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Two Novel Diketopiperazines Isolated from the Fungus *Tolypocladium* sp.

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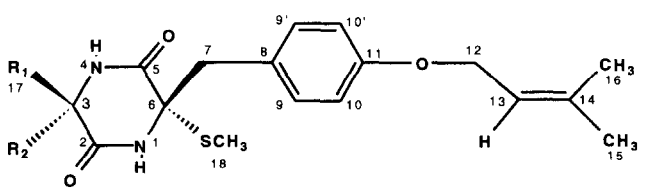
Abstract: *Diketopiperazines, Sch 54794 and Sch 54796, have been isolated from a fungal fermentation. The structures of these compounds have been established based on spectroscopic data analysis. Sch 54794 exhibited inhibitory activity in the platelet activating factor (PAF) assay.*

In the course of searching for bioactive substances from fungal fermentations,[1,2] we have isolated two new compounds, Sch 54794 **(1)** and Sch 54796 **(2)**, from the fermentation of a fungal culture, *Tolypocladium* sp. The microorganism, *Tolypocladium* sp., was isolated from dead twigs from a *Quercus virginiana* Miller, an old live oak tree in the state of Tamalupas, Mexico.[3] The structures of **1** and **2** were determined as *cis* and *trans* isomers by analysis of the spectroscopic data. This paper details the bioassay-guided isolation and structure elucidation.

The fermentation broth (8 L) was extracted with ethyl acetate at harvest pH. The oily residue from EtOAc extraction (3 g) was dissolved in MeOH-CH_2Cl_2 (1:1). After removal of insoluble material, the bioactive soluble portion was concentrated and precipitated with hexane. The precipitate was chromatographed by reversed-phase HPLC with aqueous MeOH (YMC-ODS column 30 x 500 mm, 80-90% MeOH in H_2O with linear gradient in 20 min, 20 mL/min flow rate, UV detection at 210 nm, the active peak t_R = 18.5 min). The active fraction from HPLC was found to be a mixture of two isomers by means of TLC and NMR spectral studies. Attempts to separate the isomers by using silica gel, HP-20P and LH-20 column chromatography with various solvent systems were not successful. It should be noted that these compounds decomposed on the silica gel column, and furthermore, were not separable on either a neutral or basic aluminum oxide



Sch 54794 (**1**): R_1 = H R_2 = SCH_3
Sch 54796 (**2**): R_1 = SCH_3 R_2 = H

column. The separation of the isomer mixture was successfully accomplished by utilizing a polyvinyl alcohol coated silica gel column with a methanol: 1-chlorobutane solvent system (YMC semi-preparative PVA-SIL column 20 X 250 mm, S-5, 2-10% MeOH in n-BuCl with a linear gradient

in 20 min, 8 mL/min flow rate, UV detection at 275 nm, the first peak, **1**, t_R = 22.3 min and the second peak, **2**, t_R = 25.4 min). The pure *cis* isomer, **1**, (10 mg) and *trans* isomer, **2**, (20 mg) were obtained from semi-preparative PVA-SIL column as amorphous white solids after solvent evaporation.

The major component, **2** , mp = 210 °C, $[\alpha]^{25}_D$ = -25° (c 0.1, DMSO), was extensively studied by analysis of spectroscopic data for structure elucidation. The molecular weight of **2** was found to be 380 by fast atom bombardment mass spectroscopy (FAB-MS) that showed a protonated molecular ion m/z 381 $(M+H)^+$. The elemental analysis of **2** indicated the presence of 2 nitrogen and 2 sulfur atoms. The molecular formula was established as $C_{18}H_{24}N_2S_2O_3$ by high resolution FAB-MS (Calcd. for $C_{18}H_{25}N_2S_2O_3$: 381.1307. Found: 381.1302), as well as ^{13}C NMR spectral data. A very strong peak at m/z 333 in FAB-MS was observed as a (M^++H-48) fragment revealing the lose of a methanethiol (CH_3SH) unit. The UV spectrum showed absorptions at 277 and 281 nm indicating an O-alkyl benzene moiety. The IR spectrum displayed bands at 3440, 3200 (br. 2NH) and 1670 cm^{-1} (amide).

Table 1 ^1H(300 MHz) & ^{13}C (75 MHz) NMR Data for Sch 54794 (1) and Sch 54796 (2)[a,b]

	1		**2**	
Position	^1H	^{13}C	^1H	^{13}C
2	--	165.5 s	--	165.6 s
3	4.25 (s)	58.11 d	4.93 (s)	57.80 d
5	--	166.0 s	--	165.9 s
6	--	67.62 s	--	68.06 s
7	3.49, 2.97, (d, 13.7)	44.26 t	3.60, 2.95 (d, 13.7)	42.28 t
8	--	125.2 s	--	126.1 s
9	7.16 (d, 8.6)	131.7 d	7.19 (d, 8.7)	132.0 d
9'	7.16 (d, 8.6)	131.7 d	7.19 (d, 8.87)	132.0 d
10	6.86 (d, 8.6)	114.7 d	6.83 (d, 8.7)	114.6 d
10'	6.86 (d, 8.6)	114.7d	6.83 (d, 8.87)	114.6 d
11	--	158.4 s	--	158.5 s
12	4.50 (d, 6.8)	64.70 t	4.47 (d, 6.7)	64.54 t
13	5.49 (t, 6.8)	119.3 d	5.46 (t, 6.7)	119.4 d
14	--	138.3 s	--	138.3 s
15	1.76 (s)	25.63 q	1.74 (s)	25.10 q
16	1.82 (s)	18.02 q	1.79 (s)	17.46 q
17	2.24 (s)	13.87 q	2.22 (s)	9.75 q
18	2.30 (s)	13.46 q	1.48 (s)	12.21 q

a. Recorded in CDC13 with small amount of CD_3OD, coupling constant (Hz).

b. Assignments are based on APT, COSY and HMBC data.

The ^1H NMR spectral data of **2** which were summarized in Table 1 indicated two vinyl connected methyl singlets at δ 1.74 and 1.79. The chemical shift of a thiomethyl at δ 1.48 suggested a strong shielding influence. An oxy-methylene doublet at δ 4.47 and vinyl proton triplet at δ 5.46 were observed. Two aromatic proton doublets at δ 6.83 and 7.19 represented four protons on 1,4-disubstituted benzene ring due to the symmetrical environment. In addition, a methylene group at δ 2.95 and 3.60 was observed as an AX spin system.

The ^{13}C NMR spectrum of **2** suggested the presence of a diketopiperazine moiety due to two amide carbonyl signals at δ 165.6 and 165.9. A quaternary aromatic carbon resonance at δ 158.5 displayed an oxy-substituted benzene unit. Two vinyl carbon signals at δ 119.4 and 138.3 along with as oxy-methylene carbon at δ 64.54 and two methyl carbons at δ 17.46 and 25.10 were assigned to an isopentenyl group.

The proton and carbon assignments above were supported by COSY experiments. The COSY spectral data showed the oxy-methylene doublet coupled with a vinyl methane triplet and two methyl singlets to form partial structure A. As shown in Figure 1, unit B was established based on the correlation of the AX methylene quartet to the aromatic doublet (H-9, H-9'), which was further coupled to the other aromatic doublet (H-10, H-10'). The remaining part of the molecule was assigned as a trisubstituted diketopiperazine C.

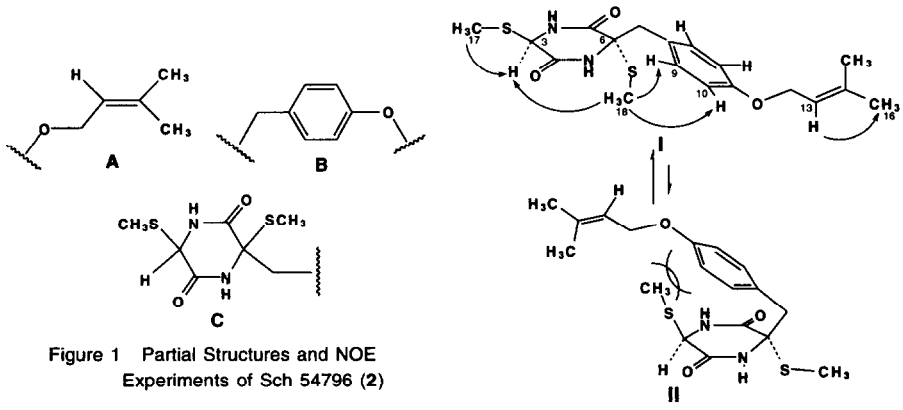


Figure 1 Partial Structures and NOE Experiments of Sch 54796 (**2**)

In order to complete the structural assignments for **2**, a heteronuclear multiple bond correlation (HMBC) experiment was conducted.[4] The presence of diketopiperazine ring was confirmed by the observation of cross peaks from the H-3 to C-2, C-5 and C-17. Diketopiperazine C and the benzene ring were connected through the methylene group because of the correlation between H-7 and C-2, C-5 and C-6, as well as C-8, C-9 and C-9'. Since the oxy-methylene CH_2-12 correlated to C-11, the isopentenyl group was obviously linked to the benzene ring at 11-position through a oxygen atom.

The stereochemistry of **2** was established by difference NOE experiments. As depicted in Figure 1 an NOE signal at H-3 was observed when the CH_3-18 protons were irradiated. This

evidence revealed the *trans* arrangement of two thiomethyl groups on diketopiperizine ring. Furthermore, the NOE resonance of H-9 and H-10 was also detected with the irradiation of CH_3-18 protons. This data suggested that the conformation **I** of **2** was dominating over the conformation **II** due to a steric hindrance between CH_3-18 group and aromatic side-chain. Therefore, the structure elucidation of **2** was completed except for the absolute stereochemistry.

The compound **1**, mp = 180-182 °C, $[\alpha]^{25}_D$ = -70° (c 0.1, DMSO), possessed the same molecular weight as **2** based on FAB-mass spectral data that showed a protonated molecular ion m/z 381 $(M+H)^+$. The UV and IR spectra of **1** were also identical to **2**. Both ^1H and ^{13}C NMR data suggested that **1** is an isomer of **2** by direct comparison of their spectra. It should be noted that the up-field chemical shift of H-3 singlet at δ 4.25 indicated a strong shielding effect in **1**. The *cis* isomer of **1** was proposed based on the difference NOE experiment. The only NOE signal of CH_3-17 was observed when H-3 singlet was irradiated. This result also revealed that conformation II was more favorable than conformation I because of the lack of a steric hindrance between H-3 and aromatic group.

The *trans* isomer, which is similar to other diketopiperazines reported as PAF inhibitors in literature,[5-7] displayed weak inhibitory activity in PAF assay with an IC_{50} value at 50 μM. However, the *cis* isomer was found to be inactive (IC_{50} >100 μM).

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3. The fungus was supplied by Dr. B. Katz from MYCOsearch.
4. HMBC correlations (^1H signal---^{13}C correlations through 2-bond, 3-bond and 4-bond, respectively): H-3---C-2 (2-bond), C-5, C-17 (3-bond); CH_2-7---C-6, C-8 (2-bond), C-5, C-9, C-9' (3-bond); H-9---C-10 (2-bond), C-7, C-9', C-11 (3-bond), C-10' (4-bond); H-9'---C-10' (2-bond), C-7, C-9, C-11 (3-bond), C-10 (4-bond); H-10---C-11 (2-bond), C-8, C-10' (3-bond); H-10'---C-11 (2-bond), C-8, C-10 (3-bond); CH_2-12---C-13 (2-bond), C-11, C-14 (3-bond), C-15 (4-bond); H-13---C-15, C16 (3-bond); CH_3-15---C-14 (2-bond), C-13, C-16 (3-bond); CH_3-16---C-14 (2-bond), C-13, C-15 (3-bond); CH_3-17---C-3 (3-bond); CH_3-18---C-6 (3-bond).
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