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The Biological Activities of 3,4-*O*-Isopropylidene-3,3′4,5′-tetrahydroxystilbene[1)]

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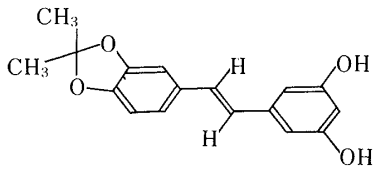
A new compound, 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene (I), chemically derived from 3,3′,4,5′-tetrahydroxystilbene (II) showed strong antifungal activity, ichthyotoxicity, coronary vasodilator action on the isolated guinea-pig heart and a hypotensive effect on rats. These activities of I were much stronger than those of the original substance (II). As regards antifungal activity, the minimal growth inhibitory concentrations (MIC) of I for *Trichophyton mentagrophytes* and *Fusarium oxysporum* f. sp. *lycopersici* were 6 and 1 μg/ml, respectively. As regards ichthyotoxic activity, the median tolerance limit (TLm) at 48 h was 14.0 ppm in *Oryzias latipes* TEMMINCK *et* SCHLEGEL. Compound (I) also had strong coronary vasodilator action on guinea-pig heart *in vitro*; the ED_{50} value was 4.5 μg/heart. Finally, I showed a strong hypotensive effect on rats (-43.0 ± 5.2 mmHg, 10 mg/kg *i.v.*).

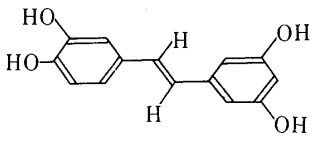
Keywords——3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene; 3,3′,4,5′-tetrahydroxystilbene; stilbene derivative; biological activity; antifungal activity; ichthyotoxicity; coronary vasodilator action; hypotensive effect

It has been reported that 3,3′,4,5′-tetrahydroxystilbene (II, Fig. 1),[2)] isolated from the heartwood of *Cassia garrettiana* CRAIB, shows antifungal, ichthyotoxic and phytogrowth-inhibitory activities. Recently, we reported that II has a strong coronary vasodilator action on guinea-pig heart *in vitro* and a hypotensive effect on rats,[3)] so II-tetraacetate, II-tetramethyl ether and 3,3′,4,5′-tetrahydroxybibenzyl were synthesized for examination of their activities. All of the activities, however, were far lower than those of II. Subsequently, many derivatives of II were synthesized in an attempt to obtain new compounds which show more potent activities. As a result, the new compound, 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene (I, Fig. 1) was found to show strong antifungal activity.[4)]

In this paper, the antifungal activity towards various organisms including plant pathogenic fungi, the ichthyotoxicity, the coronary vasodilator action on guinea-pig heart *in vitro* and the hypotensive effect on rats of the new compound, I, are reported.



3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene (I)



3,3′,4,5′-tetrahydroxystilbene (II)

Fig. 1. Chemical Structures of 3,4-*O*-Isopropylidene-3,3′,4,5′-tetrahydroxystilbene (I) and 3,3′,4,5′-Tetrahydroxystilbene (II)

Materials and Methods

Chemicals——3,4-*O*-Isopropylidene-3,3′,4,5′-tetrahydroxystilbene (I) was synthesized from 3,3′,4,5′-tetrahydroxystilbene (II) by the following method. A mixture of II (5 g), P_2O_5 (50 g) and anhydrous acetone (500 ml) was refluxed for 5 h on a water bath. The reaction mixture, after being neutralized with anhydrous Na_2CO_3, was stirred and filtered. The filtrate was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with hexane–AcOEt as the eluent to give the active fraction. The active fraction, after being concentrated *in vacuo*, was subjected to Sephadex LH-20 column chromatography with methanol as a solvent to afford a colorless crystalline powder I, 870 mg.

Compound I: I was recrystallized from hexane–$CHCl_3$ as a colorless crystalline powder, mp 118—119 °C. Thin layer chromatography (TLC) *Rf*: 0.11 (hexane : AcOEt = 3 : 1), 0.34 (benzene : AcOEt = 3 : 1), 0.32 ($CHCl_3$: MeOH = 10 : 1). *Anal.* Calcd for $C_{17}H_{16}O_4$: C, 71.80, H, 5.68. Found: C, 71.60, H, 5.84. UV λ_{max}^{EtOH} nm (log ε): 302 (4.61), 328 (4.76). IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1590, 1610 (aromatic ring). Proton nuclear magnetic resonance (^{1}H-NMR) ($CDCl_3$) δ ppm: 1.66 (6H, s), 6.34 (1H, d, J = 3 Hz, aromatic H), 6.51 (2H, d, J = 3 Hz, aromatic H), 6.66 (1H, d, J = 8.5 Hz, aromatic H), 6.71, 6.95 (1H, each d, J = 17 Hz, –CH=CH–), 6.87 (1H, dd, J = 8.5, 3 Hz, aromatic H), 6.92 (1H, d, J = 3 Hz, aromatic H), 8.20 (2H, br s, OH × 2).

Rotenone (Nakarai Chemical Co., Ltd.) was used as a standard for the ichthyotoxic activity test, and papaverine (Wako Pure Chemical Industries, Ltd.) for the test of coronary vasodilator action.

Organisms——The microorganisms used were as follows: Fungi: *Trichophyton mentagrophytes* IFO-5811, *Trichophyton rubrum* IFO-5467, *Aspergillus terreus* IFO-6346, *Aspergillus niger* IFO-4414, *Penicillium thomii* IFO-7002, *Cladosporium cladosporioides* IFO-6348, *Mucor racemosus* IFO-4581, *Trichoderma longibrachiatum* IFO-4847, *Candida albicans* IAM-4966 and *Saccharomyces cerevisiae* IFO-0203. Plant phathogenic fungi: *Botryotinia fuckeliana* IFO-9760, *Ceratocystis fimbriata* IFO-4864, *Rhizoctonia solani* IFO-30464, *Cochliobolus miyabeanus* IFO-4870, *Pyrenophora graminea* IFO-6633 and *Fusarium oxysporum* f. sp. *lycopersici* IFO-6531. Bacteria: *Staphylococcus aureus* IFO-12732, *Bacillus subtilis* PCI-219, *Escherichia coli* IFO-12734, *Proteus vulgaris* IFO-3851, *Proteus mirabilis* IFO-3849 and *Serratia marcescens* IFO-3735.

The fishes used were as follows: *Oryzias latipes* TEMMINCK *et* SCHLEGEL and *Carassius auratus* L.

The animals used were as follows: Male Hartley strain guinea-pigs weighing 400—500 g (3 guinea-pigs/group) were used for the test of coronary vasodilator action. Male Wistar strain rats weighing 230—370 g (3 rats/group) were used for the test of effect on blood pressure. Male ddY strain mice weighing 21—24 g were also used for the acute toxicity test.

Biological Tests——1) Antimicrobial Activity Test: Antifungal tests were carried out by the agar dilution method. The media used were as follows: potato sucrose agar in all cases except for *Saccharomyces cerevisiae* IFO-0203 (malt agar: Difco Laboratories), *Candida albicans* IAM-4966 (Sabouraud glucose agar: Eiken Co., Ltd.), *Trichophyton rubrum* IFO-5811, *Trichophyton mentagrophytes* IFO-5467 and *Fusarium oxysporum* f. sp. *lycopersici* IFO-6531 (potato dextrose agar: Eiken Chemical Co., Ltd.). The test fungi were applied to these media containing various concentrations of I. The plates were incubated at 27 °C for 7 d (except for *Candida albicans* IAM-4966, 2 d; *Saccharomyces cerevisiae* IFO-0203 and plant pathogenic fungi, 5 d) and the growth was observed with the naked eye. Antibacterial tests were carried out by the agar dilution method. The test bacterium was applied to nutrient agar (Eiken Chemical Co., Ltd.) containing various concentrations of I. The plates were incubated at 37 °C for 18 h and the growth was observed with the naked eye.

2) Ichthyotoxic Activity Test:[5] The method described by Sugawara and Koyama[6] was employed for the ichthyotoxicity test. The median tolerance limit (TLm) at 48 h was calculated according to the Doudoroff method.[7]

3) Coronary Vasodilator Action Test: The heart of the guinea-pig was rapidly isolated and perfused with Krebs-Hensleit solution, according to the Langendorff method. Compound I (0.1 ml in 10% dimethyl sulfoxide (DMSO)) was administered directly into the perfused solution through the connecting rubber tubing. It was shown that 10% DMSO has no effect on coronary vasodilation. Transducer: force diplacement transducer 45196 (SAN-EI Instrument Co., Ltd.) and MPU-0.5-290-0-3 (Nihon Kohden Kogyo Co., Ltd.). The potency of I was determined in terms of the dose at which the perfusion pressure was decreased by 50% of the maximum response produced by papaverine at 33 μg/heart (ED_{50}).

4) Measurement of Blood Pressure: Systemic blood pressure was measured with a pressure transducer (Nihon Kohden Kogyo Co., Ltd. RMP-6004, AP-600-G MPU 0.5 A) following cannulation of the carotid artery in rats under anesthesia with sodium pentobarbital (40 mg/kg, *i.p.*). Compound I was dissolved in 5% DMSO and administered *via* the femoral vein. It was shown that 5% DMSO has no effect on blood pressure.

Test for Toxicity of I to Mice——The mice were divided into groups of 5 mice each. Compound I was suspended in 5% gum arabic saline solution and intraperitoneally administered. The general condition of mice and mortality were followed for 7 d after administration. The LD_{50} was calculated according to the Van der Waerden method.

Temperature——All experiments were carried out at 26 to 28 °C except for the antibacterial activity tests (37 °C).

Results

Antimicrobial Activities of I

The antimicrobial activities of I were examined by the agar dilution method. The results are summarized in Table I. Compound I showed strong antifungal activity. The antifungal activity of I is much stronger than that of the original compound II.[2] The minimal growth inhibitory concentration (MIC) of I for *Trichophyton mentagrophytes* was 6 μg/ml and that for *Trichophyton rubrum* was 8 μg/ml. On the other hand, I showed no antibacterial activity even at a concentration of 500 μg/ml.

Furthermore, the antifungal activity of I on plant pathogenic fungi was investigated by the agar dilution method. As shown in Table II, I exhibited strong antifungal activity against

Table I. Antimicrobial Activities of I

Microorganism	Antimicrobial activity MIC (μg/ml)	
	I	II[a]
Fungi		
Trichophyton mentagrophytes IFO-5811	6	60
Trichophyton rubrum IFO-5467	8	50
Mucor racemosus IFO-4581	10	50
Aspergillus niger IFO-4414	20	100
Candida albicans IAM-4966	20	700
Trichoderma longibrachiatum IFO-4847	30	50
Cladosporium cladosporioides IFO-6348	30	50
Saccharomyces cerevisiae IFO-0203	30	370
Aspergillus terreus IFO-6346	50	100
Penicillium thomii IFO-7002	50	100
Bacteria		
Staphylococcus aureus IFO-12732	>500	135
Bacillus subtilis PCI-219	>500	230
Escherichia coli IFO-12734	>500	250
Proteus vulgaris IFO-3851	>500	190
Proteus mirabilis IFO-3849	>500	220
Serratia marcescens IFO-3735	>500	450

Culture conditions: Fungi—27 °C, 7 d (*Saccharomyces cerevisiae*, 5 d, *Candida albicans*, 2 d). Bacteria—37 °C, 18 h. Media: Fungi—potato sucrose agar (*Saccharomyces cerevisiae*, malt agar, *Candida albicans*, Sabouraud glucose agar, *Trichophyton rubrum* and *Trichophyton mentagrophytes*, potato dextrose agar). Bacteria—nutrient agar. Method: agar dilution method. *a*) Ref. 2.

Table II. Antifungal Activity of I on Plant Pathogenic Fungi

Fungi	Antifungal activity MIC (μg/ml)
Fusarium oxysporum f. sp. *lycopersici* IFO-6531	1.0
Pyrenophora graminea IFO-6633	5.0
Botryotinia fuckeliana IFO-9760	7.5
Rhizoctonia solani IFO-30464	10.0
Ceratocystis fimbriata IFO-4864	20.0
Cochliobolus miyabeanus IFO-4870	20.0

Culture conditions: 27 °C, 5 d. Media: potato sucrose agar (*Fusarium oxysporum* f. sp. *lycopersici* IFO-6531, potato dextrose agar). Method: agar dilution method.

TABLE III. Ichthyotoxic Activities of I

Fish	TLm (ppm, 48 h)		
	I	II[a)]	Rotenone
Oryzias latipes TEMMINCK *et* SCHLEGEL	14.0	26.5	0.030
Carassius auratus L.	18.4	31.5	0.033

Calculation of TLm: Doudoroff method. Temperature: 27 °C. Experimental size: 10 fishes/group, 2 groups. *a*) Ref. 2.

TABLE IV. Cardiac Effect of I on the Isolated Guinea-Pig Hearts

Compound	Coronary vasodilation (ED_{50}: μg/heart)	Cardiotonic effect
I	4.5	n.e.
II[a)]	13.0	n.e.
Papaverine	7.0	p.i.

Animal: male Hartley strain guinea-pigs (body weight, 400—500 g). Bioassay: Langendorff method. Experimental size: 3 guinea-pigs/group, 2 groups. n.e., no effect; p.i., positive inotropic effect. *a*) Ref. 3.

TABLE V. Effect of I on Mean Arterial Blood Pressure in Anesthetized Rats

Dose (mg/kg)	Mean arterial blood pressure (mmHg)	
	I	II[a)]
10	-43.0 ± 2.6	-34.5 ± 3.9
20	-81.3 ± 9.0	-53.7 ± 2.5

Each value represents the mean ± S.D. of 3 rats. Route: intravenous injection. *a*) Ref. 3.

all six kinds of test fungi. In particular, the MIC level of I for *Fusarium oxysporum* f. sp.*lycopersici* was 1 μg/ml.

Ichthyotoxic Activity of I

The toxicity of I to *Oryzias latipes* and *Carassius auratus* was investigated. As shown in Table III, I had toxic effects on both fishes. The ichthyotoxicity of I was stronger than that of the original compound II.[2)]

Effect of I on Isolated Guinea-Pig Hearts

The effect of I on the isolated guinea-pig heart was examined by the Langendorff method. The results are summarized in Table IV. Compound I showed rather strong coronary vasodilator action on the isolated guinea-pig heart, and its action was stronger than that of II.[3)] On the other hand, I, unlike papaverine, did not show cardiotonic action.

Effect of I on Blood Pressure in Rats

The effect of I on blood pressure in rats was investigated. As shown in Table V, I showed a potent hypotensive action, which was stronger than that of II.[3)] The group given 20 mg/kg of I showed a fall in blood pressure of 81.3 ± 5.2 mmHg. However, the hypotensive action of I was transient and the blood pressure recovered to the original level within 5 min.

TABLE VI. Toxicity Profile of Mice Injected with I

Dose (mg/kg)	Mortality (%)						
	(d)						
	1	2	3	4	5	6	7
900	100	100	100	100	100	100	100
800	80	80	80	80	80	80	100
700	40	60	80	80	80	80	80
600	20	20	20	20	40	40	40
500	0	0	0	0	40	40	40
400	0	0	0	0	0	0	0

Animals: ddY strain mice (male) 21—24 g (body weight). Route: intraperitoneal injection. Calculation of LD_{50}: Van der Waerden method. LD_{50}: 574.9 mg/kg.

General Condition and Mortality of Mice Following the Administration of I

The general condition and mortality of mice following the intraperitoneal administration of I were examined. When 1100 mg/kg of I was administered, convulsions developed at approximately 5 min after administration and all the mice died within 5 h. Table VI shows the mortality at various dosages. In the group given 800 mg/kg, reduction in spontaneous movement began at approximately 20 min after administration, then the animals showed crouching associated with eye-closing and finally they began to die at approximately 10 h after administration. The LD_{50} value of I for the mice was 574.9 mg/kg (intraperitoneal injection, Van der Waerden method).

Discussion

It was found that I showed strong antifungal activity, ichthyotoxicity, coronary vasodilator action on the isolated guinea-pig heart and a hypotensive effect on rats. These activities of I were much stronger than those of the original substance II.[2,3]

Firstly, I exhibited strong antifungal activity (Table I). It should be emphasized that I shows strong antifungal activity against *Trichophyton mentagrophytes* and *Trichophyton rubrum*. The antifungal activities of oxystilbene derivatives, *i.e.*, I, II and resveratrol,[8] are all characterized by rather strong growth-inhibitory activities against *Trichophyton* sp. Several papers have appeared on phytoallexin,[9,10] which has a stilbene skeleton in common with I and II. Thus, the antifungal activities of I on plant pathogenic fungi were investigated. Compound I showed strong activities against all six kinds of test fungi (Table II).

In addition to I and II, resveratrol,[8] pterostilbene,[9] pinosylvin and its methyl ether[10] and diethylstilbestrol[11] have a phenolic hydroxyl group on a stilbene skeleton, and show antimicrobial activities. This result suggests that the hydroxyl group attached to the benzene ring and the *trans*-olefin structure in the molecule are necessary for these stilbene derivatives to show antifungal activity. It has been shown[2] that 1) the antifungal activities of derivatives of II, *i.e.*, II-tetraacetate and II-tetramethyl ether, were weaker than that of II, and 2) the antifungal activity of 3,3′,4,5′-tetrahydroxybibenzyl obtained by reduction of the *trans*-olefin portion of II was lower than that of II.

Secondly, I had ichthyotoxic activity (Table III), which was stronger than that of II.[2] The ichthyotoxic activities of I and II were considered to be intrinsic to stilbene derivatives, because diethylstilbestrol[12] and other stilbene derivatives[12] also show strong ichthyotoxic activity against killifish (*O. latipes*). The toxicity of various stilbene derivatives to more kinds of fishes should be investigated.

Thirdly, I had a strong coronary vasodilator action on the isolated guinea-pig heart (Table IV). The coronary vasodilator action of I was much stronger than that of II[3]; the ED_{50} value of I was 4.5 μg/heart, and its effect was stronger than that of papaverine used as a standard. The coronary vasodilator actions of I and II[3] might be intrinsic to stilbene derivatives,[3] since 1) II-tetraacetate, II-tetramethyl ether and 3,3′,4,5′-tetrahydroxybibenzyl all showed coronary vasodilator action on the isolated guinea-pig heart, although their actions were weaker than that of II, and 2) oxystilbene derivatives, *i.e.*, diethylstilbestrol,[13] piceid[13] and rhapontin,[13] also show coronary vasodilator action. These results suggest that the hydroxyl group attached to the benzene ring and the *trans*-olefin structure in the molecule are necessary for stilbene derivatives to show coronary vasodilator action. Compound I meets these requirements. These considerations are supported by the following findings: 1) phloroglucinol,[14] which had a polyphenol structure in common with I and II, relaxed the smooth muscle of rats, and 2) relaxation of the smooth muscle by curcumine,[15] which has the same polyphenol and *trans*-olefin structure in the molecule as I and II, was also confirmed.

Finally, I showed a strong hypotensive effect on rats (Table V). The hypotensive effect of I was stronger than that of II.[3] In addition to I and II,[3] stilbamidine,[16] dimethylstilbamidine[16] and diethylstilbestrol,[17] which are also stilbene derivatives, show strong hypotensive effects. However, stilbamidine and dimethylstilbamidine, in spite of their antiprotozoal activity, are not used clinically because of the strong side effect of hypotensive action. These findings suggest that stilbene derivatives generally have hypotensive activity. Further studies on the hypotensive effect of many stilbene derivatives seem to be desirable.

It is noteworthy that the acute toxicity (LD_{50}: 574.9 mg/kg, *i.p.*, Van der Waerden method) of I is far lower than that (LD_{50}: 217 mg/kg, *i.p.*, Van der Waerden method) of II.[2]

From the above-mentioned results, it is clear that the biological activities of I are stronger than those of II. However, it is not clear whether the difference between the activities of I and II,[2,3] is due to 1) the acetonide group of I itself, or 2) the increase in lipid solubility caused by protection of the 3,4-dihydroxyl group. We are now investigating the mechanisms of these activities.

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References and Notes

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