

Communication

## Diketopiperazine Derivatives from the Marine-Derived Actinomycete *Streptomyces* sp. FXJ7.328

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**Abstract:** Five new diketopiperazine derivatives, (3Z,6E)-1-N-methyl-3-benzylidene-6-(2S-methyl-3-hydroxypropylidene)piperazine-2,5-dione (**1**), (3Z,6E)-1-N-methyl-3-benzylidene-6-(2R-methyl-3-hydroxypropylidene)piperazine-2,5-dione (**2**), (3Z,6Z)-3-(4-hydroxybenzylidene)-6-isobutylidenepiperazine-2,5-dione (**3**), (3Z,6Z)-3-((1H-imidazol-5-yl)-methylene)-6-isobutylidenepiperazine-2,5-dione (**4**), and (3Z,6S)-3-benzylidene-6-(2S-but-2-yl)piperazine-2,5-dione (**5**), were isolated from the marine-derived actinomycete *Streptomyces* sp. FXJ7.328. The structures of **1–5** were determined by spectroscopic analysis, CD exciton chirality, the modified Mosher's, Marfey's and the C<sub>3</sub> Marfey's methods. Compound **3** showed modest antiviral activity against influenza A (H1N1) virus with an IC<sub>50</sub> value of 41.5 ± 4.5 μM. In addition, compound **6** and **7** displayed potent anti-H1N1 activity with IC<sub>50</sub> value of 28.9 ± 2.2 and 6.8 ± 1.5 μM, respectively. Due to the lack of corresponding data in the literature, the <sup>13</sup>C NMR data of (3Z,6S)-3-benzylidene-6-isobutylpiperazine-2,5-dione (**6**) were also reported here for the first time.

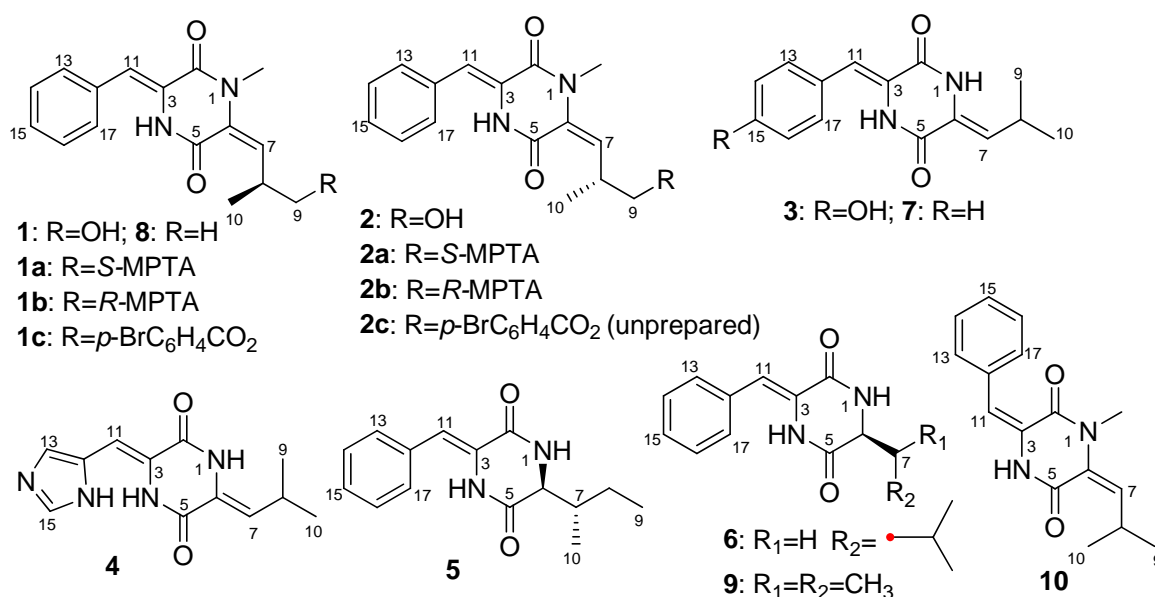
**Keywords:** *Streptomyces*; diketopiperazine derivatives; antiviral activity; H1N1

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## 1. Introduction

Marine actinomycetes are a rich source of bioactive compounds with new structures [1], e.g., the cytotoxic thiocoraline from *Micromonospora* sp. L-13-ACM2-092 [2], and the anti-inflammatory cyclomarins A–C from *Streptomyces* sp. CNB-382 [3]. As one of the most important classes of bioactive compounds, alkaloids have received much attention. As part of our ongoing research on bioactive alkaloids with new structures from marine-derived actinomycetes [4–7], *Streptomyces* sp. FXJ7.328 was isolated from marine sediment, and was found to produce alkaloids by TLC visualizing with Dragendorff's reagent in a saline culture. The ethyl acetate extract of fermentation broth exhibited antiviral activity against H1N1 influenza virus at 100 µg/mL and displayed a series of peaks with UV absorptions at 220 and 340 nm similar to those of diketopiperazine derivatives such as albonoursin and (3*Z*,6*S*)-3-benzylidene-6-isobutylpiperazine-2,5-dione by HPLC-UV analysis [8]. Chemical investigation of the extract resulted in the isolation and identification of five new diketopiperazine derivatives (Figure 1), namely (3*Z*,6*E*)-1-*N*-methyl-3-benzylidene-6-(2*S*-methyl-3-hydroxypropylidene)-piperazine-2,5-dione (**1**), (3*Z*,6*E*)-1-*N*-methyl-3-benzylidene-6-(2*R*-methyl-3-hydroxypropylidene)-piperazine-2,5-dione (**2**), (3*Z*,6*Z*)-3-(4-hydroxybenzylidene)-6-isobutylidenepiperazine-2,5-dione (**3**), (3*Z*,6*Z*)-3-((1*H*-imidazol-5-yl)methylene)-6-isobutylidenepiperazine-2,5-dione (**4**), and (3*Z*,6*S*)-3-benzylidene-6-(2*S*-but-2-yl)piperazine-2,5-dione (**5**). In addition, five known analogues were also isolated and their structures were identified as (3*Z*,6*S*)-3-benzylidene-6-isobutylpiperazine-2,5-dione (**6**) [8], albonoursin (**7**) [8–10], (3*Z*,6*E*)-1-*N*-methyl-3-benzylidene-6-isobutylidenepiperazine-2,5-dione (**8**) [9,11], (3*Z*,6*S*)-3-benzylidene-6-isopropylpiperazine-2,5-dione (**9**) [12], and (3*E*,6*E*)-1-*N*-methyl-3-benzylidene-6-isobutylidenepiperazine-2,5-dione (**10**) [13], respectively, by comparing their NMR data and specific rotation (Supplementary Information) with those reported in the literatures. Compounds **3**, **6** and **7** displayed activity against influenza A (H1N1) virus with the IC<sub>50</sub> values of 41.5 ± 4.5, 28.9 ± 2.2 and 6.8 ± 1.5 µM, respectively.

**Figure 1.** Chemical structures of compounds **1–10** from *Streptomyces* sp. FXJ7.328.



## 2. Results and Discussion

### 2.1. Structure Elucidation

The ethyl acetate extract of the fermentation broth of *Streptomyces* sp. FXJ7.328 was subjected to extensive chromatographic separations over silica gel, Sephadex LH-20 and by HPLC to yield the new compounds **1–5** and the known analogues **6–10**.

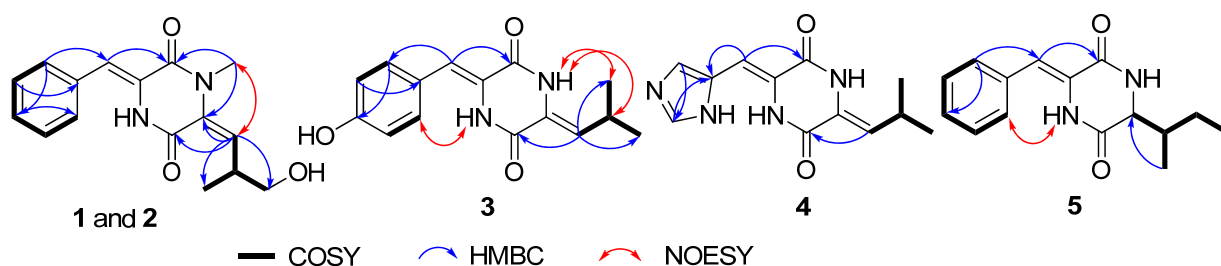
Compounds **1** and **2** were at first isolated as a racemic mixture with an excess of one enantiomer and thought to be a pure compound according to their identical NMR data and a small negative specific rotation ( $[\alpha]_D -5.8$ ). When (*R*)-Mosher's acyl chloride was used to determine the absolute configuration, the Mosher's ester was found to be a separable mixture according to  $^1\text{H}$  NMR spectrum and HPLC profile. A chiral HPLC column was used to separate the enantiomeric mixture of **1** and **2**, which yielded compounds **1** and **2** in an approximate ratio of 2:1 (Table S37). The planar structures of **1** and **2** were established based on MS and NMR data of the racemic mixture. HRESIMS gave an ion peak at  $m/z$  287.1387  $[\text{M} + \text{H}]^+$  (calcd. for  $\text{C}_{16}\text{H}_{19}\text{N}_2\text{O}_3$  287.1390), corresponding to the molecular formula  $\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_3$ . Analysis of the 1D NMR data (Table 1) revealed one methyl ( $\delta_{\text{H/C}}$  1.01/17.9), one *N*-methyl ( $\delta_{\text{H/C}}$  3.17/31.3), one  $\text{sp}^3$  oxygenated methylene ( $\delta_{\text{H/C}}$  3.36/66.7), seven  $\text{sp}^2$  methines, one  $\text{sp}^3$  methine ( $\delta_{\text{H/C}}$  3.59/34.9), and two signals ( $\delta_{\text{C}}$  158.5, 158.9) interpreted as conjugated amide carbonyls.  $^1\text{H}$  NMR signals at  $\delta_{\text{H}}$  7.52 (2H, d,  $J = 7.7$ ), 7.40 (2H, t,  $J = 7.7$ ) and 7.31 (1H, t,  $J = 7.2$ ) revealed the presence of a monosubstituted benzene nucleus that was further identified as a dehydro-phenylalanine (deh-Phe) unit from the key HMBC correlations of H-13/17 ( $\delta_{\text{H}}$  7.52) to C-11 ( $\delta_{\text{C}}$  116.1) and C-15 ( $\delta_{\text{C}}$  128.7), and of H-11 ( $\delta_{\text{H}}$  6.75) to C-13 ( $\delta_{\text{C}}$  129.9) and C-2 ( $\delta_{\text{C}}$  158.5).  $^1\text{H}$ - $^1\text{H}$  COSY correlations of H-7/H-8/H-9 and H-8/H-10 combined with the key HMBC correlations from H-7 ( $\delta_{\text{H}}$  5.56) to C-5 ( $\delta_{\text{C}}$  158.9) and C-6 ( $\delta_{\text{C}}$  130.4), and from N-CH<sub>3</sub> ( $\delta_{\text{H}}$  3.17) to C-6 ( $\delta_{\text{C}}$  130.4) suggested another amino acid unit, *N*-methyl dehydro-5-hydroxyleucine. The key HMBC correlation of N-CH<sub>3</sub> to C-2 finally established the conjugate diketopiperazine (DKP) structure of **1** and **2** (Figure 2). The NOE correlation from N-CH<sub>3</sub> to H-7 indicated the *E*-configuration of the  $\Delta^6$  double bond. The *Z*-configuration of  $\Delta^{3,11}$  double bond could be deduced from the relative downfield shift of H-11 because of the deshielding effect of the 2-carbonyl group, as e.g.,  $\delta_{\text{H-11}}$  6.85, 6.40 and 6.74 for (3*Z*,6*E*)-1-*N*-methyl-3-benzylidene-6-isobutylidenepiperazine-2,5-dione [11], (3*E*,6*E*)-1-*N*-methyl-3-benzylidene-6-isobutylidenepiperazine-2,5-dione [13], and (3*Z*,6*Z*)-3-benzylidene-6-isobutylidene piperazine-2,5-dione [10], respectively. The *S*-configuration of **1** was determined by analysis of exciton chirality CD of its *p*-bromobenzoate (**1c**) [14]. The stable conformers of the *p*-bromobenzoates of **1c** and its enantiomer **2c** (unprepared) were obtained by HyperChem Release 7.5 software [4] (Figure 3). The transition dipole orientations of two chromophores, the conjugated DKP core and the *p*-bromobenzoate in **1c** and **2c**, were oriented in counterclockwise and clockwise manners, which should result in negative and positive Cotton effects at long wavelength, respectively. The measured negative CD effect of **1c** at  $\lambda_{\text{ext}}$  313 nm ( $\Delta\epsilon -5.2$ ) indicates the *S*-configuration of **1c** (Figure 3). This deduction was further validated by modified Mosher's method for primary alcohols [15,16]. When **1** reacted with *R*- and *S*-MTPA chloride, the *S*- (**1a**) and *R*-MTPA esters (**1b**) were obtained, respectively. The chemical shift difference between two methylene protons of C-9 in *S*-MTPA ester **1a** is larger than that in *R*-MTPA ester **1b** ( $\Delta\delta$  0.08 vs. 0.01), indicating *S*-configuration of C-8 in **1**. Thus,

the structure of **1** was clearly elucidated as (3*Z*,6*E*)-1-*N*-methyl-3-benzylidene-6-(2*S*-methyl-3-hydroxypropylidene)-piperazine-2,5-dione. Compound **2** showed the opposite specific rotation and the opposite chemical shift difference between the two methylene protons of C-9 in *S*- and *R*-MTPA esters (**2a** and **2b**), indicating *R*-configuration of **2**. So compound **2** was identified as (3*Z*,6*E*)-1-*N*-methyl-3-benzylidene-6-(2*R*-methyl-3-hydroxypropylidene)piperazine-2,5-dione.

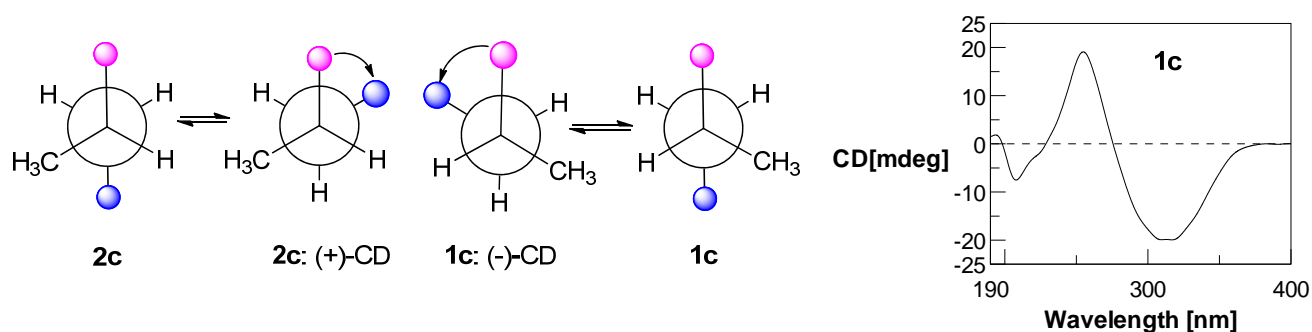
**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR Data for **1–5** (600 and 150 MHz, DMSO- $d_6$ ,  $\delta$  values).

Position	1 and 2		3		4		5	
	$\delta_{\text{C}}$ , type	$\delta_{\text{H}}$ , mult. (J in Hz)	$\delta_{\text{C}}$ , type	$\delta_{\text{H}}$ , mult. (J in Hz)	$\delta_{\text{C}}$ , type	$\delta_{\text{H}}$ , mult. (J in Hz)	$\delta_{\text{C}}$ , type	$\delta_{\text{H}}$ , mult. (J in Hz)
1	31.3, NCH <sub>3</sub>	3.17, s		10.24, s				8.47, s
2	158.5, qC		158.0, qC		157.8, qC		161.0, qC	
3	126.5, qC		125.3, qC		125.2, qC		127.3, qC	
4				9.82, s				9.93, s
5	158.9, qC		157.4, qC		156.8, qC		166.8, qC	
6	130.4, qC		125.2, qC		125.9, qC		60.2, CH	3.86, t, (3.3)
7	129.7, CH	5.56, d, (9.9)	125.1, CH	5.66, d, (10.4)	125.5, CH	5.68, d, (9.9)	40.9, CH	1.80, m
8	34.9, CH	3.59, m	23.9, CH	2.93, m	24.4, CH	2.95, m	24.8, CH <sub>2</sub>	1.46, m; 1.18, m
9	66.7, CH <sub>2</sub>	3.36	22.2, CH <sub>3</sub>	0.96, d, (6.5)	22.8, CH <sub>3</sub>	0.97, d, (6.6)	15.3, CH <sub>3</sub>	0.91, d, (7.1)
10	17.9, CH <sub>3</sub>	1.01, d, (7.1)	22.2, CH <sub>3</sub>	0.96, d, (6.5)	22.8, CH <sub>3</sub>	0.97, d, (6.6)	12.1, CH <sub>3</sub>	0.86, t, (7.7)
11	116.1, CH	6.75, s	115.0, CH	6.66, s	105.1, CH	6.60, s	114.6, CH	6.66, s
12	133.9, qC		123.9, CH		137.0, qC		133.9, qC	
13/17	129.9, CH	7.52, d, (7.7)	130.9, CH	7.36, d, (8.5)	119.8, CH	7.52, s	129.3, CH	7.45, d, (7.7)
14/16	129.2, CH	7.40, t, (7.7)	115.6, CH	6.79, d, (8.5)			129.7, CH	7.39, t, (7.7)
15	128.7, CH	7.31, t, (7.2)	157.5, qC		137.1, CH	7.94, s	128.5, CH	7.29, t, (7.7)

**Figure 2.** Selected 2D NMR correlations for **1–5**.



**Figure 3.** The stable conformers of **1c** and **2c** and the measured CD curve for *p*-bromobenzoate **1c**.



The molecular formula of compound **3** was determined to be  $C_{15}H_{16}N_2O_3$  based on HRESIMS with a peak at  $m/z$  273.1231  $[M + H]^+$  (calcd. for  $C_{15}H_{17}N_2O_3$  273.1234). The similarity of the 1D NMR spectra to those of albonoursin (**7**) [8,9] indicated a conjugated DKP as well. The difference of  $^1H$  and  $^{13}C$  NMR data between **3** and **7** pointed to a *p*-hydroxy substituted phenyl system in **3** instead of the benzene ring in **7**, which explains the obvious upfield shifts of H-13/17 ( $\delta_H$  7.36), H-14/16 ( $\delta_H$  6.79) and C-14/16 ( $\delta_C$  115.6) by the electron-donor effect of the hydroxy group.  $^1H$ - $^1H$  COSY correlations between H-13/17 and H-14/16 and the key HMBC correlations from H-11 ( $\delta_H$  6.66) to C-13/17 ( $\delta_C$  130.9) and C-2 ( $\delta_C$  158.0), from H-13/17 to C-15 ( $\delta_C$  157.5), and from H-14/16 to C-12 ( $\delta_C$  123.9) further supported the existence of a dehydro-tyrosine (deh-Tyr) unit. The  $^1H$ - $^1H$  COSY correlations from H-7 ( $\delta_H$  5.66) to H-9/10 ( $\delta_H$  0.96) through H-8 ( $\delta_H$  2.93) along with the HMBC correlations from H-7 to C-5 ( $\delta_C$  157.4) supported the existence of a dehydro-leucine (deh-Leu) unit. The NOE correlation between H-4 ( $\delta_H$  9.82) and H-13/17 indicated the *Z*-configuration of the  $\Delta^{3,11}$  double bond. The NOE correlations of H-1 ( $\delta_H$  10.24) to H-8 and H-9/10 combined with the relative downfield shift of H-7 revealed *Z*-configuration of the  $\Delta^6$  double bond. Therefore, compound **3** was determined to be (3*Z*,6*Z*)-3-(4-hydroxybenzylidene)-6-isobutylidenepiperazine-2,5-dione.

Compound **4** was found to have the molecular formula of  $C_{12}H_{14}N_4O_2$  from the HRESIMS peak at  $m/z$  247.1189  $[M + H]^+$  (calcd. for  $C_{12}H_{15}N_4O_2$  247.1190). 1D NMR (Table 1) and 2D NMR (Figure 2) data disclosed the same *Z*-deh-Leu unit as in compound **3**. The remainder  $C_6H_5N_3O$  displayed three  $sp^2$  methine signals at  $\delta_{H/C}$  6.60/105.1, 7.94/137.1 and 7.52/119.8, two  $sp^2$  quaternary carbon signals at  $\delta_C$  125.2 and 125.9, and one amide carbonyl signal at  $\delta_C$  157.8. The HMBC correlations of H-15 ( $\delta_H$  7.94) to C-13 ( $\delta_C$  119.8) and C-12 ( $\delta_C$  137.0), of H-13 ( $\delta_H$  7.52) to C-15 ( $\delta_C$  137.1), and of H-11 ( $\delta_H$  6.60) to C-2 ( $\delta_C$  157.8) and C-12 suggested a dehydro-histidine (deh-His) unit. The *Z*-configurations of both  $\Delta^{3,11}$  and  $\Delta^6$  double bonds were deduced from the relative downfield shifts of H-11 and H-7 consistent with those of (3*Z*,6*R*)-3-((1*H*-imidazol-5-yl)methylene)-6-isopropylpiperazine-2,5-dione ( $\delta_H$  6.51) [17] and compound **3**, respectively. Compound **4** was therefore elucidated as (3*Z*,6*Z*)-3-((1*H*-imidazol-5-yl)methylene)-6-isobutylidenepiperazine-2,5-dione.

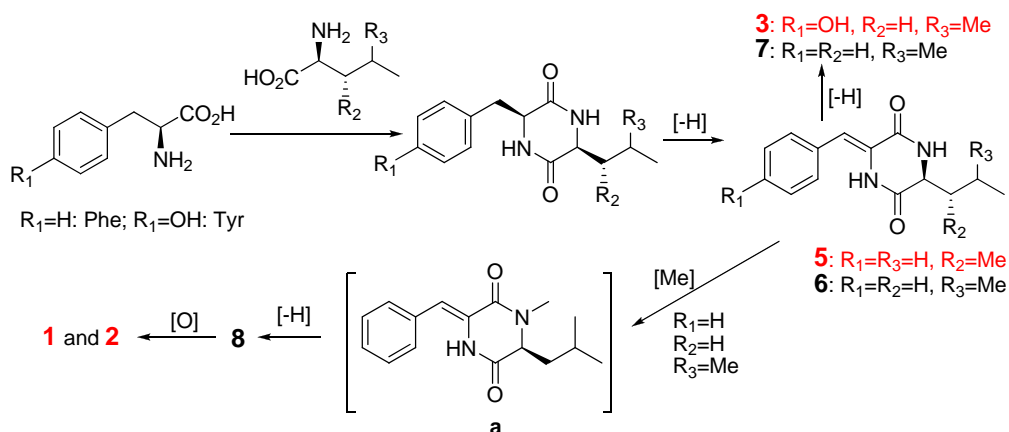
The molecular formula of  $C_{15}H_{18}N_2O_2$  was assigned to **5** according to the HRESIMS peak at  $m/z$  259.1439  $[M + H]^+$  (calcd. for  $C_{15}H_{19}N_2O_2$  259.1441), indicating an isomer of **6**. The 1D NMR (Table 1) spectra were very similar to those of **6** [8] except for the leucine moiety signals, suggesting that a deh-Phe unit was also presented in the structure of **5**. The NOE correlation from H-4 to H-13/17 and the relative downfield shift of H-11 accounted for the *Z*-configuration of the  $\Delta^{3,11}$  double bond [8]. The main differences of the  $^1H$  NMR spectra are a methyl triplet in **5** replacing the methyl doublet in **6**; further, a distinct split methylene signal in **5** substitutes the overlapped methylene proton signal in **6**. These observations combined with the separate downfield and upfield shifts of methine and methylene carbon signals revealed the existence of an isoleucine moiety in **5**, which was further confirmed by the  $^1H$ - $^1H$  COSY correlations of H-1/H-6/H-7/H-8/H-9 and H-7/H-10 (Figure 2) and the key HMBC correlation of H-10 to C-6. The absolute configuration of the isoleucine moiety was determined by Marfey's method [18] combined with  $C_3$  Marfey's method [19,20]. The 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) derivatives of the acid hydrolysates of **5** and four authentic isoleucine samples (L-, L-*allo*-, D- and D-*allo*-) were prepared. HPLC analysis over ODS column (Figure S38) revealed that acid hydrolysates of **5** displayed the same retention time ( $t_R$  23.06 min) as the authentic L-Ile ( $t_R$  23.06 min) and L-*allo*-Ile ( $t_R$  23.06 min) but were different from D-Ile ( $t_R$  28.11 min) and

D-*allo*-Ile ( $t_R$  28.11 min). The FDAA derivatives of the acid hydrolysates of **5** and the authentic L-Ile and L-*allo*-Ile were further analyzed by C<sub>3</sub> HPLC column (Figure S39). The retention time of the acid hydrolysates of **5** was the same as for the authentic L-Ile ( $t_R$  38.50 min), but different from the authentic L-*allo*-Ile ( $t_R$  37.49 min). Thus, the isoleucine moiety in **5** was unambiguously identified as L-Ile, and the structure of compound **5** was elucidated as (3*Z*,6*S*)-3-benzylidene-6-(2*S*-but-2-yl) piperazine-2,5-dione.

## 2.2. The Postulated Biosynthesis Pathway of Compounds 1–10

Compounds **1–10** were postulated to be produced biogenetically from the amino acid pathway (Figure 4). Cyclic condensation between Phe and Leu formed *cyclo*(Phe-Leu) that underwent dehydrogenation in Phe moiety to form compounds **6** and **7**. The *N*-methylation of **6** produced an un-isolated intermediate (**a**) that further underwent another dehydrogenation in the Leu moiety to form compound **8**. The oxidation of **8** at the same homoallylic positions produced compounds **1** and **2**. By a similar biosynthetic pathway, compounds **3–5** and **9** were produced from the cyclic condensation and successive dehydration of Tyr with Leu, His with Leu, Phe with Ile, and Phe with Val, respectively. The dehydrogenation is favored to form *Z*-products because of the steric hindrance of the carbonyl oxygen, while the dehydrogenation of Leu moiety in the *N*-Me substituted compounds only produced the *E*-products due to the strong steric hindrance by *N*-Me. So, compound **10** could be an artifact produced from the photo-isomerization of compound **8** under light during the extraction and the subsequent isolation steps.

**Figure 4.** The postulated biosynthetic pathway of **1–3** and **5–8**.

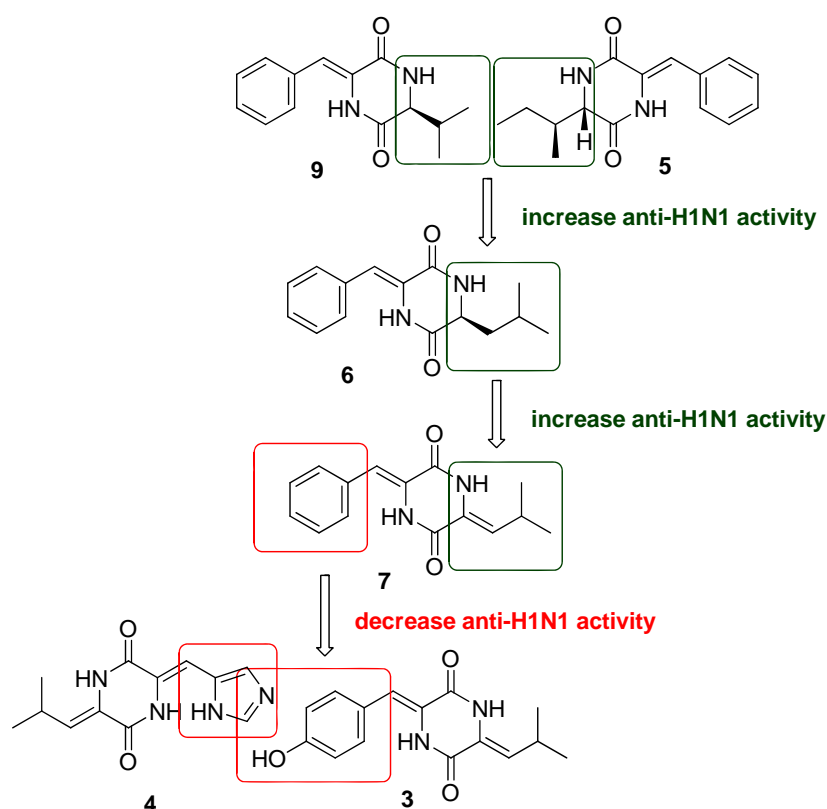


## 2.3. The Bioactivities of Compounds 1–10 from *Streptomyces* sp. FXJ7.328

Compounds **1** and **3–10** were tested for antiviral effects on H1N1 by the CPE inhibition assay [21,22], separately. The IC<sub>50</sub> values of **1**, **3–10** and ribavirin (positive control) were  $75.5 \pm 2.2$ ,  $41.5 \pm 4.5$ ,  $62.6 \pm 3.9$ ,  $106.5 \pm 4.2$ ,  $28.9 \pm 2.2$ ,  $6.8 \pm 1.5$ ,  $94.5 \pm 3.0$ ,  $113.8 \pm 4.9$ ,  $156.6 \pm 4.0$ , and  $38.8 \pm 1.5$   $\mu$ M, respectively. Except for compounds **3**, **6** and **7**, the other compounds were inactive (IC<sub>50</sub> > 50  $\mu$ M) against H1N1 influenza virus, indicating that both (*Z*)-deh-Phe and Leu or (*Z*)-deh-Leu moieties are necessary for anti-H1N1 activity (Figure 5). The dehydrogenation of Leu increases the activity, while the hydroxylation of deh-Phe or deh-Leu, the inversion of double bonds in deh-Phe and deh-Leu, and

the  $N_1$ -methylation reduce the activity. In addition, the new compounds **1–5** were tested for cytotoxicity against HL-60 and K562 cell lines by MTT method [23], and A549 cell lines by SRB method [24] and for anti-inflammatory effects by inhibition of LPS-mediated NF- $\kappa$ B transcription activity in RAW264.7 cells [25]. The antimicrobial activities of compounds **1–10** against *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Candida albicans* were also evaluated by 2-fold dilution method [26]. The results (Table S1, Supplementary Information) showed that new compounds **1–5** did not exhibit cytotoxicity ( $IC_{50} > 100 \mu\text{M}$ ) and anti-inflammatory effects ( $IC_{50} > 10 \mu\text{M}$ ); compounds **1–10** did not show antimicrobial effects as well ( $MIC > 100 \mu\text{g/mL}$ ). Although dehydro-DKPs have been reported to display diverse bioactivities, such as inhibition of protein tyrosine kinase [27], cell cycle arrest [28], inhibition of blood platelet aggregation [29], anti-bacteria [30], antitumor [30] and anti-inflammation [31], the antiviral effect on H1N1 was reported here for the first time.

**Figure 5.** The structure-activity relationship (SAR) of compounds **3–7** and **9** for anti-H1N1 viral activity.



### 3. Experimental Section

#### 3.1. General Experimental Procedures

Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were measured on a Beckman DU 640 spectrophotometer. IR spectra were recorded on a Nicolet Nexus 470 spectrophotometer as KBr disks. CD spectra were collected using a JASCO J-715 spectropolarimeter. NMR data of **1**, **2** and **4–6** were measured on a JEOL JNM-ECP 600 spectrometer, and chemical shifts

were recorded as  $\delta$  values. NMR spectra of **1a**, **1b**, **1c**, **2a**, **2b**, **3**, and NOESY spectra of all the compounds were recorded on a Bruker Avance 600 spectrometer. HRESIMS measurements were taken on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column (YMC-pak ODS-A, Kyoto, Japan, 10  $\times$  250 mm, 5  $\mu$ m, 4.0 mL/min) and chiral separation was performed on chiral column (CHIRALPAK AY-H, Kyoto, Japan, 4.6  $\times$  150 mm, 0.5 mL/min) by HPLC. Marfey's analysis and C<sub>3</sub> Marfey's analysis were implemented using ODS column (YMC-pak ODS-A, 4.6  $\times$  250 mm, 5  $\mu$ m, 1.0 mL/min) and C<sub>3</sub> column (Agilent Zorbax StableBond C<sub>3</sub>, Palo Alto, CA, USA, 4.6  $\times$  150 mm, 5  $\mu$ m, 1.0 mL/min), respectively. TLC and column chromatography (CC, 2.5  $\times$  103 cm) were performed on plates precoated with silica gel GF<sub>254</sub> (10–40  $\mu$ m, Qingdao Marine Chemical Factory, Qingdao, China), and over Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden), respectively. Vacuum-liquid chromatography (VLC, 7  $\times$  40 cm) utilized silica gel (200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, China) and RP-18 (40–63  $\mu$ m, Merck, Darmstadt, Germany). Sea salt used was made by the evaporation of seawater collected in Laizhou Bay (Weifang Haisheng Chemical Factory, Shangdong, China). Glucose (Shanghai Huixing Biochemical Reagent Co., Ltd., Shanghai, China); beef extract, yeast extract and peptone (Beijing Shuangxuan Microbe Culture Medium Products Factory, Beijing, China); soluble starch (Beijing Aoboxing Universeen Bio-Tech Co., Ltd., Beijing, China); K<sub>2</sub>HPO<sub>4</sub> (Tianjin Kermel Chemical Reagent Co., Ltd., Tianjin, China); MgSO<sub>4</sub> (Shanghai Chemical Reagent Research Institute, Shanghai, China), and CaCO<sub>3</sub> (Tianjijn Bodi Chemical Co., Ltd., Tianjin, China).

### 3.2. Actinomycete Material

The actinomycete strain *Streptomyces* sp. FXJ7.328 was isolated from coastal sediment collected at Huanghai beach (E 121.706°, N 39.007°), Dalian, China in January 2009. The sediment sample was dried at room temperature, suspended in sterile distilled water, serially diluted, heated in a water bath at 55 °C for 10 min, and spread-plated on oatmeal agar plates (ISP3, medium 3 of the International *Streptomyces* Project) [32]. After four weeks of incubation at 28 °C, the strain was purified on yeast extract-malt extract agar (ISP2, medium 2 of the International *Streptomyces* Project) [32], and was identified as a member of the genus *Streptomyces* on the basis of 16S rRNA gene sequence analysis. Genomic DNA isolation, PCR amplification of 16S rRNA gene and sequence alignment of the strain were performed as described previously [33]. Its 16S rRNA gene sequence (GenBank access No. JF346514) showed 99% similarity with type strains of *Streptomyces albus* subsp. *albus* (AB184781), *Streptomyces almquistii* (AB184258), *Streptomyces flocculus* (DQ442498), *Streptomyces gibsonii* (NR\_041180) and *Streptomyces rangoonensis* (NR\_041110). The producing strain was prepared on ISP3 medium and stored in Huang's Lab at 4 °C.

### 3.3. Fermentation and Extraction

The spores of *Streptomyces* sp. FXJ7.328 were directly cultured in 500 mL Erlenmeyer flasks containing 150 mL fermentation media consisted of 2% glucose, 0.3% beef extract, 1% yeast extract, 1% soluble starch, 1% peptone, 0.05% K<sub>2</sub>HPO<sub>4</sub>, 0.05% MgSO<sub>4</sub>, 0.2% CaCO<sub>3</sub>, and 3.3% sea salt (pH 7.0). The cultures were incubated on a rotatory shaker at 180 rpm at 28 °C for eight days. The whole fermentation broth (120 L) was divided into three equal parts that were extracted three times



with equal volumes of EtOAc separately. The EtOAc solutions were combined and evaporated under reduced pressure to give a dark brown gum (32.5 g).

### 3.4. Purification and Identification

The EtOAc extract (32.5 g) was subjected to SiO<sub>2</sub> VLC eluting with CH<sub>2</sub>Cl<sub>2</sub>-petroleum ether (0%–100%), and then with MeOH-CH<sub>2</sub>Cl<sub>2</sub> (0%–50%), to give nine fractions (Fr.1–Fr.9). Fraction 3 (3.82 g) was separated into three subfractions by gel filtration over Sephadex LH-20 with CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub> (1:1). Fraction 3-2 (442 mg) was further subjected to HPLC separation eluting with 70% MeOH to yield **7** (5.5 mg,  $t_R = 7.20$  min, 0.046 mg/L), **8** (20 mg,  $t_R = 11.5$  min, 0.17 mg/L), and **10** (3 mg,  $t_R = 9.5$  min, 0.025 mg/L). Fraction 4 (3.2 g) and Fraction 5 (2.78 g) were both separated into five parts (Fr.4-1–Fr.4-5 and Fr.5-1–Fr.5-5) by RP-18 column chromatography, eluting with CH<sub>3</sub>OH/H<sub>2</sub>O (5%–100%). Fraction 4-2 (512 mg) was further separated by Sephadex LH-20 with CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub> (1:1) to give four subfractions (Fr.4-2-1–Fr.4-2-4). Compound **3** (7 mg, 0.058 mg/L) was purified from the precipitate of Fr.4-2-2 (135 mg) after washing with MeOH, and compound **9** (6 mg,  $t_R = 7.4$  min, 0.05 mg/L) was obtained from Fr.4-2-3 (163 mg) by HPLC purification eluting with 60% MeOH. Fraction 4-4 (332.7 mg) was separated by Sephadex LH-20 with CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub> (1:1) to afford four subfractions (Fr.4-4-1–Fr.4-4-4). Fr.4-4-2 (94 mg) was also submitted to HPLC purification on ODS column eluting with 70% MeOH to yield a racemic mixture of **1/2** with an excess of one enantiomer (11 mg,  $t_R = 5.59$  min, 0.092 mg/L) which showed two peaks (1:2) by HPLC analysis on a chiral column (CHIRALPAK AY-H, Figure S37, Supplementary Information). Then the chiral separation by HPLC (Amylose tris(5-chloro-2-methylphenylcarbamate)) with EtOH yielded **1** (7 mg, 12.12 min, 0.058 mg/L) and **2** (3 mg, 8.82 min, 0.025 mg/L). Then Fr.4-4-3 (120 mg) was further subjected to HPLC separation eluted with 60% MeOH to yield **5** (3 mg,  $t_R = 10.4$  min, 0.025 mg/L) and **6** (4.7 mg,  $t_R = 11.64$  min, 0.039 mg/L). Fraction 5-2 (245 mg) was further purified by Sephadex LH-20 with MeOH to produce four subfractions (Fr.5-2-1–Fr.5-2-4). Compound **4** (2.5 mg,  $t_R = 14.7$  min, 0.021 mg/L) was obtained from Fr.5-2-3 (63 mg) by HPLC with 45% MeOH.

**Compound 1:** Yellow solid; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 238 (4.10), 323 (4.51) nm;  $[\alpha]_D^{25} -28$  ( $c$  0.05, CH<sub>3</sub>OH), IR (KBr)  $\nu_{\max}$  3496, 3208, 3073, 3022, 1675, 1617, 1494, 1454, 1376, 1033, 997, 763, 688 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, Table 1.

**Compound 2:** Yellow solid; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 238 (4.10), 323 (4.51) nm;  $[\alpha]_D^{25} +28$  ( $c$  0.05, CH<sub>3</sub>OH), IR (KBr)  $\nu_{\max}$  3496, 3208, 3073, 3022, 1675, 1617, 1494, 1454, 1376, 1033, 997, 763, 688 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, Table 1.

**Compound 3:** Yellow amorphous solid; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 248 (3.75), 347 (4.10) nm; IR (KBr)  $\nu_{\max}$  3183, 3073, 2960, 2867, 1681, 1639, 1606, 1541, 1421, 1359, 1275, 1173, 1024, 998, 829, 761 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, Table 1; HRESIMS  $m/z$  273.1231 [M + H]<sup>+</sup> (calcd. for C<sub>15</sub>H<sub>17</sub>N<sub>2</sub>O<sub>3</sub> 273.1234).

**Compound 4:** Yellow solid; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 250 (3.71), 347 (4.25) nm; IR (KBr)  $\nu_{\max}$  2973, 2937, 2879, 1708, 1638, 1577, 1459, 1379, 1273, 1122, 1092, 1025, 960 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, Table 1; HRESIMS  $m/z$  247.1189 [M + H]<sup>+</sup> (calcd. for C<sub>12</sub>H<sub>15</sub>N<sub>4</sub>O<sub>2</sub> 247.1190).

**Compound 5:** White solid; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 229 (4.05), 302 (4.17) nm;  $[\alpha]_D^{25}$   $-36$  ( $c$  0.66, CH<sub>3</sub>OH), IR (KBr)  $\nu_{\max}$  3168, 3077, 3042, 2961, 2867, 1680, 1646, 1546, 1426, 1392, 1357, 1092, 933, 800, 760, 619 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, Table 1; HRESIMS  $m/z$  259.1439 [M + H]<sup>+</sup> (calcd. for C<sub>15</sub>H<sub>19</sub>N<sub>2</sub>O<sub>2</sub> 259.1441).

**(3Z,6S)-3-Benzylidene-6-isobutylpiperazine-2,5-dione (6):**  $[\alpha]_D^{25}$   $-54$  ( $c$  0.3, DMSO); <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  8.58 (1H, s, NH-1), 9.98 (1H, s, NH-4), 3.94 (1H, m, H-6), 1.58 (2H, m, H-7), 1.80 (1H, m, H-8), 0.89 (3H, d,  $J = 6.1$ , H-9/10), 0.88 (3H, d,  $J = 5.5$ , H-9/10), 6.67 (1H, s, H-11), 7.48 (2H, d,  $J = 7.7$ , H-13/17), 7.39 (2H, t,  $J = 7.7$ , H-14/16), 7.30 (1H, t,  $J = 7.7$ , H-15). <sup>13</sup>C NMR (150 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  161.0 (C<sub>q</sub>, C-2), 127.3 (C<sub>q</sub>, C-3), 168.1 (C<sub>q</sub>, C-5), 54.2 (CH, C-6), 44.2 (CH<sub>2</sub>, C-7), 24.1 (CH, C-8), 23.3 (CH<sub>3</sub>, C-9), 22.6 (CH<sub>3</sub>, C-10), 114.9 (CH, C-11), 133.9 (C<sub>q</sub>, C-12), 129.2  $\times$  2 (CH, C-13/17), 129.8  $\times$  2 (CH, C-14/16), 128.5 (CH, C-15). HRESIMS  $m/z$  259.1456 [M + H]<sup>+</sup> (calcd. for C<sub>15</sub>H<sub>19</sub>N<sub>2</sub>O<sub>2</sub> 259.1441).

### 3.5. Preparation of *p*-Bromobenzoate (**1c**) of Compound **1** [14]

Compound **1** (2 mg, 6.99  $\mu$ mol) was dissolved in 1 mL of CH<sub>2</sub>Cl<sub>2</sub>, and triethylamine (10  $\mu$ L) and *p*-bromobenzoyl chloride (20 mg, 92.2  $\mu$ mol) were added. The mixture was stirred for 7 h at room temperature. Then 2 mL of H<sub>2</sub>O were added and the solution was extracted three times with CH<sub>2</sub>Cl<sub>2</sub> (5 mL each). The CH<sub>2</sub>Cl<sub>2</sub> solutions were combined and evaporated under reduced pressure to give a gum. *p*-Bromobenzoate **1c** (2 mg, 4.27  $\mu$ mol, 7.9 min, 61% yield) was obtained by HPLC purification eluting with 85% (MeOH-H<sub>2</sub>O).

***p*-Bromobenzoate (1c):** Yellow solid;  $[\alpha]_D^{25}$   $-50$  ( $c$  0.1, CH<sub>3</sub>OH); CD ( $c$  0.11, MeOH)  $\lambda_{\text{ext}}$  ( $\Delta\epsilon$ ) 313 ( $-5.2$ ), 254 ( $+4.78$ ), 208 ( $-1.73$ ). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  3.18 (3H, s, 1-NCH<sub>3</sub>), 5.57 (d,  $J = 9.5$  Hz, H-7), 4.09 (m, H-8), 4.20 (dd,  $J = 7.3, 10.3$  Hz, H-9a), 4.28 (dd,  $J = 6.0, 10.4$  Hz, H-9b), 1.14 (3H, d,  $J = 6.7$  Hz, CH<sub>3</sub>-10), 6.71 (s, H-11), 7.53 (2H, d,  $J = 7.6$  Hz, H-13/17), 7.38 (2H, t,  $J = 7.5$  Hz, H-14/16), 7.30 (t,  $J = 7.4$  Hz, H-15), 7.86 (2H, d,  $J = 8.5$  Hz, H-2'/6' in *p*-BrC<sub>6</sub>H<sub>4</sub>CO<sub>2</sub>-), 7.67 (2H, d,  $J = 8.5$  Hz, H-2'/6' in *p*-BrC<sub>6</sub>H<sub>4</sub>CO<sub>2</sub>-). <sup>13</sup>C NMR (150 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  31.3 (CH<sub>3</sub>, 1-NCH<sub>3</sub>), 158.5 (C<sub>q</sub>, C-2), 126.0 (C<sub>q</sub>, C-3), 158.8 (C<sub>q</sub>, C-5), 131.1 (C<sub>q</sub>, C-6), 130.4 (CH, C-7), 31.4 (CH, C-8), 69.3 (CH<sub>2</sub>, C-9), 17.5 (CH<sub>3</sub>, C-10), 116.6 (CH, C-11), 133.5 (C<sub>q</sub>, C-12), 129.7  $\times$  2 (CH, C-13/17), 129.1  $\times$  2 (CH, C-14/16), 128.0 (CH, C-15), 165.6 (C<sub>q</sub>, -CO<sub>2</sub>- in *p*-BrC<sub>6</sub>H<sub>4</sub>CO<sub>2</sub>-), 129.4 (C<sub>q</sub>, C-1' in *p*-BrC<sub>6</sub>H<sub>4</sub>CO<sub>2</sub>-), 132.3  $\times$  2 (CH, C-2'/6' in *p*-BrC<sub>6</sub>H<sub>4</sub>CO<sub>2</sub>-), 131.5  $\times$  2 (CH, C-3'/5' in *p*-BrC<sub>6</sub>H<sub>4</sub>CO<sub>2</sub>-), 127.9 (C<sub>q</sub>, C-4' in *p*-BrC<sub>6</sub>H<sub>4</sub>CO<sub>2</sub>-). ESIMS  $m/z$  469.1 and 471.1; HRESIMS  $m/z$  469.0755 [M + H]<sup>+</sup> (calcd. for C<sub>23</sub>H<sub>22</sub><sup>79</sup>BrN<sub>2</sub>O<sub>4</sub> 469.0758).

### 3.6. Preparation of *S*-MTPA and *R*-MTPA Esters **1a**, **1b**, **2a**, and **2b** of Compounds **1** and **2** [15,16]

Compound **1** (1 mg, 3.50  $\mu$ mol) was dissolved in 500  $\mu$ L of anhydrous pyridine and 4-dimethylaminopyridine (3 mg, 24.6  $\mu$ mol) and (*R*)-MTPACl (10  $\mu$ L) were added. The reaction was stirred for 12 h at room temperature. Then 1 mL of H<sub>2</sub>O was added, and the solution was extracted three times with CH<sub>2</sub>Cl<sub>2</sub> (5 mL each). After removal of CH<sub>2</sub>Cl<sub>2</sub> under reduced pressure, the residue was purified by semipreparative HPLC (70% MeOH-H<sub>2</sub>O) to yield (*S*)-MTPA ester **1a** (1.2 mg, 2.39  $\mu$ mol,  $t_R = 27.11$  min, 68% yield). By the same procedure, (*R*)-MTPA ester **1b** (1.1 mg, 2.19  $\mu$ mol,

$t_R = 24.74$  min, 63% yield), (*S*)-MTPA ester **2a** (0.8 mg, 1.59  $\mu\text{mol}$ ,  $t_R = 24.74$  min, 45% yield) and (*R*)-MTPA ester **2b** (0.9 mg, 1.79  $\mu\text{mol}$ ,  $t_R = 27.11$  min, 51% yield) were obtained from the reaction of **1** and **2** (1 mg, 3.50  $\mu\text{mol}$  each) with (*S*)-MTPACl, (*R*)-MTPACl and (*S*)-MTPACl (10  $\mu\text{L}$  each), respectively.

**(S)-MTPA ester 1a**: White solid.  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  3.07 (3H, s, 1-NCH<sub>3</sub>), 3.99 (m, H-8), 1.06 (3H, d,  $J = 6.8$  Hz, H-10), 5.51 (d,  $J = 9.6$  Hz, H-7), 6.76 (s, H-11), 4.36 (dd,  $J = 5.6, 10.5$  Hz, H-9a), 4.28 (dd,  $J = 7.4, 10.4$  Hz, H-9b), 7.51 (2H, d,  $J = 7.5$  Hz, H-13/17), 7.41 (2H, t,  $J = 7.6$  Hz, H-14/16), 7.32 (t,  $J = 7.4$  Hz, H-15). HRESIMS  $m/z$  525.1612 [ $\text{M} + \text{Na}$ ]<sup>+</sup> (calcd. for C<sub>26</sub>H<sub>25</sub>F<sub>3</sub>N<sub>2</sub>O<sub>5</sub>Na 525.1608).

**(R)-MTPA ester 1b**: White solid.  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  3.12 (3H, s, 1-NCH<sub>3</sub>), 3.99 (m, H-8), 1.07 (3H, d,  $J = 6.8$  Hz, H-10), 5.51 (d,  $J = 9.6$  Hz, H-7), 6.76 (s, H-11), 4.33 (2H, d,  $J = 6.5$  Hz, H-9), 7.50 (2H, d,  $J = 7.6$  Hz, H-13/17), 7.41 (2H, t,  $J = 7.6$  Hz, H-14/16), 7.32 (t,  $J = 7.4$  Hz, H-15). HRESIMS  $m/z$  525.1613 [ $\text{M} + \text{Na}$ ]<sup>+</sup> (calcd. for C<sub>26</sub>H<sub>25</sub>F<sub>3</sub>N<sub>2</sub>O<sub>5</sub>Na 525.1608).

**(S)-MTPA ester 2a**: White solid.  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  3.12 (3H, s, 1-NCH<sub>3</sub>), 3.99 (m, H-8), 1.07 (3H, d,  $J = 6.8$  Hz, H-10), 5.51 (d,  $J = 9.6$  Hz, H-7), 6.76 (s, H-11), 4.33 (2H, d,  $J = 6.5$  Hz, H-9), 7.50 (2H, d,  $J = 7.6$  Hz, H-13/17), 7.41 (2H, t,  $J = 7.6$  Hz, H-14/16), 7.32 (t,  $J = 7.4$  Hz, H-15). HRESIMS  $m/z$  525.1607 [ $\text{M} + \text{Na}$ ]<sup>+</sup> (calcd. for C<sub>26</sub>H<sub>25</sub>F<sub>3</sub>N<sub>2</sub>O<sub>5</sub>Na 525.1608).

**(R)-MTPA ester 2b**: White solid.  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  3.07 (3H, s, 1-NCH<sub>3</sub>), 3.98 (m, H-8), 1.06 (3H, d,  $J = 6.8$  Hz, H-10), 5.51 (d,  $J = 9.6$  Hz, H-7), 6.76 (s, H-11), 4.35 (dd,  $J = 5.6, 10.5$  Hz, H-9a), 4.27 (dd,  $J = 7.4, 10.4$  Hz, H-9b), 7.50 (2H, d,  $J = 7.5$  Hz, H-13/17), 7.41 (2H, t,  $J = 7.6$  Hz, H-14/16), 7.32 (t,  $J = 7.4$  Hz, H-15). HRESIMS  $m/z$  525.1606 [ $\text{M} + \text{Na}$ ]<sup>+</sup> (calcd. for C<sub>26</sub>H<sub>25</sub>F<sub>3</sub>N<sub>2</sub>O<sub>5</sub>Na 525.1608).

### 3.7. Preparation of FDAA Derivatives of the Acid Hydrolysates of **5** and Four Authentic Isoleucine Samples (*L*-, *L*-allo-, *D*- and *D*-allo-) and Marfey's Analysis [18] and C<sub>3</sub> Marfey's Analysis [19,20]

Compound **5** (1 mg, 3.88  $\mu\text{mol}$ ) was dissolved in 6 M HCl (1 mL) in a sealed tube and the mixture was heated at 105 °C for 17 h. Then the solution was cooled and evaporated to dryness. The residue, *L*-Ile, *L*-allo-Ile and *D*-Ile, *D*-allo-Ile, was dissolved in H<sub>2</sub>O (250  $\mu\text{L}$  each), respectively. 50  $\mu\text{L}$  of each solution was treated with 200  $\mu\text{L}$  of 1% FDAA in acetone followed by 1.0 M NaHCO<sub>3</sub> (40  $\mu\text{L}$ ). The reaction was maintained 1 h at 45 °C and then quenched by addition of 2.0 M HCl (10  $\mu\text{L}$ ). The corresponding FDAA derivatives of hydrolysate of **5**, *L*-Ile, *L*-allo-Ile, *D*-Ile and *D*-allo-Ile were analyzed by ODS HPLC column maintained at 30 °C using the following programs: solvent A, H<sub>2</sub>O + 0.2% TFA; solvent B, MeCN; linear gradient, 0 min 25% B (75% A), 40 min 60% B (40% A), 45 min 100% B; UV detection at 340 nm. The retention times for the FDAA derivatives of the hydrolysate of **5**, *L*-Ile, *L*-allo-Ile, *D*-Ile and *D*-allo-Ile were 23.06, 23.06, 23.06, 28.11 and 28.11 min, respectively (Figure S38). The FDAA derivatives of the hydrolysate of **5**, *L*-Ile, and *L*-allo-Ile were further analyzed by C<sub>3</sub> HPLC column maintained at 50 °C. The column was developed with a linear gradient of 15%–60% MeOH/water (+isocratic 5% of a 1% formic acid solution in MeCN) over 55 min with UV detection at 340 nm. The retention times for the FDAA derivatives of the hydrolysate of **5**, standard *L*-Ile, and *L*-allo-Ile, were 38.50, 38.50, and 37.49 min, respectively (Figure S39).

### 3.8. Bioassays

Cytotoxicity was assayed by the MTT [23] and SRB method [24]. In the MTT assay, HL-60 cell and K562 cell line were cultured in RPMI-1640 supplemented with 10% FBS under a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37 °C, 198 µL of cell suspensions with a density of  $4.6 \times 10^4$  cells mL<sup>-1</sup> was plated in 96-well microtiter plates and incubated for 24 h. Then, 2 µL of the test solutions in MeOH were added to each well and further incubated for 36 h. The MTT solution (20 µL, 5 mg/mL in RPMI-1640 medium) was then added to each well and incubated for 4 h. Old medium containing MTT (150 µL) was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a Spectra Max Plus plate reader at 570 nm. In the SRB assay, 200 µL of the A549 cell suspension was plated in 96-well plates at density of  $2 \times 10^5$  cell mL<sup>-1</sup>. Then, 2 µL of the test solutions (in MeOH) were added to each well and the culture was further incubated for 24 h. The cells were fixed with 12% trichloroacetic acid and the cell layer stained with 0.4% SRB. The absorbance of the SRB solution was measured at 515 nm. Adriamycin was used as positive control (IC<sub>50</sub> 0.652 µM, 0.645 µM and 0.080 µM for HL-60, K562 and A549 cell, respectively).

The antiviral activity against H1N1 was evaluated by the CPE inhibition assay [21,22]. Confluent MDCK cell monolayers were firstly incubated with influenza virus (A/Puerto Rico/8/34 (H1N1), PR/8) at 37 °C for 1 h. After removing the virus dilution, cells were maintained in infecting media (RPMI 1640, 4 µg/mL of trypsin) containing different concentrations of test compounds at 37 °C. After 48 h incubation at 37 °C, the cells were fixed with 100 µL of 4% formaldehyde for 20 min at room temperature. After removal of the formaldehyde, the cells were stained with 0.1% crystal violet for 30 min. The plates were washed and dried, and the intensity of crystal violet staining for each well was measured in a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. The IC<sub>50</sub> was calculated as the compound concentration required inhibiting influenza virus yield at 48 h post-infection by 50%. Ribavirin was used as the positive control with an IC<sub>50</sub> value of 38.8 µM.

The anti-inflammatory effects of compounds were assayed by inhibition of lipopolysaccharide (LPS)-mediated NF-κB transcriptional activity in RAW 264.7 cells [25]. RAW 264.7 cells ( $2.5 \times 10^5$  cells/well) were placed in a 24-well plate. The cells were then stably transfected with pNF-κB-Luc expression plasmid (0.5 µg/well). Transfections were performed using lipofectamine 2000 in accordance with the instructions of the manufacturer (Invitrogen, Carlsbad, NM, USA). Stably transfected cells were pretreated 2 h with the test compounds and stimulated with 0.1 µg/mL of LPS for an additional 4 h. The luciferase assay was performed with the aid of a Steady-Glo Luciferase assay system in accordance with the instructions of the manufacturer (Promega, Madison, WI, USA).

The antimicrobial activities against *E. coli*, *E. aerogenes*, *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *C. albicans* were evaluated by 2-fold dilution method [26]. The tested strains were cultivated on LB broth for bacteria and in YPD broth for *C. albicans* at 37 °C. The test compounds were dissolved in DMSO at different concentrations from 100 to 0.78 µg/mL (from 6.25 to 0.025 µg/mL for the positive controls) by the continuous 2-fold dilution methods. The minimum inhibitory concentrations (MICs) were determined in 96-well plates, and each well contains 100 µL of contents composed of 20 µL of inoculums ( $5 \times 10^5$  CFU/mL), test compounds and LB or YPD media. The microtiter plates were incubated at 35 °C for 24 h and were examined for microbes' growth by turbidity in daylight. The

MICs were defined as the lowest concentration at which no visible growth of microbes could be observed. Ciprofloxacin lactate and ketoconazole were used as positive controls for *E. coli*, *E. aerogenes*, *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *C. albicans* with MIC values of 0.05, 0.19, 0.1, 0.39, 3.12 and 0.025 µg/mL, respectively.

#### 4. Conclusions

Five new vinylidene substituted diketopiperazines (DKPs) **1–5** were isolated and their structures including absolute configurations were determined. The new compound **3** displayed modest activity and the known analogs **6** and **7** displayed potent activity against H1N1 virus with IC<sub>50</sub> values of 41.5 ± 4.5, 28.9 ± 2.2 and 6.8 ± 1.5 µM, respectively. The results showed that both *Z*-deh-Phe and Leu or *Z*-deh-Leu substitutions significantly increase the anti-H1N1 activity of DKPs, while *E*-isomerization and hydroxylation of both deh-Phe and deh-Leu moieties, and *N*-methylation reduce the activity. The substitutions of both deh-Phe or deh-Tyr with deh-His and Leu or deh-Leu with *iso*-Leu also reduce the anti-H1N1 activity. In addition, the <sup>13</sup>C NMR data of (3*Z*,6*S*)-3-benzylidene-6-isobutylpiperazine-2,5-dione (**6**) was reported here for the first time.

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