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The Biological Activities of Diethylstilbestrol and Its Derivatives[1)]

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Diethylstilbestol (I), a nonsteroidal estrogen, showed strong coronary vasodilator action on isolated guinea-pig heart, as well as ichthyotoxicity and antimicrobial activity. First, the coronary vasodilator action of I (ED_{50}: 0.26 μg/heart) on isolated guinea-pig heart was much stronger than that of papaverine (ED_{50}: 7.0 μg/heart) used as a standard. On the other hand, the activities of derivatives of I, *i.e.*, I-diphosphate (II), I-dimethyl ether (III), I-diacetate (IV), I-dipropionate (V) and hexestrol (VI) were weaker than that of I. Second, I showed strong ichthyotoxicity (median tolerance limit at 48 h: 3.30 ppm in *Oryzias latipes* and 4.50 ppm in *Carassius auratus*). On the other hand, the ichthyotoxic activities of II—V were weaker than that of I. However, VI showed the same toxicity as I on both fishes. Third, the antimicrobial activities of II—V were weaker than that of I, whereas that of VI was stronger. In particular, I and VI showed strong antifungal activity against *Trichophyton* spp.

It was concluded that both the hydroxyl groups attached to the benzene rings and the *trans*-olefin structure are necessary for coronary vasodilator action, while only the hydroxyl groups attached to the benzene ring are necessary for the ichthyotoxic and antimicrobial activities.

Keywords——diethylstilbestrol; nonsteroidal estrogen; oxystilbene derivative; 3,3′,4,5′-tetrahydroxystilbene; 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene; coronary vasodilator action; ichthyotoxicity; antimicrobial activity

It has already been reported that 3,3′,4,5′-tetrahydroxystilbene[2,3)] isolated from the heartwood of *Cassia garrettiana* CRAIB and its derivative, 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene,[4,5)] show antifungal activity, ichthyotoxic activity, coronary vasodilator action on isolated guinea-pig hearts and a hypotensive effect on rats. Subsequently, various biological activities of oxystilbene derivatives were examined in attempts to find more active substances. As a result, it was reported that diethylstilbestrol (I, Table I),[6)] a nonsteroidal estrogen, has strong ichthyotoxicity and coronary vasodilator action on isolated guinea-pig hearts.

In this paper, the coronary vasodilator action and ichthyotoxicity of I and its derivatives (II—VI, Table I) are reported. Furthermore, the relationship between the antimicrobial[7)] and other activities and the chemical structures of I and its derivatives is discussed.

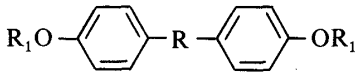
Materials and Methods

Chemicals——Chemicals used in the biological activity tests were diethylstilbestrol (I), I-diphosphate (II), I-dimethyl ether (III), I-diacetate (IV), I-dipropionate (V) and hexestrol (VI).

Diethylstilbestrol (I): Aldrich Chemical Co., Inc. Small plates, mp 170—172 °C. *Anal.* Calcd for $C_{18}H_{20}O_2$: C, 80.56; H, 7.51. Found: C, 80.34; H, 7.51. UV λ_{max}^{MeOH} nm (log ε): 240 (4.12). IR ν_{max}^{Nujol} cm^{-1}: 3400 (OH), 1592 (arom.), 1508 (arom.). ^{1}H-NMR (DMSO-d_6) δ ppm: 0.71 (6H, t, J=7.0 Hz, 2×C<u>H</u>$_3$), 2.07 (4H, q, J=7.0 Hz, 2×-C<u>H</u>$_2$-C=C), 6.76 (4H, d, J=8.2 Hz, aromatic H), 6.98 (4H, d, J=8.2 Hz, aromatic H), 9.29 (2H, s, 2×O<u>H</u>).

Diethylstilbestrol Diphosphate (II): II was prepared according to the method of Miescher and Heer.[8)]

TABLE I. Chemical Structures of Diethylstilbestrol (I) and Its Derivatives (II—VI)



Compound	R	R_1
I	$C_2H_5>C=C<C_2H_5$	H
II	$C_2H_5>C=C<C_2H_5$	O ↑ $P(OH)_2$
III	$C_2H_5>C=C<C_2H_5$	CH_3
IV	$C_2H_5>C=C<C_2H_5$	$COCH_3$
V	$C_2H_5>C=C<C_2H_5$	$COCH_2CH_3$
VI	$C_2H_5>C-C<C_2H_5$ (H) (H)	H

Voluminous white crystalline powder from dil. HCl, mp 249—252 °C. *Anal.* Calcd for $C_{18}H_{22}O_8P_2$: C, 50.48; H, 5.18; P, 14.46. Found: C, 50.14; H, 5.25; P, 14.13. UV λ_{max}^{MeOH} nm (log ε): 237 (4.16). IR ν_{max}^{Nujol} cm^{-1}: 1605 (arom.), 1500 (arom.). ^{1}H-NMR (DMSO-d_6) δ ppm: 0.73 (6H, t, J=7.3 Hz, 2×CH_3), 2.08 (4H, q, J=7.3 Hz, 2×$-CH_2-C=C$), 7.19 (8H, s, aromatic H).

Diethylstilbestrol Dimethyl Ester (III): III was prepared according to the method of Reid and Wilson.[9] Crystals from petr. ether, mp 124 °C, *Anal.* Calcd for $C_{20}H_{24}O_2$: C, 81.04; H, 8.16. Found: C, 81.30; H, 8.14. UV λ_{max}^{MeOH} nm (log ε): 237 (4.16). IR ν_{max}^{Nujol} cm^{-1}: 1762 (C=O), 1605 (arom.), 1500 (arom.). ^{1}H-NMR (DMSO-d_6) δ ppm: 0.71 (6H, t, J=7.3 Hz, 2×CH_3), 2.08 (4H, q, J=7.3 Hz, 2×$-CH_2-C=C$), 3.77 (6H, s, 2×OCH_3), 6.95 (4H, d, J=8.6 Hz, aromatic H), 7.12 (4H, d, J=8.6 Hz, aromatic H).

Diethylstilbestrol Diacetate (IV): A solution of I (1 g) in a mixture of Ac_2O (10 ml) and pyridine (1 ml) was allowed to stand at room temperature overnight. After the reaction mixture had been treated in the usual way, the product was recrystallized from EtOH to give colorless needles, mp 120 °C. *Anal.* Calcd for $C_{22}H_{24}O_4$: C, 74.97; H, 6.86. Found: C, 74.04; H, 6.87. UV λ_{max}^{MeOH} nm (log ε): 237 (4.16). IR ν_{max}^{Nujol} cm^{-1}: 1762 (C=O), 1605 (arom.), 1500 (arom.). ^{1}H-NMR (DMSO-d_6) δ ppm: 0.74 (6H, t, J=7.0 Hz, 2×CH_3), 2.10 (4H, q, J=7.0 Hz, 2×$-CH_2-C=C$), 2.29 (6H, s, 2×$COCH_3$), 7.16 (4H, d, J=8.8 Hz, aromatic H), 7.25 (4H, d, J=8.8 Hz, aromatic H).

Diethylstilbestrol Dipropionate (V): A solution of I (1 g) in a mixture of Pr_2O (10 ml) and pyridine (1 ml) was allowed to stand at room temperature overnight. After the reaction mixture had been treated in the usual way, the product was recrystallized from EtOH to give colorless needles, mp 101—104 °C. *Anal.* Calcd for $C_{24}H_{28}O_4 \cdot 1/2H_2O$: C, 74.04; H, 7.46. Found: C, 74.29; H, 7.08. UV λ_{max}^{MeOH} nm (log ε): 237 (4.20). IR ν_{max}^{Nujol} cm^{-1}: 1760 (C=O), 1608 (arom.), 1502 (arom.). ^{1}H-NMR (DMSO-d_6) δ ppm: 0.74 (6H, t, J=7.3 Hz, 2×CH_3), 1.15 (6H, t, J=7.3 Hz, 2×$COCH_2CH_3$), 2.10 (4H, q, J=7.3 Hz, 2×$-CH_2-C=C$), 2.62 (4H, q, J=7.3 Hz, 2×$COCH_2CH_3$), 7.16 (4H, d, J=8.6 Hz, aromatic H), 7.25 (4H, d, J=8.6 Hz, aromatic H).

Hexestrol (VI): Aldrich Chemical Co., Inc. Small plates, mp 186—188 °C. *Anal.* Calcd for $C_{18}H_{22}O_2$: C, 79.96; H, 8.20. Found: C, 80.18; H, 8.42. UV λ_{max}^{MeOH} nm (log ε): 279 (3.47). IR ν_{max}^{Nujol} cm^{-1}: 3400 (OH), 1600 (arom.), 1515 (arom.). ^{1}H-NMR (DMSO-d_6) δ ppm: 0.45 (6H, t, J=7.3 Hz, 2×CH_3), 1.23 (4H, m, 2×$-CHCH_2CH_3$), 2.40 (2H, m, 2×$-CHCH_2CH_3$), 6.70 (4H, d, J=8.6 Hz, aromatic H), 6.98 (4H, d, J=8.6 Hz, aromatic H), 9.12 (2H, s, 2×OH). Rotenone (Nakarai Chemical Co., Ltd.) was used as a standard for the ichthyotoxicity test, and papaverine (Wako Pure Chemical Industries, Ltd.) as a standard for the test of the coronary vasodilator action.

Organisms——1) Animals used for the test of coronary vasodilator action were male Hartley strain guinea-pigs weighing 400—500 g.

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

3) The microorganisms used were as follows. Fungi: *Trichophyton rubrum* IFO-5467, *Trichophyton mentagrophytes* IFO-5811, *Aspergillus niger* IFO-4414, *Aspergillus terreus* IFO-6346, *Cladosporium cladosporioides* IFO-6348, *Mucor racemosus* IFO-4581, *Trichoderma longibrachiatum* IFO-4847, *Penicillium citrinum* IFO-6026, *Penicillium notatum* IFO-4046, *Penicillium thomii* IFO-7002, *Rhizopus stolonifer* IFO-4781, *Candida albicans* IAM-4966 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, *Serratia marcescens* IFO-3735, *Sarcina lutea* IAM-1099 and *Pseudomonas aeruginosa* IFO-12689.

Biological Tests——1) Coronary Vasodilator Action Test: The heart of a guinea-pig was rapidly isolated and perfused with Krebs–Hensleit solution, according to the Langendorff method. Force generation was monitored using a pressure transducer, model 45196 (SAN-EI Instrument Co., Ltd.) and MPU-0.5-290-0-3 (Nihon Kohden Kogyo Co., Ltd.). Compounds I—VI (0.1 ml in 10% dimethyl sulfoxide (DMSO)) were administered directly into the perfused solution through connecting rubber tubing. It was confirmed that 10% DMSO had no effect on coronary vasodilation. The potency of I—VI was determined in terms of the dose at which the perfusion pressure decreased by 50% of the maximum response produced by papaverine at 33 μg/heart (ED_{50}).

2) Ichthyotoxicity Test:[10] A method described by Sugawara and Koyama[11] was employed for the ichthyotoxicity test. Median tolerance limit (TLm) at 48 h was calculated according to the Doudoroff method.[12]

3) Antimicrobial 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 *Trichophyton rubrum* and *Trichophyton mentagrophytes* (potato-dextrose agar: Eiken Chemical Co., Ltd.), *Saccharomyces cerevisiae* (malt agar: Difco Laboratories) and *Candida albicans* (Sabouraud glucose agar: Eiken Chemical Co., Ltd.). The fugni were applied to these media containing various concentrations of the test substances (I—VI, 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 bacteria were applied to nutrient agar (Eiken Chemical Co., Ltd.) containing various concentrations of the test substances (I—VI). The plates were incubated at 37 °C for 18 h and the growth was observed with the naked eye.

Results

Coronary Vasodilator Actions of Diethylstilbestrol (I) and Its Derivatives (II—VI) on Isolated Guinea-Pig Heart

The coronary vasodilator actions of diethylstilbestrol (I) and its derivatives (II—VI) on

Table II. Cardiac Effects of Diethylstilbestrol (I) and Its Derivatives (II—VI) on Isolated Guinea-Pig Heart

Compound	Coronary vasodilation (ED_{50} μg/heart)	Cardiotonic effect
I	0.26	n.e.
II	>100.0	n.e.
III	100.0	n.e.
IV	1.0	n.e.
V	n.e.	n.e.
VI	9.0	n.e.
Papaverine	7.0	p.i.

Animals: male Hartley strain guinea-pig (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.

Table III. Ichthyotoxic Activities of Diethylstilbestrol (I) and Its Derivatives (II—VI)

Fish	TLm (ppm, 48 h)						
	I	II	III	IV	V	VI	Rotenone
Oryzias latipes Temminck *et* Schlegel	3.30	>10	>10	>10	>10	3.25	0.030
Carassius auratus L.	4.50	>10	>10	>10	>10	6.75	0.033

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

isolated guinea-pig heart were examined by the Langendorff method. The results are summarized in Table II. Compound I showed strong coronary vasodilator action on isolated guinea-pig heart (ED_{50}: 0.26 μg/heart). The actions of I and IV were stronger than that of papaverine used as a standard (ED_{50}: 7.0 μg/heart). However, the actions of the other derivatives (II, III, V and VI) were weaker than that of I. On the other hand, unlike papaverine, I and its derivatives (II—VI) did not show a cardiotonic effect.

Ichthyotoxic Activities of Diethylstilbestrol (I) and Its Derivatives (II—VI)

The ichthyotoxic activities of diethylstilbestrol (I) and its derivatives (II—VI) were examined with *Oryzias latipes* TEMMINCK *et* SCHLEGEL and *Carassius auratus* L. As shown in Table III, the TLm at 48 h of I was 3.30 ppm in *O. latipes* and 4.50 ppm in *C. auratus*. Compound I was toxic to both fishes. On the other hand, the ichthyotoxic activities of II—V were weaker than that of I. However, VI showed the same toxicity as I on both fishes.

Antimicrobial Activities of Diethylstilbestrol (I) and Its Derivatives (II—VI)

The antimicrobial activities of diethylstilbestrol (I) and its derivatives (II—VI) were examined by the agar dilution method. The results are summarized in Table IV. Compound I showed the antifungal activity against *Trichophyton rubrum* (minimal inhibitory concentration (MIC): 70 μg/ml) and *Trichophyton mentagrophytes* (MIC: 40 μg/ml), while I did not show activity against the other fungi even at the high concentration of 1000 μg/ml. On the other hand, the activities of II—V were weaker than that of I, but the activity of VI was stronger.

TABLE IV. Antimicrobial Activities of Diethylstilbestrol (I) and Its Derivatives (II—VI)

Microorganism	Antimicrobial activity MIC (μg/ml)					
	I	II	III	IV	V	VI
Fungi						
Trichophyton mentagrophytes IFO-5811	40	>1000	>1000	>1000	>1000	14
Trichophyton rubrum IFO-5467	70	>1000	>1000	>1000	>1000	16
Aspergillus niger IFO-4414	>1000	>1000	>1000	>1000	>1000	>1000
Aspergillus terreus IFO-6346	>1000	>1000	>1000	>1000	>1000	>1000
Penicillium notatum IFO-4046	>1000	>1000	>1000	>1000	>1000	>1000
Penicillium citrinum IFO6026	>1000	>1000	>1000	>1000	>1000	>1000
Penicillium thomii IFO-7002	>1000	>1000	>1000	>1000	>1000	>1000
Mucor racemosus IFO-4581	>1000	>1000	>1000	>1000	>1000	>1000
Trichoderma longibrachiatum IFO-4847	>1000	>1000	>1000	>1000	>1000	>1000
Cladosporium cladosporioides IFO-6348	>1000	>1000	>1000	>1000	>1000	>1000
Rhizopus stolonifer IFO-4781	>1000	>1000	>1000	>1000	>1000	>1000
Candida albicans IAM-4966	>1000	>1000	>1000	>1000	>1000	>1000
Saccharomyces cerevisiae IFO-0203	>1000	>1000	>1000	>1000	>1000	>1000
Bacteria						
Staphylococcus aureus 209-P	6	>1000	>1000	>1000	>1000	9
Bacillus subtilis PCI-219	7	>1000	>1000	>1000	>1000	10
Sarcina lutea IAM-1099	7	>1000	>1000	>1000	>1000	8
Proteus mirabilis IFO-3849	7	>1000	>1000	>1000	>1000	8
Proteus vulgaris IFO-3851	>1000	>1000	>1000	>1000	>1000	>1000
Escherichia coli IFO-12734	>1000	>1000	>1000	>1000	>1000	>1000
Pseudomonas aeruginosa IFO-12689	>1000	>1000	>1000	>1000	>1000	>1000

Culture conditions: Fungi, 27 °C, 7 d (*Saccharomyces cerevisiae*, 5 d; *Candida albicans*, 2 d). Bacteria, 37 °C, 18 h. Media, 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.

Compound I also exhibited strong antibacterial activities against Gram-positive bacteria except for *Proteus mirabilis*. The MIC for *Staphylococcus aureus, Sarcina lutea, Bacillus subtilis* and *Proteus mirabilis* were 6, 7, 7 and 7 μg/ml, respectively. On the other hand, the activities of II—V were weaker than that of I, while VI showed the same activity as I.

Discussion

It has become apparent that diethylstilbestrol (I), apart from its estrogen effect, has a strong coronary vasodilator action on isolated guinea-pig heart, as well ichthyotoxicity and antimicrobial activities. Some structure–biological activity relationships were also elucidated in this work.

Compound I showed strong coronary vasodilator action on isolated guinea-pig heart (ED_{50}: 0.26 μg/heart, Table II). On the other hand, the actions of the derivatives (II—IV) were weaker than that of I (Table II). The results suggest that the hydroxyl groups attached to the benzene rings and the *trans*-olefin structure are necessary for I to show coronary vasodilator action. This relationship between activity and chemical structure is consistent with that reported for the coronary vasodilator action of 3,3′,4,5′-tetrahydroxystilbene.[3] Further support is provided by the following results: 1) phloroglucinol[13] (which has a polyphenol structure in common with I), 3,3,′,4,5′-tetrahydroxystilbene[3] and 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene[5] relax the smooth muscle of rats, and 2) relaxation of the smooth muscle by curcumine[14] and magnolol[15] (which also have the same polyphenol and *trans*-olefin structures at I) was also reported. On the other hand, unlike papaverine, I did not show a cardiotonic effect. In this respect I resembles 3,3′,4,5′-tetrahydroxystilbene[3] and 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene.[5]

In addition to I, 3,3′,4,5′-tetrahydroxystilbene,[3] 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene,[5] piceid[16] and rhapontin[16] have phenolic hydroxyl groups, are oxystilbene derivatives, and show coronary vasodilator action. Among these, I has the strongest action. These findings suggest that coronary vasodilator action might be a common pharmacological activity of oxystilbene derivatives.

Compound I had strong ichthyotoxic acitivity (TLm_{48}: 3.30 ppm in killifish and 4.50 ppm in goldfish, Table III). On the other hand, the activities of II—V were weaker than that of I (Table III), though VI had the same toxicity as I on both fishes. The results suggest that I requires at least the hydroxyl groups attached to the benzene rings in the molecule to show ichthyotoxic activity. Since 3,3′,4,5′-tetrahydroxystilbene[2] and 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene[5] are also ichthyotoxic, ichthyotoxicity may be another common biological activity of oxystilbene derivatives.

The antimicrobial activities of I[7,17,18] and synergism with antibiotics[19] have already been reported. However, the relationship between the antimicrobial activity and chemical structure has not been investigated. Compound I showed strong antifungal activity against *Trichophyton* spp. (Table IV), in accordance with the findings of Adam and Schönenberger.[7] The antifungal activities of oxystilbene derivatives, *i.e.*, I, 3,3′,4,5′-tetrahydroxystilbene,[2] 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene[4] and resveratrol[20] appear to be characteristic in that all of them have rather strong growth-inhibitory activities against *Trichophyton* spp. On the other hand, the activities of the derivatives (II—V) were weaker than that of I (Table IV), though the activity of VI was stronger. The results suggest that I requires at least the hydroxyl groups attached to the benzene rings to show antifungal activity. Compound I also showed strong antibacterial activity against Gram-positive bacteria (Table IV), in accordance with the findings of Yotis and Baman.[21] On the other hand, the antibacterial activities of II—V, like the antifungal activities, were weaker than that of I (Table IV), while the activity of VI was stronger. This again suggests that the hydroxyl groups attached to the

benzene rings are necessary for antibacterial activity.

In addition to I, 3,3′,4,5′-tetrahydroxystilbene,[2] 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene,[4,5] resveratrol,[20] pterostilbene,[22] pinosylvin and its methyl ether[23] have phenolic hydroxyl groups, are stilbene derivatives, and show antimicrobial activities.

From these results, it has became apparent that I requires at least the hydroxyl groups attached to the benzene rings in order to show the above-mentioned activities. However, it is not yet clear whether the activity is due to 1) the unchanged form or 2) its metabolites. To clarify this, further studies on the metabolites of I are in progress.

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References and Notes

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