Inhibition of *c-fos* Proto-oncogene Induction by Sch 52900 and Sch 52901, Novel Diketopiperazines Produced by *Gliocladium* sp.

MIN CHU*, IMBI TRUUMEES, MARNIE L. ROTHOFSKY, MAHESH G. PATEL, FRANK GENTILE, PRADIP R. DAS, MOHINDAR S. PUAR and STANLEY L. LIN

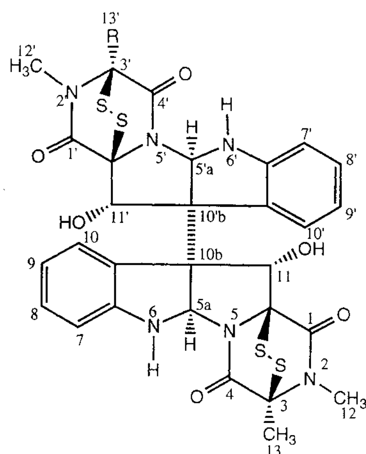
Schering-Plough Research Institute,
2015 Galloping Hill Road, Kenilworth, NJ 07033-0359, U.S.A.

(Received for publication May 24, 1995)

Sch 52900 (1) and Sch 52901 (2), two new inhibitors of *c-fos* proto-oncogene induction, have been isolated from the fermentation broth of the fungal culture (SCF-1168), *Gliocladium* sp. Along with compounds 1 and 2, a known compound verticillin A (3) was also obtained from the culture. Structure elucidation of 1 and 2, accomplished by analysis of spectral data in comparison with the data of 3, revealed both 1 and 2 were found to be closely related to the verticillin family of diketopiperazines. All three compounds prevented serum-stimulated transcription of the human *c-fos* promoter, using a *fos/lac Z* reporter gene assay, with IC_{50} values of 1.5, 18 and 0.5 μM for 1, 2 and 3, respectively. Northern analysis revealed that exposure of cells to compound 3 causes inhibition of both phorbol ester-induced *c-fos* induction and serum-induced JE induction in the absence of inhibiting RNA synthesis, as measured by [^{3}H]uridine incorporation. These results suggest that this class of compounds exerts antitumor activity by blocking a signal transduction pathway that is common to and necessary for the induction of at least a subset of immediate early genes involved in cell proliferation.

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 of mitogens, including serum, platelet derived growth factor and epidermal growth factor[1~3]. In addition, oncogene expression of *v-src*, *v-raf* and *Ha-ras* has been shown to induce *c-fos*[4~6], and certain tumor cells have been shown to possess constitutively elevated levels of *c-fos*[7]. This suggests that inhibitors of *c-fos* proto-oncogene induction could serve as therapeutic agents for the control of neoplastic disease. Utilizing a *fos/Lac Z* reporter gene system to identify inhibitors of *c-fos* proto-oncogene induction from microbial sources, we identified three diketopiperazines, isolated from a fermentation broth of the fungus (culture SCF-1168), *Gliocladium* sp., with potent activity to inhibit *c-fos* proto-oncogene induction. The minor components, Sch 52900 (1) and Sch 52901 (2), were found to be novel compounds closely related to the major component, verticillin A (3), previously identified as an antitumor compound[8]. In this paper, we report the fermentation, isolation, structure determination and mechanism of antitumor activity of 1, 2, and 3.

Fig. 1. The structures of 1, 2 and 3.



Materials and Methods

General

The optical rotations were measured on a Rudolph Research model 'AUTOPOL III' automatic polarimeter. The melting points were determined on a MEL-TEMP apparatus (Laboratory Devices, Cambridge, MA) and are uncorrected. IR spectra were obtained using a Nicolet FTIR Model 10-MX instrument. UV spectra were recorded on a Hewlett Packard '8450A' UV-vis spectrophotometer. FAB-mass spectra were produced on a

VG-ZAB-SE double focussing mass spectrometer using a 3-nitrobenzyl alcohol (NBA) matrix. High resolution FAB-MS were recorded on a JOEL HX 110A double focussing mass spectrometer. Both ^{1}H and ^{13}C NMR spectra were obtained from a Varian XL-300 instrument at 300 and 75 MHz, respectively.

Microorganism

The fungus, *Gliocladium* sp. (Culture SCF-1168), was collected and isolated from freshly fallen dicot leaf litter in the El Yunque rainforest of Puerto Rico*.

Fermentation

The fungus was grown in a two stage germination prior to inoculation into large-scale fermentation. In stage one, two milliliters of a frozen whole broth (stored at −80°C with 10% glycerol added) were inoculated in germination medium containing 70 ml of the following ingredients: proteus peptone #3 0.5%, NaCl 0.5%, KH_2PO_4 0.5%, Difco yeast extract 0.3%, cerelose 2.0%, soybean grits 0.5%, Dow Corning antifoam 2 ml in tap water, and the pH was adjusted to 7.0 (±0.2) with NaOH before autoclaving. In stage two, twenty-five milliliters of the stage one germination were inoculated into 500 ml of the same germination medium listed above. Each germination stage was shaken on a New Brunswick Scientific (NBS) two tier shaker at 300 rpm for 90 hours at 24°C. Five hundred milliliters of the pooled stage two germination was inoculated into 100 liters of fermentation medium containing the following ingredients: neopeptone 1.0%, cerelose 4.0%, and $CaCO_3$ 0.4% in tap water, and the pH was adjusted to 7 (±0.2) with NaOH before autoclaving. The fermentation stage was carried out in a Biolafitte (LSL Biolafitte) 100 liter fermentor at 350 rpm agitation, 55.0 lpm aeration, and 24°C for 6 days.

Construction of the *fos/lac Z* Reporter Gene

An intron-containing *lac Z* plasmid construct was made by inserting a favorable translation start codon, preceded by a Pst 1 site, at the Kpn 1 site of pCH110 (Pharmacia), then isolating the 3.2 kb Pst 1/Dra 1, *lac Z*-containing fragment and inserting it into the Pst 1 site 3′ to the modified SV40 intron in the Okayama-Berg pCD vector[9]. The coding sequences from the human *c-fos* gene[10] were removed by Nae 1 digestion, and replaced with a blunt-ended 3.4 kb Xho 1/Xba 1 fragment, containing the intron and *lac Z* gene. The *fos/lac Z* reporter gene was further modified to remove the cyclic AMP response element (CRE) by Nar 1 cleavage at -9 and -80, and replacing the fragment with a synthetic oligonucleotide lacking nucleotides -73 through -43. The resultant construct contains all known enhancer elements except for the CRE, contains most of the 3′ untranslated region, and utilizes the *c-fos* polyadenylation signals. BALB/c 3T3 clone A31 cells (ATCC) were grown in DULBECCO's modified EAGLE's medium supplemented with 10% bovine calf serum (D10). Cells were co-transfected with the *fos/lac Z* reporter gene plasmid and a neomycin resistance gene driven by the Herpes thymidine kinase promoter using calcium phosphate[11], and stably transfected cells were selected in D/10 supplemented with 400 μg/ml G418. Resistant colonies were isolated by overlaying colonies with 0.6% agar in D/10, scraping colonies with a 1 ml pipette, and transferring cells to 15 mm dishes until ready for assay for serum-inducible β-galactosidase activity. A clonal cell line, denoted 68-220, was utilized to identify compounds which inhibit the serum-mediated induction of β-galactosidase activity. For the assay, quiescent, serum starved Clone 68-220 cells were treated overnight with varying concentrations of each compound. The following day, replicate monolayers were stimulated by addition of serum, to a final concentration of 20%, directly to the medium. Three hours after serum addition, monolayers were lysed and assayed for β-galactosidase activity as described below. To normalize for nonspecific toxicity, the ratios of serum-stimulated *vs.* unstimulated monolayers were determined at each compound concentration, and IC_{50} values were defined as the concentration required to give a stimulated/unstimulated ratio midway between the stimulated/unstimulated controls, and the endpoint ratio of 1 : 1 (stimulated/unstimulated), and were determined by linear regression analysis.

β-Galactosidase Assays

β-galactosidase activity was measured by a modification of the procedure provided by Pharmacia. Briefly, quiescent monolayers of Clone 68-220 cells were treated with serial 1 : 2 dilutions of testing compound in medium supplemented with 0.5% bovine calf serum. The following day, serum was added to replicate monolayers and cultures were incubated an additional 3 hours at 37°C to permit β-galactosidase induction. Unstimulated and serum-stimulated monolayers were rinsed twice with phosphate-buffered saline, then lysed in 10 μl of ice-cold *lac Z* extraction buffer (Z buffer + 0.5% NP-40 + 20% glycerol) for 20 minutes on ice. Following lysis, 50 μl of a 0.8 mg/ml solution of O-nitrophenyl-β-D-galactopyranoside dissolved in Z buffer (10 mM KCl + 1 mM $MgSO_4$ + 50 mM mercaptoethanol + 0.1 M Na_2PO_4, pH 7.0) was added to each well, and plates were incubated at 37°C for 4 hours. The reaction was terminated by addition of 30 μl of a freshly made solution of 1 M Na_2CO_3 + 0.3% sodium dodecylsulphate, and absorbance at 420 nm was measured.

Effects of Compound **3** on Gene Induction

In BALB/c 3T3 cells, induction of *c-fos* occurs predominantly through a protein kinase C-mediated pathway[12]. To evaluate the ability of **3** to block protein kinase C-mediated induction of the endogenous murine

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

c-fos, quiescent monolayers of 68-220 cells were exposed to 10 and 20 nM of **3** for 3 hours. Following treatment, TPA (12-*O*-tetradecanoylphorbol-13-acetate) was added directly to the **3**-containing medium to a final concentration of 300 nM. Following an additional incubation at 37°C for 1 hour, cells were harvested. Total RNA was extracted by the method of KEATH *et al.*[13]. Briefly, cells were scraped into PBS and transferred to a 15 ml conical centrifuge tube, pelleted in a refrigerated tabletop centrifuge, and lysed in NP-40 lysis buffer (0.14 M NaCl + 10 mM Tris, pH 8.6 + 1.5 mM $MgCl_2$ + 0.5% NP-40). Nuclei were pelleted by centrifugation and the supernatant transferred to a sterile 1.5 ml reaction vial. An equal volume of 2X proteinase K buffer (0.3 M NaCl + 25 mM EDTA + 2% sodium dodecylsulphate + 0.2 M Tris, pH 7.5) was added, followed by addition of proteinase K to a final concentration of 200 μg/ml, and cell extracts were incubated at 37°C for 15 minutes. Digests were extracted four times with phenol - chloroform - isoamyl alcohol (25 : 24 : 1), and RNA was precipitated with two volumes of ethanol. Approximately 25 μg total RNA from each was separated on formaldehyde agarose gels, transferred and bound to nylon membranes (Hybond, Amersham), then probed for *fos*, using the 300 bp Ncol/Avr2 fragment of pc-fos-1 (ATCC), or JE with the 600 bp Eco R1 fragment of pcJE-1 (ATCC)[12].

Results and Discussion

Isolation

The fermentation broth (40 liters) was extracted with ethyl acetate at harvest pH. The EtOAc extract was evaporated *in vacuo*, and the residue applied to a silica gel column, and eluted with a 2 ~ 10% tetrahydrofuran (THF) gradient in dichloromethane. The active fractions (8 ~ 9% THF/CH_2Cl_2) were combined based on the *c-fos* bioassay data, and precipitated with acetonitride to obtain a light yellow-grey powder. The precipitate was separated by semi-preparative reverse-phase HPLC (YMC-ODS 50 × 500 mm column, irregular 15 μ particles, mobile phase: isocratic aqueous 90% methanol, flow rate: 20 ml/minute, detection: UV at 305 nm, sample dissolved in DMSO - MeOH (1 : 1 solution) to afford three active components **1**, **2** and **3** with the retention times of 16.3, 18.9 and 17.7 minutes, respectively. Each component was finally purified on a Sephadex LH-20 gel column eluting with $CHCl_3$ - MeOH (1 : 1) to yield pure **1** (29 mg), **2** (18 mg) and **3** (44 mg). The purification procedure is summarized in Scheme 1. Compounds **1**, **2** and **3** were white amorphous powders. The physico-chemical properties of **1** and **2** are listed in Table 1. All

Scheme 1. Isolation procedure of culture SCF-1168.

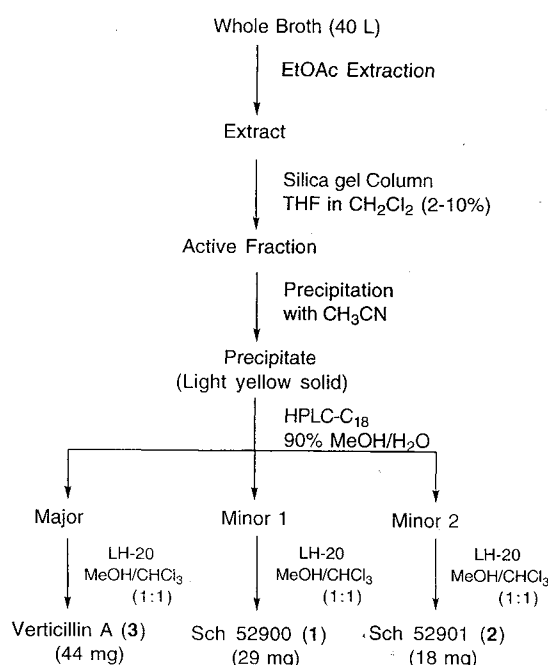


Table 1. Physico-chemical properties of **1** and **2**.

	1	2
Molecular Formula	$C_{31}H_{30}N_6O_7S_4$	$C_{31}H_{30}N_6O_6S_4$
M. P. °C	202-205 (dec.)	199-200 (dec.)
HRFAB-MS (m/z)	727 (M + H)+	711 (M + H)+
Calcd:	727.1137	711.1188
Found:	727.1124	711.1177
$[\alpha]_D^{22}$ ($CHCl_3$)	+768.9° (*c* 0.1)	+ 688.2° (*c* 0.1)
UV (MeOH) λ_{max} nm	242, 301	241, 305
IR (KBr) υ_{max} cm^{-1}	3412, 2924, 1684, 1674, 1608, 1469, 1351, 1303, 1247, 1094, 1068, 765	3390, 2923, 1690, 1668, 1610, 1469, 1349, 1302, 1247, 1203, 1093, 1065, 756

three *c-fos* inhibitors are soluble in $CHCl_3$, CH_2Cl_2, MeOH, DMSO and THF, but insoluble in hexane and water.

Structure Elucidation

The structures of **1** and **2** were determined by analysis of spectroscopic data including UV, IR, MS (Table 1), ^1H and ^{13}C NMR (Tables 2 and 3). ^1H and ^{13}C NMR spectral data indicated that both **1** and **2** belong to the verticillin family by direct comparison to the major component **3**, which is identical to verticillin A reported in literature[8] (also see Tables 2 and 3 for NMR data).

Table 2. ^1H NMR chemical shift assignment and coupling data of **1**, **2** and **3**[a].

Proton	1	2	3
5a, 5'a	5.78 s, 5.80 s[b]	5.77 S	5.77 S
7, 7'	6.70 d, 6.74 d (7.8)[b,c]	6.70 d (7.7)	6.72 d (7.8)
8, 8'	6.88 t, 6.90 t (7.4)[c]	6.88 t, 6.86 t (7.5)[c]	6.87 t (7.4)
9, 9'	7.20 t (7.5)	7.19 q (4.3)	7.19 t (7.5)
10, 10'	7.88 d, 7.86 d (7.3)[c]	7.88 d, 7.86 d (7.1)[c]	7.87 d (7.8)
11,11'	5.18 s, 5.15 s[c]	5.19 s	5.19 s
12, 12'	3.03 s, 3.21 s	3.03 s, 3.05 s[c]	3.04 s
13, 13'	1.93 s, 4.51 q (6.6)	1,92 s, 2.30 m (12-line)	1.93 s
14'	1.67 d (6.6)	1.26 t (7.4)	-------

[a] Measured at 300 MHz in $CDCl_3$; chemical shifts in ppm from TMS.
[b] Multiplicity; coupling constant in parenthesis (Hz).
[c] The assignments may be interchangeable.

The molecular weight of **1** was established as 726 from fast atom bombardment (FAB) mass spectral data that showed a protonated molecular ion, $(M+H)^+$, at *m/z* 727. A fragment ion, $(M-64)^+$, was detected in the mass spectrum which suggested the presence of a disulfide moiety in the molecule. The molecular formula was deduced as $C_{31}H_{30}N_6O_7S_4$ from high resolution FAB-MS (Calcd: 727.1137, measured: 727.1124 for $(M+H)^+$ for $C_{31}H_{31}N_6O_7S_4$), ^1H and ^{13}C NMR data. The UV spectrum showed absorbencies at 242 and 301 nm. The IR spectrum showed bands at 3412 (br. NH and OH), 1684 and 1674 cm^{-1} (two amides).

The ^{13}C NMR spectrum of **1** clearly revealed an unsymmetrical dimeric structure because some carbon signals appeared to be split due to the presence of two slightly different subunits in comparison with **3**. However, six aromatic carbon and other oxygen/nitrogen-attached carbon signals did not split. Four amide carbonyl signals from δ 161.4 to δ 166.6 indicated two different diketopiperazine units. Chemical shift differences between the two alkyl methyl ($C-CH_3$) and nitrogenated methyl ($N-CH_3$) carbons for **1** were also observed. The signal of an oxygenated methine carbon at δ 66.8 was assigned as a secondary alcohol at C-13' position.

^1H NMR data were consistent with ^{13}C NMR data, which showed an unsymmetrical dimer. A doublet of

Table 3. ^{13}C NMR chemical shift assignments of **1**, **2** and **3**[a].

Carbon	1	2	3
1, 1'	162.0 s, 161.4 s[b]	161.9 s, 161.0 s[b]	162.4 s
3, 3'	76.74 s, 76.34 s[b]	76.73 s, 76.34 s[b]	76.61 s
4, 4'	166.6 s, 165.7 s[b]	166.7 s, 165.8 s[b]	166.3 s
5, 5'	81.73 d	81.54 d	81.98 d
5a, 5'a	81.73 d	81.54 d	81.98 d
6, 6'	148.8 s	148.2 s	148.7s
6a, 6'a	148.8 s	148.2 s	148.7s
7, 7'	110.3 d	110.3 d	110.9 d
8, 8'	128.1 d, 128.0 d[b]	127.7 d	128.2 d
9, 9'	120.0 d, 119.9 d[b]	120.0 d, 119.9 d[b]	120.5 d
10, 10'	129.6 d	129.5 d	130.0 d
10a, 10'a	129.5 s, 129.4 s[b]	128.9 s	129.5 s
10b, 10'b	65.84 s, 65.76 s[b]	65.31 s, 65.24 s[b]	65.87 s
11, 11'	82.62 d, 82.58 d[b]	82.69 d, 82.53 d[b]	82.99 d
11a, 11'a	73.12 s	72.39 s	72.97 s
12, 12'	27.07 q, 28.45 q[b]	27.53 q, 26.69 q[b]	27.23 q
13, 13'	17.43 q, 66.81 d	17.00 q, 24.06 t	17.53 q
14'	19.60 q	9.35 q	---

[a] Measured at 75 MHz in $CDCl_3$, chemical shifts in ppm from TMS.
[b] Multiplicity was determined by DEPT data. The assignments are interchangeable.

methyl protons at δ 1.67 (J=6.6 Hz) was coupled with a quartet of methine proton at δ 4.51 (J=6.6 Hz) which again suggested a secondary alcohol, CH(OH)CH_3 group, in the molecule. The remaining proton assignments were comparable to **3**. Based on the above evidence, the structure of **1** was proposed as indicated in Figure 1 showing a dimeric structure consisting of two slightly differing monomers.

The molecular weight of **2** was established as 710 from FAB-MS that showed a protonated molecular ion, $(M+H)^+$, at m/z 711. The compound also contained a disulfide moiety which is supported by the mass spectrum (a loss of 64 mass units due to S_2). The molecular formula was deduced as $C_{31}H_{30}N_6O_6S_4$ from high resolution FAB-MS (Calcd: 711.1188, measured: 711.1177 for $(M+H)^+$ of $C_{31}H_{31}N_6O_6S_4$) and NMR spectral data. The UV and IR spectra were very similar to **1**, indicating that they are structurally related to each other.

As shown in Table 3, a methylene at δ 24.06 along with a methyl carbon at δ 9.35 displayed an ethyl group at C-13′ and C-14′ positions. The presence of CH_2CH_3 group was supported by ^1H NMR spectrum which showed the AB multiplets of methylene protons at δ 2.30, and the triplet of methyl protons at δ 1.26 (J=7.4 Hz). The assignments of remaining carbons and protons appeared to be comparable to **1** and a proposed structure of **2** is shown in Figure 1.

The stereochemistry for both compounds **1** and **2** was assumed to be the same as **3** by a direct comparison of their optical rotation ($[\alpha]_D$ + 727.5° for verticillin A) and circular dichroic (CD) spectral data with the same Cotton effects observed: positive maximum absorptions at 236 and 307 nm, negative maximum absorptions at 272 and 375 nm[8].

Biological Activity

It has previously been demonstrated that a *fos/lac Z* fusion gene can function as a *fos* promoter-dependent reporter gene, in a manner analogous to the endogenous *c-fos* proto-oncogene, following introduction into BH104 neuroblastoma cells[14]. We have constructed a similar *fos/lac Z* reporter gene, but lacking the cyclic AMP response element, and transfected the gene into mouse BALB/c clone A31 cells. Compounds **1**, **2** and **3** exhibited an inhibitory activity *in vitro* with IC_{50} values at 1.5, 18, and 0.5 μM, respectively.

Compound **3** was chosen for biological evaluation because it showed the most potent inhibitory activity in the *fos/lac Z* reporter gene assay. Even though **3** has been previously reported as an antitumor agent, the mechanism of action remained unknown. To further explore the mechanism of action for verticillin family of compounds, the mechanistic studies of **3** were performed.

Quiescent BALB/c 3T3 cells were stimulated by addition of TPA (300 nM final concentration) or serum (20% final concentration). One hour after stimulation, total RNA was extracted and probed for the induction of *c-fos* (Fig. 2, A) and JE (Fig. 2, B), respectively. In the absence of **3**, both *c-fos* and JE were induced greater than 20-fold. Compound **3** inhibited both TPA-mediated induction of *c-fos* and serum-mediated induction of JE to similar extents at similar concentrations, with significant inhibition occurring at 10 nM, and greater than 80% inhibition occurring at 20 nM (Fig. 2, A and B).

To determine whether **3** exerted effects by acting as a general inhibitor of RNA synthesis, quiescent monolayers of 68 ~ 220 cells were exposed to **3** for 3 hours. Then pulse-labeled for 1 hour with [5-^{3}H]uridine. The incorporation of [^{3}H]uridine into acid-precipitable counts was measured and revealed that **3** treatment slightly, but significantly increased the level of [^{3}H]uridine incorporation above the control, untreated cultures (6040 ± 300 cpm in the treated *vs.* 4200 ± 450 in the controls).

In BALB/c 3T3 cells, induction of *c-fos* occurs predominantly through the protein kinase C pathway[12]. This, plus our demonstration that **3** inhibits TPA-mediated induction of *c-fos*, suggests that the cellular target(s) for **3** is involved in protein kinase C-mediated signal transduction. Northern analysis presented here

Fig. 2. Verticillin A-mediated inhibition of TPA-induced *c-fos* proto-oncogene induction (A) and serum-induced JE induction (B).

A; TPA-induced *c-fos* proto-oncogene induction: lane 1, uninduced; lane 2, 300 nM TPA; lane 3, 300 nM TPA + 10 nM verticillin A; lane 4, 300 nM TPA + 20 nM verticillin A.

B; Serum-induced JE induction: lane 1, uninduced; lane 2, 20% serum; lane 3, 20% serum + 10 nM verticillin A; lane 4, 20% serum + 20 nM verticillin A.

demonstrates that, similiar to the ability to inhibit the induction of our *c-fos/lac Z* reporter gene, **3** inhibited TPA-mediated induction of *c-fos* at concentrations as low as 10 nM, with greater than 80% inhibition occurring at 20 nM concentrations (Fig. 2, A). In contrast, induction of JE occurs by a pathway which does not involve protein kinase C[13]. Compound **3** inhibited serum-mediated induction of the JE gene, at concentrations as low as 20 nM (Fig. 2, B). The experiments demonstrating that **3** did not inhibit [5-^{3}H]uridine incorporation indicated that the mechanism of verticillin-mediated inhibition of gene induction did not involve general transcriptional inhibition.

The ability of **3** to inhibit both protein kinase C-dependent and -independent gene induction suggests that the biological target of **3** is involved in a common signal transduction pathway that is necessary for both protein kinase C-dependent and -independent gene induction. The alternative possibility, that **3** blocks two or more biological targets, is less likely in view of the similar concentration requirements needed to inhibit both *c-fos* and JE induction. We suggest that **3** (verticillin A) exerts its antitumor effects by inhibiting a signal transduction pathway common to the induction of at least a subset of immediate early genes. Our results indicating that verticillin A inhibits activation of more than one signalling pathway suggest that the compound could be acting at a very early step in the signalling pathway, responsible for activation of multiple signalling pathways leading to *c-fos* proto-oncogene induction. In suport of this, preliminary results* indicate that at 1 μM, verticillin A inhibits p185$^{c\text{-}neu}$ phosphorylation of poly (glu, tyr) by 20%, suggesting that the compound could exert its effects by inhibiting phosphorylation by specific receptor tyrosine kinases. Identification of the verticillin target can be expected to lead to the discovery of a critical enzyme in signal transduction.

Acknowledgments

The authors thank Dr. B. KATZ for the taxonomic information, and Mr. Y-H ING for low resolution FAB mass spectral dara.

References

1) GREENBERG, M. E. & E. B. ZIFF: Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. Nature (London) 311: 433~438, 1984
2) FISCH, T.M.; R. PRYWES & R. G. ROEDER: *c-fos* sequences necessary for basal expression and induction by epidermal growth factor, 12-O-tetradecanoyl phorbol-13-acetate, and the calcium ionophore. Mol. Cell. Biol. 7: 3490~3502, 1987
3) HAYES, T. E.; A. M. KITCHEN & B. H. COCHRAN: Inducible binding of a factor to the *c-fos* regulatory region. Proc. Natl. Acad. Sci. (U.S.A.) 84: 1272~1276, 1987
4) FUJII, M.; D. SHALLOWAY & I. M. VERMA: Gene regulation by tyrosine kinases: *src* protein activates various promoters, including *c-fos*. Mol. Cell. Biol. 9: 2493~2499, 1989
5) JAMAL, S. & E. ZIFF: Transactivation of *c-fos* and beta-actin genes by raf as a step in early response to transmembrane signals. Nature 344: 463~466, 1990
6) GUTMAN, A.; C. WASYLYK & B. WASYLYK: Cell-specific regulation of oncogene-responsive sequences of the *c-fos* promoter. Mol. Cell. Biol. 11: 5381~5387, 1991
7) O'HARA, B. M.; H. P. KLINGER, T. CURRAN, Y.-D. ZHANG & D. G. BLAIR: Levels of *fos*, *ets2*, and *myb* proto-oncogene RNAs correlate with segregation of chromosome 11 of normal cells and with suppression of tumorigenicity in human cell hybrids. Mol. Cell. Biol. 7: 2941~2946, 1987
8) KATAGIRI, K.; K. SATO, S. HAYAKAWA, T. MATSUSHIMA & H. MINATO: Verticillin A, a new antibiotic from *Verticillium* sp. J. Antibiotics 23: 420~422, 1970
9) OKAYAMA, H. & P. BERG: A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. Mol. Cell. Biol. 3: 280~289, 1983
10) VAN STRAATEN, F.; R. MULLER, T. CURRAN, C. VAN BEVEREN & I. M. VERMA: Complete nucleotide sequence of a human *c-onc* gene: deduced amino acid sequence of the human *c-fos* protein. Proc. Natl. Acad. Sci. (U.S.A.) 80: 3183~3187, 1983
11) WIGLER, M.; S. SILVERSTEIN, L.-S. LEE, A. PELICER, Y.-C. CHENG & R. AXEL: Transfer of purified Herpes virus thymidine kinase gene to cultured mouse cells. Cell 11: 223~232, 1977
12) HALL, D. J. & C. D. STILES: Platelet-derived growth factor inducible genes respond differentially to at least two distinct intracellular second messengers. J. Biol. Chem. 262: 15302~15308, 1987
13) KEATH, E. J.; A. KELEKAR & M. D. COLE: Transcriptional activation of the translocated *c-myc* oncogene in mouse plasmacytomas: similar RNA levels in tumor and proliferating normal cells. Cell 34: 521~528, 1984
14) SCHILLING, K.; D. LUK, J. I. MORGAN & T. CURRAN: Regulation of a *fos/lac Z* fusion gene: A paradigm for quantitative analysis of stimulus-transcription coupling. Proc. Natl. Acad. Sci. (U.S.A.) 88: 5665~5669, 1991

* The data were provided by Ms. K. SYLVESTER and Dr. O. WILSON through private communication