Structurally Diverse Metabolites from the rare Actinobacterium *Saccharothrix xinjiangensis*

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ABSTRACT

The bioassay-guided fractionation from cultures of the actinobacterium *Saccharothrix xinjiangensis* Act24Zk, collected from the Caspian Sea beach in Iran led to the isolation of three new compounds, caerulomycin M (**1**), saccharopyrone (**2**) and saccharonoic acid (**3**), together with the known compound, caerulomycin A (**4**). Their structures were elucidated from HRESIMS and 1D and 2D NMR data. Compound **2** displayed moderate cytotoxic activity against the human cervix carcinoma HeLa cells KB3.1 with an **IC₅₀** value of 5.4 µM.

Keywords: acid hydrolysis/ cytotoxic activity/ NOE/ rhamnose/ *Saccharothrix xinjiangensis*

INTRODUCTION

✉✉† These authors contributed equally to this work.

It is estimated that by 2050 about 10 million people per year would be dying from antibiotic-resistant infections, which has led to an increasing demand of new active drugs.^{1,2} Out of the 23,000 known microbial bioactive secondary metabolites³ more than 75% are derived from actinobacteria, in particular from the genus *Streptomyces*. Many of these compounds are selective antibiotics, or anticancer, antifungal and immunosuppressive agents.^{4,5} In recent years other genera of the so-called rare actinobacteria have increasingly become attractive resources of novel biological active secondary metabolites.^{6,7,8,9,10} Of these, the genus *Saccharothrix* has been reported to produce a range of bioactive compounds such as the antibacterial agents tianchimyins A–B from *Saccharothrix xinjiangensis*,¹¹ the antitumor agent saccharothriolide B from *Saccharothrix* sp.^{12,12} and the antifungal antibiotic formamicin from *Saccharothrix tangerines* sp. nov.¹³ As part of our ongoing research to discover new bioactive compounds from our collected actinomycetes, which had been isolated from soil samples from different areas of Iran, the strain Act24Zk was selected for further study due to the strong antibacterial and antifungal activities during bioassay screening. Herein we report the isolation, spectroscopic structural elucidation and biological activities of three unprecedented metabolites, caerulomycin M (**1**), saccharopyrone (**2**), saccharonoic acid (**3**) and the known caerulomycin A (**4**) from the culture of Act24Zk (**Figure 1**).

RESULTS AND DISCUSSION

Phylogenetic analysis showed that strain Act24Zk, isolated from a beach on the Caspian Sea of Iran, has a high 16S rRNA nucleotide sequence similarity to *S. xinjiangensis* (99.5%), a species that was first isolated from Tianchi Lake, Xinjiang Uygur Autonomous Region, China.¹⁴ Strong activities against *Staphylococcus aureus* and *Mucor hiemalis* were found in the ethyl acetate extract of the cell mass of Act24Zk. After removing the lipophilic by-product by a partition between methanol and heptane, the residue of the extract was separated by Sephadex LH-20 in methanol, followed by preparative RP-18 of the LH-20 fractions to give caerulomycin M (**1**), saccharopyrone (**2**) and saccharonoic acid (**3**), along with caerulomycin A (**4**). Their structures were elucidated by HR-ESIMS and 2D NMR spectroscopy.

The molecular formula of C₁₈H₂₁N₃O₆ was identified as caerulomycin M (**1**) from the HR-ESIMS spectrum of the molecular ion cluster [M+H]⁺ at *m/z* 376.1509 (calcd for C₁₈H₂₂N₃O₆ 376.1503) and [2M+Na]⁺ at *m/z* 773.2738 (calcd for C₃₆H₄₂N₆NaO₁₂ 773.2753), implying the presence of ten degrees of unsaturation. Its complete structure was determined by 1D and 2D NMR spectroscopy. The ¹³C NMR and ¹H,¹³C HSQC NMR spectral data confirmed the presence of 18 carbons, including seven olefinic methines (δ_{H} 7.37–8.70, δ_{C} 151.3–106.9), four quaternary olefinic carbons (δ_{C} 166.8–151.9), five oxymethines (δ_{H} 3.29–5.35, δ_{C} 103.0–69.1), one methoxy carbon ($\delta_{\text{H/C}}$ 3.97/55.8) and one methyl group ($\delta_{\text{H/C}}$ 1.15/18.0). The ¹H NMR spectrum provided further three hydroxyl groups (δ_{H} 5.08, 4.90, 4.78). Analysis of the ¹H,¹H COSY and ¹H,¹³C HMBC correlations revealed the presence of the 2,2'-bipyridine

moiety (**Figure 2**). The NMR spectra of bipyridine moiety were very similar to those of caerulomycin A,¹⁵ indicating the existence of a caerulomycin A analogue. COSY correlations from H-2" to H-6", in conjunction with the HMBC correlation of H-5" to C-1" suggested the presence 6-deoxyhexose sugar moiety. This was identified to be α -rhamnopyranose by comparison of the ¹H and ¹³C spectroscopic data with those reported in the literature,¹⁶ which was confirmed by ROESY data and coupling constant analysis. Furthermore, the characteristic small coupling constant of the anomeric proton at C-1" (< 2 Hz) supported the α -configuration and an NOE correlation between H-6 and H-1" indicated the *E*-configuration of the oxime moiety. The absolute configuration of rhamnose was determined to be L after acid hydrolysis by comparison of the specific rotation of the pure sugar moiety (**1a** [α]_D²⁰ +16.0, H₂O) with that of standard L(+)-rhamnose ([α]_D²⁰ +18.8, H₂O). Thus, the structure of **1** was determined to be (4-methoxy[2,2'-bipyridin]-6-methylidene)amino)oxy-6- α - L-rhamnopyranoside.

Lahoum et al.¹⁷ have recently published two bipyridine glycosides isolated from another *Saccharothrix* strain. One of the glycosides, cyanogriside J, has a similar structure to **1**. The authors stated that the sugar moiety of cyanogriside J should be 2,4-dimethyl- α - L-mannose, but did not provide any detailed data concerning the relative and absolute configurations of the molecule. We here report, based on the NMR data and sugar analysis by specific rotation determination that **1** has different sugar moiety than cynogriside J.

Saccharopyrone (**2**) exhibited in the HR-ESIMS spectrum a molecular ion peak cluster [M+Na]⁺ at *m/z* 451.1937 (calcd for C₂₁H₃₂NaO₉ 451.1939) and [2M+Na]⁺ at *m/z* 879.3980 (calcd for C₄₂H₆₄NaO₁₈ 879.3985) which provided the elemental formula C₂₁H₃₂O₉ (6 DBE). The ¹³C NMR and ¹H, ¹³C HSQC NMR data of **2** implied the presence of five quaternary olefinic carbons (δ_C 171–101) including two carbonyl carbons (δ_C 170.4 and 164.5), one olefinic methine ($\delta_{H/C}$ 6.46/96.4), five oxymethines (δ_H 3.28–5.59, δ_C 97.9–69.7), four methylenes (δ_H 1.13–2.48, δ_C 25.3–32.8), one methine ($\delta_{H/C}$ 1.71/31.9) and three methyl groups ($\delta_{H/C}$ 2.01/20.7, 1.79/8.5, 0.86/16.6). In addition, the downfield shift of proton signals at 3.79 and 3.87 with the corresponding carbon at 68.4 suggested the fifth methylene group positioned to oxygen. The ¹H, ¹H COSY spectrum showed a contiguous aliphatic chain from H-1' to H-6' which was connected to the methyl acetate from ¹H, ¹³C HMBC correlations of H₂-6' to C-7', and of methyl H₃-8 to C-7', respectively (**Figure 3**). An aromatic unit was identified as 3-methyl-2-oxo-2*H*-pyran by detailed analysis of further correlations. The ¹H and ¹³C NMR data of the sugar moiety were very similar to those of compound **1** and of the reported literature precedent for the L-rhamnose. Detailed analysis of 2D NMR data confirmed the presence of α - L-rhamnopyranoside with the small coupling constant (1.9 Hz) of the anomeric proton (H-1"). Finally, HMBC correlations from H-1" to C-4 and from H-1' to C-6 and C-5 linked the α -pyrone unit to the sugar moiety and the side chain, completing the

structure of **2**¹. Therefore, **2** was identified to be 2-methyl-6-(3-methyl-2-oxo-2*H*-pyran-6-yl) hexylacetate- 6- α - L-rhamnopyranoside.

α -pyrones are known to be derived from the polyketide synthase (PKS) pathway.¹⁸ Compound **2** is probably assembled by the condensation of two polyketide chains by the catalytic activity of a free standing KS and subsequently the O-linked glycosylation forms the final structure. Examples for such a mechanism are corallopyronins and myxopyronins, which are biosynthesized *via* interconnection of two linear polyketide chains by the stand-alone ketosynthase enzyme CorB and MxnB, respectively.^{19,20} However, feeding studies and analysis of biosynthetic gene cluster of **2** remain necessary to prove this biosynthetic hypothesis.

The elemental formula C₂₂H₃₇NO₃ (5 DBE) of saccharonoic acid (**3**) was determined by HR-ESIMS of the molecular ion cluster [M+H]⁺ at *m/z* 364.2842 (calcd for C₂₂H₃₈NO₃ 364.2846) and [2M+H]⁺ at *m/z* 727.5601 (calcd for C₄₄H₇₅N₂O₆ 727.5620). The ¹³C and ¹H, ¹³C HSQC NMR data confirmed the carbon number and showed the presence of two carbonyl carbons (δ_C 171.2 and 165.5), two quaternary olefinic carbons (δ_C 133.1 and 130.5), three olefinic methines (δ_H 7.01–5.04, δ_C 145.7–126.0), three methines (δ_H 2.63, 1.53, 1.51 δ_C 30.6, 28.1, 26.9), five methylenes (δ_H 1.97–1.09, δ_C 47.0–25.2) and six methyl groups (δ_H 1.75–0.76, δ_C 22.4–15.5). The ¹H NMR spectrum in DMSO showed further an NH signal at 8.05. The planar structure of **3** was elucidated from the ¹H,¹H COSY and ¹H,¹³C HMBC correlations which provided the connection through secondary amide bond and the attachment of carboxyl residue (**Figure 4**). The *E* configuration of the $\Delta^{5,6}$ double bond was assigned from the coupling constant of 15.5 Hz. The methyl substituted $\Delta^{7,8}$, $\Delta^{13,14}$ double bonds were established as *trans* from the ¹H,¹H ROESY correlations of methyl-19 and H-5 and between H-6 and H-8, as well as of methyl-22 and H₂-15 and between H₂-12 and H-14.

Further, an energy minimized conformation of **3**, which was calculated by MM2 with ChemBio3D 17.1., in combination with the observed NOEs allows to propose a relative configuration of dimethylated stereo centers at C-9 and C-11 as shown in **Figure 5**. This presents the preferential conformation of the carbon chain by avoidance of the high-energy *syn*-pentane interactions.²¹ As expected from the model, significant NOEs from CH₃-19 to H-5 and H-9 positioned H-9 *syn* to H-5. On the other side, NOE correlations from H-11 to CH₃-20 and CH₃-22 and from CH₃-20 to H-8 placed H-11 on the same face as H-8. Thus, both C-9 and C-11 stereo centers occupy the relative *syn* configuration. All further NOEs are in good agreement with the conformation of compound **3**. On this basis, **3** was proposed as [(2*E*,4*E*,6*S*,8*R*,10*E*)-4,6,8,10,14-pentamethyl-pentadeca-2,4,10-trienoyl]amino}acetic acid.

1 The complete absolute configuration of compound **2** resulted in hydrolysis of acetate and subsequently derivatization of the consequence alcohol according to modified Mosher's method couldn't be determined due to the low amount of compound **2**.

The structure of **3** bearing 1,3-dimethylated hydrocarbon segment appears frequently as structural elements in polyketide natural products.²² Some macrocyclic polyketides such as narbonolide and methymycin isolated from *Streptomyces* species have been previously reported to occupy *syn* conformation of their 1,3- dimethyl substituted chain segment.^{23,24,25}

Compounds **1–4** were evaluated for their antimicrobial and cytotoxic activities. Compound **1** showed moderate activity against *M. hiemalis*, whereas **3** exhibited weak activity against *M. hiemalis* and *Candida albicans*. Compound **2** did not show any antimicrobial activities at concentrations $\leq 66.7 \mu\text{g ml}^{-1}$. Compound **4** is known to exhibit antibacterial and antifungal activity,²⁶ which is also confirmed against our test microorganisms except *C. albicans*. In the cytotoxicity assay compound **1** exhibited weak cytotoxicity against KB3.1 and L929 cell lines, whereas compound **2** showed significant inhibition against KB3.1 cell line. Compound **3** had no activity against our test cell lines. Compound **4** was most potent, showing inhibition against KB3.1 and L929 cell lines in the IC_{50} range of 0.1–0.2 μM . The presence of the sugar moiety of compound **1** in the corresponding aglycone **4** weakened the antimicrobial effects and also led to a significant decrease in cytotoxicity.

CONCLUSION

Our work on the culture broth of *Saccharotrix* strain Act24Zk resulted in the isolation and characterization of a new bipyridine glycoside, caerulomycin M (**1**), a new α -pyrone glycoside, saccharopyrone (**2**) and a new polyketide, saccharonoic acid (**3**), together with one known caerulomycin A (**4**). The evaluation of antimicrobial and cytotoxic activities of compounds **1–3** revealed that **1** and **3** exhibited moderate to weak activities against *M. hiemalis*, whereas compound **2** displayed significant activity against KB3.1 cells.

EXPERIMENTAL SECTION

General Experimental Procedures

Fractionation and analytical RP HPLC were carried out with an Agilent 1100 HPLC system equipped with a diode-array UV detector, a fraction collector and a Corona Ultra detector (Dionex). HPLC conditions: XBridge C18 column $100 \times 2.1 \text{ mm}$ (Waters), $3.5 \mu\text{m}$, solvent A [H_2O –acetonitrile (95:5, v/v), 5 mmol NH_4Ac , 0.04 ml l^{-1} CH_3COOH]; solvent B [H_2O –acetonitrile (5:95, v/v), 5 mmol NH_4Ac , 0.04 ml l^{-1} CH_3COOH]; gradient system, from 10% B to 100% B in 30 min and maintaining 100% for 10 min; flow rate 0.3 ml min^{-1} ; 40°C . Preparative HPLC purification was performed on an Agilent 1100 series preparative HPLC system [ChemStation software (Rev. B.04.03 SP1); binary pump system; diode-array UV detector; 180-fraction collector]. HRESIMS (High resolution electrospray ionization mass spectrometry) data were recorded on a MaXis ESI TOF mass spectrometer (Bruker Daltonics)

[scan range m/z 100-2500, rate 2 Hz, capillary voltage 4500 V, dry temperature 200 °C] equipped with an Agilent 1260 series HPLC-UV system (Agilent Technologies) (Santa Clara, CA, USA) [column 2.1×50 mm, 1.7 μ m, C18 Acquity UPLC BEH (Waters), solvent A: H₂O + 0.1% formic acid; solvent B: acetonitrile (ACN) + 0.1% formic acid, gradient: 5% B for 0.5 min, increasing 19.5 min to 100% B, holding 5 min at 100% B; flow = 0.6 ml min⁻¹, 40°C; DAD-UV detection at 200-600 nm]. Molecular formulas were calculated including the isotopic pattern (Smart Formula algorithm). HPLC DAD MS analysis was performed using an amaZon speed ETD ion trap mass spectrometer (Bruker Daltonics) in positive and negative ionization modes. The mass spectrometer was coupled to a Dionex UltiMate 3000 HPLC system [column 2.1×50 mm, 1.7 μ m, C18 Acquity UPLC BEH (Waters), solvent A: H₂O + 0.1% formic acid; solvent B: acetonitrile (ACN) + 0.1% formic acid, gradient: 5% B for 0.5 min, increasing to 100% B in 20 min, maintaining isocratic conditions at 100% B for 10 min, flow = 0.6 ml min⁻¹, UV-vis detection 200–600 nm]. NMR spectra were recorded on a Bruker 700 MHz Avance III spectrometer with a 5 mm TCI cryoprobe (¹H: 700 MHz, ¹³C: 175 MHz), locked to the deuterium signal of the solvent. Chemical shifts are given in parts per million (ppm), and coupling constants in hertz (Hz). UV/vis spectra were measured on a Shimadzu UV/vis-2450 spectrophotometer using methanol (Uvasol, Merk).

Strain origin and Identification

The Act24Zk strain was isolated from sand samples collected from the Caspian sea beach in north Iran on December 2016 by an isolation method originally designed for myxobacteria²⁷ using St21 agar medium (solution A: K₂HPO₄ 0.1% (w/v); yeast extract (Difco) 0.002% (w/v); Agar 1% (w/v); solution B: KNO₃ 0.1% (w/v), MgSO₄·7H₂O 0.1% (w/v), CaCl₂·2H₂O 0.1% (w/v), FeCl₃ 0.02% (w/v), MnSO₄·7H₂O 0.01% (w/v) + 100 μ g ml⁻¹ Cycloheximide). For molecular identification, the strain Act24Zk was cultured in 100 ml of GYM medium and incubated for 3 days (160 rpm, 30 °C). DNA extraction was performed using Invisorb Spin Plant Mini Kit, following the manufacturer's protocol. The 16S rRNA gene region was amplified by PCR using two universal primers, forward primers bind on the position (F27) and reverse primers bind on the position (R1541). The partial identification indicated that the strain Act24Zk has 99.5% homology with *S. xinjiangensis*.

Production, Extraction and Isolation

A seed culture of strain Act24Zk was grown in a 250-ml flask, which contained 100 ml of GYM medium (0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.2% CaCO₃; pH 7.2; sterilized for 20 min at 121°C) and incubated on a rotary shaker (160 rpm) for 7 days at 30 °C. The production culture was transferred 1:10 in ninety 250 ml flasks, filled with 100 ml of medium 5294 (1% soluble starch, 0.2% yeast extract, 1% glucose, 1% glycerol, 0.25% corn

steep liquor, 0.2% peptone, 0.1% NaCl, 0.3% CaCO₃; pH 7.2) suspended in distilled water and sterilized for 20 min at 121 °C. The flasks were incubated on a rotary shaker (160 rpm) for nine days at 30 °C. Mycelial biomass was separated from the culture broth by centrifugation (5000 rpm, 10 min). The biomass was extracted with ethyl acetate three times (1.5 l). The combined ethyl acetate extracts were centrifuged and evaporated *in vacuo*. The residue (2.0 g) was redissolved in 100 ml methanol containing 3% of water and partitioned with 100 ml *n*-heptane three times. After evaporation the methanol layer yielded 1.34 g of a dark brown oily residue, which was separated by gel chromatography in two portions [column: Sephadex LH-20 3 × 830 cm, flow rate 3.4 ml min⁻¹, solvent methanol]. Totally six fractions were collected and tested against *S. aureus* and *M. hiemalis*. Fraction 2 (240 mg) was further separated by preparative RP-HPLC [column 250 × 21.2 mm, Nucleodur Phenylhexyl, 5 µm (Macherey Nagel), solvent A: water, solvent B: acetonitrile, gradient from 35% B to 75% B in 60 min, flow rate 20 ml min⁻¹, UV detection at 220, 280 and 360 nm] to yield 1.0 mg saccharopyrone (**2**) and 1.1 mg saccharonoic acid (**3**). Fraction 4 (180 mg) was purified by preparative RP-HPLC [column: 250 × 21.2 mm, Nucleodur 100-10 C18 (Macherey-Nagel); solvent A: water, solvent B: acetonitrile, gradient from 15 % B to 35 % B in 60 min, flow rate 20 ml min⁻¹, UV detection at 220, 300 and 360 nm] and delivered 2.45 mg of caerulomycin M (**1**) and 1.1 mg caerulomycin A (**4**). The analytical HPLC profile of fr. 5 containing 8.79 mg of **4** was found to be pure.

Caerulomycin M (**1**): yellow solid; $[\alpha]_D^{20} + 54.7$ (c 0.3, MeOH); UV $\lambda_{\max}^{\text{MeOH}}$ (log ϵ) 235 (4.76), 281 (4.38 sh) nm; NMR data (¹H: 700 MHz, ¹³C 176 MHz, DMSO-*d*₆) see Table 1; HRESIMS: $[M+H]^+$ *m/z* 376.1509, calcd. 376.1503 for C₁₈H₂₂N₃O₆, $[2M+Na]^+$ *m/z* 773.2738, calcd. 773.2753 for C₃₆H₄₂N₆NaO₁₂, *t_R* = 4.2 min.

Saccharopyrone (**2**): colorless oil; $[\alpha]_D^{20} + 25.5$ (c 0.04, MeOH); UV $\lambda_{\max}^{\text{MeOH}}$ (log ϵ) 203 (4.24), 285 (3.71); NMR data (¹H: 700 MHz, ¹³C 176 MHz, DMSO-*d*₆) see Table 1; HRESIMS: $[M+Na]^+$ *m/z* 451.1937, calcd. 451.1939 for C₂₁H₃₂NaO₉, $[2M+Na]^+$ *m/z* 879.3980, calcd. 879.3985 for C₄₂H₆₄NaO₁₈, *t_R* = 7.7 min.

Saccharonoic acid (**3**): yellow solid; $[\alpha]_D^{20} + 22.7$ (c 0.3, MeOH); UV $\lambda_{\max}^{\text{MeOH}}$ (log ϵ) 205 (2.71), 264 (3.26) nm; NMR data (¹H: 700 MHz, ¹³C 176 MHz, DMSO-*d*₆) see Table 1; HRESIMS: $[M+H]^+$ *m/z* 364.2841, calcd. 364.2846 for C₂₂H₃₈NO₃, $[2M+Na]^+$ *m/z* 727.5601, *t_R* = 15.6 min.

Acid hydrolysis of **1** and sugar analysis

Determination of sugar moiety of caerulomycin A derivative was performed by hydrolysis according to Nakae et al.²⁸ Compound **1** (1.2 mg) was dissolved in 600 µl of 8% HCl-MeOH and stirred at 60 °C for 24 h. The hydrolysate was concentrated and purified by preparative

TLC (CHCl₃:MeOH:H₂O, 10:5:1) with α -naphthol as visualizing agent to give pure L-rhamnopyranose **1a** (0.2 mg) and caerulomycin A, which were confirmed by LC/MS analysis. The optical rotations of **1a** ($[\alpha]_D^{20} +16.0$ (c 0.04, H₂O)) and of the reference standard L(+)-rhamnose monohydrate (Carl Roth GmbH) ($[\alpha]_D^{20} +18.8$ (c 0.05, H₂O)) were determined.

Biological assays

Antimicrobial assay

Minimum inhibitory concentrations were determined by a serial dilution assay in 96-well plates with EBS medium for bacteria and MYC medium for fungi.²⁹ Twenty microliter aliquots of compounds **1–4** with a concentration of 1 mg ml⁻¹ (the end concentration in the first well is 67 μ g ml⁻¹) were tested against two different Gram-positive bacteria (*S. aureus*, *Micrococcus luteus*), one Gram-negative bacterium (*Escherichia coli* Tol C) and two fungi (*C. albicans*, *M. hiemalis*) with methanol as a negative control.

Cytotoxicity assay

Cytotoxicity (IC₅₀) of compounds **1–4** was determined against two human cancer cell lines (normal cell line mouse fibroblasts L929, HeLa cells KB3.1) by using a 5-day MTT assay according to an established procedure.³⁰

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature

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31. Titles and legends to figures

Figure 1. Structures of metabolites **1–4** from *S. xinjiangensis*.

Figure 2. Selected COSY (bold bonds), HMBC (plain arrows) and ROESY (dashed arrows) correlations of **1**.

Figure 3. Selected COSY (bold bonds), HMBC (plain arrows) and ROESY (dashed arrows) correlations of **2**.

Figure 4. Selected COSY (bold bonds) and HMBC (plain arrows) correlations of **3**.

Figure 5. MM2 calculated model of **3** (ChemBio3D 17.1) and selected ROESY correlations. Plain lines upper side NOEs, dashed lines lower side NOEs.

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Figure 4. Selected COSY (bold bonds) and HMBC (plain arrows) correlations of **3**.

Figure 5. MM2 calculated model of **3** (ChemBio3D 17.1) and selected ROESY correlations. Plain lines: upper side NOEs, dashed lines: lower side NOEs.

Table 1. NMR data of compounds **1–3** in DMSO- d_6 (^1H 700.4 MHz, ^{13}C 176.1 MHz)

	1		2		3	
Pos.	δ_C , type	δ_H , m (<i>J</i> in Hz)	δ_C , type	δ_H , m (<i>J</i> in Hz)	δ_C , type	δ_H , m (<i>J</i> in Hz)
1	-	-	-	-	171.2, C	-
2	157.1, C	-	164.5, C	-	41.5, CH ₂	3.73, d (5.6)
3	107.2, CH	7.96 ^a , d (2.4)	101.0, C	-	NH	8.05, br. s
4	166.8, C	-	162.9, C	-	165.5, C	
5	106.9, CH	7.37, d (2.4)	96.4, CH	6.47, s	119.5, CH	6.02, d (15.5)
6	151.9, C	-	163.1, C	-	143.9, CH	7.01, d (15.5)
7	151.3, CH	8.33, s	8.5, CH ₃	1.79, s	130.5, C	-
8	55.8, CH ₃	3.97, s			145.7, CH	5.62, d (9.7)
9					30.6, CH	2.63, m
10					44.1, CH ₂	(a) 1.09, ddd (11.2, 7.1, 3.9) (b) 1.23, m
11					28.1, CH	1.53 ^a , m
12					47.0, CH ₂	(a) 1.64, dd (13.0, 8.4) (b) 1.97 ^b , dd (13.0, 5.6)
13					133.1, C	-
14					126.0, CH	5.05, t (6.9)
15					25.2, CH ₂	1.95 ^b , dt (6.9, 6.5)
16					38.6, CH ₂	1.18, dt (7.5, 7.1)
17					26.9, CH	1.51 ^a , m
18					22.4, CH ₃	0.85, dd (6.6, 1.4)
19					12.4, CH ₃	1.75, d (0.9)
20					20.4, CH ₃	0.91, d (6.5)
21					19.8, CH ₃	0.76, d (6.5)
22					15.5, CH ₃	1.48, s
1'		-	32.8, CH ₂	2.48, t (7.3)		
2'	154.3, C	-	26.5, CH ₂	1.55, m		
3'	120.8, CH	8.39, d (7.9)	25.4, CH ₂	(a) 1.27, m (b) 1.34 ^a , m		
4'	137.4, CH	7.97 ^a , ddd (7.9, 7.5, 1.7)	32.3, CH ₂	(a) 1.13, m (b) 1.35 ^a , m		
5'	124.7, CH	7.49, ddd (7.5, 4.7, 1.1)	31.9, CH ₂	1.71, m		
6'	149.3, CH	8.70, d (4.7)	68.4, CH ₂	(a) 3.79, dd (10.7, 6.7) (b) 3.87, dd (10.7, 5.8)		
7'			170.4, C	-		
8'			20.7, CH ₃	2.01, s		
9'			16.6, CH ₃	0.86, d (6.7)		
1''	103.1, CH	5.35, d (1.7)	97.8, CH	5.59, d (1.9)		
2''	69.1, CH	3.84, t (3.4)	69.7, CH	3.83, t (3.4)		

3"	70.6, CH	3.50 ^b , dd (9.3, 3.4)	70.3, CH	3.60, dd (9.3, 3.4)
4"	71.8, CH	3.29, dd (9.3, 9.7)	71.4, CH	3.28, t (9.7)
5"	69.7, CH	3.52 ^b , dq (9.7, 6.3)	70.4, CH	3.35, dq (9.7, 6.0)
6"	18.0; CH ₃	1.15, d (6.3)	17.6, CH ₃	1.11, d (6.0)
2"- OH	-	5.08, br. s	-	5.18, br. s
3"- OH	-	4.78, br. s	-	4.84, br. s
4"- OH	-	4.90, br. s	-	4.94, br. s

^{a,b} Overlapping signals. Chemical shifts were assigned from ¹H, ¹³C HSQC spectrum.

Table 2. Antimicrobial and cytotoxic activities of **1–4**

	1	2	3	4	oxytetracycline
Microorganisms	MIC (µg ml ⁻¹)				
<i>Staphylococcus aureus</i> Newman	-	-	-	4.2	0.1
<i>Micrococcus luteus</i> DSM1790	-	-	-	4.2	0.4
<i>Escherichia coli</i> DSM1116	-	-	-	8.3	1.7
					nystatin
<i>Mucor hiemalis</i> DSM2656	16.7	-	66.7	4.2	4.2
<i>Candida albicans</i> DSM1665	-	-	33.4	-	8.8
Cell lines	IC ₅₀ (µM)				epothilon B
Mouse fibroblasts L929	50.7	30.4	-	0.1	1.9·10 ⁻³
HeLa cells KB3.1	40.0	5.4	-	0.2	0.5·10 ⁻³

-: not active. Positive control: oxytetracycline, nystatin, epothilon B