Sch 56396: A New *c-fos* Proto-oncogene Inhibitor Produced by the Fungus *Tolypocladium* sp.

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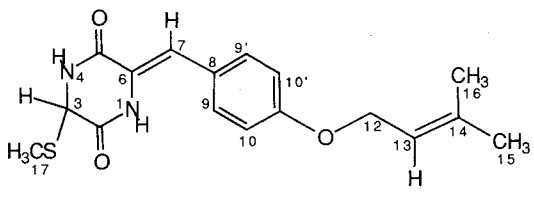
Our previous investigation of searching for novel *c-fos* proto-oncogene inhibitors led to the discovery of two new diketopiperazines related to verticillin family of compounds from the fungus, *Gliocladium* sp.[1] As a result of continued efforts of our natural product program in the anticancer therapy area, a new compound, Sch 56396 (**1**), was isolated from the fermentation broth of a fungal culture (SCF-0729), *Tolypocladium* sp. This study describes the fermentation, isolation, structure elucidation and biological activity of **1**.

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†. The fungus was grown in a two stage germination prior to inoculation into a large-scale fermentation. The first stage germination consisted of inoculating 2.5 ml of a frozen whole broth sample of the producing strain in a 250 ml Erlenmeyer flask containing 50 ml of a medium consisting of (g/liter): Difco yeast extract 3 g, proteus peptone 5 g, NaCl 5 g, KH_2PO_4 5 g, cerelose 2 g, soybean grits 5 g, Dow Corning antifoam 3 drops in 1 liter tap water, and the pH was adjusted to 7.0 ($\pm$0.2) with NaOH before autoclaving. The seed culture was incubated at 24°C for 96 hours on a New Brunswick Scientific (NBS) two tier shaker at 300 rpm. For the second stage inocula, 25 ml of the first seed was transferred to a 2 liter Erlenmeyer flask containing 500 ml of the germination media and incubated under the same conditions as described above. Fermentation was initiated by transferring 500 ml of the second stage germination to a 2 liter Erlenmeyer flask containing a media consisting of (g/liter) neopeptone 10 g and cerelose 40 g in 1 liter tap water and the pH was adjusted to 7.0 ($\pm$0.2) with NaOH before autoclaving. The fermentation was carried out at 24°C for 6 days on a NBS two tier shaker at 300 rpm.

The fermentation broth (8 liters) was extracted with ethyl acetate at harvest pH (6.6). The oily residue from EtOAc extraction (2.5 g) was dissolved in MeOH-CH_2Cl_2 (1:1) to remove insoluble material. The bioactive soluble portion (1.5 g) was concentrated and precipitated with hexane. The active precipitate was chromatographed by two consecutive Diaion CHP-20P columns eluting with a step-gradient of MeOH-H_2O and CH_3CN-H_2O solvent systems, respectively. The enriched complex was further purified by a Sephadex LH-20 column eluting with MeOH-CH_2Cl_2 (1:1, v/v). Final purification of **1** was achieved by a polyvinyl alcohol polymerized silica gel column (PVA-Sil) with a MeOH/*n*-BuCl solvent mixture (YMC semi-preparative PVA-Sil column, 20 × 250 mm, S-5, 120 Å, 2~10% MeOH in *n*-BuCl with a linear gradient in 20 minutes, 8 ml/minute flow rate, UV detection 275 nm). Pure **1** (18 mg) was obtained as a pale-yellow solid and the physico-chemical properties are listed in Table 1.

The molecular weight of **1** was determined to be 332 by fast atom bombardment mass spectroscopy (FAB-MS) that showed a protonated molecular ion *m/z* 333 $(M+H)^+$. The molecular formula was deduced as $C_{17}H_{20}N_2SO_3$ by high resolution FAB-MS (Calcd. for $C_{17}H_{21}N_2SO_3$: 333.1273. Found: 333.1264) and ^{13}C NMR spectral data. A peak at *m/z* 285 in FAB-MS was observed as a $(M+H-48)^+$ fragment indicating the loss

Fig. 1. The structure of Sch 56396 (**1**).



Sch 56396 (**1**)

Table 1. Physico-chemical properties of Sch 56396 (**1**).

Appearance:	Pale-yellow powder
Melting point:	170~172°C
Molecular formula:	$C_{17}H_{20}N_2SO_3$
FAB-MS (*m/z*):	333 $(H+H)^+$
HR FAB-MS:	Calcd. 333.1273
($C_{17}H_{21}N_2SO_3$)	Found. 333.1264
$[\alpha]_D^{26}$ ($CHCl_3$)	−9.0° (*c*, 0.2)
UV (MeOH) ν_{max} nm (ε):	231 (18,260), 323 (22,908)
IR (KBr) ν_{max} cm^{-1}:	3442, 3221, 3066, 1670, 1604, 1516, 1408, 1391, 1267, 1189, 827

† The fungus was supplied by Dr. B. KATZ from MYCOsearch Lab.

of a methanethiol (CH_3SH) unit. The IR spectrum showed bands at 3442, 3221 (br. 2NH), 1670 (amide) and 1604 cm^{-1} (conjugated amide).

The ^1H NMR spectrum of **1** (Table 2) displayed two vinyl attached methyl singlets at δ 1.70 and 1.76, and a thiomethyl singlet at δ 2.19. A methylene doublet at δ 4.55 suggested the oxygen attachment. The chemical shift of a methine singlet at δ 4.94 revealed its bis-heteroatom connectivity. A vinyl triplet at δ 5.42 and a vinyl singlet at δ 6.69 were observed. Two aromatic proton doublets at δ 6.98 and 7.48 represented four protons on the 1,4-disubstituted benzene ring due to the symmetrical environment. In addition, two NH proton singlet at δ 9.01 and 10.14 were observed as exchangeable signals using DMSO-d_6 solvent (Table 2).

The ^{13}C NMR spectrum indicated the presence of a diketopiperazine moiety due to two amide carbonyl signals at δ 163.6 and 164.4. Two vinyl carbons at δ 118.0 and 123.3 reflected a trisubstituted double bond. A quaternary aromatic carbon resonance at δ 159.6 together with the other five aromatic carbons at δ 115.5 ~ 130.4 displayed an oxy-substituted benzene unit. Two vinyl carbon signals at δ 119.2 and 138.8 along with an oxy-methylene carbon at δ 64.82 and two methyl carbon at δ 17.81 and 25.40 were assigned to an isopentenyl group.

Table 2. ^1H and ^{13}C NMR spectral data for Sch 56396 (**1**)[a].

Position	^1H (DMSO-d_6)	^1H ($CDCl_3$)[b]	^{13}C ($CDCl_3$)
1	10.14 (s, NH)	—	—
2	—	—	163.6 s
3	4.94 (br s)	5.00 (s)	58.01 d
4	9.01 (br s, NH)	—	—
5	—	—	164.4 s
6	—	—	123.3 s
7	6.69 (s)	6.99 (s)	118.0 d
8	—	—	124.9 s
9	7.48 (d, 8.8)	7.34 (d, 8.5)	130.4 d
9′	7.48 (d, 8.8)	7.34 (d, 8.5)	130.4 d
10	6.98 (d, 8.8)	6.97 (d, 8.5)	115.5 d
10′	6.98 (d, 8.8)	6.97 (d, 8.5)	115.5 d
11	—	—	159.6 s
12	4.55 (d, 6.2)	4.55 (d, 6.8)	64.82 t
13	5.42 (t, 6.2)	5.48 (t, 6.8)	119.2 d
14	—	—	138.8 s
15	1.70 (s)	1.76 (s)	25.40 q
16	1.76 (s)	1.81 (s)	17.81 q
17	2.19 (s)	2.28 (s)	12.38 q

[a] Recorded at 300 MHz for ^1H spectrum and 75 MHz for ^{13}C spectrum, respectively, coupling constant (Hz).
[b] A small amount of CD_3OD was added to enhance the solubility, NH amide protons exchanged with CD_3OD.

The assignments above were confirmed by COSY experiments. The COSY spectral data indicated the oxy-methylene doublet coupled with a vinyl methine triplet and two methyl singlets to form partial structure **A**. As shown in Figure 2, unit **B** was established based on the coupling of the vinyl methine singlet 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 diketopiperazine moiety **C**.

Selective INEPT experiments were performed to complete the structural assignments of **1**. The presence of diketopiperazine ring was confirmed by the observation of correlations of H-3 to C-2, C-5 and C-17. Diketopiperazine **C** and the benzene ring **B** were connected through the double bond (C_6-C_7) due to correlations of vinyl proton H-7 to C-2 and C-5, as well as to C-9 and C-9′. The isopentenyl **A** was linked to the **B** at position-11 through an oxygen atom based on the correlation of H-12 to C-11.

The configuration of the double bond (C_6-C_7) was determined by difference NOE experiments. The NOE correlations of NH-1 to H-9 (or H-9′) and H-7 to H-9′ (or H-9) were observed without showing the correlation of NH-1 to H-7. This evidence revealed that the vinyl proton H-7 should be *trans* to the nitrogen N-1 but not *cis*.

The stereochemistry of chiral center C-3 for **1**, however, can not be determined by spectroscopic methods. Several attempts failed to obtain single crystals of **1** for X-ray diffraction analysis using different solvent systems. Therefore, derivatization of **1** is necessary in order to provide a suitable crystal from its derivative for X-ray crystallographic study.

Intracellular induction of the *c-fos* proto-oncogene is among the earliest events in the transition of cells from a quiescent to a growing state, and is induced by a variety

Fig. 2. Partial structures of Sch 56396 (**1**).

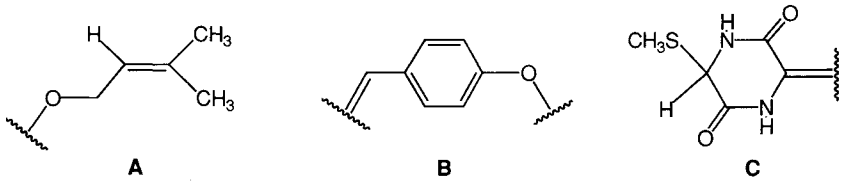
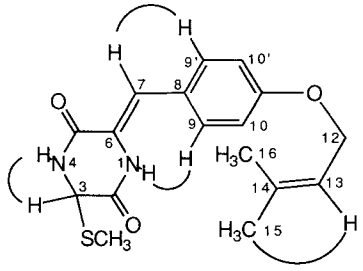


Fig. 3. Important difference NOE data for Sch 56396 (**1**).



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of mitogenes, including serum, platelet derived growth factor and epidermal growth factor.[2~4] Since oncogene expression of *v-src*, *v-raf* and *Ha-ras* has been shown to induce *c-fos*,[5~7] and certain tumor cell lines have been reported to possess constitutively elevated levels of *c-fos*,[8] inhibitors of *c-fos* proto-oncogene induction could serve as therapeutic agents for the control of neoplastic disease. Compound **1** demonstrated an inhibitory activity *in vitro* against serum-stimulated transcription of the human promoter in the *fos/lac* Z reporter gene assay with the IC_{50} value of 15 μM. The cytotoxicity study of **1** showed $IC_{50}=39$ μM in the MTT assay.[9,10]

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