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New antimicrobial biscembrane hydrocarbon and cembranoid diterpenes from the soft coral *Sarcophyton trocheliophorum*

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Abstract: A new tetracyclic biscembrane hydrocarbon, trocheliane (**1**), along with two new cembranoid diterpenes, sarcotrocheldiol A (**2**) and B (**3**), and the known diterpene cembrene-C (**4**), were isolated from the Red Sea soft coral *Sarcophyton trocheliophorum.* The structures and relative stereochemistry of the compounds were elucidated by interpretation of MS, 1 D NMR, and 2 D NMR experiments. The sensitivity of some pathogenic bacteria used as test organisms to the new compounds **1**–**3** was determined. **1** showed appreciable antimicrobial activity with the diameter of inhibition zones ranging from 11 to 18 mm. **1** was active against the two multidrug resistant bacteria *Acinetobacter baumannii* and *Staphylococcus aureus.* Minimal inhibitory concentrations (MICs) of compound **1** were recorded for all the tested bacteria using the fluorescein diacetate method and the recorded MIC values ranged from 4 to 6 μM.

Key words: Soft coral, biscembranes, diterpenoids, antibacterial activity

1. Introduction

Common, prolific, and offering potentially novel and biologically active secondary metabolites are the characteristics of the species of the soft bodied coral *Sarcophyton* (phylum, Cnidaria; class, Anthozoa; subclass, Octocorallia; order, Alcyonaceae; family, Alcyoniidae).[1] The most frequently isolated metabolites are terpenoids. Among terpenoids, diterpene dimers or biscembranoids are extremely rare compounds and are almost exclusive to four species of the genus *Sarcophyton*, i.e. *S. glaucum*, *S. elegans*, *S. tortuosum*, and *S. latum.*[2,3] Several cembranoid-type diterpenes have been shown to exhibit various pharmacological activities, such as antifeedant[4] and anti-inflammatory activities,[5] and cytotoxicity.[6] We have previously reported several cytotoxic cembranoids from *Sarcophyton glaucum* and *S. trocheliophorum* collected from the Saudi Arabian Red Sea.[7–9]

2. Results and discussion

Our further investigation of the CH_2Cl_2-soluble material of the $CHCl_3$:MeOH extract of the soft bodied organism *S. trocheliophorum* led to the isolation of several metabolites: **1** (5.3 mg, 0.0067%), **2** (3.5 mg, 0.0044%), **3** (3.6 mg, 0.0046%), and **4** (15.0 mg, 0.0190%) (Figure 1).

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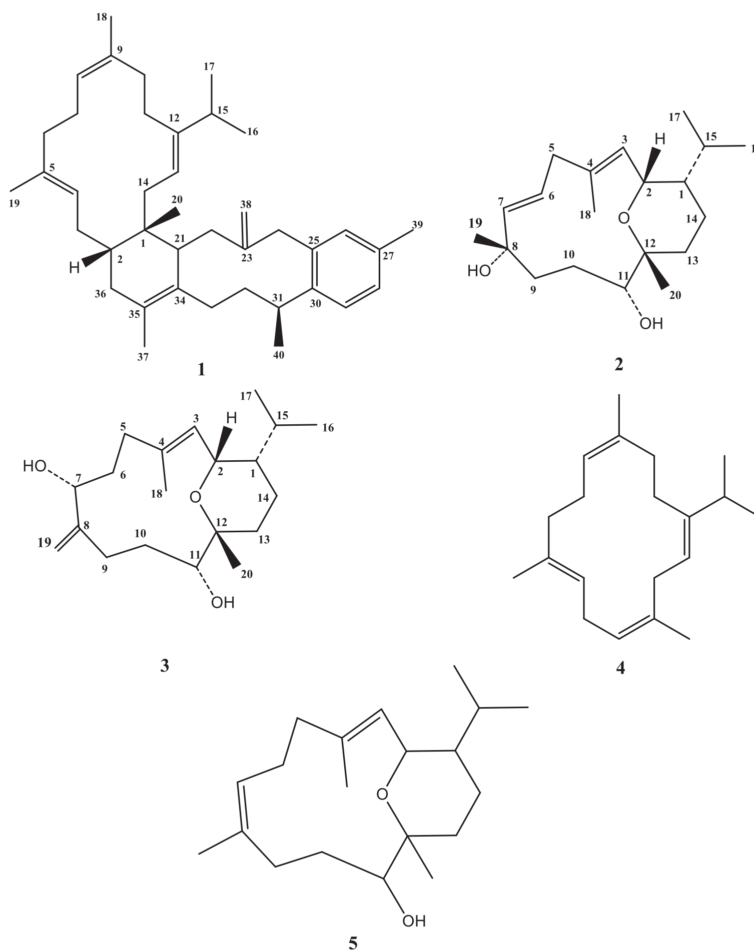

Figure 1. Structures of compounds **1**–**5**.

Compound **1** was isolated as gummy material. **1** was analyzed for $C_{40}H_{58}$ on the basis of HREIMS at m/z 538.4528 $(M)^+$ and ^{13}C NMR spectra. The IR (neat) spectrum displayed absorptions due to (C–H) 3050–2700, (C=C) 1630, (aromatic ring) 1510, and (terminal methylene) 925 functionalities cm^{-1}. The presence of a substituted benzene ring was concluded from the UV absorption maximum (λ_{max}) at 221 nm, supported by the IR absorption at 1510 cm^{-1}. The ^{13}C NMR spectrum of **1** (*cf. exp.*) showed resonances for 40 carbons, differentiated by DEPT NMR experiment into 8 methyl, 12 methylene, 10 methine, and 10 quaternary carbons. Eight of the twelve elements of unsaturation as indicated by the molecular formula of **1** are attributed to eight C=C double bonds evidenced from 16 signals between δ_C 110.1 and 149.3; thus, the molecule is a tetracarbocyclic skeleton. The ^1H NMR signals at 7.02 (br s), 6.94 (br d, J = 7.8 Hz), and 7.11 (br d, J = 7.8 Hz) together with the aromatic methyl signal at δ_H/δ_C 2.32 (s)/21.1 revealed the presence of 1,3,4-trisubstituted benzene. Moreover, ^1H, ^{13}C, and HSQC NMR spectral data featured the following: three olefinic methines resonating at δ_H/δ_C 4.98 (ddd, J = 12.0, 6.0, 1.2 Hz)/125.9, 5.06 (ddd, J = 12.0, 6.6, 1.2 Hz)/121.8, and 5.19 (ddd, J = 12.0, 7.2, 1.2 Hz)/124.0; exocyclic methylene protons at 4.65 (d, J = 2.4 Hz) and 4.71 (d, J = 2.4 Hz)/110.1; three doublet methyls at 0.71 (d, J = 6.6 Hz)/17.3, 0.99 (d, J = 6.6 Hz)/21.3, and 1.26 (d, J = 6.6 Hz)/22.3, and four singlet methyls at 1.56/18.0, 1.57/15.5, 1.59/15.3, and 1.0/16.6. The ^1H–^1H COSY and HSQC analyses led to assignment of the following proton sequences:

CH_2–CH–CH_2–CH= (C-36–C-2–C-4), CH_2–CH_2–CH= (C-6–C-8), CH_2–CH_2 (C-10–C-11), CH_3–CH–CH_3 (C-15–C-17; isopropyl moiety), =CH–CH_2 (C-13–C-14), CH–CH_2 (C-21–C-22), =CH–CH= (C-28–C-29), and CH_3–CH–CH_2–CH_2 (C-40–C-31–C-33). Extensive study of the HMBC correlations established the following connectivities: a) the correlations observed between H-15 and the quaternary carbon C-12 (δ_C 149.3), C-13 (121.8), the CH_2 carbon C-11 (33.9), and the methyl carbon C-17 (21.3) established the attachment of the isopropyl function to C-12. The correlations between H-13 and the quaternary carbon C-1 (39.9) and C-11, between H-8 and the methyl carbon C-18 (15.5) and C-10 (28.1), and between H-4 and C-19 (15.3), C-6 (32.4), and the methine carbon C-2 (45.9), together with those between H_3-20 and C-14 (39.4), C-21 (43.7), C-2, and C-1 established the 14-membered ring, which is very similar to the data of cembrene-C (**5**). b) For the remaining part of the structure we expect a tricyclic partial structure containing a 3,4-disubstituted toluene, which was unambiguously determined from the HMBC's correlations: H-21 with C-2, C-35 (δ_C 133.4), C-23 (150.3), and C-1; H_3-37 with C-34 (128.7) and C-36; H-24 with C-30 (134.8), C-38 (110.1), and C-26 (128.7); and finally H-29 with C-31 (32.5) and C-27 (134.4). Hence, the gross structure can be constructed as in Figure 1, and the trivial name trocheliane (**1**) was given. The relative stereochemistry of the asymmetric carbons (C-1, C-2, and C-31) and the geometry of the double bonds were deduced from the NOESY spectrum and studying the chemical shift (δ_C) values of the methylene carbons allylic to the carbon–carbon double bond.[10] The cross peak between H-2 and H_3-20 implies *cis* fusion between the 14-membered ring moiety and the cyclohexene ring. These protons are also correlated with protons of Me-18, Me-19, Me-40, and H-13, also indicating cofacial orientation of these groups, and the *E* configuration for C-4/C-5, C-8/C9, and C-12/C-13 double bonds as well. The downfield chemical shift value of the allylic CH_2 group δ_C is higher than 30 ppm, supporting the *E* configuration of these double bonds.

Compound **2** was obtained as gummy material. **2** was analyzed for $C_{20}H_{34}O_3$ on the basis of HREIMS at m/z 322.2500 $(M)^+$ and ^{13}C NMR spectra, implying four degrees of unsaturation. The ^{13}C (*cf. exp.*) and DEPT NMR experiments allowed the determination of 20 resonances attributable to five Me carbons (δ_C 17.6, 20.5, 20.7, 23.8, and 29.3), five CH_2 carbons (42.5, 39.9, 22.8, 34.1, and 18.6), and four sp^3 CH carbons including two oxygen-bearing (72.0 and 75.8), three sp^2 CH (124.4, 128.2, and 135.7), and three quaternary C-atoms including two O-bearing (73.6 and 74.9). The ^1H NMR and COSY NMR of **2** displayed three Me singlet signals (δ_H 1.83, 1.33, 1.04), one ^{i}Pr [δ_H 1.22 (m, 1H), 0.88 (d, J = 6.6 Hz, 3H) and 0.76 (d, J = 6.6 Hz, 3H)], three olefinic protons [δ_H 5.28 (d, J = 10.8 Hz), 5.85 (ddd, J = 15.6, 10.8, 4.2 Hz) and 5.41 (br d, J = 15.6 Hz)], and two O-bearing CH protons [δ_H 4.62 (dd, J = 10.8, 4.8 Hz), 3.48 (d, J = 9.6 Hz)]. The NMR data of **2** displayed great similarity to those of the known compound sarcotrocheliol (**5**).[9] The main difference was signals at δ_C 128.2 and 135.7, suggesting the presence of a disubstituted double bond system in **2**, instead of a trisubstituted double bond system in **5**, and a signal at 73.6, indicating an extra oxygenated quaternary carbon. The ^{13}C NMR signals at δ_C 124.4 and 141.2 indicated a nonconjugated trisubstituted double bond belonging to C-3–C-4 based on the HMBC correlation with Me-18, also ^{13}C NMR signals at δ_C 128.2 and 135.7 assigned as nonconjugated disubstituted double bonds belonging to C-6–C-7 by HMBC correlation between Me-19 and C-7. Moreover, ^1H–^1H COSY spectral analysis established three partial structures of consecutive proton spin systems from H-5 to H-7 through H-6; from H-9 to H-11 through H-10; and finally from H-3 to H-13 through H-2, H-1 (H-15), H-14, and H-13. These data, together with the HMBCs correlation, from H-16 to C-15, C-17, and C-1; from H-18 to C-3, C-4, and C-5; from H-19 to C-7, C-8, and C-9; and from H-20 to C-12, C-11, and C-13 established the connectivity within the 14-membered ring. The relative configuration of **2** was assigned

mainly from the NOESY spectrum and the coupling constant values (J). The existence of a cross peak between the H_3-18/H-6 and H_3-19, together with the absence of the correlation H_3-18/H-3, as well as the presence of a cross peak between H-3 and H-7, led to the E geometry for the C-3–C-4 double bond. This (E) geometry was also supported by the downfield chemical shift of the allylic CH_2 group of C-5 at δ_C 42.5 (i.e. $\delta_C > 30$) and the J value (10.8 Hz).[10] The E geometry of C-6=C-7 was concluded from the large coupling constant value ($J = 15.6$ Hz), and the NOE correlation H-7/H-3. The cross peak between H-1 and H-5a (m, 2.75–2.80) and H_3-20 allowed us to assign them as β-oriented and OH-11 as α-oriented, owing to the fact that the ^{i}Pr group must be α-oriented (cembranes from the order Alcyonaceae possess an α-oriented isopropyl group at C-1).[11] Analysis of all the NMR spectroscopic data confirmed **2** to be a cembranoid derivative, and the trivial name sarcotrocheldiol A was given.

Compound **3** was found to have the same molecular formula as **2**, $C_{20}H_{34}O_3$, as indicated from HREIMS and ^{13}C NMR spectra (*cf. exp.*). Comparison of the ^{1}H and ^{13}C NMR data of **3** with those of **2** revealed that **3** is analogous to those of **2** except for the replacement of a disubstituted double bond (C-6=C-7) in **2** by the substitution of a hydroxyl group at C-7 resonating at δ_C 67.8 in **3**, and the appearance of an exocyclic double bond at C-8 δ_C 147.5. The structure of **3** was established by analyses of HMBC and COSY NMR spectra. Moreover, the similar splitting pattern and J values of H-3 (10.8 Hz) in both **2** and **3**, together with the significant NOE interaction between H_3-18 and H-6a, revealed the E geometry of the trisubstituted double bond at C-3=C-4. Therefore, the structure of **3** was identified as sarcotrocheldiol B.

Compound **4** was identified by comparing its spectral data with those in the literature.[12]

The sensitivity of some pathogenic bacteria including *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, and *Streptococcus pneumoniae* to the purified fractions **1**–**3** was determined by agar well diffusion.[13] To compare the antibacterial activity results, ampicillin was used as positive control due to its broad spectrum of bactericidal activity against both gram-positive and gram-negative pathogens. **1** showed appreciable antimicrobial activity against the tested bacteria with diameter of inhibition zones ranging from 12 to 18 mm. **1** was active against the two multidrug resistant bacteria *A. baumannii* and *S. aureus.* Very weak antibacterial activity was recorded against all the tested pathogenic bacteria for compounds **2** and **3**.

Minimal inhibitory concentrations (MICs) of compound **1** were recorded for all the tested bacteria using a fluorescein diacetate assay.[14] The recorded MICs ranged from 4 to 6 μM (Table).

Table. The antibacterial activity (diameter of inhibition zone, mm) of the tested compounds (**1**–**3**) and the control (5 μg/mL), and MIC values of compound **1**.

Tested bacteria	Mean diameter of the inhibition zone (mm) ± SD				MIC (μM)	
	1	2	3	Ampicillin	1	Ampicillin
Acinetobacter baumannii	18 ± 3.2	7 ± 3.2	NF	20 ± 1.2	4.2	≥ 2.6
Escherichia coli	13 ± 1.7	8 ± 3.2	NF	15 ± 1.3	6.0	2.6
Klebsiella pneumonia	15 ± 1.2	7 ± 3.2	11 ± 3.2	24 ± 2.2	5.8	9 ± 2.1
Pseudomonas aeruginosa	16 ± 2.2	7 ± 3.2	NF	19 ± 3.0	5.2	13 ± 1.1
Staphylococcus aureus	18 ± 1.4	NF	11 ± 1.8	28 ± 1.4	4.0	10 ± 1.2
S. epidermidis	16 ± 1.4	NF	6 ± 3.2	24 ± 1.7	5.7	9 ±1.7
Streptococcus pneumoniae	13 ± 1.5	NF	NF	28 ± 1.4	6.0	11 ± 2.7

NF: Not found, Ampicillin: positive control

3. Conclusions

Herein, we report the first isolation of an unprecedented biscembranoid dimer hydrocarbon (trocheliane, **1**) and two new furan-containing cembranoid diterpenes (**2**, **3**) from the Red Sea soft coral *S. trocheliophorum.* Product **1** is a promising compound due to its activity against the two multidrug resistant bacteria *A. baumannii* and *S. aureus.* The MICs were determined using a fluorescein diacetate assay. The recorded MICs ranged from 4 to 6 μM (Table). Trocheliane (**1**) could be the addition adduct of two cembrene-C (**4**) isomers (Figure 2).

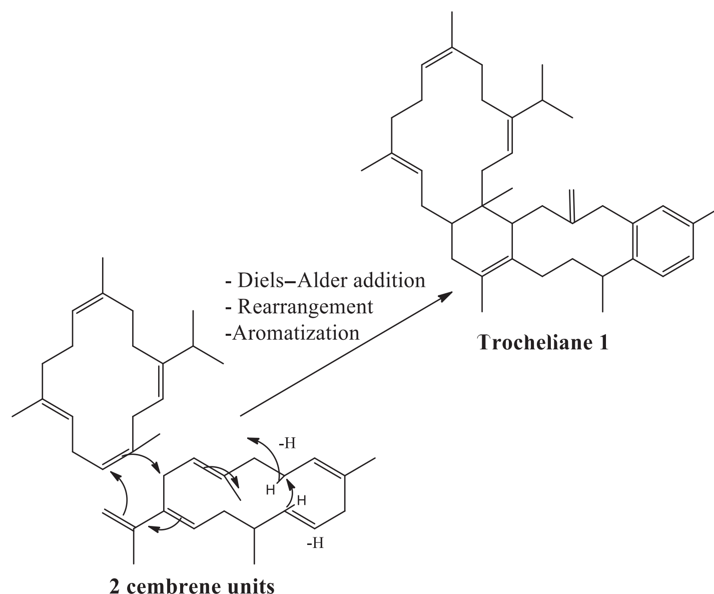

Figure 2. Hypothetical formation of trocheliane (**1**).

4. Experimental

4.1. General

Silica gel (SiO_2; Kieselgel 60 F_{254}) of 0.25 mm layer thickness. 1 D and 2 D NMR: Bruker AVANCE III WM at 600 MHz, and ^{13}C NMR at 150 MHz; δ in ppm rel. to Me_4Si as internal standard, J in Hz. High resolution mass spectra were recorded on a Finnigan FTMS 2001 instrument.

Soft coral *S. trocheliophorum* was collected (May, 2013) from the Red Sea coast (21° 29′ 31″ N 39° 11′ 24″ E), north of Jeddah, Saudi Arabia, at a depth of 5–10 m. It was identified by Dr Yahia Folos (Marine Biology Department, Faculty of Marine Sciences, KAU). A voucher sample (JAD 09060) was deposited at the Chemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, KSA.

Bacterial isolates: *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, and *Streptococcus pneumoniae* were taken from the culture collection of the Microbiology Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.

4.2. Extraction and isolation

Coral material was washed with water and dried in the shade at room temperature. The dried material (79.00 g) was exhaustively extracted with equal volumes of $CHCl_3$/MeOH (2 × 6 L, 24 h for each batch) at room temperature. The residue (20.25 g) was partitioned between CH_2Cl_2 and water; the CH_2Cl_2 soluble

material was dried (9.18 g) and chromatographed by NP (Merck, 60G) column chromatography employing n-hexane/CH_2Cl_2, followed by EtOAc/MeOH mixtures with increasing polarity. Fractions of ~100 mL were collected. TLC was carried out by employing silica gel chromatoplates, an appropriate solvent system, and 50% H_2SO_4 in MeOH as spraying reagent. Fractions containing a single compound were combined and further purified by preparative TLC of glass-supported silica gel plates (20 cm × 20 cm) of 250 μm thickness.

Fraction A, eluted with n-hexane:methylene chloride (9.5:0.5, 37.00 mg), was purified by preparative TLC using the solvent system n-hexane:methylene chloride (9:1). The band with R_f = 0.75 (pink color with sulfuric acid/methanol) was taken to give compound **4** as a colorless oil (15.00 mg). Fraction B, eluted with n-hexane (9:1, 36.00 mg), was purified by preparative TLC using the solvent system n-hexane:methylene chloride (8:2). The band with R_f = 0.70 (violet appearance under UV_{254}, and brown color with H_2SO_4/MeOH) was taken to give compound **1** (Figure 1) as a colorless oil (5.3 mg, 0.0067 dry weight). Fraction H, eluted with CH_2Cl_2:MeOH (9:1, 35.00 mg), was purified by preparative TLC using the solvent system n-hexane:EtOAc (2:8) to give two bands. The first band, with R_f = 0.38 (brown color with H_2SO_4/MeOH), was taken to give colorless oil (3.5 mg) of compound **2**. The second band, with R_f = 0.35 (brown color with H_2SO_4/MeOH), was taken to give colorless oil (3.6 mg) of compound **3**.

Trocheliane (1): Gummy material; $[\alpha]_D^{20}$ –22.0 (c 0.02, C_6H_6); IR (film) cm^{-1}: 3050–2700 (CH), 1630, 1620 (C=C), 1510, 925, 740; ^{1}H NMR (C_6D_6, 600 MHz): 2.01–2.05 (1H, m, H-2), 2.15–2.20 (1H, m, H-3a), 2.10–2.15 (1H, m, H-3b), 4.98 (1H, ddd, J = 12.0, 6.0, 1.2 Hz, H-4), 1.98–2.04 (1Hm, H-6a), 1.56–1.58 (1H, m, H-6b), 1.92–1.94 (1H, m, H-7a), 1.32–1.36 (1H, m, H-7b), 5.19 (1H, ddd, J = 15.0, 7.2, 1.2 Hz, H-8), 1.64–1.70 (1H, m, H-10a), 1.30–1.33 (1H, m, H-10b), 1.92–1.98 (1H, m, H-11a), 1.76–1.80 (1H, m, H-11b), 5.06 (1H, ddd, J = 12.6, 6.6, 1.2 Hz, H-13), 2.01–2.10 (1H, m, H-14a), 1.44–1.48 (1H, m, H-14b), 2.20–2.26 (1H, m, H-15), 0.71 (3H, J = d, 6.6 Hz, H-16), 0.99 (3H, d, J = 6.6 Hz, H-17), 1.57 (3H, s, H-18), 1.59 (3H, s, H-19), 1.00 (3H, s, H-20), 2.68–2.69 (1H, m, H-21), 1.81–1.86 (1H, m, H-22a), 1.57–1.61 (1H, m, H-22b), 2.12 (1H, d, J = 6.6 Hz, H-24a), 2.06 (1H, d, J = 6.6 Hz, H-24b), 7.02 (1H, s, H-26), 6.94 (1H, br d, J = 7.8, H-28), 7.11 (1H, J = br d, 7.8, H-29), 2.75 (1H, hex, H-31), 1.31–1.39 (1H, m, H-32a), 1.21–1.29 (1H, m, H-32b), 1.26–1.30 (1H, m, H-33a), 1.30–1.32 (1H, m, H-33b), 2.20–2.23 (1H, m, H-36a), 2.16–2.20 (1H, m, H-36b), 1.56 (3H, s, H-37), 4.65 (1H, d, J = 2.4, H-38a), 4.70 (1H, d, J = 2.4, H-38b), 2.32 (3H, s, H-39), 1.26 (3H, d, 6.6, H-40), ^{13}C NMR (C_6D_6, 150 MHz): 39.9 (C, C-1), 45.9 (CH, C-2), 38.9 (CH_2, C-3), 125.9 (CH, C-4), 133.9 (C, C-5), 32.4 (CH_2, C-6), 30.8 (CH_2, C-7), 124.0 (CH, C-8), 140.0 (C, C-9), 28.1 (CH_2, C-10), 33.9 (CH_2, C-11), 149.3 (C, C-12), 121.8 (CH, C-13), 39.4 (CH_2, C-14), 31.8 (CH, C-15) 17.3 (CH_3, C-16), 21.3 (CH_3, C-17), 15.5 (CH_3, C-18), 15.3 (CH_3, C-19), 16.6 (CH_3, C-20), 43.7 (CH, C-21), 21.4 (CH_2, C-22), 150.3 (C, C-23), 23.7 (CH_2, C-24), 139.9 (C, C-25), 128.7 (CH, C-26), 134.4 (C, C-27), 126.1 (CH, C-28), 126.7 (CH, C-29), 134.8 (C, C-30), 32.5 (CH, C-31), 29.6 (CH_2, C-32), 22.7 (CH_2, C-33), 128.7 (C, C-34), 133.4 (C, C-35), 24.8 (CH_2, C-36), 18.0 (CH_3, C-37), 110.1 (CH_2, C-38), 21.1 (CH_3, C-39), 22.3 (CH_3, C-40); HRESIMS data m/z 538.4528 [M]+ (Calculated 538.4539 for $C_{40}H_{58}$).

Sarcotrocheldiol A (**2**): Colorless oil; $[\alpha]_D^{20}$ 62.4 (c 0.012, $CHCl_3$); IR (film) cm^{-1}: 3423 (OH), 3180 (OH), 2937 (C–H), 1645 (C=C), 1378, 1221, 1045; ^{1}H NMR ($CDCl_3$, 600 MHz): 1.34–1.36 (1H, m, H-1), 4.62 (1H, dd, J = 10.8, 4.8, H-2), 5.28 (1H, d, J = 10.8, H-3), 2.77 (1H, dd, J = 12.0, 10.8 Hz, H-5a), 2.56 (1H, dd, J = 12.0, 4.2 Hz, H-5b), 5.87 (1H, ddd, J = 15.6, 10.8, 4.2 Hz, H-6), 5.41 (1H, br d, J = 15.6 Hz, H-7), 1.86–1.88 (1H, m, H-9a), 1.54–1.56 (1H, m, H-9b), 1.74–1.76 (1H, m, H-10a), 1.26–1.30 (1H, m, H-10b), 3.48

(1H, d, 9.6 Hz, H-11), 1.62–1.64 (1H, m, H-13a), 1.35–1.36 (1H, m, H-13b), 2.28–2.32 (1H, m, H-14a), 1.20–1.22 (1H, m, H-14b), 1.21–1.23 (1H, m, H-15), 0.88 (3H, d, J = 6.6 Hz, H-16), 0.76 (3H, d, J = 6.6 Hz, H-17), 1.83 (3H, s, H-18), 1.33 (1H, s, H-19), 1.04 (1H, s, H-20), ^{13}C NMR ($CDCl_3$, 150 MHz): 45.6 (CH, C-1), 72.0 (CH, C-2), 124.4 (CH, C-3), 141.1 (C, C-4), 42.5 (CH_2, C-5), 128.2 (CH, C-6), 135.7 (C, C-7), 73.6 (C, C-8), 39.9 (CH_2, C-9), 22.8 (CH_2, C-10), 75.8 (CH_2, C-11), 74.9 (CH_2, C-12), 18.6 (C, C-13), 34.1 (CH, C-14), 29.4 (CH, C-15), 20.7 (CH_3, C-16), 20.5 (CH_3, C-17), 17.6 (CH_3, C-18), 29.3 (CH_3, C-19), 23.8 (CH_3, C-20); HRESIMS data m/z 322.2500 $[M]^+$ (Calculated 322.2508 for $C_{20}H_{34}O_3$).

Sarcotrocheldiol B (**3**): Colorless oil $[\alpha]_D^{20}$ 89.1 (c 0.010, $CHCl_3$); IR (film) cm^{-1}: 3383 (OH), 3180 (OH), 2937 (C–H), 1645 (C=C), 1378, 1221, 1045; ^{1}H NMR ($CDCl_3$, 600 MHz): 1.29–1.32 (1H, m, H-1), 4.52 (1H, dd, J = 10.8, 5.4, H-2), 5.23 (1H, d, J = 10.8, H-3), 2.13–2.15 (1H, m, H-5a), 2.17–2.19 (1H, m, H-5b), 1.88–1.90 (1H, m, H-6a), 2.12–2.15 (1H, m, H-6b), 3.88 (1H, dd, J = 10.8, 1.2 Hz, H-7), 2.48–2.51 (1H, m, H-9a), 2.28–2.32 (1H, m, H-9b), 1.95–1.97 (1H, m, H-10a), 1.28–1.30 (1H, m, H-10b), 3.76 (1H, d, 9.6 Hz, H-11), 1.62–1.64 (1H, m, H-13a), 1.38–1.40 (1H, m, H-13b), 2.26–2.28 (1H, m, H-14a), 1.26–1.28 (1H, m, H-14b), 1.18–1.20 (1H, m, H-15), 0.86 (3H, d, J = 6.6 Hz, H-16), 0.71 (3H, d, J = 6.6 Hz, H-17), 1.73 (3H, s, H-18), 5.07 (1H, br d, J = 1.2 Hz, H-19a), 5.06 (1H, br d, J = 1.2 Hz, H-19b), 1.05 (1H, s, H-20), ^{13}C NMR ($CDCl_3$, 150 MHz): 45.9 (CH, C-1), 70.4 (CH, C-2), 125.3 (CH, C-3), 137.7 (C, C-4), 36.4 (CH_2, C-5), 30.1 (CH_2, C-6), 67.8 (CH, C-7), 147.5 (C-8), 30.9 (CH_2, C-9), 27.1 (CH_2, C-10), 70.6 (CH, C-11), 74.6 (C, C-12), 18.6 (CH_2, C-13), 33.7 (CH_2, C-14), 29.0 (CH_3, C-15), 20.7 (CH_3, C-16), 20.3 (CH_3, C-17), 14.2 (CH_3, C-18), 112.4 (CH_3, C-19), 23.5 (CH_3, C-20); HRESIMS data m/z 322.2501 $[M]^+$ (Calculated 322.2508 for $C_{20}H_{34}O_3$).

4.3. Antibacterial activity assay

The growth inhibition of the isolated compounds against several pathogenic bacteria was carried out using agar well diffusion.[13] First 0.1 mL of suspended bacterium in sterile medium (1.5×10^8 CFU/mL) was spread on Mueller–Hinton agar. Then 50 μL of each sample (10 μg/mL) was poured into the wells (6-mm diameter). For yeast and fungi, cell and spore suspensions (2×10^4 CFU/mL) were spread on potato dextrose agar. All plates were left for 1 h at 48 °C and then incubated for 24 h at 37 °C for bacteria and 4 days at 25 °C for fungi. Inhibition zone diameters formed around the well were measured and the mean diameter of three replicates was calculated. DMSO was used as a negative control and ampicillin as a positive control.

MICs were determined by a modified version of the method described by Chand et al. and Aly and Gumgumji.[15,16] First 175 μL of an exponentially growing culture ($\times 10^6$–10^7 CFU/mL) was poured into each well of the microtiter plate along with 20 μL of solution of each concentration of the isolated compounds. The appropriate solvent was used as control. Then 5 μL of a solution of fluorescein diacetate (FDA) 0.2% (w/v) in acetone was added to the microtiter plate after incubation for 40 min, and incubation was continued for 90 min. The green color produced from the hydrolysis of FDA was measured at 490 nm (MR7000 automatic ELISA tray reader) along with the blanked wells and control wells containing microbial cultures.

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