1'-(2-Phenyl-ethylene)-ditryptophenaline, a New Dimeric Diketopiperazine from Aspergillus flavus

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1′-(2-PHENYL-ETHYLENE)-DITRYPTOPHENALINE, A NEW DIMERIC DIKETOPIPERAZINE FROM *ASPERGILLUS FLAVUS*

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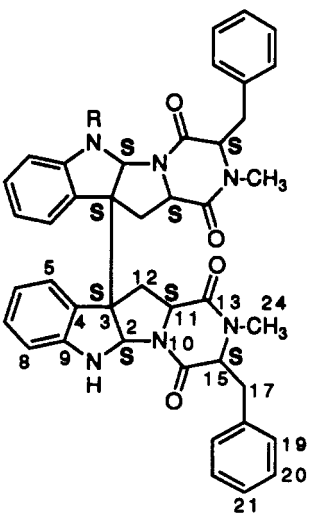
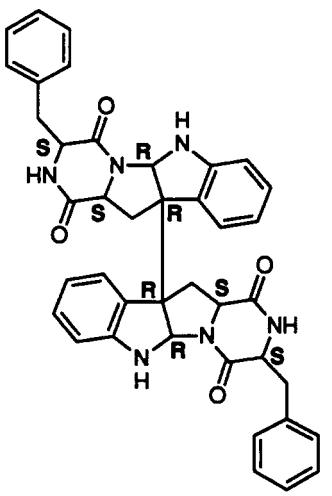
ABSTRACT.—A new diketopiperazine dimer, 1′-(2-phenyl-ethylene)-ditryptophenaline [**1**], was isolated together with ditryptophenaline [**2**] from the fungus *Aspergillus flavus*. The structure of **1** was determined by analysis of spectroscopic data. In contrast to the structurally related substance-P antagonist WIN 64821 [**3**], both **1** and **2** are weak substance-P inhibitors, indicating that stereochemistry at the position of dimerization is an important determinant of biological potency for these molecules.

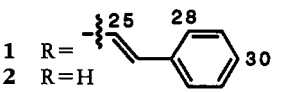
Ditryptophenaline [**2**], a dimeric dipeptide derivative, has been identified as a secondary metabolite from several strains of *Aspergillus flavus* (1,2). Recently, we reported the isolation of a related compound, WIN 64821 [**3**], as a potent substance-P antagonist (3). For compound **3** the ring junction stereochemistries at carbons C-2, C-2′, C-3 and C-3′ are *R*, while for **2** the corresponding carbons have the *S* configuration. In order to investigate the importance of the ring junction chiralities for biological activity, as part of our study of structure-activity relationships for **3** (4), we reisolated **2** from a regrowth of the original MIT strain of *A. flavus* (MIT-M26). Co-produced with **2** in culture, MIT-M26 was a new nonsymmetrical ditryptophenaline analogue, 1′-(2-phenyl-ethylene)-ditryptophenaline [**1**]. We herein report the isolation and structure determination of **1**. We also report structure-activity data indicating the importance of the *R* chirality at the ring junction positions for the biological potency of **3**.

RESULTS AND DISCUSSION

A. flavus culture SC1661, originally MIT M26, was fermented in a cracked corn/







3

glycerol-dextrin medium to give a yield of 50 mg/flask of **2**, as detected by hplc. From an EtOAc extract of the whole culture **1** and **2** were isolated as white solids. The structure of **2** was confirmed by spectroscopic comparison with published data (1,2). The uv spectrum of **1** indicated that it was structurally related to **2**. The molecular formula of **1** was established as $C_{50}H_{46}N_6O_4$ by hrfabms. Lrfabms of **1** gave fragments at *m/z* 346, 317, 157, and 130, identical to those observed for **2**, indicating that **1** contained the monomeric subunit of **2** as part of its structure. Other fragment ions present in the lrfabms of **1**, at *m/z* 448, 420, 259, and 232, were 102 daltons higher in mass than the corresponding fragments in the ms of **2**. Because the peaks at *m/z* 130 and 157 correspond to indoline-containing fragments, the presence of peaks at *m/z* 232 and 259 suggests that **1** is equivalent to **2**, with the exception of an additional C_8H_6 attached to one indoline ring.

In the ^{1}H- and ^{13}C-nmr spectra of **1** (Table 1) the signals corresponding to the non-alkylated subunit were identified and assigned with the aid of COSY, HMQC, HMBC, and NOESY experiments. NOe correlations from H-2 to H-12b, from H-5 to H-12a, from H-12a to H-11, and from H-11 to H-15 established the relative stereochemistry within this subunit of **1** to be the same as that of **2**. Only one exchangeable hydrogen (H-1) was present in the ^{1}H-nmr spectrum of **1**, consistent with *N*-alkylation of one of the indolines in **1**.

In addition to two indoline and two benzyl systems, the ^{1}H- and ^{13}C-nmr spectra of **1** showed the presence of a double bond and a phenyl ring. HMBC couplings from H-25 to C-27, and from H-26 to C-27 and C-28, indicated a 2-phenyl-ethylene moiety. A 14.6 Hz coupling indicated a trans arrangement for the olefinic protons of the double bond. NOes from the olefinic protons (H-25 and H-26) to both H-2′ and H-8′, and HMBC couplings from H-2′ to C-9′ and C-25, and from H-25 to C-9′, indicated that the 2-phenyl-ethylene group was attached at the indoline nitrogen (N-1′). The above, together with nOe correlations from H-2′ to H-12b′, from H-5′ to H-12a′, from H-12a′ to H-11′, and from H-11′ to H-15′, established the structure of **1** to be as shown. An optical rotation of $-125°$ for **1** indicated that the absolute stereochemistry is probably the same as that of **2** (1).

For **1**, nOes from methyl-24 to H-17b but not to H-17a, and from methyl-24 to H-17b′ but not to H-17a′, and nondegeneracy of the phenyl hydrogens, indicated that each benzyl ring was constrained in close proximity to a diketopiperazine nucleus, consistent with reported data for **2** (2). Although the crystal structure of **2** has been reported (1), the solution structure could not be completely defined due to the symmetry of this molecule. Molecular modeling, starting with the published crystal structure coordinates for **2**, and using a three-bond MULTIC method with MACROMODEL as previously described for **3** (3), indicated that three low-energy structures exist (Figure 1) which are consistent with the nmr data obtained for **2** (2). These three structures differ in the C-4–C-3–C-3′–C-4′ dihedral angle which defines the monomer-monomer spatial relationship.

Molecular modeling for **1** gave similar results to those obtained for **2**, indicating that *N*-alkylation has little influence on conformation. For **1**, an intense nOe was observed between H-2 and H-2′ indicating that these two hydrogens are close in space. Of the three conformations obtained for **1** by modeling, only the one similar to the crystal structure of **2** (Figure 1A) would place H-2 and H-2′ close in space (2.1 Å). This indicates that in solution compounds **1** and **2** exist in a conformation defined by a C-4–C-3–C-3′–C-4′ dihedral angle of $-75°$ (Figure 1A), which is quite different from **3** which has a solution conformation defined by a C-4–C-3–C-3′–C-4′ dihedral angle of 165° (Figure 2) (3).

TABLE 1. ^{1}H- and ^{13}C-Nmr Data for 1′-(2-Phenylethylene)-ditryptophenaline [**1**].[a]

Position	^{1}H (Hz) (mult.)	^{13}C
1	4.78 s	
2	4.91 s	78.47 d
3		59.06 s
4		125.94 s
5	6.94 d (7.5)	125.48 d
6	6.66 t (7.4)	118.83 d
7	7.03 t (7.6)	129.81 d
8	6.50 d (7.7)	109.61 d
9		149.96 s
11	3.73 m	58.26 d
12a	2.23 dd (12.3, 5.0)	36.16 t
12b	1.54 t (12.0)	
13		165.30 s
15	4.22 t (3.4)	63.01 d
16		164.24 s
17a	3.53 dd (15.0, 3.1)	36.25 t
17b	3.24 m	
18		134.56 s
19(23)	7.08 d (7.4)	129.12 d
20(22)	7.28 t (7.5)	128.91 d
21	7.15 t (7.5)	127.81 d
24	2.98 s	32.28 q
2′	5.46 s	80.92 d
3′		58.48 s
4′		128.13 s
5′	7.04 d (7.5)	125.80 d
6′	6.78 t (7.5)	120.26 d
7′	7.13 t (7.6)	129.81 d
8′	6.92 d (7.7)	109.53 d
9′		146.48 s
11′	3.72 m	58.42 d
12a′	2.13 dd (12.1, 4.6)	37.79 t
12b′	1.48 t (12.1)	
13′		164.77 s
15′	4.37 t (3.5)	63.23 d
16′		163.49 s
17a′	3.54 dd (15.1, 3.2)	36.62 t
17b′	3.24 m	
18′		134.97 s
19′(23′)	7.18 d (7.5)	129.53 d
20′(22′)	7.51 t (7.6)	129.45 d
21′	7.36 t (7.6)	127.62 d
24′	3.02 s	32.91 q
25	7.32 d (14.6)	127.62 d
26	6.56 d (14.6)	108.94 d
27		138.04 s
28(32)	7.40 d (7.5)	125.02 d
29(31)	7.33 t (7.5)	128.62 d
30	7.28 m	125.39 d

[a]Values in ppm, referenced to δ_C=77.0 and δ_H=7.25 for $CDCl_3$ solutions, relative to TMS.

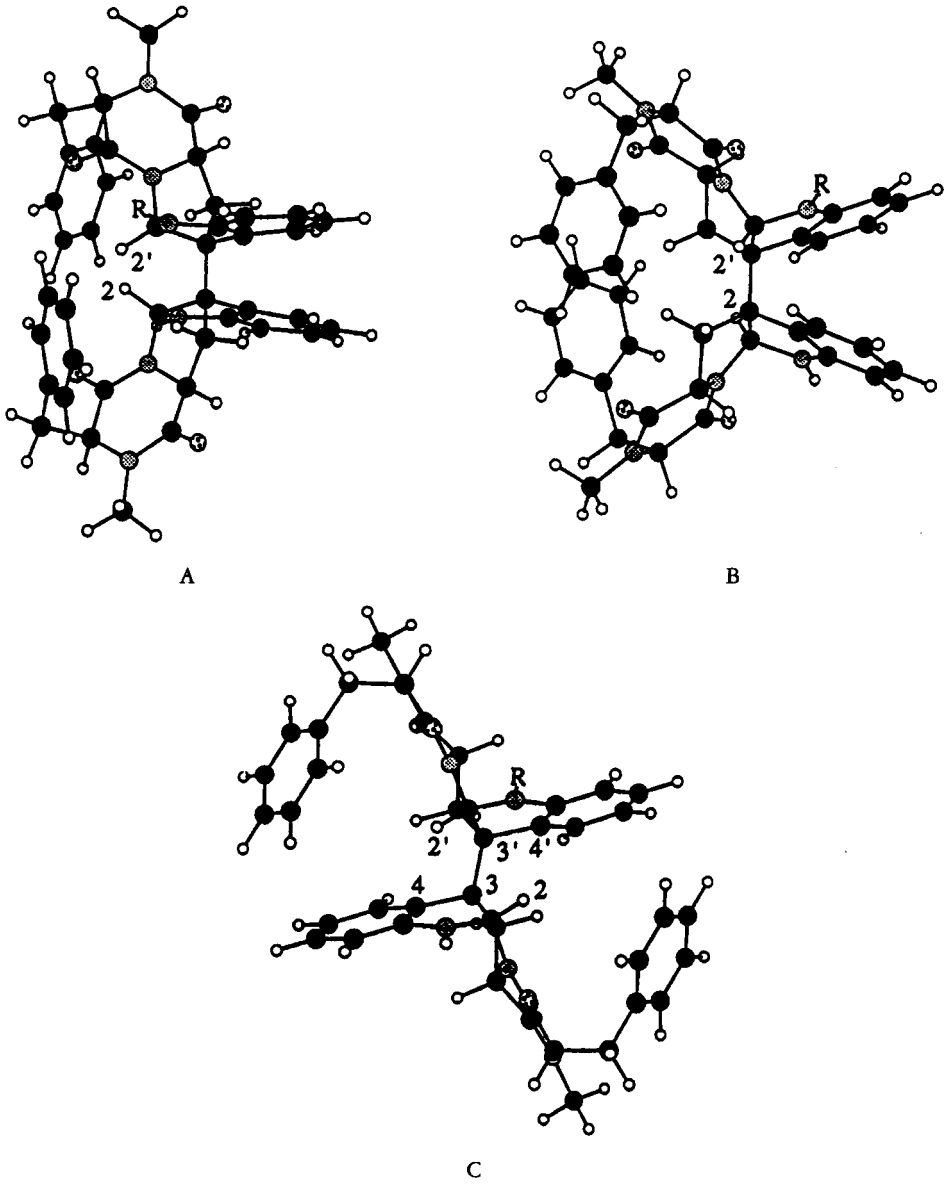


FIGURE 1. Low-Energy Structures of **1** and **2** Obtained Using MACROMODEL. (For **1**, R=CHCHPh; for **2**, R=H. Structures differ in the C-4–C-3–C-3′–C-4′ dihedral angle; −75° for A, 40° for B, 165° for C. An nOe between H-2 and H-2′ for **1** in solution indicates that the predominant solution conformation is A, which is the same as the crystal structure of **2**.)

Compound **1** was inactive as a substance-P inhibitor in our binding assay (5) at concentrations as high as 100 μM. Compound **2** had an IC_{50} of 12 μM, making it considerably less potent than **3** which has an IC_{50} of 0.23 μM. The low potency of compounds **1** and **2** is probably due to their inability to form a low energy conformation resembling that of the probable binding conformation of **3**, which has one indoline and both phenyl groups on the same side of the molecule (Figure 2) (3). Therefore, stereochemistry at the position of dimerization appears to be an important determinant of biological activity in these molecules.

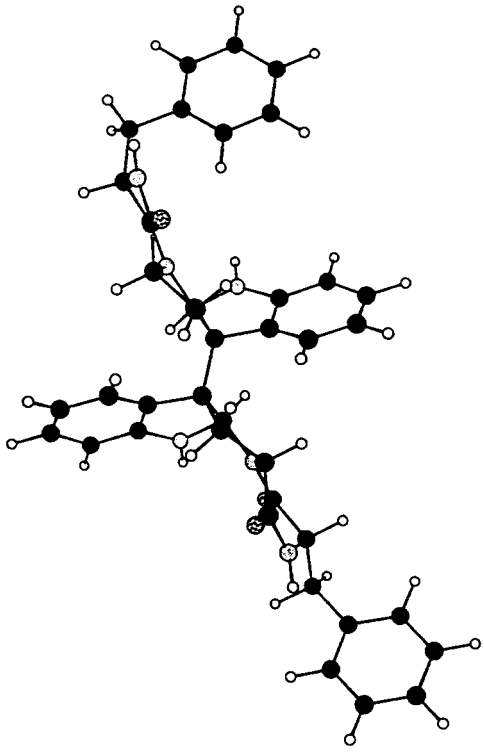


FIGURE 2. Low-Energy Structure and Probable Binding Conformation of **3** (3).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—One- and two-dimensional nmr spectra were recorded on a Bruker AMX 500 spectrometer. Chemical shifts are reported as δ values in ppm referencing $CHCl_3$ relative to TMS. Uv spectra were recorded on a Shimadzu UV160U spectrophotometer. The optical rotation was determined on a Perkin-Elmer 241 polarimeter. Ir spectra were recorded on a Nicolet IBM IR/3X spectrometer. Ms was performed on a Finnigan MAT TSQ-700 mass spectrometer and high-resolution ms data were obtained from M-Scan (Malvern, PA) using a VG ZAB 2-SE mass spectrometer. Hplc was performed on a Waters system with 990 photodiode array detection, 510 pump, 715 ultra WISP, automated gradient controller, and Powermate 386/20 computer.

CULTURE FERMENTATION.—The *Aspergillus flavus* culture MIT-M26 was kindly provided by A.L. Demain (MIT, Cambridge, MA) as a preserved soil culture and added to the Sterling culture collection as SC1661. Stock culture was generated by inoculating either Bennett's or Sabouraud dextrose agar medium with a small amount of the preserved soil suspended in 1 ml of liquid medium consisting of 2% glycerol, 2% dextrin, 1% bacto soytane, 0.3% yeast extract, 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$, and incubating for 5–9 days at 27° (60–70% humidity; New Brunswick Psycrotherm). After purity was established, the plates were washed with sterile freezing medium containing 5% lactose and 10% glycerone, and the resulting cell suspension was stored in 1-ml sample aliquots at −70°.

Seed cultures for inoculation of the production stage were prepared by quick-thawing a 1-ml stock culture suspension and adding this to 30 ml of seed medium in a 250-ml Erlenmeyer flask. The seed medium consisted of 2% glucose, 1.5% Pharmamedia, 0.5% yeast extract, 0.4% $CaCO_3$, 0.3% $(NH_4)_2SO_4$, and 0.003% $ZnSO_4.7H_2O$. All flasks were incubated in a New Brunswick Psycrotherm incubator at 27° with 60–70% humidity for 2 days. Production of compounds **1** and **2** was accomplished by transferring 15 ml of seed culture to a solid-state fermentation medium consisting of 250 g cracked corn plus 250 ml of glycerol-dextrin medium contained in 2.8-liter Fernbach flasks. The flasks were incubated for 10 days at 27° with 60–70% humidity.

Isolation of 1'-(2-Phenyl-ethylene)-ditryptophenaline [**1**] *and ditryptophenaline* [**2**].—*Aspergillus flavus* culture SC1661, grown in two Fernbach flasks, was extracted with EtOAc (1000 ml×2) to give 7 g of extract.

Fatty material, mostly corn oil, was removed by trituration with hexane and the remaining material (1.0 g) was subjected to flash reversed-phase chromatography (MeOH/H_2O gradient) followed by reversed-phase hplc (isocratic, 80:20 MeOH-H_2O). Ditryptophenaline (2) was obtained as a white solid (95 mg). Spectroscopic data including uv, ms, nmr, and optical rotation were in agreement with literature data (1,2). 1′-(2-Phenyl-ethylene)-ditryptophenaline was obtained as a white solid (18 mg). Mp 183–186° (uncorrected) $[\alpha]_D$ −125° (c=0.05, $CHCl_3$); hrfabms m/z M^+ 794.3571, glycerol/thioglycerol ($C_{50}H_{46}N_6O_4$ requires 794.3580); fabms m/z 794 (38), 448 (18), 420 (10), 346 (40), 317 (35), 259 (36), 232 (43), 157 (63), 130 (100); uv λ max (ε) (MeOH) 221 (33800), 244 (11100), 324 (14690), 338 (14180) nm; ir ν max 3348, 3019, 2954, 1666, 1600, 1488, 1460, 1402, 1308, 1216, 1151, 1085, 944, 755, 703 cm^{-1}; ^1H- and ^{13}C-nmr spectra, see Table 1.

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