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Antimitotic Diterpenes from *Erythropodium caribaeorum* Test Pharmacophore Models for Microtubule Stabilization

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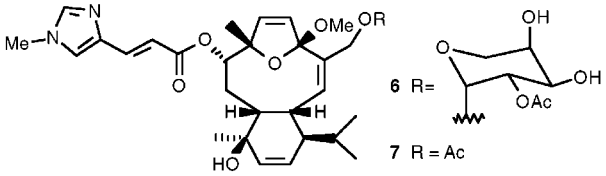
ABSTRACT



Six new antimitotic diterpenes, 2–7, have been isolated from the Caribbean octocoral *Erythropodium caribaeorum*. Structural variations encountered in this group of natural products test recently proposed pharmacophore models for microtubule stabilizing compounds.

Antimitotic compounds interfere with the dynamic assembly and disassembly of α- and β-tubulin into microtubules, causing cells to arrest in mitosis.[1] Prolonged arrest in mitosis eventually leads to cell death, mainly by apoptosis. Two chemical classes of antimitotic agents, the vinca alkaloids (vinblastine, vincristine, and vinorelbine) and the taxanes (paclitaxel and docetaxel), are clinically useful anticancer drugs. Most known antimitotic agents, including the vinca alkaloids, induce mitotic arrest by inhibiting the polymerization of tubulin into microtubules. Paclitaxel was the first chemical entity shown to cause mitotic arrest by stabilizing microtubules against depolymerization. Since the initial discovery of paclitaxel's mechanism of action and its introduction into clinical use, there has been much interest in finding other chemical structural types that also stabilize microtubules. Four additional chemotypes that have paclitaxel-like effects have subsequently been identified. These include the myxobacterium metabolites epothilones A and B,[2] the marine sponge metabolites discodermolide,[3] laulimalide, and isolaulimalide,[4] and the soft coral metabolite eleutherobin.[5] Ojima et al. have recently proposed a common pharmacophore for the microtubule stabilizing compounds that effectively accommodates nonataxel, paclitaxel, disco-

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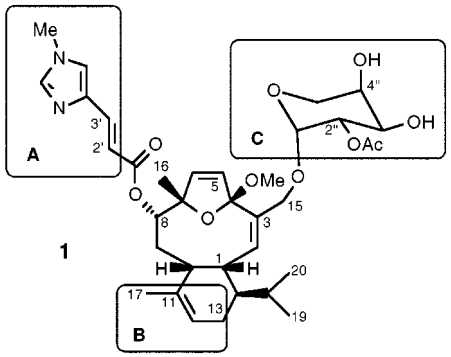
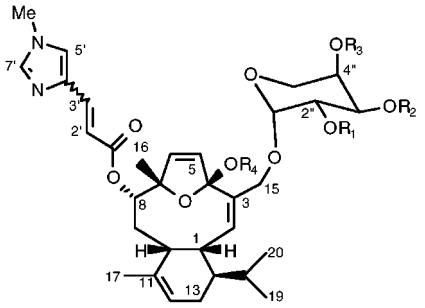
dermolide, eleutherobin, and the epothilones.[6] This model predicts that three regions of eleutherobin (**1**) (see boxes A, B, and C below) are important domains for binding to tubulin.



Most known antimitotic natural products were initially isolated because they exhibited potent in vitro cytotoxicity, and only subsequent mechanism of action studies revealed that they interfered with tubulin assembly and disassembly dynamics. Two classes of antimitotic agents, the epothilones[2] and the laulimalides,[4] have been discovered by rational screening, illustrating the significant potential for assay-directed identification of novel antimitotic chemotypes. Recently, a new cell-based antimitotic assay that is rapid and reliable has been developed in one of our laboratories.[7] Extracts of the octocoral *Erythropodium caribaeorum* collected at several sites in the Southern Caribbean showed potent activity in the assay.[8] Microscopic examination of cells arrested in mitosis by the *E. caribaeorum* extract showed evidence of tubulin bundling, similar to the effects of paclitaxel.

Bioassay guided fractionation of the *E. caribaeorum* extract led to the isolation of eleutherobin (**1**) and the new antimitotic diterpenoids desmethyleleutherobin (**2**), desacetyleleutherobin (**3**), isoeleutherobin A (**4**), *Z*-eleutherobin (**5**), caribaeoside (**6**), and caribaeolin (**7**). Compounds **3**−**7** all differ from eleutherobin in the proposed A, B, and C tubulin binding regions. In particular, caribaeoside (**6**) provides the first test for the B region in Ojima's model of the eleutherobin pharmacophore.

Freshly collected specimens of *E. caribaeorum* were frozen on site and transported to Vancouver over dry ice. Thawed samples (5.3 kg wet wt) were extracted multiple times with MeOH, and the combined MeOH extracts were concentrated to a gum in vacuo. Fractionation of the crude gum (280 g) by sequential application of vacuum reversed-phase flash (gradient elution: 80:20 H_2O/MeOH to MeOH in 10% increments), normal-phase flash (gradient elution: EtOAc to 80:20 EtOAc/MeOH in 2% increments), and normal-phase high-performance liquid chromatographies (eluent: 93:7 CH_2Cl_2/MeOH) gave pure samples of **1** (50 mg), **2** (7 mg), **3** (6 mg), **4** (3 mg), and **5** (2 mg). Compounds **6** (1 mg) and **7** (1 mg) partially decomposed on silica gel so they were isolated using only vacuum reversed-phase flash chromatography and cyano-bonded-phase HPLC (eluent: 56:42:2 EtOAc/hexane/$(^i\text{Pr})_2$NH).



2 R_1 = Ac; R_2 = R_3 = R_4 = H; $\Delta^{2',3'}$ (E)
3 R_1 = R_2 = R_3 = H; R_4 = Me; $\Delta^{2',3'}$ (E)
4 R_1 = R_3 = H; R_2 = Ac; R_4 = Me; $\Delta^{2',3'}$ (E)
5 R_1 = Ac; R_2 = R_3 = H; R_4 = Me; $\Delta^{2',3'}$ (Z)
8 R_1 = R_2 = R_3 = Ac; R_4 = Me; $\Delta^{2',3'}$ (E)

All NMR data for the *E. caribaeorum* diterpenes were recorded in DMSO-d_6 at 500 MHz. Tables of the NMR assignments are available in the Supporting Information. Eleutherobin (**1**) was identified by comparison of its spectroscopic data with literature values.[5a] Desmethyleleutherobin (**2**) was isolated as a clear oil that gave a $[M + H]^+$ ion in the HRFABMS at *m/z* 643.32230, appropriate for a molecular formula of $C_{34}H_{46}N_2O_{10}$ (ΔM −1.21 ppm), that differed from the molecular formula of eleutherobin simply by the loss of CH_2. The ^{1}H NMR spectrum of **2** differed from the ^{1}H NMR spectrum of eleutherobin (**1**) only by the absence of a methyl resonance at $\sim\delta$ 3.1 that could be assigned to the C-4 methoxyl substituent. This evidence indicated that **2** was identical to eleutherobin (**1**) except for the presence of a hydroxyl group instead of a methoxyl group at C-4. The 2D NMR data obtained for **2** was in complete agreement with this assignment.

Desacetyleleutherobin (**3**) was isolated as a clear oil that gave a $[M + H]^+$ ion at *m/z* 615.32813 in the HRFABMS corresponding to a molecular formula of $C_{33}H_{46}N_2O_9$ (ΔM −0.05 ppm), which differed from the formula of eleutherobin (**1**) by the loss of C_2H_2O. The ^{1}H NMR spectrum of **3** showed a strong resemblence to the ^{1}H NMR spectrum of eleutherobin (1) except for the absence of a methyl singlet at $\sim\delta$ 2 that could be assigned to an acetyl residue and the chemical shifts of the resonances assigned to the arabinose protons. These NMR differences suggested that **3** was simply the

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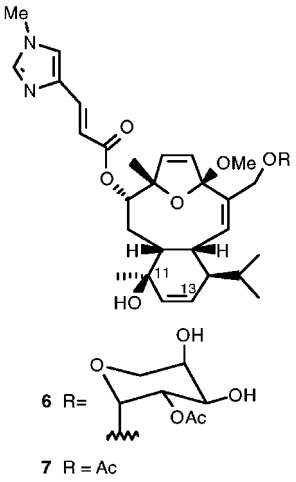
desacetyl analogue of eleutherobin (**1**). Acetylation of **3** with acetic anhydride in pyridine converted it to diacetyleleutherobin (**8**), which was identical to **8** prepared by acetylation of eleutherobin using the same reaction conditions, confirming the proposed structure of **3**.

Isoeleutherobin A (**4**), isolated as a clear oil, gave an $[M + H]^+$ ion at *m*/*z* 657.33834 in the HRFABMS corresponding to a molecular formula of $C_{35}H_{48}N_2O_{10}$ (ΔM -0.58 ppm), which was identical to the molecular formula of eleutherobin (**1**). Comparison of the ^{1}H 1D and 2D NMR data for isoeleutherobin A (**4**) with the data for eleutherobin (**1**) showed that the molecules differed only in the position of acetylation on the arabinose fragment. COSY correlations observed between resonances at δ 3.38 and 3.62 (both broad doublets: $J = 12.2$ Hz), assigned to the C-5″ methylene protons, and a methine at δ 3.83 (H-4″: m) showed that the acetate was not at C-4″. The H-4″ resonance in turn showed a COSY correlation to a resonance at δ 4.80 (dd, $J = 2.5$, 10.1 Hz), assigned to H3″, which was significantly deshielded relative to the corresponding H3″ resonance (δ 3.73) in eleutherobin (**1**). Therefore, isoeleutherobin A was assigned structure **4**. Acetylation with acetic anhydride in pyridine converted isoeleutherobin A (**4**) to diacetyleleutherobin (**8**), confirming the assigned structure of **4**.

Z-Eleutherobin (**5**) gave a $[M + H]^+$ ion at *m*/*z* 657.33830 in the HRFABMS appropriate for a molecular formula of $C_{35}H_{48}N_2O_{10}$ (ΔM -0.65 ppm), again identical to the molecular formula of eleutherobin (**1**). Comparison of the NMR data obtained for **5** with the data for **1** showed that the molecules differed only in the configuration of the $\Delta^{2',3'}$ olefin. In the ^{1}H NMR spectrum of *Z*-eleutherobin (**5**), the uroconic acid olefinic proton resonances appeared at δ 5.75 (H-2′) and 6.94 (H-3′) with a coupling constant of 12.6 Hz, whereas in the spectrum of eleutherobin (**1**) they were found at δ 6.35 (H-2′) and 7.53 (H-3′) with a coupling constant of 15.6 Hz. The NMR sample of *Z*-eleutherobin (**5**) partially isomerized over time to eleutherobin (**1**), confirming the assigned structure.



7 R = Ac

Caribaeoside (**6**), obtained as a colorless glass, gave a $[M + H]^+$ ion in the HRFABMS at *m*/*z* 673.33474 appropriate for a molecular formula of $C_{35}H_{48}N_2O_{11}$ (ΔM 1.64 ppm), that only differed from the molecular formula of eleutherobin (**1**) by the presence of one additional oxygen atom. Analysis of the NMR data obtained for caribaeoside (**6**) revealed that it too was a diterpene glycoside with the same *N*-(6′)-methyluroconic acid and 2″-*O*-acetylarabinose substituents that are attached to the central core of eleutherobin (**1**).

A number of features of the NMR data revealed that caribaeoside (**2**) and eleutherobin (**1**) differed in the C-11 to C-13 regions of their diterpene cores. The C-17 olefinic methyl resonance at δ 1.47 and the H-12 olefinic methine resonance at δ 5.27 in the ^{1}H NMR spectrum of eleutherobin (**1**) were both missing in the ^{1}H NMR spectrum of caribaeoside (**6**). In their place, the ^{1}H NMR spectrum of **6** had a singlet methyl resonance at δ 0.82 and a pair olefinic methine resonances at δ 5.52−5.54 (H-12 and H-13). The two-proton olefinic resonance showed correlations in the HMQC spectrum to carbon resonances at δ 125.6 (C-13) and 137.4 (C-12). HMBC correlations observed between the Me-17 singlet at δ 0.82 and the C-12 olefinic resonance at δ 137.4, a quaternary carbon resonance at δ 68.3, and a methine resonance at δ 45.7 (HMQC to δ 2.07) confirmed the proximity of Me-17 and C-12 and indicated that there was a hydroxyl substituent at C-11 and a methine carbon at C-10.[9] A pair of overlapping doublets (6H) at δ 0.94−0.95, that showed COSY correlations to a methine resonance at δ 1.68, were assigned to the Me-19 and Me-20 isopropyl protons, and a multiplet at δ 4.00, that showed COSY correlations to an olefinic doublet at δ 5.38 (H-2) and a methine resonance at δ 2.07 (H-10), was assigned to H-1. The H-1 resonance in the spectrum of **6** had a chemical shift and multiplicity nearly identical to the H-1 resonance in eleutherobin (**1**) (δ 3.88), consistent with the proposal that the C-1, C-2, C-10, and C-14 centers in **6** were identical to the corresponding sites in **1**.

ROESY and scalar coupling constant data established the relative stereochemistry about the cyclohexene ring in caribaeoside (**6**). The resonances assigned to H-1 (δ 4.00) and H-2 (δ 5.38) in **6** had chemical shifts and a vicinal coupling constant ($J = 9.7$ Hz) nearly identical with their counterparts in eleutherobin (**1**) (δ H-1, 3.88; H-2, 5.39: $J = 9.4$ Hz), indicating that the dihedral angle between them in **6** was essentially identical to that in **1**. ROESY correlations observed between the isopropyl methyl proton resonances at δ 0.94−0.95 and the H-1 (δ 4.00) and H-10 (δ 2.07) resonances in **6**, demonstrated that the isopropyl group, H-1, and H-10 were on the same face of the molecule, as in eleutherobin (**1**). The Me-17 resonance at δ 0.82 in **6** showed a strong ROESY correlation to the H-2 (δ 5.38) resonance demonstrating that Me-17 and C-2 were cis. Models indicate that the Me-17 protons can sit in the shielding region of the $\Delta^{2,3}$ olefin, consistent with their unusually shielded chemical shift of δ 0.82. ROESY correlations observed between Me-16 (δ 1.33) and both H-8 (δ 4.85) and OMe-21 (δ 3.08), and between H-8 and H-10 (δ 2.07), confirmed that car-

(9) Sarcodictyin F, a related 4,7-oxaeunicellane diterpenoid, has a similarly functionalized cyclohexene ring, but with the opposite stereochemistry at C-11. See: D'Ambrosio, M.; Guerriero, A.; Pietra, F. *Helv. Chim. Acta* **1988**, *71*, 964−976.

ibaeoside (**6**) and eleutherobin (**1**) had identical relative stereochemistries at C-4, C-7, C-8, and C-10.

Caribaeolin (**7**) was isolated as a clear oil that gave a $[M + H]^+$ ion in the HRFABMS at *m/z* 541.29111 corresponding to a molecular formula of $C_{30}H_{40}N_2O_7$ (ΔM −0.49 ppm). Analysis of the 1D and 2D ^1H detected NMR data obtained for **7** revealed that it contained the diterpene core and *N-(6′)*-methyluroconic acid fragments that constitute the aglycon of caribaeoside (**6**) but was missing the arabinose sugar residue. COSY and ROESY correlations were observed between an olefinic methine resonance at δ 5.38, assigned to H-2, and a broad two proton singlet at δ 4.46, assigned to the H-15 methylene protons. HMBC correlations were observed between a carbonyl resonance at δ 169.9 and both the H-15 methylene proton resonance at δ 4.46 and a singlet methyl resonance at δ 1.97. These HMBC correlations demonstrated that in caribaeolin (**7**) a C-15 acetyl substituent was present in place of the C-15 arabinose sugar residue found in caribaeoside (**6**). Strong ROESY correlations were observed between the Me-17 resonance at δ 0.77 and the H-2 olefinic proton resonance at δ 5.38, indicating that Me-17 and C-2 were cis to each other as in caribaeoside (**6**). Additional ROESY correlations observed between the C-19/C-20 isopropyl methyl proton resonances at δ 0.95−0.96 and the H-1 (δ 4.01) and H-10 (δ 2.07) resonances, between the Me-16 (δ 1.34) and both of the H-8 (δ 4.85) and OMe-21 (δ 3.08) resonances, and between the H-8 and H-10 (δ 2.07) resonances confirmed that the relative sterochemistry in caribaeolin (**7**) was the same as in caribaeoside (**6**).

All of the *E. caribaeorum* diterpenoids **1**−**7** reported above showed antimitotic activity in the cell-based assay.[7] Desmethyleleutherobin (**2**) (IC_{50} 20 nM) and isoeleutherobin A (**4**) (IC_{50} 50 nM) were both slightly more potent than eleutherobin (**1**) (IC_{50} 100 nM), *Z*-eleutherobin (**5**) (IC_{50} 250 nM) was comparable in activity to eleutherobin (**1**), desacetyleleutherobin (**3**) (IC_{50} 400 nM) was slightly less potent, while caribaeoside (**6**) (IC_{50} 20 μM) and caribaeolin (**7**) (IC_{50} 20 μM) were considerably less potent that eleutherobin (**1**).

Many synthetic analogues of eleutherobin (**1**) have been prepared as part of SAR studies;[10] however, to date they have all been based on the eleutherobin diterpenoid core. The Ojima pharmacophore proposal implies that changes in the C-11−C-13 region of eleutherobin should have an impact on the ability of analogues to stabilize tubulin polymers. Caribaeoside (**6**) represents the first such analogue to be tested for antimitotic activity. The significant decrease in antimitotic potency of caribaeoside (**6**) relative to eleutherobin (**1**), resulting from introduction of a hydroxyl group at C-11 and migration of the olefin to the $\Delta^{12,13}$ position, provides further support for Ojima's pharmacophore model. The structural changes in **6** alter both the shape and polarity of the diterpene core in the tubulin binding region B of the proposed pharmacophore.

A number of other features of the antimitotic potencies are also noteworthy. Altering the $\Delta^{2',3'}$ configuration (i.e., **5**), a change in the A region of the pharmacophore, has little effect, while alterations in the arabinose fragment, representing changes in the C region of the pharmacophore, can either enhance (i.e., **4**) or decrease (i.e., **3**) the potency. Changing the C-4 substituent from methoxyl (i.e., **1**) to hydroxyl (i.e., **2**), an alteration that is formally outside of the Ojima pharmacophore binding regions, leads to a slight increase in potency. Replacement of the arabinose fragment in caribaeoside (**6**) with a simple acetate residue (i.e., **7**) results in no additional loss of potency, which is consistent with previous observations.[6]

In summary, the current study has identified a new and relatively high-yielding source of eleutherobin (**1**), whose preclinical development has been impeded by its scarcity, as well as providing a series of new eleutherobin analogues **2**−**7** that serve as a further test of recent pharmacophore models for microtubule stabilization.

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Supporting Information Available: Tables of optical rotations, UV data, and ^1H and ^{13}C NMR assignments for compounds **1**−**8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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