New Cytotoxic Cembranes from the Sea Pen *Gyrophyllum sibogae*

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Two new cembrane-type diterpenoids have been isolated from the 2-propanol extract of the sea pen *Gyrophyllum sibogae* collected in South Africa: 7,8-dihydroflabellatene A (**1**) and 7,8-dihydroflabellatene B (**2**). Their structures were determined on the basis of detailed spectroscopic analysis and by single-crystal X-ray analysis of the major metabolite **1**, which showed strong in vitro cytotoxicity against a panel of 13 tumor cell lines.

Cembrane diterpenes have been isolated from several sources, including marine and terrestrial organisms, since 1960.[1] Soft corals constitute a particularly rich source of these natural products that have been of great interest because of their unusual structural features and remarkably wide range of biological activities. The structural elucidation of this type of diterpenes has involved NMR spectroscopy with the help of X-ray crystallography in several cases.[2]

In the course of our screening program for the search for new marine anticancer agents, we have focused our attention on the sea pen *Gyrophyllum sibogae* Hickson (1916) (family Pennatulidae, order Pennatulacea, subclass Octocorallia, class Anthozoa) because of the high cytotoxicity found in its organic extracts. Bioassay-guided isolation afforded two new diterpenes possessing a cembrane skeleton: 7,8-dihydroflabellatene A (**1**) and 7,8-dihydroflabellatene B (**2**). The isolation, structural determination, and bioactivity of these novel compounds are described below.

The 2-propanol extract of the sea pen *G. sibogae* collected in South Africa was subjected to reversed-phase C18 and silica gel VLC chromatographies and finally was purified by semipreparative RP-HPLC to yield compounds **1** and **2**.

Compound **1**, isolated as a white solid, showed a molecular formula of $C_{20}H_{32}O_4$ as deduced from its HRFABMS and NMR spectra. The IR spectrum of **1** showed absorptions due to two hydroxyl groups (3447 and 3370 cm^{-1}) and a conjugated dienone moiety (1646 and 1616 cm^{-1}). The presence of this conjugated dienone unit was also evident in the UV spectrum [λ_{max} 298 nm]. Twenty signals (four quaternaries, six methines, six methylenes, and four methyl groups) were observed in the ^{13}C NMR and DEPT-135 spectra, confirming the diterpenoid nature of the compound. Methyl groups were distinguished from methyne groups by running an HSQC experiment. Carbon resonances observed at δ_C 203.5 (C), along with those at δ_C 139.5 (C), 135.1 (CH), 120.1 (CH), and 151.2 (C) in the ^{13}C NMR and DEPT spectra, confirmed the presence of the expected $\alpha,\beta,\gamma,\delta$-unsaturated ketone, while the quaternary carbon signal at δ_C 212.5 (C) accounted for the presence of an additional ketone functionality. Signals for two oxygenated methine and methylene carbons were observed at δ_C 71.8 (CH) and 64.0 (CH$_2$), respectively. The presence of five

methylene groups (one allylic) was deduced from the observation of five CH$_2$ signals between δ_C 25.4−34.3, and finally, four methyl (one allylic) groups were inferred from the presence of four CH$_3$ signals between δ_C 14.0−26.2. These data were consistent with the deduced empirical formula and indicated that **1** had a cembrane-type skeleton. The ^{1}H NMR spectrum confirmed the presence of one oxygenated methine at δ_H 4.89 (br d, J = 4.8 Hz) and one oxygenated methylene at δ_H 3.78 (m). One intense singlet signal was also observed at δ_H 1.94, corresponding to the allylic methyl group, and three doublets at δ 1.13, 1.24, and 0.66 were assigned to three methyl groups.

Comparison of all of the spectroscopic data of **1** with those of related cembrane-like diterpenoids suggested a similar structure to flabellatene A (**3**), previously isolated from the Antarctic sponge *Lissodendoryx flabellate*.[3] Indeed, the ^{1}H and ^{13}C NMR chemical shifts for C-1 to C-4, C-14, the isopropyl group (C-15 to C-17), and Me-18 in **1** were almost identical to those reported for **3**. The spectra of **1** lacked the resonances attributable to the Δ^7 olefin, but instead showed resonances attributable to two sp^3 carbons observed at δ_C 29.3 (C-7) and 57.5 (C-8). The downfield shift of the ketone carbon at C-9 (δ_C 212.5 ppm) in the ^{13}C spectrum of **1** with respect to **3** (δ_C 206.0 ppm) confirmed the absence of the Δ^7 double bond. 2D NMR spectroscopy confirmed the planar structure as well as the assignments proposed for compound **1**.

The relative stereochemistry of **1** at C-12 and C-13 and the double bond configurations were elucidated by a NOESY experiment, coupling constant analysis, and comparison of the spectroscopic data with those of flabellatene A (**3**). However, it was not possible to deduce the configuration at C-8 on the basis of these data. Fortunately, an X-ray structure analysis of a suitable crystal unequivocally allowed us to establish the complete structure and relative stereochemistry of **1**. A computer-generated drawing of the X-ray results of **1** is shown in Figure 1.

Several attempts were performed to establish the absolute stereochemistry of **1**. We tried to determine the configuration at the secondary hydroxyl group at C-13 using both the modified Mosher's method[4] as well as the methodology developed by Riguera et al. based on the single derivatization to the corresponding ester and subsequent acquisition of the NMR spectra at different temperatures.[5] Selective protection of the primary alcohol at C-19 as a *tert*-butyldiphenylsilyl derivative (**1a**) allowed the preparation of the corresponding esters at C-13 with (*S*)- and (*R*)-methoxyphenylacetic acids (Scheme 1). Comparison of key
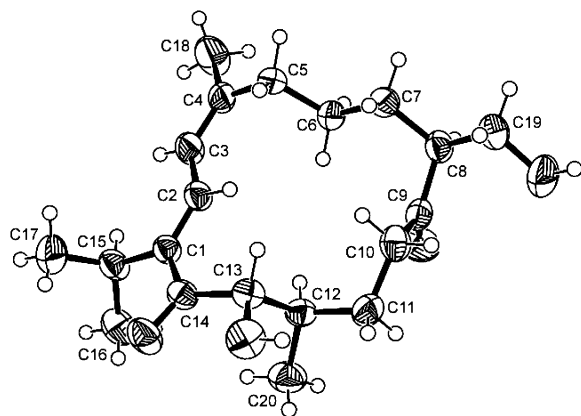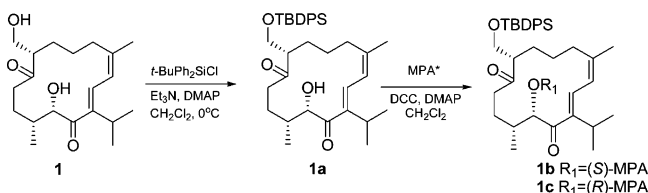
Figure 1. Computer-generated ORTEP diagram of **1**.

Scheme 1

Chart 1

NMR signals, assigned by COSY experiments, of the resulting diasteroisomeric (*S*)- and (*R*)-MPA esters **1b** and **1c** showed that the $\Delta\delta^{SR}$ values were irregularly arranged (see Table 2, Supporting Information) and therefore were not useful. Similarly, irregular $\Delta\delta^{T1-T2}$ values were obtained by comparison of the NMR spectrum of the (*S*)-MPA ester **1b** taken at room temperature ($T1 = 298$ K) and spectra recorded at lower temperatures ($T2 = 223$ and 263 K). All the protons on the left and right sides of the MPA plane, with the exception of H-8, were upfield-shifted in the range of 0.019 to 0.094 ppm (Table 3, Supporting Information).

The fact that the positive and negative $\Delta\delta^{SR}$ or $\Delta\delta^{T1-T2}$ values were found to be not systematically arranged suggested that steric interference of the *tert*-butyldiphenylsilyl protecting group might prevent adoption of the ideal conformation of the MPA and thereby cause irregular arrays of the $\Delta\delta$ values. The interference of the aromatic rings of the protecting group at C-19 (TBDPS) can be another possible explanation for the irregular $\Delta\delta$ values obtained. Remarkably, this was not the case for compound **3**, where Fontana et al. applied a similar approach using the methoxytrifluoromethylphenylacetic acid (MTPA), and a clear differentiation around both sides of C-13 were observed, leading to the absolute configuration assignment of **3**. On the basis of these data, we defined compound **1** as 7,8-dihydroflabellatene A.

In additon to **1**, a second compound **2** was present in minor quantities in the extract of *G. sibogae*. HRFABMS and ^{13}C NMR spectra supported a molecular formula of $C_{20}H_{32}O_5$ for **2**. The major structural difference of this compound with respect to **1** was the replacement of the C-3/C-4 double bond by an oxirane ring. This difference could be explained by the presence in the ^{1}H NMR spectrum of **2** of a signal at δ 3.50 (d, $J = 9.5$ Hz) and the upfield shifts of H-2 (δ 5.69), both H-5 protons (δ 1.86 and 1.38), and Me-18 (δ 1.39). In the ^{13}C NMR, the sp[2] carbons at 120.1 (CH) and 151.1 (C) in **1** were replaced by two oxygenated carbons in **2** at δ_C 58.4 (CH) and 64.0 (C), characteristic of an epoxide group. The stereochemistry at C-3/C-4 was established on the basis of the similarity of chemical shifts and coupling constants between **2** and flabellatene B (**4**)
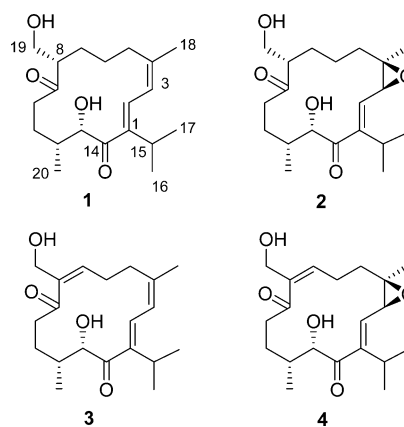
(Chart 1).[3] An intense NOE between Me-18 and H-3 supported the cis stereochemistry for the epoxide. Finally, the structure proposed was corroborated by 2D NMR experiments, and the relative stereochemistry of the remaining chiral centers was determined by 2D NOESY, coupling constant analysis, and spectral comparison with compounds **1** and **4**. Therefore, compound **2** was defined as 7,8-dihydroflabellatene B.

Cytotoxicity assays were performed for compounds **1** and **2** against a panel of 13 different tumor cell lines.[6] Compound **1** showed the strongest activity against all of the lines tested. GI$_{50}$ values (nM) for some of the lines evaluated are: DU-145 (82.0), IGROV (90.3), K-562 (62.7), and HT-29 (14.5).

This note constitutes the first chemical study of a species belonging to the genus *Gyrophyllum*. Interestingly, the isolation of structurally similar compounds from a sponge (**3** and **4**) and a Cnidaria (**1** and **2**) raises the question of the origin of these metabolites and may indicate that the microbial fauna and flora associated with these organisms are responsible for the production of these metabolites. The significant cytotoxic activity reported for flabellatane A (**3**)[3] and now for its 7,8-dihydro derivative, compound **1**, expands the potential antitumor activity for this type of structure.

Experimental Section

General Experimental Procedures. Melting points were measured on a Büchi 535 apparatus and are uncorrected. Optical rotations were determined in CHCl$_3$ using a Jasco P-1020 polarimeter. UV spectra were obtained with a Perkin-Elmer Lambda 15 UV/vis spectrophotometer. IR spectra were recorded on a Perkin-Elmer 881 infrared spectrophotometer. X-ray data was collected on a Nonius-MACH3 difractometer. Intensity data were collected using graphite monochromated copper radiation and an $\omega-2\theta$ variable scan speed technique. Reflections were used in the solution and refinement, and the structure was solved using WinGX software (SIR-92 for solution and SHELXL-97 for refinement).[7] NMR spectra were recorded on a Varian "Unity 500" spectrometer at 500/125 MHz (^{1}H/^{13}C) and on a Bruker AC-200 NMR spectrometer at 200/50 MHz (^{1}H/^{13}C). Chemical shifts were reported in parts per million using residual CDCl$_3$ (δ 7.26 for ^{1}H and 77.0 for ^{13}C) as internal reference. (+)-FABMS was performed on a VGAutoSpec spectrometer employing *m*-NBA as matrix. ESIMS was recorded using an Agilent 1100 series LC/MSD spectrometer.

Animal Material. *G. sibogae* Hickson (1916) was collected in October 2001 by trawling at depths ranging from 666 to 943 m in international waters near Madagascar (33° 39.0′ S, 44° 50.7′ E). The material was identified by Dr. Pablo López (University of Seville, Spain). A voucher specimen (ORMA002073) is deposited at PharmaMar.

Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Chemical Shifts for Compounds **1** and **2** in CDCl$_3$

	1		**2**	
C	δ_C mult^a	δ_H mult (J in Hz)	δ_C mult^a	δ_H mult (J in Hz)
1	139.5 C		150.3 C	
2	135.1 CH	6.84 d (11.8)	134.5 CH	5.69 d (9.5)
3	120.1 CH	6.25 br d (11.8)	58.4 CH	3.50 d (9.5)
4	151.2 C		64.0 C	
5	34.3 CH$_2$	2.50 m/1.95 m	35.8 CH$_2$	1.86 td (13.5, 4.0)/1.38 m
6	28.8 CH$_2$	1.68 m/1.04 m	24.4 CH$_2$	1.54 m/1.04 m
7	29.3 CH$_2$	1.68 m (2H)	29.2 CH$_2$	1.71 ddd (12.0, 8.0, 4.0)/1.50 m
8	57.5 CH	2.64 m	57.4 CH	2.62 m
9	212.5 C		212.4 C	
10	34.3 CH$_2$	2.93 ddd, (19.2, 7.9, 5.4)/2.50 m	34.5 CH$_2$	2.74 ddd (19.0, 9.5, 4.5)/2.50 ddd (19.0, 6.0, 3.5)
11	25.4 CH$_2$	1.81 m (2H)	26.3 CH$_2$	1.78 m (2H)
12	37.3 CH	1.68 m	35.9 CH	1.65 dq (2.0, 7.0)
13	71.8 CH	4.89 br d (4.8)	73.9 CH	4.62 dd (6.5, 2.0)
14	203.5 C		205.2 C	
15	27.9 CH$_3$	3.03 hept (7.0)	29.2 CH$_3$	3.01 (hept, 7.0)
16	19.9 CH$_3$	1.13 d (7.0)	20.3 CH$_3$	1.15 (d, 7.0)
17	22.2 CH$_3$	1.24 d (7.0)	23.3 CH$_3$	1.30 (d, 7.0)
18	26.2 CH$_3$	1.94 s	22.7 CH$_3$	1.39 (s)
19	64.0 CH$_2$	3.78 m (2H)	64.1 CH$_2$	3.83 dd (10.5, 9.0)/3.78 dd (10.5, 5.5)
20	14.0 CH$_3$	0.66 d (6.7)	13.9 CH$_3$	0.70 d (7.0)
OH-13		3.78 m		3.39 d (6.5)

^{a} Multiplicity by DEPT and HSQC.

Extraction and Isolation. The frozen specimen (32 g) was triturated and exhaustively extracted with 2-propanol (1.5 L). The extract was concentrated under vacuum to yield a crude of 1.19 g, which was subjected to vacuum-liquid chromatography (VLC) on Lichroprep RP-18 with a stepped gradient from H$_2$O to MeOH. Fractions eluted with MeOH−H$_2$O (1:1) were purified by semipreparative HPLC (SymmetryPrep C-18, 7.8 × 150 mm, gradient H$_2$O/MeCN from 5 to 30% MeCN in 15 min and then to 100% MeCN in 7 min, UV detection) to yield compound **2** (1.3 mg). Fractions eluted with MeOH were chromatographed on silica gel with *n*-hexane/EtOAc mixtures of increasing polarity to give compound **1** (29.0 mg) in a fraction eluted with *n*-hexane/EtOAc (1:1).

7,8-Dihydroflabellatene A (1): pale yellow crystals; mp 175−176 °C; [α]^{25}D +84.6° (*c* 0.100, CHCl$_3$); UV(CHCl$_3$) λ_{max} (log ε) 298 (4.30) nm; IR (KBr) ν_{max} 3447, 3370, 2960, 2918, 2876, 1711, 1646, 1616 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; (+)-HRFABMS *m/z* 337.2374 [M + H]$^+$ (calcd for C$_{20}$H$_{33}$O$_4$ 337.2379, Δ 0.5 mmu).

7,8-Dihydroflabellatene B (2): pale yellow oil; [α]^{25}D +77.0° (*c* 0.013, CHCl$_3$); UV (CHCl$_3$) λ_{max} (log ε) 281 (2.31) nm; IR (KBr) ν_{max} 3450, 2934, 2877, 1735, 1675 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; (+)-HRFABMS *m/z* 375.2134 [M + Na]$^+$ (calcd for C$_{20}$H$_{32}$O$_5$Na 375.2147, Δ 1.3 mmu).

(*S*)- and (*R*)-MPA Esters of 7,8-Dihydroflabellatene A (1b and 1c). A solution of **1** (7 mg) in dry CH$_2$Cl$_2$ (1.5 mL) and dry Et$_3$N (7 μL) was treated with *tert*-butyldiphenylsilyl chloride (7 μL) and DMAP (1 mg). The mixture was stirred at 0 °C for 24 h. A volume of 2 mL of 10% HCl was added, the organic phase was washed with brine, and the solvent was removed under vacuum. The residue was subjected to a small silica gel column (5 × 0.5 cm) eluted with *n*-hexane/EtOAc (4:6) to give 15 mg of **1a**. A catalytic amount of DMAP was added to a solution of **1a** (6 mg), methoxyphenylacetic acid (2 mg) [(*S*)- or (*R*)-MPA], and dicyclohexylcarbodiimide (2 mg) in dry CH$_2$Cl$_2$ (2 mL). Each reaction mixture was allowed to stand at room temperature for 48 h. The dicyclohexylurea was removed by filtration, and the solvent was removed under vacuum. The resulting solid was washed with Et$_2$O (3 times), and the combined filtrates were washed with cold 1N HCl (twice), saturated aqueous NaHCO$_3$ (twice), and aqueous NaCl (twice). The combined organic extracts were filtered, and the solvent was removed to afford a residue that was purified by reversed-phase HPLC in MeOH/H$_2$O (9:1) to give **1b** (3 mg) and **1c** (2 mg). ^{1}H NMR (500 MHz), see Table 2.

Crystallographic Data and X-ray Structure Analysis of 1. A suitable colorless plate, 0.50 × 0.50 × 0.45 mm^3, of **1** was grown by the slow evaporation of a MeOH solution. Crystal data: C$_{20}$H$_{32}$O$_4$, orthorhombic; space group $P2_12_12_1$;

$Z = 4$, unit cell parameters $a = 9.6188(7)$, $b = 11.2192(8)$, $c = 18.3999(13)$ Å, $V = 1985.638$ Å^3, $d_{calcd} = 1.125$ Mg m^{-3}, $T = 293(2)$ K, $F(000) = 736$, $\lambda = 71073$ Å, μ (Mo Kα) = 0.076 mm^{-1}. Final refinement with 4730 reflections led to $R(F)$, $R(F > 2\sigma)$, and GOF of 0.1158, 0.0442, and 1.034. Crystallographic data have been deposited with the Cambridge Crystallographic Data Center (deposit number CCDC 224044). Copies of the data can be obtained, free of charge, on application to CDCC, 12 Union Road, Cambridge, CB2 1EZ, U.K. (fax: +44(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

Biological Activity. A colorimetric type of assay using sulforhodamine B reaction has been adapted for a quantitative measurement of cell growth and viability following the technique described in the literature.[6] The in vitro activity of the compounds was evaluated against a panel of 13 tumor cell lines, including prostate (DU-145), ovary (IGROV and IGROV-ET), breast (SK-BR3), melanoma (SK-MEL-28), NSCL (A549), leukemia (K-562), pancreas (PANC1), colon (HT29, LOVO, and LOVO-DOX), and cervix (HeLa and HeLa-APL).

Supporting Information Available: Key ^{1}H NMR signals, final coordinates, and bond distances and angles for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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