NOTE

Oxysporizoline, an antibacterial polycyclic quinazoline alkaloid from the marine-mudflat-derived fungus *Fusarium oxysporum*

Viviane Nenkep, Keumja Yun and Byeng Wha Son

The Journal of Antibiotics (2016) 69, 709–711; doi:10.1038/ja.2015.137; published online 6 January 2016

During the course of our studies on bioactive constituents of marine-derived microorganisms, we isolated antibacterial cerebrosides, flavusides A and B, from the marine-derived fungus *Aspergillus flavus*, and determined their absolute stereostructures.¹

In a subsequent study of the antibacterial chemical constituents of the marine-derived fungi, we found that a crude extract from a small-scale culture (100 ml) of the marine-mudflat-derived fungus *Fusarium oxysporum* displayed *in vitro* antimicrobial activity against methicillin-resistant and multidrug-resistant *Staphylococcus aureus* (MRSA and MDRSA) in a primary screen. We used a large-scale culture (10 l) to purify the antibacterial metabolites from the broth extract. By fractionating the extract, we isolated a new antibacterial alkaloid, a *tris*-anhydrotetramer of anthranilic acid (α -aminobenzoic acid), named oxysporizoline (**1**); the alkaloid 1*H*-indol-3-butanamide (**2**)² and two polyketides, chlamydosporel (**3**)³ and butenolide (4-acetamido-4-hydroxy-2-butenic acid γ -lactone) (**4**).⁴ In this paper, we report the structural elucidation of these alkaloids and polyketides.

The fungal strain, *F. oxysporum*, was isolated from the marine mudflat collected at Suncheon Bay, Korea, and identified based on 18S ribosomal RNA analyses (SolGent, Daejeon, Korea), with an identity of 98%. A voucher specimen is deposited at Pukyong National University (code MSA392).

The fungus was cultured in 2.8 l Fernbach flasks (10 × 1 l) in culture medium consisting of soytone (0.1%), soluble starch (1.0%) and seawater (100%). The cultures were incubated at 29 °C for 20 days under constant conditions. The mycelium and broth were separated by filtration through cheesecloth, and the whole broth was extracted with EtOAc (10 l) to afford the broth extract (270 mg). A portion of this extract (250 mg) was subjected to Si gel flash chromatography with *n*-hexane-EtOAc (stepwise, 0–100% EtOAc) to yield six fractions. Fractions 4 and 5 exhibited antibacterial activity and were purified further by medium-pressure liquid chromatography [octadecyl silica (ODS) column], with a step gradient from 10:1 MeOH/H₂O to MeOH to yield the crude compound **1** (8 mg) from fraction 4, and compounds **2** (7 mg) and **3** (10 mg) from fraction 5. Fractions 4

and 6 also displayed radical scavenging activity, and fraction 6 was purified again by the same method to furnish crude compound **4** (6 mg). Crude compounds **1** and **3** displayed antibacterial activity and were purified by HPLC (Gemini C18, 4.6 × 250 mm, 1 ml min⁻¹) with a 30-min gradient program of 50–100% MeOH in H₂O to obtain compounds **1** (5 mg) and **3** (7 mg), respectively. Crude compounds **2** and **4** were also purified by this method to furnish compounds **2** (5 mg) and **4** (4 mg), respectively.

Oxysporizoline (**1**): a colorless amorphous solid; [α]_D +23 (c 0.12, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 285 (3.2) (sh), 315 (3.3) nm; IR (KBr) $\nu_{\max}$ 3500 ~ 2600, 3425, 1728, 1611, 1581, 1498, 1376, 1276, 755 cm⁻¹; ¹H NMR (DMSO-*d*₆, 400 MHz) and ¹³C NMR (DMSO-*d*₆, 100 MHz) (see Table 1); LR-EI-MS: *m/z* 446 [M]⁺ (rel. int. 14), 355 (14), 310 [M-(anthranilic acid-H)]⁺ (91), 206 [310-C₇H₆N]⁺ (30), 180 [310-C₈H₆N₂]⁺ (65), 179 [310-C₈H₇N₂]⁺ (32), 151 (47), 137 [anthranilic acid]⁺ (19), 131 [C₈H₇N₂]⁺ (18), 97 [M-CO₂H-(C₆H₄ × 3)]⁺ (42), 83 [97-N]⁺ (62), 76 [C₆H₄]⁺ (11), 71 (100), 57 (79); HR-EI-MS: *m/z* 446.1740 [M]⁺ (calcd for C₂₈H₂₂N₄O₂, 446.1743), (–0.5 p.p.m./–0.2 m.m.u.).

Oxysporizoline (**1**) was isolated as a colorless amorphous solid, identified as C₂₈H₂₂N₄O₂ by HR-EI-MS and ¹³C NMR (Supplementary Figures S2 and S4). The IR spectrum of **1** showed bands characteristic of carboxylic acid (3500 ~ 2600, 1728, 1376 cm⁻¹), aromatic amine (3425, 1498, 755 cm⁻¹) and aromatic (1611, 1581 cm⁻¹) functionalities. The broad carboxylic acid and aromatic amine bands in the IR spectrum and the UV absorption at 285 (3.2) (sh) and 315 (3.3) nm indicate the presence of anthranilic acid and quinazoline moieties,⁵ respectively, in compound **1**. Detailed analysis of the ¹H and ¹³C NMR spectra of **1**, including two-dimensional-NMR (DEPT, COSY, HMQC, HMBC and NOESY) experiments, revealed diagnostic signals for *N*-substituted α -amino-benzoic acid, three 1,2-disubstituted benzenes, one 1,4-dihydro-2,3,4-trisubstituted quinazoline and two 2,4-dihydro-1,2,3,4-tetrasubstituted quinazolines (see Table 1, Figure 1 and Supplementary Figures S1 ~ S6). The fragment ions, *m/z* 76 [C₆H₄]⁺ and 97 [M-CO₂H-(C₆H₄ × 3)]⁺, in the fragmentation pattern of **1** also

indicated the presence of four 1,2-disubstituted benzenes and benzoic acid moieties (see Supplementary Figure S1). In the fragmentation pattern of **1**, the fragment ions m/z 310 $[M-(\text{anthranilic acid-H})]^+$ and m/z 137 $[\text{anthranilic acid}]^+$ were consistent with the *N*-substituted α -aminobenzoic acid moiety, and m/z 180 $[310-\text{C}_8\text{H}_6\text{N}_2]^+$, 179 $[310-\text{C}_8\text{H}_7\text{N}_2]^+$ and 131 $[\text{C}_8\text{H}_7\text{N}_2]^+$ were consistent with the

Table 1 NMR spectra data for oxysporizoline (**1**)^a

Oxysporizoline (1)			
Position	δ_H (mult, J)	δ_C (mult)	HMBC (H to C)
1		121.6 (s)	
2		148.5 (s)	
3	7.53 (d, 8.1)	113.4 (d)	C-1, C-5
4	7.33 (dd, 8.1, 7.0)	130.3 (d)	C-2, C-6
5	6.68 (dd, 7.5, 7.0)	115.6 (d)	C-1, C-3
6	7.98 (d, 7.5)	131.7 (d)	C-2, C-4, C-7
7		172.0 (s)	
8	10.45 (br s)		
1'	7.20 (d, 7.5)	129.4 (d)	C-3', C-4a', C-13'
2'	7.01 (m)	123.6 (d)	C-4', C-13a'
3'	7.24 (m)	123.0 (d)	
4'	7.25 (m)	123.9 (d)	C-2', C-4a', C-13a'
4a'		145.4 (s)	
5'-N			
6'	5.36 (d, 3.2)	69.0 (d)	C-4a', C-7a', C-11b', C-2'', C-6''
7'	7.03 (m)		C-6', C-8', C-11a', C-1''
7a'		142.1 (s)	
8'	6.59 (d, 7.5)	115.4 (d)	C-10', C-11a'
9'	6.94 (dd, 7.0, 7.0)	128.1 (d)	
10'	6.49 (dd, 7.5, 7.0)	117.3 (d)	C-8', C-11a'
11'	6.58 (d, 7.5)	128.8 (d)	C-7a', C-9', C-11a', C-11b'
11a'		121.2 (s)	
11b'	5.51 (s)	62.5 (d)	C-4a', C-6', C-7a', C-11', C-13', C-2''
12'-N			
13'	5.64 (d, 5.4)	73.9 (d)	C-2, C-1', C-4a', C-11b'
13a'		129.6 (s)	
1''		128.4 (s)	
2''		143.4 (s)	
3''	7.24 (m)	128.4 (d)	
4''	7.15 (m)	128.2 (d)	C-2'', C-6''
5''	6.92 (dd, 7.6, 7.0)	123.4 (d)	
6''	7.13 (m)	128.4 (d)	C-6', C-2'', C-4''

^aRecorded in DMSO- d_6 at 400 MHz (^1H) and 100 MHz (^{13}C).

Figure 1 Molecular structure of oxysporizoline (**1**), 1*H*-indol-3-butanamide (**2**), chlamydosporel (**3**) and butenolide (**4**).

1,4-dihydro-2,3,4-trisubstituted quinazoline and 2,4-dihydro-1,2,3,4-tetrasubstituted quinazolines groups (see Supplementary Figure S1).

The connections and positions of the functional groups in **1** were determined based on HMBC and NOESY spectral data (Table 1, and Supplementary Figures S5 and S6). The HMBC correlations from H-13' to C-2 showed the connection (NH8-C13') between anthranilic acid moiety and the condensed quinazoline moiety, which was also supported by the NOESY correlation between H-3 and H-13' (Supplementary Figure S6). The HMBC correlations (H-13' $\rightarrow$ C-1', C-4a' and C-11b'; H-11b' $\rightarrow$ C-4a', C-6', C-7a', C-11', C-13' and C-2'; H-6' $\rightarrow$ C-4a', C-7a', C-11b', C-2'' and C-6''; and NH-7' $\rightarrow$ C-8', C-11a' and C-1'') indicated the presence of a quinazoline bis-anhydrotrimer moiety, which was further supported by the chemical shift of the azaacetal protons at δ 5.36 (1H, d, J = 3.2 Hz, H-6'), 5.51 (1H, s, H-11b') and 5.64 (1H, d, J = 5.4 Hz, H-13')⁶ (Table 1 and Supplementary Figure S6). Thus, oxysporizoline (**1**) was characterized as an *N*-(7,11b-dihydro-13*H*-6,12-*o*-benzeno-6*H*-quinazolino[3,4-*a*]quinazolin-13-yl)anthranilic acid. The relative configurations at the asymmetric centers C-6', C-11b' and C-13' were assigned by molecular modeling based on the NOESY experiments. The NOESY correlations between H-3 and H-13', between H-6' and H-6'', and between H-11' and H-11b', and molecular modeling suggested that the quinazoline rings were half chair-half chair-half chair conformations (Supplementary Figure S6). The absence of a NOESY correlation between H-11b' and H-13' suggested that both protons favored α -pseudoequatorial and β -pseudoaxial conformations, respectively. Therefore, the relative configurations at C-6', C-11b' and C-13' in **1** were assigned as 6'S*, 11b'R* and 13'S*, respectively (Figure 1 and Supplementary Figure S6). The known alkaloid, 1*H*-indol-3-butanamide (**2**),² and two polyketides, chlamydosporel (**3**)³ and butenolide (**4**),⁴ were also isolated in this study, and were identified by comparing their NMR and MS spectra with literature data.

We examined compounds **1**–**4** for antibacterial activity against *S. aureus*, MRSA and MDRSA, and for radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) (see Supplementary p. 8).⁷ Compounds **1** and **3** displayed weak antibacterial activity against MRSA and MDRSA, with MICs of 6.25 $\mu\text{g ml}^{-1}$ and 31.5 $\mu\text{g ml}^{-1}$, respectively. Compounds **1** and **4** also exhibited a moderate radical scavenging activity against DPPH, with IC₅₀ values of 10 and 12 μM , respectively, and **1** and **4** were more active than the positive control, ascorbic acid (IC₅₀, 20 μM).

Polycyclic quinazoline alkaloid derivatives such as compound **1** have been reported as synthetic intermediates in the development of tribenzo[*b,f,j*][1,5,9]triazacyclodecine (TRI) complexes and tetrabenzo[*b,f,j,n*][1,5,9,13]tetraazacyclohexadecine (TAAB) complexes

containing various metals. These complexes have interesting biological activities, including nuclease and superoxide dismutase activities, and antineoplastic activity.⁶ Based on the biological activity of the TRI and TAAB complexes derived from the polycyclic quinazoline alkaloid reported above, compound **1** warrants further study for development of potential chemotherapeutic compounds against cancer and diseases related to nucleases and superoxide dismutase.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2014R1A1A2053912).

- 1 Yang, G. *et al.* Flavusides A and B, antibacterial cerebroside from the marine-derived fungus *Aspergillus flavus*. *Chem. Pharm. Bull.* **59**, 1174–1177 (2011).
- 2 Mewshaw, R. E. *et al.* Studies toward the discovery of the next generation of antidepressants. 3. Dual 5-HT_{1A} and serotonin transporter affinity within a class of *N*-aryloxyethylindolylalkylamines. *J. Med. Chem.* **47**, 3823–3842 (2004).
- 3 Nenkep, V. N. *et al.* New production of halogenated pyranopyranones, bromochlamydosporols A and B, from the marine-derived fungus *Fusarium tricinctum*. *J. Nat. Prod.* **73**, 2061–2063 (2010).
- 4 Cole, R. J. & Cox, R. H. (eds). *Handbook of Toxic Fungal Metabolites* 898–901, (Academic Press, New York, NY, USA, 1981).
- 5 Manske, R. H. F. & Holmes, H. L. (eds). *The Alkaloids—Chemistry and Pharmacology*. Vol. III, 101–111 (Academic Press, New York, NY, USA, 1953).
- 6 Xiao, X., Fanwick, P. E. & Cushman, M. Synthesis, crystal structure, and conversion of the polycyclic tris-anhydrotetramer of *o*-aminobenzaldehyde to Cu(TAAB)²⁺. *Synth. Commun.* **34**, 3901–3907 (2004).
- 7 Li, Y., Li, X. & Son, B. W. Antibacterial and radical scavenging epoxycyclohexenones and aromatic polyols from a marine isolate of the fungus *Aspergillus*. *Nat. Prod. Sci.* **11**, 136–138 (2005).

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