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New diketopiperazine dimer from a filamentous fungal isolate of *Aspergillus sydowii*

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Graphical abstract

Keywords

NMR; ^{1}H NMR; ^{13}C NMR; Diketopiperazine; Fungi; Secondary metabolites

Introduction

Prior investigations of filamentous fungi in our group have resulted in the isolation of several new and biologically active natural products.[1–3] Continuing these investigations, we have now analyzed the metabolites from a fungal isolate of *Aspergillus sydowii* (MSX19583) that was obtained from spruce litter collected in 1984 in Colorado, USA. The

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Supporting Information Additional supporting information may be found in the online version of this article at the publisher's website.

extracts from solid-substrate fermentation cultures exhibited cytotoxic activity against MDA-MB-435 (human melanoma) cells and were therefore pursued for further analysis. Chemical separation of the CH_3CN/CH_3OH extract afforded a new diketopiperazine dimer (**1**) in addition to three known compounds including cyclo-(*L*-phenylalaninyl-*L*-tryptophanyl) [**2**],[4, 5] *S*-sydonic acid (**3**), and *S*-sydonol (**4**) (Figure 1).[6]

Results and Discussion

Compound **1** was assigned the molecular formula $C_{40}H_{36}N_6O_4$ (Index of Hydrogen Deficiency of 26) based on the HRESIMS data. The ^1H and ^{13}C NMR spectra of **1** displayed signals for only 18 protons and 20 carbons, respectively, suggesting a symmetrical dimeric structure (Table 1). The ^1H signals were attributed to nine aromatic protons, a pair of methylene units, three methine protons, and two exchangeable protons (Table 1; Figure S1). In addition to the ^{13}C NMR signals expected for the above structural features, one quaternary (δ_C 59.1, C-10), five non-protonated sp^2-hybridized carbons [three aromatic (δ_C 126.5, 135.6, and 150.1 for C-11, C-18, and C-16, respectively) and two carbonyl carbons (δ_C 165.9, C-4 and 168.7, C-7)] were also observed (Table 1; Figure S2). Phenylalanine and tryptophan-derived subunits were readily identified after analysis of HSQC and HMBC NMR data (Figure 2). Presence of phenylalanine was also confirmed by amino acid analysis using Marfey's method.[7] HMBC correlations from H-6 (δ_H 5.45) to C-4, C-5 (δ_C 56.3), C-7, and C-8 (δ_C 58.9) supported the diketopiperazine ring system in **1**. A C–N covalent bond between C-2 and N-3 was identified by HMBC correlations from H-2 (δ_H 5.26) to C-8, C-9 (δ_C 35.4), C-11, and C-16. Finally, the monomeric units were linked at the only remaining position, C-10, thereby completing the gross structure of **1**.

A search of the Dictionary of Natural Products[8] identified two compounds with identical molecular formulae, a symmetric diketopiperazine, WIN 64821, and an asymmetric analogue, asperazine.[9, 10] Although the gross structure of **1** was found to be similar to WIN 64821 after analysis of ^1H, ^{13}C, HSQC, and HMBC NMR data (Table 1 and Figure 2), the ^1H NMR signals for **1** were not fully consistent with those reported in the literature for WIN 64821 or a synthetic analogue, *ent*-WIN 64821,[11] suggesting differences in configuration. Four additional secondary metabolites with identical molecular formulae and structural skeletons are known in the literature; however, the lack of reported NMR data did not permit comparisons between these and **1**,[12, 13] which is a general challenge with this class of compounds.

The absolute configuration of **1** was determined using a suite of techniques. Analysis of the NOESY data for **1** did not provide sufficient information for complete assignment of the relative configuration. Marfey's method established the absolute configuration at C-5. Briefly, a sample of **1** was hydrolyzed in 6N HCl (110 °C; 24 h), and Marfey's derivative of the resulting phenylalanine standard sample was prepared using the methods reported previously.[7] HPLC analysis of the product (t_R = 5.06 min) and comparison with standards (L-Phe: t_R = 5.06 min and D-Phe: t_R = 5.92 min) prepared in an analogous manner revealed the presence of L-phenylalanine. By analogy to compound **2**, the C-8 stereocenter was presumed to be an *S*-configuration based on biosynthetic considerations. Due to the limited number of protons, a NOESY correlation to assign the configuration at C-2 relative to other

stereocenters could not be observed; however, the absence of a correlation between H-2 and H-8 was consistent with the orientation of these protons on opposite faces of the ring system. More convincingly, the ECD spectrum of **1** resembled that of a closely related analogue, ditryptophenaline (**5**),[14] where both compounds, each with an anti orientation for H-2 and H-8, exhibited a pair of negative Cotton effects near 245 and 300 nm (Figure 3).[9] In the same literature,[9] WIN 64821 was shown to have an opposite Cotton effect at 245 nm. Thus, the relative orientation of these two protons, anti in **1** and syn in WIN 64821, accounted for the key difference between these compounds, supporting the absolute configuration of **1** as shown (Figure 1).

The extract of MSX19583 exhibited moderate cytotoxic activity (54% cell viability at 20 μg/mL) against MDA-MB-435 human melanoma cells using procedures described in detail previously.[15–17] As such, compounds **1**–**4** were tested against two cancer cell lines, MDA-MB-435 and HT-29 (human colon cancer). All compounds were found to be inactive, displaying IC_{50} values >20 μM. Although the initial activity observed with the extract could not be attributed to the major compounds, it is possible that minor components present in trace amounts could contribute to the moderate cytotoxic effects. Also, various other biological activities have been reported for the known compounds isolated during this study. For example, *S*-sydonol has been reported to have anti-diabetic and anti-inflammatory activities.[18] *R*-sydonol has been reported to show selective antibacterial activity against *Staphylococcus albus* and *Micrococcus tetragenus*, while *R*-sydonic acid exhibited broad spectrum activity against several species.[19] Cyclo-(*L*-phenylalaninyl-*L*-tryptophanyl) has been previously reported as a plant growth regulator.[4]

Experimental

General experimental procedures

Optical rotation data were acquired on a Rudolph Research Autopol III polarimeter (Rudolph Research Analytical, Flanders, NJ, USA). ECD data were collected using an Olis DSM 17 CD spectrophotometer (Olis, Bogard, GA, USA). UV data were obtained using a Varian Cary 100 Bio UV-vis spectrophotometer (Varian Medical Systems, Palo Alto, CA, USA). HRESIMS data were collected using an electrospray ionization (ESI) source coupled to a LTQ Orbitrap XL system (Thermo Fisher Scientific, San Jose, CA, USA) in positive and negative ionization modes via a liquid chromatography/autosampler system comprised of an Acquity UPLC system (Waters Corp., Milford, MA, USA). A CombiFlash Rf system using a 12 g RediSep Rf Si-gel Gold column (both from Teledyne-Isco, Lincoln, NE, USA) was employed for normal-phase flash column chromatography. High-performance liquid chromatography (HPLC) separations were performed utilizing Varian ProStar HPLC systems equipped with ProStar 210 pumps and a ProStar 335 photodiode array detector, using Galaxie Chromatography Workstation software (version 1.9.3.2, Varian Inc.). YMC ODS-A (Waters Corp.; 5μm; 250 × 10 mm for semi-preparative HPLC column) or Kinetex C_{18} (Phenomenex, Torrance, CA, USA; 5μm; columns of dimensions 250 × 21.2 mm for preparative HPLC and 250 × 4.6 mm for analytical HPLC) were used for HPLC. For UPLC analysis, a BEH C_{18} (Waters Corp.; 1.7 μm; 50 × 2.1mm column) was used with data

collected and analyzed using Empower 3 software (Waters Corp.). The solvents were obtained from Fisher Scientific.

Fungal strain and fermentation

Mycosynthetix fungal strain MSX19583 (*Aspergillus sydowii*; See Supporting Information and Figure S5) was isolated from spruce litter collected in 1984 near Cumbres Pass, Colorado, USA at an elevation of 2985 m. A fresh culture of this isolate was grown on a malt extract slant, and a piece was transferred to a medium containing 2% soy peptone, 2% dextrose, and 1% yeast extract (YESD media). After incubation at 22 °C for 7 days (with agitation), the culture was used to inoculate 50 mL of rice medium [containing rice, vitamin solution and water (twice the volume of rice)] in a 250 mL Erlenmeyer flask. The culture was incubated at 22 °C until sufficient fungal growth (~ 14 d) was observed. The scaled-up culture was grown in a 2.8 L Fernbach flask containing 150 g of rice and 300 mL of H_2O and inoculated using a seed culture grown in YESD medium followed by incubation at 22 °C for 14 d. Details for molecular identification and phylogenetic analysis of fungal strain MSX19583 can be found in the supporting information (Figure S5).

Extraction and isolation

To one Fernbach flask containing rice with fungal growth (MSX19583) was added 500 mL of 1:1 $CH_3OH/CHCl_3$. The culture was chopped with a spatula and shaken overnight (~16 h; rt) at ~100 rpm. After filtration, the remaining residues were washed with CH_3OH. To the filtrate, 900 mL of $CHCl_3$ and 1.5 L of H_2O were added. The mixture was stirred for 30 min and then transferred to a separatory funnel. The lower layer was drawn off into round-bottom flasks and evaporated to dryness. This dried material was re-constituted in 200 mL of 1:1 CH_3OH/CH_3CN and 200 mL of hexanes and transferred to a separatory funnel. The biphasic solution was shaken vigorously. The CH_3OH/CH_3CN layer was evaporated to dryness under vacuum to obtain 795 mg of the organic extract.

A portion of the organic extract (770 mg) was adsorbed onto a minimal amount of Celite 545 (Acros Organics, Geel, Belgium). After drying, the adsorbed mixture was loaded into a cartridge and subjected to normal-phase silica gel flash column chromatography (RediSep Rf Gold Si-gel column; 12g) using a step gradient with hexanes, $CHCl_3$, and CH_3OH (30 mL/min flow rate and 61.0 column volumes over 31.4 min). The resulting fractions were pooled according to UV and ELSD data to afford four fractions. Fraction three (80 mg) was subjected to preparative RP-HPLC (gradient elution using CH_3CN in H_2O (w 0.1% HCOOH): 40–80% for 20 min and 80–100% CH_3CN for 10 min; λ = 210 and 254 nm; flow rate = 21.2 mL/min) affording cyclo-(*L*-phenylalaninyl-*L*-tryptophanyl) [**2**; 1.2 mg, t_R 4.0 min], **1** (3.0 mg, t_R 7.5 min), *S*-sydonol (**3**; 2.4 mg, t_R 9.7 min), and *S*-sydonic acid (**4**; 12.2 mg, t_R 10.5 min). Compounds **1** and **2** were further subjected to semi-preparative HPLC [isocratic elution using 50% CH_3CN in H_2O for 30 min in the case of **2** and 60% CH_3CN in H_2O for 30 min in the case of **1**; flow rate = 3 mL/min; YMC ODS-A (Waters Corp.; 5μm; 250 × 10 mm)] affording 1.1 mg (t_R 7.5 min) and 0.51 mg (t_R 6.0 min), respectively. Purity was determined by UPLC using a gradient elution of 20% CH_3CN in H_2O (w 0.1% HCOOH) to 100% CH_3CN over 3 min. All of the known compounds (**2**–**4**) were identified by comparison of their ^1H NMR, ^{13}C NMR, and/or MS data with literature values.[4–6]

Compound **1**: White powder; $[\alpha]^{23}_{D}$ −25 [*c* 0.04, 4:1 CH_3OH: $(CH_3)_2SO$], $[\alpha]^{24}_{D}$ −343 [*c* 0.04, CH_2Cl_2]; UV/Vis (CH_3OH) λ_{max} (log ε) 303 (3.5), 245 (3.8), 218 (3.7) nm; ECD (50 μM, CH_3OH) λ_{max} (Δε) 303 (−18), 246 (−27), 226 (+12), 220 (+23) nm; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see table 1; Key NOESY data: H-5 ↔ H-6, H-17_a, H-17_b; H-8 ↔ H-9_a, H-9_b; H-9_a ↔ H-2; H-19/H-23 ↔ H-6, H-17_a, H-17_b; HRESIMS obsd. *m/z* 665.2839 $[M+H]^+$ (calcd. for $C_{40}H_{37}N_6O_4$, 665.2871).

Cyclo-(*L*-phenylalaninyl-*L*-tryptophanyl) [**2**]: White powder; $[\alpha]^{23}_{D}$ −110 (*c* 0.05, CH_3OH); HRESIMS obsd. *m/z* 334.1548 $[M+H]^+$ (calcd. for $C_{20}H_{20}N_3O_2$, 334.1550); ^1H NMR data were fully consistent with those reported in literature.[4, 5]

S-Sydonic acid (**3**): Colorless oil; $[\alpha]^{23}_{D}$ +4 (*c* 1.22, CH_3OH); HRESIMS obsd. *m/z* 249.1476 $[M-H_2O+H]^+$ (calcd. for $C_{15}H_{21}O_3$, 249.1485); ^1H NMR data were fully consistent with those reported in literature; structure was also confirmed by analysis of 2D NMR data.[6] *S*-Sydonol (**4**): Colorless oil; $[\alpha]^{23}_{D}$ +6 (*c* 0.24, CH_3OH); HRESIMS obsd. *m/z* 235.1684 $[M-H_2O+H]^+$ (calcd. for $C_{15}H_{23}O_2$, 235.1693); ^1H NMR data were fully consistent with those reported in literature.[6]

NMR data

NMR spectra (^1H, ^{13}C, ^1H-^{13}C HSQC, and ^1H-^{13}C HMBC) were recorded at 25 °C in $CDCl_3$ on a JEOL ECS-400 NMR spectrometer (399.78 MHz for ^1H and 100.53 MHz for ^{13}C; JEOL Ltd., Tokyo, Japan) equipped with an auto tune 5 mm field gradient tunable Royal probe (NM-03810RO5/UPG). The ^1H and ^{13}C chemical shifts were referenced to the residual solvent peak of $CDCl_3$ at 7.24 ppm and 77.2 ppm, for proton and carbon, respectively. The ^1H sweep width was set at 5997 Hz for all experiments with a 90° pulse for ^1H of 6.4 μs and ^{13}C sweep width 25131 Hz with a 90° pulse for ^{13}C of 11.6 μs. The digital resolution of ^1H NMR was 0.37 Hz and that of ^{13}C NMR was 0.77 Hz. The edited-gradient ^1H-^{13}C HSQC was acquired with ^{13}C sweep width of 16084 Hz and 256 t1 increments. Each increment was acquired with 16 transients. The one-bond coupling constant delay was set using 145 Hz and MPF8 decoupling was applied during acquisition. The gradient ^1H-^{13}C HMBC was acquired using 64 transients per increment with 256 t1 increments. A sweep width of 20105 Hz was used for the ^{13}C dimension. One-bond coupling constant of 145 Hz and long-range coupling constant of 8 Hz were used to set the delays in the pulse sequence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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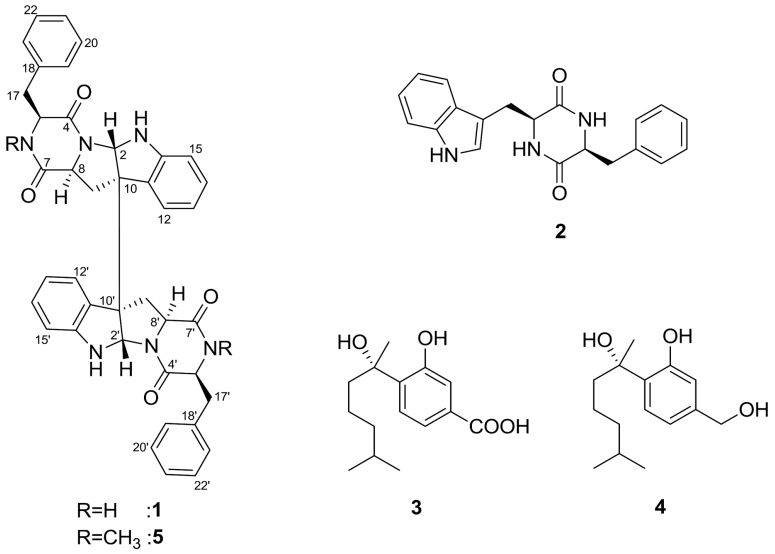

Figure 1.
Structures of compounds **1**–**5**.

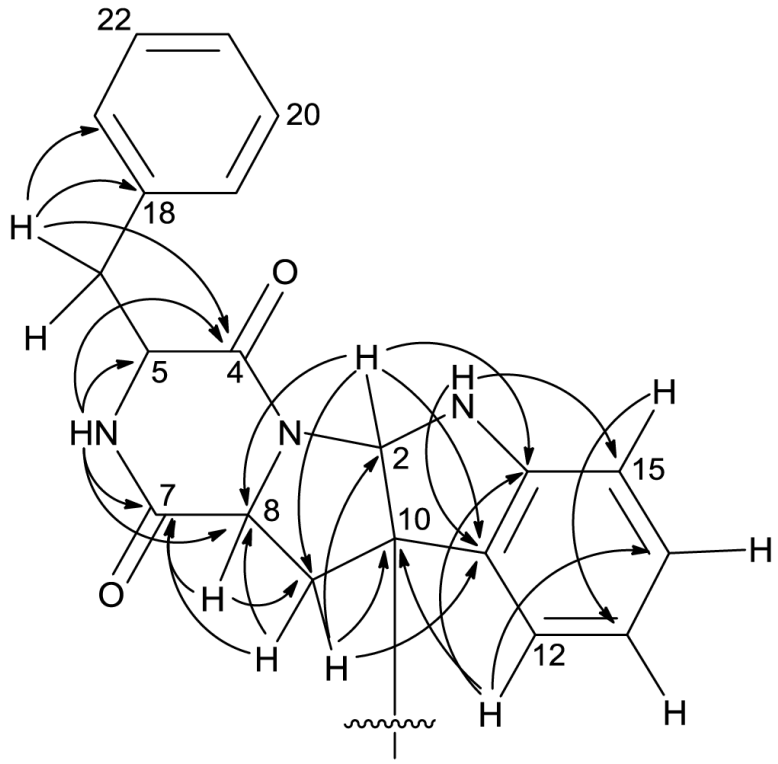

Figure 2.
Key HMBC correlations for **1**.

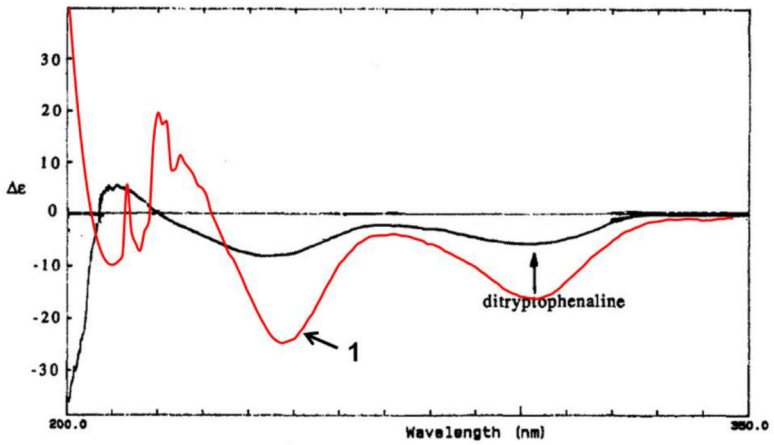

Figure 3.

Experimental ECD spectrum for **1** overlaid with ECD data reported for ditryptophenaline (**5**).

Table 1

^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of **1** in $CDCl_3$.

#	δ_H (mult., *J*)	δ_C	HMBC (H→ #C)
1, 1′	5.17 (s)		10, 11
2, 2′	5.26 (s)	78.9	8, 9, 11, 16
4, 4′		165.9	
5, 5′	4.15 (br d; 11.3)	56.3	4, 17, 18
6, 6′	5.45 (s)		4, 5, 7, 8, 17
7, 7′		168.7	
8, 8′	3.87 (dd, 11.0, 5.9)	58.9	7, 9
9_a, $9'_a$	2.67 (dd, 12.9, 11.0)	35.4	7, 8, 10, 11
9_b, $9'_b$	2.56 (dd, 12.9, 5.9)		2, 8, 10, 11
10, 10′		59.1	
11, 11′		126.5	
12, 12′	7.20 (d, 7.6)	125.5	10, 14, 16
13, 13′	6.79 (t, 7.6)	119.6	11, 15
14, 14′	7.15 (m)	130.2	12, 16
15, 15′	6.65 (d, 7.9)	110.5	11, 13
16, 16′		150.1	
17_a, $17'_a$	3.55 (dd, 14.4, 3.4)	36.7	4, 5, 18, 19/23
17_b, $17'_b$	2.70 (dd, 14.4, 11.3)		4, 5, 18, 19/23
18, 18′		135.6	
19, 19′	7.15 (m)	129.1	17, 21, 23
20, 20′	7.32 (m)	129.6	18, 22
21, 21′	7.28 (m)	127.9	19/23
22, 22′	7.32 (m)	129.6	18, 20
23, 23′	7.15 (m)	129.1	17, 19, 21