Determination of Oenothein B as the Active 5-α-Reductase-Inhibiting Principle of the Folk Medicine Epilobium parviflorum

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Determination of Oenothein B as the Active 5-α-Reductase-Inhibiting Principle of the Folk Medicine *Epilobium parviflorum*

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Several extracts from *Epilobium parviflorum*, a plant used in Central Europe for the treatment of prostate disorders, were evaluated in a biochemical assay with 5-α-reductase. The aqueous extract displaying inhibition of the enzyme was analyzed, the fraction responsible for this activity was purified, and the active compound identified as a macrocyclic tannin, oenothein B (**1**).

Epilobium parviflorum Schreb., the small-flowered willow herb, a plant of the Onagraceae,[1] was disclosed 15 years ago by the Austrian herbalist Maria Treben[2] as useful in the treatment of all kinds of bladder, kidney, and particularly prostate disorders, including cancer.[3] More recently, P. Bohinsky, a Slovak herbalist, briefly evoked the curative effects of *Epilobium montanum* and *Epilobium roseum* in the case of a benign tumor and hypertrophy of the prostate gland in men.[4] Several authors have tried to relate this property to an anti-inflammatory activity and have analyzed extracts of the plant to this end, which resulted in the identification of several compounds of *Epilobium*, including flavonoids,[5−7] β-sitosterol derivatives,[8] fatty acids,[7] and gallic, chlorogenic, and ellagic acids.[6] We hypothesized that the above biological effect of these herbs might be explained in terms of inhibition of 5-α-reductase, the enzyme reponsible for the biosynthesis of dihydrotestosterone from testosterone,[9] and undertook the preparation and biological evaluation of several extracts of *E. parviflorum*.

Several extracts of *E. parviflorum* were prepared and evaluated in a competition assay with 5-α-reductase obtained from prostate homogenates prepared from radical prostatectomies of benign prostatic hyperplasia.[10] The activity was measured after incubation with the enzyme and its substrate testosterone by quantitative HPLC determination of the 5-α-reduced metabolites dihydrotestosterone and 5-α-androstane-3α,17β-diol and 5-α-androstane-3β,17β-diols. Table 1 presents the results of this first analysis. The organic extracts (no. 1−4) did not exhibit 5-α-reductase inhibition. On the other hand, most of the activity resulted from the aqueous extract (no. 5).

The aqueous fraction responsible for the 5-α-reductase inhibition (no. 5) was studied by HPLC. The fraction (no. 37) exhibiting 56% of 5-α-reductase inhibition was subjected to chemical study. A mass spectrometric analysis of this very small sample revealed the presence of gallic acid (mol wt 152). A cross search of the literature between gallic acid and Onagraceae and *Epilobium* points to it being a possible tannin.[11,12] A preparative HPLC purification of the active compound contained in the aqueous fraction was performed to obtain additional sample for analysis. A total of 9 g of lyophilized aqueous extract of *E. parviflorum* was purified over 10 runs to afford 314 mg of active fraction.

Table 1. Evaluation of the Extracts of *Epilobium parviflorum* on the Inhibition of 5-α-Reductase from Human Benign Prostatic Hyperplasia

extracts/fractions (no.)	amount tested (solvent)	% inhibition
1	30 μg (EtOH)	5
2	30 μg (EtOH)	10
3	30 μg (EtOH)	15
4	30 μg (EtOH)	14
5	10 μL	90
6	30 μg (H2O)	17
7	30 μg (DMSO)	52
8	10 μL	92
9	30 μg (H2O)	43

A 2D ^{1}H-^{13}C HMQC NMR experiment in D_2O allowed the identification of two glucose cores, one in the α-form and the other in the β-form. A COSY experiment along with a mass spectrometric analysis using matrix-assisted laser desorption ionization (MALDI) and liquid secondary mass spectrometry (LSIMS) suggested that the structure was a macrocyclic dimer incorporating two gallic acid, two valoneoic acid, and two glucose moieties.

A new cross search of the literature between tannic acids and macrocyclic structures led to a limited number of compounds; one of them, **1**, displayed spectroscopic features[13] analogous to those exhibited by the active compound; therefore, it was identified as oenothein B. Oenothein B (**1**) was first reported as a dimeric hydrolyzable tannin from an extract of *Lythrum anceps* by Okuda.[14] Later, this compound was extracted from *Oenothera erythrosepala* (Onagraceae), and the corrected structure was published as the first example of

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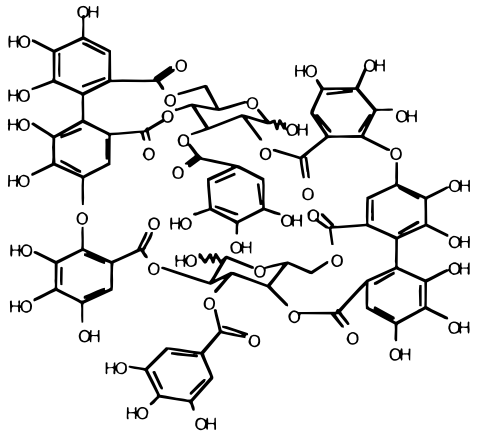

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a macrocyclic hydrolyzable tannin.[13,15] This compound was also isolated from *Eucalyptus alba* (Myrtaceae).[16,17]

The 5-α-reductase inhibitory potency of the pure isolated **1** was confirmed, and the IC_{50} value was found to be 2.2 10^{-5} M. Finasteride,[18] the reference compound for this class of inhibitors, displayed an IC_{50} of 10^{-8} M. The same measurement was performed with the crude lyophilized aqueous extract, which gave an IC_{50} of 0.16 g/L.

This work demonstrates once more that folk medicine can be of precious value to the medicinal chemist. We have shown[19,20] that a decoction, traditionally used in Central Europe for the treatment of some prostate disorders, displayed inhibition of the enzyme 5-α-reductase, the current target in the search for drug treatment of benign prostatic hyperplasia. The active principle responsible for this inhibition has been identified and characterized as a macrocyclic tannin, oenothein B (**1**). This molecule has previously been described in the literature as possessing antiviral[21] and antitumor activities.[22,23] The conjunction of these effects with the inhibition of 5-α-reductase could possibly be of value in the treatment of prostatic adenoma and carcinoma.

Experimental Section

General Experimental Procedures. Analytical HPLC was performed using a Waters 600 pump and a Waters 991 Diode array UV detector. Preparative HPLC was performed on a Waters 3000. NMR was performed on Bruker AM400 and AM250 spectrometers. The samples were solubilized in D_2O (99.96%, purchased from Euriso-top) using sodium tetradeutero-2,2,3,3-(trimethylsilyl)-3-propionate (TSP-d_4, purchased from MSD Isotopes) as an internal reference or in Me_2CO-d_6 (99.97%, purchased from Spectrométrie, Spin et Techniques). Mass spectrometry was first performed on a Fisons Instrument TofSpec (VG Analytical Ltd, UK). A pulsed N_2 laser beam (wavelength 337 nm) was employed to impinge on the analyte, which is supported in a strongly UV-absorbing matrix. Samples and matrix were prepared at concentrations 10−200 pmol/μL sample solution and 10 μg/μL of DHB matrix dissolved in H_2O−MeCN 70:30. The MALDI analyzer was operated in linear mode and calibration performed using gramicidin S (mol wt 1142) and bovine insulin (mol wt 5734). LSIMS was performed on a VG Autospec E (Fisons Instrument) (acceleration 8 kV under positive and negative modes). The primary ions were obtained with a Cs^+ beam and accelerated at 35 kV with an output of 3 μA. The compound (100 μg) was dissolved in the matrix (Magic bullet + 1% TFA). The fragmentation spectra were obtained under First Field Free Region (1FFR) collision with air. Circular dichroism was studied on a Dichrographe Jobin-Yvon Mark V (cell paths 1 and 0.5 cm). UV measurements were performed on a Perkin-Elmer Lambda 9 spectrophotometer. Homogenization of the human prostates was achieved with a Polytron (Kinematica GmbH, Switzerland) and a glass−glass Potter tube (Wheaton, USA). Centrifugation was performed on a T2080 (Kontron Instruments) ultracentrifuge equipped with a 45 Ti angular rotor (Beckman Instruments). All chemical and biochemical reactants were from Sigma Chemicals unless otherwise noted. The metabolites of [1,2,6,7-^{3}H]testosterone (NET 370, DuPont -NEN) were separated by HPLC (Kontron Instruments) equipped with an ODS-Hypersil 5 μm (Waters Inc.) filled (in house) column (4.6 mm × 25 cm). The detection of radioactive metabolites of testosterone was achieved by an on-line radiodetector (LB507A, Berthold Instruments) using Quickszint Flow 302 (Zinsser-Analytic) as scintillating phase.

Plant Material. Seeds of *E. parviflorum* Schreb. from the medicinal plants collection of the Station Fédérale de Recherche Agronomique, Centre d'Arboriculture et d'Horticulture des Fougères, 1964 Conthey, Switzerland, were planted in Conthey, Switzerland, during the spring of 1991. Aerial parts were collected in August 1992. Voucher specimens of the seeds and the plant are on file at the Centre de Recherche sur les Plantes Médicinales et Aromatiques (CRPMA), Centre des Fougères, Conthey, Switzerland (voucher no. 93-114).

Preparation of *E. parviflorum* Extracts. Dried stems and leaves (420 g) were chopped in a mixer and extracted sequentially with ligroin, $CHCl_3$, MeOH, and H_2O. The ligroin and $CHCl_3$ extracts were concentrated, and aliquots were placed in test tubes (no. 1 and no. 2). Upon concentration of the MeOH extract, precipitation occurred; the resulting solid was separated (no. 4). The filtrate was further concentrated (no. 3) . A fraction of the aqueous extract (no. 5) was concentrated to afford in a similar way a solid (no. 7) and a filtrate concentrated to dryness (no. 6). Some fraction of the aqueous extract (no. 5) was centrifuged, and the supernatant and pellet were labelled samples 8 and 9, respectively. All samples (with the exception of no. 5) were thoroughly dried under vacuum at 40 °C.

HPLC Fractionation of the Aqueous Extract. Sample 5 was lyophilized, and 20 mg of the resulting powder was dissolved in 10 mL of distilled H_2O. The solution was injected on a preparative column (Novapak (C-18 6 μm 7.8 × 300 mm). Elution was performed by a gradient of solvents (5 mL/min) under the following conditions (% vol): 0−2 min: sample; 2−7 min: 100% H_2O containing 0.1% vol trifluoroacetic acid (TFA); 7−57 min: linear gradient of 0−10% vol MeCN in H_2O containing 0.1% vol TFA; 57−77 min: linear gradient of 10−25% vol MeCN in H_2O containing 0.1% vol TFA; 77−97 min: linear gradient of 25−90% vol MeCN in H_2O containing 0.1% vol TFA. The absorbance of the eluate was monitored on a diode array detector system at wavelengths between 210 and 400 nm. In all, 43 fractions were collected.

Extraction and Isolation of Oenothein B. Stems

and leaves of *E. parviflorum* (19 g) dried at ambient temperature in the dark were covered by 200 mL of warm distilled H_2O (65 °C). The mixture was stirred for 48 h and filtered. The filtrate was lyophilized to afford 3.8 g of crude extract as a pale green powder. Lyophilizate (1 g) was dissolved in 50 mL of distilled H_2O and injected on a preparative Microbondapack column (C-18 10 μm 20 × 500 mm). Elution was performed by a linear (15% vol in 30 min) gradient of H_2O and MeCN containing 0.1% vol of TFA, detection with UV (305 nm). Oenothein B was eluted at retention time of 27 min, which, following lyophilization, was obtained as an off-white powder (35 mg).

Preparation of 5 α-Reductase from Human Prostates. The homogenate of human hyperplastic prostate was prepared as described by Liang *et al.*[10] Frozen human prostates were thawed on ice and minced with a pair of scissors. The minced tissue was homogenized in 3 tissue volumes of medium A (20 mM potassium phosphate, pH 6.5, containing 0.32 M sucrose, 1 mM dithithreitol [DTT], and 50 μM NADPH) with a Polytron and then with a glass−glass homogenizer. The homogenate was centrifuged at 140 000*g* for 60 min. The pellets were suspended in medium B (20 mM potassium phosphate, pH 6.5, containing 1 mM DTT and 20% glycerol), dispatched in 1 mL cryotubes, and stored at −80 °C. The suspension (≈10 mg protein/ml)[24] was referred to as the particulate preparation and was used as the source of 5α-reductase.

5α-Reductase Assay and IC_{50} Measurements. For each fraction, inhibition of the conversion of testosterone to the 5 α-reduced products dihydrotestosterone and androstane-3α,17β-diol and androstane-3β,17β-diol was evaluated. Each test was done in duplicate tubes. Substrate (2 μM [1,2,6,7-^{3}H]-testosterone) in 40 mM citrate trisodium salt, pH 5.0, was preincubated at 37 °C for 3 min with the fraction to be tested. Prostatic particulate (1 mg/mL protein) in a 40 mM potassium phosphate buffer, pH 6.5 containing 1 mM DTT and 500 μM NADPH) was also preincubated at 37 °C for 3 min. Reaction was started by mixing 0.5 mL of substrate solution with 0.5 mL of enzyme suspension. The final pH was 5.5 . The mixture was incubated for 30 min at 37 °C under slight agitation. The reactions were stopped in ice with 2 mL EtOAc. After vortexing for 2 min and centrifugation (1000*g*, 5 min), the upper phase was transferred to a tube and evaporated under air to dryness. The steroids were taken up in the HPLC mobile phase as described by Le Goff *et al.*[25] Briefly, 50 μL were injected in MeOH-THF-H_2O (45:15:40, v/v/v) onto an ODS Hypersil column (5 μM, 4.6 mm × 250 mm). The radioactive metabolites were detected by an on-line radiodetector and quantified by the automatic measurement of the peak areas. Results were expressed as the percentage of 5 α-reductase activity remaining vs control as indicated below:

$$\frac{\text{pmol of 5 }\alpha\text{−reduced metabolites (+ inhibitor) } 100}{\text{pmol of 5 }\alpha\text{−reduced metabolites (control)}}$$

The concentration of product required to inhibit 5α-reductase by 50% (IC_{50}) was estimated from its inhibition curve using linear regression analysis.

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