The Synthesis, Structure, and Anticancer Activity of *cis*- and *trans*-4′,7-Dihydroxyisoflavan-4-ols

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cis-4′,7-Dihydroxyisoflavan-4-ol (**4**) and *trans*-4′,7-dihydroxyisoflavan-4-ol (**5**), two proposed metabolites of daidzein (4′,7-dihydroxyisoflavone), have been synthesized and fully characterized for the first time. The vicinal coupling constants of the pyran ring protons are compatible with a half-chair conformation. The *cis* isomer is anancomeric while the *trans* isomer consists of a 68:32 mixture of two ring inversion conformers. Molecular mechanical calculations are in agreement with the half-chair conformation of the pyran ring and suggest that the *cis* isomer is biased because of an unfavorable gauche interaction of the equatorial hydroxyl and the axial phenyl group. The isoflavanols **4** and **5** are comparable to genistein (4′,5,7-trihydroxyisoflavone) in antitumor activity against human prostate cancer cells.

Introduction

There is a growing interest in plant isoflavonoids which commonly occur in health diets in the Western countries, particularly in the United States. Daidzein (**1**) and genistein (**2**), abundant in soy and other legumes, have been shown to possess a variety of biological effects such as oestrogenic and antitumor activity.[1] There is also evidence of their beneficial role in the fight against cardiovascular diseases.[2] Although numerous plant isoflavonoids have been isolated, much less is known of their metabolism in man. Metabolites of daidzein, reliably identified[1] by comparison with authentic synthetic samples, include dihydrodaidzein (**3**, 4′,7-dihydroxyisoflavanone), equol (4′,7-dihydroxyisoflavan), and *O*-demethylangolensin [1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-1-propanone]. The latter two also show a significant oestrogenic effect in animal and in man.[1] Additional metabolites of daidzein and genistein have been tentatively identified by gas chromatography/mass spectrometry (GC/MS) from human urine after a soy diet,[3,4] and from daidzein after incubation with fecal bacteria.[5,6] Among the new metabolites, the rare tetrahydrodaidzein (isoflavan-4-ol) structures **4** and **5** were suggested on the basis of ms data.[7,8] We now report the synthesis,[9] 2D ^{1}H and ^{13}C NMR characterization, and conformational aspects of *cis*- and *trans*-4′,7-dihydroxyisoflavan-4-ol (**4** and **5**, respectively). Although a few mentions of isoflavan-4-ols appear in the literature, there exists no systematic and detailed study of their spectra or structure. Finally, we make here the preliminary announcement that **4** and **5** inhibit the growth of the LNCaP 90 prostate cancer cells in culture.

Results and Discussion

Synthesis. To our knowledge there are no literature methods for reducing polyhydroxy-substituted isoflavones to the isoflavan-4-ols, which would be an attractive approach, as there is an easy access to hydroxy/methoxy-substituted isoflavones by a one-pot procedure.[10] Unsubstituted or methoxy-substituted isoflavones have been reduced by catalytic hydrogenation or $NaBH_4$ to give isoflavanones, isoflav-3-enes, occasionally *cis*- and *trans*-isoflavan-4-ols, and isoflavans, but the results published by various workers are contradictory to a large extent.[11] In our hands, catalytic Pd/C hydrogenation of polyhydroxyisoflavones in aqueous ethanol furnished polyhy-

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(7) In one of the papers[6] it was stated that the isoflavanols were identified by NMR comparison with published values in the literature;[3] however, ref 3 does not have any NMR information related to this matter.
(8) In addition to the known metabolite dihydrodaidzein (**3**), its enol form has also been suggested as an independent metabolite from human urine[4] and from daidzein incubated with fecal bacteria.[5] We feel it is not meaningful to view the enol form as an independent metabolite because the keto−enol tautomerism is quite facile; in any case, the keto form only appears in the NMR spectra, there being no trace of the free enol. The fact that some enol silyl ether[4] or acetate[6] is formed on derivatization of the ketone is only to be expected. Furthermore, the characterization[5] of the claimed enol was based on a 2-H singlet due to 2-CH_2 at δ 4.10 and on comparison with literature data;[3] the latter paper, however, does not have any structural data on the free enol. Additionally, the δ value cited is not in line with the 2-CH_2 value for the corresponding enol acetate (5.03)[6] or the silyl ether (4.94).[9] Crucially, no ^{13}C NMR data was given.[5] Altogether, we consider insecure the identification of dihydrodaidzein enol as a metabolite of daidzein.
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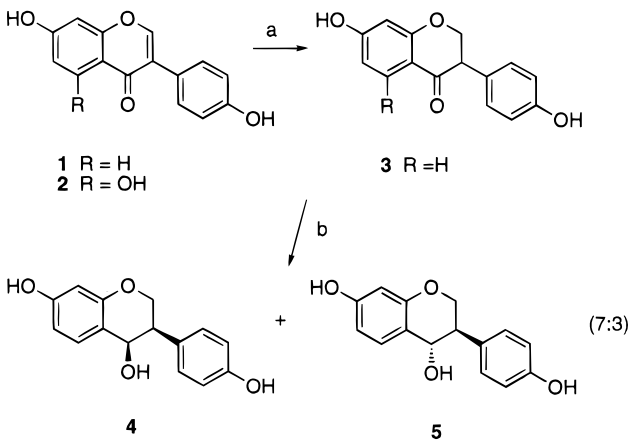
Table 1. Selected NMR Data on **4** and **5**

	δ_{H-2a}	δ_{H-2e}	δ_{H-3}	δ_{H-4}	$J_{2a,2e}$	$J_{2a,3}$	$J_{2a,4}$	$J_{2e,3}$	$J_{2e,4}$	$J_{3,4}$
4	4.52	4.14	3.12	4.64	−10.24	12.07	−0.28	3.65	−1.36	3.04
5	4.18	4.26	3.01	4.77	−11.03	8.27	−0.47	3.56	−0.35	7.05

Scheme 1[a]



[a] (a) Pd/C, NH_4OCHO, MeOH; (b) $LiBH_4$, THF.

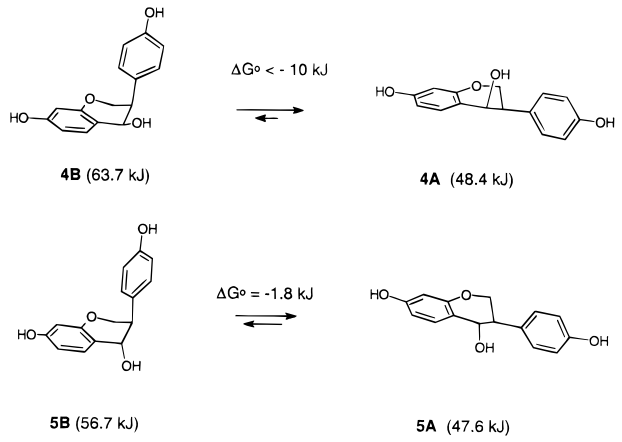


Figure 1.

droxyisoflavans in high yields and purity, but no intermediate corresponding isoflavanols were detected.[12] We found that although the $NaBH_4$ reduction of the unsubstituted isoflavone leads to the *cis*- and *trans*-isoflavanols in 1:3 ratio, hydroxy-substituted isoflavones, particularly the 7-hydroxy-substituted derivatives, react very sluggishly with the borohydride reducing agents.[13]

Thus the two isoflavanol stereoisomers of tetrahydrodaidzein (**4**, **5**) were synthesized by reducing dihydrodaidzein (4′,7-dihydroxyisoflavanone) (**3**) with excess lithium borohydride in THF to furnish a mixture of *cis*- and *trans*-4′,7-dihydroxyisoflavan-4-ols (**4**, **5**) in a 7:3 ratio. The slight difference in their R_f values made them detectable by TLC but this was not enough to allow separation by preparative TLC or flash chromatography. Thus **4** and **5** were isolated by preparative HPLC using an RP18 column and MeOH−H_2O elution. Dihydrodaidzein (**3**) was obtained by the catalytic transfer hydrogenation[14] of the corresponding isoflavone[10] with ammonium formate (Scheme 1).

Structural and Molecular Mechanical Studies. The key signals and the associated coupling constants in the ^1H NMR spectra of the *cis*- and *trans*-isoflavanols **4** and **5** are given in Table 1. The assignment of stereochemistry to the two isoflavanols is readily made on the basis of the vicinal coupling constant $J_{3,4}$.[15] Overall, the coupling constants in the pyran rings are fully compatible with the half-chair conformation where the phenyl group is in the equatorial position. Further support for this is provided by the long-range coupling of 1.4 Hz between H-2e and H-4 in **4**. Moreover, certain related isoflavans, proven[16] by X-ray work to have the pyran ring in a half-chair conformation, have very similar H-2,H-3 coupling constants. Comparison of the *cis* and *trans* isomers reveals that while in the *cis* series the larger $J_{2,3}$ (corresponding to the axial−axial coupling) is 11−12 Hz, in the *trans* compounds this coupling is only 8−9 Hz. Also, a $J_{3,4}$ of 6−7 Hz is too small to be an axia− pseudoaxial coupling in the *trans* series. We interpret these values as arising from the conformational mobility of the *trans*-isoflavanols, in contrast to the anancomeric nature of the *cis* compounds, a fact that has not been reported previously.

All ^{13}C NMR signals could be assigned by the C−H COSY and long-range C−H COSY techniques. The spectra of the *cis* and *trans* isomers are very similar, the only notable difference between **4** and **5** being in the chemical shifts of C-2. In the *cis*-isoflavanol (**4**) this carbon (at δ 65.3) is shifted upfield from the corresponding carbon (at δ 68.9) in the *trans* isomer (**5**). This upfield shift is evidently due to the γ-gauche effect of the axial hydroxyl in the *cis* isomer. The observed chemical shift difference, 3.6 ppm, is smaller than would be expected for single conformations (4.7 ppm[17]). Assuming that this deviation is caused by the conformational equilibrium of **5,** the conformer ratio can be estimated as 77:23.

The conformational energies of the ring substituents in benzodihydropyrans (chromans) are expected to be small because the heterocyclic ring is flattened. Thus one would expect that the *trans* isomer, with two equatorial substituents, would prefer mainly the diequatorial conformation, whereas the *cis* isomer, with one equatorial and one axial substituent, would be mobile, contrary to what is observed. This unexpected behavior was examined by MacroModel molecular mechanics calculations.[18] There are two low-energy interconverting half-chair conformations for **4** and **5** each (Figure 1). Other conformations are merely high-energy rotamers of hydroxyls and/or phenyls. Calculated structures indicate that the pyran ring is a somewhat flexible half-chair[19]

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Table 2. Calculated[a] and Observed ^3J for 4 and 5

	2a,3	2e,3	3,4
4A calcd	11.2	3.1	2.0
4B calcd	1.6	2.7	5.8
4 observed	12.07	3.65	3.05
5A calcd	11.2	3.2	10.5
5B calcd	1.9	2.3	1.8
5A:5B 68:32	8.2	2.9	7.7
5 observed	8.27	3.56	7.05

[a] Coupling constants obtained by applying Karpus equation on MacroModel or SYBYL structures (identical values).

and that no sofalike minima were found, in contrast to some related flavanols.[20] The axial−axial H-2,H-3 coupling constants were calculated for each isomer by applying the Karplus equation[21] (Table 2). Comparison with the experimental values shows that **4** ($J_{2a,3}$ = 12.1 Hz) clearly exists in the biased conformation **4A,** whereas **5** ($J_{2a,3}$ = 8.3 Hz) is a 68:32 mixture of the conformers **5A** and **5B**. This estimate of the equilibrium is in reasonable agreement with the value (77:23) obtained on the basis of carbon chemical shifts.

Molecular mechanics calculations suggest an explanation for the different conformational behavior of the isoflavanol isomers. It appears that the higher-energy conformer of the *cis* compound **4B** is strained because of the axial phenyl and the equatorial OH come too close to each other. To relieve strain, the phenyl group is rotated away from the hydroxyl to an almost eclipsing position with respect to the C-3 hydrogen [ϕ(C-2′−C-1′−C-3−H3) = 5°]. In comparison, the axial phenyl of **5B** adopts the normal, less strained conformation (torsion angle 37°). This explanation is supported by the fact that isoflavans for which the crowding hydroxyl is lacking are conformationally mobile to some extent. Enthalpies for the ring inversion (15 kJ/mol for **4**, 9 kJ/mol for **5**) calculated by MacroModel agree qualitatively with the experimental estimates (ΔG > 10 kJ for **4**, ΔG = 1.8 kJ for **5**). The discrepancies may be attributed to entropy factors and to possibly invalid MM3 force field parameters. The Tripos force field of the SYBYL[22] program gave a somewhat better agreement with the experimental energies (2.3 kJ/mol for **4**, 0.4 kJ/mol for **5**).

Anticancer Activity. Owing to the notable anticancer activity of daidzein,[1,23] the biological precursor of **4** and **5**, and the oestrogenic activity[24] of the daidzein metabolites equol and *O*-demethylangolensin, a preliminary survey of possible anticancer properties of **4** and **5** was undertaken. Specifically, the two isoflavanols were tested as to their effect on the growth of two types of malignant cells, the LNCaP 90 prostate cancer cells and the Colo 205 colon cancer cells (both from ATCC cell lines, Rockville, MD) in culture, using the Amar blue technique.[25] The Colo 205 cell line was resistant to treatment with either compound up to concentrations of 100 μmol/L insofar that no effect on the proliferation was seen and no toxicity was observed. As regards the prostate cancer cells, however, both **4** and **5** showed an inhibition on cell growth, the IC_{50} being about 40−50 μmol/L. Furthermore, already at the 5−10 μmol/L level a significant decrease in cell growth was found. At lower concentrations the *trans*-4′,7-dihydroxyflavan-4-ol appeared more effective than the *cis* form. No toxicity could be seen in concentrations of up to 100 μmol/L. *Thus **4** and **5** are more effective than the parent daidzein* and are comparable to genistein (4′,5,7-trihydroxyisoflavone) with regard to their activity against prostate cancer cells.[26]

Experimental Section

Melting points were obtained in open capillary tubes and are uncorrected. NMR spectra were recorded on a 400 MHz spectrometer with Me_4Si as the internal standard. The heteronuclear correlation experiments were optimized for 145 Hz coupling constants and the long-range experiments for 10 Hz coupling constants. Mass spectra were obtained using EI ionization at 70 ev. Microanalyses were performed by the Analytische Laboratorien, Lindlar, Germany.

Dihydrodaidzein (3). Daidzein[10] (0.255 g, 1 mmol) was dissolved in methanol (20 mL) and 0.255 g of 10% Pd/C and ammonium formate (0.252 g, 4 mmol) were added under argon. After refluxing for 2 h the reaction mixture was cooled and filtered, the solvent evaporated, and the crude product recrystallized from CH_2Cl_2−EtOAc to give **3** (0.211 g, 82%): mp 250 °C (lit.[27] mp 250−1 °C); IR υ_{max} 3262 (OH) 1658 cm^{-1} (CO); ^{1}H NMR (200 MHz; acetone-d_6) δ 3.86 (t, 1H), 4.62 (d, J = 6.7 Hz, 2H), 6.41 (d, J = 2.3 Hz, 1H), 6.57 (dd, J = 8.8, 2.3 Hz, 1H), 6.80 (d, J = 8.2 Hz, 2H), 7.15 (d, J = 8.2 Hz, 2H), 7.75 (d, J = 8.7 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 50.03, 71.15 , 102.16, 110.52, 113.52, 115.09, 126.08, 128.92, 129.49, 156.38, 162.97, 164.34, 190.50; λ_{max} (94% EtOH) nm (ϵ) 214 (21 900), 278 (13 600), 313 (7850); *m*/*z* 256 (M^+, 22), 137 (100), 120 (35), 108 (5) and 91 (7); HRMS calcd for $C_{15}H_{12}O_4$ 256.0736, found 256.0744.

***cis*- and *trans*-4′,7-Dihydroxyisoflavan-4-ol (4, 5)**. Dihydrodaidzein (**3**, 0.150 g, 0.59 mmol) in THF (12 mL) was added slowly to a solution of $LiBH_4$ (0.089 g, 40 mmol) in THF (6 mL) at 0° under Ar. The reaction was monitored by TLC, and after 27 h at rt the reaction mixture was poured cautiously into a cold saturated NH_4Cl solution, extracted with EtOAc, dried with Na_2SO_4 and evaporated giving a mixture (0.14 g, 94%) of two isomers (7:3 ratio by NMR) which were separated by preparative HPLC.

***cis*-4′,7-Dihydroxyisoflavan-4-ol (4) (the major isomer)**: mp 167 °C (dec); ^{1}H NMR (acetone-d_6) δ 3.12 (dt, J = 12.4, 3.2 Hz, 1H), 3.89 (d, J = 4.8 Hz, 1H), 4.13 (ddd, J = 10.3, 3.7, 1.3 Hz, 1H), 4.51 (dd, J = 10.3, 12.1 Hz, 1H), 4.64 (br t, J = 4.0 Hz, 1H), 6.29 (d, J = 2.4, 1H), 6.39 (dd, J = 8.2, 2.4 Hz, 1H), 6.79 (d, J = 8.4 Hz, 2H), 7.07 (d, J = 8.2 Hz, 1H), 7.19 (d, J = 8.4 Hz, 2H); ^{13}C NMR (acetone-d_6) δ 44.74 , 65.30, 67.26, 103.30, 108.81, 115.85, 118.31, 130.58, 130.87, 132.33, 156.04, 157.12, 159.18; λ_{max} (94% EtOH) nm (ϵ) 199 (68 200), 224s (19 700), 280 (4850); *m*/*z* 240 (90), 239 (100), 223 (7), 210 (5), 181 (5), 165 (5), 147 (15), 120 (18), 71 (10); 258 (M^+) was detected at 19 eV; HRMS calcd for $C_{15}H_{14}O_4$ 258.0892, found 256.0744. Anal. Calcd for $C_{15}H_{14}O_4$: C, 69.76; H, 5.46. Found: C, 69.44; H, 4.97.

***trans*-4′,7-dihydroxyisoflavan-4-ol (5)**: mp 124 °C (dec); ^{1}H NMR (acetone-d_6) δ 3.00 (dt, J = 4.0, 7.2 Hz, 1H), 4.18 (dd, J = 7.9, 10.5 Hz, 1H), 4.25 (dd, J = 11.0, 4.0 Hz, 1H) 4.77 (br t, J = 6.4 Hz, 1H), 6.25 (d, J = 2.4 Hz, 1H), 6.42 (dd, J = 8.4, 2.4 Hz, 1H), 6.72 (d, J = 8.6 Hz, 2H), 7.16 (d, J = 8.6 Hz, 2H), 7.26 (d, J = 8.4 Hz, 1H); ^{13}C NMR (acetone-d_6) δ 47.22, 68.87,

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69.26, 103.06, 109.13, 116.08, 118.47, 129.93, 130.81, 131.71, 156.33, 157.17, 158.71; λ_{max} (94% EtOH) nm (ϵ) 199 (44 600), 225s (14 100), and 280 (3550); *m*/*z* 258 (M^+, 2), 254 (8), 240 (90), 239 (100), 238 (62), 226 (7), 210 (27), 197 (5), 181 (18), 165 (8), 147 (14), 120 (15); HRMS calcd for $C_{15}H_{14}O_4$ 258.0892, found 258.0901. Anal. Calcd for $C_{15}H_{14}O_4$: C, 69.76; H, 5.46. Found: C, 69.46; H, 4.97.

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