SCH 45752—AN INHIBITOR OF CALMODULIN-SENSITIVE CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY

V. R. HEGDE*, J. R. MILLER†, M. G. PATEL, A. H. KING, M. S. PUAR, A. HORAN, R. HART, R. YARBOROUGH and V. GULLO

Schering-Plough Research Institute,
2015 Galloping Hill Rd, Kenilwooth, NJ 07033, USA

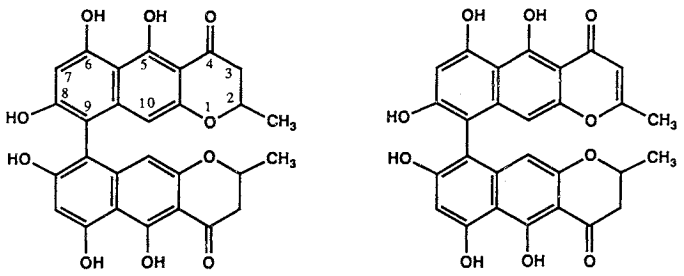
(Received for publication August 24, 1992)

A highly potent inhibitor of calmodulin-sensitive phosphodiesterase (PDE) activity was isolated from the culture broth of an unidentified fungal isolate, SCF-125. A chemically defined medium was developed for production of this compound. The PDE inhibitor was isolated from the fermentation filtrate by adsorption on a macro-reticular resin and further purified by gel filtration chromatography and reverse-phase HPLC. The major PDE inhibitor was identified as cephalochromin, a bis-naphthopyrone, by spectral data analysis. The compound, SCH 45752, inhibited calmodulin-sensitive PDE activities with IC_{50} values of 40~47 nM. It inhibited the activities of calmodulin-independent PDE and various protein kinases with higher IC_{50} values (2~40 μM). SCH 45752 does not appear to be a calmodulin antagonist. Furthermore, SCH 45752 affects smooth muscle contraction at a concentration of 30 μM; it potentiated the relaxing effect of sodium nitroprusside on carotid artery media contracted by histamine. Thus SCH 45752 is one of the most potent inhibitors of calmodulin-sensitive PDE activity known, and it is capable of exerting a pharmacological effect in at least one intact tissue model.

Cyclic nucleotides have long been known to play important roles as second messengers regulating many cell functions. Cyclic nucleotide phosphodiesterases (PDE), which influence intracellular cyclic AMP and cyclic GMP concentrations by regulating the rate of cyclic nucleotide breakdown, exist in multiple isozymic forms, which differ not only in their biochemical properties but also in their tissue distributions. The ability to selectively inhibit particular PDE isozymes carries the promise of tissue-specific pharmacological effects.

In the course of our screening program to obtain a novel calmodulin-sensitive PDE inhibitor, we have isolated a bis-naphthopyrone, SCH 45752 (**1**) from a fungal culture (SCF-125). SCH 45752 was isolated

Fig. 1. Structures of SCH 45752 (cephalochromin; **1**) and its dehydro derivative (**2**).



1 **2**

† Present address: Whitby Research Inc., 2801 Reserve Street, Box 27426, Richmond, VA 23261, U.S.A.

and purified. Based on the spectral data, the structure of SCH 45752 appears to be the same as cephalochromin (Fig. 1). In this paper we describe the fermentation, isolation, structure and the physico-chemical and pharmacological properties of SCH 45752.

Materials and Methods

Fermentation

Stock cultures were maintained as frozen whole broths at −20°C in a final concentration of 10% glycerol. A 250-ml Erlenmeyer flask containing 70 ml of seed medium was inoculated with 3.0 ml of stock culture. The flask was incubated at 24°C on a rotary shaker at 300 rpm for 48 hours. The seed medium consisted of proteus peptone 0.5%, sodium chloride 0.5%, potassium dihydrogen phosphate 0.5%, Difco yeast extract 0.3%, Cerelose 0.2%, soybean grits 0.5%, and Dow-Corning antifoam B 0.2 ml per liter of tap water. Twenty-five ml of this seed culture was used to inoculate a 2-liter Erlenmeyer flask containing 500 ml of the same germination medium and incubated as above. This second stage germination inoculum (17.5 ml) was used to inoculate a 2-liter Erlenmeyer flask containing 350 ml of fermentation production medium consisting of Neopeptone 1.0% and Cerelose 4%. The pH was adjusted to 7.0±0.2 prior to autoclaving. The fermentation was carried out at 24°C on a rotary shaker at 300 rpm. 500 ml of this third stage inoculum was used to inoculate 10-liter fermentors using the same conditions as before. The production of active compound was monitored by PDE inhibition. A typical time-course study of fermentation in a 10-liter tank is shown in Fig. 2. The optimum PDE inhibitory activity was achieved at 90~96 hours before depletion.

Isolation

The steps leading to isolation and purification of the PDE inhibitor are outlined in Fig. 3. After fermentation (4 liters), the culture broth was filtered to remove cells. The active compound from the filtrate was adsorbed onto Amberlite XAD-16 resin and the inactive spent filtrate was decanted off. The charged resin was eluted with 75% methanol - water. Purification of the PDE inhibitor was achieved by gel filtration on a Sephadex LH-20 column in methanol and purification on a reverse-phase C-18 silica column (1.9×30 cm) eluting with a mixture of methanol and 0.01 N NaH_2PO_4 (pH 3.0 with phosphoric acid, 65:35). Methanol was removed from the active peak eluate, and the activity was separated from the aqueous solution by ethyl acetate extraction. Two active components were separated by preparative HPLC using a reverse-phase phenyl column (1.9×30 cm) and elution with 0.01 N NaH_2PO_4 (adjusted to pH 3.0

Fig. 2. Fermentation profile for the production of SCH 45752 complex.

● Packed cell volume, ■ glucose, ○ pH, □ dissolved oxygen, △ inhibition of PDE activity.

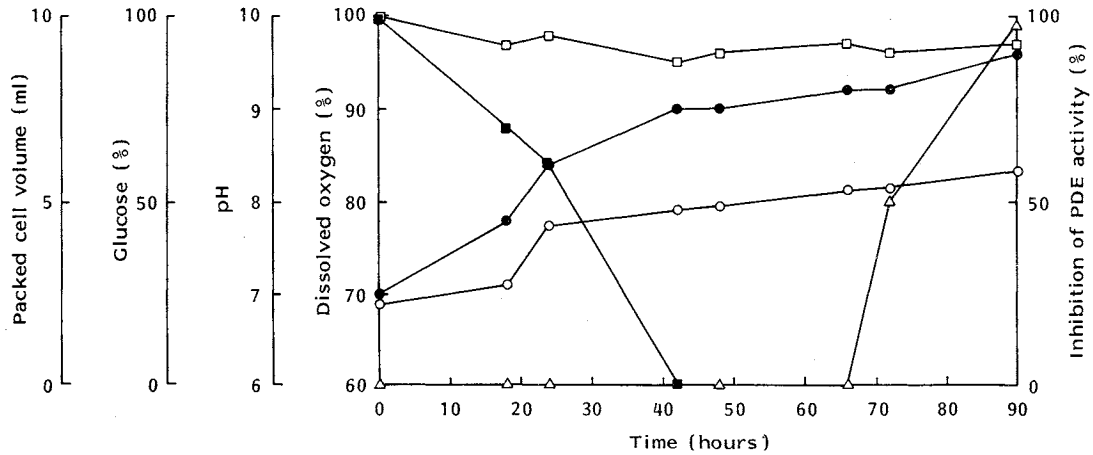


Fig. 3. Scheme for the isolation and purification of SCH 45752.

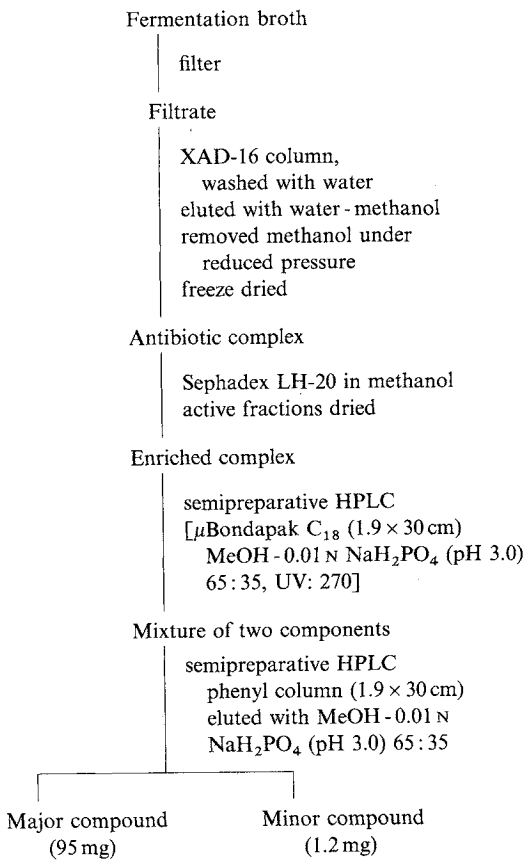
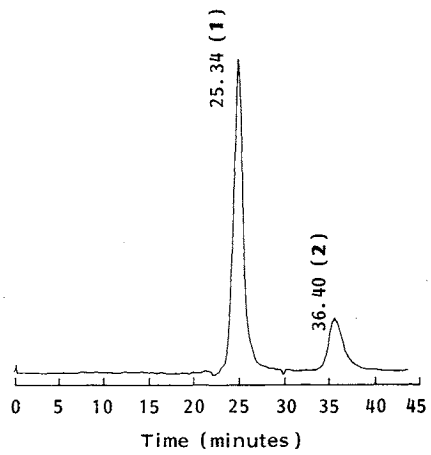


Fig. 4. Analytical HPLC profile of mixture of SCH 45752 (**1**) and dehydro-SCH 45752 (**2**).



Column: Waters μBondapak phenyl 0.39×30 cm. Mobile phase: MeOH-0.01 N NaH_2PO_4 (adj. pH 3.0 by phosphoric acid), 65:35, 1 ml/minute. Detection: UV 270 nm.

with phosphoric acid) and methanol mixture (35:65). The components from the active peak eluates were obtained as above to yield 95 mg of SCH 45752 (**1**) and 1.2 mg of a dehydro derivative (**2**). The analytical HPLC profile of the mixture of two compounds (enriched with **2**) are shown in Fig. 4.

Phosphodiesterase Assays

Calmodulin-sensitive PDE was purchased from Boehringer-Mannheim Biochemicals (Calmodulin-Deficient Phosphodiesterase from Beef Heart, Cat. No. 108 235). Calmodulin-sensitive (Peak I) and calmodulin-independent (Peak III) PDEs, prepared from rabbit aortic media, were provided by Dr. Ho Sam Ahn, Schering-Plough Research Institute. Other materials and measurement of hydrolysis of either 1 μM cyclic AMP or 1 μM cyclic GMP were as described by Ahn *et al.*[1]

Myosin Light Chain Kinase Assays

Bovine aortic smooth muscle myosin light chain kinase (MLCK) was provided by Dr. M. Cable, Schering-Plough Research Institute. MLCK activity was measured in a 100-ml reaction mixture containing 50 mM 3-(*N*-morpholino)-propanesulfonic acid, 100 mM calcium acetate, 10 mM magnesium acetate, 100 nM calmodulin (Sigma), 30 mM MLCK substrate (Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2; Peninsula Laboratories, Cat. No. 8654), 25 mM [^{32}P]ATP (3×10^4 Bq/nmol; New England Nuclear), SCH 45752 at various concentrations, and sufficient MLCK to catalyze approximately 10% maximal phosphorylation of the substrate in 30 minutes at 25°C, pH 7.0. The reaction was terminated with the addition of 100 ml of 150 mM H_3PO_4. Aliquots of the reaction mixture were spotted on Whatman P-81 phosphocellulose paper squares, which were then processed for counting as described elsewhere.[2]

Cyclic AMP-dependent Protein Kinase Assays

Bovine heart cyclic AMP-dependent protein kinase catalytic subunit was supplied by Dr. Jackie Corbin, Vanderbilt University. Phosphorylation of the substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly (Peninsula

Cat. No. 8650) was measured in a 250-μl reaction mixture containing 5 mM Tris, 5 mM Tris-HCl, 1 mM magnesium acetate, 60 mg/ml BSA, 16 mM peptide substrate, 16 mM [^{32}P]ATP, SCH 45752 at various concentrations, and sufficient kinase to catalyze approximately 10% maximal phosphorylation of substrate in 10 minutes at 25°C, pH 7.4. The reaction was terminated with H_3PO_4, and the product was quantitated as described elsewhere.[2]

Cyclic GMP-dependent Protein Kinase Assays

Beef heart cyclic GMP-dependent protein kinase was supplied by Dr. CORBIN. Phosphorylation of the substrate Arg-Lys-Arg-Ser-Arg-Ala-Glu (Peninsula Cat. No. 8659) was measured in a 250-μl reaction mixture containing 5 mM Tris, 5 mM Tris-HCl, 1 mM magnesium actate, 60 mg/ml BSA, 2 mM cyclic GMP, 29 mM peptide substrate, 25 mM [^{32}P]ATP, SCH 45752 at various concentrations, and sufficient kinase to catalyze approximately 10% maximal phosphorylation of kemptamide in 10 minutes at 25°C, pH 7.4. The reaction was terminated with H_3PO_4, and the product was quantitated as described elsewhere.[2]

Protein Kinase C Assays

Rat brain protein kinase C was partially purified (through the DEAE-Sephacel step) and its activity was assayed as described previously.[3]

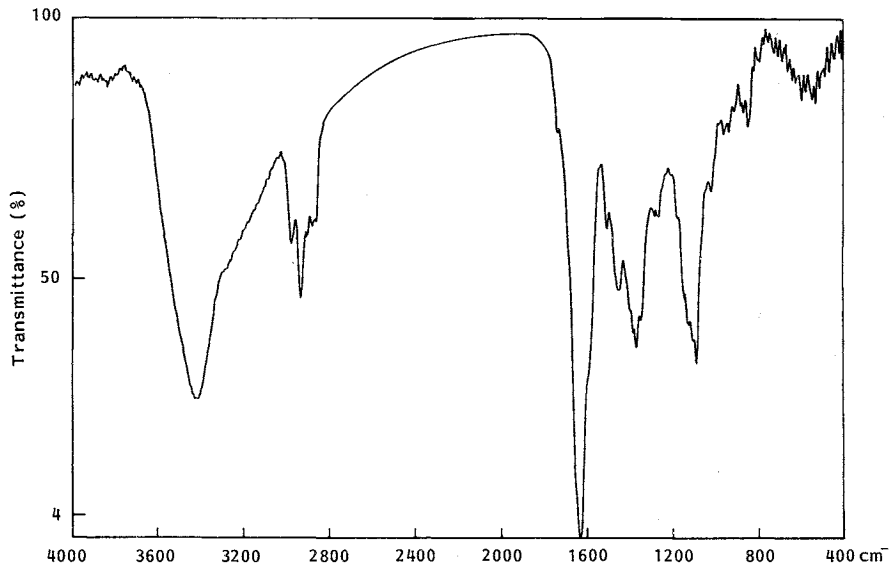
Contracting Smooth Muscle Preparation from Rabbit Aorta

Circumferential strips of porcine carotid artery media, approximately 2 × 10 mm, were mounted and equilibrated for contraction measurements according to the procedure of KATSUKI and MURAD.[4] The muscle strips were contracted to steady-state force levels with 1 mM histamine, then relaxed by addition of sodium nitroprusside in increasing concentration, 1 nM to 1 mM, added cumulatively. Dose-response curves for sodium nitroprusside were measured in the absence and in the presence of 30 μM SCH 45752.

Results and Discussion

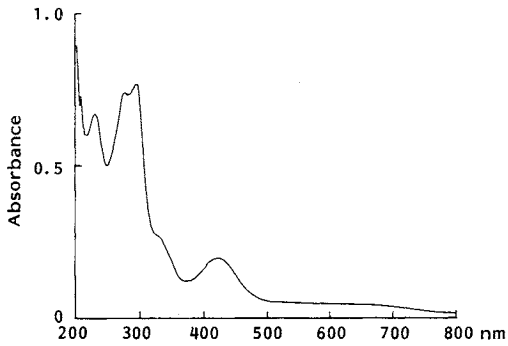
The physico-chemical properties of SCH 45752 are summarized in Table 1. SCH 45752 was isolated as a yellow solid, MP > 300°C, and it showed an optical rotation $[\alpha]_D^{22.5}$ +541.1°. The IR and UV spectrum of SCH 45752 are shown in Figs. 5 and 6, respectively. The IR spectrum showed the presence of hydroxyl groups (3400 cm^{-1}) and ketonic function (1640 cm^{-1}). The UV spectrum of this compound showed five

Fig. 5. IR spectrum of SCH 45752.



absorption maxima, at 227, 275, 293, 327, and 420, which are characteristic of a naphthopyrone class of compounds, similar to that of ustilaginoidins. The FAB mass spectrum showed the molecular weight to be 518 and the high-resolution mass measurements revealed the molecular formula to be $C_{28}H_{22}O_{10}$. However, the ^{13}C NMR spectrum showed only 14 carbon signals indicating a symmetrical bis-naphthopyrone. ^1H NMR exhibited proton signals at δ 1.4 (6H, due to 3-CH_3's), δ 2.7 (4H due to two 3-CH_2's), δ 4.5 (2H, due to 2-CH's), δ 5.92 (2H, 10-CH's), δ 6.55 (2H, 7-CH's), and two phenolic and two hydroxyl protons at δ 5.7 (2H), δ 9.7 (1H) and δ 10.1 (1H), respectively. From analysis of the ^1H NMR signals and the other spectral data, we deduced structure **1** for SCH 45752 (Fig. 1). From the spectral data, the structure of SCH 45752 appears to be the same as cephalochromin.[5,6] However, we could not obtain a sample of cephalochromin for direct comparison. We have also isolated a closely related compound having molecular weight 516 (revealed by mass spectrum), indicative of a dehydro derivative. ^1H NMR of compound **2** (1.2 (dd, 3H), 2.2 (s, 3H), 2.7 (dd, 2H), 4.5 (dq, 1H), 5.7 (br s, 1H), 5.95 (s, 1H), 6.35 (s, 1H), 6.67 (s, 1H), 9.7 (br s, 1H)), showed that it contains a double bond at the 2, 3 position of one of the pyrone rings as shown in structure **2** (Fig. 1), the same as dihydroiso-ustilaginoidin A.

Fig. 6. UV spectrum of SCH 45752.



SCH 45752 was a highly potent inhibitor of calmodulin-sensitive PDE activities from either

Table 1. Physico-chemical properties of SCH 45752.

MP	>300°C
UV (MeOH) λ_{max} nm	227 (14,030), 275 (15,330), 293 (15,870), 327 (5,800), 420 (4,120)
+HCl	229 (15,425), 273 (16,875), 292 (17,220), 327 (5,075), 420 (4,050)
+NaOH	222 (13,468), 281 (17,700), 335 (5,800), 420 (4,725)
IR (KBr) ν_{max} cm^{-1}	3400, 2860, 2570, 1640, 1590, 1365, 1150
$[\alpha]_D^{22.5}$	+541.1° (dioxane)
FAB-MS	519 $(M+H)^+$
HR-MS	Found 519.1294 $(M+H)^+$, calcd for $C_{28}H_{23}O_{10}$ 519.1291
^1H NMR ($CDCl_3$) δ	1.4 (dd, J=6.5 and 2 Hz, 6H), 2.7 (dd, J=8 and 2 Hz, 4H), 4.5 (dq, J=8 and 6.5 Hz, 2H), 5.7 (br s, 2H), 5.92 (s, 2H), 6.55 (s, 2H), 9.7 (br s, 1H), 10.1 (br s, 1H)
^{13}C NMR ($CDCl_3$) ppm	20.43 (d, CH_3), 42.73 (t, 3-C), 72.85 (d, 2-C), 99.04 (d, 10-C), 99.39 (d, 7-C), 101.33 (s, 5a-C), 102.04 (s, 4a-C), 105.06 (s, 9-C), 141.52 (s, 9a-C), 155.90 (s, 5-C), 159.5 (s, 6-C), 160.53 (s, 8-C), 164.08 (s, 10a-C), 197.9 (s, 4-C)

Table 2. Inhibition of enzyme activity by SCH 45752.

Enzyme	Substrate	n	IC_{50} (μM)
Calmodulin-sensitive PDE from bovine heart	1 μM Cyclic GMP	2	0.040
Calmodulin-sensitive (Peak I) PDE from bovine aorta	1 μM Cyclic GMP	2	0.047
Calmodulin-independent (Peak III) PDE from bovine aorta	1 μM Cyclic AMP	2	4.0
Myosin light chain kinase	Synthetic peptide	2	14
Protein kinase C	Synthetic peptide	1	11
Cyclic AMP-dependent protein kinase	Synthetic peptide	2	1.9
Cyclic GMP-dependent protein kinase	Synthetic peptide	1	5

Fig. 7. Inhibition of calmodulin-sensitive and calmodulin-independent PDE activities by SCH 45752.

■ +Calmodulin, □ −calmodulin.

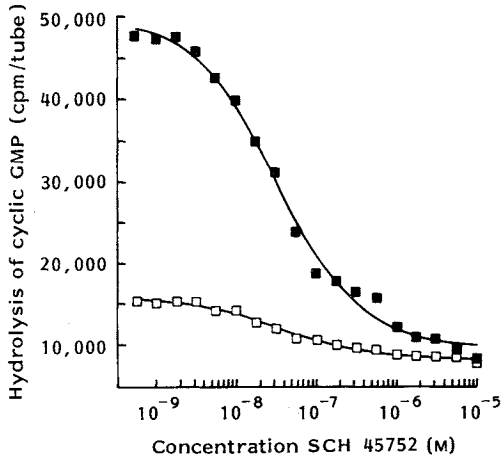
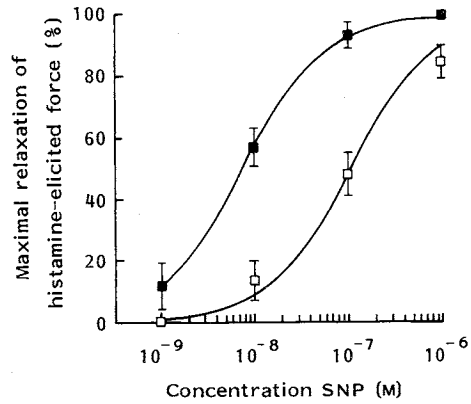


Fig. 8. The effect of SCH 45752 on relaxation of carotid artery smooth muscle by sodium nitroprusside (SNP).

■ SCH 45752 (30 μM), □ control.



bovine heart or bovine aorta (Table 2). To evaluate the selectivity of SCH 45752, we compared the potencies of SCH 45752 for inhibiting calmodulin-sensitive PDEs with the potencies for inhibiting another PDE isozyme as well as some other enzymes that utilize purine-containing substrates. For the calmodulin-independent enzyme, Peak III PDE, cyclic AMP was used as substrate because that enzyme is specific for the hydrolysis of cyclic AMP. As judged by IC_{50} values (Table 2), SCH 45752 was far more potent as an inhibitor of calmodulin-sensitive PDEs than as an inhibitor of calmodulin-independent (Peak III) PDE or of the four protein kinases tested.

The concentration-effect curve for inhibition of calmodulin-sensitive PDE activity by SCH 45752 (Fig. 7) suggests that SCH 45752 is not specifically a calmodulin antagonist. Hydrolysis of 1 mM cyclic GMP by that enzyme was stimulated 6-fold by calcium·calmodulin. SCH 45752 inhibited the PDE activities measured in the presence and in the absence of calcium·calmodulin with indentical IC_{50} values (33 nM for the experiment shown in Fig.7). Such would not be the case of a specific calmodulin antagonist, which would inhibit only activity stimulated by calmodulin.

The ability of SCH 45752 to inhibit cyclic nucleotide PDEs suggests the possibility of influencing smooth muscle contraction through manipulation of intracellular cyclic nucleotide concentrations. LORENZ and WELLS have reported that the abilities of xanthines to potentiate the relaxant effects of sodium nitroprusside on vascular smooth muscle are predicted by their abilities to inhibit calmodulin-sensitive PDE.[7] Consistent with those findings, SCH 45752 shifted to the left the concentration-effect curve for relaxation of bovine carotid artery smooth muscle by sodium nitroprusside (Fig. 8). Thus SCH 45752 may permeate smooth muscle cells and influence their contraction by inhibiting cyclic nucleotide hydrolysis that proceeds *via* calmodulin-sensitive PDE activity.

Conclusions

SCH 45752 (cephalochromin), isolated from the culture broth of a fungal isolate, is a highly potent (nM range) inhibitor of at least some PDEs that are regulated by calmodulin. The potencies of SCH 45752 to inhibit the other enzymes tested, including another PDE isoenzyme and various protein kinases, are

substantially lower. SCH 45752, at μM concentrations, potentiates relaxation of smooth muscle by sodium nitroprusside *in vitro*, possibly by inhibiting calmodulin-sensitive PDE activity in that tissue.

References

1) AHN, H. S.; W. CRIM, M. ROMANO, E. SYBERTZ & B. PITTS: Effects of selective inhibitors of cyclic nucleotide phosphodiesterase of rabbit aorta. Biochem. Pharmacol. 38: 3331~3339, 1989
2) CORBIN, J. D.; T. W. GETTYS, P. F. BLACKMORE, S. J. BEEBE, S. H. FRANCIS, D. B. GLASS, J. B. REDMON, V. S. SHERAIN & L. R. LANDISS: Purification and assay of cAMP, cGMP, and cyclic nucleotide analogs in cells treated with cyclic nucleotide analogs. Methods Enzymol. 159: 74~82, 1988
3) BISHOP W. R.; J. PETRIN, L. WANG, U. RAMESH & R. J. DOLL: Inhibition of protein kinase C by the tyrosine inhibitor erbastatin. Biochem. Pharmacol. 40: 2129~2135, 1990
4) KATSUKI, S. & F. MURAD: Regulation of adenosine cyclic 3′,5′-monophosphate and guanosine cyclic 3′,5′-monophosphate levels and contractibility in bovine tracheal smooth muscle. Mol. Pharmacol. 13: 330~341, 1977
5) MATSUMOTO, M.; H. MINATO, E. KONDO, T. MITSUGI, & K. KATAGIRI: Cephalochromin, dihydroisoustilaginoidin A, and iso-ustilaginoidin A from *Verticillium* sp. K-113. J. Antibiotics 28: 602~604, 1975
6) TERTZAKANIAN, G.; R. H. HASKINS, G. R. SLATER & L. R. NESBITT: The structure of cephalochromin. Proc. Chem. Soc. 1964: 195~196, 1964
7) LORENZ, K. L. & J. N. WELLS: Potentiation of the effects of sodium nitroprusside and isoproterenol by selective phosphodiesterase inhibitors. Mol. Pharmacol. 23: 424~430, 1983