Tolypodiol, an Antiinflammatory Diterpenoid from the Cyanobacterium *Tolypothrix nodosa*

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A new diterpenoid, tolypodiol (**1**), has been isolated from the terrestrial cyanobacterium *Tolypothrix nodosa* (HT-58−2) and identified by NMR and mass spectral analysis. The monoacetate derivative **2** was prepared. Tolypodiol (**1**) and its monoacetate derivative **2** show potent antiinflammatory activity in the mouse ear edema assay.

Reports of the occurrence of terpenoids in cyanobacteria (blue-green algae) are uncommon. The few examples known are the bacteriohopanes,[1−3] triterpenes isolated from several species of cyanobacteria that act as membrane stabilizers;[4−5] the antifungal hapalindoles,[6−9] hapalindolinones[10−11] and ambiguines,[12] and the welwitindolinones.[13] All of these compounds, with the exception of the bacteriohopanes, are metabolites of mixed biosynthetic origin, contain isoprene units, and were isolated from several species of the Stigonemataceae family (order Stigonematales).

We have previously reported the structures of tolyporphin A[14] and eight analogues, the tolyporphins B−I,[15] isolated from an extract of the cyanophyte *Tolypothrix nodosa*, which was isolated from a soil sample collected at Nan Madol, Pohnpei. In a continuing investigation of this extract, we report here the isolation and structure elucidation of the diterpenoid, tolypodiol (**1**). This is the first reported occurrence of a diterpenoid compound in a cyanobacterium. The structurally related compounds taondiol[16,17] and epitaondiol[17] have been isolated previously from marine brown algae.

Isolation and culture of the cyanobacterium was carried out by a general procedure that has been described elsewhere.[18] The freeze-dried cyanophyte was extracted with CH_2Cl_2/2-propanol (1:1) and the extract subjected to reversed-phase and repeated normal-phase chromatography to give tolypodiol (**1**) in 0.14% yield.

The structure of tolypodiol was deduced by spectral analysis and confirmed by chemical modification. HREIMS established the molecular formula $C_{28}H_{40}O_5$. Most of the structure was determined by the use of 1D and 2D NMR spectroscopic techniques. The ^{1}H-NMR spectrum of tolypodiol contained five methyl signals, a methoxy resonance, signals indicative of a 1,2,4-substituted aromatic ring, and protons attached to oxygenated carbons, in addition to a number of aliphatic proton signals. Quaternary carbon signals in the ^{13}C-NMR spectrum of tolypodiol at 167.30 and 157.65 ppm were characteristic of an acetate carbon and an oxygenated aromatic carbon, respectively. Additional quaternary carbon signals at 121.44 and 122.13 ppm and three methine carbon signals at 116.97, 129.06, and 131.99 ppm confirmed the presence of a trisubstituted aromatic ring.

A DEPT NMR spectrum established the multiplicity of each carbon. Chemical shifts of a quaternary carbon at 77.85 ppm and methine carbons at 68.91 and 80.59 ppm, respectively, indicated that these carbons were oxygenated. The remainder of the structure was pieced together by COSY, TOCSY, HMQC, and HMBC NMR experiments.

The relative stereochemistry of tolypodiol was established from H−H coupling constant considerations and from a NOESY experiment. The stereochemistry of fusion of the ring junctions was trans in all cases, with the methyl groups at these junctions being β. That the hydroxyl group at C-1 was also β was deduced from examination of the coupling constants of H-1. Values of 5.4 and 10.6 Hz for the H-1 coupling constants, corresponding to couplings to H-2α and H-2β protons, respectively, implied that H-1 was in an axial, and therefore α, orientation. The NOEs observed from H-1 to H-2α, H-2β, H-5α, and H-9α confirmed this stereochemical assignment.

Similarly, the C-6 hydroxyl was determined to be β, because the values of the coupling constants of H-6 were 2.2, 2.6, and 5.9 Hz. Three small coupling constants could only be observed if H-6 were equatorial (and therefore α), whereas if it were β, two large diaxial and one small axial−equatorial coupling would be observed. Observed NOE correlations from H-6 to H-5α and to α methyl protons at C-22 again confirmed the stereochemical assignment.

Hence, the structure **1** was postulated. To check that there was indeed a dihydropyran ring fused between ring c of the diterpene nucleus and the aromatic ring, and that there were not instead two hydroxyl groups attached to C-13 and C-17, with resultant loss of H_2O from the parent under mass spectral conditions, a

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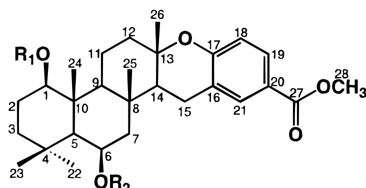
sample of tolypodiol was acetylated with acetic anhydride/pyridine (1:1). The ^{1}H-NMR spectrum of the product contained only one acetate resonance, confirming the presence of the dihydropyran ring as opposed to two hydroxyl groups. The ^{13}C-NMR spectrum contained an additional signal for the acetyl carbonyl resonance at 170.52 and for the acetate methyl carbon at 21.98 ppm.

The site of acetylation was determined to be C-1, as opposed to C-6, by comparison of ^{1}H- and ^{13}C-NMR chemical shifts in the parent **1** and in the monoacetate derivative **2**. In the ^{1}H-NMR spectrum of tolypodiol monoacetate (**2**), the H-1 resonance had shifted downfield from 3.30 to 4.52 ppm. Similarly, in the ^{13}C-NMR spectrum of **2**, the C-1 resonance shifted from 80.59 to 82.93 ppm. The chemical shifts of the H-1 and C-1 resonances on acetylation are entirely consistent with acetylation occurring at the 1-position. Lack of acetylation at C-6 can be explained by the extreme hinderance of the position from adjacent methyl groups, and this corroborates the stereochemical assignment. Treatment of **1** with acetic anhydride/pyridine for longer periods and addition of dimethylamino pyridine (DMAP) as a catalyst, failed to produce any acetylation at C-6.



Overlap of signals in the ^{1}H-NMR spectra of compounds **1** and **2**, even at 500 MHz, prevented complete determination of coupling constants. The ^{13}C-NMR spectra were, however, completely assigned with the aid of HMQC and HMBC experiments.

Tolypodiol showed strong antiinflammatory activity in the mouse ear edema assay, with an ED_{50} of 30 μg/ear. The monoacetate **2**, by comparison, was still active but considerably less potent, with an ED_{50} of 60 μg/ear. The ED_{50} value of tolypodiol compares very favorably with those obtained for the standards, hydrocortisone (20 μg/ear) and manoalide (100 μg/ear) in the same assay.[19] Further studies to determine the mechanism of inhibition of **1** are underway.

Experimental Section

General Experimental Procedures. NMR spectra were determined on an 11.75 T instrument, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. ^{1}H-NMR chemical shifts are referenced in $CDCl_3$ to residual $CHCl_3$ (7.26 ppm). ^{13}C-NMR chemical shifts are referenced in $CDCl_3$ to the solvent (77.0 ppm). Homonuclear ^{1}H- connectivities were determined with the COSY experiment, and heteronuclear ^{1}H-^{13}C connectivities were determined by HMQC and HMBC experiments. Mass spectra were recorded on a VG 7070E mass spectrometer, operating at a nominal accelerating voltage of 70 eV.

Organism and Culture Conditions. *T. nodosa*, designated strain HT-58-2, was isolated from a soil sample collected at Nan Madol, Pohnpei. The culture is deposited in the culture collection at the Department of Chemistry, University of Hawaii. Clonal cultures were prepared by repeated subculture on solidified media. The cyanophyte was cultured in 20-L glass bottles containing a modification of inorganic medium BG-11.[20] Prior to autoclaving, the pH of the medium was adjusted to 7.0 with NaOH. Cultures were illuminated continuously at an incident intensity of 25 μmol photons PAR (photosynthetically active radiation) m^{-2} s^{-1} from banks of cool-white fluorescent tubes, aerated at a rate of 5 L/min with a mixture of 0.5% CO_2 in air, and incubated at a temperature of 24 $\pm$ 1 °C. After 28 days, the cyanophyte was harvested by filtration and freeze-dried. Yields of lyophilized cells ranged from 0.17 to 0.33 g/L.

Isolation of Tolypodiol (1). Freeze-dried cyanophyte (192 g) was extracted in batches in a blender with 4 $\times$ 500 mL portions of CH_2Cl_2/2-propanol (1:1). The combined extract was filtered and the solvent removed *in vacuo*. The crude extract (15.5 g) was divided in two, and each batch was subjected to step-gradient, reversed-phase flash chromatography on a C-18 column (80 g, YMC Gel ODS-A, 12-nm particle size) and then to repeated normal-phase column chromatography on Si gel (Davisil 200−425 mesh, 60Å) using a hexane/EtOAc gradient to yield **1** (276 mg) as an amorphous white solid, pure by TLC analysis (si gel, hexane/EtOAc 3:1, R_f = 0.3), α_D^{25} −2.9° (*c* 0.002, MeOH), ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.30 (1H, dd, J = 10.6, 5.4 Hz, H-1α), 1.65* (1H, m, H-2α), 1.68* (1H, m, H-2β), 1.31 (2H, m, H_2-3), 0.76 (1H, d, J = 2.2 Hz, H-5α), 4.46 (1H, ddd, J = 2.2, 2.6, 5.9 Hz, H-6α), 2.04 (1H, m, H-7α), 1.23 (1H, m, H-7β), 1.21 (1H, obscured, H-9α), 2.78 (1H, dddd, J = 14.1, 6.0, 3.0, 4.0 Hz, H-11α), 1.21 (1H, obscured, H-11β), 1.72 (1H, m, H-12α), 2.08 (1H, m, H-12β), 1.53 (1H, dd, J = 6.3, 11.7 Hz, H-14α), 2.65 (2H, m, J = 6.3, 11.7 Hz, H_2-15), 6.73 (1H, dd, J = 8.5, 0.6 Hz, H-18), 7.72 (1H, dd, J = 8.5, 2.1 Hz, H-19), 7.76 (1H, dd, J = 2.1, 0.6 Hz, H-21), 0.92 (3H, s, H-22), 1.19* (3H, s, H-23), 1.28 (3H, s, H-24), 1.25 (3H, s, H-25), 1.20* (3H, s, H-26), 3.84 (3H, s, H-28); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 80.59 d (C-1), 30.14 t, (C-2), 41.83 t (C-3), 33.90 s (C-4), 55.77 d (C-5), 68.91 d (C-6), 48.20 t (C-7), 37.53 s (C-8), 61.73 d (C-9), 43.24 s (C-10), 21.17 t (C-11), 41.09 t (C-12), 77.85 s (C-13), 52.54 d (C-14), 22.28 t (C-15), 122.13 s (C-16), 157.65 s (C-17), 116.97 d (C-18), 129.06 d (C-19), 121.38 s (C-20), 131.99 d (C-21), 32.65 q (C-22), 23.51 q (C-23), 13.21 q (C-24), 16.52 q (C-25), 20.87 q (C-26), 167.30 s (C-27), 51.82 q (C-28). *Sequential values may be interchanged; HREIMS m/z found $[M]^+$ 456.28706 ($C_{28}H_{40}O_5$ requires 456.28758).

Acetylation of Tolypodiol (1). A solution of tolypodiol (5.9 mg) in Ac_2O/pyridine (1:1) was stirred overnight at room temperature. The solvent was removed, toluene (2 mL) added, and the solution evaporated to dryness ($\times$3) to yield the monoacetate **2** (5.6 mg) as a brown oil, pure by TLC analysis (silica gel, hexane/EtOAc 3:1, R_f = 0.5), ^{1}H-NMR (500 MHz, $CDCl_3$) δ 4.52 (1H, m, H-1α), 1.76 (2H, m, H_2-2), 1.33 (2H, m, H_2-3), 0.89 (1H, d, J = 2.5 Hz, H-5α), 4.52 (1H, m, C-6α), 2.01 (1H, m, H-7α), 1.31 (1H, m, H-7β), 1.23 (1H, m, H-9α), 2.68 (1H, m, H-11α), 1.20 (1H, obscured, H-11β), 1.75 (1H, m, H-12α), 2.03 (1H, m, H-12β), 1.53 (1H, dd, J = 6.6, 11.5 Hz, H-14α), 2.67 (2H, m, H_2-15), 6.74 (1H, d, J = 8.5 Hz, H-18), 7.74 (1H, dd, J = 8.5, 2.1 Hz, H-19),

7.78 (1H, d, J = 2.1 Hz, H-21), 0.97 (3H, s, H_3-22), 1.24 (3H, s, H_3-23), 1.41 (3H, s, H_3-24), 1.26 (3H, s, H_3-25), 1.20 (3H, s, H_3-26), 3.86 (3H, s, H_3-28), 2.04 (3H, s, H_3-30); ^{13}C-NMR (500 MHz, $CDCl_3$) δ 82.93 d (C-1), 25.10 t (C-2), 41.21 t (C-3), 33.87 s (C-4), 55.80 d (C-5), 68.83 d (C-6), 48.01 t (C-7), 37.45 s (C-8), 60.94 d (C-9), 42.16 s (C-10), 20.67 t (C-11), 40.86 t (C-12), 77.29 s (C-13), 52.38 d (C-14), 22.21 t (C-15), 121.87 s (C-16), 157.47 s (C-17), 116.95 d (C-18), 129.02 d (C-19), 121.44 s (C-20), 131.89 d (C-21), 32.57 q (C-22), 23.57 q (C-23), 14.55 q (C-24), 16.51 q (C-25), 20.82 q (C-26), 167.17 s (C-27), 51.75 q (C-28), 170.53 s (C-29), 21.98 q (C-30); HREIMS m/z found $[M]^+$ 498.29862 ($C_{30}H_{42}O_6$ requires 498.29814).

Mouse Ear Edema Assay. Test samples in an Me_2CO solution containing an edema-causing irritant, phorbol 12-myristate 13-acetate (PMA), were topically applied to the inside pinnae of mouse ears. PMA alone (2 μg/ear) or in combination with 100, 50, 25, 12.5, or 6.25 μg/ear of test compound was applied to left ears (5 mice per treatment group) and Me_2CO applied to all right ears. After 200 min incubation, mice were sacrificed, ears removed, and bores taken and weighed. Edema is measured by subtracting the weight of right ear (Me_2CO control) from the weight of the left ear (treated). Results are recorded as percent decrease (inhibition) or percent increase (potentiation) in edema relative to a PMA control group edema, and the ED_{50} is calculated from these results.

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