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Phenolics from extracts of *Brahea armata* with inhibitory effect against 5α-reductase type-II

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Received February 28, 2006, accepted April 4, 2006

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Pharmazie 61: 1034–1037 (2006)

Fractions of the aqueous alcohol extracts of the rind and kernel of *Brahea aramata* fruits have been investigated for their activity against 5α-reductase type II, which is expressed predominantly in the prostate. This isozyme represents a major target for drugs against benign prostate hyperplasia (BPH) and prostate cancer. Also, a structural analysis of the phytophenolics, present in both aqueous alcohol extracts as the major constituents, has led to the isolation of five phenolics, including the new natural product, 4′,6′-dimethoxy β,4,2′-trihydroxy chalcone from the rind extract and three phenolics, including the new natural product, 1-*p*-hydroxybenzoyl glycerol from the kernel extract. All structures were confirmed by ESI-MS and NMR analysis.

1. Introduction

The genus *Brahea* (Arecaceae) comprises about 12–16 species, including which *Brahea serrulata* (synonym: *Serenoa repens*) has a long folk history of use for improving the signs and symptoms of benign prostatic hyperplasia (BPH), (Wilt et al. 1998; Marks et al. 2000). The berry of another *Brahea* species, namely, *Brahea armata* – S. Watson (synonym: *Erythea armata*), known in English as blue hesper palm, has not been investigated previously for its biological activity or constitutive phenolics. However, the leaf material of the plant has been reported previously to contain *C*-glycosy flavones, the flavones, tricin and luteolin in addition to the anthocyanin, cyanidin (Williams and Harborne 1973).
B. armata has stiff, blue costapalmate leaves and cream colored flowers in arching inflorescences reach four to six meters long. Its fruit (18–24 mm long) is ovid, shiny and brownish-yellow with white strips or speckles (Bailey 1976). In the present study, the methanol and the residue fractions of the aqueous alcohol extract of the rind and the ethanol, methanol and the residue fractions of the aqueous alcohol extract of the kernel of *B. armata* fruits have been investigated for their activity against 5α-reductase type II (Table 1), which converts testosterone into the much more active androgen, dihydrotestosteron (DHT) (Strauch et al. 1994). As DTH plays a crucial role in the development of BPH, 5α-reductase type II, which is the isoform predominantly expressed in the prostate, appears to be a promising therapeutic target. On the other hand, a phytochemical screening, including color reactions and chromatographic analysis (Harborne 1973) of both parent aqueous alcohol extracts have shown that they contain mainly phenolics. It

Table 1: Inhibition of 5α-reductase type II by fractions of the aqueous alcohol extract of the rind and that of the kernel of *Brahea armata* fruits

Sample No.	Fraction specification	Inhibition of 5α-reductase type II in human HEK cells Crude fractions 10 mg/ml	Inhibition of 5α-reductase type II in human HEK cells Clarified fractions 10 mg/ml	Inhibition of 5α-reductase type II in human HEK cells Clarified fractions 1 mg/ml		
				Single values (%)	Mean +/− SD (%)	Variance coefficient (%)
1	Rind MeOH fraction	42.6%	69%	55% 36%	45 +/− 9,5	21
2	Rind residue	93.4%	n.d.	51% 53%	52 +/− 1	2
3	Kernel EtOH fraction-1	58.9% (1 mg/ml)	–	64.2% 32%	48 +/− 16	33
4*	Kernel EtOH extract-2	58.7% (1 mg/ml)	–	67.2% 50%	59 +/− 9	15
5	Kernel MeOH fraction-1	94.2%	77%	56% 43%	49,5 +/− 6,5	13
6*	Kernel MeOH fraction-2	91.2%	61%	52% 55%	53,5 +/− 1,5	2,8
7	Kernel residue-1	100.0%	79.5%	45% 57%	51 +/− 6	12
8*	Kernel residue-2	82.6%	71%	59% 52%	42 +/− 17	40

* Repetition (= two independent determinations)

was therefore found reasonable to perform a comprehensive structural analysis of these phytophenolics. Five pure phenolics (**1**–**5**), including the new natural product, 4′,6′-dimethoxy β,4,2′-trihydroxy chalcone (**5**) have been isolated and purified from the rind material. The kernel material afforded three pure isolated phenolics (**6**–**8**), including the new natural product, 1-*p*-hydroxybenzoyl glycerol (**8**). All structures were confirmed by ESI-MS and NMR analysis.

2. Investigations, results and discussion

2.1. Preparation of extracts

Crushed material of the rind and kernel has been extracted, separately by being shaken for 24 h at room temperature together with aqueous (aqu) EtOH (75%). The filtered aqueous alcohol extract of the rind, dried *in vacuo* was fractionated through shaking with MeOH, then filtered off. Removal of the solvent *in vacuo* afforded a dry MeOH fraction in addition to the residue fraction left after filtration. Successive fractional extraction of the aqueous alcoholic extract of the kernel by EtOH, followed by MeOH afforded, after removal of the solvents under vacuum, an EtOH and a MeOH fraction beside the residue fraction obtained by filtration after the MeOH extraction.

2.2. 5α-Reductase type II inhibition

The inhibitory activity of the different extracts on 5α-reductase type II was determined using a cell line (HEK293-5αII), that expressed the human recombinant enzyme and which has been used in previous investigations for the identification of potent inhibitors (Hartmann et al. 2000; Reichert, et al. 2001a, b). For this purpose solutions of the different extracts in cell culture medium (DMEM) were prepared and subjected to the enzyme inhibition assay as described below. As can be seen in Table 1 the most active extracts of the fruit parts, showing up to 100% inhibition at 10 mg/ml and between 48 to 59% inhibition at 1 mg/ml were those obtained after extracting the kernel with EtOH or with MeOH. The most active extract was kernel EtOH extract-2, which showed 59% inhibition at a concentration of 1 mg/ml. It can be considered as very active, as previous investigations on the inhibition of human 5α-reductase by plant extracts reported much higher concentrations to be necessary to obtain 50% inhibition (Rhodes et al. 1993). Out of the 30 phytotherapeutic compounds available to date in Europe, which were mostly derived from eight different plant sources, in no less than 15 preparations the active constituent is derived from the American dwalf palm *Brahea serrulata (Sėrenoa repens)*, also referred to as *Sabal serrulata* (Buck 1996). Extracts from this saw palmetto plant has been successfully used for the treatment of men with symptomatic BPH (Wilt et al. 1998; Marks and Tyler 1999; Marks et al. 2000). A commercially available preparation from Serenoa repens has the trade name Strogen S (or forte) and exhibited 50% inhibition of 5α-reductase at a concentration of 31 mg/ml in an *in vitro* assay (Rhodes et al. 1993). Another commercially available extract known as Tadenan, is derived from the African prune, *Pygeum africanum* and was shown to inhibit human 5α-reductase to a degree of 50% at a concentration 63 mg/ml *in vitro* (Buck 1996; Rhodes et al. 1993).

To resume, all extracts from *Brahea armata* prepared and subjected to inhibition studies in this investigation showed significant inhibition of human 5α-reductase type II. This could be an indication, that the berry of *Brahea armata* or extracts thereof could be an alternative phytotherapeutic agent for the treatment of the symptoms and the effects of BPH.

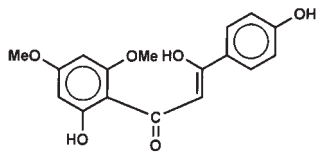
2.3. Isolation and structure elucidation

The parent aqueous alcoholic extracts of the rind and kernel were individually inspected for their constitutive phytochemicals (Harborne 1973) to prove the presence of a high percent of phenolic constituents (intense green $FeCl_3$ color reaction and chromatographic analysis). Each of these extracts were then subjected to repeated Sephadex LH-20 column fractionation, using H_2O followed by a H_2O/MeOH mixture (50%) and MeOH to afford five phenolic compounds (**1**–**5**) from the rind extract and three (**6**–**8**) from the kernel extract. The known compounds (**1**–**4**) and **6** and **7** gave chromatographic, UV, ESIMS, ^1H and ^{13}C NMR analytical data identical with those reported for orientin, isovitexin, isoorietin (Chopin et al. 1982), naringenin (Nawwar et al. 1999) catechin and epicatechin (Nawwar et al. 2000).

Compound **5** is an amorphous yellowish brown powder that exhibits chromatographic characteristics and UV spectral maxima in MeOH and after addition of diagnostic shift reagents (Bohm 1975; Mabry et al. 1970) similar to those reported for chalcones (Bohm 1994; Kiuchi et al. 1990). It showed in negative ESIMS $[M-H]^-$ ion peak at m/z 315, corresponding to a molecular mass of 316, thus suggesting the molecular formula $C_{17}H_{16}O_6$, which was further confirmed by HRESIMS. The compound was recovered unchanged after normal aqueous acid hydrolysis (aqu 2 N HCl, for 2 h, at 100 °C). The ^1H NMR spectrum (measured at 270 MHz, $CDCl_3$, room temp.) displayed five aromatic resonances at δ ppm 7.45 (1 H, s), 7.55 (2 H, d, J = 8 Hz), 6.85 (2 H, d, J = 8 Hz), 6.1 (1 H, d, J = 2 Hz) and 5.98 (1 H, d, J = 2 Hz) along with two methoxyl group resonances at δ ppm 3.9 and 3.84 (each 3 H, s) in addition to a hydrogen bonded proton sharp singlet at δ ppm 14.5. These resonances were assigned to the α-chalcone proton lacking a vicinal olefinic proton at the β-position (Waterman and Elhadi 1985), the equivalent H-2 & H-6, the equivalent H-3 & H-5, H-5′, H-3′, OMe-6′, OMe-4′ and to the hydrogen bonded proton of the OH-2′, respectively in the chalcone molecule. Direct correlation observable in the HMBC spectrum of **5** confirmed the presence of the H-α as a singlet resonance at δ ppm 7.45 which was found correlated to the resonances of the hydroxylated β-carbon (δ ppm 168.2) and the carbonyl carbon (δ 192.6 ppm). In the 1D-^1H spectrum, the relatively low field location of the resonances of the H-3′ and H-5′ (δ ppm 5.98 & 6.1, respectively) together with the presence of two methoxyl proton resonances at δ 3.9 and 3.84 ppm proved methyl etherification at the chalcone positions 4′ and 6′. This conclusion was confirmed by the recognized ^2J and ^3J set of correlation in the HMBC spectrum (Table 2). The resonances in the ^{13}C spectrum (measured at 67.5 MHz) at δ ppm 104.2, 168.2 and 192.6 ppm were assigned to the α-, the hydroxylated β-carbons and the chalcone carbonyl carbon, respectively. The correlations recognized in HMQC and HMBC spectra together with the data presented by the PENDANT spectrum led to the complete assignments of all of the carbon resonances (Table 2), thus confirming the structure of **5** to be 4′,6′-dimethoxy β,4,2′-trihydroxy chalcone, which represents a new natural product as far as the available literature is concerned.

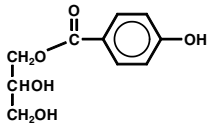
Table 2: 1D and 2D NMR spectral data of compound 5

Carbon number	5			
	δ_H (Hz)	δ_C	PENDANT	HMBC
α	7.45 s	104.2	CH	β, C=O, 1′, 1
β		168.2		
carbonyl		192.6		
1		127.7		
2	7.55 d (8 Hz)	130.3	CH	1, β, 3, 4
3	6.85 d (8 Hz)	115.9	CH	1, 2, 4, 5
4		158.5		
5	7.55 d (8 Hz)	115.9	CH	1, 3, 4, 6
6	6.85 d (8 Hz)	130.3	CH	1, 2, 4, 5, β
1′		106.8		
2′	14.5 s (hydrogen bonded proton of OH 2′)	168.3		
3′	5.98 d (2 Hz)	93.7	CH	1′, 2′, 4′, 5′
4′		165.7		4′
4′-OMe	3.84 s	55.5	CH	1′, 3′, 4′, 6′
5′	6.1 (2 Hz)	91.2		
6′		162.6		6′
6′-OME	3.9 s	55.8		



Compound **5**: 4′,6′-dimethoxy β,4,2′-trihydroxy chalcone

Compound **8**, a cream colored amorphous powder, exhibits chromatographic properties, UV absorption maxima in MeOH (λ_{max}: 255 nm) and an $[M-H]^-$ ion at m/z: 211 in negative ESIMS, corresponding to a molecular mass of 212. It hydrolysed by an aqueous 5% alcoholic KOH solution (2 h, 100 °C) to glycerol (comparative PC) and *p*-hydroxy benzoic acid (comparative PC, ^{1}H and ^{13}C NMR spectral analysis). The ^{1}H NMR spectrum ($CDCl_3$, room temp.) of **8** showed resonances assignable to the *p*-hydroxy benzoyl and the glyceride moieties, [*p*-hydroxy benzoyl moiety: δ ppm 6.66 (d, J = 8 Hz, H-3′ & H-5′); 7.72 (d, J = 8 Hz, H-2′ & H-6′). Glyceride moiety: δ ppm 4.15 (m, 2 H-1); 3.66 (m, H-2 & 2 H-3)]. The ^{13}C NMR spectrum proved the presence of free OH groups at C-2 and C-3 in the glyceride moiety of **8** (δ ppm 74.5 & 62.7, respectively), but suggested esterification at C-1 of this moiety (Barakat et al. 1987), (δ ppm 64.5) by the carboxyl group of the *p*-hydroxy benzoic acid whose carbonyl carbon resonance was shifted upfield to δ ppm 165.4, in comparison with that of the corresponding signal in the spectrum of free *p*-hydroxy benzoic acid (δ ppm 167.5). This suggestion was confirmed through HMBC spectral analysis. Direct correlation observable in the received spectrum allowed unambiguous determination of the glyceride C-1 methylenic protons resonance which was recognized at δ ppm 4.2 (2 H, m) and was found correlated to the carbonyl carbon resonance (δ ppm 165.4) of the *p*-hydroxybenzoyl moiety. This and the above given data finally confirmed the structure of compound **8** to be 1-(*p*-hydroxybenzoyl) glycerol, which has not been reported before as a natural product.



Compound **8**: 1-[*p*-hydroxybenzoyl] glycerol

3. Experimental

3.1. Instruments and materials

Reversed phase HPLC analysis was performed by means of a high pressure solvent delivery pump (Waters M6000A, Milford, USA), a radioactive detector (LB506C, Berthold,Wildbad, Germany) and an autosampler system 9581-as, Jasco, Tokyo, Japan). Nucleosil 120-3-C_8 was applied as stationary phase using repacked columns (125 × 4 mm; Macherey-Nagel, Düren, Germany). The injection volume was 13 µl, the flow rate was 0.4 ml/min and MeOH/H_2O (50/50) was used as the mobile phase for separation of the steroid metabolites. Data acquisition and integration was carried out by the use of the HALABE 1.6.5 software (Berthold, Wildbad, Germany).

^{1}H NMR spectra were measured by a Jeol EX-270 NMR spectrometer, at 270 MHz. ^{1}H chemical shifts were measured relative to TMS and ^{13}C NMR chemical shifts to $CDCl_3$ and converted to TMS scale by adding 77. Typical conditions: spectral width = 4 KHz for ^{1}H and 19 KHz for ^{13}C, 32 K data points and a flip angle of 45°. ESIMS spectra were measured on SSQ Finnigan MAT 4600 quadrupol mass spectrometer (Institut für Chemie, Humboldt Universität, Berlin). Paper chromatographic analysis was carried out on Whatman no. 11 paper, using the following solvent systems: (1) 15% HOAc; (2) 60% isopropanol; (3) BAW (*n*-BuOH−HOAc−H_2O, 4 : 1 : 5, upper layer). Solvent 3 were used for PPC.

3.2. Plant material

Fresh fruits of *Brahea armata* − S. Watson (Arecaceae) were collected from a mature tree growing in Orman Garden, Cairo, Egypt, during May 2003 and authenticated by Dr. M. El-Gibali, National Research Centre, Cairo, Egypt. A voucher specimen is deposited at the NRC.

3.3. Extraction

Crushed materials of the rind (2 kg) and kernel (1.3 kg) have been extracted, separately by being shaken for 24 h at room temperature together with 3 L aqueous EtOH (75%) for each. The filtered extract of the rind was dried *in vacuo* to yield 288 g of a sticky dark brown, aqueous alcohol rind extract, from which 28 g were taken into fractionation through shaking with MeOH (1 L) at room temperature for 8 h and then filtered off. Removal of the solvent *in vacuo* afforded a dry MeOH fraction (11.5 g), beside the residue obtained by filtration after the MeOH extraction. A part (22 g) of the received dried aqu alc kernel extract (153) was subjected to successive fractional extraction by 1 L EtOH (95%, room temperature, 8 h) followed by MeOH (1 L, room temperature, 8 h) to afford, after removal of the solvents under vacuum, an EtOH and a MeOH fraction (3.4 g and 7.8 g, respectively), beside the residue obtained by filtration after the MeOH extraction. A concentrated aqueous solution of the parent aqueous alcoholic extract of the rind (50 g in 100 ml H_2O) was applied to a Sephadex LH-20 column (125 × 5 cm) and eluted with H_2O followed by a H_2O−MeOH (50–50) mixture and finally by MeOH. Compounds **1**–**3** were isolated pure (87 mg, 54 mg and 46 mg, respectively) from the 50% MeOH fraction by repeated Sephadex LH-20 column fractionation, using *n*-butanol saturated with H_2O as an eluent. Compounds **4** and **5** were separated from the MeOH fraction by Sephadex LH-20 column fractionation, using H_2O−MeOH (30–70) mixture for elution followed by Preparative PC of the crude materials of **4** and **5,** using BAW as solvent to afford pure samples of **4** (28 mg) and **5** (43 mg). A concentrated methanolic solution of the parent aqu alc extract of the kernel (22 g in 50 ml H_2O) was applied to a Sephadex LH-20 column (100 × 3.5 cm) and eluted with H_2O followed by H_2O−MeOH (50–50) mixture and finally with MeOH. Compounds **6** and **7** were isolated pure (23 mg and 41 mg, respectively) from the dried 50% MeOH fraction by being extracted with ether followed by preparative PC for the ether extract, using BAW as solvent. Compound **8** was separated pure (31 mg) from the dried MeOH column fraction by being extracted by ether followed by precipitation (thrice) from ether by adding excess *n*-hexane.

3.4. Inhibition assay

The inhibitory activity of the extracts on human 5α-reductase II was determined as described previously (Hartmann et al. 2000; Reichert et al. 2001a). Briefly, HEK293-5αII cells (300,000/well) which were obtained by stable transfection of HEK293 cells with 5α-reductase expression plasmid pRcCMV-II, were seeded in a 24-well tissue culture plate (Nunc, Wiesbaden, Germany) and incubated overnight to allow the cells to attach. Culture medium was removed by aspiration and replaced by 0.5 ml of a freshly prepared substrate/inhibitor solution. The solution was comprised of complete DMEM-medium with 5 nM[^{3}H] androstenedione (NEN Du-pont, Köln, German) and a final concentration of either 10 mg/ml or 1 mg/ml of the different extracts as indicated in Table 1. For this purpose the dried extracts were first dissolved in DMEM at an adequate dilution, treated with sonification and then supplied with the substrate. As in case of the final extract concentrations of 10 mg/ml, unsolvable particles were observed, these extracts were also subjected to centrifugation in order to obtain a clarified solution before the inhibitory potential was determined, as indi-

cated in Table 1. As a consequence, with the exceptions of kernel EtOH-extract 1 and kernel EtOH extract 2 fraction, the extracts in a final concentration of 1 mg/ml were exclusively measured after clarification by centrifugation. Controls were performed using complete DMEM containing the substrate without extracts. After 20 min incubation time 500 µl supernatant was removed from the cells and the steroids were extracted with 800 µl diethylether. The organic phase was transferred and dried. The obtained dry material was re-suspended in 50 µl MeOH and subjected to reversed phase HPLC analysis as described (Reichert et al. 2001a; Hartmann et al. 2000).

3.5. New natural products

3.5.1. 4′,6′-Dimethoxy β,4,2′-trihydroxy chalcone (**5**)

R_f-values (×100): 05 (1), 82 (2), 88 (3). UV spectral data λ_{max} nm: MeOH 257 shoulder, 291, 364; NaOAc 287, 364; $AlCl_3$ 318,357 shoulder, 422; NaOMe 285, 320, 370 shoulder, 436. Normal acid hydrolysis [11 mg of **5**, heated with aqu. 2 N HCl (5 ml), at 100 °C, 2 h] whereby the compound was extracted unchanged by EtOAc (comparative PC). For ESIMS and ^{1}H NMR see section 2. 2D and ^{13}C NMR: Table 2.

3.5.2. 1-(p-Hydroxybenzoyl) glycerol (**8**)

R_f-values (×100): 65 (1), 79 (2), 85 (3). UV spectral data λ_{max} nm: MeOH 255. Alkaline hydrolysis [21 mg of **8**, heated with aqu. 5% KOH (5 ml) at 100 °C, 2 h, followed by acidification] yielded (a) *p*-hydroxybenzoic acid, extracted with ether, R_f-values (×100): 54 (1), 72 (2), 88 (3); ^{1}H δ ppm: 6.8 (d, J = 8 Hz, H-3 and H-5), 7.75 (d, J = 8 Hz, H-2 and H-6), ^{13}C NMR δ ppm: 167.5 (C=O), 123.0 (C-1), 132.2 (C-2 & C-6), 115.5 (C-3 & C-5), 161.3 (C-4) and (b) glycerol, extracted with EtOAc (comparative PC). For ESIMS and ^{1}H NMR of **8** see section 2. ^{13}C NMR of **8** δ ppm: glycerol moiety: (C-1) 64.5, 74.5 (C-2), 62.7 (C-3); *p*-hydroxybenzoyl moiety: 165.4 (C=O), 122.8 (C-1), 131.9 (C-2 & C-6), 115.4 (C- & C-5), 160.5 (C-4).

Acknowledgement: The authors thank Anja Palusczak, Institut für Pharmazeutische und Medizinische Chemie, Heinrich-Heine-Universität, Düsseldorf, Universitätsstr. 1 for skillful technical assistance.

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