Two New Cytotoxic Peroxide-Containing Acids from a New Guinea Sponge, Callyspongia sp.

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TWO NEW CYTOTOXIC PEROXIDE-CONTAINING ACIDS FROM A NEW GUINEA SPONGE, *CALLYSPONGIA* SP.

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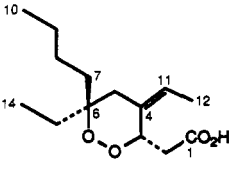
ABSTRACT.—Two new C_{14} cytotoxic, branched-chain acids, **1** and **2**, containing a cyclic peroxide moiety were isolated from a sponge, *Callyspongia* sp., collected in New Guinea. The structures were elucidated through extensive spectral analysis.

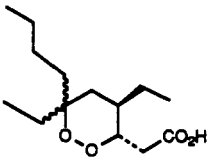
A number of straight-chain (1–5) and branched-chain fatty acids (6–10) containing a cyclic peroxide moiety have been isolated from marine sponges. Steroidal and terpenoid peroxides have also been reported from marine organisms (11). Many of the cyclic-peroxide-containing acids have been reported to be cytotoxic or exhibit other types of biological activity. We describe here two new, cytotoxic cyclic-peroxide-containing acids isolated from a sponge, *Callyspongia* sp. Duchassaing & Michdotti (family Callyspongidae), collected in New Guinea in the course of our continuing search for potential tumor-inhibitory compounds.

Freshly-thawed sponge specimens were extracted with MeOH and $CHCl_3$/MeOH several times and the combined, concentrated extracts then partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ solubles were chromatographed over Si gel and the fractions of interest were finally purified by C_{18} reversed-phase hplc using a H_2O-MeOH mixture (1:3) containing 1% of HCO_2NH_4. This gave excellent resolution of the acid components into sharp bands whereas aqueous MeOH alone produced broad peaks with poor resolution. The elution order was **1**, **2**, with **1** being the major component.

Compound **1** did not yield a molecular ion peak in its eims, but a peak at 279.1577 $[M+Na]^+$ was observed in the hrfabms corresponding to a formula of $C_{14}H_{24}O_4$ (calcd 279.1572) implying three degrees of unsaturation. This was corroborated by a negative-ion fabms peak corresponding to $(M-H)^-$ at 255.1605 (calcd for $C_{14}H_{23}O_4$, 255.1596). The presence of a carboxyl group was inferred from the infrared spectrum [3450–2300 (br), 1712 cm^{-1}] and this was substantiated by a ^{13}C-nmr peak at 174.2 ppm. The DEPT spectrum revealed the presence of a trisubstituted double bond [131.1 (s), 122.2 (d)], two oxygenated carbons [-C-O, 84.6 (s); -CHO, 75.8 (d) ppm], three methyl groups [14.2, 12.7, 7.4, ppm], and six methylene groups [38.1, 36.0, 31.5, 29.0, 25.2, 23.2 ppm]. Because the double bond and carboxyl group account for only two degrees of unsaturation, one ring must be present. From the COSY spectrum, decoupling, and chemical shift considerations, partial structures **A**–**C** outlined below could be deduced. The chemical shift of the methine proton of partial structure **A** suggested that the carbon to which this proton is attached may be allylic as well as oxygenated. Furthermore, an nOe was observed with the vinyl methyl group when the proton resonating at 5.20 ppm was irradiated. Hence, the olefinic carbon of fragment **B** could be attached to the open valence of partial structure **A**. The methylene protons of partial structure **B** exhibited geminal



1



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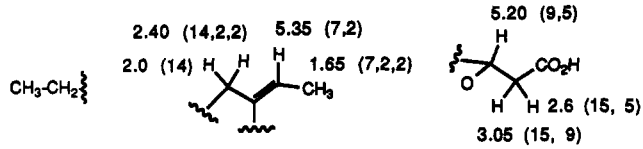

C B A

coupling (14 Hz) and one of them (2.4 ppm) was also coupled to both the vinyl proton and the vinyl methyl group. The lack of further coupling for the methylene group of **B** indicates it must be attached to a quaternary center on one side, hence to the oxygenated quaternary carbon resonating at 84.6 ppm.

From a COSY spectrum measured at 40° in $CDCl_3$ after exchange with D_2O, one could observe that the methyl triplet at 0.83 ppm was coupled with two methylene protons, 1.56 (J=14.4, 7.2 Hz) and 1.42 (J=14.4, 7.2) ppm, each a doubled quartet, and these were not further coupled. Hence, an ethyl group attached to the oxygenated quaternary carbon (84.6 ppm) must be present and by difference the remaining methyl group and three methylenes must comprise an *n*-butyl group, also attached to the oxygenated quaternary carbon. Because this accounts for all of the elements in the molecular formula, the required ring must be formed by joining the oxygens on the quaternary and methine carbons to give structure **1** containing a peroxide moiety.

The double bond configuration was deduced to be *Z* from nOe data obtained in $CDCl_3$; irradiation of H-3 induced an enhancement of the vinyl methyl signal. Because the H-3 proton exerts a strong nOe on the signal for the vinyl methyl group although neither of the H-2 protons show nOe effects with the vinyl methyl, we conclude that H-3 is pseudoequatorially disposed and the acid side-chain is pseudoaxially oriented. The chemical shifts of the H-2 protons (δ 2.6 and 3.1) of **1** are also more consistent with the shifts of the corresponding protons in plakortin (δ 2.35, 3.05; axial -CH_2-CO_2Me group) (6) than with those of epiplakortin (δ 2.38, 2.66; equatorial -CH_2CO_2Me group) (7).

Energy minimization calculations on structure **1** (Chem 3D; substituting a CH_2 for the peroxide oxygen at C-6) revealed that the ring adopts a conformation in which the dihedral angle between H-5ax (2.40 ppm, J=14, 2.5, 2.2 Hz) and the vinyl proton is ca. 98°, in good agreement with the large allylic coupling observed between these protons (12).

Irradiation of the H-5ax signal induced a small nOe in two of the signals due to the C-6 ethyl group, i.e., a 0.83 ppm triplet and a 1.42 ppm doublet of quartets. Hence the ethyl group would seem to be equatorial, and thus the acid side-chain and C-6 ethyl group are assigned a cis relationship.

The molecular formula of acid **2** was indicated from hr positive-ion, lr negative-ion fabms and thermospray ms to be $C_{14}H_{26}O_4$, two mass units more than that of **1**. All its spectral characteristics indicated that it was a 4,11-dihydro analog of **1**. The only unsaturation evident from spectral data was due to a carboxyl group [ir 3450–2400 (br), 1710 cm^{-1}, ^{13}C nmr 177.5 (s) ppm]. Hence **2** possessed one ring. Like **1**, acid **2** also possessed only two oxygenated sp^3 carbons: a quaternary carbon absorbing at 83.7 ppm and a methine carbon resonating at 82.5 ppm, the latter confirmed by an HMQC experiment. From the H/H COSY nmr spectrum, it was clear that the only downfield signal, 4.02 ppm (J=10.5, 9.7, 2.4 Hz, H-3) was coupled to a mutually coupled pair of protons absorbing at 2.18 and 2.35 ppm (H-2, -2′), and another proton whose absorption appeared at ca. 1.39 ppm (coincident with two other proton signals). The latter unresolved multiplet (see below) was in turn correlated with two sharp, well-resolved, and mutually coupled signals at 1.55 (dd, 13.4, 4.5 Hz, H-5α) and 0.98 (dd,

13.4, 12 Hz, H-5β) ppm. The large mutual coupling between the latter two protons and the large (12 Hz) vicinal coupling of the 0.98 proton indicate that this methylene group is in a fixed ring system in which one of the protons has a vicinal axial neighboring proton, i.e., H-4. Since all other protons are accounted for by upfield methyl and methylene signals, the final two oxygens present in **2** must form a peroxide ring as in **1**.

Although the H-4 signal at ca. 1.39 ppm coincides with two other proton resonances, the HMQC nmr spectrum clearly shows that one of these signals is a methine proton. The other two signals could be assigned to one of the H-9 protons and one of the H-11 protons by a combination of H/H COSY nmr data and proton carbon correlations (HMQC).

A broad triplet, $J=13.8$ Hz, was observed at 1.71 ppm, clearly separated from other signals, and this was assigned to one of the H-7 protons since this signal showed correlation with only three other resonances: 1.39 (H-7′), 1.22 (H-8) and 1.03 (H-8′) ppm. These assignments were supported by the HMQC nmr spectrum.

Because both H-3 and H-5β show large couplings to H-4 (10–12 Hz), all of these protons must be axially oriented; hence both the acid side-chain at C-3 and the ethyl group at C-4 must be equatorial. In agreement with this assignment, it may be noted that the chemical shifts of the H-2 protons of **2**, 2.18 and 2.35 ppm, are more similar to the corresponding signals in epiplakortin (2.38, 2.66 ppm, equatorial acid side-chain) (7) than for plakortin (2.35, 3.05 ppm, axial acid side-chain) (6).

Additional support for structure **2** was derived from results of a COSY nmr spectrum and nOe measurements taken in C_6D_6 (all above data for **2** were obtained in $CDCl_3/C_6D_6$ mixture). In C_6D_6, the three methyl triplet signals of **2** were well-resolved. The ^{1}H-nmr assignments for these signals could best be made by correlation with their respective carbon signals. Two of the methyl ^{13}C-nmr signals in **2** (14.7 and 7.9 ppm) are nearly the same as in **1** (14.2, 7.4 ppm) as would be expected for C-10 and C-14, respectively. The 7.9 or 7.4 ppm absorptions fit very well for the ^{13}C-nmr signals of a methyl of an ethyl group attached to a quaternary center, while the ca. 14 ppm signal is typical for that of the methyl of an *n*-butyl chain (10,13). The corresponding proton resonances for H-10 and H-14 could then be assigned as 0.87 and 0.73 ppm from an H/C correlation spectrum. The remaining methyl resonance, 0.61 ppm assigned to H-12, correlated with the carbon signal at 11.2 ppm. The COSY nmr spectrum for **2** in C_6D_6 showed that H's-12 were correlated with two protons resonating at 0.99 and 0.68 (H's-11) and these showed correlation with the three proton multiplet at ca. 1.38–1.42, which in turn showed correlation with H's-5 and H-3. These correlations are consistent with structure **2**. Irradiation of H-12 (0.61 ppm), which unavoidably also caused some irradiation of H-11 at 0.68 ppm, enhanced a complex multiplet at ca. 1.42 ppm (assigned to H-4), an enhancement that is consistent with structure **2**.

Due to partial overlap of the signals for various protons, especially H-14, H-12, and H-11, the results of nOe experiments did not provide unambiguous proof of the relative stereochemistry at C-6. A similar difficulty has been noted in related peroxy aliphatic acids (10).

Acids **1** and **2** inhibited murine leukemia cell growth: ED_{50} (P-388)=5.5 and 2.6 μg/ml, respectively, for **1** and **2**.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were obtained using Varian VXR-500 and XL-300 spectrometers (500 and 300 MHz for ^{1}H and 100 and 75.4 MHz for ^{13}C, respectively) in the solvents specified. Ir spectra were taken on a Bio-Rad FTS-7 Ft-ir spectrophotometer. Fabms were obtained using a VG ZAB-E spectrometer.

Flash chromatography columns were made using Merck Si gel 60H. Thin-layer chromatograms were run on precoated Kodak chromatogram sheets with silica adsorbent and fluorescent indicator, or on Whatman MKC18F reversed-phase tlc plates. Alltech Econosphere C_{18} 5μm, 10 mm×30 cm, and Alltech Econosphere silica 5μm, 10 mm×30 cm, columns were used in the hplc separations. All solvents used in the extraction and separations were distilled prior to use.

EXTRACTION AND ISOLATION.—The sponge was collected off the coast of New Guinea in the vicinity of Madang using Scuba at a depth of 15 m and frozen. A voucher specimen, 24-NG-87, is kept at the University of Oklahoma. The frozen specimen (224 g wet wt) was homogenized and extracted sequentially for 24 h periods with MeOH (1 liter), distilled H_2O (1 liter), MeOH (1 liter), and $CHCl_3$/MeOH (1:1,4×1 liter). The organic extracts were combined and the solvents removed by evaporation *in vacuo*. This residue was then combined with the distilled H_2O extract and the mixture partitioned between H_2O and $CHCl_3$ (5×500 ml). The combined $CHCl_3$ extracts were evaporated *in vacuo* to give a dark brown gum (2.0 g). This residue was then redissolved in $CHCl_3$-MeOH (1:1) and preabsorbed onto Si gel by evaporation of the solvent. The preabsorbed extract was then applied to a Si gel column and eluted, using vacuum flash chromatography with step gradients of $CHCl_3$ to EtOAc followed by step gradients of $CHCl_3$ to $CHCl_3$-MeOH (1:1).

Relevant fractions containing the peroxide compounds (tlc analysis) were then pooled and rechromatographed over Si gel eluting with a step gradient varying from $CHCl_3$ to $CHCl_3$-MeOH (2:1). Fractions containing the peroxides were finally resolved by C_{18} reversed-phase hplc (1% HCO_2NH_4/33% H_2O/MeOH), with the elution order being **1**, **2**.

Acid **1**.—17.7 mg: faint yellow gum; [α]D +50° (*c*=0.72, $CHCl_3$); ir ($CHCl_3$) ν max 3450-2300 (br), 1712 cm^{-1}; positive-ion hr fabms calcd for $C_{14}H_{24}O_4$+Na, 279.1572, found 279.1572; negative-ion hr fabms calcd for $C_{14}H_{23}O_4$, 255.1596, found 255.1605; lr positive-ion fabms *m/z* $[M-H+2\,Na]^+$ 301 (32), $[M+Na]^+$ 279 (100), lr negative-ion fabms *m/z* $[M-H]^-$ 255 (100); ^1H nmr (40° in $CDCl_3$/1 drop D_2O) δ 5.35 (dq, *J*=2.2, 7.0 Hz, H-11), 5.20 (dd, *J*=8.8, 5.0 Hz, H-3β), 3.05 (dd, *J*=15.5, 8.8 Hz, H-2α), 2.60 (dd, *J*=15.5, 5.0 Hz, H-2β), 2.40 (ddd, *J*=14, 2.5, 2.2 Hz, H-5α), 2.00 (d, *J*=14 Hz, H-5β), 1.72 (br m, H-7), 1.65 (dd, *J*=7, 2.5 Hz, H-12), 1.56 (dq, *J*=14.4, 7.2 Hz, H-13), 1.42 (dq, *J*=14.4, 7.2 Hz, H-13′), 1.40 (m, H-7′), 1.30 (m, H-8, H-9, H-9′), 1.17 (m, H-8′), 0.86 (t, *J*=7.2 Hz, H-10), 0.83 (t, *J*=7.2 Hz, H-14) ppm; ^{13}C *nmr* (40° in $CDCl_3$/1 drop D_2O) 174.2 (s, C-1), 131.1 (s, C-4), 122.2 (d, C-11), 84.6 (s, C-6), 75.8 (d, C-3), 38.1 (t, C-5), 36.0 (t, C-2), 31.5 (t), 29.0 (t), 25.2 (t), 23.2 (t), 14.2 (C-10), 12.7 (C-12), 7.4 (C-14) ppm.

Acid **2**.—12.4 mg; clear gum; [α]D +48° (*c*=0.55, $CHCl_3$); ir ($CHCl_3$) ν max 3450-2400 (br), 1710 cm^{-1}; positive-ion hrfabms calcd for $C_{14}H_{26}O_4Na$ 281.1729, found 281.1760; positive-ion lcms *m/z* $[M+NH_4]^+$ 276 (100); lr negative-ion fabms *m/z* $[M+HCO_2]^-$ 303 (22), $[M-H]^-$ 275 (5), 257 (5), 247 (5), 229 (6), 201 (5), 159 (14), 137 (100); ^1H nmr ($CDCl_3/C_6D_6$) δ 4.02 (ddd, *J*=10.5, 9.7, 2.4, Hz, H-3β), 2.35 (dd, *J*=16.5, 2.4 Hz, H-2), 2.18 (bs, H-2′), 1.71 (bt, *J*=13.8 Hz, H-7), 1.55 (dd, *J*=13.4, 4.5, H-5eq), 1.39 (m, H-4α, H-7′, H-13), 1.22 (m, H-8, 9, 9′, 11, 13), 1.03 (m, H-8′), 0.98 (dd, *J*=13.4, 12.0, H-5ax), 0.82 (t, *J*=7.2 Hz, H-10), 0.81 (m, H-11′), 0.70 (t, *J*=7.4 Hz, H-14), 0.68 (t, *J*=7.4 Hz, H-12) ppm; ^{13}C nmr ($CDCl_3/C_6D_6$) 177.5 (s, C-1), 83.7 (s, C-6), 82.5 (d, C-3), 37.0 (d, C-4), 37.0 (t, C-2), 36.8 (t, C-5), 32.1 (t, C-7), 30.2 (t, C-13), 26.1 (t, C-8), 24.9 (t, C-11), 23.9 (t, C-9), 14.7 (q, C-10), 11.1 (q, C-12), 7.90 (q, C-14) ppm.

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