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Original article

Neopeapyran, an unusual furo[2,3*b*]pyran analogue and turnagainolide C from a soil *Streptomyces* sp. S2236

Hao Zhou [a,1], Ya-Bin Yang [a,1], Rong-Ting Duan [a], Xue-Qiong Yang [a], Ju-Cheng Zhang [a], Xiao-Guang Xie [a], Li-Xing Zhao [b], Zhong-Tao Ding [a,*]

[a] *Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education, School of Chemical Science and Technology, Yunnan University, Kunming 650091, China*
[b] *Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, China*

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ABSTRACT

Neopeapyran (**1**), an unusual furo[2,3*b*]pyran analogue, together with a new cyclopeptide, turnagainolide C (**2**), were isolated from *Streptomyces* sp. S2236 associated with the rhizosphere soil of *Panax notoginseng*. The planar structure and relative configuration of neopeapyran (**1**) were elucidated on the basis of spectroscopic techniques, while the absolute configuration was determined by TDDFT calculation. The absolute configuration of turnagainolide C (**2**) was determined by partial hydrolysis, together with the advanced Marfey's method and spectroscopic analysis. The antimicrobial activities of these two compounds were also investigated.

1. Introduction

The actinomycetes of genus *Streptomyces* have attracted the attention of the scientific community due to their enormous biosynthetic capabilities of producing secondary metabolites showing novel scaffolds and biologically actives [1–3]. In particular, terrestrial soil-derived *Streptomyces* species have been extensively studied because they can potentially provide bioactive natural products [1,4]. They have been ascertained to afford clinically useful antibiotics, such as streptomycin and neomycin [5,6]. Although actinomycetes are a major source of microbial compounds, the chemical redundancy of compounds isolated from these actinomycetes has become one of the current challenges in the discovery of novel secondary metabolites with biologically actives [7]. Investigation of unique niches harbouring chemically new actinomycetes, rather than typical environments, is one approach to overcome this problem [8,9]. Many recent investigations have exposed compelling evidence that the *Streptomyces* species derived from unique habitats might lead to the discovery of novel natural products with significant biological activity, such as the rhizosphere soil of hosts [8,10], saltern [3], sulphur mine [11], and so on.

As part of our programme to discover structurally unique and biologically active secondary metabolites from *Streptomyces* associated with the rhizosphere soil of medicine plants, *Streptomyces* sp. S2236 had drawn the most interest of us which was isolated from the rhizosphere soil of *Panax notoginseng* in Wenshan, Yunnan Province, China. The strain was most likely a *Streptomyces* sp. based on the 99.22% 16S rRNA sequence similarity with the *Streptomyces neopeptinius* KNF 2047^T (EU258679). Our chemical investigation of this strain yielded two new natural products, neopeapyran (**1**), an unusual furo[2,3*b*]pyran analogue, and a new cyclopeptide, turnagainolide C (**2**) (Fig. 1). The planar structure and relative configuration of compound **1** were elucidated on the basis of spectroscopic techniques, while the absolute configuration was determined by TDDFT calculation. The planar structure of compound **2** was determined by spectroscopic analysis, and the absolute configuration of constituent amino acid residues was determined by the advanced Marfey's method. Differentiation of L-Val and D-Val in the sequence was established by the advanced Marfey's analysis of fragment peptides obtained from the partial hydrolysate. In the antimicrobial assays, compound **2** displayed moderate antimicrobial activities against

* Corresponding author.
E-mail address: ztding@ynu.edu.cn (Z.-T. Ding).
[1] These authors contributed equally to this work.

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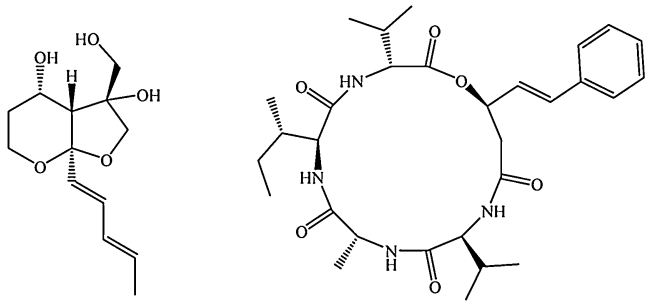

Fig. 1. Structures of compounds **1**, **2**.

Candida albicans, *Escherichia coli* and *Staphylococcus aureus*. Herein, we report the discovery and characterization of these two compounds and their antimicrobial activities.

2. Experimental

2.1. Biological material and cultivation

The bacterial strain S2236 was isolated using modified ISP4-medium from the rhizosphere soil of *P. notoginseng* in Wenshan, Yunnan Province, China. The strain was most likely a *Streptomyces* sp. based on the 99.22% 16S rRNA sequence similarity with the *S. neopeptinius* KNF 2047^T (EU258679), and it was identified as *Streptomyces* sp. S2236. The strain has been preserved at Yunnan Institute of Microbiology, Yunnan University, China. This bacterium was cultivated on 40 L scale using 1 L Erlenmeyer flasks containing 250 mL of the seed medium (yeast extract 0.4%, glucose 0.4%, malt extract 1.0%, decavitamin 0.01%, pH 7.2) and the fermentation medium (soluble starch 1%, glucose 1%, peptone 0.5%, yeast extract 0.5%, soybean flour 0.3%, NaCl 0.4%, K_2HPO_4 0.05%, $MgSO_4{\cdot}7H_2O$ 0.05%, $CaCO_3$ 0.2%, pH 7.0) at 28 °C for 7 d on rotary shaker (250 rpm).

2.2. Extraction and isolation

After 7 days of growth, the mycelia were removed from the cultures (40 L) by filtration. The filtrate was extracted with ethyl acetate (EtOAc, 3× 40 L), and the solvent was removed under vacuum. The EtOAc extract (31.0 g) was separated into four fractions (Fr 1–Fr 4) by a chromatographic column (3 cm × 50 cm) on silica gel (200–300 mesh), eluting with stepwise $CHCl_3$/MeOH gradient ($CHCl_3$, $CHCl_3$/MeOH = 30:1 v/v, $CHCl_3$/MeOH = 10:1 v/v, MeOH, 1.5 L each). The Fr 2 (18.5 g, eluted with $CHCl_3$/MeOH = 30:1 v/v) was placed in a silica gel column (2 cm × 30 cm) cm) and eluted with petroleum ether/ethyl acetate mixture (10:1) to ethyl acetate, then MeOH, which gave three fractions (Fr 2-1 to Fr 2-3). Fr 2-1 (3.4 g) was separated by a chromatographic column (1 cm × 150 cm) on Sephadex LH-20 (MeOH) and then subjected to further elution on a silica gel column (1 cm × 15 cm) with $CHCl_3$/MeOH (50:1–9:1) to afford **2** (10.2 mg). Fr 2-2 (2.1 g) was subjected to further elution on a silica gel column (1 cm × 20 cm) with $CHCl_3$/MeOH (40:1–9:1) and then separated by a chromatographic column (1 cm × 150 cm) on Sephadex LH-20 (MeOH) to give **1** (2.1 mg).

2.3. Determination of absolute configuration by the advanced Marfey's method

Details may be found in Supporting information and the references cited therein [12,13].

2.4. Partial hydrolysis and the advanced Marfey's analyses of the fragments

Each of **2** (0.5 mg) was hydrolyzed in 6 mol/L HCl at 100 °C for 3 h or 4 h. The dried hydrolysates were redissolved in 50 μL of MeOH. 20 μL portions were separated by RP-HPLC (Agilent Eclipse XDB-C18 column 4.6 mm × 150 mm 5 μm) using a gradient elution from 10 to 100% MeCN containing 0.2% AcOH. After the assignments of peptide sequences by extensive MS/MS analyses, the fractions were identified as Ala-Val-Hppa, Ile-Val-Hppa, Val-Ile, together with other fragments (Fig. 2). Then 2.0 mg of **2** was hydrolyzed in 6 mol/L HCl at 100 °C for 3 h. The hydrolysate was redissolved in 100 μL of MeOH, and each of 20 μL portions was separated by RP-HPLC (Agilent Eclipse XDB-C18 column 4.6 mm × 150 mm 5 μm) using the same gradient elution, and the eluate was collected according to the retained time which was analyzed by the LC–MS/MS data. The fractions of Ala-Val-Hppa, Ile-Val-Hppa and Val-Ile were hydrolyzed in 6 mol/L HCl at 110 °C overnight. The solution was dried, converted to the FDLA derivative, and analyzed as described previously.

3. Results and discussion

Neopeapyran (**1**) was obtained as colourless oil, and the results of TCL chromogenic reactions certified the inexistence of *N* in compound **1**. Its molecular formula of $C_{13}H_{20}O_5$ was confirmed on the basis of a prominent ion peak at *m/z* 257.1388 $[M+H]^+$ (calcd. for $C_{13}H_{21}O_5$, 257.1389) observed in the HRESIMS spectrum (Fig. S7 in Supporting information) and ^{13}C NMR spectra, and that indicated four degrees of unsaturation. IR spectrum exhibited absorption at 3433, 2923, 1632, 1450 and 1047 cm^{-1} which indicated that the structure of compound **1** contained at least one hydroxyl group and one olefin group. The ^1H NMR and ^{13}C NMR spectra of **1** displayed resonances that were assigned to one methyl (δ_C 18.2), one methylene (δ_C 26.6), three oxygen-bearing methylenes (δ_C 55.8, 78.0, 77.9), six methines [among them four vinyl methines (δ_C 132.1, 130.8, 130.6, 130.3)], and two quaternary carbons (δ_C 104.8, 88.4) (Table 1). The ^1H–^1H COSY correlations between H-10 and H-11, H-11 and H-12, H-12 and H-13, H-13 and H-14, revealed the carbon skeletons for C-10 connected to C-11, C-11 connected to C-12, C-12 connected to C-13, and C-13

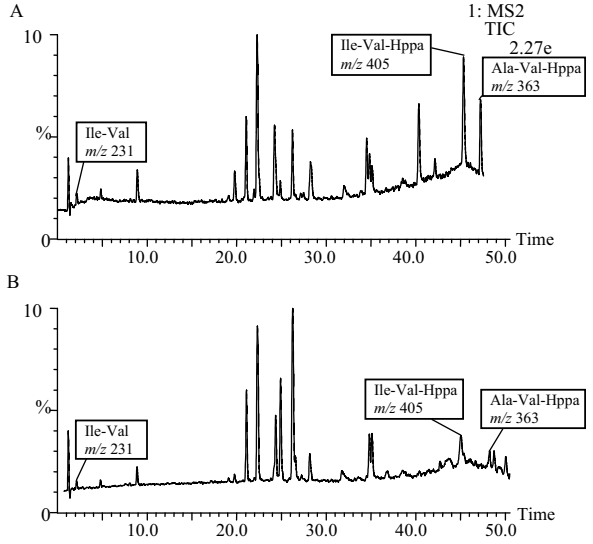

Fig. 2. Total ion current (TIC) chromatogram of the partial hydrolysate of **2**. (A) The hydrolysis time was 3 h, (B) the hydrolysis time was 4 h.

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Table 1
NMR data for compound **1** (δ in ppm, J in Hz).[a]

Position	δ_C	δ_H (mult.; J)
2	55.8	4.12 (m, 1H), 3.63 (m, 1H)
3	26.6	1.86 (m, 2H)
4	74.7	4.32 (m, 1H)
5	52.1	2.40 (d, 1H, 7.6)
6	88.4	–
7	78.0	4.17 (d, 1H, 9.6), 3.82 (dd, 1H, 9.7, 1.8)
9	104.8	–
10	130.6	5.58 (d, 1H, 15.4)
11	130.8	6.39 (dd, 1H, 15.4, 10.5)
12	130.3	6.06 (ddd, 1H, 15.1, 12.2, 3.0)
13	132.1	5.80 (m, 1H)
14	18.2	1.76 (d, 3H, 6.5)
15	77.9	4.08 (d, 1H, 10.0), 3.71 (dd, 1H, 9.1, 1.6)

[a] Data in $CDCl_3$ at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR.

connected to C-14. The HMBC correlations from H-14 to C-12 and C-13, together with $J_{10,11}$ = 15.4 Hz and $J_{12,13}$ = 15.1 Hz for H-10, 11 *trans* and H-12, 13 *trans*, respectively, indicated the presence of a (2*E*,4*E*)-pentylene residue. A tetrahydrofuran moiety was deduced by the HMBC correlations from H-5 to C-6 and C-9, H-7 to C-5, C-6 and C-9, together with the chemical shifts of C-7 (δ_C 78.0) and C-9 (δ_C 104.8). The HMBC correlations from H-15 to C-5, C-6 and C-7, together with the chemical shifts of C-6 (δ_C 88.4) and C-15 (δ_C 77.9), indicated that C-6 was substituted by a hydroxymethyl and a hydroxyl. At the same time, the HMBC correlations from H-10 and H-11 to C-9, H-10 to C-5, indicated that the (2*E*,4*E*)-pentylene residue was linked to C-9. The ^1H–^1H COSY correlations between H-2 and H-3, H-3 and H-4, H-4 and H-5, revealed the carbon skeletons for C-2 connected to C-3, C-3 connected to C-4, and C-4 connected to C-5, and the chemical shift of C-4 (δ_C 74.7) indicated that C-4 was substituted by a hydroxyl. However, the HMBC correlations from H-2 and H-4 to C-9, along with one additional degree of unsaturation and the molecular formula of $C_{13}H_{20}O_5$ deduced by the HRESIMS spectrum, indicated the existed of a tetrahydropyrane moiety (Fig. 3), and the upfield shifts of H-2 (δ_H 3.63, 4.12) and C-2 (δ_C 55.8) cased by the shielding effect of the double bond between C-10 and C-11. Finally, the structure of compound **1** was deduced as shown in Fig. 1. The skeleton of furo[2,3*b*]pyran with a (2*E*,4*E*)-pentylene side chain in compound **1** was reported for the first time.

The relative stereochemistry of **1** was deduced from the ROESY experiments (Fig. 3). ROESY correlations between H-4, H-5 and H-15, and no significant correlations between H-10 and H-4/H-5/H-15, suggested that there were two possible isomers [(4*S*, 5*R*, 6*R*, 9*R*)-**1** and (4*R*, 5*S*, 6*S*, 9*S*)-**1**] of compound **1**. The absolute configuration of **1** was determined by comparison of quantum chemical TDDFT calculated and experimental specific rotations. Each isomer was optimized using DFT at the B3LYP/6-311 + G(d,p) level in the Gaussian 09 program. Then, the optimized isomer was calculated using TDDFT/GIAOs at the B3LYP/6-311 + G(d,p) in the Gaussian 09 program to generate its specific rotation. The calculated specific rotation of (4*S*, 5*R*, 6*R*, 9*R*)-**1** was negative, and the calculated specific rotation of (4*R*, 5*S*, 6*S*, 9*S*)-**1** was positive. Therefore, the absolute configuration of **1** was determined as (4*S*, 5*R*, 6*R*, 9*R*)-**1** (Fig. 1) by compared with the experimental specific rotation of **1** which was $[\alpha]_D^{25}$ −5.8 (*c* 0.1, MeOH).

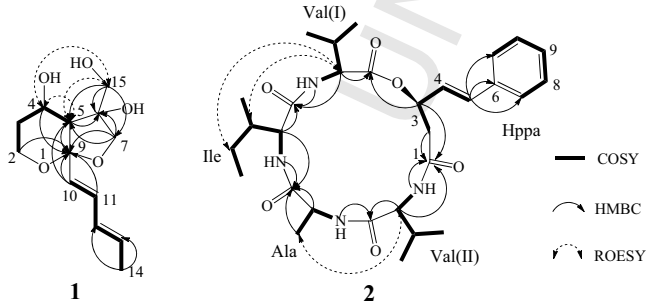

Fig. 3. Key COSY, HMBC and ROESY correlations of **1** and **2**.

Turnagainolide C (**2**) obtained as a white powder, and its molecular formula of $C_{30}H_{44}N_4O_6$ was assigned by positive HRESIMS (*m/z* 579.3156 for $[M+Na]^+$, calcd. for $C_{30}H_{44}N_4O_6Na$: 579.3159, Fig. S14 in Supporting information) and ^{13}C NMR data (Table 2). The molecular formula of **2** indicated 11 degrees of unsaturation. IR spectrum exhibited intense N–H and CO absorption at 3392 and 1665 cm^{-1}, respectively. The appearance of four amino carbonyl signals (δ_C 173.1, 172.4, 170.4, and 168.8) in ^{13}C NMR spectrum and four NH signals (δ_H 7.59, 7.73, 8.09, and 8.56) in ^1H NMR spectrum, suggesting the molecule contained a peptide component. The amino acid residues were identified as two valine groups (Vals), one alanine group (Ala), and one isoleucine group (Ile) by ^1H–^1H COSY, HSQC and HMBC spectra. The sequence of amino acid residues in **2** was determined from HMBC correlations between the carbonyl group and amide of the adjacent residues (Fig. 3). HMBC correlations from αH-Val(I) and NH-Val(I) to CO-Ile, from αH-Ile and NH-Ile to CO-Ala, from NH-Ala to CO-Val(II), demonstrated the sequence of the four amino acid residues was -Val(I)-Ile-Ala-Val(II)-. Compared with the molecular formula of **2** ($C_{30}H_{44}N_4O_6$), the molecule still had an elemental composition of $C_{11}H_{10}O_2$ by subtracted the atoms attributed to the four amino acids residues ($C_{19}H_{34}N_4O_4$). This fragment was identified as a 3-hydroxy-5-phenylpent-4-enoic acid residue (Hppa) by ^1H–^1H COSY, HSQC and HMBC spectra (Fig. 3). The 16.0 Hz scalar coupling observed between H-4(Hppa) and H-5(Hppa) deduced the moiety

Table 2
NMR data for compound **2** (δ in ppm, J in Hz).[a]

Residue	Position	δ_C	δ_H (mult.; J)
Val(I)	CO	168.8	–
	α	58.3	4.24 (m, 1H)
	β	28.5	2.25 (m, 1H)
	γ	18.9	0.90 (d, 3H, 6.2)
		19.7	0.90 (d, 3H, 6.2)
	NH	–	7.59 (d, 1H, 8.5)
Ile	CO	170.4	–
	α	57.3	4.26 (m, 1H)
	β	35.7	2.04 (m, 1H)
	γ	23.6	1.23 (m, 2H)
		11.9	0.81 (m, 3H)
	δ	15.6	0.82 (m, 3H)
	NH	–	8.09 (d, 1H, 9.4)
Ala	CO	173.1	–
	α	48.9	4.32 (m, 3H)
	β	16.4	1.18 (d, 3H, 6.6)
	NH	–	8.56 (d, 1H, 5.6)
Val(II)	CO	172.4	–
	α	57.6	4.14 (t, 1H, 7.2)
	β	29.9	1.96 (m, 1H)
	γ	18.3	0.88 (d, 3H, 5.6)
		19.4	0.88 (d, 3H, 5.6)
	NH	–	7.73 (d, 1H, 8.3)
Hppa	1	168.8	–
	2	40.1	2.88 (dd, 1H, 13.7, 11.4)
			2.42 (d, 1H, 13.4)
	3	73.0	5.50 (m, 1H)
	4	126.8	6.28 (dd, 1H, 16.0, 7.0)
	5	132.5	6.68 (d, 1H, 15.9)
	6	135.8	–
	7, 11	126.6	7.45 (d, 2H, 9.0)
	8, 10	128.8	7.35 (t, 2H, 7.3)
	9	128.2	7.28 (t, 1H, 7.3)

[a] NMR data in DMSO-d_6 at 400 MHz for ^1H and 100 MHz for ^{13}C.

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Table 3
MICs of the extract and compounds from *Streptomyces* sp. S2236 (μg/mL).

Sample	*C. albicans*	*E. coli*	*S. aureus*
Extract	128	128	256
1	128	64	128
2	32	32	32
Nystain	16	–	–
Kanamycin	–	8	4

was (*E*)-3-hydroxy-5-phenylpent-4-enoic acid residue. Finally, the HMBC correlations from αH-Val(II) and NH-Val(II) to CO-Hppa, from H-3(Hppa) to CO-Val(I) established that **2** was cyclo(Val(I)-Ile-Ala-Val(II)-Hppa).

^{1}H NMR and ^{13}C NMR spectra of **2** displayed the similar resonances as EGM-556 [14] and turnagainolides A-B [15], but the absolute stereochemistry of the amino acid residues in **2** were identified as L-Val, D-Val, L-Ile and D-Ala by the advanced Marfey's method. There was no correlations between αH-Ile and αH-Val(I), αH-Ala and αH-Val(II) in ROESY spectrum, but observed the correlations between βH-Ile and αH-Val(I), γH-Ile(CH_2) and αH-Val(I), βH-Ala and αH-Val(II) in ROESY spectrum, together with contrasting the NMR spectra of **2** with turnagainolide A, established the configurations of Val(I), Val(II) and Ile as (*R*)-Val(I), (*S*)-Val(II) and (2*S*,3*S*)-Ile (Fig. 3). In the same way, the configuration of Hppa was determined as (3*R*)-Hppa by comparing the chemical shifts of Hppa residue with turnagainolide A, and there was no ROESY correlation between H-3(Hppa) and αH-Val(I). Therefore, the structure of **2** was determined as cyclo((*R*)-Val(I)-(2*S*,3*S*)-Ile-(*R*)-Ala-(*S*)-Val(II)-(3*R*)-Hppa), which had a different stereochemical structure from turnagainolides A-B, named as turnagainolide C.

In order to confirm the configuration elucidation of **2**, we used LC–MS/MS analysis to set up proper hydrolysis conditions and get suitable peptide fragments by partial hydrolysis [16]. We hoped to isolate peptides to determine the chirality of each Val in the peptide sequence. To do this we chose harsher conditions of 6 mol/L HCl at 100 °C and set the hydrolysis time for 3 h on the basis of the LC–MS data. The LC–MS/MS data allowed us to identify fragments that contain Val residues of specific position, i.e., Ala-Val-Hppa, Ile-Val-Hppa and Val-Ile, together with other fragments (Fig. 2). Marfey's analysis of fragment Ala-Val-Hppa indicated that the Val(II) was a L-Val. Due to the little amount of **2**, we did not get enough fragments of Ile-Val-Hppa or Val-Ile to determined the absolute stereochemistry of Val(I). However, the Marfey's analysis result of partial hydrolysis of **2** was consistent with the determined structure.

The extract of *Streptomyces* sp. S2236 and the isolates (**1** and **2**) were tested for their antimicrobial activity against *C. albicans*, *E. coli*, and *S. aureus*. The results were showed in Table 3. Both **1** and **2** showed activity against *E. coli* with MICs of 64 and 32 μg/mL, respectively. At the same time, **2** also showed moderate antimicrobial activities against *C. albicans* and *S. aureus*.

4. Conclusion

Two new compounds, neopeapyran (**1**) and turnagainolide C (**2**) were isolated from the fermentation broth of *Streptomyces* sp. S2236. The skeleton of furo[2,3*b*]pyran with a (2*E*,4*E*)-pentylene side chain has never been reported from natural resources or synthesis. The skeleton of **1** was reported for the first time. The relative configuration of **1** was elucidated by spectroscopic techniques, while the absolute configuration was determined by TDDFT calculated specific rotation. Compound **2** showed moderate antimicrobial activities against *C. albicans*, *E. coli* and *S. aureus* all with the MIC of 32 μg/mL.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cclet.2016.03.018.

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