Aromatase Inhibitors from *Urtica dioica* Roots

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Abstract

Methanolic extracts of stinging nettle (*Urtica dioica* L.) roots were investigated for aromatase inhibition. Enzyme inhibition was detected only after appropriate chromatographic separation. Inhibitory effects on aromatase could be demonstrated *in vitro* for a variety of compounds belonging to different classes. The following compounds developed weak to moderate activity: secoisolariciresinol (**1**), oleanolic and ursolic acid (**2** and **3**), (9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid (**4**), and 14-octacosanol (**5**). Inhibitory effects on aromatase have been known to date neither for pentacyclic triterpenes nor for secondary fatty alcohols. The potential physiological significance of the above findings is discussed. Compound **5** is a previously unknown constituent of plants.

Key words

Urtica dioica, Urticaceae, roots, benign prostatic hyperplasia, aromatase inhibition, secoisolariciresinol, (9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid, oleanolic acid, ursolic acid, 14-octacosanol.

Abbreviations and Symbols

BPH:	Benign prostatic hyperplasia
CH:	cyclohexane
R_i:	Retention index on GC (according to Kováts)

Introduction

Benign prostatic hyperplasia (BPH) is the most commonly occurring neoplastic disease in the aging human male (1). Etiology of BPH is hardly understood (2), but estrogens seem to be involved (3, 4). Recently, estradiol was shown to exert strong influences on the human prostate (5).

Aromatase is a key enzyme in steroid hormone metabolism. It mediates the conversion of androgens to estrogens (6, 7). Inhibition of the aromatase system leads to decreased estrogen-levels (4, 8) and thereby, according to the above-mentioned theory, would improve the patients prostatic disorder. Consequently, positive effects of aromatase inhibitors on human BPH were reported (9).

Since *Urtica dioica* L. (Urticaceae) root extracts are successfully applied (10) in the treatment of stages I and II of BPH [according to Vahlensieck (11)], their effects could (at least in part) be based on the action of aromatase inhibitors. Therefore we investigated *U. dioica* roots in order to find aromatase inhibitors. Previously we reported on the identification of (10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid as an aromatase inhibitor (12). This paper deals with the continuation of this investigation.

Materials and Methods

Analytical methods

M.p.s: uncorrected. Preparative TLC was performed on self-made silica gel plates (20 × 20 cm; thickness 0.75 mm; silica gel 60 PF_{254}, Merck, Darmstadt/Germany). For HPLC separation we used a Beckman system with an Si 100-column (250 × 7.1 mm, 3 µm, Serva, Mannheim/Germany) and a Zorbax Sil pre-column (20 × 4.6 mm, 5 µm, DuPont, Bad Nauheim/Germany). Eluting compounds were detected at 212 nm and the solvent was programmed as follows (A: hexane; B: hexane/isopropanol, 1 : 9): 0 % B isocratically for 2 min → linear gradient to 30 % B in 30 min → linear gradient to 100 % B in 8 min → 100 % B isocratically for 15 min.

Analytical GC was carried out with a Carlo Erba HRGC Fractovap 4160 chromatograph equipped with a flame ionization detector, using a DB-1 fused silica glass capillary column (30 m × 0.32 mm i.d.; film thickness 0.1 µm) [temperature programmed from 80 to 280 °C at 3 °C min^{-1}]. The temperatures of the injector and the detector were kept at 270 °C and 290 °C, respectively. Carrier gas was hydrogen and the splitting ratio was 1 : 30. Retention indices (R_i) were calculated according to Kováts (13) with *n*-alkanes as reference compounds.

GC-MS was performed on a Finnigan MAT 95 system. EI-mass spectra were recorded at an ionization energy of 70 eV. An HP 5890 gas chromatograph was used for sample separation. Carrier gas was hydrogen and the temperature programme was the same as used for analytical GC.

^{1}H-NMR spectra were recorded on a Bruker AM 500 instrument at 500 MHz with TMS as internal standard (solvent as indicated).

Plant material

Dried and ground *U. dioica* roots were purchased from Chr. Finzelbergs Nachf. GmbH & Co. KG, Andernach/Germany (CH.-Nr. 2630596). A voucher specimen is kept in our institute.

Work-up procedure: 600 g of *U. dioica* roots were extracted 3 × with 4 l MeOH each (24 h, room temperature). Ex-

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tracts were combined and the solvent removed in vacuo. The residue was suspended in 1000 ml H_2O. This suspension was successively extracted with cyclohexane (CH), Et_2O, EtOAc, *n*-BuOH (500 ml × 4 each – solvent partition). Combined organic layers were washed with 200 ml H_2O and evaporated to dryness to give CH-, Et_2O-, EtOAc-, and *n*-BuOH-fractions (3.6 g, 1.2 g, 1.2 g, 4.8 g, respectively). These fractions were subjected to CC or preparative TLC separation for further fractionation: The CH-fraction contained **2** and **3** (see Scheme **1**) as well as **5** (1. preparative TLC, CH_2Cl_2-EtOAc, 9 : 1, R_f = 0.8–0.9; 2. preparative TLC, CH-EtOAc, 98 : 2 R_f = 0.05–0.15; 3. preparative TLC, CH-EtOAc, 8 : 2 R_f = 0.7–0.75). The Et_2O-fraction contained **4** (CC on 150 g silica gel (diameter of the column 3 cm), CH-EtOAc, 7 : 3, elution volume between 350 and 450 ml; 1. preparative TLC, toluene-Et_2O-MeOH, 50 : 40 : 10, R_f = 0.45–0.48; 2. TLC, CH-EtOAc, 7 : 3, R_f = 0.58–0.61), while the EtOAc-fraction contained **1** (CC on 80 g silica gel (diameter 3 cm), EtOAc, elution volume between 500–700 ml; 1. preparative TLC, CH-EtOAc 1 : 2, R_f = 0.15–0.2; 2. preparative TLC, EtOAc, R_f = 0.23–0.25). As the resulting fractions in each case contained impurities, compounds **1–5** were isolated, purchased, or synthesized in pure form (see below).

Origin of individual compounds

Isolation of **1**: 400 g dried and cut *U. dioica* roots were extracted × 3 with 2.5 l MeOH for 48 h each time. The crude extract (28 g) was suspended in 700 ml H_2O and extracted with 300 ml EtOAc × 5. After removal of the solvent, the extract (2.7 g) was separated by CC (150 g silica gel, CH-EtOAc, 1 : 2, elution volume of **1** 1.8–2.2 l). Three subsequent preparative TLC processes (silica gel, EtOAc) yielded 3.1 mg of pure **1** (R_f = 0.23–0.25). M.p.: 106–109 °C; $[\alpha]_D^{20}$: –13.44° {mixture of (+)and (–)-enantiomers 3 : 7, as $[\alpha]_D^{20}$ is reported to be –30.8° for (–)-**1** (14)}; R_i (TMSi-derivative): 3051; ^{1}H-NMR (acetone-d_6) and MS data corresponded with those given in the literature (15, 16).

Compounds **2** and **3** (purity 97% each) were purchased from Sigma, Deisenhofen/Germany and Roth, Karlsruhe/Germany, respectively.

Compound **4** was synthesized from linoleic acid by oxidation with O_2/soybean-lipoxygenase, subsequent reduction with $NaBH_4$ and finally purified by preparative TLC (17). Spectroscopic data (IR, MS, ^{1}H-NMR) corresponded with those in the literature (18, 19).

Compound **5** was synthesized by a Grignard reaction (20) and subsequently purified by preparative TLC (silica gel, CH-EtOAc, 9 : 1, R_f = 0.49–0.52). M.p. 79–80 °C; IR (KBr) cm^{-1}: 3400 (br), 2900, 2850, 1470, 1380, 1150, 710; R_i (TMSi-derivative) 2965; ^{1}H-NMR ($CDCl_3$): δ 0.86 (t, J = 7 Hz, 6 H), 1.08–1.35 (m, ca. 46 H), 1.35–1.46 (m, 4 H), 3.57 (m, 1 H); GC-MS (trimethylsilylated): 482 $[M]^+$ (2), 467 (7), 392 (2), 299 (100), 285 (96), 97 (4), 83 (4), 75 (6), 73 (4), 69 (3), 55 (3).

Biological assay

The aromatase assay was performed according to Thompson Jr. and Siiteri (6). Briefly, test buffer contained (final concentrations in a total volume of 540 µl) an NADPH-regenerating system (250 µM $NADP^+$, 3.8 mM glucose-6-phosphate, 250 U/l glucose-6-phosphate-dehydrogenase), 70 mg/l human placental microsomal protein, 8.1 mM nicotinamide, 6.6 mM $MgCl_2$, 1.3 mM dithiothreitol in 0.1 M potassium phosphate buffer. Incubation was started by addition of 7 nM [1β,2β-^3H(N)]-androst-4-ene-3,17-dione (NEN, Dreieich/Germany, sp. act. 50.5 Ci/mmol) and 325 nM radioinert androst-4-ene-3,17-dione together with test compounds. Temperature was held at 37 °C and incubation period was 15 min. Thereafter 200 µl of a 5% (w/v) charcoal suspension were added. After centrifugation, liberated tritiated water in the supernatant was counted. Its quantity is an index of the enzymatic reaction's progress. Inhibition of aromatase is indicated by reduction of the concentration of liberated tritiated water compared to reference incubations.

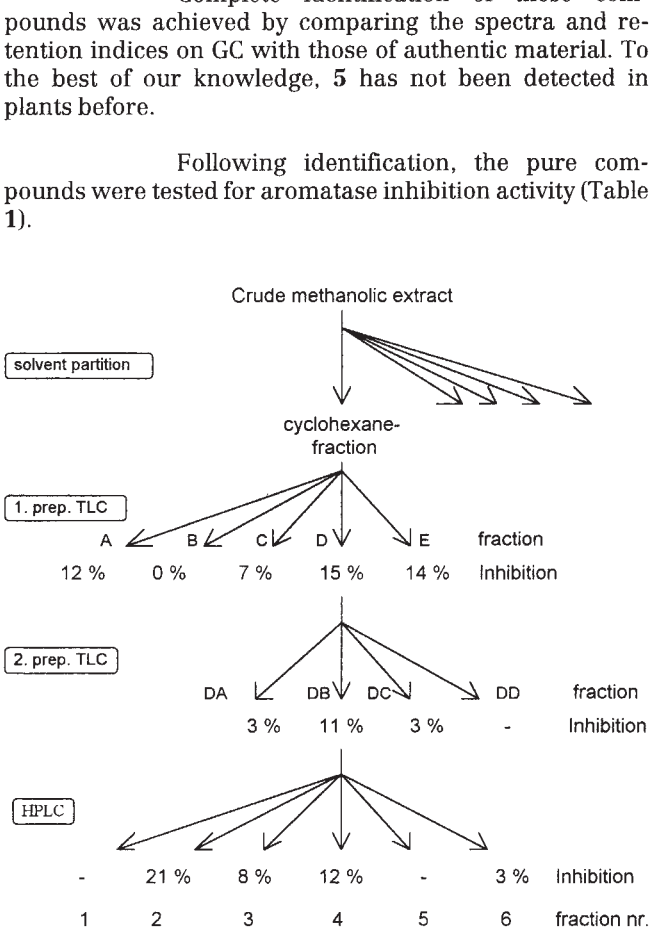
Results

The investigation of *U. dioica* roots was carried out as reported previously (12). Briefly, extracts were separated by chromatographic means (CC, preparative TLC, HPLC). Obtained fractions were tested in an *in vitro*-aromatase assay (see Materials and Methods). Active compounds were accumulated by further chromatographic steps and resulting fractions were tested again (as exemplified in Scheme **1**). Structures of individual compounds finally were deduced from their mass spectra.

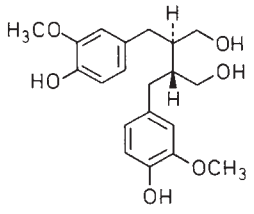
Results obtained by this procedure suggested compounds **1–5** to be aromatase inhibitors (Fig. **1**).

Complete identification of these compounds was achieved by comparing the spectra and retention indices on GC with those of authentic material. To the best of our knowledge, **5** has not been detected in plants before.

Following identification, the pure compounds were tested for aromatase inhibition activity (Table **1**).



Scheme 1 Example of a fractionation during the search for aromatase inhibitors in *U. dioica* root extracts.
Fraction 2 mainly contained compounds **2** and **3**, indicating that they might be responsible for the aromatase inhibition activity.
Inhibition: Aromatase inhibition as defined in Materials and Methods. Biological assays: c (A–D) = 50 mg/l; c (DA–DD; 1–6) = 25 mg/l; other experimental conditions as given in Materials and Methods.
Solvent partition: see Materials and Methods.
1. preparative TLC: solvent CH_2Cl_2/EtOAc, 9 : 1; R_f (D) = 0.15–0.3.
2. preparative TLC: solvent CH_2Cl_2/EtOAc, 8 : 2; R_f (DB) = 0.45–0.7.
HPLC: Experimental conditions see Materials and Methods; R_t (fraction 2) = 23.4–27.2 min.



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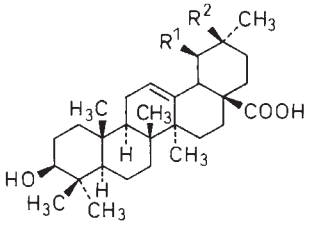
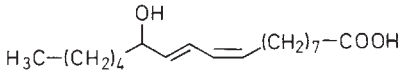


2 R^1 = H, R^2 = CH_3
3 R^1 = CH_3, R^2 = H



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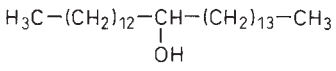

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Fig. 1 Aromatase inhibitors from *U. dioica* roots (formula **1** represents (−)-secoisolariciresinol).

Table 1 Effect of **1**–**5** on aromatase activity.

compound	concentration (μM)	aromatase inhibition (mean ± s.d.)
1	409.0	10.9 ± 4.5 %
2	40.7	12.4 ± 4.6 %
3	81.5	30.4 ± 1.3 %
	40.7	12.2 ± 2.9 %
4	313.0	15.9 ± 8.6 %
5	29.6	24.3 ± 8.9 %
	14.8	11.0 ± 0.7 %

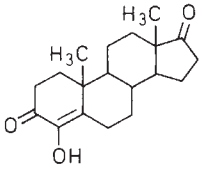
Aromatase inhibition without addition of test compounds = 0 %.
4-Hydroxy-androst-4-ene-3,17-dione **6** was used as a positive control (200 nmol/l gave 23.2 ± 6.1 % inhibition; n = 5).
c (androst-4-ene-3,17-dione) = 332 nmol/l; 70 μg/ml microsomal protein; total incubation volume 540 μl; Incubation period 15 min at 37 °C.
Each value represents 3 to 8 individual tests.
Due to their limited solubility in the aqueous test medium compounds **2**, **3**, and **5** could not be tested in higher concentrations than indicated.

Discussion

The tested compounds significantly inhibited the aromatase system *in vitro*. Their activity is weak to moderate.

Inhibitory effects on aromatase were unknown to date for pentacyclic triterpenes and secondary fatty alcohols (**2**, **3**, and **5**). As other classes also show similar (and partly much more pronounced) influences on aromatase [e.g. flavones (21)], aromatase exhibits relatively low specifity. Thus aromatase can be inhibited by a series of structurally different compounds at least to a low extent. It must be expected that some more compounds developing aromatase inhibitory effects will be added to this collection in the future.

Compounds **1**–**5** are present in *U. dioica* roots in relatively low concentrations, as judged from GC-chromatograms [ranges (per 1000 g dry weight): free **1** – i.e. not glycosidically bound, **2** and **3** 15 mg each, **4** 350 mg, and **5** 1 mg].



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Furthermore, compared to the potent synthetic aromatase inhibitor **6** (22), their activity *in vitro* is weak (< 1 %). So for each individual compound a distinct effect on aromatase *in vivo* seems unlikely. Nevertheless it is conceivable that cooperation of many weakly active agents might lead to a considerable influence on an organism. Factors like metabolism, accumulation in target tissues or synergism do not allow a final statement about the effect of *U. dioica* root extracts on aromatase *in vivo*.

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