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NOTE

New diketopiperazine derivatives with cytotoxicity from *Nocardiopsis* sp. YIM M13066

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The diketopiperazines (DKPs)-containing natural products represent a large class of secondary metabolites mainly produced by bacteria, fungi, marine invertebrates and higher organisms. DKPs were biosynthesized by condensation of two amino acids including tryptophan, proline, histidine and phenylalanine.[1–3] Interest in DKPs is due to their activities in various pharmacological assays ranging from antibacterial, antifungal, antiviral to immunosuppressive.[4–7] In recent studies, the nocazines are a newly defined family of antibacterial and cytotoxic cyclic dipeptides produced by *Nocardiopsis dassonvillei* and *N. alba*.[8,9] Our investigation with a deep-sea sediment strain, YIM M13066, identified as a *Nocardiopsis* sp., was found to produce six DKPs, including two new DKPs, nocazines F (**1**) and G (**2**), and four known DKPs (**3–6**). In this study, the fermentation, isolation, and structural elucidation and bioactivity of compounds **1–6** (Figure 1) from *Nocardiopsis* sp. YIM M13066 were described.

The strain YIM M13066 was first cultured in Petri dishes with ca. 20 ml MP agar medium for 7 days to obtain seed cultures. The seeds were inoculated on MP agar media (20 l) by streaking plate method and cultured for 10 days at 28 °C. The fermented culture was extracted with EtOAc–MeOH (85:15, volume per volume, 20 l) at room temperature to obtain a crude extract. The crude extract was partitioned with equal volume EtOAc (0.5 l) and H_2O. The EtOAc portion was concentrated under vacuum to afford the EtOAc extract (7.8 g). The extract was subjected to column chromatography over Sephadex LH-20 (25–100 μm; GE Healthcare, Sweden; column dimensions: 200 × 20 mm) eluted with MeOH and pooled on the basis of TLC detection to obtain Fr.1–10. Fr.7 (1.1 g) was separated by MPLC (310 × 2.6 mm) over RP-18 silica gel (80 g) to afford Fr.7a–7h. Compounds **5** (30.0 mg) and **6** (40 mg) were obtained by crystallization from Fr.7d and Fr.7e, respectively. Fr.7f was purified by HPLC (SunFire Prep C_{18} OBD, 19 × 150 mm, 15 ml min^{-1}, UV 365 nm) eluted with 70% acetonitrile to afford **4** (t_R 11.7 min, 6.8 mg) and **3** (t_R 13.5 min, 7.1 mg). Fr.10 (108.0 mg) was separated by MPLC over RP-18 silica gel (80 g) to afford Fr.10a–10j. Fr.10e was purified by HPLC (SunFire Prep C_{18} OBD, 19 × 150 mm, 15 ml min^{-1}, UV 365 nm) eluted with 40% acetonitrile to yield **1** (t_R 7.1 min, 29.2 mg) and **2** (t_R 9.2 min, 20.1 mg), respectively.

Nocazine F (**1**) was obtained as a yellow amorphous powder with the molecular formula $C_{20}H_{18}N_2O_4$ according to its HRESI-MS at $m/z = 351.1297$ $[M+H]^+$ (calcd 351.1339). Its ^1H and ^{13}C NMR (in DMSO-d_6, Table 1) corresponded to 1 NH group (δ 9.86 (s)), 2 OCH_3 (δ_H/δ_C 3.98/54.8 and 3.79/55.7), 10 olefinic CH, 7 quaternary C-atoms and 1 amidocarbonyl (δ 160.2). The presence of two 1,4-disubstituted benzene rings was determined by two sets of coupled ^1H signals at δ 8.02 (*d*, $J = 8.7$ Hz), 6.83 (*d*, $J = 8.7$ Hz) and δ 7.50 (*d*, $J = 8.7$ Hz), 6.99 (*d*, $J = 8.8$ Hz). HMBC correlations from 18-OH (δ 9.91) to C-17/19 (δ 115.9) and C-18 (δ 158.8), and from 11-OCH_3 (δ 3.79) to C-11 (δ 159.5) revealed that the two 1,4-disubstituted benzene rings were connected with hydroxyl and methoxyl, respectively. HMBC correlations from 5-OCH_3 (δ 3.98) to C-5 (δ 154.6), from NH-1 (δ 9.86) to C-5 and C-3, from H-7 (δ 6.46) to C-5 and C-9/13 (δ 131.2), and from H-14 (δ 7.03) to C-2 (δ 160.2) and C-16/20 (δ 133.8) indicated a specific DKP unit formed from a *p*-hydroxyl-Phe (Tyr) and a *p*-MeO-Phe moieties, similar to nocazine A.[8] The *Z* configurations of both Δ^6 and $\Delta^{3(14)}$ double bonds were determined by the NOE correlations between 5-OCH_3 (δ 3.98) and H-20 (δ 8.02), NH-1 (δ 9.86), and H-13 (δ 7.50) (Figure 2). Finally, the structure of compound **1** was determined

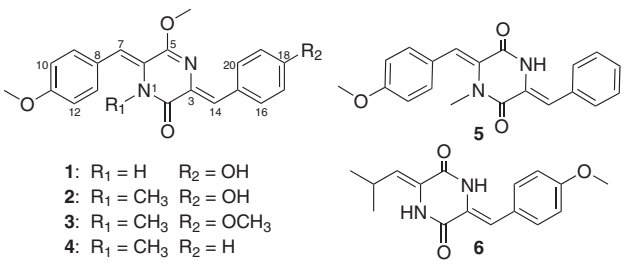

Figure 1 The chemical structures of compounds **1**–**6**.

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Table 1 The ^{1}H and ^{13}C NMR data for 1 and 2 (δ in p.p.m., J in Hz)

	1[a]		*2*[b]	
Position	δ_C	δ_H	δ_C	δ_H
1		9.86 (brs)		
1-NCH_3			36.3	3.00 (s)
2	160.2		163.0	
3	131.8		130.5	
5	154.6		156.1	
5-OCH_3	54.8	3.98 (s)	54.4	4.04 (s)
6	130.0		127.1	
7	110.9	6.46 (s)	114.9	6.80 (s)
8	122.9		126.8	
9/13	131.2	7.50 (*d*, 8.7)	130.9	7.19 (*d*, 8.6)
10/12	114.7	6.99 (*d*, 8.8)	113.7	6.90 (*d*, 8.8)
11	159.5		159.4	
11-OCH_3	55.7	3.79 (s)	55.3	3.83 (s)
14	126.7	7.03 (s)	127.4	7.22 (s)
15	126.2		128.4	
16/20	133.8	8.02 (*d*, 8.7)	133.4	8.05 (*d*, 8.7)
17/19	115.9	6.83 (*d*, 8.7)	115.4	6.87 (*d*, 8.8)
18	158.8		156.3	
18-OH		9.91 (s)		
18-OCH_3				

[a]Compound **1** in DMSO-d_6.
[b]Compound **2** in $CDCl_3$.

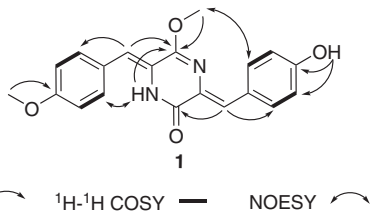

Figure 2 Selected HMBC, COSY and NOESY correlations of compound **1**.

Table 2 IC_{50} values (μM) of compounds 1 and 2 tested against human tumor cell lines

Compounds	*H1299*	*HeLa*	*HL7702*	*MCF-7*	*PC3*	*U251*
1	3.87	4.47	7.10	3.86	8.17	22.5
2	2.60	3.97	8.73	6.67	16.7	>40
VP-16	16.1	7.69	5.85	5.75	1.69	6.04

as 3-((*Z*)-4-hydroxybenzylidene)-5-methoxy-6-((*Z*)-4-methoxybenzylidene)-3,6-dihydropyrazin-2(1H)-1.

Nocazine G (**2**) was obtained as a yellow amorphous powder. Its molecular formula was determined as $C_{21}H_{20}N_2O_4$ according to its HRESI-MS at $m/z = 365.1503$ $[M+H]^+$ (calcd 365.1496). The NMR spectra of **2** were very similar to those of nocazine A.[8] The difference is that the 18-OCH_3 in nocazine A was replaced by 18-OH in **2**. The *Z* configurations of both Δ^6 and $\Delta^{3(14)}$ double bonds were determined by the NOE correlations between 5-OCH_3 (δ 4.04) and H-20 (δ 8.05), 1-N-CH_3 (δ 3.00), and H-13 (δ 7.19). Furthermore, the *Z* configurations of the two double bonds in **2** also can be deduced from the similarity of the chemical shifts of H-7 (δ 6.80) and H-14 (δ 7.22) to those of nocazine A and B[8] (in $CDCl_3$). Therefore, compound **2** was determined as 3-((*Z*)-4-hydroxybenzylidene)-5-methoxy-6-((*Z*)-4-methoxybenzylidene)-1-methyl-3,6-dihydropyrazin-2(1H)-1.

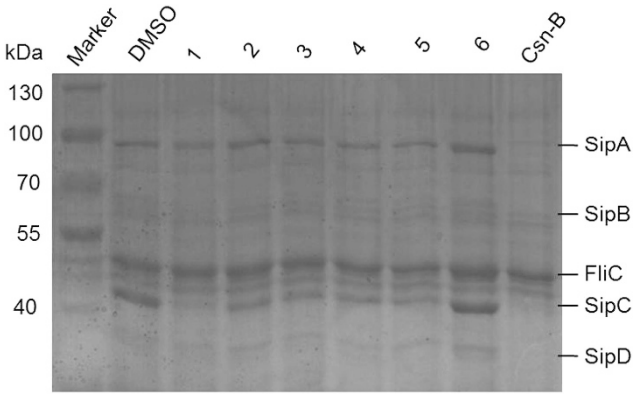

Figure 3 The effects of compounds **1–6** on secretion of SPI-1 effector proteins. Csn-B, cytosporone B; FliC, flagellar filament protein; SipA/B/C/D, SPI-1 effector proteins. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

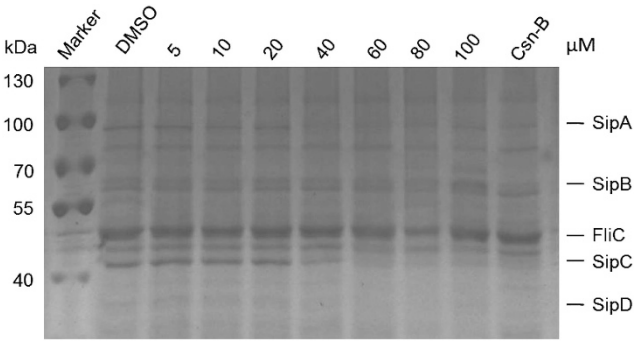

Figure 4 The effect of compound **1** on secretion of SPI-1 effector SipC in a dose-dependent manner. Csn-B, cytosporone B; FliC, flagellar filament protein; SipA/B/C/D, SPI-1 effector proteins. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Compounds **3–6** were found to be nocazine A (**3**),[8] nocazine B (**4**),[8] XR 330 (**5**)[9,10] and (3*Z*,6*Z*)-3-(4-methoxybenzylidene)-6-(2-methylpropylidene)-piperazine-2,5-dione (**6**),[9,11] respectively, by comparing their NMR and MS data with those previously reported.

The new compounds (**1** and **2**) were evaluated for *in vitro* cytotoxic activities against human cancer cell lines H1299, HeLa, HL7702, MCF-7, PC3 and U251 using the sulforhodamine B method,[12] and VP-16 was used as a positive control, and for antibacterial activities against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6051, *Pseudomonas aeruginosa* PA01 and *Salmonella enterica* serovar Typhimurium UK-1 χ8956. Compounds **1** and **2** showed broad spectrum and excellent cytotoxicity to all tested cell lines (Table 2). As for antimicrobial activity, only **2** showed moderate activities against *B. subtilis* ATCC 6051 with an MIC of 25.8 μM. Meanwhile, compounds **1–6** were further assayed for their activities of inhibiting the type three secretion system (T3SS) of *S. enterica* serovar Typhimurium UK-1 χ8956 using cytosporone B as the positive control, as described.[13] Compounds **1–6** exhibited inhibitory activity against the secretion of SPI-1 effector SipA/B/C/D and no effects on FliC at the concentration of 100 μM (Figure 3). Among them, compound **1** exhibited a strong inhibition on the secretion of the SPI-1 effector SipC in a dose-dependent manner (Figure 4).

In summary, the chemical study on strain YIM M13066 led to the isolation and characterization of six DKPs (**1–6**). Compared with nocazine A-D,[8,9] XR330 and 334,[10] nocazines F (**1**) and G (**2**) showed a broad spectrum of cytotoxicity against the human tumor cell lines, which suggested that the *O*-methyl group at C-5, 18-OH group and the $\Delta^{6(7)}$ double bond may be the key active groups of nocazines. Besides, nocazine F exhibited a strong inhibition on the T3SS of *S. enterica* serovar Typhimurium, which could be a promising candidate for a novel type of antibiotics.

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)