Isolation of Flavonoids from *Populus nigra* as Δ^4-3-Ketosteroid (5α) Reductase Inhibitors

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Inhibitors of Δ^4-3-ketosteroid (5α) reductase, which had been prepared from rat prostate and converted testosterone to 5α-dihydrotestosterone and 4-androstene-3,17-dione, were isolated from 50% ethanol extract of *Populus nigra*. They were identified as pinobanksin (I, 3,5,7-trihydroxyflavanone), 3,7-dimethylquercetin (II, 3′,4′,5-trihydroxy-3,7-dimethoxyflavone) and pinocembrin (III, 5,7-dihydroxyflavanone). Compound III showed the most potent inhibitory activity among them.

Keywords Δ^4-3-ketosteroid (5α) reductase; testosterone; 5α-dihydrotestosterone; *Populus nigra*; pinocembrin; pinobanksin; 3,7-dimethylquercetin; inhibitor; flavonoid

Δ^4-3-Ketosteroid (5α) reductase prepared from rat prostate converts testosterone mainly to 5α-dihydrotestosterone and slightly to 4-androstene-3,17-dione, and the enzymatic activity is inhibited by estradiol.[1] 5α-Dihydrotestosterone is a more active circulating androgen in man than testosterone. Therefore, inhibitors of this enzymatic conversion of testosterone may be useful as drugs for androgen-dependent diseases, such as prostatomegaly.

In the course of research on the isolation of biologically active substances from natural products, the 50% ethanol extract of a mixture of flowers and buds of *Populus nigra* showed inhibitory activity on this enzymatic conversion. In this paper, we report the isolation of three active constituents from *P. nigra* and their identification as pinobanksin (I, 3,5,7-trihydroxyflavanone), 3,7-dimethylquercetin (II, 3′,4′,5-trihydroxy-3,7-dimethoxyflavone) and pinocembrin (III, 5,7-dihydroxyflavanone).

Isolation and Identification of the Active Constituents The 50% ethanolic extract, which showed 11% inhibition of the enzymatic conversion of testosterone to 5α-dihydrotestosterone and 4-androstene-3,17-dione was partitioned between ethyl acetate and water. The ethyl acetate fraction, which exhibited an inhibitory activity of 15%, was preliminarily separated into eight fractions by high-performance liquid chromatography (HPLC). These fractions were further investigated for inhibitory activity and it was found that frs. 1-3, 1-4 and 1-5 exhibited potent activity (22%, 21% and 44%, respectively), as shown in Chart 1. The HPLC pattern showed that frs. 1-3 and 1-4 each contained mainly a single compound, tentatively named compound I and compound II, respectively, while fr. 1-5 contained two compounds, compound III and compound IV.

Therefore, fractionation of the ethyl acetate fraction was carried out to isolate these four compounds as shown in Chart 2, guided by HPLC analysis.

Compound I, colorless plates, mp 175—177 °C, $[\alpha]_D^{20}$ 14.3° ($c=0.5$, methanol) was indicated to be pinobanksin (3,5,7-trihydroxyflavanone) from the mass spectrum (MS) and the proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra.

Compound II was obtained as yellow needles, mp 242—243 °C and its MS and ^{1}H- and ^{13}C-NMR spectra indicated it to be 3,7-dimethylquercetin (3′,4′,5-trihydroxy-3,7-dimethoxyflavone). Analysis of the ^{1}H- and ^{13}C-NMR spectra and MS indicated that compound III, colorless minute needles, mp 201—202 °C, $[\alpha]_D^{20}$ −46.0° ($c=0.9$, acetone), was pinocembrin (5,7-dihydroxyflavanone).

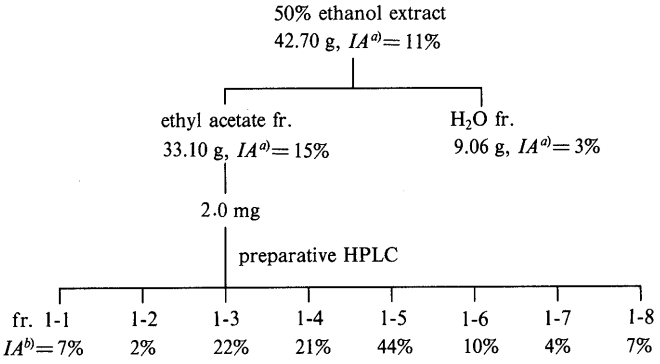

Chart 1. Fractionation of 50% Ethanol Extract and Inhibitory Activity in Enzymatic Assay

$IA^{a)}$: Inhibitory activity of 18.2 μg. Estradiol showed $IA=27.5\%$. $IA^{b)}$: The amount of each fraction assayed was equivalent to 182 μg of ethyl acetate fraction. Conditions of preparative HPLC: column, Nova-PAK C_{18} Radial-PAK; mobile phase A, CH_3COONH_4 (10 mM, pH 4.0); mobile phase B, CH_3CN, 0% B/A to 100% B/A linear gradient; elution time, 20 min at 2.0 ml/min; detector, 254 nm.

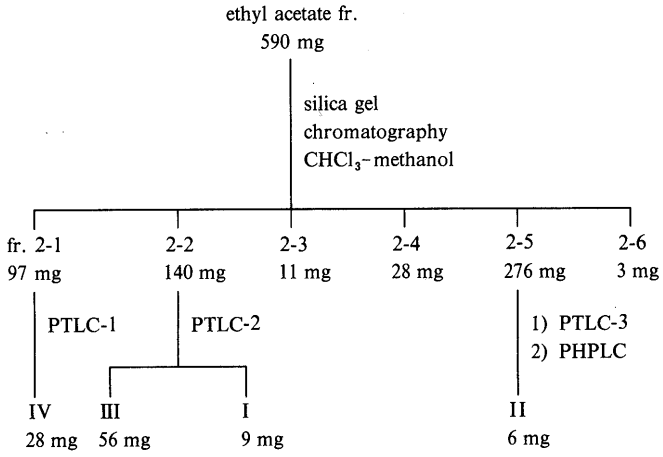

Chart 2. Isolation of Compounds I, II, III and IV from the Ethyl Acetate Fraction by Fractionation

PTLC, preparative thin layer chromatography; PTLC-1, silica gel with 1% methanol–$CHCL_3$; PTLC-2, silica gel with 2% methanol–$CHCl_3$; PTLC-3, silica gel with 5% methanol–$CHCl_3$; PHPLC, preparative HPLC [column, Nova-PAK C_{18} Radial-PAK; mobile phase, 40% CH_3CN–CH_3COONH_4 (10 mM, pH 4.0) at 2.0 ml/min].

These three compounds isolated from the ethyl acetate fraction were finally identified by comparisons of the

Table I. Inhibitory Activity of Compounds I, II, III and IV

Compound	Dose (nmol)	Inhibitory activity (%)
I	4	5
	20	6
	100	14
II	4	6
	20	7
	100	16
III	4	3
	20	21
	100	31
IV	4	2
	20	6
	100	4
Estradiol	4	14
	20	20
	100	33

spectral data with the literature values (compound I,[2] compound II[3] and compound III[4]).

Compound IV, yellow plates, mp 286—287 °C was indicated from ^{1}H- and ^{13}C-NMR spectra and MS to be chrysin (5,7-dihydroxyflavone), and this was confirmed by direct comparison with an authentic sample.

Inhibition of the Enzymatic Activity Compounds I, II, III and IV were tested for inhibitory activity on the conversion of testosterone to 5α-dihydrotestosterone and 4-androstene-3,17-dione by Δ^4-3-ketosteroid (5α) reductase prepared from rat prostate. The results are summarized in Table I.

Compound III showed the most potent inhibition among them and the potency was almost the same as that of estradiol, which was used as a positive control. Compounds I and II exhibited milder inhibitory activity than compound III. Compound IV showed almost no activity, and this result may be attributed to the low solubility of compound IV in the bioassay medium.

Studies are in progress on the inhibitory activity of various other flavonoids to investigate the structure–activity relationship.

Experimental

Materials Dried flowers and buds of *P. nigra* collected in Budapest, Hungary, were extracted with 50% ethanol and the filtrate was concentrated to give 50% ethanolic extract. [4-^{14}C]Testosterone (52 mCi/mmol) was purchased from New England Nuclear Corporation, U.S.A. Unlabelled steroids (testosterone, 5α-dihydrotestosterone, 4-androstene-3,17-dione and estradiol; guaranteed reagent grade) were purchased from Tokyo Kasei Kogyo Co., Ltd., Japan.

Preparation of Prostate Juice Male Wistar rats (200—220 g) were killed by decapitation and the prostates were immediately removed and immersed in cold (0—4 °C) 50 mM Tris buffer, pH 7.4, containing NaCl (61 mM), KCl (1.5 mM), $MgCl_2 \cdot 6H_2O$ (1 mM), nicotinamide (15 mM) and fumaric acid (1 mM). The prostates were blotted with filter papers, weighed, cut into small pieces with scissors, added to four volumes of the same Tris buffer and homogenized in a glass homogenizer (30 strokes) under cooling with ice-water. The homogenate was centrifuged at 1000 rpm for 3 min. After separation of the supernatant, the pellet was washed with 1.4 volumes of the buffer and centrifuged under the same conditions as before. The combined supernatant was centrifuged again and the final supernatant (called the prostate juice) was used for enzymatic experiments.

Enzymatic Assay For the enzymatic conversion assay, [4-^{14}C]-testosterone (0.2 μCi, 3.8 nmol) and cold testosterone (2.9 nmol) dissolved with ethanol were added to test tubes. Estradiol as a positive control, and fractionated and isolated substances as test samples, were dissolved in methanol and further added to the test tubes. After evaporation of the solvent to dryness under a nitrogen stream, each residue was redissolved with methanol (50 μl) and mixed with Tris buffer (2 ml) and reduced nicotinamide-adenine dinucleotide phosphate tetrasodium (NADPH-4Na) (1 mg/1 ml in Tris buffer). The mixture was preincubated at 37 °C for 5 min and mixed with the prostate juice (1 ml). Incubation was performed at 37 °C in a shaking water bath for 30 min.

The reaction was terminated by the addition of 5 ml of ethyl acetate followed by shaking at 250 rpm for 20 min and centrifuging at 3000 rpm for 4 min. The ethyl acetate layer was separated from the aqueous phase, which was extracted further with ethyl acetate (4 ml × 2). The combined ethyl acetate extract was washed with saturated sodium bicarbonate solution (10 ml) and then 50% sodium chloride solution (10 ml). The ethyl acetate phase was dried over anhydrous sodium sulfate (2 g) overnight, filtered and evaporated to dryness under vacuum at 45 °C.

Each dry extract was supplemented with 200 μg of testosterone, 5α-dihydrotestosterone and 4-androstene-3,17-dione as markers, and separated into three fractions by means of preparative thin-layer chromatography (TLC) (plates, Silica gel 60 (Merck); developing solvent, chloroform–diethyl ether, 85 : 15). Adsorbent containing each of the three steroids was extracted with methanol (2 ml) at 300 rpm for 10 min, and centrifuged at 3000 rpm for 3 min. The methanol extract (1 ml) was added to Aquasol-2 (10 ml) and counted with a liquid scintillation counter (LSC 700: Aloka).

From the final ^{14}C counts, the inhibitory activity (*IA*) of each sample was calculated as follows.

$$\text{synthetic rate of 5}\alpha\text{-dihydrotestosterone } (S, \%) = \frac{D}{T+D+A} \times 100$$

D, counts of 5α-dihydrotestosterone; *T*, counts of testosterone; *A*, counts of 4-androstene-3,17-dione.

$$\text{inhibitory activity } (IA, \%) = \left(1 - \frac{S_{\text{sample}}}{S_{\text{control}}}\right) \times 100$$

S_{sample}, *S* in the presence of test sample or estradiol; S_{control}, *S* in the presence of testosterone alone.

Chemical Analysis All melting points were taken with a Yanagimoto microscope hot stage and are uncorrected. MS were obtained by the electron impact ionization method on a JEOL JMS-D300 mass spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were obtained using a JEOL FX-270 spectrometer at 270 and 67.8 MHz, respectively. Chemical shifts (δ) are reported in ppm downfield from internal tetramethylsilane (TMS) and the following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and dd = double doublets. Column chromatography was carried out using silica gel (Wakogel C-200, Wako). TLC was performed on Silica gel 60 F_{254} plates (Merck).

Compound I (Pinobanksin, 3,5,7-Trihydroxyflavanone) Colorless plates, mp 175—177 °C ($CHCl_3$). $[\alpha]_D^{20}$ 14.3° (*c* = 0.5, methanol). ^{1}H-NMR (in DMSO-d_6): 4.61 (1H, d, *J* = 11.2 Hz, H-3), 5.18 (1H, d, *J* = 11.2 Hz, H-2), 5.88 (1H, d, *J* = 2.0 Hz, H-6), 5.92 (1H, d, *J* = 2.0 Hz, H-8), 7.30—7.50 (3H, m, H-3′, H-4′, H-5′), 7.52 (2H, d, *J* = 6.1 Hz, H-2′, H-6′). ^{13}C-NMR (in DMSO-d_6): 71.5 (d, C-3), 82.8 (d, C-2), 95.1 (d, C-8), 96.1 (d, C-6), 100.3 (s, C-10), 128.0 (d, C-2′, C-6′), 128.1 (d, C-3′, C-5′), 128.6 (d, C-4′), 137.2 (s, C-1′), 162.4 (s, C-9), 163.3 (s, C-5), 167.0 (s, C-7), 197.4 (s, C-4). MS *m/z*: 272 (M^+), 243, 153, 120, 91.

Compound II (3,7-Dimethylquercetin, 3′,4′,5-Trihydroxy-3,7-dimethoxyflavone) Yellow needles, mp 242—243 °C (methanol). ^{1}H-NMR (in DMSO-d_6): 3.80 (3H, s, 7-OCH_3), 3.86 (3H, s, 3-OCH_3), 6.37 (1H, d, *J* = 2.0 Hz, H-6), 6.71 (1H, d, *J* = 2.0 Hz, H-8), 6.91 (1H, d, *J* = 8.0 Hz, H-5′), 7.49 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′), 7.59 (1H, d, *J* = 2.0 Hz, H-2′). ^{13}C-NMR (in DMSO-d_6): 56.0 (q, 7-OCH_3), 59.6 (q, 3-OCH_3), 92.1 (d, C-8), 97.6 (d, C-6), 105.1 (s, C-10), 115.4 (d, C-2′), 115.6 (d, C-5′), 120.4 (d, C-6′), 120.6 (s, C-1′), 137.8 (s, C-3), 145.3 (s, C-3′), 149.0 (s, C-4′), 155.9 (s, C-2), 156.2 (s, C-5), 160.9 (s, C-9), 165.0 (s, C-7), 177.9 (s, C-4). MS *m/z*: 330 (M^+), 301, 287.

Compound III (Pinocembrin, 5,7-Dihydroxyflavanone) Colorless minute needles, mp 201—202 °C ($CHCl_3$). $[\alpha]_D^{20}$ −46.0° (*c* = 0.9, acetone). ^{1}H-NMR (in DMSO-d_6): 2.78 (1H, dd, *J* = 17.1, 3.0 Hz, H-3α), 3.24 (1H, dd, *J* = 12.5, 17.1 Hz, H-3β), 5.58 (1H, dd, *J* = 3.0, 12.5 Hz, H-2), 5.92 (1H, d, *J* = 2.0 Hz, H-6), 5.95 (1H, d, *J* = 2.0 Hz, H-8), 7.30—7.50 (3H, m, H-3′, H-4′, H-5′), 7.52 (2H, d, *J* = 6.6 Hz, H-2′, H-6′). ^{13}C-NMR (in DMSO-d_6): 42.1 (t, C-3), 78.3 (d, C-2), 95.0 (d, C-8), 95.9 (d, C-6), 101.8 (s, C-10),

126.5 (d, C-2′, C-6′), 128.5 (d, C-3′, C-4′, C-5′), 138.6 (s, C-1′), 162.7 (s, C-9), 163.2 (s, C-5), 166.5 (s, C-7), 195.7 (s, C-4). MS *m/z*: 256 (M^+), 179, 152, 124.

Compound IV (Chrysin, 5,7-Dihydroxyflavone) Yellow plates, mp 286—287 °C (methanol). ^{1}H-NMR (in DMSO-d_6): 6.23 (1H, d, J=2.0 Hz, H-6), 6.53 (1H, d, J=2.0 Hz, H-8), 6.96 (1H, s, H-3), 7.50—7.70 (3H, m, H-3′, H-4′, H-5′), 8.06 (2H, d, J=6.2 Hz, H-2′, H-6′). ^{13}C-NMR (in DMSO-d_6): 94.1 (d, C-8), 98.9 (d, C-6), 103.9 (s, C-10), 105.1 (d, C-3), 126.3 (d, C-2′, C-6′), 129.1 (d, C-3′, C-5′), 130.6 (s, C-1′), 132.0 (d, C-4′), 157.4 (s, C-5), 161.1 (s, C-9), 163.2 (s, C-2), 164.1 (s, C-7), 181.7 (s, C-4). MS *m/z*: 254 (M^+), 226, 152, 124, 103.

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