Three New Arylobenzofurans from *Onobrychis ebenoides* and Evaluation of Their Binding Affinity for the Estrogen Receptor

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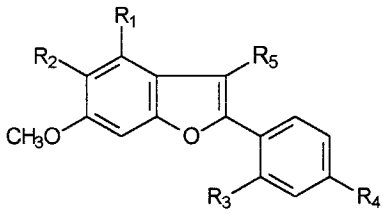
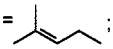
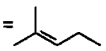
Three new 2-phenyl-benzofurans, ebenfuran I, ebenfuran II, and ebenfuran III, were isolated from *Onobrychis ebenoides*. Their structures were elucidated on the basis of chemical and spectral data as 2-(2,4-dihydroxyphenyl)-5-hydroxy-6-methoxy-benzofuran (**1**), 2-(2,4-dihydroxyphenyl)-3-formyl-4-hydroxy-6-methoxy-benzofuran (**2**), and 2-(2,4-dihydroxyphenyl)-3-formyl-4-hydroxy-6-methoxy-5-(3-methyl-buten-2-yl)-benzofuran (**3**). The affinity of these compounds for the estrogen receptor was studied using a receptor-binding assay.

An exciting group of substances known as phytoestrogens has been detected in plants belonging mainly to the Leguminosae family. These compounds bind with high affinity to estrogen receptors, in some tissues mimicking the effect of estrogens (synergism) and in others antagonizing the effect of estrogens (antagonism).[1] In the context of a program aimed at discovering new phytoestrogens, the phytochemical study of *Onobrychis ebenoides* Boiss & Spruner Leguminosae was undertaken. This plant has a silvery gray appearance due to the abundance of adpressed hairs and is distributed in Central and Southern Greece.[2] It has not undergone any previous phytochemical analysis. In the present study on *O. ebenoides*, three new 2-phenyl-benzofurans, **1**, **2**, and **3** were isolated, together with the two known sterols, *β*-sitosterol and stigmast-5-en-3-ol. All these compounds were identified by means of spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, 2D NMR, HREIMS, and EIMS) and chemical data. The affinity of these compounds for the estrogen receptor was studied using a receptor-binding assay as a preliminary indication of possible estrogenic activity.



1. $R_1 = R_5 = H$; $R_2 = R_3 = R_4 = OH$
2. $R_1 = R_3 = R_4 = OH$; $R_2 = H$; $R_5 = CHO$
3. $R_1 = R_3 = R_4 = OH$; R_2 =  ; $R_5 = CHO$
4. $R_1 = R_5 = H$; $R_2 = R_3 = R_4 = OCOCH_3$
5. $R_1 = R_3 = R_4 = OCOCH_3$; $R_2 = H$; $R_5 = CHO$
6. $R_1 = R_3 = R_4 = OCOCH_3$; R_2 =  ; $R_5 = CHO$

Ebenfuran I (**1**) was obtained as an amorphous yellow solid, and its empirical formula was determined by HREIMS as $C_{15}H_{12}O_5$. The UV spectrum of this compound was characteristic of 2-arylbenzofurans,[3,4] with maxima at 326 and 342 nm that shifted in alkali to 340 and 352 nm, respectively. The ^{1}H NMR (Table 1) spectrum indicated the presence of one aromatic methoxyl group, five aromatic protons (an ABX system correlated in the ^{1}H NMR 2D COSY and two singlets), and a furanic proton. The ^{13}C NMR (Table 1) spectrum showed the presence of a methoxy group and 14 carbons, eight trisubstituted and six disubstituted. The ^{1}H NMR data obtained were similar to the ones previously reported for another aryl-benzofuran, sainfuran,[5] isolated from *Onobrychis viciifolia*. The main difference was that sainfuran has two hydroxy groups and two methoxy groups, whereas compound **1** contains one methoxy group and three hydroxy groups. This was confirmed by the preparation of a triacetate of **1**. The location of the methoxy group was assigned to the C-6 position from HMBC correlations (Table 1).

Ebenfuran II (**2**) was also obtained as an amorphous yellow solid. The molecular formula of **2** was determined as $C_{16}H_{12}O_6$ by HREIMS. The UV spectrum of **2** shows absorption maxima at 265 and 349 nm that shift in alkali to 277 and 406 nm, respectively, closely resembling a 2-aryl-3-carbamoylbenzofuran derivative.[6,7] The ^{1}H NMR spectrum (Table 1) of compound **2** shows a signal at δ 9.91 corresponding to the carbaldeyde moiety, a methoxy signal at δ 3.78, and five aromatic protons (an ABX system and two meta-coupling signals, which correlated in the ^{1}H NMR 2D COSY). The ^{13}C NMR (Table 1) spectrum indicated the presence of a methoxy group, a carbonyl group (which corresponds to the carbaldehyde moiety), and 14 carbons, nine trisubstituted and five disubstituted. The location of the methoxy group was determined to be at the C-6 position from the HMBC spectrum (Table 1), whereas the presence of three hydroxy groups was demonstrated by the preparation of the triacetate of compound **2**.

Ebenfuran III (**3**) was similar to ebenfuran II. It has the molecular formula $C_{21}H_{20}O_6$, as deduced by HREIMS. The UV spectrum was similar to that of **2** with maxima at 266 and 362 nm, which shifted in alkali to 273 and 402 nm, respectively. Comparison of the ^{1}H and ^{13}C NMR spectral

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Table 1. ^{1}H and ^{13}C NMR Data and HMBC Correlation of **1**, **2**, and **3** in MeOD

	compound **1**			compound **2**			compound **3**		
postion	δ ^{1}H (m, *J* in Hz)	δ ^{13}C	HMBC	δ ^{1}H (m, *J* in Hz)	δ ^{13}C	HMBC	δ ^{1}H (m, *J* in Hz)	δ ^{13}C	HMBC
2		154.6	H-3/H-6′		166.4	H-6′		165.8	H-6′
3	7.02 (s)	104.5	H-4		122.5	CH=O		119.3	CH=O
3a		124.4	H-3/H-7		109.6	CH=O/H-5/H-7		109.2	CH=O/H-7
4	6.91 (s)	106.5			152.7	H-5		150.2	H-1″
5		144.7	H-4/H-7	6.29 (d, 1.83)	99.5	H-7		113.6	H-7/H-1″
6		147.7	H-4/H-7/CH30		162.3	CH3O/H-5/H-7		160.1	OCH3/H-7/H-1″
7	7.10 (s)	96.3		6.61 (d, 1.83)	89.2	H-5	6.66 (s)	87.3	
7a		149.7	H-3/H-4/H-7		157.8	H-7		156.2	H-7
1′		112.1	H-3′/H-5′		109.6	H-3′/H-5′		109.5	H-3′/H-5′
2′		157.3	H-3′/H-6′		158.5	H-3′/H-6′		158.8	H-3′/H-6′
3′	6.42 (d, 1.96)	104.3	H-5′	6.46 (d, 1.83)	104.5	H-5′	6.49 (m)	104.5 or 109.5	H-5′
4′		159.8	H-3′/H-5′/H-6′		163.6	H-3′/H-5′/H-6′		163.4	H-3′/H-5′/H-6′
5′	6.41 (dd, 8.31, 1.96)	108.5	H-3′	6.47 (dd, 8.53, 1.83)	110.8	H-3′	6.49 (m)	104.5 or 109.5	H-3′
6′	7.86 (d, 8.31)	128.6		7.40 (d, 8.53)	134.0		7.42 (d, 8.80)	134.0	
1″							3.36 (d, 6.36)	23.7	
2″							5.22 (t, 6.36)	125.0	H-1″/H-4″/H-5″
3″								131.7	H-1″/H-4″/H-5″
4″							1.80 (s)	18.4	H-5″/H-2″
5″							1.65 (s)	26.5	H-4″/H-2″
CH=O				9.91 (s)	192.3		9.90 (s)	192.2	
OCH3	3.89 (s)	57.3		3.78 (s)	56.7		3.83 (s)	56.8	

data of **3** with that of **2** (Table 1) indicated the presence of an additional 1,1-dimethylallyl group in the former compound. From the HMBC spectrum this side chain was assigned to the C-5 position. The preparation of the triacetate indicated that **3** possesses three hydroxyl groups.

From previous studies it has been found that natural benzofurans and benzopyrans have binding affinity for the estrogen receptors and thus may behave as phytoestrogens. Evidence is accumulating suggesting that phytoestrogens may protect against a variety of disorders, such as osteoporosis, breast cancer, and cardiovascular disease, so they are currently at the center of research interest.[8−11] The similarity of ebenfurans I, II, and III to the aforementioned compounds suggested they may possess estrogenic activity. Therefore, these compounds were tested for estrogen receptor affinity by a receptor binding assay. The principle of the assay is the determination of the relative binding affinity (RBA) of a test compound to the estrogen receptor.[12,13] RBA is the ratio of the molar concentrations of estradiol to that of the tested compound required to decrease the total bound radioactivity by 50% (disintegrations per minute of radioactive estradiol: DPM), multiplied by 100. The RBA for ebenfuran I (**1**) was 0.29 and for ebenfuran II (**2**), 0.28, although ebenfuran III (**3**) showed no significant binding affinity with the estrogen receptor. The RBA of tamoxifen, which was used as a control, was 0.69. IC_{50} values (which are the concentration necessary for 50% displacement of ^{3}H-estradiol binding to the estrogen receptor) of compounds **1** and **2** were found to be 0.046 μM and 0.043 μM, respectively. Further biological evaluation of the new compounds is currently in progress.

Experimental Section

General Experimental Procedures. UV spectra were obtained using spectroscopic grade EtOH/MeOH on a Shimadzu-160A spectrophotometer. A Bruker AC200 spectrometer and a Bruker AC400 spectrometer were used in obtaining the NMR spectra. Chemical shifts are given in δ values, with TMS as internal standard. The 2D experiments (COSY, HMBC, and HMQC) were performed using standard Bruker microprograms. EIMS were run on HP-6890 spectrometer and HRMS were run on a AEI MS-902 spectrometer. Column chromatography was carried out using Si gel [Merck, 0.04−0.06 mm (flash) and 0.015−0.04 mm], with an applied pressure of 300 mbar. MPLC was performed with a Büchi model 688 apparatus on columns containing Si gel (Merck, 0.015−0.040 mm).

Plant Material. Whole plants were collected in May 1998, from Mount Ymitos, Attica (Greece). A voucher specimen (no. NEK 006) was deposited in the herbarium of the Laboratory of Pharmacognosy, Department of Pharmacy, University of Athens.

Extraction and Isolation. The whole plant, dried and pulverized (1.8 kg), was extracted with CH_2Cl_2 (2 L × 3) and MeOH (2 L × 5). The MeOH-soluble extract was concentrated under reduced pressure to give a residue (50 g), which was subjected to vacuum−liquid chromatography on Si gel (0.015−0.04 mm). Elution with a CH_2Cl_2/MeOH gradient yielded 11 fractions. Fraction 2 was chromatographed (1.15 g) over a flash Si gel column using a system consisting of *c*-hexane/CH_2Cl_2 (50−50) to afford β-sitosterol (7.1 mg) and stigmast-5-en-ol (4.5 mg). Fraction 4 (1.15 g) and fraction 5 (1.78 g) were combined and subjected to MPLC. Elution with increasing polarity of CH_2Cl_2/MeOH gave ebenfuran I (20 mg), ebenfuran II (26 mg), and ebenfuran III (28 mg).

Tissue and Cytosol Preparation. Breast tumor specimens were processed immediately after surgery and stored at −70 °C. The tumor homogenization procedure was identical to that used for the routine determination of estrogen receptor; tissue was weighed, a 5-fold volume of ice-cold buffer (10 mM Tris, 1.5 mM EDTA, 5 mM Na_2MoO_4, 0.49 mM Dithiothreitol) was added, and the tissue was homogenized in an Ultra Turrax T25 apparatus. Cytosol was prepared by centrifugation at 100000*g*, for 60 min, at 4 °C. The supernatant (cytosol) was then used for determining the affinity of compounds for the estrogen receptor. The protein concentration of the cytosol was calculated at 6.505 mg/mL, using the Lowry-protein assay method.

Competition Assay. To determine the binding parameters, cytosol, prepared as above, was used throughout. Incubation of 150 μL cytosol with 40 nM (50 μL) of ^{3}H-estradiol in the absence and presence of various concentrations of competitors (40 mM−20 mM−4 mM−40 μM) was performed at 0 °C for 18 h. Then 0.5 mL of dextran-coated charcoal (DCC) slurry (0.05% dextran, 0.5% charcoal) was added to the tubes, and the contents were mixed. The tubes were incubated for 15 min at 0 °C and then centrifuged at 3000 rpm for 10 min to pellet the charcoal. An aliquot (200 μL) of the supernatant was removed, and the radioactivity was determined by liquid−scintillation spectrometry after addition of 4 mL of Lumagel Plus (Packard). Nonspecific binding was calculated with 40 μM 17β-estradiol as competing ligand. The RBA was calculated as the ratio of the molar concentrations of estradiol and

compound required to decrease the amount of bound radioactivity by 50%, multiplied by 100.

Ebenfuran I (1): yellow solid; UV (EtOH) λ_{max} (log ϵ) 211 (4.55), 231 sh (4.27), 243 sh (4.03), 274 (4.17), 283 sh (4.18), 312 sh (4.20), 326 (4.45), 341 (4.45) nm; (EtOH + NaOH) λ_{max} (log ϵ) 206 (4.58), 244 sh (4.81), 279 sh (4.11), 290 (4.17), 311 sh (4.15), 319 sh (4.23), 326 sh (4.30), 340 (4.43), 352 sh (4.40) nm; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 1; HREIMS *m*/*z* 272.0579, calcd for $C_{15}H_{12}O_5$ 272.0577.

Ebenfuran II (2): yellow solid; UV (MeOH) λ_{max} (log ϵ) 214 (4.29), 265 (4.01), 349 (3.66) nm; (EtOH + NaOH) λ_{max} (log ϵ) 207 (4.34), 277 (3.95), 406 (3.69) nm; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 1; HREIMS *m*/*z* 300.0416, calcd for $C_{16}H_{12}O_6$ 300.0414.

Ebenfuran III (3): yellow solid; UV (MeOH) λ_{max} (log ϵ) 2.16 (4.39), 266 (4.30), 362 (3.75) nm (MeOH + NaOH) λ_{max} (log ϵ) 204 (4.82), 273 (4.30), 402 (3.84) nm ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 1; HREIMS *m*/*z* 368.0928, calcd for $C_{21}H_{20}O_6$ 368.0927.

Acetylation of 1. Treatment of **1** (5 mg) with Ac_2O (0.5 mL) and pyridine (0.5 mL) at room temperature overnight gave the triacetate **4** (91%): UV ($CDCl_3$) λ_{max} (log ϵ) 272 (3.75), 282 (3.87), 292 (3.94), 303 (4.11), 318 (4.33), 332 (4.25) nm; ^{1}H NMR data ($CDCl_3$, 400 MHz) δ 7.90 (1H, d, J = 8.0 Hz, H-6′), 7.21 (1H, s, H-4), 7.09 (1H, s, H-7), 7.08 (1H, d J = 8 Hz, H-5′), 7.01 (1H, d, J = 2 Hz, H-3′), 6.90 (1H, s, H-3), 3.86 (3H, s, OCH_3), 2.37 (3H, s, OCOCH_3), 2.32 (3C, s, OCOCH_3), 2.28 (3H, s, OCOCH_3); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 170.4 (O*C*OCH$_3$), 168.7 (O*C*OCH$_3$), 168.5 (O*C*OCH$_3$), 151.8 (C-5), 151.8 (C-7a), 150.0 (C-2), 149.8 (C-4′), 149.0 (C-6), 146.5 (C-2′), 136.2 (C-3a), 128.2 (C-6′), 120.9 (C-1′), 118.8 (C-5′), 116.9 (C-3′), 114.8 (C-4), 105.0 (C-3), 95.5 (C-7), 55.9 (OCH_3), 21.0 (3 × OCO*C*H$_3$).

Acetylation of 2. Treatment of **2** (5 mg) with Ac_2O (0.5 mL) and pyridine (0.5 mL) under the same conditions gave the triacetate **5** (95%): UV ($CHCl_3$) λ_{max} (log ϵ) 249 (3.53), 325 (3.25) nm; ^{1}H NMR data ($CDCl_3$, 400 MHz) δ 9.84 (CHO), 7.65 (1H, d, J = 8.3 Hz, H-6′), 7.19 (1H, dd, J = 8.3, 2.1 Hz, H-5′), 7.13 (1H, d, J = 2.1 Hz, H-3′), 6.94 (1H, d, J = 2 Hz, H-7), 6.67 (1H, d, J = 2 Hz, H-5), 3.87 (3H, s,OCH_3), 2.48 (3H, s, OCOCH_3), 2.31 (3H, s, OCOCH_3), 2.14 (3H, s, OCOCH_3); ^{13}C NMR ($DCCl_3$, 50 MHz) δ 185.0 (CHO) 170.9 (O*C*OCH$_3$), 168.8 (O*C*OCH$_3$), 168.8 (O*C*OCH$_3$), 161.8 (C-2), 159.8 (C-6), 157.2 (C-7a), 153.8 (C-4′), 149.5 (C-2′), 145.0 (C-4), 133.8 (C-6′), 119.5 (C-1′), 119.2 (C-3), 118.2 (C-5′), 117.0 (C-3′), 111.5 (C-3a), 106.7 (C-5), 93.8 (C-7), 56.4 (OCH_3), 22.1 (OCO*C*H$_3$), 22.0 (OCO*C*H$_3$), 21.8 (OCO*C*H$_3$).

Acetylation of 3. The same procedure was followed for ebenfuran III. Acetylation of the hydroxyl groups gave the triacetate **6** (89%): UV ($CHCl_3$) λ_{max} (log ϵ) 249 (3.61), 327 (3.47) nm; ^{1}H NMR data ($CDCl_3$ 400 MHz) δ 9.8 (1H, s, CHO), 7.62 (1H, d, J = 8.3 Hz, H-6′), 7.19 (1H, dd, J = 8.3, 2.4 Hz, H-5′), 7.11 (1H, d, J = 2.4 Hz, H-3′), 6.93 (1H, s, H-7), 5.1 (1H, t, J = 6.8, Hz, H-2″), 3.86 (3H, s, OCH_3), 3.45 (2H, d, J = 6.8 Hz, H-1″), 2.48 (3H, s, OCOCH_3), 2.32 (3H, s, OCOCH_3), 2.13 (3H, s, OCOCH_3), 1.75 (3H, s, OCH_3), 1.64 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 186.0 (CHO), 170.2 (O*C*OCH$_3$), 169.7 (O*C*OCH$_3$), 169.7 (O*C*OCH$_3$), 161.7 (C-2), 158,5 (C-6), 154.3 (C-7a) 152.2 (C-4′), 150.2 (C-2′), 132.5 (C-3″), 132.0 (C-4), 131.9 (C-6′), 122.0 (C-5), 121.9 (C-2″), 120.0 (C-1′), 119.0 (C-5′), 118.0 (C-3), 117.2 (C-3a), 116.2 (C-3′), 92.2 (C-7), 56.0 (OCH_3), 26.0 (C-4″), 23.0 (C-1″), 21.5 (OCO*C*H$_3$), 20.7 (OCO*C*H$_3$), 20.7 (OCO*C*H$_3$), 18 (C-5″).

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