor for this section of the molecule.

RESULTS AND DISCUSSION

A collection of *L. majuscula* from St. Croix in the U.S. Virgin Islands was repeatedly extracted to yield a brine shrimp toxic lipid extract. Curacin D was isolated, guided by the brine shrimp toxicity assay, through gradient silica gel vacuum liquid chromatography (25% ethyl acetate/hexanes, v/v), RP C-8 column chromatography (2% MeOH/CH_2Cl_2, v/v), and NP-HPLC (10% ethyl acetate/hexane, v/v) to yield **2** (brine shrimp $LD_{50} = 40$ ng/mL).

Comparison of the ^{1}H-NMR and ^{13}C-NMR data obtained for pure curacin D (**2**) with those of curacin A (**1**) showed these to be similar metabolites [3]. However, by LR-EIMS, compound **2** had a mass equivalent to one less $-CH_2-$ than that of curacin A (**1**) (obs. $[M^+]$ for **2** at m/z 359 by GC EIMS), suggesting a molecular composition of $C_{22}H_{33}NOS$. This was confirmed by ^{13}C NMR analysis of metabolite **2** which showed 22 resonances, one carbon less than that of curacin A. The nature of this difference between **2** and **1** was best revealed by ^{1}H-NMR in which the C-17 methyl group present in curacin A was absent, ostensibly replaced by a highly coupled olefinic proton at δ 5.55 (m). This assignment was confirmed by ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR data which allowed sequential connection from H_2-1 to H-10, as well as from H-10 to H_2-16. Finally, the methyl cyclopropyl chain of this new curacin derivative (C-17 to C-21) had spectroscopic features identical to the comparable positions (C-18 to C-22) in curacin A [3]. The geometry of the C-7 to C-10 conjugated diene in curacin D was shown to be *trans, trans* ($J_{9-10} = 14.5$ Hz, $J_{7\ 8} = 14.5$ Hz)

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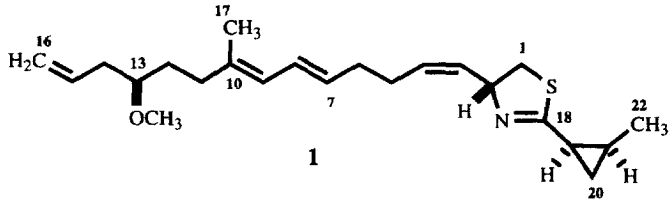

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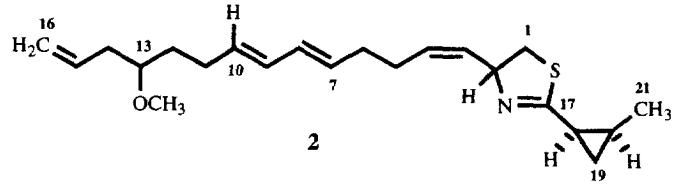

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and the stereochemistry of the methyl cyclopropyl moiety was determined as *cis* ($J_{18-20}=8.3$ Hz, $J_{18-19a}=8.3$ Hz, $J_{18-19b}=5.5$ Hz, $J_{19a-20}=8.1$ Hz). The remaining stereochemical features of curacin D (**2**), while not experimentally determined, are likely the same as in curacin A (**1**) [4].

While curacin D was comparable to curacin A as a potent inhibitor of colchicine binding (**2**, $53\pm10\%$ inhibition at 5.0 μM; **1**, $94\pm2\%$ inhibition at 5.0 μM) [5], it was 7-fold less active than curacin A in its ability to inhibit tubulin polymerization (IC_{50} 4.8 ± 0.4 μM) [5], 10-fold less active in inhibiting MCF-7 breast cancer cell growth (IC_{50} 0.34 ± 0.1 μM) [5], and 13-fold less active as a brine shrimp toxin [3]. It is intriguing that the biosynthetic pathway to the curacins is flexible in the incorporation of a methyl group at C-10. Of the two most likely origins for this carbon atom (the *S*-methyl of *S*-adenosyl methionine or C-3 of a propionate unit), this biosynthetic flexibility may indicate that it is incorporated during a late or "tailoring" stage in the biosynthesis, thus arguing a SAM origin. Recently, we have initiated pathway biosynthetic studies with this curacin A-producing strain of *L. majuscula*, and our preliminary results from feeding [S-$^{13}CH_3$]methionine strongly support this hypothesis [6].

EXPERIMENTAL

General

Ultraviolet spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM 400 MHz NMR spectrometer. All ^1H NMR chemical shifts are reported relative to an internal tetramethylsilane (TMS) standard and ^{13}C spectra are referenced to the center line C_6D_6 at 128.39 ppm. Low resolution mass spectra (LRMS) were obtained using a Hewlett-Packard GC (Model 5810) connected to a Hewlett-Packard mass selective detector (Model 5971), while high resolution mass spectra (HRMS) were obtained on a Kratos MS 50 TC. High performance liquid chromatography (HPLC) was performed using a M-6000 pump and a Lambda-Max 480 UV-detector. TLC-grade (10–40 μm) silica gel was used for vacuum chromatography and Merck aluminum-backed TLC sheets (silica gel 60 F_{254}) were used for thin layer chromatography.

Collection

One liter of alga was collected from −1 m on the northeast end of St. Croix, Virgin Islands on 4 August 1989 and preserved in isopropyl alcohol at −10° until extraction. A voucher specimen is available from WHG under the code STX-4Aug89-1.

Extraction and isolation

The defrosted alga (294 g dry wt.) was homogenized in CH_2Cl_2/MeOH (2:1, v/v), filtered, and the solvents removed *in vacuo* to yield a residue which was partitioned between CH_2Cl_2 and H_2O. The algal residue was then repeatedly extracted with CH_2Cl_2/MeOH (2:1, v/v) and the CH_2Cl_2 layers combined to give a dark-green extract (970 mg). Fractionation of the crude extract was achieved by silica gel vacuum chromatography using a step-wise gradient from 100% hexanes to 100% EtOAc. The brine shrimp toxic fraction (62 mg; 100% lethality at 1 ppm), eluted with 25% EtOAc in hexanes, was further fractionated by flash RP C-8 silica gel column chromatography using 2% MeOH in CH_2Cl_2 to give an impure brine shrimp toxin (12.5 mg) ($LD_{50}=ca.$ 0.2 ppm). Final purification was achieved by HPLC using 10% EtOAc in hexanes, 2×4.1 mm $\times$ 30 cm Versapack Silica 10 μm, UV detection at 254 nm, to give pure curacin D (**1**, 1.2 mg, 0.12% of extract).

Bioassays

In a method slightly modified from the original description for measurement of brine shrimp

toxicity [7], about 15 newly hatched brine shrimp (*Artemia salina*) in *ca.* 0.5 mL seawater were added to each well containing different concentrations of samples in 50 μL EtOH and 4.5 mL artificial seawater to make a total volume of *ca.* 5.0 mL. Samples and controls were run in duplicate. After 24 h at 28°C, the brine shrimp were observed and counted with a dissecting microscope. The percentage of live shrimp vs total shrimp was used to determine LD_{50} values.

The methods used in the colchicine binding to tubulin assay, inhibition of tubulin polymerization assay, and MCF-7 cancer cell growth inhibition assay were recently described [7].

Curacin D (**2**)

Curacin D (**2**) was isolated as a pale yellow oil showing the following: $[\alpha]_D + 33°$ (c 0.14, $CHCl_3$)]; IR (neat) 3300, 2928, 1750, 1725, 1700, 1500, 1490, 1400, 1110, 1020, 980 cm^{-1}; UV (hexanes) λ_{max} 224 (ε 9,000); ^{1}H-NMR (C_6D_6, 400 MHz) δ 6.1 (1H, m, H-9), 6.0 (1H, m, H-8), 5.79 (1H, *ddt*, J = 16.2, 11.0, 7.2 Hz, H-15), 5.64 (1H, *dd*, J = 10.5, 10.4 Hz, H-3), 5.55 (1H, *dt*, J = 14.5, 7.1 Hz, H-10), 5.49 (1H, *bdt*, J = 14.5, 7.3 Hz, H-7), 5.38 (1H, m, H-4), 5.05 (2H, m, H-16), 5.03 (1H, m, H-2), 3.12 (3H, *s*, –OMe), 3.05 (1H, m, H-13), 3.03 (1H, *dd*, J = 10.3, 8.3 Hz, H-1b), 2.74 (1H, *dd*, J = 10.3, 10.3 Hz, H-1a), 2.2 (4H, m, H-11,14), 2.1 (2H, m, H-5), 2.0 (2H, m, H-6), 1.67 (1H, *td*, J = 8.3, 5.5 Hz, H-18), 1.58 (1H, m, H-12a), 1.54 (1H, m, H-12b), 1.17 (3H, *d*, J = 6.3 Hz, H-21), 1.15 (1H, m, H-19b), 0.95 (1H, m, H-20), 0.67 (1H, *ddd*, J = 8.1, 8.1, 4.3 Hz, H-19a); ^{13}C-NMR (C_6D_6, 100 MHz) δ 168.44 (C17), 135.09 (C15), 132.29 (C10), 131.39 (C9), 131.12 (C3), 131.05 (C7), 130.87 (C8), 130.57 (C4), 116.54 (C16), 79.56 (C13), 74.1 (C2), 56.05 (–OMe), 39.69 (C1), 37.91 (C14), 33.39 (C12), 32.51 (C6), 28.51 (C11), 27.67 (C5), 19.86 (C18), 15.71 (C20), 13.96 (C19), 12.06 (C21); GC EIMS (70 eV, rel. intensity) obs. [M^+] at m/z 359 (4), 344 (12), 328 (21), 318 (20), 274 (9), 260 (18), 246 (5), 232 (5), 206 (4), 194 (15), 181 (21), 180 (100), 166 (22), 141 (14), 140 (18), 117 (11), 105 (21), 99 (25), 91 (32), 79 (40), 67 (36), 65 (23), 55 (21).

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