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Characterization of the Interaction of the Marine Cyanobacterial Natural Product Curacin A with the Colchicine Site of Tubulin and Initial Structure-Activity Studies with Analogues

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SUMMARY

Curacin A, the major lipid constituent of a strain of the marine cyanobacterium *Lyngbya majuscula* obtained off the coast of Curaçao, is a potent antimitotic agent that we have previously shown to inhibit microtubule assembly and colchicine binding to tubulin. In the present study, we report that curacin A probably binds in the colchicine site because it competitively inhibits the binding of [^{3}H]colchicine to tubulin with an apparent K_i value of 0.6 μM and stimulates tubulin-dependent GTP hydrolysis, as do most other colchicine-site agents. The binding of curacin A to tubulin resembled the binding reactions of combretastatin A-4 and podophyllotoxin in contrast to that of colchicine in that it occurred as extensively on ice as at higher temperatures. However, once bound, the dissociation rate of curacin A from tubulin is very slow, more closely resembling that observed with colchicinoids (thiocolchicine was the drug examined) than the faster dissociation that occurs with combretastatin A-4 and podophyllotoxin. Because the molecular structure of curacin A is so different from that of previously described colchicine-site drugs (e.g., there is no aromatic moiety, and there are only two conjugated double bonds in its linear hydrocarbon chain), we have been examining the activities of natural isomers and synthetic derivatives. So far, only modest enhancement or reduction of activity has been observed with a variety of structural changes.

The subunit protein of microtubules, known as tubulin, interacts with a wide array of natural products and synthetic compounds that either inhibit or promote microtubule assembly in cell-free systems and in intact cells, causing the latter to arrest in the mitotic phase of the cell cycle. A major source of interest in these agents lies in their potential for the treatment of both neoplastic and infectious, particularly parasitic, diseases. Equally important, the structural and mechanistic diversity of antimitotic compounds and their interactions with tubulin have provided insights into the role of microtubules in cells and the biochemistry of tubulin.

We recently reported the isolation and chemical characterization of curacin A (structure in Fig. 1) (1, 2). This compound is the major lipid component of a strain of the marine cyanobacterium *Lyngbya majuscula* obtained off the coast of Curaçao, and we found that curacin A potently inhibited mitosis in cultured cells. We have been able both to recollect the organism in mass from Curaçao and to grow it in culture, obtaining high yields of curacin A in both cases.

In initial studies with purified tubulin on the mechanism of action of curacin A, we noted perturbations in turbidity development that varied with reaction conditions and drug concentration, but electron microscopic evaluation of reaction mixtures convincingly demonstrated that curacin A inhibited microtubule assembly (3). The chemical structure of curacin A differs substantially from that of other similarly potent antimitotic agents. Ligand inhibition studies showed little effect of curacin A on vinblastine binding and strong inhibition of colchicine binding to tubulin. This latter finding was unexpected because inhibitors of vinblastine binding show substantially more structural variation than do inhibitors of colchicine binding (for reviews, see Refs. 4–6).

During our work with curacin A, we observed considerable variability in the inhibitory effects of different samples of the compound. These observations resulted in our establishment that the compound was unstable as a solid, but we could detect no breakdown once it was dissolved in dimethyl sulfoxide (3). The ligand inhibition studies were repeated, and

ABBREVIATIONS: CS-A4, combretastatin A-4; CS-A2, combretastatin A-2; CPRO curacin A, a chemically prepared derivative of curacin A in which the methylcyclopropyl substituent at position C18 has been converted to a butenyl substituent; MTPT, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone; HPLC, high performance liquid chromatography.

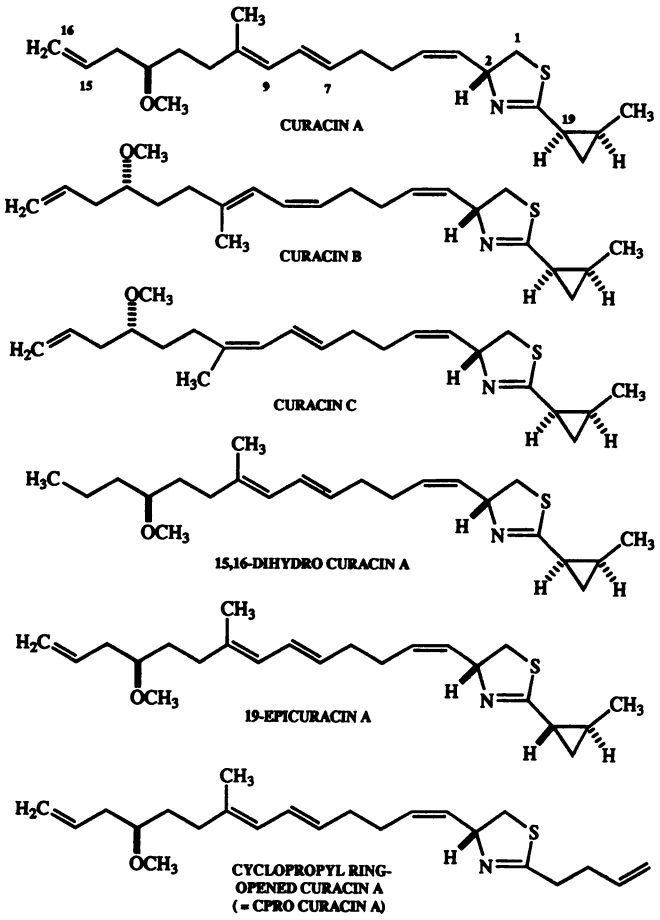

Fig. 1. Molecular formulas of the natural products curacins A, B, and C and of the synthetic derivatives 15,16-dihydrocuracin A, 19-epicuracin A, and CPRO curacin A. Note that in all compounds configuration at C13 is *R*. The α orientation in the diagram of the methoxy substituent in curacins B and C is necessitated by *E*-to-*Z* transition at the C7–8 and C9–10 olefinic bonds, respectively.

we found that curacin A was quite potent as an inhibitor of colchicine binding to tubulin, with activity comparable to that of podophyllotoxin and almost as great as that of CS-A4.

We subsequently began a detailed study of the interaction of curacin A with tubulin to establish whether it bound in the colchicine site and to obtain information about the binding reaction, particularly its relative speed and whether it was reversible. As this work was progressing, structurally related new lipid components were obtained from *L. majuscula*, and chemically modified derivatives of curacin A were prepared. In the present report, we present our findings that curacin A binds in the colchicine site as a competitive inhibitor of colchicine, that its binding rate to tubulin is relatively rapid, and that it dissociates from tubulin relatively slowly. In addition, we describe our initial structure-activity findings with the naturally occurring curacins B and C and with three chemical derivatives of curacin A (structures in Fig. 1).

Experimental Procedures

Materials. The isolation of curacin A from *L. majuscula* (1) and the preparation of electrophoretically homogeneous bovine brain tubulin (7) were described previously. Isolation of curacins B and C from *L. majuscula* extracts and their structural elucidation will be described elsewhere.[1] Podophyllotoxin was obtained from Aldrich Chemical Co., nonradiolabeled colchicine from Sigma Chemical Co., and [^{3}H]colchicine from DuPont-NEN. We received the generous gifts of MTPT (frequently described as the "A-C" analogue of colchicine) from Dr. M. G. Banwell, thiocolchicine from Dr. A. Brossi, and CS-A4 from Dr. G. R. Pettit. The antimitotic carbamate NSC 181928 was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institutes of Health. Stock 2.0 M solutions of monosodium glutamate (from USB) were adjusted to pH 6.6 with HCl.

Preparation of 15,16-dihydrocuracin A. Curacin A (14.4 mg, 38.6 μmol) was dissolved in 0.5 ml ethanol, and 0.2 mg Pd on activated carbon (5%, 0.094 μmol) was added. A balloon filled with H_2 was attached to the reaction vessel, and the mixture was stirred for 3 hr. The reaction mixture was passed through a 1-cm Celite plug, which was washed with hexanes. The combined filtrate solvents were removed under vacuum, and the residue was purified with reverse-phase HPLC on a 5-μm Partisil ODS C_{18} silica column (25 × 0.5 cm) developed at 0.8 ml/min with 95% (v/v) methanol. The 15,16-dihydrocuracin A (0.21 mg, 0.56 μmol, 1.5% yield) was recovered as a colorless oil, and its chemical characterization is as follows: IR ν_{max} (film) 2929, 1617, 1449, 1439, 1380, 1093, 1074, 1053, 962 cm^{-1}; $[\alpha]_D^{30}$ +60° (*c*0.22, $CHCl_3$); UV λ_{max} (methanol) 241 nm (logϵ, 4.57); UV λ_{max} (CH_3CN) 242 nm (logϵ, 4.53); CD (CH_3CN) $\Delta\epsilon$ = −7.24, +13.1 (λ_{max} = 257, 229 nm); ^{1}H NMR (C_6D_6, 400 MHz) δ 6.41 (dd, 1 H, *J* = 15.0 and 10.8, H-8), 6.05 (bd, 1 H, *J* = 10.8, H-9), 5.70 (dd, 1 H, *J* = 10.7 and 9.0, H-3), 5.60 (dt, 1 H, *J* = 15.0 and 6.2, H-7), 5.46 (dt, 1 H, *J* = 11.1 and 6.3, H-4), 5.11 (bq, 1 H, *J* ~8–9, H-2), 3.22 (s, 3 H, -OCH_3), 3.10 (dd, 1 H, *J* = 10.7 and 8.4, H-1b), 3.1 (m, 1 H, H-13), 2.80 (dd, 1 H, *J* = 10.7 and 9.8, H-1a), 2.2 (m, 2 H, H-11), 2.1 (m, 4 H, H-5 and H-6), 1.74 (bs, 3 H, H-17), 1.6–1.72 (m, 5 H, H-12, H-15, and H-19), 1.41 (m, 2 H, H-15), 1.23 (d, 3 H, *J* = 6.1, H-22), 1.2 (m, 1 H, H-20b), 1.00 (m, 1 H, H-21), 0.94 (t, 3 H *J* = 6.9, H-16), 0.76 (td, 1 H, *J* = 8.1 and 4.3, H-20a); ^{13}C NMR (C_6D_6, 100 MHz) δ 168.61 (C18), 136.62 (C10), 131.31 (2C, C3 and C7), 130.88 (C4), 127.72 (C8), 125.42 (C9), 80.20 (C13), 74.35 (C2), 56.18 (-OCH_3), 39.95 (C1), 35.98 (C14), 35.80 (C11), 33.13 (C6), 32.21 (C12), 28.15 (C5), 20.12 (C19), 18.79 (C15), 16.64 (C17), 15.97 (C21), 14.49 (C16), 14.23 (C20), 12.32 (C22); GC EIMS (% rel. int.) obs. $[M]^+$ *m/z* 375 (6), 360 $[M - CH_3]^+$ (8), 344 $[M - OCH_3]^+$ (6), 302 (3), 288 (12), 274 (11), 180 $[M - C_{13}H_{23}O]^+$ (100), 166 (22), 140 (15), 107 (14), 93 (21), 79 (26), 67 $[C_5H_7]^+$ (12), 55 $[C_4H_7]^+$ (13); HR EIMS obs. $[M]^+$ *m/z* 375.2594 ($C_{23}H_{37}NOS$, deviation of −0.2 milliatomic mass units).

Preparation of 19-epicuracin A. Pyridine and acetic anhydride (1.0 ml each) were added successively to curacin A (20 mg, 54 μmol), and the mixture was stirred overnight at room temperature. After the solvents were removed under vacuum, the residue was dissolved in 4% (v/v) ethyl acetate in hexanes and filtered through sintered glass. HPLC purification was performed on a Phenomenex Maxil 10-μm silica column (50 × 1.0 cm) developed at 9 ml/min with 4% (v/v) ethyl acetate in hexanes. The chemical characterization of the recovered 19-epicuracin A (4.2 mg, 11.3 μmol, 21% yield) is as follows: IR ν_{max} (film) 2928, 2867, 2853, 1614, 1441, 1379, 1096, 1080, 999, 964, 912 cm^{-1}; $[\alpha]_D^{25}$ +122° (*c*0.635, $CHCl_3$); UV λ_{max} (methanol) 242 nm (logϵ, 4.40); ^{1}H NMR (C_6D_6, 300 MHz) δ 6.38 (dd, 1 H, *J* = 15.0 and 10.8, H-8), 6.02 (d, 1 H, *J* = 10.8, H-9), 5.87 (m, 1 H, H-15), 5.61 (dd, 1 H, *J* = 10.5 and 9.0, H-3), 5.58 (dt, 1 H, *J* = 15.0 and 10.0, H-7), 5.45 (dt, 1 H, *J* = 10.5 and 7.1, H-4), 5.12 (ddt, 1 H, *J* = 9.0, 8.7, and 1.1, H-2), 5.06 (m, 2 H, H-16), 3.18 (s, 3 H, -OCH_3), 3.09 (m, 1 H, H-13), 3.09 (dd, 1 H, *J* = 10.8 and 8.2, H-1b), 2.75 (dd, 1 H, *J* = 10.7 and 8.7, H-1a), 2.26 (m, 2 H, H-14), 2.23 (m, 2 H, H-11), 2.11 (m, 4 H, H-5 and H-6), 1.70 (s, 3 H, H-17), 1.65 (m, 2 H, H-12), 1.55 (m, 1 H, H-19), 1.49 (m, 1 H, H-21), 1.34 (dt, 1 H, *J* = 8.6 and 4.4, H-20a), 0.85 (d, 3 H, *J* = 5.7, H-22), 0.48 (m, 1 H, H-20b); ^{13}C NMR (C_6D_6, 75 MHz) δ 170.84 (C18), 136.07 (C10), 134.99 (C15), 131.03 (C3), 130.65 (C7), 130.59 (C4), 127.54 (C8), 125.19 (C9), 116.45 (C16), 79.59 (C13), 73.85 (C2), 55.97 (-OCH_3), 39.25 (C1), 37.69 (C14), 35.44

[1] H.-D. Yoo and W. H. Gerwick, unpublished observations.

(C11), 32.81 (C6), 31.82 (C12), 27.81 (C5), 23.21 (C19), 17.74 (C22), 17.50 (C21), 17.34 (C20), 16.25 (C17); GC EIMS (% rel. int.) obs. $[M]^+$ m/z 373 (5), 342 (13), 332 (13), 274 (13), 181 (27), 180 (100), 166 (30), 98 (20), 91 (24), 85 (16), 79 (28), 67 (14), 55 (12); HR FAB MS (positive) obs. $[M + H]^+$ at m/z 374.2534 ($C_{23}H_{36}NOS$, deviation of +1.7 milliatomic mass units).

Preparation of CPRO curacin A. Curacin A (15 mg, 40 μmol) was added to 2.5 ml of toluene containing a boiling stone at room temperature. The reaction mixture was heated at reflux for 4 hr. After the reaction mixture was again at room temperature, the solvent was removed under vacuum. The residue was dissolved in 4% (v/v) ethyl acetate in hexanes and filtered through sintered glass. HPLC purification was performed on a Phenomenex Maxil 10-μm silica column (50 × 1.0 cm) developed at 9 ml/min with 4% (v/v) ethyl acetate in hexanes. The chemical characterization of the recovered CPRO curacin A (see Fig. 1 for structural details) (1.1 mg, 3.0 μmol, 7.3% yield) is as follows: IR ν_{max} (film) 2977, 2925, 2868, 1621, 1437, 1096, 963, 913 cm^{-1}; $[\alpha]_D^{22}$ +110° (c0.1, $CHCl_3$); UV λ_{max} (methanol) 242 nm (logϵ, 4.40); ^1H NMR (C_6D_6, 300 MHz) δ 6.38 (dd, 1 H, J = 15.0 and 10.8, H-8), 6.05 (d, 1 H, J = 10.8, H-9), 5.87 (m, 1 H, H-15), 5.76 (m, 1 H, H-21), 5.62 (dd, 1 H, J = 10.7 and 8.4, H-3), 5.60 (dt, 1 H, J = 15.0 and 7.0, H-7), 5.46 (dt, 1 H, J = 10.7 and 7.1, H-4), 5.14 (m, 1 H, H-2), 5.08 (m, 2 H, H-16), 5.02 (ddt, 1 H, J = 17.0, 1.7, and 1.7, H-22a), 4.96 (ddt, 1 H, J = 10.0, 1.7, and 1.1, H-22b), 3.18 (s, 3 H, -OCH_3), 3.09 (m, 1 H, H-13), 3.09 (dd, 1 H, J = 10.7 and 8.4, H-1b), 2.77 (dd, 1 H, J = 10.7 and 8.7, H-1a), 2.50 (m, 2 H, H-19), 2.42 (m, 2 H, H-20), 2.25 (m, 4 H, H-11 and H-14), 2.15 (m, 4 H, H-5 and H-6), 1.70 (s, 3 H, H-17), 1.65 (m, 2 H, H-12); ^{13}C NMR (C_6D_6, 75 MHz) δ 168.68 (C18), 137.27 (C21), 136.50 (C10), 135.35 (C15), 131.32 (C7), 131.14 (C3), 130.78 (C4), 127.93 (C8), 125.53 (C9), 116.78 (C16), 115.39 (C22), 79.97 (C13), 74.53 (C2), 56.31 (-OCH_3), 39.83 (C1), 38.06 (C14), 35.80 (C11), 33.98 (C19), 33.13 (C6), 32.19 (C12), 31.62 (C20), 28.19 (C5), 16.59 (C17); GC EIMS (% rel. int.) obs. $[M]^+$ m/z 373 (4), 332 (15), 181 (32), 180 (100), 140 (16), 119 (17), 105 (20), 93 (20), 91 (27), 85 (20), 79 (33), 67 (16), 55 (14); HR FAB MS (positive) obs. $[M + H]^+$ at m/z 374.2494 ($C_{23}H_{36}NOS$, deviation of −2.4 milliatomic mass units).

Methods. NMR spectra were recorded with Bruker AM 400 and AC 300 spectrometers. Chemical shifts were referenced to the solvent C_6D_6 signals at 7.2 ppm for ^1H NMR and at 128 ppm for ^{13}C NMR. Mass spectra were recorded with Kratos MS 50 TC and Finnigan 4023 mass spectrometers. Gas chromatography/mass spectrometry was carried out with a Hewlett-Packard 5890 Series II gas chromatograph connected to a Hewlett-Packard 5971 mass selective detector. UV and IR spectra were obtained, respectively, with Hewlett-Packard 8452A and Nicolet 510 spectrophotometers.

The binding of [^{3}H]colchicine to tubulin was measured by the DEAE-cellulose filter method as described previously (8). Reaction mixtures contained 1.0 μM (0.1 mg/ml) tubulin, 1.0 M monosodium glutamate, 0.1 M glucose-1-phosphate, 1.0 mM $MgCl_2$, 1.0 mM GTP, 0.5 mg/ml bovine serum albumin, and [^{3}H]colchicine, inhibitors, and dimethyl sulfoxide as indicated. These reaction conditions were used because they strongly stabilize the colchicine binding activity of tubulin (9). In general, each data point represents the average of triplicate samples.

We used Sephadex G-50 (superfine) columns (1.5 × 25 cm) for gel filtration chromatography to separate tubulin-drug complex from unbound drug. Columns were equilibrated and developed with 1.0 M monosodium glutamate.

Hydrolysis of GTP was measured by following formation of [8-^{14}C]GDP from [8-^{14}C]GTP by thin layer chromatography on polyethyleneimine-cellulose and autoradiography (10). Both product and reactant spots were cut from the plate and quantified by counting with a liquid scintillation spectrometer.

Tubulin polymerization was followed turbidimetrically at 350 nM in Gilford model 250 spectrophotometers equipped with electronic temperature controllers. All concentrations refer to the final reaction volume of 0.25 ml, although the preincubation was performed in 0.24 ml, followed by the addition of 10 μl of 10 mM GTP. Reaction mixtures contained 1.0 mg/ml tubulin, 0.8 M monosodium glutamate, 4% (v/v) dimethyl sulfoxide, and varying drug concentrations. Samples were preincubated for 15 min at 30° and chilled on ice. GTP was added to each reaction mixture, and these were placed in cuvettes held at 0°. Base-lines were established, the temperature was raised to 30° (~0.5°/sec), and polymerization was followed for 20 min. IC_{50} values were determined by graphical interpolation of experimental points, with drug-containing samples compared with control reaction mixtures containing dimethyl sulfoxide but no drug. Each experimental series contained two control samples, with readings usually within 5% of their average. At least three independent values were obtained with each compound.

IC_{50} values were obtained for murine L1210 leukemia and human CA46 Burkitt lymphoma cells grown in suspension culture for 16 and 32 hr, respectively, at 37° under 5% CO_2 atmosphere. The culture medium was RPMI 1640 supplemented with 10% fetal bovine serum, 0.3% L-glutamine, and 0.01 mg/ml gentamicin sulfate. The dimethyl sulfoxide concentration was 0.1%.

Results

Curacin A is a competitive inhibitor of colchicine binding to tubulin. Fig. 2A represents a study comparing the relative activities of curacin A, podophyllotoxin, and CS-A4 as inhibitors of the binding of [^{3}H]colchicine to tubulin in reaction mixtures containing 1.0 μM tubulin and 5.0 μM colchicine. When equimolar with the radiolabeled colchicine, inhibition by curacin A and CS-A4 was near total, and ~90% inhibition occurred with podophyllotoxin. At inhibitor concentrations below 1.5 μM, however, the effect of curacin A was more comparable to that of podophyllotoxin.

When the inhibitory effect of curacin A was examined at several inhibitor and colchicine concentrations and examined according to the method of Hanes (11), differing curacin A concentrations produced a set of parallel lines (Fig. 2B). This indicates a competitive mode of inhibition (11). Data analysis according to the Dixon method (11) (Fig. 2C) yielded an apparent K_i value for curacin A of 0.6 μM (mean ± standard deviation, 0.58 ± 0.07; four experiments). In previous experiments with the same reaction condition, we obtained apparent K_i values of 0.1 and 0.5 μM for CS-A4 and podophyllotoxin, respectively (12).

Curacin A binds rapidly to tubulin. Among the antimitotic drugs, the colchicinoids are unique in binding very slowly to tubulin (5). Thus, the rate of binding of [^{3}H]colchicine to tubulin is negligible at 0°, and at warmer temperatures it is not difficult to find reaction conditions where binding is not complete for 1 hr or longer. In the absence of a radiolabeled version of a new colchicine-site drug, one approach to determining whether the agent mimics this property of colchicine is to preincubate the drug with tubulin and determine whether the concentration of drug required to inhibit microtubule assembly is lowered. With colchicine, a 3–4-fold reduction in the apparent IC_{50} value can be obtained with a preincubation, whereas with colchicine-site drugs that interact rapidly with tubulin, such as CS-A4, there is no enhancement of activity with a preincubation (12). Curacin A, however, could not be evaluated by this procedure because inhibition of turbidity development occurs only under some reaction conditions and with a narrow window of drug concentrations (3).

We therefore explored alternate assays to gain information about the interaction of curacin A with tubulin. Most colch-

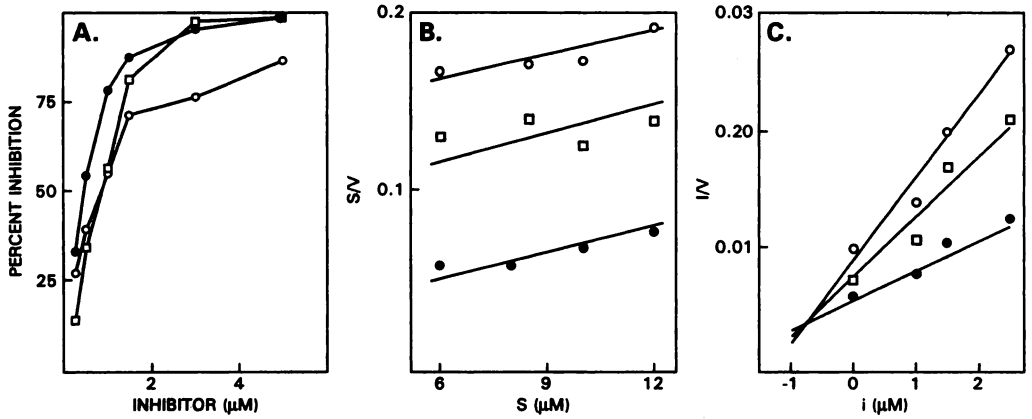

Fig. 2. Inhibition of [^{3}H]colchicine binding by curacin A. Incubation time was 10 min at 37°. A, Comparison of curacin A with CS-A4 and podophyllotoxin. Reaction mixtures contained the indicated inhibitor concentration, 5.0 μM [^{3}H]colchicine, 1.0 μM tubulin, and 5% dimethyl sulfoxide. Incubation was for 10 min at 37°. □, Curacin A; ●, CS-A4; ○, podophyllotoxin. B, Hanes analysis of the inhibitory effect of curacin A on [^{3}H]colchicine binding to tubulin. Reaction mixtures contained the [^{3}H]colchicine concentrations indicated on the abscissa (*S*), 1.0 μM tubulin, and curacin A as follows: ●, none; □, 1.5 μM; ○, 2.5 μM. The ordinate (*S/V*) units are μM colchicine·min·mg tubulin/pmol of colchicine bound. C, Dixon analysis of the inhibitory effect of curacin A on [^{3}H]colchicine binding to tubulin. Reaction mixtures contained the curacin A concentrations indicated on the abscissa (*i*), 1.0 μM tubulin, and [^{3}H]colchicine as follows: ●, 6.0 μM; □, 10 μM; ○, 12 μM. The ordinate (*I/V*) units are (min) (mg tubulin)/(pmol colchicine bound).

TABLE 1

Preincubation of tubulin with curacin A does not enhance subsequent hydrolysis of GTP

Each 100-μl reaction mixture contained 1.0 mg/ml tubulin, 1.0 M monosodium glutamate, 1.0 mM $MgCl_2$, the indicated drug at 2.0 μM, 10% (v/v) dimethyl sulfoxide, and 100 μM [8-^{14}C]GTP. GTP was the last component added in 10 μl. Before GTP addition, reaction mixtures were either incubated for 20 min at 37° and subsequently placed on ice (preincubated) or left on ice for 20 min (not preincubated). After GTP addition, incubation was for 20 min at 37°. Values are the average of a minimum of five experiments.

Drug added	Not preincubated	Preincubated
	nmol/ml GDP formed	
None	12 ± 0.3	15 ± 0.7
Colchicine	18 ± 1	32 ± 0.9
Curacin A	37 ± 0.7	39 ± 1
NSC 181928	33 ± 0.5	33 ± 0.5

Values are given as mean ± standard error.

icine-site drugs stimulate tubulin-dependent GTP hydrolysis even as they inhibit the polymerization reaction, usually closely coupled to hydrolysis (4). This drug-dependent hydrolysis reaction is a sigmoidal function of tubulin concentration, suggesting that tubulin/tubulin interactions are required for it to occur, but the nature of such interactions is uncertain (13).[2]

Preliminary experiments demonstrated that curacin A, like colchicine, induced GTP hydrolysis. We therefore examined the effect of a drug-tubulin preincubation on the extent of subsequent GTP hydrolysis, comparing the effects of curacin A with those of colchicine and of the colchicine-site carbamate drug NSC 181928, which we have previously shown to interact rapidly with tubulin (14) (Table 1). Glutamate was used in the preincubation because it prevents tubulin decay (9) and can replace microtubule-associated proteins as an inducer of both assembly and assembly-dependent GTP hydrolysis (15). Preincubation of the tubulin alone had little effect on subsequent GTP hydrolysis. In the presence of colchicine, there was little enhancement of GTP hydrolysis unless the drug was preincubated with the tubulin. This preincubation resulted in >2-fold enhancement of GTP breakdown. This preincubation was not required with either NSC 181928 or curacin A, providing indirect evidence for a considerably more rapid interaction with tubulin of curacin A compared with colchicine.

In addition, we studied the effect of gel filtration chromatography of drug-tubulin mixtures on the ability of the recovered protein to bind [^{3}H]colchicine, using inhibitor concentrations sufficient to completely inhibit colchicine binding in the initial reaction mixture (Table 2). These studies also demonstrated that curacin A bound to tubulin at 0°. There was little difference in the ability of the tubulin to bind [^{3}H]colchicine when the protein had been incubated with

TABLE 2

Ability of drug-treated tubulin to bind [^{3}H]colchicine after gel filtration chromatography

The preincubation reaction mixtures (1.0 ml) contained 2.0 mg/ml (20 μM) tubulin, the indicated drug at 160 μM (except for control reaction mixtures without drug), 1.0 M monosodium glutamate, and 4% (v/v) dimethyl sulfoxide and were incubated for 30 min at the indicated temperature. The samples were applied to Sephadex G-50 (superfine) columns (25 × 1.5 cm) equilibrated and developed with 1.0 M glutamate. The peak protein fractions (concentrations ranging from 0.5 to 0.8 mg/ml) were stored in liquid nitrogen. Reaction mixtures for binding of [^{3}H]colchicine contained the components described in the text, [^{3}H]colchicine at 5.0 μM, and the indicated drug-treated and gel-filtered tubulin preparation (or a preincubated, gel-filtered control tubulin) at 0.1 mg/ml (1.0 μM). Incubation was at 32°, as indicated. Three experiments were performed with the tubulin preincubated with curacin A and podophyllotoxin (standard error presented), and two experiments were performed with the other agents (average ± standard deviation).

	Preincubation temperature			
	0° Incubation time		32° Incubation time	
	5 min	40 min	5 min	40 min
	% Inhibition			
Drug in preincubation				
Curacin A	87 ± 10	78 ± 8	92 ± 7	91 ± 3
Podophyllotoxin	75 ± 4	39 ± 1	82 ± 4	28 ± 2
Thiocolchicine	0	0	100	88 ± 3
CS-A4	47 ± 1	14 ± 1	47 ± 2	13 ± 1
MTPT			0	0

[2] E. Hamel, unpublished observations.

curacin A on ice or at 32° before gel filtration. In this study, curacin A was compared with thiocolchicine, podophyllotoxin, CS-A4, and MTPT. Thiocolchicine, like colchicine, binds to tubulin readily only at higher temperatures (8, 16), and with this agent, we found no inhibition of colchicine binding with a cold preincubation and nearly complete inhibition after the 32° preincubation. In contrast, the binding of MTPT is so weak (17–19) that gel filtration routinely yields a protein preparation that differs little from the control in its ability to polymerize or to bind colchicine (20), as was again observed here. Previously, we had found with CS-A4 that preincubation temperature did not affect inhibition of colchicine binding to the tubulin after gel filtration (12), and this was again observed. Podophyllotoxin behaved like curacin A and CS-A4 in that preincubation temperature had little effect on subsequent inhibition of colchicine binding.

Curacin A dissociates relatively slowly from tubulin. The data presented in Table 2 confirm earlier observations that the binding reactions of podophyllotoxin (21) and of CS-A4 (12) to tubulin are reversible because inhibition after gel filtration is substantially weaker than that which occurs in unfiltered reaction mixtures. Moreover, with these drugs, the extent of inhibition markedly declined as a function of incubation time once the [^{3}H]colchicine was mixed with the tubulin. These findings are most consistent with substoichiometric amounts of both compounds being retained by the tubulin after gel filtration. With curacin A and thiocolchicine, the data in Table 2 are at most suggestive of drug dissociation in that after gel filtration, inhibition of [^{3}H]colchicine binding was no longer complete, especially after the 40-min incubations (compare with Fig. 2A for curacin A).

We have shown previously that the [^{3}H]colchicine binding assay can be used to obtain indirect evidence about whether agents binding in the colchicine site can dissociate from tubulin (12, 20), based on the slow dissociation rate of colchicine from its binding site (5, 8). Such studies can also provide suggestive evidence about relative dissociation rates among colchicine-site agents, although interpretation is usually limited by uncertainty regarding relative association rates, for both inhibitor and [^{3}H]colchicine remain in the reaction mixture. This analysis is facilitated by the use of reaction conditions in which tubulin retains its ability to bind colchicine for prolonged periods of time (9). Thus, if [^{3}H]colchicine is added to a stabilizing reaction mixture after preincubation with a colchicine-site agent, apparent inhibition, relative to a control without drug, should decrease as a function of incubation time if the nonradiolabeled drug dissociates from active tubulin. We performed two experiments: the first over a 3-day period, and the second over a 14-day period. The longer incubation time was required to observe extensive apparent dissociation of curacin A from the tubulin (see later).

The inset of Fig. 3 shows the amount of [^{3}H]colchicine bound in the absence of inhibitor in the two experiments. As described previously (9), the amount of colchicine bound by tubulin changed little for 72 hr, followed by a slow decay that appeared to stabilize at ~50% activity loss (within the time frame of the experiment). If confirmed by further experimentation, this decay pattern merits more detailed study, as it could indicate differential stability of tubulin isotypes (22) that could be used for preparative purposes.

Fig. 3 presents a comparison of the effects of preincubating tubulin with curacin A, podophyllotoxin, CS-A4, and thiocolchicine on subsequent binding of [^{3}H]colchicine in the 14-day experiment, with extent of inhibition determined for the inhibitors at each time point using the control value (shown in the inset) for the same time point. With all compounds, inhibition decay (plotted on a logarithmic scale) was a linear function of time, consistent with dissociation of the nonradiolabeled inhibitor from a tubulin/drug complex in a first-order reaction. The apparent half-lives (average of results from the 3-day experiment, by extrapolation, and from the 14-day experiment presented in Fig. 3) of these complexes were as follows: curacin A, 173 hr (obtained twice); thiocolchicine, 263 ± 1 hr; CS-A4, 59 ± 1 hr; and podophyllotoxin, 41 ± 5 hr.[3] Thus, the apparent dissociation rate of curacin A from tubulin more closely resembles that of the colchicinoids, represented by thiocolchicine, than any other colchicine-site agent yet examined (see Discussion). Its apparent dissociation rate is only ~1.5 times as fast as that of thiocolchicine but one fourth that of podophyllotoxin and one third that of CS-A4.

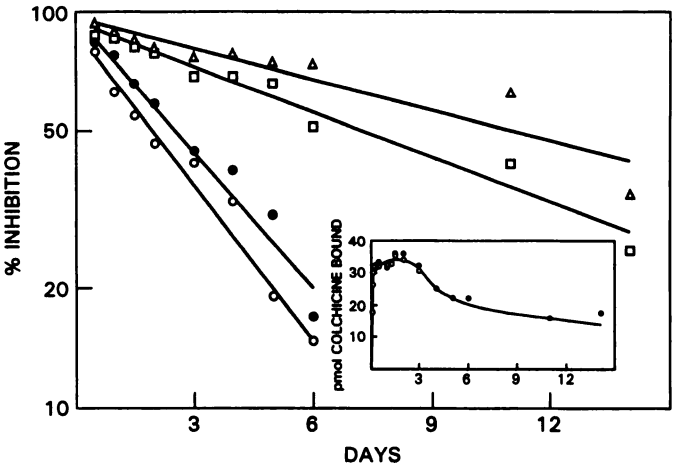

Fig. 3. Effect of prolonged incubation time on inhibition of [^{3}H]colchicine binding after preincubation of inhibitor with tubulin. The reaction volume was 4.5 ml during the preincubation and, initially, 5.0 ml after addition of [^{3}H]colchicine. Concentrations refer to the final reaction volume of 5.0 ml. Each reaction mixture contained 0.1 mg/ml (1.0 μM) tubulin, the components described in the text, 1% (v/v) dimethyl sulfoxide, and, except for the control, the following inhibitors at 10 μM: □, curacin A; ●, CS-A4; ○, podophyllotoxin; △, thiocolchicine. After 30 min at 32°, the reaction mixtures were placed on ice, and 0.5 ml containing sufficient [^{3}H]colchicine for a final concentration of 2.0 μM was added to each mixture. The incubation was resumed at 32°, and at the indicated times triplicate 0.1-ml aliquots were removed from the reaction mixtures for determination of the amount of colchicine bound. *Inset: filled symbols*, values for the amount of colchicine bound by the control samples for the experiment shown in the main plot; *open symbols*, control samples from an independent experiment in which shorter incubation times were studied, with a total elapsed time of 3 days.

Structure-activity studies. The molecular formula of curacin A differs greatly from that of other agents that bind in the colchicine site, but presumably the conformation of the drug in aqueous solution shares important features with the conformations of such drugs as CS-A4, podophyllotoxin, and colchicine. In an initial effort to gain insight into how curacin

[3] These half-lives correspond to apparent dissociation rate constants as follows: curacin A, 1.1×10^{-6}/sec; thiocolchicine, 7.3×10^{-7}/sec; CS-A4, 3.3×10^{-6}/sec; and podophyllotoxin, 4.7×10^{-6}/sec. These are unlikely to represent true dissociation rate constants (see Discussion).

A resembles these other drugs, we have been evaluating naturally occurring analogues and semisynthetic derivatives.

In addition to curacin A, we have been able to define the structures for two other natural products, which we have named curacins B and C (see Fig. 1). So far, we have not been able to completely separate curacins B and C, but we have been able to define their relative proportions in different preparations. These molecules differ from curacin A in configuration about the 7–8 and 9–10 olefinic bonds, which are both *E* in curacin A. In curacin B, the 7–8 olefin is *Z* and the 9–10 olefin is *E*, whereas in curacin C, the 7–8 olefin is *E* and the 9–10 olefin is *Z*. To obtain information about these two agents, we have used preparations in which the B/C ratios are 19:1 (presumably representing the activity of curacin B) and 1:1 (to provide some insight into whether curacin C is either highly active or almost inactive, by comparing the activity of the 1:1 preparation with that of the 19:1 preparation).

In addition, we found that gentle hydrogenation resulted in selective reduction of the terminal 15–16 olefinic bond, yielding 15,16-dihydrocuracin A. Heat treatment of curacin A caused disruption of the C18 cyclopropyl substituent, yielding CPRO curacin A, with a butenyl group at C18. Acetylation conditions were effective in the epimerization of the C19 cyclopropyl methine center, presumably through formation of a labile *N*-acetate. The analytical data for each of these compounds (see above), in concert with two-dimensional NMR data, established the structures of these semisynthetic derivatives.

We examined these compounds as cytotoxic agents in two cell lines, murine L1210 leukemia and human CA46 Burkitt lymphoma (Table 3). Although CPRO curacin A was almost 1 order of magnitude less active than curacin A, the remaining agents had similar potency. In particular, the nearly identical results with the two B/C mixtures indicate that both isomers have significant activity.

We examined the agents for their effects on tubulin polymerization, using for the most detailed studies the only reaction condition where we had observed complete inhibition of turbidity development with curacin A (0.8 M glutamate; no exogenous Mg^{2+}; 30° incubation), even though higher concentrations of the drug had induced formation of bifilamentous spiral fibers (3). With all five preparations, we obtained turbidity data similar to the patterns obtained with curacin A. At low concentrations, there was progressive and, ultimately, complete inhibition of assembly. As the drug concentration was raised further, turbidity development again occurred and became increasingly prominent with increased drug concentration. The initial phase of progressive inhibition allowed us to obtain quantitative comparisons of the drug effects in terms of IC_{50} values, and these are presented in Table 3 with data obtained in the same series of experiments with curacin A, colchicine, podophyllotoxin, and CS-A4. The only unequivocal difference obtained was with CPRO curacin A, which yielded an IC_{50} value approximately twice that of curacin A. Although the differences are small, 15,16-dihydrocuracin A and 19-epicuracin A appeared to be slightly more active than curacin A, and the curacin B/C mixtures appeared to be slightly less active. There was no significant difference between the two B/C mixtures, as in the cytotoxicity experiments, indicating that curacins B and C have similar potencies.

We also examined the series of curacin preparations for inhibitory effects on [^{3}H]colchicine binding to tubulin (Table 3), with potential inhibitor and colchicine present in equimolar (5 μM) amounts and at 5-fold excess over tubulin (1 μM). Relative to curacin A, reduced inhibition was observed with the two B/C mixtures and with CPRO curacin A. Nevertheless, these preparations were >50% inhibitory with equimolar colchicine and thus retain significant ability to bind to tubulin. Again, the two B/C mixtures had similar activity, suggesting that the two isomers are nearly equivalent. Further comparison of 19-epicuracin A and 15,16-dihydrocuracin A with curacin A as inhibitors of colchicine binding was performed with inhibitor concentrations as low as 1 μM. No significant differences were observed among the three compounds, with inhibition of [^{3}H]colchicine binding ranging from 66% to 70%. As with curacin A, the isomers and derivatives stimulated tubulin-dependent GTP hydrolysis, and this reaction was not enhanced by preincubating drug with tubulin before the addition of GTP to the reaction mixture (data not presented).

Discussion

Interaction of curacin A in the colchicine site of tubulin. Curacin A is the most recent of a series of potently

TABLE 3

Effects of curacin A analogues and derivatives on cell growth, tubulin polymerization, and colchicine binding

IC_{50} values for cell growth and tubulin polymerization were determined as described in the text. Inhibition of colchicine binding was measured after a 10-min incubation at 37° with the inhibitor and colchicine present in equimolar concentrations (5.0 μM) with 1.0 μM tubulin. Dimethyl sulfoxide concentration was 5% (v/v).

Compound	Inhibition cell growth[a]		Inhibition tubulin polymerization[b]	Inhibition colchicine binding
	L1210	Burkitt CA46		
	nM		μM	% Inhibition
Curacin A	1	1	1.4 ± 0.2	96
Curacins B plus C (19:1)	2	3	1.6 ± 0.1	65
Curacins B plus C (1:1)	2	4	1.7 ± 0.2	58
19-Epicuracin A	1	4	1.1 ± 0.1	97
CPRO curacin A	7	9	3.3 ± 0.6	55
15,16-Dihydrocuracin A	1	4	1.3 ± 0.2	96
CS-A4			1.3 ± 0.2	96
Colchicine	3	15	1.7 ± 0.09	
Podophyllotoxin			1.6 ± 0.03	83

[a] IC_{50}.
[b] IC_{50} ± standard deviation.

cytotoxic antimitotic natural products obtained from marine organisms (23–25), and it is the first derived from a marine cyanobacterium.[4] Unlike the marine peptides and polyethers, which noncompetitively inhibit the binding of vinca alkaloids to tubulin, curacin A potently inhibits colchicine binding to tubulin and has no significant effect on vinblastine binding (1). The molecular structure of curacin A differs substantially from that of previously described colchicine-site agents (4, 5). This structural novelty may indicate that the compound has greater therapeutic potential than previously evaluated colchicine-site agents have thus far shown. In this regard, the reproducible isolation of the compound from *L. majuscula* obtained in Curaçao, the abundance of curacin A in extracts of the organism, and the successful culture of the organism in the laboratory are important factors in its evaluation.

In the present study, we have shown that curacin A competitively inhibits colchicine binding, indicating that binding occurs in the same site on tubulin as colchicine. From indirect evidence based on the effects of curacin A on GTP hydrolysis and on colchicine binding after various experimental manipulations, we attempted to gain insight into the rates of association and dissociation of curacin A with tubulin by comparing the new agent with previously studied drugs.

To place our work into context, it is worthwhile first to summarize the parameters of the binding of colchicine and MTPT to tubulin at 37° (see Ref. 5 for further details). These two drugs have been studied most thoroughly because of the intense fluorescence that occurs when they bind to tubulin. Colchicine and MTPT also appear to represent extremes of behavior, despite their structural similarities, and thus provide limits for values expected for other agents. Despite similar equilibrium constants of 220/M and 273/M for binding of colchicine and MTPT, respectively, to tubulin, overall binding of MTPT is ~400 times faster (apparent binding rate constant of 5.2×10^4 M^{-1} sec^{-1} versus 1.4×10^2 M^{-1} sec^{-1} for colchicine), and its dissociation is ≈3,000–12,000 times faster (apparent dissociation rate constant of $\sim 6 \times 10^{-2}$/sec versus $\sim 0.5–2 \times 10^{-5}$/sec for colchicine). The range of colchicine values corresponds to a half-life for the tubulin-colchicine complex of 10–39 hr. We obtained 37° half-lives of 27 and 24 hr for the tubulin complexes of colchicine and thiocolchicine, respectively (8).

The results of the present study demonstrate that curacin A binds relatively rapidly to tubulin and, once bound, dissociates relatively slowly. Rapid binding was first demonstrated by showing that curacin A, unlike colchicine, did not require a 37° preincubation with tubulin to obtain maximum stimulation of GTP hydrolysis when addition of the nucleotide to the reaction mixture was delayed. This finding was confirmed by showing that after either a 0° or 32° incubation with curacin A, tubulin had little ability to bind [^{3}H]colchicine after gel filtration chromatography. Curacin A shares this ability to bind rapidly to tubulin at low temperatures with many other colchicine-site agents, including MTPT (17, 19, 26), CS-A4 and CS-A2 (12), carbamate agents (14, 27), 6-member B ring thiocolchicine analogues (28), and styrylquinazolinone derivatives (20).

[4] Although their interaction with tubulin has not yet been detailed, the cryptophycins are antimitotic natural products isolated from a terrestrial cyanobacterium (29, 30).

Relative dissociation rates for colchicine-site drugs are more readily determined, at least to a first approximation. A method for obtaining such estimates exploits the very slow dissociation of [^{3}H]colchicine from tubulin. As a consequence of this property, with most colchicine-site drugs that bind more rapidly than colchicinoids, the extent of inhibition declines as incubation time increases. A major factor in this inhibition decay probably is dissociation of the competing inhibitor from tubulin, and different agents have significantly different rates of inhibition decay (12, 20).

In previous studies, we evaluated inhibition decay with colchicine-site drugs by two methods: (a) after isolation of tubulin with bound inhibitor by gel filtration (12), with unknown initial stoichiometry of binding; and (b) by simply determining the extent of inhibition of colchicine binding as a function of incubation time after preincubation of tubulin with excess inhibitor (20). A limitation in both methods is that the half-life obtained is substantially greater than the true half-life of the tubulin-inhibitor complex because the reaction mixture contains unbound inhibitor (thus, relative association rates affect the results obtained) and the [^{3}H]colchicine that binds to the newly liberated tubulin can in turn dissociate, albeit very slowly, from the protein. In our study (12) using the first method, with isolated complexes of tubulin with CS-A4 and CS-A2, we found that progressively shorter apparent half-lives of the complexes were obtained as the concentration of [^{3}H]colchicine was increased. In our previous study using the second method, we found that the excess CS-A2 led to a much longer apparent half-life than that obtained with the isolated tubulin-CS-A2 complex: 5 hr (20) versus 2.4 min (12). Analogously, the apparent half-life for the tubulin-CS-A4 complex at 32° obtained in the present study (59 hr) was far greater than the 3.6-min half-life obtained previously at 37° using the isolated tubulin/drug complex (12). At the opposite extreme, we previously directly measured the dissociation of radiolabeled thiocolchicine from tubulin at 30°, inhibiting rebinding with nonradiolabeled colchicine, and obtained an apparent half-life of 84 hr (8) compared with the 263 hr half-life obtained during the present study at 32°.

Thus, the long inhibition decay half-life of 173 hr obtained with curacin A cannot be viewed as a true protein/drug complex dissociation half-life. Rather, this experiment only provides insight into relative activities, demonstrating that curacin A binds relatively tightly to tubulin. Its tight binding appears to be more closely related to that of the colchicinoids than to that of more rapidly reversible agents such as MTPT, CS-A4, and podophyllotoxin. If the possible effects of drug rebinding are ignored, the data of Fig. 3, together with the directly measured dissociation rate of thiocolchicine from tubulin (8), indicate that the actual half-life of the curacin A/tubulin complex at 32° is 50–70 hr.

Structure-activity analysis. The structural analogies of curacin A to colchicine-site drugs are unknown at the present, but speculation is irresistible. Fig. 4 represents a comparison of conformations of curacin A and colchicine derived with an energy minimization program (Allinger's MM2 Force Field algorithm), but this relatively linear conformation of curacin A is unlikely to exist in significant concentrations in aqueous solution. Like MTPT and CS-A4, curacin A binds rapidly at 0° to tubulin, leading us to speculate that the compound takes on a folded conformation that must closely

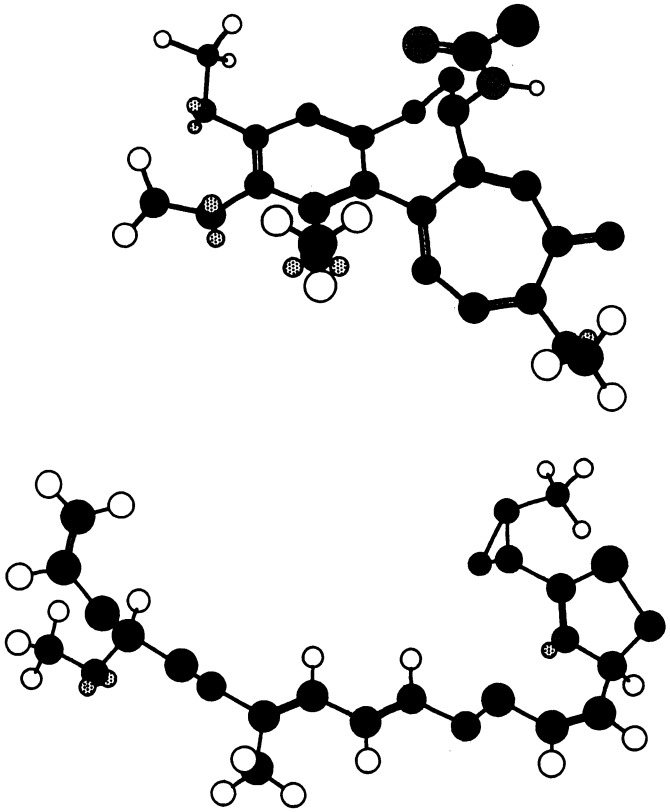

Fig. 4. Comparison of energy-minimized conformations of colchicine (*top*) and curacin A (*bottom*). The diagrams were obtained with the use of Allinger's MM2 Force Field algorithm on Chem3D-Plus 3.1 (Cambridge Scientific Computing). *Shaded*, carbon; *coarse stippled*, oxygen; *fine stippled*, nitrogen; *striped*, sulfur. Selected hydrogens were deleted for clarity.

resemble those of the phenyltropolone and the *cis*-stilbene. Presumably, this involves strong hydrophobic interactions between different domains of the curacin A molecule.

The two ends of the linear molecule are not critical in the interactions that yield the active conformation. Our results with 19-epicuracin A and 15,16-dihydrocuracin A, both at least as potent as curacin A, demonstrate this. This conclusion is further supported by the persistent, but reduced, activity obtained even after disruption of the cyclopropyl ring. The midportion of the hydrocarbon backbone of curacin A also does not appear to be critical for its interaction at the colchicine site. Our data indicate significant, and equivalent, activity with curacins B and C, which differ from curacin A in being in the *Z*-configuration about the C7–8 or C9–10 olefinic bonds.

In addition to the positive results presented in Table 3 with curacin A isomers and derivatives, we failed to find any activity in a large number of fatty acid derivatives (data not presented). Thus, it is not a "nonspecific" hydrophobic folding of the curacin A hydrocarbon backbone that results in its binding in the colchicine site. By elimination, these structure-activity observations suggest that the conformational analogy of curacin A to CS-A4/MTPT lies in the C10 and C13 substituents and/or possibly the thiazoline ring. Work with synthetic and semisynthetic derivatives are under way to determine whether this speculation is correct.

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