Biocatalytic and Antimetastatic Studies of the Marine Cembranoids Sarcophine and 2-*epi*-16-Deoxysarcophine

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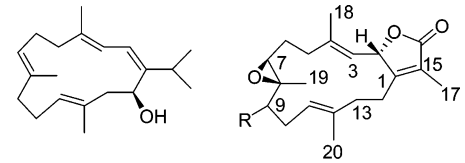
The soft coral *Sarcophyton glaucum* is a rich resource of several bioactive cembranoids. Sarcophytol A (**1**) and sarcophine (**2**) are cembranoid diterpenes reported from this soft coral and extensively investigated for their cancer chemopreventive properties. This study aimed at investigating the antimetastatic potential of the major cembranoids, sarcophine (**2**) and 2-*epi*-16-deoxysarcophine (**3**), from the Red Sea soft coral *S. glaucum*. Biocatalytic transformation of **3** using *Rhizopus stolonifer* ATCC 6227a and *Absidia spinosa* ATCC 6648 afforded four new metabolites, **5**−**7** and **9**, along with the known 9α-hydroxysarcophine (**8**). Sarcophine, 2-*epi*-16-deoxysarcophine, and metabolites **5**−**9** revealed significant antimetastatic activity against the highly metastatic mouse melanoma cell line (B16B15b). Cembranoids demonstrate a great potential for further development as antimetastatic agents.

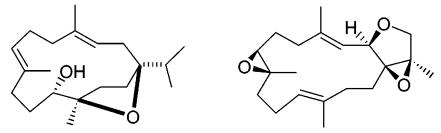
Soft corals are marine invertebrates possessing a vast range of terpenoid metabolites.[1] These terpenes, mainly cembranoids, represent the animal's main chemical defense tools against its natural predators.[2,3] Cembranoids exhibit a wide range of biological activities including neuroprotective, antimicrobial, and antitumor properties.[2,3] The soft coral *Sarcophyton* is one of the most abundant coral reef animals with a high cembranoid content.[4] The cancer chemopreventive properties of the two major *Sarcophyton* cembranoids, sarcophytol A (**1**) and sarcophine (**2**), are well documented.[5−8] Several attempts to optimize the anticancer potential of sarcophine have previously been reported.[6−8] However, no similar studies were reported for other major cembranoids from this soft coral. Biocatalysis demonstrates many advantages including high regio-, stereo-, and chemoselectivity and the ability to generate novel and diverse derivatives.[9] Biocatalysis was successfully used to enhance the anticancer potential of sarcophine.[6] The related olibanum cembranoid incensole (**4**) was patented for its antiangiogenic activity.[10] Based on correlations between angiogenesis and cancer metastasis as well as the reported chemopreventive activities of sarcophytol A and sarcophine, cembranoids could possess antimetastatic properties.

Hence, this study aims at (1) isolation of major cembranoids from *Sarcophyton glaucum*; (2) optimization of the major cembranoid 2-*epi*-16-deoxysarcophine (**3**) using biocatalysis to generate structurally diverse analogues; and (3) evaluating the ability of cembranoids to inhibit the migration of the highly metastatic melanoma cells (B16B15b).



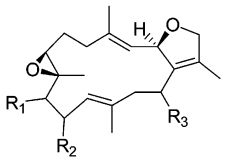
1 Sarcophytol A

2 Sarcophine, R = H
8, R = α-OH



4 Incensole

5



	R_1	R_2	R_3
3	H	H	H
6	α-OH	H	H
7	H	β-OH	H
9	H	H	β-OH

Results and Discussion

Reinvestigation of the Red Sea soft coral *S. glaucum* led to the isolation of two known cembranoids, sarcophine (**2**) and 2-*epi*-16-deoxysarcophine (**3**), in high yields. The identification of **2** and **3** was based on extensive analysis of their NMR data and comparison with the literature.[11−13] There have been discrepancies in the nomenclature of 2*S*,7*S*,8*S*-16-deoxysarcophine and 2*R*,7*S*,8*S*-16-deoxysarcophine. Irrespective of the C-2 configuration, this compound has been referred to as sarcophytoxide, 16-deoxosarcophine, and 16-deoxysarcophine.[13−15] The name 16-deoxysarcophine should be used to describe 2*S*,7*S*,8*S*-16-deoxysarcophine in order to unify the literature nomenclature and to correlate its structure with sarcophine (**2**), whose absolute configuration is well established as 2*S*,7*S*,8*S* by X-ray crystallography and modified Mosher's method.[11,16] 2*R*,7*S*,8*S*-16-Deoxysarcophine (**3**) should then be named 2-*epi*-16-deoxysarcophine, to clearly denote the opposite C-2 configuration compared to sarcophine.

The success of the previous bioconversion study of sarcophine that led to metabolites with enhanced anticancer activity encouraged a similar study for **3**.[6] Thirty growing cultures were screened for their ability to bioconvert 2-*epi*-16-deoxysarcophine. Of these, *Rhizopus stolonifer* ATCC 6227a and *Absidia spinosa* ATCC 6648 were selected for the scale-up fermentation based on the diversity of the generated metabolites.

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Table 1. ^{13}C and ^{1}H NMR Data of Compounds **5**−**7**[a]

	5[a]		**6**[a]		**7**[a]	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	72.4, C		133.6, C		133.5, C	
2	78.2, CH	4.82, d (10.9)	85.3, CH	5.40, m	85.3, CH	5.37, m
3	122.5, CH	5.27, d (9.8)	125.5, CH	5.29, d (9.5)	124.9, CH	5.17, d (9.6)
4	142.5, C		140.2, C		139.8, C	
5	35.5, CH_2	2.34, m, 2.23, m	34.7, CH_2	2.30, m, 2.25, m	35.0, CH_2	2.29, m, 2.24, m
6	29.5, CH_2	1.87, m, 1.66, m	27.0, CH_2	1.87, m, 1.71, m	26.4, CH_2	2.10, m, 1.77, m
7	62.4, CH	2.67, t (5.5)	62.1, CH	2.68, t (5.5)	61.6, CH	2.68, t (5.7)
8	60.0, C		63.4, C		59.4, C	
9	38.5, CH	1.62, m, 1.23, m	78.8, CH	2.98, dd (11.7, 4.7)	45.4, CH_2	2.22, m, 1.50, m
10	23.2, CH_2	2.20, m, 2.08, m	31.5, CH_2	2.45, m, 2.05, m	65.0, CH	4.50, m
11	123.0, CH	5.08, t (7.0)	118.2, CH	4.88, t (9.2)	127.0, CH	5.18, d (7.3)
12	135.2, C		138.9, C		138.5, C	
13	34.5, CH_2	1.95, m, 1.89, m	39.2, CH_2	2.38, m, 2.35, m	38.4, CH_2	2.10, m, 1.60, m
14	25.9, CH_2	1.60, m	28.9, CH_2	1.90, m, 1.52, m	23.0, CH_2	2.40, m, 2.30, m
15	67.8, C		128.2, C		128.5, C	
16	70.1, CH_2	3.86, d (9.9), 3.75, d (9.9)	78.5, CH_2	4.50, dq (12.0, 5.5), 4.47, dq (12.0, 5.5)	78.5, CH_2	4.45, m, 4.41, m
17	12.8, CH_3	1.45, 3H, s	10.5, CH_3	1.65, 3H, brs	10.6, CH_3	1.67, 3H, brs
18	18.6, CH_3	1.90, 3H, s	17.8, CH_3	1.85, 3H, s	17.6, CH_3	1.78, 3H, s
19	16.4, CH_3	1.20, 3H, s	10.0, CH_3	1.26, 3H, s	19.5, CH_3	1.38, 3H, s
20	15.7, CH_3	1.62, 3H, s	15.5, CH_3	1.62, 3H, s	16.2, CH_3	1.62, 3H, s

[a] In $CDCl_3$, 400 MHz for ^{1}H and ^{13}C NMR. Coupling constants (*J*) are in Hz.

Fourteen-day fermentation of **3** with *R. stolonifer* afforded four more polar metabolites, **5**−**8**. The HRMS data of compound **5** suggested a molecular formula of $C_{20}H_{30}O_3$ and six degrees of unsaturation. The ^{1}H and ^{13}C NMR data (Table 1) suggested 1,15-epoxidation and the presence of two macrocyclic double bonds. The quaternary oxygenated carbons at δ 72.4 and 67.8 were assigned to C-1 and C-15, respectively. This was aided by their ^{3}J- and ^{2}J-HMBC correlations with the H_3-17 methyl singlet. The upfield shift of H_3-17 (δ 1.45), compared with H_3-17 in the parent compound **3**, indicated the replacement of $\Delta^{1,15}$ in **3** with an epoxy functionality in **5**. This was further confirmed by the ^{2}J- and ^{3}J-HMBC correlations of the H-2 doublet (δ 4.82) with C-1 and C-15, respectively. The 14-membered macrocycle of **3** is identical to that of sarcophine (**2**), which has restricted mobility on the basis of X-ray crystallography.[11] The unique nature of sarcophine's 14-membered ring macrocycle was previously reported.[11] The existence of the segment C-7−C-11 in a half-chair conformer, as proved by X-ray crystallography and NOE difference data, restricts the mobility freedom in the entire macrocycle.[6,11] This was further supported by the high *J* and dihedral angle values between H-2 and H-3 in sarcophine (10.5 Hz and ~160°) and the paramagnetic deshielding of the C-18 methyl functionality by the C-2 oxygen.[6,11] This is true whenever the epoxy group is intact; hence, the relative stereochemistry of the biocatalytic products of **3** was assigned on the basis of NOESY correlations. The presence of the C-7/C-8 epoxide forces the functional groups on C-7 and C-8 to be eclipsed and the methylene groups C-6 and C-9 to be in an anti-configuration around the C-7−C-8 bond.[6,11] NOESY is widely employed for the assignment of relative stereochemistry of structurally diverse cembrane diterpenes isolated from soft corals.[17,18] The α-oriented H_3-19 methyl singlet (δ 1.20) in **5** showed a strong NOESY correlation with H_3-17, indicating a similar stereo-orientation. Thus, the C-1−C-15 epoxide group of **5** should be *β*-oriented.

The HRMS and NMR data of **6** (Table 1) suggested a monohydroxylated analogue of **3**. The newly oxygenated doublet of doublets (δ 2.98, J = 11.7 and 4.7 Hz) was assigned to H-9. The location of the new C-9 hydroxy group was based on HMBC data. The methyl singlet at H_3-19 showed a ^{3}J-HMBC correlation with the new oxygenated methine at δ 78.8, which was then assigned to C-9. This assignment was also confirmed by the ^{3}J-HMBC correlations of H-7 (δ 2.68) with C-9. The stereochemistry assignment of C-9 was based on comparison of the ^{13}C NMR chemical shift and coupling constants with those of the previously reported 9α-hydroxysarcophine.[6] The reported chemical shift value of C-9 in 9α-hydroxysarcophine is at δ 78.6 and H-9 resonates at δ 2.95 (dd, J = 11.8, 4.1 Hz).[6] By contrast, the reported C-9 chemical shift value in 9*β*-hydroxysarcophine is at δ 70.7 and H-9 resonates at δ 3.92 (dd, J = 4.8, 2.1 Hz).[6]

The HRMS and NMR data of **7** (Table 1) indicated monohydroxylation. The olefinic proton doublet at H-11 (δ 5.18) showed a COSY correlation with the new oxygenated proton multiplet at H-10 (δ 4.50). Proton H-11 also showed a ^{2}J-HMBC correlation with C-10 (δ 65.0). The α-orientation of H-10 was based on its NOESY correlation with the α-oriented H_3-19 (δ 1.38).

The HRMS and NMR data (Table 2) of **8** suggested the molecular formula $C_{20}H_{28}O_4$, monohydroxylation, and an α,*β*-unsaturated lactone system. The most downfield carbon signals at δ 174.9 (C-16) and 162.5 (C-1) and the downfield shifted H-2 at δ 5.44 (dq, J = 9.7, 1.8) indicated a sarcophine-like α,*β*-unsaturated lactone. The hydroxylated proton at H-9 (δ 2.96, dd, J = 11.7, 4.7) showed a close resemblance to that of 9α-hydroxy-2-*epi*-16-deoxysarcophine (**6**). Compound **8** was identical to the previously reported sarcophine bioconversion product 9α-hydroxysarcophine.[6] Comparison of the TLC and ^{1}H NMR of the total fermentation mixture of **3** with *R. stolonifer* versus substrate control, 2-*epi*-16-deoxysarcophine in blank compound medium α, indicated that **8** is a true microbial metabolite and not an artifact formed by air oxidation of **3** to sarcophine. Faulkner and co-workers previously suggested a possible C-2 epimerization after oxidation of C-16.[14] The present data provide a strong support of this prediction.

Bioconversion of **3** using *A. spinosa* ATCC 6648 predominantly yielded compound **9**. The HRMS and NMR data (Table 2) of **9** suggested a monhydroxylated analogue. The oxygenated proton multiplet at H-14 (δ 4.69) showed a COSY correlation with the methylene protons at H_2-13 (δ 2.45). The oxygenated proton multiplet at H-2 (δ 5.70) showed a ^{3}J-HMBC correlation with C-14 (δ 66.2), confirming the hydroxylation at this position. The α-oriented H-2 showed a NOESY correlation with H-16a (δ 4.60), which in turn showed a NOESY correlation with H-14, suggesting a similar α-orientation.

Antimigratory Activity. Migration and proliferation are events underlying cancer metastasis. The antimigratory potential of three different concentrations of compounds **2**−**9** was evaluated using wound-healing assays.[19−21] The highly metastatic mouse melanoma cell line (B16B15b) was used.[19−21] All cembranoids showed a dose-dependent increase in the inhibition of migration (Figures 1 and 2). Sarcophine (**2**) was the most potent of all tested compounds. The 9α-hydroxy derivative (**6**) showed an improved activity

Table 2. ^{13}C and ^{1}H NMR Data of Compounds **8** and **9**[a]

	8[a]		**9**[a]	
position	δ_C	δ_H	δ_C	δ_H
1	162.5, C		137.1, C	
2	79.7, CH	5.44, dq (9.7, 1.8)	84.1, CH	5.70, m
3	119.3, CH	5.04, d (9.7)	125.9, CH	5.29, d (9.5)
4	145.3, C		138.5, C	
5	34.6, CH_2	2.41, m, 2.37, m	37.4, CH_2	2.35, m, 2.28, m
6	26.6, CH_2	1.80, m, 1.62, m	25.6, CH_2	1.75, m, 1.65, m
7	61.2, CH	2.63, t (5.5)	61.0, CH	2.63, t (5.5)
8	63.4, C		60.8, C	
9	78.6, CH	2.96, dd (11.7, 4.7)	35.9, CH_2	2.08, m, 1.95, m
10	31.5, CH_2	2.48, m, 2.08, m	23.4, CH_2	1.54, 2H, m
11	119.2, CH	4.96, dd (10.3, 6.6)	128.0, CH	5.15, t (7.0)
12	137.6, C		128.3, C	
13	37.6, CH_2	2.30, m, 1.82, m	45.8, CH_2	2.45, 2H, m
14	28.9, CH_2	2.84, m, 1.95, m	66.2, CH	4.69, m
15	123.2, C		130.9, C	
16	174.9, C		78.5, CH_2	4.60, dq (12.1, 5.5), 4.42, d (12.1)
17	9.1, CH_3	1.84, 3H, brs	10.7, CH_3	1.64, 3H, s
18	18.3, CH_3	1.93, 3H, s	15.9, CH_3	1.78, 3H, s
19	9.7, CH_3	1.29, 3H, s	18.1, CH_3	1.23, 3H, s
20	16.9, CH_3	1.64, 3H, s	16.8, CH_3	1.66, 3H, s

[a] In $CDCl_3$, 400 MHz for ^{1}H and ^{13}C NMR. Coupling constants (*J*) are in Hz.

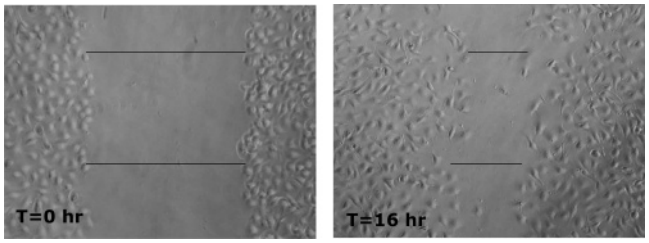

Figure 1. Relative gap closure in the presence of 10 μM 9α-hydroxy-2-*epi*-16-deoxysarcophine (**6**) at 0 and 16 h after wounding of B16B15b mouse melanoma cells.

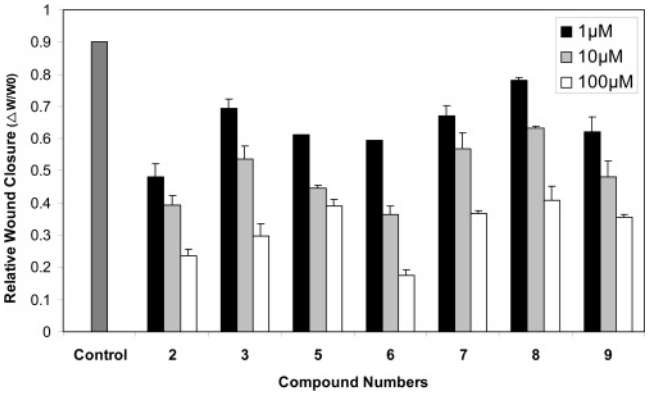

Figure 2. Effect of different concentrations of sarcophine, 2-*epi*-16-deoxysarcophine, and cembranoid derivatives on the migration of highly metastatic B16B15b mouse melanoma cells.

compared to its parent compound 2-*epi*-16-deoxysarcophine (**3**). Cembranoids (1 and 10 μM) did not show a significant effect on cell proliferation, indicating that cembranoids' tumor cell migration inhibition was not attributed to cytotoxicity but due to the inhibition of migratory pathways.

Conclusions. Biocatalysis of 2-*epi*-16-deoxysarcophine afforded four new and one known metabolites. The transformation of **3** to 9α-hydroxysarcophine provided evidence for Faulkner's previously proposed hypothesis of possible C-2 epimerization after C-16 oxidation of deoxysarcophine. The antimigratory effect of cembranoids is reported for the first time. C-16-oxygenated cembranoids show better antimigratory activity compared to 16-deoxycembranoids.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Analytical Autopol III polarimeter. IR spectra were recorded on a Nicolet Impact 400D FTIR spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, on a JEOL Eclipse NMR spectrometer operating at 400 MHz for proton and 100 MHz for carbon. The HRESITOF experiments were conducted at the University of Michigan on a Micromass LCT spectrometer and the University of Minnesota on a Bruker BioTOF II spectrometer. TLC analyses were carried out on precoated silica gel G_{254} 500 μm, using the developing systems *n*-hexane/EtOAc (3:2) and $CHCl_3$/MeOH (4:1). For medium-pressure liquid column chromatography (MPLC), Si gel <63 μm particle size mesh was used.

Materials. The soft coral *S. glaucum* was collected by scuba in June 2003 from Hurghada, at the Egyptian Red Sea coast. A voucher specimen (03RS24) was deposited in the Department of Basic Pharmaceutical Sciences, College of Pharmacy, University of Louisiana at Monroe. The frozen soft coral (680 g) was extracted four times with 2-propanol in a percolator at room temperature. The extract (86 g) was then concentrated under vacuum and chromatographed on silica gel using *n*-hexane/EtOAc to yield 1.5 g of sarcophine (**2**), which was further recrystallized from EtOAc. Further chromatographic separation of the extract afforded the less polar 2-*epi*-16-deoxysarcophine (**3**, 4.1 g, R_f 0.50 *n*-hexane/EtOAc, 7:3).

Biocatalysis of 2-*epi*-16-Deoxysarcophine. Biocatalysis studies were conducted as described elsewhere.[6] Thirty growing ATCC microbial cultures were used for screening of **3**. These organisms were similar to those previously reported in addition to *Absidia spinosa* ATCC 6648, *Cunninghamella homothallica* ATCC 16161, *Cunninghamella verticillata* ATCC 8986, *Kluyveromyces africanus* ATCC 22294, *Lipomyces starkeyi* ATCC 58680, *Phanerochaete chrysosporium* ATCC 24725, *Rhizopus niveus* ATCC 200757, *Rhizopus oligosporus* ATCC 76011, *Saccharomyces pastorianus* ATCC 2366, and *Streptomyces malaysiensis* BAA-13.[6]

R. stolonifer ATCC 6227a and *A. spinosa* ATCC 6648 were selected for scale-up. Each of these organisms was inoculated in 10 1 L flasks containing compound medium α.[6] After 72 h, 500 mg of **3** was dissolved in 3 mL of ethanol and evenly distributed in all flasks (250 mg per organism, 25 mg/flask). After 14 days, the growth medium was filtered and extracted with EtOAc (4 × 1000 mL). The EtOAc layer was then concentrated under vaccuum to give 350 and 415 g of crude extracts, respectively. Fermentation extract of *A. spinosa* was subjected to silica gel 60 column chromatography to yield 15 mg of metabolite **9** (R_f 0.25, *n*-hexane/EtOAc, 3:2). *R. stolonifer* fermentation extract was subjected to normal-phase MPLC to collect fractions rich in metabolites **5**−**8**. The least polar fraction was subjected to preparative TLC on silica gel to afford compound **5** (5 mg, R_f 0.28, $CHCl_3$/MeOH, 3:2). Subsequent MPLC of other fractions afforded metabolites **6** (7

mg, R_f 0.22, $CHCl_3$/MeOH, 3:2), **7** (9 mg, R_f 0.21, $CHCl_3$/MeOH, 3:2), and **8** (3 mg, R_f 0.18, $CHCl_3$/MeOH, 3:2).

Wound-Healing Assays. Murine melanoma cells (B16B15b) were maintained in DMEM F12 + 5% FBS. The method used to detect migration by wound-healing assay was previously described.[19−21] Briefly, the cells were allowed to grow to 100% confluency in 24-well plates. Once the monolayer developed, a wound was made with a 100 μL pipet tip. The detached cells were then washed three times with serum-free medium (SFM). The cells were treated with 1, 10, or 100 μM solutions of each of the tested cembranoids in Dulbecco's modified Eagle's medium (DMEM/F12) + 5% FBS + penicillin/streptomycin (1%) for a period of 16 h. Wound width was measured immediately before (W_0) and after the 16 h (W_{16}) incubation ($\Delta W = W_0 - W_{16}$) (Figure 1). Figure 2 represents wound closure values for different concentrations of compounds (**2**, **3**, **5**−**9**) in millimeters, relative to the control (time 0). All experiments were conducted independently in triplicate.

Cell Proliferation Assays by Fluorescence-Based Analysis. B16B15b cells were maintained as mentioned above and were plated in 24-well tissue culture plates at 2500 cells/well. This was followed by an incubation period of 24 h at 37 °C to allow for cell attachment. On the day of the treatment, medium was replaced with fresh growth medium containing 1, 10, or 100 μM cembranoid derivatives + 1% penicillin and streptomycin. Cells were then incubated for 24 h, after which the medium was replaced with fresh growth medium containing 10% Alamar Blue (BioSource International, Camarillo, CA). This safe, nontoxic dye is used to monitor and assess the innate metabolic activity of the cells.[22] Cells were then incubated for 4 h, and fluorescence was detected using a CytoFluor multiwell-plate reader (CytoFluor 4000, Applied Biosystems, Foster City, CA).[22] Cell numbers were calculated using a standard curve. All experiments were conducted independently and in triplicate.

Analytical Data. 1,15β-Epoxy-2-*epi*-16-deoxysarcophine (5): colorless oil; $[\alpha]_D^{25}$ −10.2 (*c* 0.22, MeOH); UV λ_{max} (log ϵ) (MeOH) 204 (3.7); IR ν_{max} (neat) 3017, 2931, 2857, 1662, 1516, 1449, 1385, 1215, 1033, 755 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESITOF *m*/*z* 341.2093 $(M + Na)^+$ (calcd for $C_{20}H_{30}O_3Na$, 341.2093).

9α-Hydroxy-2-*epi*-16-deoxysarcophine (6): colorless oil; $[\alpha]_D^{25}$ +52.0 (*c* 0.05, MeOH); UV λ_{max} (log ϵ) (MeOH) 204 (4.4); IR ν_{max} (neat) 3018, 2930, 2858, 2399, 1521, 1436, 1215, 1032, 771, 669 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESITOF *m*/*z* 341.2089 $(M + Na)^+$ (calcd for $C_{20}H_{30}O_3Na$, 341.2093).

10β-Hydroxy-2-*epi*-16-deoxysarcophine (7): colorless oil; $[\alpha]_D^{25}$ −62.1 (*c* 0.19, MeOH); UV λ_{max} (log ϵ) (MeOH) 204 (4.7); IR ν_{max} (neat) 3683, 3019, 2929, 2857, 2400, 1521, 1425, 1385, 1215, 1032, 929, 755, 669 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESITOF *m*/*z* 341.2090 $(M + Na)^+$ (calcd for $C_{20}H_{30}O_3Na$, 341.2093).

14β-Hydroxy-2-*epi*-16-deoxysarcophine (9): colorless oil; $[\alpha]_D^{25}$ +69.3 (*c* 0.08, MeOH); UV λ_{max} (log ϵ) (MeOH) 204 (4.2); IR ν_{max} (neat) 3019, 2935, 2865, 2399, 1535, 1433, 1394, 1215, 928, 759, 669 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRESITOF *m*/*z* 341.2079 $(M + Na)^+$ (calcd for $C_{20}H_{30}O_3Na$, 341.2093).

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References and Notes

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