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NOTE

Oleamycins A and B: new antibacterial cyclic hexadepsipeptides isolated from a terrestrial *Streptomyces* sp.

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DESCRIPTION

Actinomycetes have been a source for anti-infectives for more than 50 years, and continue to have a very important role as the source organisms for the discovery of new antibiotics. In the course of our screening program for the discovery of new antibacterial compounds, one such strain (Lv20-58), recovered from the root zone of the plant *Oleaceae europea* came to our attention.

The strain (Lv20-58), later identified to belong to the genus *Streptomyces* was cultivated in M medium (6 l) for 8 days at 30 °C and then extracted with ethyl acetate (6 l) to give a crude extract of 138.9 mg. The crude extract was partitioned between 20 ml of hexane, CH_2Cl_2 and MeOH to afford 49.8, 80.9 and 10.6 mg fractions, respectively. The CH_2Cl_2 fraction was subsequently purified by semipreparative reverse-phase HPLC to yield compounds **1** ($t_R = 30.5$ min; 2.5 mg) and **2** ($t_R = 28.6$ min, 0.4 mg). A detailed account of the spectroscopic analysis leading to the assignment of structures to oleamycin A (**1**) and oleamycin B (**2**) are presented below.

HR-ESI(+)MS analysis of oleamycin A (**1**) (Table 1) revealed a pseudomolecular ion ($[M+Na]^+$) indicative of a molecular formula ($C_{39}H_{66}N_8O_{11}Na$) requiring 11 double bond equivalents. The NMR ($CDCl_3$) (Table 2) data revealed resonances for seven ester/amide carbonyls (δ_C 169.2–177.1) requiring **1** to incorporate four rings. Early on in the structural elucidation phase we noticed the presence of two distinct set of resonances in the NMR (Table 2). As a result, we focused on the interpretation of the major set of resonances that led to the structural assignment of oleamycins. The 39 carbons and their assorted proton resonances were attributed to 2 primary methyls (δ_C 9.6 and 11.9), 3 secondary methyls (δ_C 18.3, 19.6 and 18.9), 3 tertiary methyls from which 2 were attributed to *N*-methyls (δ_C 33.4 and 34.3), 14 methylenes (δ_C 20.0–52.3), 7 methines (δ_C 29.9–82.1) and 9 quaternary carbons (δ_C 77.4–177.1)). Further analysis of the NMR data (Table 2, Figure 1) proposed that **1** was a new cyclic hexapeptide consisting of a 3-hydroxyleucine residue, three glycine residues (two of which were *N*-methylated) and two piperazic-acid residues. The sequence of the amino acids was determined by interpretation of key correlations observed in the HMBC spectra. Specifically, HMBC correlations from the methine H-3 (δ_H 4.62) and methylene H_2-8 (δ_H 4.28, 3.63) to the ester carbonyl C-7 (δ_C 170.6). Additional correlations from N-Me (δ_H 2.83) and the methylene H_2-10 (δ_H 5.32, 3.36) to the amide carbonyl C-9 (δ_C 169.3), extended by similar correlations from N-Me (δ_H 3.07) and the methylene H_2-12 (δ_H 4.54, 3.69) to the amide carbonyl C-11(δ_C 169.2) linked the *N*-Me-glycine residues 2 and 3. The amide NH (δ_H 7.53) of the third glycine residue and the methine H-14 (δ_H 5.15) of the piperazic-acid residue (Pip1) showed correlations to C-13 (δ_C 169.8), whereas H-14 and H-19 (δ_H 5.50) showed correlations to C-18 (175.0). Finally, correlations from H-3 and H-19 to C-1, led to the construction of the 19-membered cyclic hexapeptide fragment (Figure 1).

The second structural feature of **1** was identified to be a polyketide side chain (Figure 1). The ^1H NMR (500 MHz, $CDCl_3$) (Table 2, Figure 1) and COSY data revealed a single isolated spin system, a primary methyl H_3-35 (δ_H 0.84) with correlations to a methylene H_2-34 (δ_H 1.09–1.24), methine H-33 (δ_H 1.30–1.44), secondary methyl H_3-36 (δ_H 0.98) and a methylene H_2-32 (δ_H 0.98). A second primary methyl H_3-31 (δ_H 0.79) extended to a methylene H_2-30 (δ_H 1.25–1.37), a deshielded methine H-29 (δ_H 3.53, δ_C 76.1) and finally to the methylenes H_2-27 (1.30–1.38) and H_2-26 (1.61–0.1.76). In the HMBC spectrum, a tertiary methyl H_3-37 (δ_H 1.27) showed correlations to an amide carbonyl C-23 (δ_C 177.1), oxycarbon C-24 (δ_C 77.4) and hemiketal carbon C-25 (δ_C 99.1). The absence of a hydroxyl signal

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Table 1 Physicochemical properties of 1 and 2

	1	***2***
Appearance	*Yellow solid*	*Yellow solid*
HR-ESI-MS (m/z)		
Found	845.4742 $[M+Na]^+$	831.4597 $[M+Na]^+$
Calcd	845.4749 ($C_{39}H_{66}N_8O_{11}Na$)	831.4592 ($C_{38}H_{64}N_8O_{11}Na$)
$[\alpha]_D$ (MeOH)	(+)18.5	(+)15.6°
UV λ_{max} nm (log ε) (MeOH)	210 (4.67)	210 (4.67)

Table 2 NMR (500 MHz, CDCl3) data for 1

Position	δ_H, *mult (J in Hz) (**1**)*	δ_C[a]	*COSY*	*HMBC*	*ROESY*
3-Hydroxyleucine					
1		172.5			
2	5.84, dd (9.7, 2.9)	48.4	3, 2*N*-H	1	5
3	4.62[b], dd (6.7, 2.9)	82.1	2, 4	1, 2, 4, 5, 6, 7	2*N*-H, 4, 5, 6
4	1.89, m	29.9	3, 5, 6	5, 6	3
5	1.02, d (6.9)	19.6	4	3, 4, 6,	2, 3
6	0.94, d (6.9)	18.3	4	3, 4, 5	3
2*N*-H	8.00, d (9.7)		2	2, 23	3
Glycine (1)					
7		170.6			
8a	4.28, d (17.0)	52.3	8b	7, 9, 38	
8b	3.63, d (17.0)		8a	7, 9, 38	38
Glycine (2)					
9		169.3			
10a	5.32	50.4	10b	9, 39	8a, 10b
10b	3.36, d (14.7)		10a	9, 39	
Glycine (3)					
11		169.2			
12a	4.54, dd (16.6, 9.7)	40.8	12b, 12*N*-H	11	
12b	3.69		12a, 12*N*-H		12*N*-H, 39
12*N*-H	7.53, d (9.7)		12a/b	13	12b, 14
Piperazic acid (1)					
13		169.8			
14	5.15, m	52.8	15a/b	13, 15, 16, 18	12*N*-H, 15a/b
15a	2.51, m	23.6	14, 15b, 16		14
15b	1.64, m		14, 15a, 16		14
16	1.56, m	22	15a/b, 17a/b	12, 15, 16	
17a	3.14[c], m	48.1	16, 17*N*-H, 17b	13, 14, 16	
17b	2.73, m		16, 17*N*-H, 17a		
17*N*-H	5.18, d (12.8)		17a/b		
Piperazic acid (2)					
18		175			
19	5.50, dd (6.4, 5.9)	50.7	20	1, 18, 20, 21	20
20	1.95, m	23.2	19, 21a/b		19
21a	1.77, m	20	20, 21b, 22a/b		
21b	1.57, m		20, 21a, 22a/b		
22a	3.15[c], m	46.2	21a/b, 22b, 22*N*-H	20	
22b	2.93, m		21a/b, 22b, 22*N*-H		
22*N*-H	4.62[b]		22a/b		
Polyketide					
23		177.1			
24		77.4			
25		99.1			
26	1.61–1.76, m	27.9	27	25	
27	1.30–1.38	24.1	26, 28		
28	1.17–1.29, m	36.8	27, 29		
29	3.53, dt (10.1, 2.5)	76.1	28, 30	31	

Table 2 (Continued)

Position	δ_H, *mult (J in Hz) (**1**)*	δ_C[a]	*COSY*	*HMBC*	*ROESY*
30	1.25–1.37, m	25.5	29, 31		
31	0.79	9.6	30	29, 30	
32	0.98[d]	38.6	28, 33		
33	1.30–1.44, m	31.3	32, 34, 36		
34	1.09–1.24	31.4	33, 35		
35	0.84	11.9	34	33, 34	
36	0.98[d], d	18.9	33	32, 33, 34	
37	1.27, s	20		23, 24, 25	
38	2.83, s	34.3		8, 9	8b
39	3.07, s	33.4		10,11	12b

[a]Assignments supported by HSQC and HMBC.
[b]Overlapping signals.
[c]Overlapping signals.
[d]Overlapping signals.

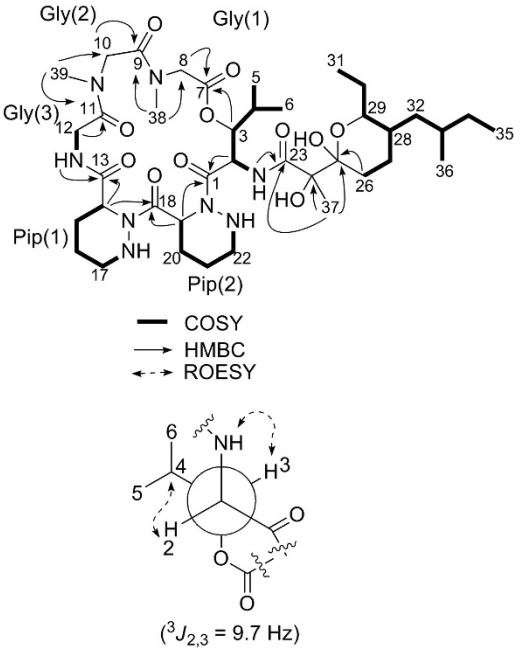

Figure 1 Key 2D NMR (500 MHz, $CDCl_3$) correlations for **1** and Newman projection showing relative configuration of C2/C3.

associated with C-29 suggested that it has an ether linkage, which was attributed to the likely presence of a heterocyclic system. This was supported by HMBC correlations from the methylene H_2-26 (δ_H 1.61–1.76) to C-25, suggesting the presence of a tetrahydropyran ring. A large coupling ($J_{29,28} = 10.1$ Hz) established a *trans*-diequatorial relationship orientation of the substituents at C-28 and C-29. The point of attachment of the polyketide unit to the peptide was established on the basis of HMBC correlations of the amide 2*N*-H (δ_H 8.00) and the tertiary methyl H_3-37 (δ_H 1.27) to the amide carbonyl C-23 (δ_C 177.1), leading to the overall planar structure of **1** (Figure 2). The relative configuration of the two stereogenic centers in β-OH-Leu was established as 2*S** and 3*S** by ROESY and *J*-based configuration analysis[1] (Figure 1). Despite the absence of a ROESY correlation from the methine H-4 to 2*N*-H, we have drawn the C2-C3 rotamer as follows, based on the absolute configuration identified as (2*S*, 3*S*) of the β-OH-Leu residue in the known 19-membered cyclic depsipeptides (A83586C, L-156,602, aurantimycins, polyoxypeptins, GE3 and dentigerumycin; Figure 2). HRESI(+)MS analysis of oleamycin B (**2**) (Supplementary Table S1) revealed a pseudomolecular ion ($[M+Na]^+$) indicative of a molecular formula ($C_{38}H_{64}N_8O_{11}Na$) requiring 11 double bond equivalents (Table 1). The principle difference of **2** over **1** was the substituent on the tetrahydropyran ring from an isobutyl to an isopropyl residue (C32-C-35) (Figure 2). The closest known natural product analogs to **1** and **2** are the rare class of 19-memebered cyclic hexadepsipeptides azinothricin,[2] A83586C,[3] L-156,602,[4] citropeptin,[5] variapeptin,[6] verucopeptin,[7] aurantimycins,[8] polyoxypeptins,[9] piplamycin,[10] IC101,[11] GE3[12] (Figure 2) and the recently reported dentigerumycin.[13] Oleamycin A (**1**) displayed significant biological activity against a panel of Gram-positive bacteria and a cancer cell line (HCT-116). It is noteworthy that MIC's were against strains *S. aureus* (0.23 μg ml^{-1}) and *Micrococcus luteus* (0.03 μg ml^{-1}), whereas an IC_{50} of 6.5 ng ml^{-1} was recorded against HCT-116 cells (human colon carcinoma). Owing to the limited supply of oleamycin B (**2**) and reproducibility issues in the fermentation, we were unable to screen for its biological activity. In summary, we have isolated, characterized and evaluated the biological activity of two new members, oleamycin A (**1**) and B (**2**), of the well-described 19-membered cyclic depsipeptides.

EXPERIMENTAL PROCEDURE

NMR spectra were obtained on a Bruker Ascend 500 MHz spectrometer equipped with a cryoprobe system (Bruker Biospin GmbH, Rheinstetten, Germany) in the solvents indicated and referenced to residual ^{1}H signals in deuterated solvents. ESI-MS were acquired using an Agilent 1100 Series separations module equipped with an Agilent 1100 Series LC/MSD mass detector (Agilent, Waldbronn, Germany) in both positive and negative ion modes under the following conditions: Zorbax C_8 column (Crawford Scientific, Lanarkshire, UK), 150 × 4.6 mm^2, eluting with 0.4 ml min^{-1} 95% H_2O/MeCN to 5% H_2O/MeCN (with isocratic 0.01% trifluoroacetic acid) over 22 min, and then held for 5 min. HR-MS was carried out using an UltiMate 3000 rapid separation LC system (Dionex RSLC, Idstein, Germany) coupled to an ultra-high resolution time-of-flight MS (Bruker Daltonik MaXis, Bremen, Germany) operating in the positive ESI mode.

Sampling was performed in the Nikitsky Botanical Garden of Crimea (Ukraine). The soil (1 g) was collected from the root zone of *O. europea* and resuspended in sterile water, followed by serial dilutions leading to the inoculation onto the oatmeal agar (oatmeal 40 g l^{-1}, agar 15 g l^{-1}, pH 7.5). The plates were incubated for 20 days at 28 °C. Individual colonies were transferred onto new oatmeal agar plates for further analysis and cryopreservation. The 16S ribosomal DNA sequence analysis of strain Lv20-58 classified it

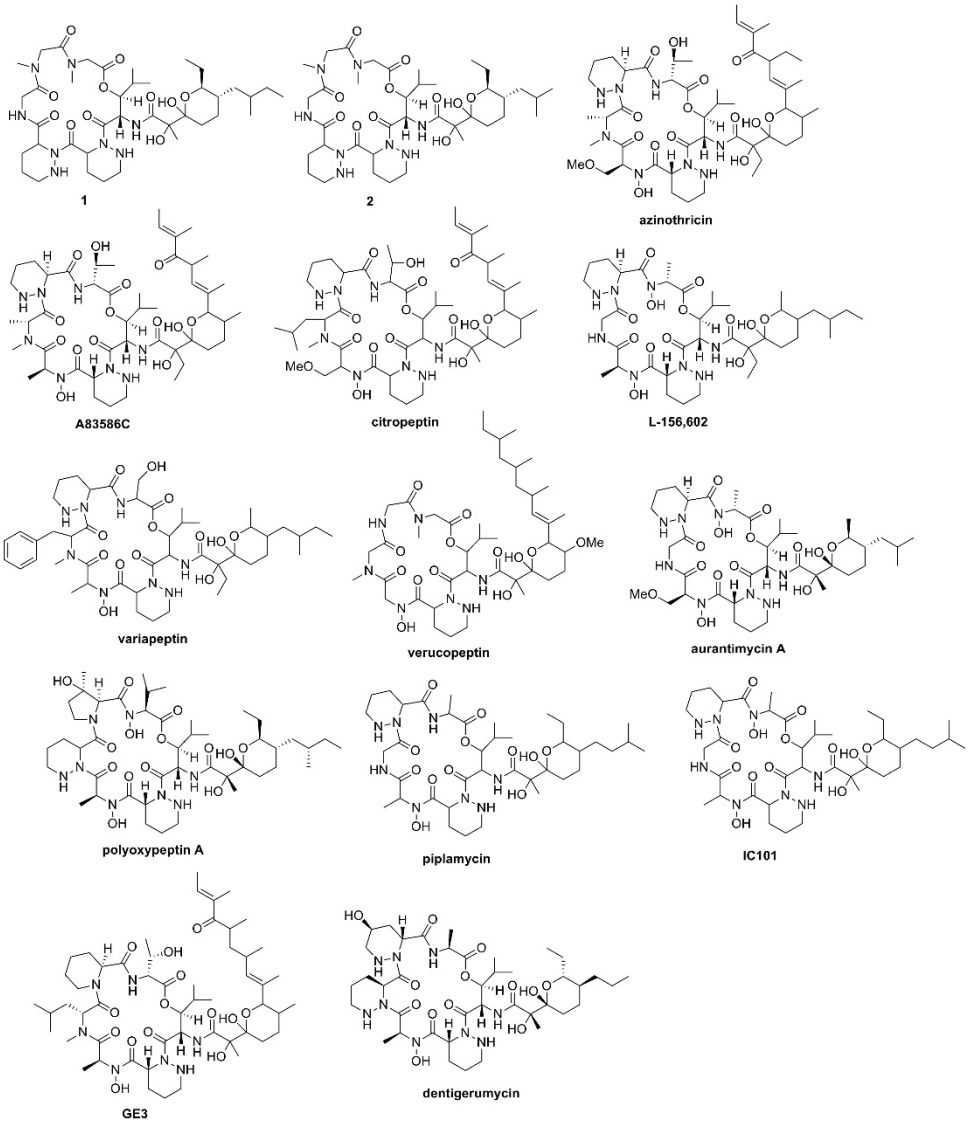

Figure 2 Structures of **1**,**2** and related metabolites.

to belong to the genus *Streptomyces*. The strain *Streptomyces* sp., Lv20-58, is deposited in the microorganism collection of Ivan Franko Lviv National University.

Strain Lv 20-58 was cultivated in a (250 ml) Schott flask containing M1 (1% starch, 0.4% yeast extract and 0.2% peptone) prepared in distilled water (80 ml). The strains were shaken at 150 r.p.m. for 8 days at 30 °C, extracted with EtOAc (50 ml) and the organic phase was concentrated *in vacuo* to yield a crude extract of 3.4 mg. The crude extracts were redissolved in MeOH generating a concentration of 1 mg ml^{-1} and analyzed using HPLC-DAD-ESI(±)MS.

Five 5 l Erlenmeyer flasks containing M1 broth (1.2 l) were inoculated with starter culture (20 ml) of *Streptomyces* sp. The flasks were incubated at 30 °C on a rotary shaker at 150 r.p.m. for 8 d, extracted with EtOAc (2×500 ml^2 per flask) and the organic phases were concentrated *in vacuo* to yield a combined EtOAc extract (167.6 mg). The EtOAc extract was sequentially triturated with hexane, CH_2Cl_2 and MeOH (40 ml aliquots), which were concentrated *in vacuo*, to yield 37.8, 77.9 and 30.6 mg partitions, respectively. The CH_2Cl_2 soluble material was further fractionated by HPLC (Zorbax, C_8 column, 250×9.4 mm^2, 5 μm, 3 ml min^{-1}, gradient from 10 to 100% acetonitrile–H_2O over 30 min, with a 100% acetonitrile hold for 5 min) to afford oleamycin A (**1**) ($t_R = 30.5$ min, 1.6 mg) and oleamycin B (**2**) ($t_R = 28.6$ min, 0.4 mg).

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