JOURNAL OF NATURAL PRODUCTS

ARTICLE

pubs.acs.org/jnp

Estrogenic Activity of Chemical Constituents from *Tephrosia candida*

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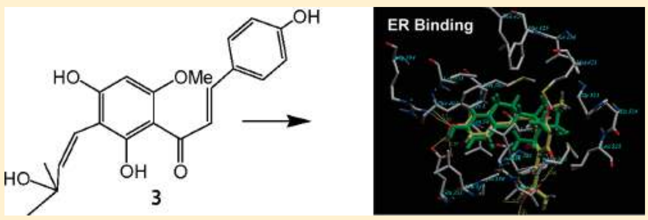
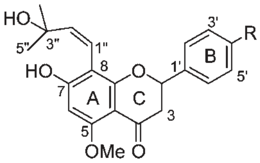
S *Supporting Information*

ABSTRACT: In a continued investigation of medicinal plants from the genus *Tephrosia*, phytochemical analysis of a methylene chloride−methanol (1:1) extract of the air-dried aerial parts of *Tephrosia candida* afforded two new 8-prenylated flavonoids, namely, tephrocandidins A (**1**) and B (**2**), a new prenylated chalcone, candidachalcone (**3**), a new sesquiterpene (**4**), and a previously reported pea flavonoid phytoalexin, pisatin (**5**). The structures of **1**−**4** were established by spectroscopic methods, including HREIMS, and ^{1}H, ^{13}C, DEPT, HMQC, and HMBC NMR experiments. The most potent estrogenic activity of these isolated natural products in an estrogen receptor (ERα) competitive-binding assay was for **3**, which exhibited an IC_{50} value of 80 μM, compared with 18 nM for the natural steroid 17β-estradiol. Results were interpreted via virtual docking of isolated compounds to an ERα crystal structure.



The genus *Tephrosia* (Leguminosae; subfamily Papilinoideae; tribe *Tephrosieae*) includes approximately 400 species.[1] Several reports have indicated that extracts of some species of the genus have antibacterial, antifungal,[2] insecticidal,[3] antiviral,[4] antiprotozoal,[5,6] antiplasmodial,[7] antioxidant,[8] and cytotoxic[9] activities. Phytochemical investigations have revealed the presence of glycosides, rotenoids, isoflavones, chalcones, flavanones, flavanols, flavones, and prenylated flavonoids[12−20] of chemotaxonomic importance in the genus.[21] Flavonoids can act as phytoestrogens, as some bind to estrogen receptor (ER) subtypes and activate their signaling pathways.[22,23] In humans, two ER isoforms have been identified (ERα and ERβ), and physiological responses to estrogen are thought to be mediated through these two receptors. In response to estrogens or estrogen mimics, ER isoforms are activated and stimulate DNA synthesis and cell proliferation.[24] Estrogen signaling can regulate health-related biological processes including cancer proliferation and bone mineral density.[25] There is a current interest in naturally occurring phytoestrogens as potential alternatives to hormonal replacement therapy (HRT).[26] With the observation that *Tephrosia candida* can produce unusual prenylated flavonoids,[27] whether or not such modified flavonoids can act as estrogen mimics was investigated.[28] Herein we report the elucidation and biological evaluation of a series of flavonoids as estrogen receptor mimics isolated from the dried aerial parts of *T. candida*, inclusive of two prenylated flavonoids (**1** and **2**), a prenylated chalcone (**3**), a sesquiterpene (**4**), and a previously reported flavonoid (**5**). The structures of the new compounds **1**−**4** were established by comprehensive spectroscopic analysis and by comparison of NMR data with related literature-reported structures. Estrogen receptor and in silico binding studies of isolated natural products were performed.



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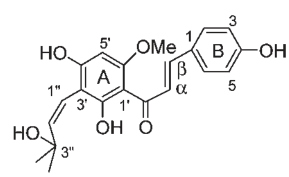
2 R = OH



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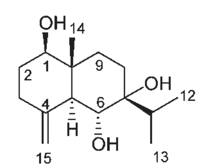

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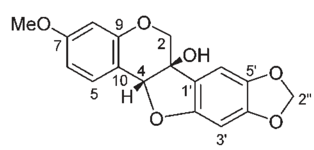

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Received: June 23, 2010
Published: April 21, 2011

Table 1. ^{1}H NMR Spectroscopic Data (500 MHz, $CDCl_3$) for Compounds 1−4

	1	2	3	4
position	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1				3.38, brs
2	5.42, dd (13.0, 3.0)	5.35, (dd, 13.0, 3.0)	7.51, (d, 8.0)	1.88, tdd (14, 5, 3)
				1.75, ddd (14, 5, 2.5)
3	2.82, dd (16.5, 3.0)	2.78, dd (16.5, 3.0)	6.86, (d, 8.0)	2.39, td (13, 5, 5)
	2.98, dd (16.5, 13.0)	2.99, dd (16.5, 13.0)		2.18, m
5			6.86, (d, 8.0)	2.71, d (10.5)
6	6.06, s	6.04, s	7.51, (d, 8.0)	3.92, d (10.5)
8				2.16, m
				1.00, m
9				1.49, td (14.5, 4.5)
				1.62, ddd (14.5, 3, 3)
α, β			7.76, s	
11				2.13, m
12				0.97, d (7.0)
13				0.95, d (7.0)
14				0.75, s
15				5.00, s; 4.74, s
2′	7.46, d (8.5)	7.34, d (8.0)		
3′	7.42, t (8.5)	6.87, d (8.0)		
4′	7.37, d (8.5)			
5′	7.42, t (8.5)	6.87, d (8.0)	5.92, s	
6′	7.46, d (8.5)	7.34, d (8.0)		
1″	6.60, d (10.0)	6.57, d (8.0)	6.68, d (9.5)	
2″	5.46, d (10.0)	5.45, d (10.0)	5.46, d (9.5)	
Me_2	1.44, s	1.43, s	1.46, s	
	1.46, s	1.46, s		
OMe	3.89, s	3.88, s	3.91, s	
OH			14.61, brs	

Tephrocandidin A (**1**) was obtained as a colorless powder, $[\alpha]_D^{25}$ −55 (c 0.1, MeOH). The HRMALDITOFMS exhibited a molecular ion peak $[M + Na - H_2O]^+$ at m/z 359.1467 (10%) (calcd 359.1449) and $[M - OH]^+$ at m/z 337.1438 (100%) (calcd 337.1429), indicating the molecular formula $C_{21}H_{22}O_5$, which was supported by ^{13}C and DEPT NMR analysis and by comparison with other prenylated flavonoids.[25] The presence of a flavanone structure was deduced from the ^{1}H (Table 1) and ^{1}H−^{1}H COSY NMR spectra. ^{13}C NMR signals (Table 2) were assigned on the basis of chemical shifts, DEPT, and HMBC data. An ABX pattern included signals resonating at δ_H 5.42 (dd, J = 13, 3.0 Hz, 1H, H-2), 2.98 (dd, J = 16.5, 13.0 Hz, 1H, H-3_{ax}), and 2.82 (dd, J = 16.5, 3.0 Hz, 1H, H-3_{eq}). The large coupling constant of H-2 (J = 13.0 Hz) was indicative of an axial orientation of this proton, and coupling was observed between the doublet doublet at δ_H 5.42 (H-2) and the doublet doublet signals at δ_H 2.98 and 2.82 (H-3_{ax} and H-3_{eq}, respectively).

The ^{1}H−^{1}H COSY spectrum of **1** exhibited coupled signals integrating for five protons, indicating the presence of an unsubstituted B-ring: a doublet at δ_H 7.46 (d, J = 8.5 Hz, 2H, H-2′,6′), a triplet at δ_H 7.42 (t, J = 8.5 Hz, 2H, H-3′,5′), and a doublet at δ_H 7.37 (d, J = 8.5 Hz, 1H, H-4′). HMBC data showed confirmation correlations between H-3 and C-1′ at δ_C 138.9; H-3 and C-2 at δ_C 78.9; H-3 and C-4 at δ_C 189.2; and H-2′,6′ and C-2 at δ_C 78.9. A 3-methyl-3-hydroxy-1-butenyl prenyl moiety was observed with a proton signal at δ_H 6.60 (d, J = 10.0 Hz, 1H, H-1″) correlated with a doublet at δ_H 5.46 (d, J = 10.0 Hz, 1H, H-2″) in the ^{1}H−^{1}H COSY spectrum. The proton coupling constant indicated a *cis* orientation for the C-1″/C-2″ double-bond linkage. Two geminal methyl groups appeared as two singlet signals at δ_H 1.44 (s, 3H) and 1.46 (s, 3H), with the chemical shift indicative of an adjacent oxygen functionality. The NOESY spectroscopic data were also consistent with a prenyl unit with correlations between H-1″ and H-2″ and the tertiary methyl groups. The prenyl carbon signals appeared at δ_C 116.0 (d, C-1″), 126.3 (d, C-2″), 78.0 (s, C-3″), 28.2 (q, Me), and 28.5 (q, Me). The placement of the prenyl moiety at C-8 was deduced from HMBC signals that showed a correlation between H-2″ and C-8 at δ_C 105.7. Prenyl moiety correlations were also observed between H-1″ and C-3″ at δ_C 78.7 and C-7 at δ_C 160.0 as well as H-4″,5″ and C-2″ at δ_C 126.3, in addition to an oxygenated carbon signal correlation at δ_C 78.0 (C-3″) with proton signals for H-2″, H-4″, and H-5″, at δ_H 5.46 (d, J = 10.0), 1.44 (s), and 1.46 (s), respectively. The remaining singlet aromatic signal at δ_H 6.06 (s, 1H, H-6) was assigned to position 6 of the A-ring, with correlations observed between H-6 and C-8 at δ_C 105.7 as well as H-6 at δ_C 93.8 and C-10 at δ_C 102.9.

The methoxy group at δ_H 3.89 (3H) was assigned to C-5 on the basis of HMBC data showing a correlation between the methoxy protons and C-5 at δ_C 162.1. The remaining hydroxy

Table 2. ^{13}C NMR Spectroscopic Data (125 MHz, $CDCl_3$) for Compounds 1−4

	1	2	3	4
carbon	δ_C	δ_C	δ_C	δ_C
1			128.5, C	74.5, CH
2	78.9, CH	78.6, CH	130.3, CH	30.7, CH_2
3	45.6, CH_2	45.6, CH_2	115.8, CH	31.8, CH_2
4	189.2, C	189.7, C	157.4, C	147.6, C
5	162.1, C	162.8, C	115.8, CH	45.1, CH
6	93.8, CH	93.6, CH	130.3, CH	67.8, CH
7	160.0, C	160.4, C		75.3, C
8	105.7, C	105.3, C		27.7, CH_2
9	158.8, C	158.8, C		22.4, CH_2
10	102.9, C	102.9, C		41.3, C
α			125.3, CH	
β			142.2, CH	
11				33.8, CH
12				17.8, CH_3
13				16.4, CH_3
14				17.1, CH_3
15				106.9, CH_2
1′	138.9, C	131.9, C	107.0, C	
2′	128.7, CH	127.6, CH	162.5, C	
3′	125.9, CH	115.9, CH	106.0, C	
4′	128.5, CH	155.9, C	162.5, C	
5′	125.9, CH	115.9, CH	91.5, C	
6′	128.7, CH	127.6, CH	160.1, C	
1″	116.0, CH	115.8, CH	116.1, CH	
2″	126.3, CH	126.1, CH	125.3, CH	
3″	78.0, C	78.7, C	78.2, C	
Me_2	28.5, CH_3	28.3, CH_3	28.4, CH_3	
	28.2, CH_3	28.2, CH_3		
OMe	56.2, CH_3	56.2, CH_3	55.9, CH_3	
CO			192.6, C	

group was assigned at the last open position at C-7 (δ_C 160.0). The specific optical rotation of **1** (−55.0) together with the *trans*-diaxial coupling constant of H-2 and H-3 ($J_{2,3ax}$ = 13.0 Hz) suggested an *S* configuration at C-2, like those of known flavanones.[27,29−31] Indeed, from the genus *Tephrosia*, a total of 18 prenylated flavanones with a chiral center at C-2 of the γ-pyrone ring have been reported, of which all have the *S* configuration. Examples include (−)-isolonchocarpin,[12,21] 5-methoxy-8,8-dimethyl-2-phenyl-2,3-dihydro-8*H*-pyrano[2,3-*f*]chromen-4-one, 5-hydroxy-8,8-dimethyl-2-phenyl-2,3-dihydro-8*H*-pyrano[2,3-*f*]chromen-4-one,[33] 7-methylglabranin,[34] ephroleocarpin A, tephroleocarpin B, quercetol C,[34] 8-prenylpinostrobin,[35] spinoflavanone A,[36] spinoflavanone B,[36] fulvinervin A,[37] 5,7-dimethoxy-8-(3-methylbut-2-enyl)-2-phenylchroman-4-one,[38] 5-methylobovatin,[39] dehydroisoderricin,[40] (−)-dehydroisoderricin,[41] maxima flavanone A,[42] 5-hydroxy-7-methoxy-8-[(*E*)-3-oxo-1-butenyl]flavanone,[43] and 7-*O*-methylglabranin.[44] Compound **1** was established as 2,3-dihydro-7-hydroxy-8-[(*Z*)-3-hydroxy-3-methylbut-1-enyl]-5-methoxy-2-phenylchroman-4-one, a new natural product.

Tephrocandidin B (**2**) was obtained as a white powder, $[\alpha]^{25}_D$ −4 (*c* 0.1, MeOH). The HRMALDITOFMS exhibited a molecular ion peak $[M + Na - H_2O]^+$ at *m/z* 375.1188 (10%) (calcd 375.1175) and $[M - OH]^+$ at *m/z* 353.1377 (100%) (calcd 353.1362), in accordance with a molecular formula of $C_{21}H_{22}O_6$. The ^1H and ^{13}C NMR data were quite similar to those of **1** (Tables 1 and 2), except for differences in the B-ring signals. The $^1H-^1H$ COSY spectrum exhibited coupled signals integrating for four protons, indicating an AA′BB′ symmetrically substituted B-ring with a set of coupled protons at δ_H 7.34 (d, *J* = 8.0 Hz, 2H, H-2′,6′) and δ_H 6.87 (d, *J* = 8.0 Hz, H-3′,5′). HMBC data showed diagnostic correlations between H-3 and C-1′ at δ_C 131.9 and of H-2′,6′ with C-2 at δ_C 78.6. Again similar to **1**, the presence of a flavanone structure was determined from the ^1H NMR spectrum, which showed three ABX signals as double doublets at δ_H 5.35 (dd, *J* = 13.0, 3.0 Hz, 1H, H-2), 2.99 (dd, *J* = 16.5, 13.0 Hz, 1H, H-3ax), and 2.78 (dd, *J* = 16.5, 3.0 Hz, 1H, H-3eq). The same placement of the 3-methyl-3-hydroxy-1-butenyl prenyl moiety at C-8 as in **1** was deduced from HMBC signals that showed correlations between H-1″ and C-3″ at δ_C 78.7 and C-7 at δ_C 160.4 as well as H-2″ and C-8 (δ_C 105.3). A prenyl correlation was also observed between H-4″,5″ and C-2″ at δ_C 126.1. The methoxy group at δ_H 3.88 (3H) was assigned to C-5 on the basis of HMBC data with a correlation between the methoxy protons and C-5 at δ_C 162.8. The remaining three hydroxy groups were assigned at the last open positions at C-7, C-4′, and C-3″ (δ_C 160.4, 155.9, and 78.7, respectively). Thus, compound **2** was established as 2,3-dihydro-7-hydroxy-8-[(*Z*)-3-hydroxy-3-methylbut-1-enyl]-2-(4-hydroxyphenyl)-5-methoxychromen-4-one, a new natural product.

Candidachalcone (**3**) was isolated as a yellowish powder; $[\alpha]^{25}_D$ −3 (*c* 0.1, MeOH). HREIMS exhibited a molecular ion peak at *m/z* 370.1425 (calcd 370.1416), in accordance with a molecular formula of $C_{21}H_{22}O_6$, which was supported by ^{13}C and DEPT NMR analysis. The ^1H NMR fingerprint signals for a *p*-substituted phenyl ring as part of a prenylated chalcone[45] were observed at δ_H 3.91 (s, 3H, OMe) and δ_H 5.92 (s, 1H, H-5′). ^{13}C NMR signals were assigned (Table 2) on the basis of chemical shifts, DEPT, and HMBC data as well as structurally related prenylated chalcones previously reported.[45,46] The $^1H-^1H$ COSY spectrum exhibited coupled signals integrating for four protons, indicating an AABB symmetrically substituted B-ring with coupled protons at δ_H 7.51 (d, *J* = 8.0 Hz, 2H, H-2,6) and δ_H 6.86 (d, *J* = 8.0 Hz, 2H, H-3,5). The HMBC data showed diagnostic correlations between H-α and C-1 at δ_C 128.5, H-α and C-β at δ_C 142.2, H-α and C=O at δ_C 192.6, and H-2,6 and C-β at δ_C 142.2.

A 3-methyl-3-hydroxy-1-butenyl prenyl moiety was observed for **3** with a proton-correlated signal at δ_H 6.68 (d, *J* = 9.5 Hz, 1H, H-1″) and C-3″ at δ_C 78.2 by HMBC analysis. The proton coupling constant for H-1″ and H-2″ indicated a *cis* orientation for the carbon−carbon double-bond linkage. Two germinal methyl groups appeared as a singlet signal at δ_H 1.46 (s, 6H) with the chemical shift indicative of a proximal hydroxy group. The placement of the prenyl moiety at C-3′ was deduced from HMBC signals that showed a correlation between δ_H 5.46 (d, *J* = 9.5 Hz, H-2″) and C-5′ at δ_C 106.0. Other A-ring correlations included H-5′ and C-3′ at δ_C 106.0, H-β and C-1′ at δ_C 107.0, and C-4′ and C-6′ at δ_C 160.1. The two olefinic protons, Hα and Hβ, resonated as a second-order singlet at δ_H 7.76 (s, 2H, Hα, Hβ), as has been reported by Herath et al. with structurally similar chalcone-type prenyl-flavonoids.[46] The methoxy group at δ_H 3.91 (3H) was assigned to C-6′ on the basis of HMBC data showing a correlation between the methoxy protons and C-6′ at δ_C 160.1. The remaining three hydroxy groups were assigned to the last open positions at C-4, C-4′, and C-3″ (δ_C 157.4, 162.5, and 78.2, respectively). Thus, compound **3** was identified as (2*E*)-1-(2,4-dihydroxy)-3-[(*E*)-3-hydroxy-3-methylbut-1-enyl]-6-methoxyphenyl-3-(4-hydroxyphenyl)prop-2-en-1-one, a new natural product.

Compound **4** was isolated as a colorless powder, $[\alpha]_D^{25}$ +52 (*c* 0.1, MeOH). The IR spectrum exhibited absorption bands at 3500 (br) and 1650 cm^{-1}. The HREIMS showed a molecular ion peak $[M]^+$ at *m/z* 254.1895 (calcd 254.1882), in accordance with a molecular formula of $C_{15}H_{26}O_3$. The ^1H and ^{13}C NMR data of **4** established the presence of a eudesmane-type sesquiterpene. The ^1H NMR spectrum showed exomethylene protons as two singlet signals at δ_H 4.74 and 5.00 for H-15$_a$ and H-15$_b$, respectively. These two protons correlated with an olefinic methylene carbon at δ_C 106.9 in the HMQC spectrum (C-15). The HMQC spectrum also exhibited a one-proton doublet at δ_H 3.92 (*J* = 10.5 Hz, H-6) correlating with a one-carbon doublet at δ_C 67.8 (C-6). The H-6 signal also showed a correlation with a doublet at δ_H 2.71 (*J* = 10.5 Hz, H-5) in the ^1H ^1H COSY spectrum. In the HMQC spectrum a hydroxy proton appearing as a broad singlet at δ_H 3.38 correlated with an oxygenated carbon signal at δ_C 74.5 (C-1), and a second hydroxy proton exhibited two doublets at δ_H 0.97 and 0.95 (*J* = 7.0 Hz), both of which were coupled to a multiplet signal at δ_H 2.13 (1H, m), in accordance with an isopropyl group. The ^{13}C NMR spectrum, with the aid of a DEPT experiment, indicated 15 carbons in the molecule (Table 2), classified as three methyls (C-12, C-13, and C-14), four methylenes (C-2, C-3, C-8, and C-9), four methines (C-1, C-5, C-6, and C-11), two quaternary carbons (C-7 and C-10), and two olefinic carbons (C-4 and C-15), with the latter protonated. The hydroxy group positions were confirmed by HMBC analysis. Correlations were observed between δ_H 3.38 (H-1) and δ_C 30.7 (C-2), 31.8 (C-3), 45.1 (C-5), 41.3 (C-10), and 17.1 (C-14); δ_H 3.92 (H-6) and δ_C 147.6 (C-4), 45.1(C-5), 75.3 (C-7), and 33.8 (C-11); and δ_H 4.74, 5.00 (H-15$_{a,b}$) and δ_C 31.8 (C-3) and 45.1 (C-5). The relative configuration of **4** was established from coupling constants and NOE experiments. The relative configuration at C-5 and C-6 was derived from coupling constants ($J_{5,6}$ = 10.5 Hz), indicating the orientation of the protons as H-5 (α) and H-6 (β). NOE effects supported these results, since irradiation of the signal at δ_H 2.71 (H-5) enhanced the signal at δ_H 3.38 (H-1), suggesting the α-configuration of H-1 and H-5. Moreover, irradiation of the signal at δ_H 3.92 (H-6) enhanced the signal at δ_H 0.75 (H-14), supporting a β-configuration of H-6 and H-14. All these data established compound **4** as a new natural product determined as 1β-hydroxy-6,7α-dihydroxyeudesm-4(15)-ene.

Each isolated compound from *T. candida* was tested for estrogen reception ERα binding at a 5×10^{-3} M concentration; compounds showing a 50% inhibition of estradiol binding with ERα at this concentration were additionally tested at lower concentrations to calculate the 50% inhibitory concentration (IC_{50}). 17β-Estradiol (E_2) was used as the positive control with an IC_{50} of 1.8×10^{-8} M (Table 3). 17β-Estradiol (E2) is the human endogenous estrogen and known to be the most active estrogen receptor agonist.[47] Although the binding affinity of phytoestrogens such as genistein and daidzein for estrogen receptors is only 1/1000−1/10 000 that of estradiol, these natural products can effectively compete with estradiol for receptor sites because plasma levels can rise to 1000 to 10 000 times the circulating concentration of estradiol in the human body.[48] Compounds **1**, **2**, and **4** showed moderate binding ability to ERα with

Table 3. Inhibition of Fluorescence-Labeled Estradiol Binding[a] to ERα by Compounds Isolated from *T. candida*

compound	5 mM (%)	IC_{50} (M)[b]
1	70.1 ± 1.2	$3.5 \times 10^{-3} \pm 0.7 \times 10^{-3}$
2	86.2 ± 0.8	$1.0 \times 10^{-3} \pm 1.5 \times 10^{-3}$
3	95.1 ± 1.1	$8.0 \times 10^{-5} \pm 0.9 \times 10^{-5}$
4	67.3 ± 0.3	$2.8 \times 10^{-3} \pm 2.2 \times 10^{-3}$
5	36.2 ± 0.4	$>5.0 \times 10^{-3}$
17β-estradiol[c]		$1.8 \times 10^{-8} \pm 0.3 \times 10^{-8}$

[a] Average reading ± standard error (*n* = 3). [b] IC_{50} is the concentration of compound that can decrease the binding of fluorescent-labeled estradiol to ERα by 50%. [c] An inhibition of 100% was observed for E2 at 10^{-7} M.

IC_{50} values of 3.5×10^{-3}, 2.8×10^{-3}, and 10^{-3} M, respectively. However, compound **3** exhibited more potent phytoestrogen activity with an IC_{50} of 8×10^{-5} M (Table 3). Although the binding affinity of **3** for the estrogen receptor is substantially lower than E2, this chalcone natural product is in the range to serve as a promising phytoestrogen receptor agonist.

Virtual docking of the assayed compounds in the crystal structure of ERα revealed that compound **1** binds to Ala_{350} and Glu_{353}, while compound **2** binds to Arg_{394}, Leu_{391}, and Gly_{521} (Figure S1, Supporting Information). Interestingly, both compounds failed to bind to Glu_{353} and Arg_{394} simultaneously, as in the case of the natural estradiol 17β-estradiol, due to the presence of a bulky substituent adjacent to the phenolic OH (Figure S2, Supporting Information). On the other hand, compound **3** showed a similar positioning of its A-ring to that of estradiol (Figure 1), enabling **3** to bind to both Arg_{394} and Glu_{353} via H bonding (1.97 and 2.07 Å), which could be due to the flexibility of the chalcone core of **3** over that of the flavanones **1** and **2**. It has been established that estradiol docking in ERα occurs via a minimum of three interactions: (a) A-ring OH 3-hydrogen bonding with Arg_{394} and Glu_{353}; (b) 17β-OH hydrogen bonding with His_{524}; and (c) hydrophobic core attraction with the ERα hydrophobic pocket.[49] A distance of 10.9 Å between the two hydroxy groups in estradiol has been found to be essential for activity, through binding to the right amino acid residues in the estrogen receptor active site.[49]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer model 341 polarimeter with a 1 dm cell. IR spectra were recorded on a JASCO FT/IR-5300 spectrometer. ^1H NMR (500 MHz, $CDCl_3$), ^{13}C NMR (125 MHz, $CDCl_3$), and the 2D NMR spectra were recorded on a JEOL 500 MHz Lambda spectrometer, with TMS as an internal standard. EIMS were recorded on a JEOL SX102A mass spectrometer. Column chromatography was carried out on silica gel 60 (Merck; 230−400 mesh) and Sephadex LH-20 (Pharmacia Co. Tokyo, Japan). TLC was performed on silica gel 60 F_{254} plates (0.25 mm, Merck), and spots were detected under UV light and colored by spraying with 10% H_2SO_4 solution followed by heating.

Plant Material. The aerial parts of *Tephrosia candida* were collected in April 2007 in Limbe (South West) Province, Cameroon. The plant identification was made by Dr. Jean Michel Onana, and a voucher specimen (No. 42711/HNC/Cam) has been deposited in the Cameroon National Herbarium, Yaoundé, Cameroon.

Extraction and Isolation. Air-dried aerial parts (500 g) were crushed and extracted with CH_2Cl_2−MeOH (1:1) at room temperature. The extract was concentrated in vacuo to give a residue (50 g),

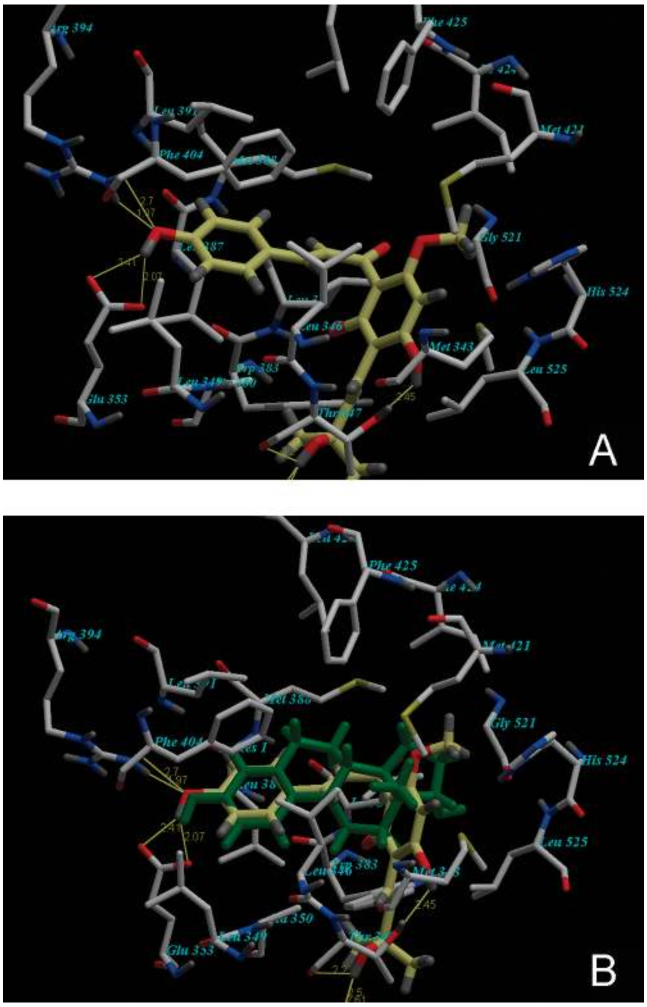

Figure 1. Computer modeling of compound docking to the estrogen receptor ERα: for **3** (A) and **3** (yellow) with estradiol (green) superimposed (B).

which was chromatographed using flash column chromatography on silica gel eluted with *n*-hexane (2 L), followed by a gradient of *n*-hexane−CH_2Cl_2 to CH_2Cl_2 and CH_2Cl_2−MeOH to 15% MeOH (2 L of each solvent or solvent mixture). The *n*-hexane−CH_2Cl_2 fraction (1:3) was carefully chromatographed on a Sephadex LH-20 column (4 × 35 cm), eluting with *n*-hexane−CH_2Cl_2−MeOH (7:4:0.25). Further purification of each subfraction through repeated chromatography using ether−petroleum ether (1:1) as a developer for preparative TLC separation afforded compounds **1** (9.0 mg) and **2** (8.0 mg). The CH_2Cl_2 fraction (100%) was chromatographed on a Sephadex LH-20 column eluted with *n*-hexane−CH_2Cl_2−MeOH (7:4:0.5), followed by further purification using preparative TLC chromatography with ether−petroleum ether as a developer (2:1), affording compounds **3** (11.0 mg), **4** (13.0 mg), and **5** (9.0 mg).

Ligand Binding Assay. A competition assay was employed to determine the binding of **1**−**5** to the estrogen receptor (ERα), using a specific assay kit (ERα assay kit; Wako Chemical Japan, Inc.). Direct comparisons were performed with a labeled estrogen mixture. The amount of the ligand that bound to ERα coated on microplate wells was determined by a dynamic equilibrium among all the ligand concentrations in the mixture, the difference of their binding affinities to the receptor, and incubation time. A reduction in fluorescence intensities from the labeled estrogens provided a measure of the affinity of the added compounds to the estrogen receptor. The isolated compounds were tested at concentrations of 10^{-5}, 10^{-4}, 5×10^{-4}, and 5×10^{-3} M. Estradiol (17β-estradiol) was used as a positive control at concentrations of 10^{-9}, 10^{-8}, and 10^{-7} M. The test compounds were pipetted together with the fluorescent-labeled estrogen (reaction mixture) to the ER-precoated plates at a 10% ratio. The microplate was incubated at room temperature for 2 h. The plate was washed several times with the wash solution followed by the addition of the assay solution to release the fluorescent substance to be measured. The fluorimetric analysis was performed on an automated TECAN GENios plate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Results were calculated as percentages of labeled estrogen mixture binding.

Docking Studies. The crystal structure of estrogen receptor α (ERα) bound to 17β estradiol (protein data bank ID 1A52) was downloaded from www.pdb.org. The crystal structure was prepared for a docking study using the Internal Coordinate Mechanics (ICM-Pro) software version 3.4-8 C (MolSoft LLC, San Diego, CA).[50] The crystal structure was first transformed to ICM object, and water molecules were eliminated. The protein model was adjusted (regularized) so that optimal positions of polar hydrogens were identified, missing hydrogen and heavy atoms were added, and atom types and partial charges were assigned. 3D structures of the ligand molecules were generated and energy minimized using Merck Molecular Force Field (MMFF). The active site of the regularized protein was identified and adjusted using ICM small-molecule docking procedures (MolSoft ICM manual). Receptor energy maps were constructed including energy terms for electrostatic, directional hydrogen bond, hydrophobic interactions, and two van der Waals interactions for steric repulsions and dispersion attractions. Docking was performed one ligand at a time using interactive docking (interactive docking/Mol table ligand), and the ICM scores were calculated. Redocking of the cocrystal structure ligand (17β-estradiol) and rmsd results were compared to literature values to validate the docking process.

*Tephrocandidin A (**1**).* 2,3-Dihydro-7-hydroxy-8-[(*Z*)-3-hydroxy-3-methylbut-1-enyl)-2-(4-hydroxyphenyl]-5-methoxychromen-4-one: colorless powder; $[\alpha]_D^{25}$ −55 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3449, 2927, 1690, 1583, 1516, 1450, 1266, 1122 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Tables 1 and 2, respectively; HRMALDITOFMS *m/z* 359.1467 $[M + Na]^+$ (calcd for $C_{21}H_{22}O_5$ 359.1449).

*Tephrocandidin B (**2**).* 2,3-Dihydro-7-hydroxy-8-[(*Z*)-3-hydroxy-3-methylbut-1-enyl-2-(4-hydroxyphenyl]-5-methoxychromen-4-one: white powder; $[\alpha]_D^{25}$ −4 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3453, 2935, 1665, 1520, 1450, 1253, 1116 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively; HRMALDITOFMS *m/z* 375.1188 $[M + Na]^+$ (calcd for $C_{21}H_{22}O_6$ 375.1175).

*Candidachalcone (**3**).* [(2*E*)-1-(2,4-dihydroxy)-3-[(*E*)-3-hydroxy-3-methylbut-1-enyl]-6-methoxyphenyl-3-(4-hydroxyphenyl)prop-2-en-1-one]: colorless powder; $[\alpha]_D^{25}$ −3 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3452, 1650, 1557, 1511, 1462, 1422, 1260, 1145 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively; HREIMS *m/z* 370.1425 $[M]^+$ (calcd for $C_{21}H_{22}O_6$ 370.1416).

*1β-Hydroxy-6,7α-dihydroxyeudesm-4(15)-ene (**4**):* yellowish oil; $[\alpha]_D^{25}$ +52 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3449 (br), 1644, 1266, 1122 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively; HREIMS *m/z* 254.1895 $[M]^+$ (calcd for $C_{15}H_{26}O_3$ 254.1882).

*(+)-Pisatin (**5**):* colorless powder; $[\alpha]_D^{25}$ +20 (*c* 0.1, MeOH); ^1H NMR ($CDCl_3$, 500 MHz) δ_H 3.89 (3H, s, OMe), 4.01 (1H, d, *J* = 11.5 Hz, H-2a), 4.18 (1H, d, *J* = 11.5 Hz, H-2b), 5.29 (1H, s, H-4), 5.91 (1H, d, *J* = 1.5 Hz, H-2″a), 5.95 (1H, d, *J* = 1.5 Hz, H-2″b), 6.40 (1H, s,H-3′), 6.46 (1H, d, *J* = 2.5 Hz, H-8), 6.66 (1H, dd, *J* = 8.5 and 2.5 Hz, H-6), 6.81 (1H, s, H-6′), 7.38 (1H, d, *J* = 8.5 Hz, H-5); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 55.4 (OMe), 69.5 (C-2), 77.0 (C-3), 84.9 (C-4), 94.2 (C-3′), 101.5 (C-8), 101.6 (C-2″), 103.0 (C-6′), 109.8 (C-6), 112.3 (C-10),

118.9 (C-1′), 131.8 (C-5), 142.4 (C-5′), 149.9 (C-4′), 154.6 (C-2′), 155.7 (C-9); EIMS *m/z* 314 $[M]^+$.

■ ASSOCIATED CONTENT

Supporting Information. Computer modeling diagrams of estrogen receptor for compounds **1** and **2** and NMR spectra (^{1}H, ^{13}C NMR, DEPT, HMQC, and HMBC) for reported compounds are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

The authors would like to thank Dr. J. M. Onana for identification of plant material. Financial assistance was provided in part by a grant from the Robert Welch Foundation (D-1478).

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