Lignans from the Roots of *Urtica dioica* and their Metabolites Bind to Human Sex Hormone Binding Globulin (SHBG)

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Abstract: Polar extracts of the stinging nettle (*Urtica dioica* L.) roots contain the lignans (+)-neoolivil, (−)-secoisolariciresinol, dehydrodiconiferyl alcohol, isolariciresinol, pinoresinol, and 3,4-divanillyltetrahydrofuran. These compounds were either isolated from *Urtica* roots, or obtained semisynthetically. Their affinity to human sex hormone binding globulin (SHBG) was tested in an *in vitro* assay. In addition, the main intestinal transformation products of plant lignans in humans, enterodiol and enterolactone, together with enterofuran were checked for their activity. All lignans except (−)-pinoresinol developed a binding affinity to SHBG in the *in vitro* assay. The affinity of (−)-3,4-divanillyltetrahydrofuran was outstandingly high. These findings are discussed with respect to potential beneficial effects of plant lignans on benign prostatic hyperplasia (BPH).

Key words: *Urtica dioica*, Urticaceae, roots, benign prostatic hyperplasia, BPH, sexual hormone binding globulin, SHBG, SBP, (+)-neoolivil, (−)-secoisolariciresinol, dehydrodiconiferyl alcohol, (−)-isolariciresinol, (−)-pinoresinol, (±)-enterodiol, (±)-enterolactone, (±)-enterofuran, (−)-3,4-divanillyltetrahydrofuran, (−)-shonanin.

Abbreviations

ERU: extractum radicis urticae
R_I: retention index according to van den Dool et al. (1)
BPH: benign prostatic hyperplasia
MSD: mass selective detector
DHT: dihydrotestosterone

Introduction

SHBG is the major plasma sex hormone transport protein with a high affinity to androgens and estrogens (2). Beside transporting and storing of sex steroid hormones SHBG interacts with typical sex hormone target tissues, e.g., through its receptor on the prostate (3). The influence of SHBG on the physiology of the prostate suggests an involvement in the genesis of benign prostatic hyperplasia (4), an abundant neoplastic disease in ageing of men (5). SHBG was shown to be an allosteric protein in which the protein-receptor interaction depends on the occupancy of the steroid binding site (6), whereby any ligand of SHBG is expected to exert an effect on the prostate.

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Polar extracts of the roots of *Urtica dioica* L. are used in the treatment of BPH (7). A previous screening of ERU for SHBG-binding compounds resulted in the identification of the lignan (−)-secoisolariciresinol (**1**) as an active constituent (8). The binding of enterodiol (**2**) and enterolactone (**3**) (9), intestinal transformation products of (−)-secoisolariciresinol (10), emphasises the effectiveness of this molecule. Preliminary experiments indicated the occurrence of other lignans from stinging nettle roots to develop affinities towards SHBG. We report in this paper on the binding of stinging nettle lignans to SHBG.

Materials and Methods

Plant extract

Extractum Radicis Urticae L. (Finzelberg, Andernach, Germany) H_2O : MeOH = 4 : 1; percolation, drug : extract 14 : 1. (CH.-B. 1313200). A voucher specimen is kept in our institute.

Analytical methods

EI-MS: MAT 95, 70 eV (Finnigan). *GC* (H_2 at 50 kpa, 3 min 80 °C, 80 °C to 280 °C at 3 °C min^{-1}, 280 °C for 15 min) was performed on a Carlo Erba HRGC Fractovap 4160 chromatograph using a fused silica capillary column coated with DB 1 phase (30 m × 0.32 mm, film 0.1 μm, J&W, Folsom, CA, USA). *NMR*: Bruker AC 300. *HPLC*: Beckman system gold was employed with a variable wavelength detector operating at 280 nm. *Prep. HPLC column*: Li Chroprep RP 18 5–10 μm (240 mm × 20 mm). *Semi-prep. HPLC column*: Spherisorb ODS-2, 5 μm (240 mm × 8 mm). *MSD*: Hewlett Packard MSD 5980, 70 eV. *Optical rotation*: Perkin Elmer 124 (l = 10 cm), solvent as indicated.

Trimethylsilylation

Trimethylsilylation was obtained by reaction with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Machery & Nagel, Düren, Germany) for 8 h at room temperature.

Hydrolysis of glycosidic compounds

ERU (100 g) and 1 g Cellulase CT (Merck, Darmstadt, Germany) were dissolved in 500 ml water and stirred at 23 °C for 2 d.

Preparation of SHBG

SHBG was obtained as described earlier (8).

Testing conditions

Testing conditions were the same as reported previously (8) with minor modifications. Briefly, human SHBG [9.7 nM] was incubated with [1,2-$^3H(N)$]-dihydrotestosterone (NEN, Dreieich/Germany, sp. act. 45.5 Ci/mmol) [8.8 nM] and the investigated lignan [250 mg/l] for 3 h at 4 °C. Non-bound ^3H-DHT was removed by the addition of dextran-coated charcoal (5 min). The remaining activity was corrected for non-specific binding which was measured by using a 300-fold excess of unlabeled DHT instead of the lignan (8).

Determination of SHBG concentrations

The concentration of SHBG were determined by an immunoenzymometric assay. (SR1, BioChem ImmunoSystems GmbH Freiburg Germany). The value of 9.7 nM was in good agreement with the one calculated by saturation of SHBG with ^3H-DHT in the charcoal assay 9.6 ± 0.6 nM (n = 10).

Origin of the individual compounds

Isolation of lignans from ERU: ERU (200 g) was dissolved in 500 ml H_2O and 1 g Cellulase CT (Merck, Darmstadt, Germany) was added. After stirring for 2 d at room temperature the aqueous solution was extracted subsequently with cyclohexane and EtOAc (2 × 300 ml). The EtOAc extract was directly used for preparative HPLC (solvent: MeOH/H_2O; flow: 18 ml/min, gradient: 20 % MeOH to 50 % MeOH in 30 min).

(+)-Neoolivil (**5**): Prep. HPLC R_t = 10.4 min. After semiprep. rechromatography of the crude neoolivil (solvent: MeCN/H_2O; flow: 2 ml/min, isocratic 20 % MeCN; R_t = 13 min) 8.3 mg pure (+)-neoolivil was obtained. $[\alpha]_D^{23}$: +49° (EtOH, *c* 0.159); R_i (trimethylsilylated) = 3230; EIMS: *m/z* (rel. int.) = 376 [M$^+$] (66), 224 (34), 196 (61), 194 (12), 193 (88), 180 (42); 176 (63), 175 (100), 151 (29), 137 (44); ^{1}H-NMR and ^{13}C-NMR are in agreement with (15).

(−)-Secoisolariciresinol (**1**): Prep. HPLC R_t = 21 min. Semiprep. rechromatography (solvent: MeCN/H_2O; flow 2.0 ml/min; gradient 20 % to 50 % MeCN in 30 min; R_t = 15.2 min) yielded 8.3 mg pure (−)-secoisolariciresinol. $[\alpha]_D^{23}$: +29° (EtOH, *c* 0.17); R_i (trimethylsilylated) = 3050; EIMS (trimethylsilylated): *m/z* (rel. int.) = 651 (10), 650 (19), 561 (18), 560 (38), 471 (8), 470 (17), 262 (15), 261 (68), 248 (20), 247 (18), 235 (15), 210 (73), 209 (100), 180 (10), 179 (32), 73 (94); (The ratio of *m/z* 474: *m/z* 470 was determined to be 5 × 10^{-3}).

(±)-9,9′-d_4-Secoisolariciresinol (**4**): d_4-(±)-Secoisolariciresinol was synthesised according to (11) using $LiAlD_4$ for reduction; purification was achieved by semiprep. HPLC [see (−)-secoisolariciresinol]; R_i (trimethylsilylated) = 3050; EIMS (trimethylsilylated): *m/z* (rel. int.) = 655 (8), 654 (M$^+$, 15), 565 (15), 564 (32), 475 (7), 474 (16), 266 (12), 265 (49), 264 (32), 263 (15), 250 (15), 249 (18), 248 (11), 237 (16), 211 (56), 210 (85), 209 (100), 180 (13), 179 (35), 147 (20), 105 (23), 74 (24), 73 (95) (The ratio of *m/z* 474: *m/z* 470 was determined to be 3 × 10^4).

Dehydrodiconiferyl alcohol (**6**): Prep. HPLC R_t = 22 min. Semiprep. rechromatography [same conditions as for (−)-secoisolariciresinol; R_t = 16.4 min] afforded 0.88 mg pure dehydrodiconiferyl alcohol. R_i (trimethylsilylated) = 3250; EIMS (trimethylsilylated): *m/z* (rel. int.) = 574 [M$^+$] (22), 559 (4), 544 (8), 484 (100), 297 (19), 209 (13), 147 (10), 103 (7), 75 (31), 73 (35). The sample displayed no optical rotation.

(−)-Isolariciresinol (**7**): (−)-Conidendrin (3.8 mg; a generous gift from Prof. K. Weinges, Heidelberg, Germany) was dissolved in 5 ml dry THF, added to 1 mg $LiAlH_4$ suspended in 5 ml THF at 0 °C and stirred for 4 h at 23 °C. After hydrolysis with 5 drops of a saturated K_2CO_3 solution and acidification with 0.1 M HCl (pH 5) the lignans were partitioned between 50 ml water and 2 × 50 ml CH_2Cl_2. Evaporation of the organic phase yielded 2.1 mg (−)-isolariciresinol (purity >97 %, detd. by GC). R_i (trimethylsilylated) = 3010; EIMS (trimethylsilylated): *m/z* (rel. int.) = 648 [M$^+$] (21), 633 (16), 558 (62), 527 (64), 486 (15), 486 (39), 455 (100), 437 (55), 428 (29), 209 (26), 147 (17), 103 (10), 73 (78).

(−)-Pinoresinol (**8**): Crude (+)-neoolivil (12 mg) was dissolved in 5 ml trimethyl orthoformate, 20 µl H_2SO_4 (96 %) were added, the mixture was heated to 100 °C for 2 h, and stirred for 10 h. After neutralisation with saturated $NaHCO_3$ the solution was diluted with 50 ml H_2O and extracted two times with 100 ml CH_2Cl_2. The two main compounds were separated by semipreparative HPLC (solvent MeCN/H_2O, flow 2 ml/min, gradient 20 % to 50 % MeCN in 30 min; R_t = 14 min); 3.2 mg pure (−)-pinoresinol was obtained. R_i (trimethylsilylated) = 3257; EIMS: *m/z* (rel. int.) = 358 [M$^+$] (84), 327 (13), 180 (16), 163 (39), 153 (12), 152 (42), 151 (100), 150 (33), 137 (64), 131 (34). ^{1}H-NMR, ^{13}C-NMR and $[\alpha]_D^{23}$ are in agreement to (12, 13).

(−)-3,4-Divanillyltetrahydrofuran (**9**): (−)-Secoisolariciresinol (5.8 mg) was dissolved in 2 ml methanol and a solution of 10 µl H_2SO_4 in 2 ml trimethyl orthoformate was added. After stirring for 8 h at room temperature 30 ml of saturated $NaHCO_3$ were added. Extraction with 2 × 20 ml CH_2Cl_2 yielded 4.2 mg pure (−)-3,4-divanillyltetrahydrofuran. $[\alpha]_D^{23}$: −62° (EtOH, *c* 0.088), R_i (trimethylsilylated) = 2920; EIMS (trimethylsilylated): *m/z* (rel. int.) = 489 (33), 488 (100), 473 (4), 261 (3), 223 (3), 210 (40), 209 (59), 179 (16), 73 (13). ^{1}H-NMR and ^{13}C-NMR is in accordance to (14).

(±)-Enterolactone (**3**) and *(±)-enterodiol* (**2**) were generous gifts from Prof. M. Metzler and E. Jacobs (Lebensmittelchemie, University Karlsruhe, Germany).

Enterofuran (**10**): Enterodiol (0.72 mg) was dehydrated to enterofuran (analogous to 3,4-divanillyltetrahydrofuran). R_i (trimethylsilylated) = 2600; EIMS (trimethylsilylated): *m/z* = 429 (8), 428 [M$^+$] (22), 231 (7), 179 (53), 180 (100), 165 (34), 73 (62); ^{1}H-NMR: (300 MHz, $CDCl_3$): δ = 2.18 (m, 2H, H-8), 2.55 (m, 4H, H-7), 3.50 (dd, 2H, ^2J = 8.7 Hz, ^3J = 6.2 Hz, H-9$_a$), 3.89 (dd, 2H, ^2J = 8.7 Hz, ^3J = 6.7 Hz, H-9$_b$), 6.54 (t, 2H, ^4J = 2 Hz, H-2), 6.65 (m, 4H, H-4, H-6), 7.11 (t, 1H, ^3J = 8 Hz, H-5).

Results

The phenolic constituents of ERU have already been the subject of investigations (15, 16), but still there is little knowledge about the amount of the lignans. Determination of the concentration of the active (−)-secoisolariciresinol (**1**) was achieved by addition of (±)-d_4-secoisolariciresinol (**4**) as an internal standard to an aqueous ERU solution, used for pharmaceutical applications. The solution was extracted subsequently with cyclohexane and EtOAc. The EtOAc fraction was trimethyl-

silylated and separated by GC using MSD and FID for detection. From the intensity of the ions $m/z = 474$ and $m/z = 470$ in a dual ion monitoring mode the amount of (−)-secoisolariciresinol (**1**) was calculated presuming identical recovery rates and fragmentation of both compounds. ERU was found to contain 40.9 ± 1.1 mg/kg ERU (n = 3) free (−)-secoisolariciresinol (**1**). After hydrolysis of glycosidic bonds with cellulase the total content of **1** was determined as 250 ± 20 mg/kg (n = 3).

Since pure lignans were needed for our *in vitro* test, the EtOAc fraction of ERU was separated by two step HPLC resulting in the isolation of the abundant (+)-neoolivil (**5**), (−)-secoisolariciresinol (**1**), and dehydrodiconiferyl alcohol (**6**). Minor components were obtained semisynthetically. $LiAlH_4$ reduction of (−)-conidendrin gave (−)-isolariciresinol (**7**). (−)-Pinoresinol (**8**) and (−)-3,4-divanillyltetrahydrofuran (**9**) were obtained by acid catalysed dehydration from (+)-neoolivil (**5**) and (−)-secoisolariciresinol (**1**). Conversion of **1** to (−)-3,4-divanillyltetrahydrofuran (**8**) was observed, although to a small extent, in HPLC systems employing 0.1 % trifluoroacetic acid in the solvent mixture. The amounts of these lignans (**5–8**) in ERU were estimated by GC-FID of the trimethylsilylated EtOAc fraction.

Table 1 Concentrations of the tested lignans in ERU after enzymatic hydrolysis.

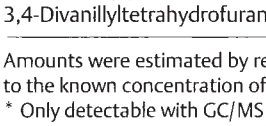
Compound	FID-signal related to **1**	mg/kg ERU
(−)-Secoisolariciresinol (**1**)	100 %	250 ± 20
(+)-Neoolivil (**5**)	150 ± 7 %	380 ± 35
Dehydrodiconiferylalcohol (**6**)	9 ± 3 %	25 ± 8
Isolariciresinol (**7**)	8 ± 2 %	20 ± 7
Pinoresinol (**8**)	8 ± 2 %	20 ± 7
3,4-Divanillyltetrahydrofuran (**9**)	–	traces*

Amounts were estimated by relating the FID signal of the different lignans to the known concentration of **1**.
* Only detectable with GC/MS using the 488 [M⁺] ion trace.

Table 2 Binding affinity of lignans to SHBG.

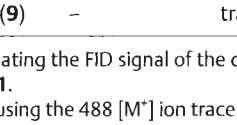
Compound	displacement of ^3H-DHT	n
Enterodiol (**2**)	16 ± 6 %	3
Enterolacton (**3**)	55 ± 3 %	5
Enterofuran (**10**)	73 ± 5 %	3
(+)-Neoolivil (**5**)	22 ± 7 %	3
(−)-Secoisolariciresinol (**1**)	60 ± 7 %	5
Dehydrodiconiferylalcohol (**6**)	34 ± 6 %	3
(−)-Isolariciresinol (**7**)	10 ± 5 %	3
(−)-Pinoresinol (**8**)	0 ± 5 %	3
(−)-3,4-Divanillyltetrahydrofuran (**9**)	95 ± 5 %	5

Incubation of 9.7 nM SHBG with 8.8 nM ^3H-DHT and 250 mg/l lignan at 4 °C for 3 h.



1 R_1 = OMe; R_2 = OH

2 R_1 = OH; R_2 = H



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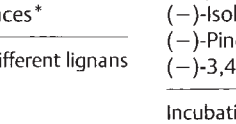
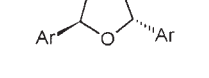


9 R_1 = OMe; R_2 = OH

10 R_1 = OH; R_2 = H

Fig. 1 Lignans tested for their binding affinity toward SHBG.



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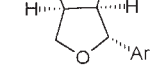

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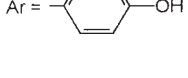

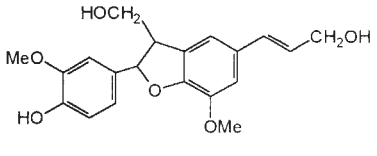

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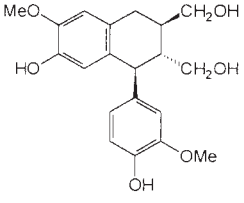

7

The peak areas of the individual compounds were related to that of (−)-secoisolariciresinol (Table **1**). Notable are the high concentrations of (+)-neoolivil (**5**) and (−)-secoisolariciresinol (**1**). The peak area of all other lignans is less than 10% compared to (−)-secoisolariciresinol. Compound **8** can only be detected by GC/MS using the ion trace $m/z = 488$ [M^+].

Investigations on the physiological effects of lignans must consider their intestinal microbial transformation products. Typical are the 3′-demethylation and 4′-dehydroxylation reactions of secoisolariciresinol (**1**) and matairesinol leading to enterodiol (**2**) and enterolactone (**3**) (17). These compounds were kindly provided by Prof. M. Metzler. An analogous transformation of (−)-3,4-divanillyltetrahydrofuran would lead to enterofuran (**10**), which was synthesised by dehydration of enterodiol.

For comparison of the binding affinities to SHBG, the above-mentioned lignans [even those with a known binding affinity (9, 8)] were investigated in an *in vitro* test system under identical testing conditions. We used a charcoal assay described previously (8). Briefly, test compounds were incubated with human SHBG in the presence of ^3H-DHT, which is a highly active androgen with the highest known binding affinity towards SHBG. The separation of bound and non-bound ^3H-DHT was achieved by adding dextran coated charcoal. With the exception of (−)-pinoresinol (**8**) all tested lignans displayed binding affinities to SHBG. While (±)-enterodiol (**3**), (+)-neoolivil (**5**), (−)-isolariciresinol (**7**), and dehydrodiconiferyl alcohol (**6**) developed only weak affinities, binding affinities of (−)-secoisolariciresinol and enterolactone were of a comparable moderate intensity. A higher binding affinity was observed with enterofuran (**10**) (Table **2**).

(−)-3,4-Divanillyltetrahydrofuran (**9**) was able to prevent completely ^3H-DHT from binding to SHBG under these testing conditions. Preliminary concentration-dependent tests revealed a significant effect of **9** in this *in vitro* test system down to concentrations of 1–2 mg/l.

Discussion

Various explanations are given concerning the action of ERU on BPH: Besides an interaction with SHBG (8), constituents of ERU are able to inhibit the binding of epidermal growth factor (EGF) to its receptor (18); furthermore an inhibition of aromatase was described (19). Relating to the SHBG-hypothesis (4), the positive effect of ERU may be due to its content of lignans. They occur predominantly in glycosidic forms. The glycosides are probably cleaved by digestion. Even the intestinal microbial transformation products of the lignans display a binding affinity to SHBG. The outstanding affinity of 3,4-divanillyltetrahydrofuran (**9**) is a further proof for the physiological effectiveness of this class of compounds (20).

According to the physiological role of SHBG, a twofold effect of lignans on the prostate is to be considered. Lignans may influence the blood level of free, i.e., active, steroid hormones by displacing them from the SHBG binding site (2). Furthermore lignans may influence the interaction of the prostate receptor with SHBG. Like steroid hormones lignans may inhibit the binding of SHBG to its receptor. Bound to the receptor SHBG is still able to bind sex steroids which results in the generation of the second messenger cAMP inside the cell (6). This reaction depends on the ligands of SHBG.

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