Zahramycins A-B, Two New Steroids from the Coral *Sarcophyton trocheliophorum*

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Two new poly-hydroxy steroids, zahramycins A (**1**) and B (**2**), have been isolated from the polar fraction of the extract of the coral *Sarcophyton trocheliophorum*. Compound **1** was confirmed to bear an oxirane ring at C-5 and C-6, while **2** has a keto-hydroxy sterol structure. The known DNA primary metabolites uracil, thymine, adenine, uridine, 2′-deoxyuridine, and thymidine were also isolated and identified. Structures of the new sterols **1** and **2** were confirmed by NMR (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY) spectroscopy, mass spectrometry (EI, ESI, and HRMS), and by comparison with related structures. The antimicrobial and cytotoxic activities of compounds **1** and **2** along with that of the coral extract were also determined. Zahramycin B (**2**) showed high (15 mm) and moderate (12 mm) antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*, and fungus *Pythium ultimum* (12 mm), while zahramycin A (**1**) and the crude extract of *Sarcophyton trocheliophorum* were inactive. Both steroids and the crude extract of *Sarcophyton trocheliophorum* showed no cytotoxicity.

Key words: Zahramycins, Polyoxygenated Sterols, *Sarcophyton trocheliophorum*, Biological Activity

Introduction

Soft corals are a productive source of chemically interesting and biologically significant secondary metabolites [1, 2]. They represent a rich source of terpenoids, polyoxygenated sterols and prostaglandin derivatives [1, 3, 4]. Soft corals of the genus *Sarcophyton* and *Sinularia* have been found to be among the most rich sources of bioactive secondary metabolites [5 – 7], such as acylated spermidine [8, 9], lipids and fatty acids [10], cyclic sesquiterpene peroxides [11], in addition to polyoxygenated sterols [12 – 14], among them epoxy steroids [15], polyhydroxy steroids [16] and norditerpenes [17, 18]. A number of them showed an array of biological activities such as cytotoxicity [8, 9] and inhibitory effects on LPS-induced TNF-α production [17, 18].

We have isolated two new hydroxy sterols, zahramycin A (**1**) containing the rare oxirane ring at C-5 and C-6, and zahramycin B (**2**), a keto-hydroxy sterol, from the polar fraction of the extract of the coral *Sarcophyton trocheliophorum*. Additionally, uracil, thymine, adenine, uridine, 2′-deoxyuridine, and thymidine were also isolated and identified. The soft coral *Sarcophyton trocheliophorum* was collected from the Red Sea (Hurghada) coast of Egypt. Structures of the newly isolated compounds **1** and **2** were determined through extensive use of NMR (1D and 2D) spectroscopy and mass spectrometry, and by comparison of their spectroscopic data with those of related structures.

Results and Discussion

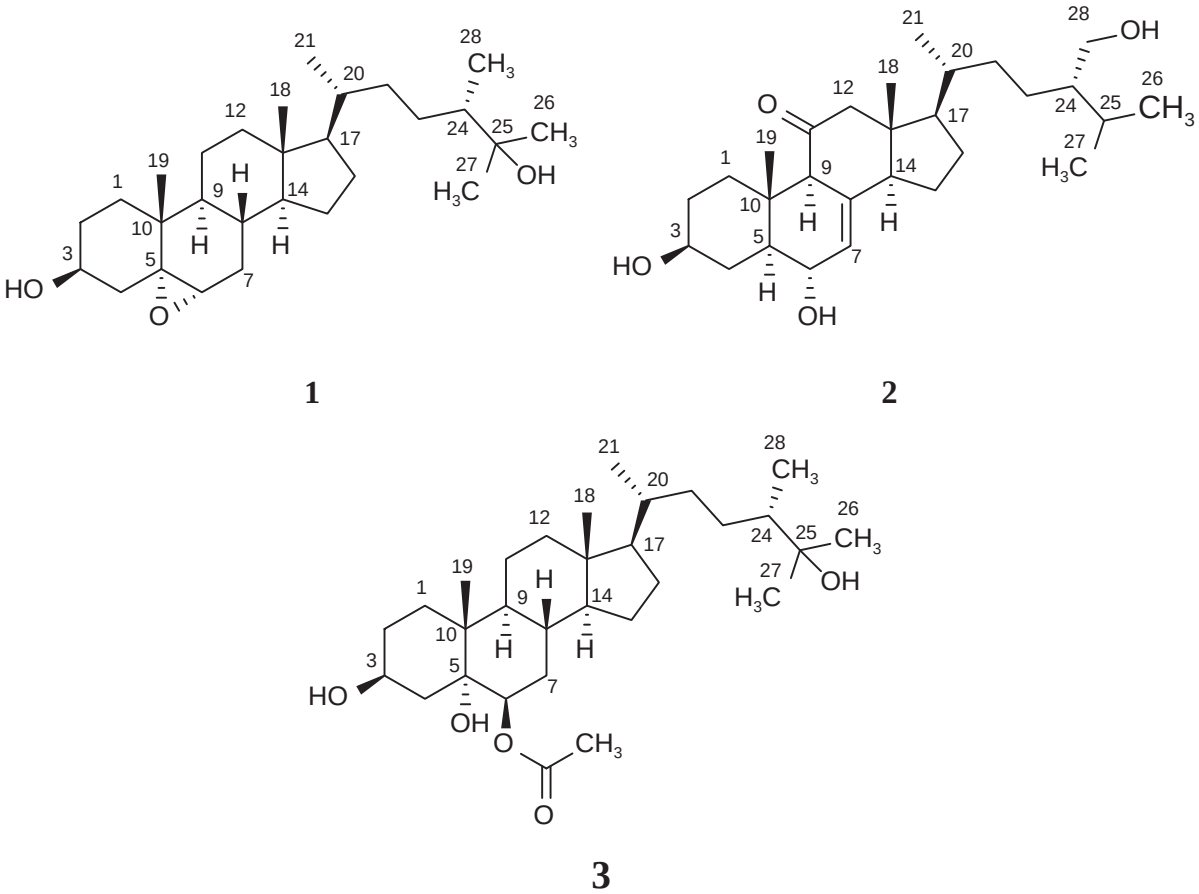
Structures of the well-known primary metabolites uracil, thymine, adenine, uridine, 2′-deoxyuridine, and

thymidine were determined by comparing their chromatographic properties and spectral data (NMR and MS) with the corresponding authentic spectra [19]. The physico-chemical properties of the new compounds zahramycins A (**1**) and B (**2**) are listed in Table 1.

Zahramycin A

Compound **1** was obtained as a colorless powder from sub-fraction F9a, applying a series of chromatographic techniques. The compound did not show UV absorption (254 nm) on TLC, however it turned blue and later changed to brown on spraying with anisaldehyde/sulfuric acid, suggesting the presence of a steroidal component [20]. The molecular weight of **1** was established as 432 Daltons, and *via* HRESI MS, the molecular formula was deduced as $C_{28}H_{48}O_3$, corresponding to five double bond equivalents. Expectedly, the EI mass spectrum of **1** showed a parent molecular ion at $m/z = 432$ together with two fragment ions at $m/z = 414$ and 396, as a result of the elimination of two water molecules (Table 1). This is indicative of the formation of a stable system after consecutive expulsion of two water molecules from the parent structure.

In the NMR spectra, compound **1** showed signals for 6 methyl, 10 methylene, 8 methine and 4 quaternary carbons. The combination of ^{1}H, ^{13}C and HMQC NMR spectra of **1** (Table 2) revealed two 1H multiplets at $\delta = 4.00$ ($\delta_C = 68.1$) and 3.45 ($\delta_C = 76.5$), representing two sp^3 oxy-methines. Four methyl singlets and two methyl doublets were found in the region of $\delta = 1.15 - 0.71$ ($\delta_C = 27.2 - 12.7$), in addition to eight sp^3 methines and ten methylenes. The presence of further four quaternary carbons was established based on the ^{13}C and HMQC NMR spectroscopic data.



Scheme 1. Formula of compounds **1 – 3**.

Table 1. Physico-chemical properties of zahramycins A (**1**) and B (**2**).

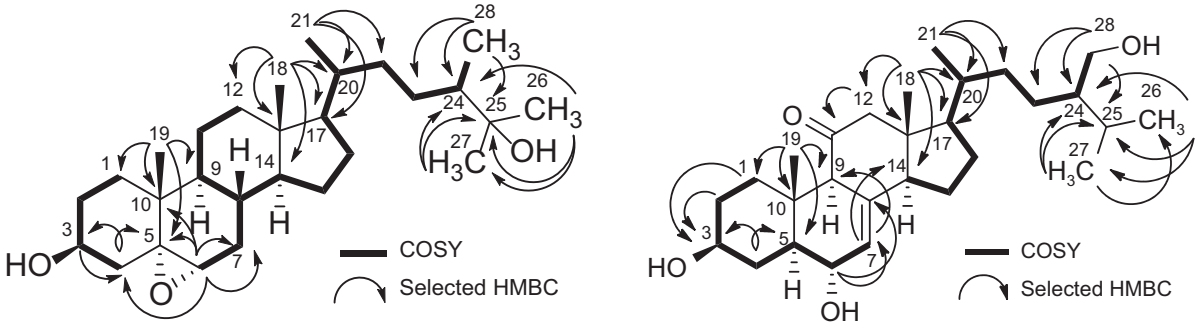
		Zahramycin A (**1**)	Zahramycin B (**2**)
Appearance		colorless solid	colorless solid
R_f^a		0.15[b], 0.35[c]	0.21[d]
Anisaldehyde/sulfuric acid[e]		blue, turned later to brown	blue, turned later to brown
Molecular formula		$C_{28}H_{48}O_3$	$C_{28}H_{46}O_4$
EI-MS: m/z (%)		432 ($[M]^{+\bullet}$, 48), 414 ($[M–H_2O]^{+\bullet}$, 48), 396 ($[M–2H_2O]^{+\bullet}$, 14), 374 (42), 356 (36), 333 (13), 305 (26), 289 (100), 271 (72), 229 (16), 211 (10), 197 (6), 173 (10), 159 (10), 133 (10), 95 (14)	–
HRMS ((+)-ESI): (m/z)			
	found	433.36775 $[M+H]^+$	447.34682 $[M+H]^+$ and 469.32877 $[M+Na]^+$
	calcd.	433.36762 for $C_{28}H_{49}O_3$, $[M+H]^+$	447.34687 for $C_{28}H_{47}O_4$, $[M+H]^+$ and 469.32882 for $C_{28}H_{46}O_4Na$, $[M+Na]^+$
$[\alpha]_D^{20}$ (MeOH)		−13 °C ($c = 0.1$)	+16 °C ($c = 0.1$)

[a] Silica gel 60 F254, Merck; [b] $CHCl_3$ – MeOH, 95 : 5; [c] $CHCl_3$ – MeOH, 93 : 7; [d] $CHCl_3$ – MeOH, 90 : 10; [e] staining reagent.

Of these, two sp^3 oxygenated carbons were observed at $\delta = 76.7$ and 74.1 ppm, while the remaining two appeared at $\delta = 39.3$ and 43.9 ppm.

According to the 2D correlations of **1** (Fig. 1), a 3β-hydroxy group is attached to ring A, as a ^2J HMBC cross signal between 3-H ($\delta_H = 4.00$) and the neighboring methylene carbon C-4 ($\delta_C = 41.4$) and an H,H COSY coupling between CH_2-4 ($\delta = 1.52$, 2.10) and the vicinal 3-oxymethine ($\delta_H = 4.00$) were observed together with those found between the latter and CH_2-2 ($\delta_H = 1.80$). The methyl singlet of CH_3-19 ($\delta_H = 1.15$, $\delta_C = 17.4$) at C-10 ($\delta_C = 39.3$) displayed four correlations with C-10, the oxygenated quaternary atom C-5 ($\delta_C = 76.7$), the methine carbon C-9 ($\delta_C = 46.5$) and CH_2-1 ($\delta_C = 33.5$). The proton signal of the second oxy-methine CH-6 ($\delta_H = 3.45$) showed four HMBC correlations with C-10 ($\delta_C = 39.3$), C-4 ($\delta_C = 41.4$), C-5 ($\delta_C = 76.7$), and CH_2-7 ($\delta_C = 31.6$), and the latter correlation was established further by H,H COSY. Consequently, C-5 and C-6 are oxygenated carbons, and hence, compound **1** carries an oxirane (an epoxy) ring between C-5 and C-6. Proton signals of the methylene CH_2-7 showed H,H COSY couplings with H-8 ($\delta_H = 2.01$), and the latter (H-8) showed two further couplings with H-9 ($\delta_H = 2.01$) and H-14 ($\delta_H = 1.10$), thus completing ring B.

In the HMBC experiment (Fig. 1), the second methyl singlet CH_3-18 ($\delta_H = 0.71$, $\delta_C = 12.7$) showed four correlations towards CH_2-12 ($\delta_C = 41.9$), C-13 ($\delta_C = 43.9$), CH-14 ($\delta_C = 57.4$), CH-17 ($\delta_C = 57.4$), and CH-20 ($\delta_C = 37.7$) connecting the remaining two rings C and D *via* C-13 and C-14. The connection of the two methylene groups CH_2-11 ($\delta_H = 1.40$) and CH_2-12 ($\delta_H = 1.88$, 1.55) was deduced from the H,H COSY correlations, closing ring C. Similarly, the connectivity between CH-14 ($\delta_H = 1.10$), CH_2-15 ($\delta_H = 1.60$, 1.30), CH_2-16 ($\delta_H = 1.78$, 1.40), and CH-17 ($\delta_H = 1.17$) was verified by H,H COSY (Fig. 1), and hence ring D was confirmed. The methyl doublet CH_3-21 ($\delta_H = 0.95$, $\delta_C = 19.6$) displayed three HMBC cou-



1 **2**

Fig. 1. Selected HMBC (⟶) and H,H COSY (▬) couplings of zahramycins A (**1**) and B (**2**).

Table 2. ^{13}C and ^{1}H NMR data of zahramycins A (**1**) and B (**2**) (125/600 MHz, $CDCl_3$; number of protons, multiplicities and coupling constants *J* in Hz in parentheses).

	Zahramycin A (**1**)		Zahramycin B (**2**)	
Position	δ_C	δ_H	δ_C	δ_H
1	33.5	1.30 (1H, m), 1.60 (1H, m)	33.2	1.82 (1H, m), 1.42 (1H, m)
2	31.7	1.80 (2H, m)	31.3	1.84 (1H, m), 1.42 (1H, m)
3	68.1	4.00 (1H, m)	70.6	3.48 (1H, m)
4	41.4	1.52 (1H, m), 2.10 (1H, m)	33.6	1.40 (1H, m), 2.26 (1H, m)
5	76.7	–	32.7	1.72 (1H, m)
6	76.5	3.45 (1H, m)	69.6	4.20 (1H, dd, 9.9, 1.9)
7	31.6	1.70 (1H, m), 1.50 (1H, m)	149.1	6.58 (1H, d, 2.1)
8	29.3	2.01 (1H, m)	137.2	–
9	46.5	2.01 (1H, m)	49.8	1.72 (1H, m)
10	39.3	–	46.0	–
11	22.3	1.10 (2H, m)	205.8	–
12	41.5	1.88 (1H, m), 1.55 (1H, m)	42.2	1.20 (1H, m), 1.55 (1H, m)
13	43.9	–	47.1	–
14	57.4	1.10 (1H, m)	43.5	3.28 (1H, m)
15	25.3	1.60 (1H, m), 1.30 (1H, m)	27.2	1.68 (2H, m)
16	35.3	1.78 (1H, m), 1.40 (1H, m)	28.0	1.62 (2H, m)
17	57.4	1.17 (1H, m)	51.5	1.72 (1H, m)
18	12.7	0.71 (3H, s)	17.8	0.71 (3H, s)
19	17.4	1.15 (3H, s)	16.6	1.14 (3H, s)
20	37.7	1.40 (1H, m)	36.4	1.42 (1H, m)
21	19.6	0.95 (3H, d, 6.6)	19.7	1.01 (3H, d, 6.8)
22	36.2	1.62 (1H, m), 1.50 (1H, m)	34.2	1.20 (1H, m), 1.00 (1H, m)
23	29.1	1.88 (2H, m)	27.2	1.68 (2H, m)
24	46.3	1.26 (1H, m)	40.4	2.35 (1H, m)
25	74.1	–	32.5	1.60 (1H, m)
26	27.2	1.12 (3H, s)	21.0	0.88 (3H, d, 6.8)
27	26.1	1.11 (3H, s)	18.2	0.81 (3H, d, 6.8)
28	15.3	0.88 (3H, d, 6.8)	59.1	3.61 (1H, m), 3.73 (1H, m)

plings to CH-17 ($\delta_C = 57.7$, ^3J), CH-20 ($\delta_C = 37.7$, ^2J) and methylene CH_2-22 ($\delta_C = 36.2$, ^3J), confirming the direct connection between ring D and the side chain *via* CH-17 ($\delta_H = 1.17$) and CH-20 ($\delta_H = 1.40$). The complementary methylene CH_2-23 ($\delta_H = 1.88$) was assigned *via* an H,H COSY correlation to H-22 ($\delta_H = 1.62$, 1.50). CH_2-23 was in turn connected to a terminal hydroxy-*iso*-pentyl fragment. The last fragment was structurally confirmed by the presence of an HMBC correlation between both singlet methyls CH_3-26 ($\delta_H = 1.12$, $\delta_C = 27.2$) and CH_3-27 ($\delta_H = 1.11$, $\delta_C = 26.1$), through the in-between oxygenated quaternary carbon C-25 ($\delta_C = 74.1$). Consequently, the presence of a terminal 2-hydroxy-isopropyl group was deduced. Both two methyls CH_3-26 and CH_3-27 displayed further ^3J HMBC connectivity towards CH-24 ($\delta_C = 46.3$), and the latter was connected to the doublet methyl CH_3-28 ($\delta_H = 0.83$, $\delta_C = 15.3$) and CH_2-

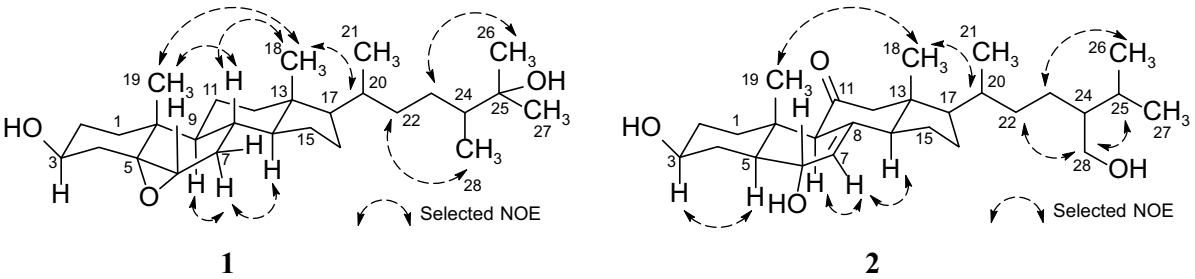


Fig. 2. Selected NOE (↙ ↘) couplings of zahramycins A (**1**) and B (**2**).

23 ($\delta_H = 1.62, 1.50$; $\delta_C = 36.2$) *via* both HMBC and H,H COSY correlations. Based on the detailed discussion of the spectroscopic data, compound **1** was deduced to bear an oxirane ring between C-5 and C-6, and a terminal hydroxy-isopropyl group in the side chain. Structure **1** was determined as (24*S*)-ergostane-5α,6α-epoxy-3β,25-diol, a new steroid, which was named zahramycin A (**1**). Compound **1** has structural similarity to the recently reported (24*S*)-ergostane-6-acetate-3β,5α,6β,25-tetraol (**3**) isolated from the soft coral *Sinularia* sp. [5], and other related structures from *Sarcophyton glaucum* [16], except that the two oxygenated carbons C-5 and C-6 in **3** bearing hydroxy (C-5) and acetoxy (C-6) groups [5] were replaced by an oxirane ring in **1**. The relative configuration of **1** was deduced on the basis of a 2D NOESY experiment. Correlations from H_3-18 to H-20 were observed, indicating that H-20 was in β-orientation. The α-orientation of H-9 and H-14, and the β-orientation of H-8 were also determined by the NOESY experiment (Fig. 2). Since almost all 24-methylsterols isolated from corals exhibit 24*S* configuration according to the literature [5, 16, 21 – 23], zahramycin A (**1**) was concluded to have the same configuration.

Zahramycin B

Closely related to zahramycin A (**1**), compound **2** was obtained as a colorless amorphous powder from sub-fraction F9b, showing weak UV absorbance (254 nm) on the TLC in addition to a similar reactivity towards anisaldehyde/sulfuric acid, indicating its steroidal nature. The molecular formula was established as $C_{28}H_{46}O_4$ based on HRESIMS (Table 1) corresponding to six double bond equivalents. The ^{1}H and ^{13}C NMR spectra (Table 2) implied furthermore that compound **2** was a polyhydroxylated keto-sterol. The ^{1}H NMR spectrum of **2** revealed two singlet methyl signals at $\delta = 0.71$ (H_3-18), and 1.14 ppm (H_3-19) and three doublet methyl signals at $\delta = 0.88$ (H_3-26), 0.81 (H_3-27) and 1.01 ppm (H_3-21), as well as two oxymethines ($\delta = 3.48$, H-3; 4.20, H-6), two 1H multiplets of an oxymethylene ($\delta = 3.61$, 3.73, H_2-28) and an olefinic doublet proton signal at $\delta = 6.58$ ppm (H-7).

The ^{13}C NMR and HMQC spectra of **2** displayed 28 carbon signals comprising four quaternary carbons, ten methines, nine methylenes, and five methyls. The olefinic carbon signals appeared at $\delta_C = 149.8$ (CH-7) and 137.2 ppm (C_q-8) corresponding to one trisubstituted double bond. The carbon signal at $\delta =$ 205.8 ppm (C-11) is corresponding to a ketone carbonyl group. The carbon signals at $\delta_C = 70.6$ (CH-3), 69.6 (CH-6) and 59.1 ppm (CH_2-28) confirmed the presence of two oxygenated methine carbons and one oxy-methylene, respectively. Detailed analysis of the ^{1}H, ^{1}H COSY spectrum in combination with HMQC and HMBC (Fig. 1) experiments allowed the assignment of all signals due to the chemical shifts in the ^{1}H and ^{13}C NMR spectra and led to structure **2**. The relative stereochemistry of **2** was assigned on the basis of a 2D NOESY experiment (Fig. 2). In the NOESY spectrum of **2**, NOE correlations observed from H_3-18 to H-20 indicated similarly that H-20 was in β-position. Likely, the α-orientation of H-9 and H-14 were also determined by a NOESY experiment (Fig. 2). Based on the coupling constants of H-6 (dd, $J = 9.9$, 1.9 Hz) and H-7 (d, $J = 2.1$ Hz), and by comparison with the reported data of hirsutosterol G [24], the protons H-5 and H-6 are in axial orientation confirming the α-OH group at C-6. Since almost all the 24-methylsterols isolated from corals have 24*S* stereochemistry according to the literature [5], the absolute configuration of **2** was determined as (3*S*,6*S*,24*S*)-ergost-7-ene-11-oxo-3β,6α-diol, which was named zahramycin B (**2**).

Biological activity

The crude extract of *Sarcophyton trocheliophorum* and the new steroid zahramycin A (**1**) were antibiotically inactive against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptomyces viridochromogenes* (Tü 57), *Escherichia coli*, *Candida albicans*, *Mucor miehi*, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Scenedesmus subspicatus*, *Rhizoctonia solani*, and *Pythium ultimum* at 40 μg per disk. In contrast, zahramycin B (**2**) showed high (15 mm) and moderate (12 mm) activity against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, respectively. In addition, compound **2** displayed a moderate activity against the plant pathogenic fungus *Pythium ultimum* (12 mm). The new steroids **1** and **2** and the crude extract were further examined for cytotoxicity against brine shrimp at a concentration of 40 μg mL^{-1} (24 hr). Both steroids exhibited no cytotoxicity (0 %), while the crude extract of *Sarcophyton trocheliophorum* showed weak cytotoxicity with a mortality rate of 1.7 %.

Experimental Section

Optical rotations: Polarimeter (Perkin-Elmer, model 343; Waltham, MA, USA). The NMR spectra were measured on Varian (Palo Alto, CA, USA) Unity 300 (300.145 MHz) and Varian Inova 600 (150.820 MHz) spectrometers. HR-ESIMS was recorded by ESI MS on an Apex IV 7 Tesla FT Ion Cyclotron Resonance MS (Bruker Daltonics, Billerica, MA, USA). EI mass spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV). Flash chromatography was carried out on silica gel (230 – 400 mesh). R_f values were measured on Polygram SIL G/UV$_{254}$ TLC cards (Macherey-Nagel). Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd; purchased from Sigma-Aldrich Chemie, Steinheim, Germany).

Animal material

The soft coral *Sarcophyton trocheliophorum* (1.6 kg wet weight) was collected from the Red Sea about ∼ 1 km off the coast of Hurghada, east Egypt, at a depth of ∼ 30 m, and stored in a freezer until extraction. The *Sarcophyton trocheliophorum* was morphologically characterized by Dr. Mohamed A. Ghani, Hurghada, Egypt, and a specimen was deposited at Red Sea Marine Parks, P. O. Box 363, Hurghada, Red Sea, Egypt.

Extraction, isolation and purification

The *Sarcophyton trocheliophorum* was homogenized in a blender, macerated with 6 L of chloroform-methanol (8 : 2) and kept at ∼ 5 celsius for 8 days. The solid material was filtered off, and the chloroform layer was evaporated *in vacuo*. The remaining aqueous methanol solution was reextracted with *n*-butanol. This butanol extract was similarly evaporated to dryness. Both extracts (chloroform and *n*-butanol) were combined according to TLC similarity and dried under vacuum, affording 62.8 g of a dark-green crude extract. The extract was subjected to column chromatography on silica gel (70 cm × 10 cm) and eluted with a cyclohexane-DCM-MeOH gradient (cyclohexane 1 L, cyclohexane-20 % DCM 1 L, cyclohexane-40 % DCM 1 L, cyclohexane-50 % DCM 1 L, cyclohexane-80 % DCM 1 L, DCM 1 L, DCM-2 % MeOH 1 L, DCM-5 % MeOH 1 L, DCM-10 % MeOH 1 L, DCM-20 % MeOH 1 L, DCM-50 % MeOH 1 L, MeOH 1 L). Based on TLC evaluation, 10 fractions were obtained F1 (1.7 g), F2 (1.2 g), F3 (7.2 g), F4 (3.4 g), F5 (2.1 g), F6 (21.2 g), F7 (1.6 g), F8 (1.9 g), F9 (4.2 g), and F10 (5.3 g). The polar fraction F9 was subjected to silica gel column chromatography (column 3 × 100 cm), eluting with an *n*-hexane-DCM-MeOH gradient (*n*-hexane 0.5 L, *n*-hexane-50 % DCM 0.25 L, DCM 0.5 L, DCM-1 % MeOH 0.2 L, DCM-2 % MeOH 0.2 L, DCM-5 % MeOH 0.5 L, DCM-10 % MeOH 0.5 L, MeOH 0.5 L). Based on TLC monitoring, visualized by UV and anisaldehyde/sulfuric acid as spraying reagent, four further sub-fractions were obtained: F9a (0.5 g), F9b (0.6 g), F9c (1.2 g), and F9d (0.8 g). Purification of F9a on Sephadex LH-20 (MeOH) followed by PTLC (DCM/10 % MeOH) and then Sephadex LH-20 (MeOH) gave zahramycin A (**1**, 15.3 mg) as a colorless powder. In the same manner, purification of sub-fraction F9b on Sephadex LH-20 (MeOH) followed by PTLC (DCM/10 % MeOH) and further Sephadex LH-20 (MeOH) afforded zahramycin B (**2**, 12.7 mg). Sub-fractions F9c and F9d were individually purified on silica gel (column 2 × 50 cm, DCM-MeOH), followed by Sephadex LH-20 (MeOH) to afford colorless solids of uracil (10.0 mg), thymine (7.0 mg) and adenine (8.0 mg) from F9c, while uridine (12.0 mg), 2′-deoxyuridine (11.0 mg) and thymidine (9.0 mg) were obtained from F9d as colorless solids.

Antimicrobial activity

The extract of the soft coral *Sarcophyton trocheliophorum* was dissolved in CH_2Cl_2-10 % MeOH at a concentration of 1 mg mL^{-1}. Aliquots of 40 μL were soaked on filter paper discs (9 mm diameter, no. 2668, Schleicher & Schüll, Einbeck, Germany) and dried for 1 h at room temperature under sterile conditions. The paper discs were placed on inoculated agar plats and incubated for 24 h at 38 °C for bacteria and 48 h (30 °C) for fungal isolates, while the algal test strains were incubated at ∼ 22 °C in day light for 8 ∼ 10 days. The crude extract of the soft coral and the new steroids zahramycins A (**1**) and B (**2**) were examined against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptomyces viridochromogenes* (Tü 57), *Escherichia coli*, *Candida albicans*, *Mucor miehi*, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Scenedesmus subspicatus*, *Rhizoctonia solani*, and *Pythium ultimum*.

Brine shrimp microwell cytotoxic assay

The cytotoxicity assay was performed according to Takahashi *et al.* [25] and Sajid *et al.* [26].

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