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ORIGINAL ARTICLE

New anti-inflammatory metabolites produced by *Streptomyces violaceoruber* isolated from *Equus burchelli* feces

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Three new metabolites (2–4), together with one known compound, GTRI-02, (1) were isolated from a fermentation broth of *Streptomyces violaceoruber* derived from *Equus burchelli* feces. The structures of the new compounds 2–4 were established using comprehensive NMR spectroscopic data analysis as well as UV, IR and MS data. The anti-inflammatory activity of compounds 1–4 was tested by examining their ability to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. Compound 2 showed a moderate inhibition of NO production with IC_{50} value of 51.2 μM.
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INTRODUCTION

Animal intestinal microorganisms have been studied for decades, and the investigation mainly focused on the food digestion and absorption, immunity, pathogens resistance and health maintaining.[1] Over the past few years, the authors have dedicated to research on the diversity, bioactivities and the secondary metabolites of cultivable actinobacteria from animal feces and several new microbial species and new bioactive substances have been found.[2–7] These findings showed animal intestinal and fecal microorganisms could be considered as a rich and important microbial resource for discovering new bioactive natural products.

In the course of our continuous search for potential structurally novel and bioactive natural products from fecal actinobacteria associated with animal feces, we investigated the secondary metabolites of *Streptomyces violaceoruber* (YIM 101131), which was isolated from *Equus burchelli* (zebra) feces. Separation of the fermentation broth of *S. violaceoruber* led to the isolation of three new compounds, a tetralone derivative, (*R*)-7-acetyl-3,6-dihydroxy-8-propyl-3,4-dihydronaphthalen-1(2*H*)-one (**2**), a tryptophan derivative, (*S*)-2-(5-(2,3-dihydroxy-3-methylbutyl)-1*H*-indol-3-yl) acetonitrile (**3**), a fatty acid amide (5*Z*,8*Z*,11 R^*,12 R^*)-11,12-dihydroxytetradeca-5,8-dienamide (**4**), as well as a known compound GTRI-02 (**1**)[8–10] (Figure 1). The structures of these metabolites were determined on the basis of comprehensive spectroscopic analysis and $Mo_2(OAc)_4$ induced CD effects. The anti-inflammatory activity of isolated compounds **1–4** were evaluated by examining their ability to inhibit NO production in LPS-stimulated RAW 264.7 macrophage cells. Details of the isolation, structure elucidation and anti-inflammatory activity of the new compounds are described in the present paper.

RESULTS AND DISCUSSION

Compound **2** was isolated as a white, amorphous solid. The molecular formula of **2** was determined to be $C_{15}H_{18}O_4$ based on the HRESIMS at *m/z* 261.1218 $[M\text{-}H]^-$. The IR spectrum showed absorptions for hydroxyl (3270 cm^{-1}), unsaturated carbonyl (1698 cm^{-1}) and benzene ring (1582 cm^{-1}). The ^1H NMR data (Table 1) of **1** displayed signals for an aromatic proton (δ_H 6.59, s), an oxygenated methine proton (δ_H 4.09, m) and two methyl groups at δ_H 2.39 (3H, s) and δ_H 0.88 (3H, t, J = 7.2 Hz). Additional signals showed six aliphatic methylene protons (δ_H 3.03, dd, J = 15.8, 2.6 Hz; δ_H 2.78, dd, J = 15.8, 7.2 Hz; δ_H 2.71, brd, J = 16.0 Hz; δ_H 2.46, dd, J = 16.0, 7.9; δ_H 1.39, 2H, m). The ^{13}C NMR spectrum of **2** showed 15 carbon signals (Table 1). Except for the ^{13}C NMR signals corresponding with the ^1H NMR data, seven quaternary carbons, including two ketone carbonyls at δ_C 205.8, 196.8, five aromatic quaternary carbons at δ_C 159.1, 146.1, 142.9, 131.4, 122.1 were observed. Its ^1H and ^{13}C NMR spectroscopic data of **2** were very similar to those of the known compound GTRI-02 (**1**),[8–10] apart from the presence of two additional methylenes. Moreover, the ^1H-^1H COSY experiment gave a propyl fragment in **2**. These findings suggested a propyl instead of a methyl located at C-8 in **2**. HMBC correlations between H-11 (δ_H 2.72, 2.66) and C-7 (δ_C 131.4), C-8 (δ_C 142.9) and C-8a (δ_C 122.1), between H-12 (δ_H 1.39) and C-8 (δ_C 142.9) confirmed the propyl was linked to C-8. The configuration of the chiral carbon C-3 was assigned as *R* by comparing the optical rotation value ($[\alpha]_D^{20}$ −10 (*c* 0.4, MeOH)) with that of (*R*)-GTRI-02 ($[\alpha]_D^{20}$ −16 (*c* 2.5, MeOH)) which had been obtained by biomimetic asymmetric synthesis and the absolute configuration was determined by CD spectroscopy.[11] Consequently,

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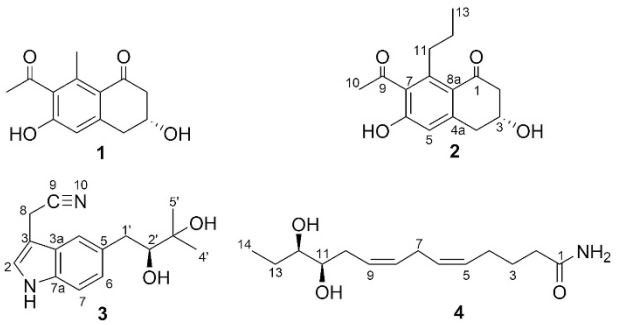

Figure 1 Structures of compounds **1**–**4**.

Table 1 ^{1}H (600 MHz, DMSO-d_6) and ^{13}C (150 MHz, DMSO-d_6) NMR data of compound **2**

No.	δ_C	δ_H	*HMBC*
1	196.8 C		
2	50.0 CH_2	2.71, brd (16.0)	1, 3, 4, 8a
		2.46, dd (16.0, 7.9)	
3	65.1 CH	4.09, m	
4	40.5 CH_2	3.03, dd (15.8, 2.6)	2, 3, 4a, 5, 8a
		2.78, dd (15.8, 7.2)	
4a	146.1 C		
5	114.7 CH	6.59, s	4, 6, 7, 9, 8a
6	159.1 C		
7	131.4 C		
8	142.9 C		
8a	122.1 C		
9	205.8 C		
10	32.9 CH_3	2.39, s	7, 9
11	33.5 CH_2	2.72, m	7, 8, 8a, 12, 13
		2.66, m	
12	25.0 CH_2	1.39, m	8, 11, 13
13	15.0 CH_3	0.88, t (7.2)	11, 12

2 was identified as (*R*)-7-acetyl-3,6-dihydroxy-8-propyl-3,4-dihydronaphthalen-1(2*H*)-one.

Compound **3** was obtained as a colorless, amorphous solid. The HRESIMS exhibited a prominent ion peak at *m/z* 257.1345 $[M-H]^-$, which established the molecular formula of **3** as $C_{15}H_{18}N_2O_2$. ^{1}H NMR spectrum (Table 2) of **3** indicated signals of four aromatic protons (δ_H 7.41, brs; δ_H 7.28, d, $J=8.3$ Hz; δ_H 7.28, brs; δ_H 7.05, d, $J=8.3$ Hz), one oxygenated methine (δ_H 3.37, m), two methylene groups (δ_H 4.00, 2H, s; δ_H 3.02, brd, $J=13.6$ Hz; δ_H 2.43, dd, $J=13.6$, 10.3 Hz), two singlet methyls (δ_H 1.14; δ_H 1.11). Moreover, three active protons were observed (δ_H 10.96, brs; δ_H 4.30, d, $J=6.0$ Hz; δ_H 4.24, s). ^{13}C NMR spectrum (Table 2) of **3** presented 15 carbon signals including six carbons without directly bonded protons, five methines, two methylenes, two methyls with the aid of HSQC spectrum. These data showed **3** was a tryptophan derivative and related to 5-dimethylallylindole-3-acetonitrile.[12] The major difference between these two compounds was the side chain at C-5. Two more oxygenated carbons were present and a double bond was absent in **3**, which indicated the double bond in 5-dimethylallylindole-3-acetonitrile was oxygenated to vicinal diol in **3**. The planar structure of **3** was further determined by HMBC correlations (Figure 2). The absolute configuration of C-9 was elucidated by $Mo_2(AcO)_4$-induced CD method.[13] The CD spectrum showed a positive Cotton effect at 313 nm suggesting S configuration for C-2′ (Figure 3). Thus, **3** was identified as (*S*)-2-(5-(2,3-dihydroxy-3-methylbutyl)-1*H*-indol-3-yl) acetonitrile.

Table 2 ^{1}H (600 MHz, DMSO-d_6) and ^{13}C (150 MHz, DMSO-d_6) NMR data of compound **3**

No.	δ_C	δ_H	*HMBC*
1		10.96, s	3, 3a, 7a
2	124.2 CH	7.28, brs	3, 7a
3	103.6 C		
3a	126.5 C		
4	118.4 CH	7.41, brs	3, 6, 7a, 1′
5	132.2 C		
6	124.1 CH	7.05, brd (8.3)	4, 7a
7	111.6 CH	7.28, d (8.3)	3a, 5
7a	135.3 C		
8	13.7 CH_2	4.00, s	2, 3, 3a, 9
9	120.0 C		
1′	38.1 CH_2	3.02, brd (13.6) 2.43, dd (13.6, 10.3)	4, 5, 6, 2′
2′	80.1 CH	3.37, m	5, 3′
3′	72.3 C		
4′	24.8 CH_3	1.11, s	2′, 3′, 5′
5′	27.2 CH_3	1.14, s	2′, 3′, 4′
2′-OH		4.30, d (6.0)	1′, 2′
3′-OH		4.24, s	2′, 3′, 4′

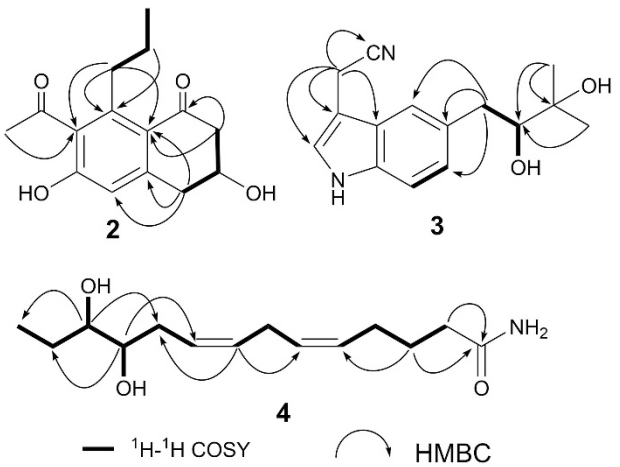

Figure 2 ^{1}H-^{1}H COSY and key HMBC correlations for compounds **2**–**4**.

Compound **4** was isolated as a white, amorphous solid. Its molecular formula was determined as $C_{14}H_{25}NO_3$ by HRESIMS at *m/z* 256.1992 $[M+H]^+$. The ^{1}H and ^{13}C NMR data indicated **4** was a fatty acid amide, consisting of 14 carbons including four olefinic carbons and two oxygenated carbons. HSQC experiment distinguished the protons linked to carbons. The uninterrupted ^{1}H-^{1}H COSY correlations from H-2 to H_3-14 determined the linkage of all the carbons. The planar structure of **4** was further confirmed by HMBC correlations from H-8 (δ_H 5.49) to C-6 (δ_C 128.9), C-7 (δ_C 25.9), C-10 (δ_C 31.5), from H-11 (δ_H 3.21) to C-9 (δ_C 128.7), C-12 (δ_C 75.3), C-13 (δ_C 25.9), from H-12 (δ_H 3.12) to C-10 (δ_C 31.5), C-11 (δ_C 74.2), C-14 (δ_C 10.7), from H-3 (δ_H 1.53) to C-1 (δ_C 174.6), C-5 (δ_C 129.7), from H-2 (δ_H 2.04) to C-1 (δ_C 174.6). Both the double

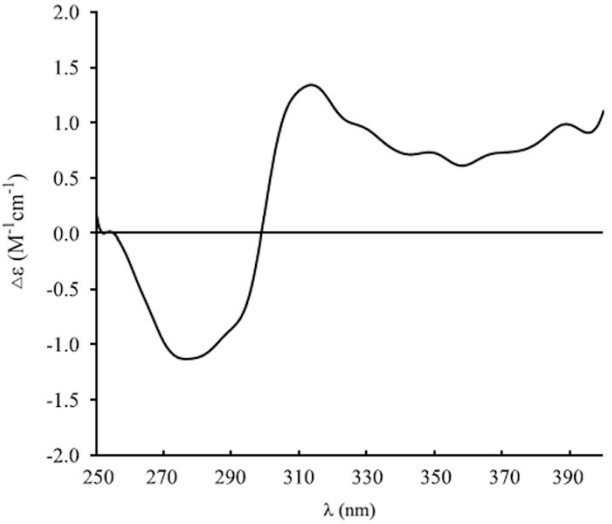

Figure 3 $Mo_2(AcO)_4$-induced CD spectrum of **3**.

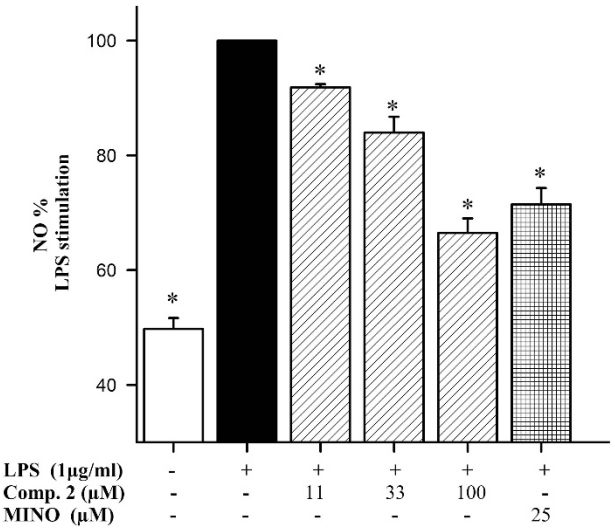

Figure 4 Effect of compound **2** on the inhibition of LPS-induced NO in Raw 264.7 cells.

bonds at C-5/C-6, C-8/C-9 elucidated as *Z* geometry based on the coupling constant of H-8 (δ_H 5.49, dt, $J = 10.8$, 7.2 Hz) and the ^{13}C NMR data of C-7 (δ_C 25.9).[14,15] The relative configuration of C-11 and C-12 was deduced as threo on the basis of the coupling constant $^3J_{H\text{-}11/H\text{-}12} = 6.2$ Hz.[16–18] Thus, the structure of **4** was established as (5*Z*, 8*Z*, 11 *R**, 12 *R**)-11,12-dihydroxytetradeca-5,8-dienamide.

Compounds **1–4** were tested for anti-inflammatory activity by examining their ability to inhibit NO production in LPS-stimulated RAW 264.7 macrophage cells. The effects of these compounds on cell viability were also determined by the MTT method, and none of these compounds exhibited cytotoxicities at 100 μM. Compared to the vehicle-treated group, compound **2** exhibited a moderate dose-dependent decrease in the NO production ($P < 0.01$) at the concentrations of 100, 33.3 and 11.1 μM (Figure 4). While compounds **1**, **3**, **4** didn't show obvious inhibition at the tested concentrations. The IC_{50} value of compound **2** was further determined to be 51.2 μM. Minocycline was used as a positive control with IC_{50} value of 25.3 μM.

METHODS

General experimental procedures

Optical rotations were determined using an Anton Paar MCP200 automatic polarimeter (Graz, Austria). IR spectrum was recorded with a Bruker Tensor 27 FT–IR spectrometer (film). UV detection was carried out with a Beckman Coulter DU 730 nucleic acid/protein analyzer (Brea, CA, USA). CD spectra were acquired on a Biologic MOS-450 spectra polarimeter (Biologic Science, Claix, France). 1D and 2D NMR spectra were recorded on a Bruker AV-600 spectrometer (Faellanden, Switzerland). ESI-MS were recorded on an Agilent 1290-6420 Triple Quadrupole LC-MS spectrometer (Santa Clara, CA, USA). HRESI-MS was measured with an Agilent G6230 TOF mass spectrometer. Biological assays were analyzed using a microplate reader (BioTek Synergy H1, BioTek Instruments, Winooski, VT, USA).

Microbial material

The producing organism was isolated from fecal samples excreted by healthy adult *E. burchelli* living in Yunnan Wild Animal Park, Kunming, Yunnan province, China. The strain was identified as *S. violaceoruber* on the basis of morphological characteristics and 16 S ribosomal RNA gene sequences analysis. Phylogenetic analysis suggested that the strain shared a higher 16 S ribosomal RNA gene sequences similarity (100%) with the closely related strain *S. violaceoruber* (accession number AB184174). The strain (No. YIM 101131) was deposited at the Yunnan Institute of Microbiology, Yunnan University, China.

Fermentation, extraction and isolation

The strain, grown on agar plate, was prepared to inoculate 500 ml Erlenmeyer flasks each containing 100 ml of sterile seed medium composed of glucose 4.0%, malt extract 5.0%, multiple vitamin solution 1.0 ml per liter and trace element solution 1.0 ml per liter at a pH of 7.2 with no adjustment. These flasks cultures were incubated at 28 °C for 2 days on a rotary shaker set at 180 r.p.m. For large-scale fermentation, 20 ml of seed medium was used to transferred into 1000 ml Erlenmeyer flasks containing 200 ml of sterile fermentation medium composed of soybean meal 10 g, peptone 2.0 g, glucose 20 g, soluble starch 5.0 g, yeast extract 2.0 g, NaCl 4 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g and $CaCO_3$ 2.0 g in 1 l tap water at a pH of 7.8 with no adjustment. The fermentation batches were cultured at 28 °C for 7 days on a rotary shaker set at 180 r.p.m.

The mycelium and broth filtrate (70 l) were separated by centrifugation (4000 r.p.m, 5 min). The resultant aqueous phase filtrate was extracted with EtOAc for three times. The organic partition layer was collected and evaporated *in vacuo* to yield 13 g of residue. The dried crude extract was further separated by chromatography on a silica gel column (CH_2Cl_2–MeOH, gradient 50:1-1:1) to obtain 10 fractions (Fr. 1–10). Fraction 4 was subjected to Sephadex LH-20 chromatography (MeOH) to afford six subfractions (Fr. 4.1–4.6). Fraction 4.3 was purified using semi-preparative reversed-phase HPLC (MeOH–H_2O 55:45, 10 ml min^{-1}) to yield compound **3** (5.5 mg). Fraction 8 was subjected to Sephadex LH-20 chromatography (MeOH) to produce six subfractions (Fr. 8.1–8.6). Subfraction 8.5 was applied to semi-preparative reversed-phase HPLC (MeOH-H_2O 40:60, 10 ml min^{-1}) to afford **2** (2.0 mg) and **1** (10 mg), respectively. Fraction 10 was separated by silica gel column chromatography (petroleum ether–EtOAc 1:5) to afford six subfractions (Fr. 10.1–10.6). Subfraction 10.2 was purified by semi-preparative reversed-phase HPLC (MeOH–H_2O 57:43, 10 ml min^{-1}) to yield **4** (4.2 mg).

(R)-7-acetyl-3,6-dihydroxy-8-propyl-3,4-dihydronaphthalen-1(2H)-one (**2**): white, amorphous solid; $[\alpha]_D^{20}$ −10 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.40), 275 (4.22) nm; IR (film) ν_{max} 3270, 2959, 2927, 1698, 1667, 1582, 1276, 1236, 1211, 1026 cm^{-1}; ESI-MS *m/z* 263 $[M+H]^+$, 285 $[M+Na]^+$; HRESI-MS *m/z* 263.1286 $[M+H]^+$(calcd for $C_{15}H_{19}O_4$, 263.1283). For ^1H and ^{13}C NMR data see Table 1.

(S)-2-(5-(2,3-dihydroxy-3-methylbutyl)-1H-indol-3-yl) acetonitrile (**3**): colorless, amorphous solid; $[\alpha]_D^{20}$ −51.8 (*c* 0.54, MeOH); UV (MeOH) λ_{max} (log ε) 219 (4.48), 274 (3.72) nm; IR (film) ν_{max} 3351, 2971, 2923, 2251, 1660, 1604, 1429, 1381, 1348, 1069 cm^{-1}; $Mo_2(OAc)_4$ induced CD λ_{max} (Δε) 313

(1.34); ESI-MS *m/z* 281 $[M+Na]^+$; HRESI-MS *m/z* 281.1265 $[M+Na]^+$ (calcd for $C_{15}H_{18}N_2O_2$ Na, 281.1266); For ^1H and ^{13}C NMR data see Table 2.

(5Z, 8Z, 11 R, 12 R*)-11,12-dihydroxytetradeca-5,8-dienamide* (**4**): white, amorphous solid; $[\alpha]_D^{20}$ −25 (*c* 0.36, MeOH); UV (MeOH) λ_{max} (log ε) 219 (3.90) nm; IR (film) ν_{max} 3331, 2927, 1662, 1612, 1405 cm^{-1}; ESI-MS *m/z* 256 $[M+H]^+$, 278 $[M+Na]^+$; HRESI-MS *m/z* 278.1719 $[M+Na]^+$ (calcd for $C_{14}H_{25}NO_3Na$, 278.1732); ^1H NMR (600 MHz, DMSO-d_6) δ: 7.23 (1H, brs, NH), 6.69 (1H, brs, NH), 5.49 (1H, dt, *J* = 10.8, 7.2 Hz, H-8), 5.34 (3H, m, H-5, H-6, H-9), 4.40 (1H, d, *J* = 5.9 Hz, 11-OH), 4.33 (1H, d, *J* = 5.9 Hz, 12-OH), 3.21 (1H, m, H-11), 3.12 (1H, m, H-12), 2.75 (2H, m, H-7), 2.35 (1H, m, H-10), 2.04 (2H, t, *J* = 7.5 Hz, H-2), 2.01 (3H, m, H-10, H-4), 1.58 (1H, m, H-13), 1.53 (2H, m, H-3), 1.25 (1H, m, H-13), 0.87 (3H, t, *J* = 7.5 Hz, H-14); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 174.6 (C-2, s), 35.1 (C-3, t), 25.6 (C-4, t), 26.8 (C-5, t), 129.7 (C-5, d), 128.9 (C-6, d), 25.9 (C-7, t), 128.3 (C-8, d), 128.7 (C-9, d), 31.5 (C-10, t), 74.2 (C-11, d), 75.3 (C-12, d), 25.9 (C-13, t), 10.7 (C-14, q). ^1H NMR (600 MHz, CD_3OD) δ: 5.42 (1H, brdt, *J* = 10.8, 7.3 Hz, H-9), 5.34 (1H, brdt, *J* = 10.8, 7.3 Hz, H-8), 5.32–5.26 (2H, m, H-5, H-6), 3.31 (1H, ddd, *J* = 8.6, 6.2, 3.7 Hz, H-11), 3.23 (1H, m (overlapped), H-12), 2.74 (2H, m, H-7), 2.35 (1H, brddd, *J* = 14.9, 7.3, 3.7 Hz, H-10), 2.12 (2H, t, *J* = 7.3 Hz, H-2), 2.07 (1H, ddd (quint-like), *J* = 14.9, 8.6, 7.3 Hz, H-10), 2.02 (2H, q, *J* = 7.3 Hz, H-4), 1.60 (1H, m, H-13), 1.57 (2H, quint, *J* = 7.3 Hz, H-3), 1.29 (1H, m, H-13), 0.89 (3H, t, *J* = 7.3 Hz, H-14).

Inhibition of NO production assay

The nitrite concentration in the medium was measured using the Griess reaction as previously described.[19] Compounds **1–4** were tested for their inhibitory activity of NO production at various concentrations (100, 33.3 and 11.1 μM for dose-dependent assay; 10, 20, 40, 60, 80 and 100 μM for IC_{50} analysis). Minocycline was used as the positive control. Statistical analysis was performed by SPSS (SPSS Inc., Chicago, IL, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)