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Physiological Activities of 3,3′,4,5′-Tetrahydroxystilbene Isolated from the Heartwood of *Cassia garrettiana* CRAIB.[1)]

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3,3′,4,5′-Tetrahydroxystilbene (**1**) isolated from the heartwood of *Cassia garrettiana* CRAIB. showed a broad spectrum of physiological activities, including antifungal, phytogrowth-inhibitory and ichthyotoxic activities. Firstly, **1** showed antifungal activity; the minimal growth-inhibitory concentration (MIC) was 25 μg/ml against *Penicillium citrinum* and *Rhizopus nigricans*. Secondly, **1** showed rather strong inhibitory activity on the growth of five plant species at a concentration of 500 ppm. Thirdly, **1** had ichthyotoxic activity. The median tolerance limit (TLm) at 48 h was 26.5 ppm in *Oryzias latipes* TEMMINCK *et* SCHLEGEL and 31.5 ppm in *Carassius auratus* LINNE.

Studies were made on the activities of derivatives of **1**, *i.e.*, 3,3′,4,5′-tetrahydroxystilbene-tetraacetate (**2**), 3,3′,4,5′-tetrahydroxystilbene-tetramethyl ether (**3**), and 3,3′,4,5′-tetrahydroxybibenzyl (**4**). The activities of these derivatives, however, were lower than those of **1**, except for the phytogrowth-inhibitory and ichthyotoxic activities of **4**. It is clear that for antifungal activity, both the hydroxyl groups attached to the benzene rings and the *trans*-olefin structure are necessary, while for the phytogrowth-inhibitory and ichthyotoxic activities, only the hydroxyl groups attached to the benzene rings are necessary.

Keywords——*Cassia garrettiana*; 3,3′,4,5′-tetrahydroxystilbene; physiological activity; antifungal activity; ichthyotoxic activity; phytogrowth-inhibitory activity; 3,3′,4,5′-tetrahydroxystilbene-tetraacetate; 3,3′,4,5′-tetrahydroxystilbene-tetramethyl ether; 3,3′,4,5′-tetrahydroxybibenzyl

The heartwood of *Cassia garrettiana* CRAIB. (Leguminosae) is called "Sa mae sarm" in Thailand and is used as a crude drug. Kozawa,[2,3)] one of the present authors, has isolated a number of substances from the heartwood. However, little is as yet known about the physiological activity of this crude drug. In this paper, the antimicrobial, phytogrowth-inhibitory, and ichthyotoxic activities of 3,3′,4,5′-tetrahydroxystilbene (**1**) and its derivatives (**2**—**4**) are reported (Table I).

Materials and Methods

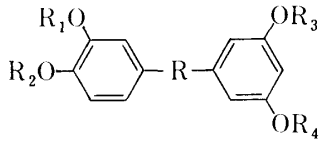
Chemicals——The chemicals used were 3,3′,4,5′-tetrahydroxystilbene (**1**),[3)] 3,3′,4,5′-tetrahydroxystilbene-tetraacetate (**2**),[3)] 3,3′,4,5′-tetrahydroxystilbene-tetramethyl ether (**3**),[3)] and 3,3′,4,5′-tetrahydroxybibenzyl (**4**)[3)] (Table I). Rotenone (Nakarai Chemicals Co., Ltd.) was used as a standard for the ichthyotoxicity test, and 2,4-dichlorophenoxyacetic acid sodium salt (Tokyo Kasei Co., Ltd.) for the phytogrowth-inhibitory activity test.

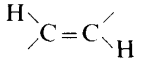
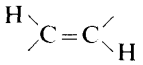
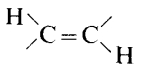
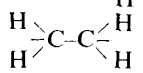
Organisms——The microorganisms used were as follows:

Fungi: *Trichophyton rubrum* IFO-5467, *Trichophyton mentagrophytes* IFO-5811, *Aspergillus niger* IFO-4414, *Aspergillus terreus* IFO-6346, *Cladosporium herbarum* IFO-6348, *Mucor racemosus* IFO-4581, *Trichoderma viride* OUT-4049, *Candida albicans* IAM-4966, *Penicillium citrinum* IFO-6026, *Penicillium thomii* OUT-2099, *Rhizopus nigricans* IFO-4781 and *Saccharomyces cerevisiae* IFO-0203.

Bacteria: *Staphylococcus aureus* 209-P, *Bacillus subtilis* PCI-219, *Escherichia coli* IFO-12734, *Proteus vulgaris* IFO-3851, *Proteus mirabilis* IFO-3849, and *Serratia marcescens* IFO-3735.

Table I. Chemical Structures of 3,3′,4,5′-Tetrahydroxystilbene (**1**) and Its Derivatives (**2**—**4**)



Compound	R	R_1	R_2	R_3	R_4
1		H	H	H	H
2		$COCH_3$	$COCH_3$	$COCH_3$	$COCH_3$
3		CH_3	CH_3	CH_3	CH_3
4		H	H	H	H

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

The plants used were as follows: *Raphanus sativus* L. var. *raphanistroides* Makino, *Cucumis sativus* L., *Brassica rapa* L., *Medicago sativa* L., and *Phaseolus hirtus* Retz.

Male mice of ddY strain, weighing 21—25 g, were also used.

Physiological Tests——1) Antimicrobial Tests: Antifungal tests were carried out by the agar dilution method. The media used were as follows: Potato Dextrose Agar (Eiken Co., Ltd.) in all cases except for *Saccharomyces cerevisiae* IFO-0203 (Malt Agar; Difco Laboratories) and *Candida albicans* IAM-4966 (Sabouraud Glucose Agar; Eiken Co., Ltd.). The test fungi were applied to these media containing various concentrations of the substances (**1**—**4**, Table I). The plates were incubated at 27 °C for 7 d (*Candida albicans* IAM-4966, 2 d; *Saccharomyces cerevisiae* IFO-0203, 5 d) and the growth was observed with the naked eye. Antibacterial tests were also carried out by the agar dilution method. The test bacterium was applied to Nutrient Agar (Eiken Co., Ltd.) containing various concentrations of the substances (**1**—**4**). The plates were incubated at 37 °C for 18 h and the growth was observed with the naked eye.

2) Phytogrowth-Inhibitory Activity Test[4]: Cotton was placed in a tall Petri dish (diameter 9 cm) and then sterilized by the dry heat sterilization method. Compound **1** was dissolved in H_2O and compounds **2**—**4** in 5% acetone. The concentrations of **1**—**4** were made up to 100, 500 and 1000 ppm. The solutions of these substances (**1**—**4**), 2,4-dichlorophenoxyacetic acid sodium salt, and the control (H_2O and 5% acetone), 2 ml each, were added to the sterilized cotton. Subsequently, twenty seeds of each plant were placed on the cotton and left for 7 d. The lengths of the roots and stems were measured and averaged. The phytogrowth-inhibitory activity was expressed as a ratio to the length of the control (to 1.00).

3) Ichthyotoxicity Test[4]: A method described by Sugawara and Koyama[5] was employed for the ichthyotoxicity test. Median tolerance limit (TLm) at 48 h was calculated according to the Doudoroff method.[6]

Test for Toxicity of 3,3′4,5′-Tetrahydroxystilbene (1) to Mice——The mice were divided into groups of 5 mice each. Compound **1** was suspended with 5% gum arabic saline solution. The suspension was intraperitoneally administered. The general condition and mortality were followed for 6 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 test (37 °C).

Results

Antimicrobial Activities of 3,3′,4,5′-Tetrahydroxystilbene (1) and Its Derivatives (2—4)

The antimicrobial activity of 3,3′,4,5′-tetrahydroxystilbene (**1**) was examined. The results are summarized in Table II. **1** showed strong antifungal activity. The minimal growth-inhibitory concentration (MIC) for *Penicillium citrinum* and *Rhizopus nigricans* was 25 μg/ml, and that for *Trichophyton rubrum* was 50 μg/ml. On the other hand, the antibacterial activity of **1** was weaker than the antifungal activity.

The antifungal activities of the derivatives (**2**—**4**) are shown in Table II. The antifungal activities of the derivatives were weaker than those of **1**.

TABLE II. Antifungal and Antibacterial Activities of 3,3′,4,5′-Tetrahydroxystilbene (**1**) and Its Derivatives (**2**—**4**)

Microorganism	MIC (μg/ml)			
	1	**2**	**3**	**4**
Fungi				
Trichophyton rubrum IFO-5467	50	>500	>200	>200
Trichophyton mentagrophytes IFO-5811	60	>500	>200	>200
Aspergillus terreus IFO-6346	100	>500	>500	>500
Aspergillus niger IFO-4414	100	>500	>500	>500
Cladosporium herbarum IFO-6348	50	>500	>500	>500
Mucor racemosus IFO-4581	50	>500	>500	>500
Penicillium thomii OUT-2099	100	>500	>500	>500
Penicillium citrinum IFO-6026	25	>500	>500	>500
Rhizopus nigricans IFO-4781	25	>500	>500	>500
Trichoderma viride OUT-4049	50	>500	>500	>500
Candida albicans IAM-4966	700	>2000	>2000	>2000
Saccharomyces cerevisiae IFO-0203	370	>2000	>2000	>2000
Bacteria				
Staphylococcus aureus 209-P	135	>1000	>1000	120
Bacillus subtilis PCI-219	230	>1000	>1000	280
Escherichia coli IFO-12734	250	>1000	>1000	280
Proteus vulgaris IFO-3851	190	>1000	>1000	120
Proteus mirabilis IFO-3849	220	>1000	>1000	280
Serratia marcescens IFO-3735	450	>1000	>1000	350

Culture conditions: Fungi——27 °C, 7 d (*Saccharomyces cerevisiae*, 5 d; *Candida albicans*, 2 d). Bacteria——37 °C, 18 h. Media: Fungi——Potato Dextrose Agar (*Saccharomyces cerevisiae*, Malt Agar; *Candida albicans*, Sabouraud Glucose Agar). Bacteria——Nutrient Agar. Method: Agar dilution method.

Phytogrowth-Inhibitory Activities of 3,3′,4,5′-Tetrahydroxystilbene (1) and Its Derivatives (2—4)

The phytogrowth-inhibitory activity of 3,3′,4,5′-tetrahydroxystilbene (**1**) was examined with five kinds of plant. The results are summarized in Table III. Compound **1** showed strong phytogrowth-inhibitory activity at a concentration of 500 ppm on all of the plants. Particularly, strong growth inhibition for the root of *Medicago sativa* L. and *Brassica rapa* L. was found.

Next, the phytogrowth-inhibitory activities of the derivatives (**2**—**4**) were examined. As shown in Table III, the inhibitory activities of **2** and **3** were weaker than that of **1**. On the other hand, the phytogrowth-inhibitory activity of **4** was as strong as that of **1**.

Ichthyotoxic Activities of 3,3′,4,5′-Tetrahydroxystilbene (1) and Its Derivatives (2—4)

The ichthyotoxic activity of 3,3′,4,5′-tetrahydroxystilbene (**1**) was examined with *Oryzias latipes* TEMMINCK *et* SCHLEGEL and *Carassius auratus* L. As shown in Table IV, the TLm (48 h) of **1** was 26.5 ppm in *Oryzias latipes* and 31.5 ppm in *Carassius auratus*. The substance (**1**) was toxic to both fishes. On the other hand, the ichthyotoxic activities of the derivatives **2** and **3** were less than that of **1**. However, **4** showed the same toxicity as **1** on both fishes.

General Condition and Mortality of Mice Following the Administration of 3,3′,4,5′-Tetrahydroxystilbene (1)

The general condition and mortality of mice following the intraperitoneal administration of 3,3′,4,5′-tetrahydroxystilbene (**1**) were examined. When 2000 mg/kg of **1** was administered, convulsions developed at approximately 30 min after administration and all the mice died

TABLE III. Inhibitory Effects of 3,3′,4,5′-Tetrahydroxystilbene (**1**) and Its Derivatives (**2**—**4**) on Plant Growth

Plant	Concentration (ppm)	Growth (Ratio)[a]									
		1		**2**		**3**		**4**		2,4-D[b]	
		Root	Stem	Root	Stem	Root	Stem	Root	Stem	Root	Stem
Raphanus sativus var. *raphanistroides* MAKINO	1000	0.25	0.85	0.85	1.10	1.01	1.01	0.36	0.33	0.05	0.12
	500	0.55	0.93	0.80	1.13	0.89	1.08	0.53	0.37	0.07	0.13
	100	0.65	0.87	1.01	1.19	0.95	1.30	0.39	0.59	0.08	0.13
Cucumis sativa L.	1000	0.59	0.56	1.33	1.15	1.39	0.96	0.66	0.29	0.06	0.09
	500	0.65	0.56	0.92	1.06	1.03	0.87	0.60	0.35	0.08	0.08
	100	0.82	0.57	1.06	1.22	1.25	1.06	0.61	0.58	0.11	0.09
Medicago sativus L.	1000	0.23	0.77	1.08	1.01	0.74	0.76	0.41	0.73	—	—
	500	0.32	0.74	0.96	1.19	1.20	0.92	0.43	0.74	0.06	0.05
	100	0.84	0.96	1.11	0.96	1.11	0.89	0.98	1.14	0.08	0.07
Brassica rapa L.	1000	0.11	0.80	0.91	0.96	0.68	0.92	0.20	0.68	—	—
	500	0.35	0.77	0.92	1.01	1.08	0.95	0.28	0.65	—	—
	100	0.89	1.00	1.00	0.98	1.31	1.04	0.73	0.84	0.05	0.16
Phaseolus hirtus RETZ.	1000	0.50	0.51	1.10	0.88	0.80	1.07	0.59	0.15	—	—
	500	0.56	0.50	1.06	0.94	0.91	1.04	0.77	0.23	—	—
	100	0.60	0.59	0.97	0.95	1.06	0.93	0.91	0.86	0.12	0.04

a) Growth in control experiments after 7d was taken as 1.00.
b) 2,4-Dichlorophenoxyacetic acid sodium salt.
Temperature: 25 °C.
Experimental size: 20 seeds/group, 2 groups.
—: no budding.
Quantity of light: 600 Lux.

TABLE IV. Ichthyotoxic Activities of 3,3′,4,5′-Tetrahydroxystilbene (**1**) and Its Derivatives (**2**—**4**)

Fish	TLm (ppm, 48 h)				
	1	**2**	**3**	**4**	Rotenone
Oryzias latipes TEMMINCK *et* SCHLEGEL	26.5	>100	>100	25.0	0.030
Carassius auratus L.	31.5	>100	>100	31.5	0.033

Calculation of TLm: Doudoroff method.
Temperature: 27 °C.
Experimental size: 10 fish/group, 2 groups.

within 1 h. Table V shows the mortality at various dosages. In the group given 225 mg/kg, the reduction in voluntary movement began about 30 min after administration, and then crouching accompanied with eye-closing developed. The animals began to die at approximately 6 h later.

The LD_{50} value of **1** in mice was 217 mg/kg (intraperitoneal administration).

Discussion

It has become apparent that 3,3′,4,5′-tetrahydroxystilbene (**1**) isolated from the heart-

TABLE V. Toxicity Profile of Mice Injected with 3,3′,4,5′-Tetrahydroxystilbene (**1**)

Dose (mg/kg)	Mortality (%) (d)					
	1	2	3	4	5	6
300	100	100	100	100	100	100
250	80	80	80	80	80	80
225	80	80	80	80	80	80
200	20	20	20	20	20	20
175	0	0	0	0	0	0

Animals: ddY strain mice (male) 22.9 ± 0.9 g (body weight).
Route: Intraperitoneal injection.
Calculation of LD_{50}: Van der Waerden method.

wood of *Cassia garrettiana* CRAIB. (Leguminosae) has antifungal, ichthyotoxic, and phytogrowth-inhibitory activities.

Firstly, the antifungal activity of **1** as a crude drug was relatively strong. Compound **1** had strong antifungal activities on *Penicillium citrinum*, *Rhizopus nigricans*, and *Trichophyton rubrum* (Table II). These findings accord with the observation that the extract of the heartwood of *Cassia garrettiana* CRAIB., unlike that of other crude drugs, did not moldy in spite of being left at room temperature for a long time. Other stilbene derivatives which have antifungal activity include resveratrol,[7] which was isolated from roots of *Polygonum cuspidatum* (Polygonaceae), and 4-stilbazole[8] as well as stilbamidine,[9] which was synthesized chemically. The antifungal activity of **1** was not less than that of resveratrol.

The antifungal activities of the derivatives (**2**—**4**) were weaker than that of **1**. This result suggests that the hydroxyl groups attached to the benzene rings and the *trans*-olefin structure are necessary for **1** to show antifungal activity.

Secondly, at a concentration of 500 ppm **1** showed strong phytogrowth-inhibitory activity against all five kinds of test plants (Table III). A number of reports have been made on the substances produced by higher plants which have growth-inhibitory activity on other kinds of plant. Among these, caffeic acid,[10] chlorogenic acid,[10] and ferulic acid[10] have partial structures in common with **1**.

The phytogrowth-inhibitory activities of the derivatives (**2** and **3**) were reduced as compared with that of **1**. On the other hand, **4** had the same activity as **1** on all five kinds of test plants (Table III).

Thirdly, **1** showed ichthyotoxic activity on *O. latipes* TEMMINCK *et* SCHLEGEL and *C. auratus* L. (Table IV). A number of reports have appeared on rotenone, isoflavones, and flavones, which are all plant constituents with ichthyotoxic activities. The ichthyotoxic activities of the stilbene derivatives, however, have been reported for the first time in this paper.

The ichthyotoxic activities of the derivatives **2** and **3** were reduced as compared with that of **1**. However, **4** showed the same activity as **1** on both fishes. In other words, the pattern of the activity was very similar to that of the phytogrowth-inhibitory activity. The results suggest that **1** requires at least the hydroxyl groups attached to the benzene rings in the molecule to show the phytogrowth-inhibitory and the ichthyotoxic activities.

The LD_{50} value of **1** in mice was 217 mg/kg (intraperitoneal administration) (Table V). In conjunction with the above-mentioned physiological activities, this result suggests that **1** has strong activities against organisms with eukaryotic cells.

Further studies on the activities of **1** on mammals are in progress, together with chemical modification studies.

References and Notes

1) This work was presented at the 103rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1983.
2) K. Hata, K. Baba, and M. Kozawa, *Chem. Pharm. Bull.*, **26**, 3792 (1978).
3) K. Hata, K. Baba, and M. Kozawa, *Chem. Pharm. Bull.*, **27**, 984 (1979).
4) T. Takemoto, Y. Inamori, Y. Kato, M. Kubo, K. Morimoto, K. Morisaka, M. Sakai, Y. Sawada, and H. Taniyama, *Chem. Pharm. Bull.*, **28**, 2884 (1980).
5) H. Sugawara and K. Koyama, "Noyaku no Seibutsu Kenteiho," Nankodo Co., Ltd., Tokyo, 1971, p. 242.
6) P. Doudoroff, *Sewage Ind. Wastes*, **23**, 1830 (1951).
7) M. Kubo, Y. Kimura, H. Shin, T. Haneda, T. Tani, and K. Namba, *Shoyakugaku Zasshi*, **35**, 58 (1981).
8) J. L. R. Williams, R. E. Adel, J. M. Carlson, G. A. Reynolds, D. G. Borden, and J. A. Ford, *J. Org. Chem.*, **28**, 387 (1963).
9) J. N. Ashley, H. J. Barber, A. J. Ewins, G. Newbery, and A. D. H. Self, *J. Chem. Soc.*, **1942**, 103.
10) R. del Moral and C. H. Muller, *Am. Midl. Nat.*, **83**, 254 (1970).