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Studies on Zoospore-Attracting Activity. I. Synthesis of Isoflavones and Their Attracting Activity to *Aphanomyces euteiches* Zoospore

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Prunetin (**2**) and its derivatives were synthesized in order to study the relationship between structure and attracting activity to *Aphanomyces euteiches* zoospore. All of the derivatives (**1**, **2**, **4** and **6**), which had a hydroxyl group at the C-5 position in the isoflavone, showed attracting activity, but the methyl ether derivatives (**3**, **5**, **7** and **8**) do not have or have very weak attracting activity. The isoflavones (**1**, **2** and **4**) with strong attracting activity also had estrogenic activity.

Keywords——isoflavone; prunetin; genistein; biochanin A; 5-methylgenistein; 5-hydroxy-4′,7-dimethoxyisoflavone; *Aphanomyces euteiches*; zoospore; attracting activity; estrogenic activity

Introduction

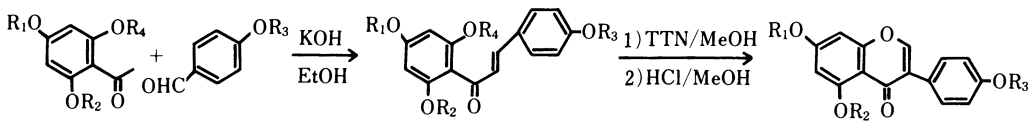
It is well-known that zoospores of zoosporic plant pathogens are attracted to the underground parts of plants and this attraction is mediated by chemical substances.[1-5] We reported the isolation of 7-methoxy-4′,5-dihydroxyisoflavone (prunetin) (**2**) from pea root, and showed that it strongly attracted *Aphanomyces euteiches* zoospore at the concentration of 5×10^{-7} M in aqueous solution.[6] It was also reported that prunetin (**2**) has estrogenic activity,[7,8] hypolipidemic activity,[9] antioxidative activity for edible oil[10] and a stimulative effect on indole acetic acid oxidase.[11] The attraction test, which was used for the previous study,[6] is simple, rapid and precise. We are interested in the relationship between the structure and attracting activity, and in the application of this attraction test for pre-screening of pharmacologically useful compounds. This report deals with the synthesis of prunetin (**2**) and its derivatives shown in Table I, and with the relationship between the structure of these compounds and their attracting activity to *A. euteiches* zoospore. The possiblity of a correlation between zoospore-attracting activity and estrogenic activity is also discussed.

Synthesis

The seven isoflavones were prepared as follows and identified on the basis of their physical properties and spectroscopic analyses, as well as by direct comparison with authentic samples.

4′,5,7-Trihydroxyisoflavone (genistein) (**1**), 5-hydroxy-4′,7-dimethoxyisoflavone (**6**) and 4′,5,7-trimethoxyisoflavone (**8**) were derived from the commercial 5,7-dihydroxy-4′-methoxyisoflavone (biochanin A)(**4**) by demethylation with aluminium chloride in benzene, by partial methylation with diazomethane in ether and by permethylation with dimethyl sulfate and anhydrous potassium carbonate in acetone, respectively.

4′-Hydroxy-5,7-dimethoxyisoflavone (**5**) was synthesized by oxidative rearrangement of the 2′-hydroxychalcone (**10**), which was prepared by the usual potassium hydroxide-catalyzed condensation of the 2′-hydroxy-4′,6′-dimethoxyacetophenone (**9**) and 4-hydroxybenzaldehyde (**11**) according to Heitz and Mentzen[12] with thallium(III) nitrate trihydride (TTN) in



9: $R_1=R_2=Me$, $R_4=H$ **11**: $R_3=H$ **16**: $R_3=CH_2Ph$
12: $R_1=R_2=R_4=H$
13: $R_1=H$, $R_2=R_4=Ac$
14: $R_1=R_4=Ac$, $R_2=H$
15: $R_1=R_4=Ac$, $R_2=Me$
10: $R_1=R_2=Me$, $R_3=R_4=H$
17: $R_1=R_4=H$, $R_2=Me$, $R_3=CH_2Ph$
19: $R_1=R_4=H$, $R_2=R_3=Me$
5: $R_1=R_2=Me$, $R_3=H$
18: $R_1=H$, $R_2=Me$, $R_3=CH_2Ph$
7: $R_1=H$, $R_2=R_3=Me$

Chart 1

methanol then acid-catalyzed ring closure of the resulting acetal according to Farkas *et al.*[13] Prunetin (**2**) was derived from **5** by partial demethylation with aluminium chloride in nitrobenzene.

4′,7-Dihydroxy-5-methoxyisoflavone (5-methylgenistein) (**3**) was prepared by the route shown in Chart 1. Acetylation of phloroacetophenone (**12**) by Büchi *et al.*'s procedure[14] gave 2′,6′-diacetoxy-4′-hydroxyacetophenone (**13**) and 2′,4′-diacetoxy-6′-hydroxyacetophenone (**14**). Methylation of the **14** with diazomethane afforded 2′,4′-diacetoxy-6′-methoxyacetophenone (**15**). The usual potassium hydroxide-catalyzed condensation of the acetophenone (**15**) and 4-benzyloxybenzaldehyde (**16**) afforded the benzyloxychalcone (**17**). Oxidative rearrangement of **17** with TTN in methanol then acid-catalyzed ring closure produced the 4′-benzyloxy-7-hydroxy-5-methoxyisoflavone (**18**). Deprotection of **18** with 10% Pd-on-carbon afforded 5-methylgenistein (**3**). 4′,5-Dimethoxy-7-hydroxyisoflavone (**7**) was also prepared by a procedure similar to that described above using the *p*-anisaldehyde.

Zoospore Formation

A. euteiches isolate (AE-F3) was grown for 3—4 d on a corn meal agar plate at 25 °C. The agar plate bearing mycelium was transferred to a Petri dish containing distilled water, and it was rinsed with four changes of distilled water then incubated for 12—20 h at 25 °C to make zoospore.

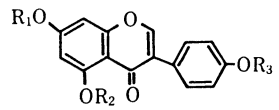
Attraction Test

A 10^{-5}—10^{-9} M methanolic solution of a test sample was introduced into a glass capillary tube and the solvent was allowed to evaporate spontaneously in order to avoid the effect of methanol on zoospores. Then the capillary tube was filled with distilled water and one end was sealed. The unsealed end of the capillary test tube was inserted into a zoospore suspension and the behavior of zoospores was observed under a microscope. The attracting activity was judged positive if the zoospores swam to mass within a few seconds at the end of the test capillary tube. The extent zoospore migration and persistence of attractiveness were variable, depending on the concentration or compound used, as shown in Table I.

Results and Discussion

Genistein (**1**), prunetin (**2**), 5-methylgenistein (**3**) and biochanin A (**4**) are naturally occurring isoflavones, and were synthesized by Baker *et al.*[15] Genistein (**1**) was originally isolated as a glycoside from *Genista trictoria* (Leguminosae)[16] and was well investigated in connection with the estrogenic properties of *Trifolium* spp. (Leguminosae).[17,18] Prunetin (**2**) was originally isolated as a glucoside from the bark of a species of wild cherry closely related

TABLE I. Attracting Activity of Isoflavones to Zoospores of *Aphanomyces euteiches* (AE-F3)



Compd. No.	R_1	R_2	R_3	Degree of attracting activity[a] and concentration (M)				
				10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
1	H	H	H	+++	++	+	−	−
2	Me	H	H	++++	++++	++	+	−
3	H	Me	H	+	−	−	−	−
4	H	H	Me	+++	++	+	−	−
5	Me	Me	H	−	−	−	−	−
6	Me	H	Me	+++	++	+	−	−
7	H	Me	Me	−	−	−	−	−
8	Me	Me	Me	−	−	−	−	−

a) −, no effect; +, attractive effect vanishes with 10 min; ++, attractive effect persists 10 to 30 min; +++, attractive effect persists 30 to 60 min; ++++, attractive effect persists more than 60 min.

to *Prunus emarginata* (Rosaceae).[19] 5-Methylgenistein (**3**) was originally isolated from *Ormosa excelsa* (Leguminosae) by Gottieb and Darocha.[20] Biochanin A (**4**) was the first nonglucosidic isoflavone from a plant[21] and was also investigated in connection with the estrogenic properties of meadow.[17,18]

4′-Hydroxy-5,7-dimethoxyisoflavone (**5**),[22] 5-hydroxy-4′,7-dimethoxyisoflavone (**6**),[23] 7-hydroxy-4′,5-dimethoxyisoflavone (**7**)[23] and 4′,5,7-trimethoxyisoflavone (**8**)[17] were synthetic substances.

As shown in Table I, the naturally occurring isoflavones (**1**—**4**) had attracting activity to *A. euteiches* zoospores, and prunetin (**2**), which was attractive at the concentration of 10^{-8} M in aqueous solution, was the most potent among them. Genistein (**1**) and biochanin A (**4**) were active at the concentration of 10^{-7} M in aqueous solution, and were slightly less potent than prunetin (**2**), while 5-methylgenistein (**3**) had very weak activity. Another four synthetic isoflavones (**5**—**8**) were significantly less active, except the 5-hydroxy-4′,7-dimethoxyisoflavone (**6**).

These results showed that the hydroxyl group at the C-5 position in 4′,5,7-trioxygenated isoflavones is necessary for strong attracting activity. The methylation of the C-7 hydroxyl group increased the attracting activity and that of the C-4′ hydroxyl group had no effect, but methylation of the C-5 hydroxyl group decreased the attracting activity.

On the other hand, it was reported that genistein (**1**), prunetin (**2**) and biochanin A (**4**) have estrogenic properties.[7,24] The estrogenic activity and attracting activity of isoflavones seem to be correlated, and we intent to carry out further work in order to judge the usefulness of this attraction test for the pre-screening of phyto-estrogenic compounds.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were taken on a Hitachi 270-30 infrared spectrophotometer. Ultraviolet (UV) spectra were obtained with a Shimadzu UV-210 spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL FX-90Q spectrometer and the chemical shifts are expressed in ppm from tetramethylsilane as an internal standard; s, singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet. Mass spectra (MS) were taken on

Shimadzu LKB-9000 and JEOL PM-60 spectrometers by the direct inlet method; ionization voltage 70 eV. Column chromatography was carried out with Kanto silica gel.

Biochanin A (4)——Biochanin A (**4**), purchased from Aldrich Co., was used after purification through silica gel column chromatography (benzene–ethyl acetate; 1 : 1). mp 214—5 °C (EtOH).

Genistein (1)——Biochanin A (**4**) (142 mg, 0.5 mmol) was suspended in 7 ml of dry benzene, and 165 mg (1.25 mmol) of anhydrous aluminium chloride was added to the suspension to yield a clear yellowish brown solution. The reaction mixture was refluxed for 18 h and then poured into ice-water containing dilute hydrochloric acid. The acidic solution was extracted with ethyl acetate and washed successively with saturated aqueous sodium chloride and water. The solvent was distilled off and the residue was purified by silica gel column chromatography (benzene–ethyl acetate; 2 : 1) to yield 63.1 mg (47%) of **1** as slightly yellow needles, mp 279—298 °C (EtOH).

5-Hydroxy-4′,7-dimethoxyisoflavone (6)——Biochanin A (**4**) (200 mg, 0.7 mmol) was suspended in 3 ml of ether, and an excess of diazomethane solution in ether was added. The reaction mixture was left standing for 30 h at room temperature. The solvent was distilled off and the residue was purified by silica gel column chromatography (benzene–ethyl acetate; 1 : 1) to yield 175 mg (84%) of **6** as slightly yellow needles, mp 139—140 °C (EtOH).

4′,5,7-Trimethoxyisoflavone (8)——Biochanin A (**4**) (142 mg, 0.5 mmol) was dissolved in 5 ml of dry acetone. Anhydrous potassium carbonate (345 mg, 2.5 mmol) was added to the solution, and then dimethyl sulfate (0.1 ml, 1.1 mmol) was added. The mixture was refluxed for 18 h, and then the solvent was distilled off. The residue was dissolved in water and extracted with ethyl acetate. The extract was washed successively with saturated aqueous sodium chloride and water. The solvent was distilled off and the residue was purified by silica gel column chromatography (benzene–ethyl acetate; 6 : 1) to yield 154 mg (98%) of **8** as slightly yellow needles, mp 162—163 °C (EtOH).

4′-Hydroxy-5,7-dimethoxyisoflavone (5)——The chalcone (**10**) (1.8 g, 6 mmol) was dissolved in 400 ml of methanol, and 5.86 g (13.2 mmol) of TTN was added at room temperature. The reaction mixture was stirred for an additional 15 min, and then 20 ml of 3 N hydrochloric acid was added to yield a small amount of yellow precipitate, and the reaction mixture was refluxed for 4.5 h. The solvent was distilled off and the residue was dissolved in a mixture of ethyl acetate and saturated aqueous sodium chloride. The organic layer was separated from the aqueous layer, and washed with saturated aqueous sodium chloride and water. The solvent was distilled off and the residue was purified by silica gel column chromatography (benzene–ethyl acetate; 1 : 1) to yield 1.362 g (75%) of **5** as slightly yellow needles, mp 180—182 °C (EtOH).

Prunetin (2)——Compound **5** (143 mg, 0.48 mmol) was suspended in 15 ml of nitrobenzene and 2.5 g (18.8 mmol) of anhydrous aluminium chloride was added to the suspension to yield a clear black solution. The reaction mixture was stirred for 2.5 h at room temperature. The reaction mixture was then poured into ice-water containing 4 ml of 3 N hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with saturated aqueous sodium chloride. The solvent was distilled off and the residue was steam-distilled until nitrobenzene could not be detected in a mixture. The solid residue was collected by filtration, washed with water and purified by silica gel column chromatography (benzene–ethyl acetate; 2 : 1) to yield 98 mg (72%) of **2** as colorless needles, mp 246—248 °C (MeOH).

2′4′-Diacetoxy-6′-methoxyacetophenone (15)——2′4′-Diacetoxy-6′-hydroxyacetophenone (**14**) (2.9 g, 11.5 mmol) was dissolved in 30 ml of ether and an excess diazomethane solution in ether was added. The reaction mixture was left standing for 12 h at room temperature. The solvent was distilled off and the oily residue was purified by silica gel column chromatography (benzene–ethyl acetate; 15 : 1) to yield 2.5 g (82%) of **15** as a colorless viscous oil. UV λ_{max}^{EtOH} nm (log ε): 275 (sh, 3.45), 250 (3.57). IR ν_{max}^{neat} cm^{-1}: 1773, 1698, 1611, 1371, 1257, 1197, 1134, 1098, 1065, 1026. MS *m/z* (%): 266 (M^+, 4), 224 (26), 182 (40), 167 (100). ^{1}H-NMR ($CDCl_3$) δ: 2.19 (3H, s, –OCOCH$_3$), 2.35 (3H, s, –OCOCH$_3$), 2.45 (3H, s, –COCH$_3$), 6.49 (1H, d, J=2.2 Hz, H-3′ or H-5′), 6.75 (1H, d, J=2.2 Hz, H-3′ or H-5′).

4-Benzyloxy-2′,4′-dihydroxy-6′-methoxychalcone (17)——Compound **15** (1.255 g, 4.7 mmol) and *p*-benzyloxybenzaldehyde (**16**) (953 mg, 4.7 mmol) were dissolved in 10 ml of ethanol. An aqueous solution of potassium hydroxide (2.07 g, 31.5 mmol) in 2 ml of water was added to the mixture under ice-cooling and stirring to yield a clear reddish brown solution. The reaction mixture was allowed to react at room temperature for 24 h and was then poured into ice-water and acidified with 11 ml of 3 N hydrochloric acid. The acidic solution was extracted with ethyl acetate. The organic layer was separated from the aqueous layer, and washed with saturated aqueous sodium chloride and water. The solvent was distilled off and the residue was purified by silica gel column chromatography (benzene–ethyl acetate; 6 : 1) to yield 832 mg (47%) of **17** as yellow needles, mp 228—229 °C (EtOH). UV λ_{max}^{EtOH} nm (log ε): 363 (4.58), 230 (sh, 4.20). IR ν_{max}^{KBr} cm^{-1}: 3142, 1641, 1626, 1602, 1539, 1512, 1485, 1464, 1335, 1257, 1230, 1170, 1113. MS *m/z* (%): 376 (M^+, 57), 285 (44), 197 (56), 167 (63), 147 (58), 65 (100). ^{1}H-NMR (CD_3OD) δ: 3.93 (3H, s, OCH$_3$), 5.14 (2H, s, –CH$_2$Ph), 5.92 (1H, d, J=2.2 Hz, H-3′), 6.01 (1H, d, J=2.2 Hz, H-5′), 6.98 (2H, d, J=8.8 Hz, H-3 and H-5), 7.39 (5H, m, PhH), 7.65 (2H, d, J=8.8 Hz, H-2 and H-6), 7.72 (1H, s, H-α or H-β), 7.77 (1H, s, H-α or H-β).

4′-Benzyloxy-7-hydroxy-5-methoxyisoflavone (18)——Compound **17** (189.6 mg, 0.5 mmol) was suspended in 50 ml of methanol, and 488.4 mg (1.1 mmol) of TTN was added to the suspension to yield a clear yellow solution. The reaction mixture was stirred for 2 h at room temperature. Then 3 ml of 3 N hydrochloric acid was added to the clear solution to yield a small amount of yellow precipitate, and the reaction mixture was refluxed for 4.5 h. The solvent

was distilled off and the residue was dissolved in a mixture of ethyl acetate and saturated aqueous sodium chloride. The organic layer was separated from the aqueous layer, and washed with saturated sodium chloride and water. The solvent was distilled off and the residue was purified by silica gel column chromatography (benzene–ethyl acetate; 1 : 1) to yield 123.3 mg (66%) of **18** as slightly yellow fine needles, mp 296—298 °C (EtOH). UV λ_{max}^{EtOH} nm (log ε): 390 (3.56), 310 (sh, 3.69), 255 (4.33). IR ν_{max}^{KBr} cm^{-1}: 3196, 1632, 1578, 1512, 1470, 1290, 1239, 1206, 1176, 1152, 1086, 831, 746. MS *m/z* (%): 374 (M^+, 28), 283 (22), 91 (100). ^{1}H-NMR ($CDCl_3$: DMSO-d_6; 5 : 1) δ : 3.90 (3H, s, OCH_3), 5.09 (2H, s, $-CH_2Ph$), 6.39 (1H, d, J=2.2 Hz, H-6), 6.46 (1H, d, J=2.2 Hz, H-8), 6.97 (2H, d, J=8.8 Hz, H-3′ and H-5′), 7.40 (5H, m, PhH), 7.45 (2H, d, J=8.8 Hz, H-2′ and H-6′), 7.78 (1H, s, H-2).

5-Methylgenistein (3)——Compound (**18**) (78.6 mg, 0.21 mmol) was dissolved in 20 ml of ethanol and the solution was stirred under atmospheric pressure of H_2 in the presence of 10% Pd-on-carbon (70.5 mg) for 48 h at room temperature. The catalyst was filtered off and the filtrate was distilled off. The residue was purified by silica gel column chromatography (ethyl acetate) to yield 59.7 mg (81%) of **3** as slightly yellow fine needles, mp 218—220 °C (EtOH).

7-Hydroxy-4′,5-dimethoxyisoflavone (7)——The chalcone **19**[24] (294 mg, 1.04 mmol) was dissolved in 70 ml of methanol, and 234 mg (2.3 mmol) of TTN was added. The mixture was stirred for 30 min at room temperature. Then 3.5 ml of 3 N hydrochloric acid was added to the clear solution to yield a small amount of precipitate, and the reaction mixture was refluxed for 6 h. The solvent was distilled off and the residue was dissolved in a mixture of ethyl acetate and saturated aqueous sodium chloride. The organic layer was separated from the aqueous layer, and washed with saturated aqueous sodium chloride and water. The solvent was distilled off and the residue was purified by silica gel column chromatography (ethyl acetate) to yield 132.5 mg (45%) of **7** as colorless needles, mp 291—292 °C (EtOH).

Fungus and Zoospore Formation——An *A. euteiches* isolate (AE-F3), which was isolated from pea root rot field soils, was used. The fungus was grown for 3—4 d on corn meal agar plates at 25 °C. Each agar plate bearing mycelia was transferred to a Petri dish, 15 cm in diameter, containing 400 ml of distilled water and it was rinsed with four changes of distilled water within 2 h to remove nutrient material inhibitory to zoospore formation. The material thus rinsed was then incubated for 12—20 h at 25 °C. Zoospore concentration was adjusted to 5000/ml with distilled water.

Attraction Test——A methanolic solution of the test sample (10^{-5}—10^{-9} M) was introduced into a glass capillary tube (2.5 cm long, 0.8 mm outer diameter) and the solvent allowed to evaporate spontaneously. Then the capillary tube was filled with distilled water and one end was sealed with vaseline. A 2 cm part from the unsealed end of the capillary tube was inserted stanting into the zoospore suspension in a small plastic vessel (44 mm long, 22 mm wide and 5 mm high) on a microscope stage, and the behavior of the zoospores was observed at a magnification of 40×.

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