

[Chem. Pharm. Bull.]
29(12)3586—3592(1981)

Inhibition of Cyclic AMP Phosphodiesterase by Lignans¹⁾

TAMOTSU NIKAIDO,*^a TAICHI OHMOTO,^a TAKESHI KINOSHITA,^b USHIO SANKAWA,^b
SANSEI NISHIBE,^c and SUEO HISADA^c

Faculty of Pharmaceutical Sciences, Toho University,^a Funabashi, Chiba, 274, Japan,

Faculty of Pharmaceutical Sciences, University of Tokyo,^b Tokyo, 113, Japan

and Faculty of Pharmaceutical Sciences, Higashi Nippon Gakuen

University,^c Ishikari-Tobetsu, Hokkaido, 061-02, Japan

(Received June 12, 1981)

Cyclic adenosine monophosphate (AMP) phosphodiesterase inhibitors contained in *Forsythia suspensa* were identified as lignans, (+)-pinoresinol (Ja) and (+)-pinoresinol- β -D-glucoside (Id), and their structure-activity relationship was investigated. In pinoresinol congeners, the configuration of the two phenyl rings is very important in relation to the activity. When both of the *p*-hydroxyl groups in matairesinol congeners are substituted by methyl or glucose, the activities are decreased as compared with those of the unsubstituted compounds.

Keywords—*Forsythia suspensa*; cAMP phosphodiesterase; inhibitors; lignans; (+)-pinoresinol; (+)-pinoresinol- β -D-glucoside; (–)-matairesinol

Introduction

Since Sutherland found cyclic adenosine monophosphate (AMP) as a second messenger inside cells, compounds that act to alter cyclic AMP metabolism have been the subject of studies not only from a biochemical point of view but also with the aim of development of new medicinal drugs. Extensive screening tests aiming to find inhibitors of cyclic AMP phosphodiesterase have shown that a variety of synthetic compounds and natural products possess inhibitory effect against this enzyme.²⁻⁷⁾ Weinryb *et al.* reported that a considerable number of therapeutic agents tested showed inhibitory effect against phosphodiesterase.⁸⁾ In addition to well-known phosphodiesterase inhibitors such as papaverine and theophylline, ethynyl estradiol, testosterone, diazepam, dipyrindamol and oxytocin are potent inhibitors of phosphodiesterase.⁸⁾ Although such a finding does not necessarily mean that pharmacological activity *in vivo* is due to the alteration of cyclic AMP metabolism,^{4,8)} these observations indicate that phosphodiesterase inhibitors found in the screening of natural products might show a variety of pharmacological activities. Cyclic AMP phosphodiesterase is a valuable tool for screening to detect biologically active compounds contained in medicinal plants used in traditional medicine, since biological activities of traditional medicinal drugs are sometimes very difficult to detect by *in vivo* tests such as blind screening due to the mildness of their actions.

In a previous paper,¹⁾ we reported the results of our screening studies to identify cyclic AMP phosphodiesterase inhibitors contained in Chinese medicinal drugs. Of 250 hot aqueous extracts of Chinese medicinal drugs tested, 34 showed reproducible inhibitory activity and inhibitors contained in the root of *Anemarrhena asphodeloides* were shown to be norlignans.¹⁾ The main inhibitor, *cis*-hinokiresinol, exhibited a prolongation effect on hexobarbital sleeping time in mice. This is consistent with the sedative action of this drug which is expected in Chinese medicine. The present paper deals with the identification of phosphodiesterase inhibitors contained in *Forsythia* fruit (used as an anti-inflammatory, diuretic and detoxicant agent in Chinese medicine) and also with the structure-activity relationship of compounds related to the inhibitors of *Forsythia* fruit.

Results and Discussion

The original plants of *Forsythia* fruits available in the Japanese market are *Forsythia suspensa* Vahl., *F. viridissima* Lind and *F. koreana* Nakai. Since the composition of lignans, which are the main constituents contained in *Forsythia* fruits, varies by plant species, it is easy to identify the original plants by thin-layer chromatography (TLC) of constituents as well as by inspection of the external shape of the fruit.⁹⁾ Hot aqueous extracts of *Forsythia* fruits that showed inhibition of beef heart phosphodiesterase in the previous screening study were extracted with chloroform. The TLC pattern of the chloroform extract indicated that the original plant is *Forsythia suspensa*.¹⁰⁾ Both the chloroform-soluble and insoluble fractions showed considerable inhibition of phosphodiesterase; *i.e.* 46.4 and 32.8%. In order to identify the phosphodiesterase inhibitors, *Forsythia* fruits originating from *Forsythia suspensa* were extracted with hot methanol and the extracts were fractionated as shown in Chart 1. The fractions that showed relatively high inhibitory activity were further fractionated by silica gel column chromatography and gel filtration, and each fraction was tested for phosphodiesterase inhibition. (+)-Pinoresinol and (+)-pinoresinol- β -D-glucoside were identified as active compounds in the chloroform and ethyl acetate fractions, respectively. If the lignans contained in *Forsythia suspensa* are active principles, other lignans contained in *Forsythia* plants used in Chinese medicine should possess inhibitory effect against phosphodiesterase. Therefore, lignans isolated from *F. viridissima* and *F. koreana* were tested for inhibitory effect against phosphodiesterase (Table I). In addition to (+)-pinoresinol and (+)-pinoresinol- β -D-glucoside, (–)-matairesinol and (–)-arctigenin showed higher inhibitory action than other lignans.

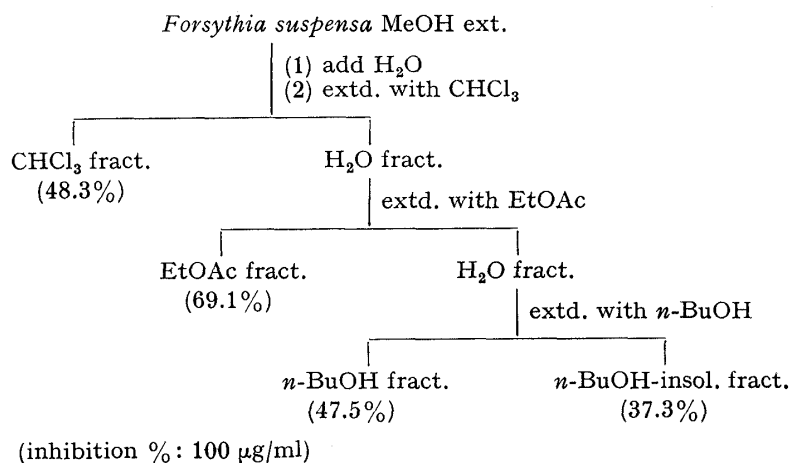
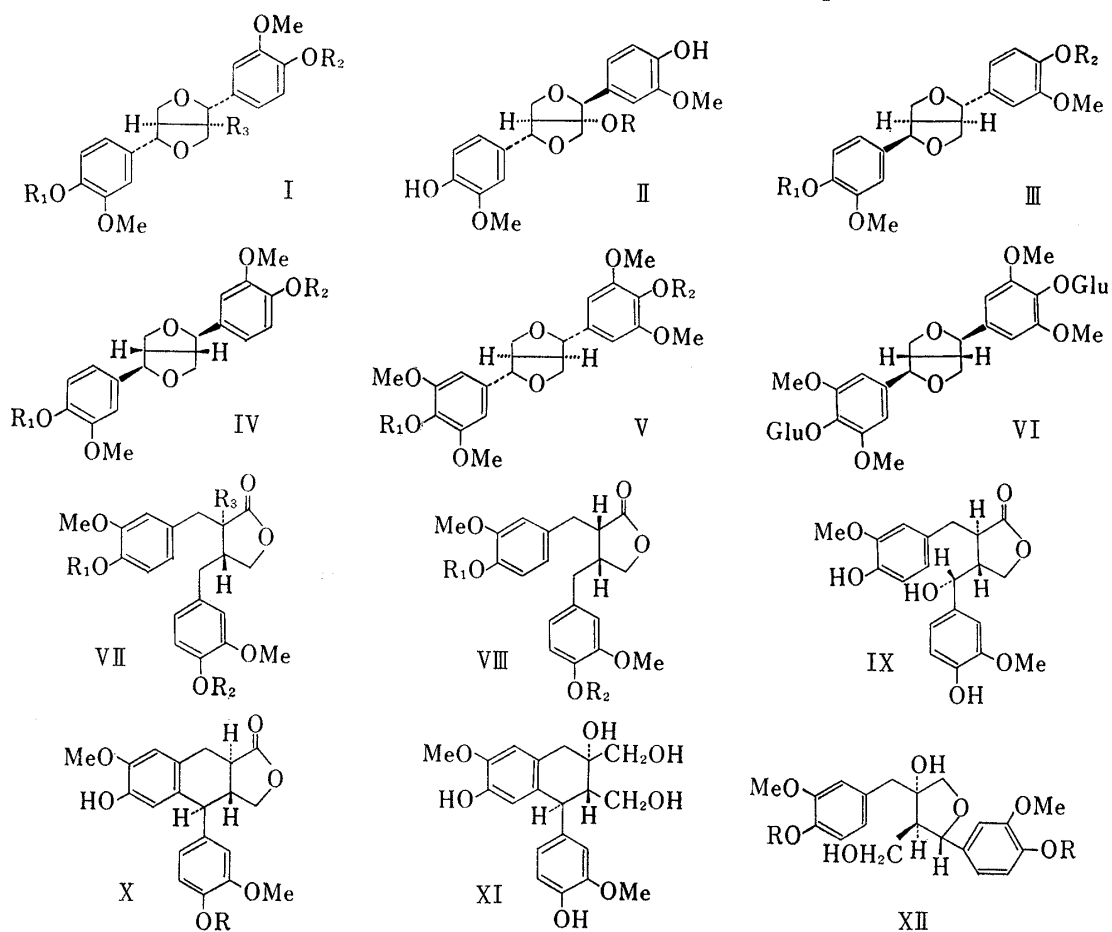


Chart 1

TABLE I. Inhibitory Activity of *Forsythia* Fruit Lignans
on Cyclic AMP Phosphodiesterase

Compound	Ic 50($\times 10^{-5}$ M)	Source
(+)-Pinoresinol (Ia)	7.5	<i>F. suspensa</i> and <i>F. koreana</i>
(+)-Pinoresinol- β -D-glucoside (Id)	14.2	<i>F. suspensa</i> and <i>F. koreana</i>
Phillygenin (IIIb)	>50	<i>F. suspensa</i> and <i>F. koreana</i>
Phillyrin (IIIe)	>50	<i>F. suspensa</i> and <i>F. koreana</i>
(–)-Matairesinol (VIIa)	9.8	<i>F. viridissima</i> and <i>F. koreana</i>
(–)-Arctigenin (VIIb)	13.9	<i>F. viridissima</i> and <i>F. koreana</i>
(–)-Matairesinol- β -D-glucoside (VIId)	>50	<i>F. viridissima</i> and <i>F. koreana</i>
(–)-Arctiin (VIIe)	>50	<i>F. viridissima</i> and <i>F. koreana</i>

TABLE II. Inhibitory Activity of Lignans on cAMP Phosphodiesterase



Compound No.	R ₁	R ₂	R ₃	R	Ic 50 ($\times 10^{-5}$ M)	Compound No.	R ₁	R ₂	R ₃	R	Ic 50 ($\times 10^{-5}$ M)
Ia	H	H	H		7.5	IVb	Glu	H			>50
Ib	Me	H	H		9.7	Va	H	H			17.5
Ic	Me	Me	H		48.0	Vb	Glu	Glu			12.7
Id	Glu	H	H		14.2	VI					>50
Ie	Glu	Me	H		21.0	VIIa	H	H	H		9.8
If	Glu	Glu	H		8.9	VIIb	H	Me	H		13.9
Ig	H	H	OAc		3.2	VIIc	Me	Me	H		28.7
Ih	Me	H	OAc		11.5	VIIId	Glu	H	H		>50
Ii	Me	Me	OAc		>50	VIIe	Glu	Me	H		>50
Ij	H	Glu	OAc		4.4	VIIIf	Glu	Glu	H		11.1
Ik	Me	Glu	OAc		>50	VIIg	H	H	OH		19.5
Il	H	H	OH		21.3	VIIh	H	Me	OH		22.7
Im	Me	H	OH		40.7	VIIi	Me	Me	OH		23.4
In	Me	Me	OH		>50	VIIj	Glu	H	OH		>50
Io	H	Glu	OH		28.6	VIIk	Glu	Me	OH		>50
Ip	Me	Glu	OH		23.2	VIIl	Glu	Glu	OH		14.3
Iq	H	H	OGlu		>50	VIIIa	H	Me			7.8
IIa				H	38.8	VIIIb	Me	Me			40.6
IIb				Glu	24.4	IX					>50
IIIa	H	H			23.0	Xa				H	>50
IIIb	Me	H			>50	Xb				Me	>50
IIIc	Me	Me			>50	XI					>50
IIId	Glu	H			>50	XIIa				H	20.1
IIIE	Me	Glu			>50	XIIb				Me	>50
IVa	H	H			>50						

In the course of screening to find enzyme inhibitors from microbial cultures, Umezawa *et al.* isolated dihydrodicaffeic acid dilactone (DDCAD) as an inhibitor of phosphodiesterase. The structure of DDCAD is very similar to that of pinoresinol.¹¹⁾ Synthetic derivatives of DDCAD have been tested for phosphodiesterase inhibition and compounds possessing two *para* hydroxyl groups showed higher activity.¹¹⁾ The results so far obtained in our studies indicate that the presence of two *p*-hydroxyl groups is not essential, since (+)-pinoresinol- β -D-glucoside and (–)-arctigenin showed relatively high inhibition. Next we tested phosphodiesterase inhibition by various lignans to clarify the structure–activity relationship. The results are summarized in Table II. Among (+)-pinoresinol congeners, the 1-acetoxy derivative (Ig) and its β -D-glucoside (Ij) were more active than (+)-pinoresinol (Ia) and its glucoside (Id). (+)-Pinoresinol monomethylether (Ib), (+)-pinoresinol-di- β -D-glucoside (If) and (+)-syringaresinol-di- β -D-glucoside (Vb) showed considerable activities. DDCAD showed an antihypertensive effect in spontaneous hypertensive rats⁶⁾ and (+)-pinoresinol-di- β -D-glucoside was isolated as an antihypertensive constituent contained in the bark of *Eucommia ulmoides*, which has been used as an hypotensive drug among Chinese people.¹²⁾

In contrast, (–)-pinoresinol (IVa), (–)-pinoresinol- β -D-glucoside (IVb), (–)-syringaresinol-di- β -D-glucoside (VI), (+)-epipinoresinol (IIIa) and (+)-epipinoresinol monomethyl ether (phillygenin, IIIb) were less active than the corresponding (+)-pinoresinol derivatives, indicating that the configuration of the two phenyl rings is very important to the activity. The structure–activity relationship in (–)-matairesinol congeners is broadly similar to that in (+)-pinoresinol congeners. When both of the *p*-hydroxyl groups are substituted with methyl or glucose, phosphodiesterase inhibitory activity became less than in the compounds with free hydroxyl groups. Recently Kato *et al.* isolated (+)-nortrachelogenin from *Wikstroemia indica*, which has been used as a traditional medicine in Taiwan, and reported that the lignan possesses sedative effect.¹³⁾

Lignans other than (+)-pinoresinol and (–)-matairesinol congeners showed little activity, as can be seen in Table II. The results so far obtained indicate that the presence of two *p*-hydroxyl groups or substituted hydroxyl groups is essential for the phosphodiesterase inhibitory activity of the lignans.

Since lignans of pinoresinol and matairesinol types possess antihypertensive and sedative effects, as described above, similar pharmacological effects are expected in the lignans obtained from *Forsythia* fruits. Further pharmacological studies to clarify the biological activities of these lignans are in progress in our laboratory.

Experimental

The following instruments were used for obtaining physical data. The liquid scintillation counter used was an Aloka LSC-903. Silica gel 60 (Merck, precoated plate, 0.25 mm) was used for TLC and detection was achieved by illumination with an ultraviolet (UV) lamp or by spraying 10% H₂SO₄ followed by heating. On preparative TLC (silica gel B-5, Wako, 0.5 mm), detection was also achieved by UV illumination. For column chromatography, silica gel C-200 (Wako) was used and for gel filtration, Sephadex LH-200 (Pharmacia Fine Chemicals). Infrared (IR) spectra were recorded on a Shimadzu IR-400 spectrometer. Gas-liquid chromatography (GLC) was carried out on a Shimadzu GC-6AM gas chromatograph equipped with a hydrogen flame ionization detector. Optical rotations were determined with a Yanagimoto OR-10.

Assay of Phosphodiesterase—Phosphodiesterase activity was assayed by a modification of the method by Thompson¹⁴⁾ and Brooker¹⁵⁾ as described in the previous paper.¹⁾

Extraction and Separation—The dried fruits of *Forsythia suspensa* (100 g, purchased from Uchida Pharmacy for Oriental Medicine, Tokyo) were crushed in a mixer and extracted three times with hot water (500 ml each) for 6 h. The extract was concentrated, frozen and dried to give a powder (10.6 g), which was dissolved in water and extracted with CHCl₃ (100 ml $\times$ 3). Removal of the solvent under reduced pressure gave a solid residue (CHCl₃ fraction; 1.6 g). The aqueous layer was frozen and dried to give a powder (CHCl₃-insoluble fraction; 8.2 g). These extracts were tested for inhibitory effect on phosphodiesterase. The original plant of the *Forsythia* fruits used in the extraction was identified as *Forsythia suspensa* by observation of the external shape of the fruits⁹⁾ as well as by TLC investigation of the components. Phillyrin (IIIe), which showed a spot of *R*_f 0.51 [solvent; CHCl₃–EtOH (4: 1)] in the CHCl₃-soluble fraction,¹¹⁾ was the main

TABLE III. The Sources of Lignans assayed for Inhibitory Activity on Cyclic AMP Phosphodiesterase

Compound	Source	Reference
Ia	<i>Forsythia suspensa</i>	16
Ib	Enzymatic hydrolysis of Ie	16
Ic	Methylation of Ia with CH ₂ N ₂	16
Id	<i>Forsythia suspensa</i>	16
Ie	Methylation of Id with CH ₂ N ₂	17
If	<i>Encommia ulmoides</i>	12
Ig	<i>Olea europaea</i>	18
Ih	<i>Olea europaea</i>	18
Ii	Methylation of Ih with CH ₂ N ₂	18
Ij	<i>Olea europaea</i>	18
Ik	<i>Olea europaea</i>	18
Il	<i>Olea europaea</i>	18
Im	<i>Olea europaea</i>	18
In	Methylation of Im with CH ₂ N ₂	18
Io	<i>Olea europaea</i>	19
Ip	<i>Olea europaea</i>	19
Iq	<i>Olea europaea</i>	20
IIa	Isomerization of Il by acid treatment	20
IIb	Isomerization of Iq by acid treatment	20
IIIa	Isomerization of Ia by acid treatment	16
IIIb	<i>Forsythia suspensa</i>	16
IIIc	Methylation of IIIb with CH ₂ N ₂	16
IIId	<i>Forsythia spp.</i>	21
IIIe	<i>Forsythia suspensa</i>	16
IVa	Enzymatic hydrolysis of IVb	22
IVb	<i>Symplocos lucida</i>	22
Va	Enzymatic hydrolysis of Vb	23
Vb	<i>Liriodendron tulipifera</i>	23
VI	<i>Eleuterococcus senticosus</i>	24
VIIa	<i>Forsythia viridissima</i>	17
VIIb	<i>Forsythia viridissima</i>	17
VIIc	Methylation of VIIb with CH ₂ N ₂	17
VIIId	<i>Forsythia viridissima</i>	17
VIIe	<i>Forsythia viridissima</i>	17
VIIIf	<i>Trachelospermum asiaticum</i> var. <i>intermedium</i>	25
VIIg	<i>Trachelospermum asiaticum</i> var. <i>intermedium</i>	26
VIIh	<i>Trachelospermum asiaticum</i> var. <i>intermedium</i>	26
VIIi	Methylation of VIIh with CH ₂ N ₂	26
VIIj	<i>Trachelospermum asiaticum</i> var. <i>intermedium</i>	26
VIIk	<i>Trachelospermum asiaticum</i> var. <i>intermedium</i>	26
VIII	<i>Trachelospermum asiaticum</i> var. <i>intermedium</i>	27
VIIIa	Isomerization of VIIb by alkaline treatment	28
VIIIb	Methylation of VIIIa with CH ₂ N ₂	28
IX	<i>Picea jezoensis</i>	29
Xa	<i>Trachelospermum asiaticum</i> var. <i>intermedium</i>	27
Xb	Synthesis from VIIb	29
XI	<i>Olea europaea</i>	18
XIIa	<i>Olea europaea</i>	18
XIIb	Methylation of XIIa with CH ₂ N ₂	18

constituent. In order to identify biologically active compounds, dried fruits of *Forsythia suspensa* (100 g) were extracted with MeOH (500 ml $\times$ 3) under reflux for 4 h. The MeOH extract (12.3 g) was fractionated with CHCl_3 and water. The CHCl_3 extract gave a solid residue (CHCl_3 -soluble fraction; 3.7 g) on removal of the solvent by evaporation, and the CHCl_3 -insoluble fraction was extracted three times with EtOAc (ca. 10 times by volume). On removal of the solvent under reduced pressure, the EtOAc extract gave a residue (EtOAc fraction; 1.9 g). The EtOAc-insoluble fraction was extracted with *n*-BuOH (ca. 10 volumes, three times). The *n*-BuOH fraction gave a solid residue (*n*-BuOH fraction; 2.2 g), and the aqueous layer was frozen and dried to give a powderlike residue (*n*-BuOH-insoluble fraction; 2.1 g). These extracts were tested for inhibitory effect against phosphodiesterase.

(+)-Pinoresinol (Ia)—The CHCl_3 -soluble fractions were fractionated by means of silica gel chromatography using CHCl_3 –EtOAc. Each fraction was monitored by TLC [solvent: CHCl_3 –EtOAc (1:1)] and also by determination of inhibitory activity against phosphodiesterase. Active fractions eluted with CHCl_3 –EtOAc (1:1) were refractionated by gel filtration with LH-20 using MeOH as an eluent. The main constituent of this fraction, *R_f* 0.46 on TLC, was identified as (+)-pinoresinol (Ia) by comparison (TLC, IR, GLC and mass spectra) with an authentic sample.¹¹⁾ $[\alpha]_D^{25} + 61.6^\circ$ ($c=0.26$, CHCl_3).

(+)-Pinoresinol- β -D-glucoside (Id)—The EtOAc-soluble fraction was fractionated by silica gel column chromatography using CHCl_3 –MeOH. Each fraction was monitored by TLC [solvent: CHCl_3 –EtOH (4:1)] as well as by bio-assay. The active fraction eluted with CHCl_3 –MeOH (9:1) was refractionated on LH-20 using MeOH. The main active compound in this fraction showed *R_f* 0.35 on TLC and was identified as (+)-pinoresinol- β -D-glucoside (Id) by TLC and UV spectroscopy. $[\alpha]_D^{25} + 8.6^\circ$ ($c=0.79$, EtOH). Furthermore, this compound was hydrolyzed enzymatically by emulsin and usual acetylation of the product by treatment with acetic anhydride in pyridine gave (+)-pinoresinol pentaacetate, which was identified by comparison with an authentic sample¹⁰⁾ (mixed mp, IR and UV spectra).

Authentic Lignans—The authentic samples which were used for tests of inhibitory action on phosphodiesterase were isolated or prepared during structural studies (Table III).

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