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Lignan and Flavonoid Phytoestrogens from the Seeds of *Cuscuta chinensis*

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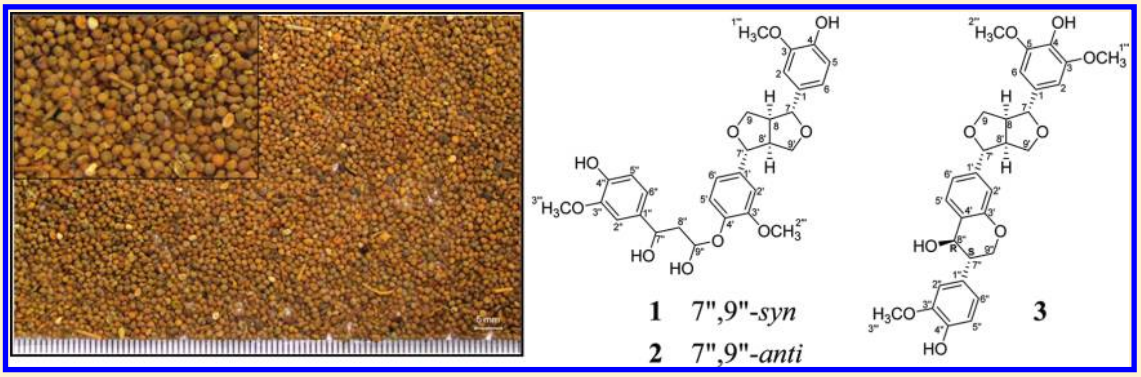
Supporting Information



ABSTRACT: In a search for natural phytoestrogens, 130 traditional Chinese medicinal extracts related to gynecological disorders were investigated by the *Arabidopsis* pER8:GUS reporter assay system. The EtOH extract of *Cuscuta chinensis* showed estrogenic activity (100 μg/mL) and afforded three new lignans, cuscutaresinols A−C (**1**−**3**), and 16 known compounds. Cuscutaresinols A−C (**1**−**3**), (+)-sesamin (**4**), (+)-xanthoxylol (**5**), 9-hydroxysesamin (**6**), (+)-pinoresinol (**7**), kaempferol (**8**), and isorhamnetin (**9**) showed estrogenic activity, with **8** and **9** exhibiting the most potent activity. Kaempferol (**8**) and isorhamnetin (**9**) are the major components of *C. chinenesis* EtOH extract and the key contributors to its estrogenic activity in the *Arabidopsis* pER8:GUS reporter assay system.

Phytoestrogens are natural secondary metabolites that exhibit several positive effects on human health, such as improving menopausal syndrome,[1,2] resisting oxidative damage accompanying neurological and cardiovascular diseases,[3−6] and inhibiting tumor growth.[6−8] Currently, phytoestrogens are one of the most important nutritional supplements. Phytoestrogens either are absorbed directly from the gastrointestinal tract or undergo metabolic conversion to the active form by gut microflora before absorption.[9] Despite the wealth of evidence showing the biological importance of dietary phytoestrogens, their use for postmenopausal syndrome is still a controversial issue.[10] Thus, finding new sources of phytoestrogens and developing feasible protocols for their detection in natural products will promote our understanding of their actual role.

In 2005, we introduced a cross-kingdom bioassay to test animal biological functions utilizing a higher plant bioassay model.[11] In this system, the transgenic *Arabidopsis thaliana* plant with the pER8:GUS reporter, bearing a human estrogenic receptor, is used as a tool for screening phytoestrogens from natural sources. The system was utilized for screening different plant extracts used to treat postmenopausal syndrome in traditional Chinese or folk medicine.[11,12] Among the tested extracts, an EtOH extract of *Cuscuta chinensis* Lam.

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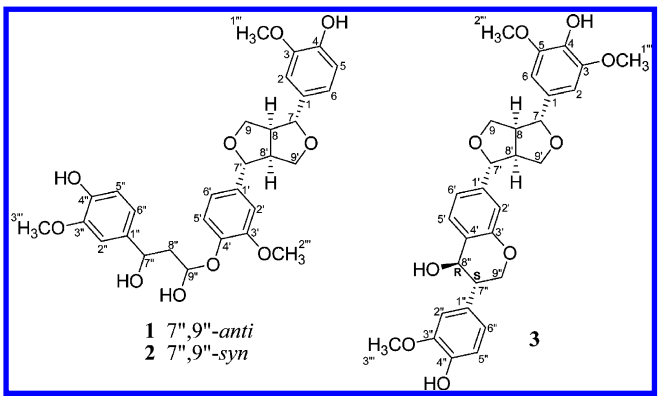
(Convolvulaceae) seeds showed estrogenic activity and was chosen for further investigation.

C. chinensis seeds, a well-known traditional Chinese medicine (TCM), are used for tonifing the liver and kidneys, improving eyesight, invigorating yang, and reducing urination. The plant material is also prescribed by gynecological TCM practitioners to prevent abortion.[10] The *Cuscuta* genus was the subject of several phytochemical studies revealing its richness in flavonoids: kaempferol, quercetin, and isorhamnetin;[13−16] benzenoids: coumaric acid, arbutin, caffeoylquinic acid, and swarnalin;[15,17−20] steroids: stigmasterol and β-sitosterol;[14,15] lignans: pinoresinol, epipinoresinol, and cuscutosides A and B;[14,15] and coumarins and alkaloids.[13,18,20] The species belonging to this genus were reported to have antibacterial,[21] antihypertensive,[19] antioxidative,[20] immune modulatory,[22] antidiabetic,[18] neuroprotective, and hepatoprotective activities.[23,24]

The EtOH extract of *C. chinensis* seeds exhibited estrogenic activity at a minimum active concentration (MAC) of 100 μg/mL in the pER8:GUS reporter assay system and was subsequently partitioned into different solvents.[11,25] The extract was partitioned between H_2O and EtOAc, providing two fractions. The H_2O fraction was inactive (>200 μg/mL), but the EtOAc fraction showed estrogenic activity (100 μg/mL) and was further fractionated between 75% aqueous MeOH (MeOH/H_2O = 75:25) and *n*-hexane. The aqueous MeOH fraction showed significant estrogenic activity (50 μg/mL) and was purified utilizing bioactivity-guided fractionation, resulting in the isolation of three new and 16 known compounds. The structures of the isolates were established by spectroscopic analysis and comparison with reported physical data. The estrogenic activity was evaluated by the pER8:GUS reporter assay system.[11,12,25]



RESULTS AND DISCUSSION

Compounds **1** and **2** were separable utilizing recycle HPLC equipped with a reversed-phase silica gel column (C_{18}) (retention time t_R: 16.03 min for **1** and 17.19 min for **2** in the first run, Thermo Hypersil column, 250 × 10 mm eluted with MeOH/H_2O = 50:50, flow rate 2 mL/min, UV detection at λ 254 nm, Figure 1).

Compound **1** was isolated as an amorphous powder. Its molecular formula was calculated as $C_{30}H_{34}O_{10}$ from analysis of its HRESIMS data (*m/z* 577.2047 $[M + Na]^+$, calcd for 577.2050), corresponding to 14 degrees of unsaturation. The IR spectrum indicated the presence of hydroxy (3417 cm^{-1}) and aromatic (1603, 1514 cm^{-1}) functionlities. In ^{13}C and DEPT NMR experiments, three methoxy groups and three methylene, 15 methine (six sp^3 and nine sp^2 carbons), and nine aromatic quaternary carbons were observed. As shown in Table 1, the ^1H and ^{13}C NMR data revealed that **1** contained three 1,3,4-trisubstituted aromatic rings, which were characterized by three ABX spin systems: δ_H 6.77 (d, J = 8.4 Hz, H-5), 6.81 (dd, J = 8.4, 1.8 Hz, H-6), and 6.95 (d, J = 1.8 Hz, H-2); δ_H 7.03 (d, J = 8.4 Hz, H-5′), 6.87 (dd, J = 8.4, 1.8 Hz, H-6′), and 7.01 (d, J = 1.8 Hz, H-2′); δ_H 6.74 (d, J = 8.4 Hz, H-5″), 6.84 (dd, J = 8.4, 1.8 Hz, H-6″), and 7.02 (d, J = 1.8 Hz, H-2″). Together with the COSY data (Figure 2), a hexahydrofuro[3,4-*c*]furan system was confirmed by the HMBC correlations from H-7 (δ_H 4.71) to C-9′ (δ_C 72.7) and H-7′ (δ_H 4.75) to C-9 (δ_C 72.6).[26,27] The connection of the hexahydrofuro[3,4-*c*]furan moiety to the two phenolic moieties was verified by the correlations of H-7/C-2, C-6 (δ_H 4.71/δ_C 111.0, 120.1), as well as H-7′/C-2′, C-6′ (δ_H 4.75/δ_C 111.5, 119.9) (Figure 2). Compared to the NMR data of (+)-de-4′-*O*-methyleudesmin, the partial structure of **1** was established as a 7,9′-7′,9-diepoxylignan.[28,29] The NMR data also suggested the presence of an oxymethine (δ_H 4.87, δ_C 74.0, CH-7″), a methylene (δ_H 3.48 and 3.73, δ_C 61.9, CH_2-8″), and an unusual hemiacetal (δ_H 4.29, δ_C 87.3, CH-9″) moiety.[30] Except for the methoxy groups, the observed nine carbons in the ^{13}C NMR data indicated the presence of a $C_6{\cdot}C_3$ phenylpropanoid moiety. A 1,3-propandiol moiety was confirmed by the COSY cross-peak between H-7″ (δ_H 4.87) and H-9″ (δ_H 4.29) and the HMBC correlation from H-9″ (δ_H 4.29) to C-7″ (δ_C 74.0). The HMBC correlations of H-7″/C-2″, C-6″ (δ_H 4.87/δ_C 111.7, 120.7) suggested that the aromatic ring is attached to the 1,3-propandiol moiety at C-7″. Additionally, the cross-peak between H-9″ (δ_H 4.29) and C-4′ (δ_C 149.2) implied that the phenylpropanoid moiety is connected to C-4′ by an ether bridge, forming a hemiacetal functionality (Figure 2). Furthermore, three methoxy groups were assigned at C-3, 3′, and 3″ based on HMBC and NOESY correlations (Figures 2 and 3). The configuration of **1** was determined by comparing ECD (electronic circular dichroism) data (Cotton effects at 211, 221, 232, and 282 nm) with that of (+)-pinoresinol,[31,32] which agreed with the assignment of the 7*R*,8*S*,7′*R*,8′*S* absolute configuration for the 7,9′-7′,9-diepoxylignan moiety. Significant NOESY correlations between H-7″ (δ_H 4.87) and H-9″ (δ_H 4.29) and between H-5′ (δ_H 6.74)/H-6″ (δ_H 6.84) and H-6′ (δ_H 6.87)/H-5″ (δ_H 7.03) (Figure 3) indicated that H-7″ and H-9″ have the same orientation, implying that the two hydroxy groups may possess an intramolecular hydrogen bonding. Thus, the 1,3-configuration of the propanediol moiety was proposed to be 7″,9″-*R*,*R* or 7″,9″-*S*,*S* (*syn* configuration as shown in Figure 4).[33] Compound **1** was named cuscutaresinol A.

Compound **2** was assigned the molecular formula $C_{30}H_{34}O_{10}$ by HRESIMS (*m/z* 577.2048 $[M + Na]^+$, calcd for 577.2050), corresponding also to 14 degrees of unsaturation as in **1**. The NMR data of **2** were similar to **1** (Table 1), indicating that **2** also possessed a 7,9′-7′,9-diepoxylignan moiety, a phenylpropanoid moiety, and three methoxy groups. The molecular structure of **2** was elucidated in a similar way to **1** by a combination of COSY, HSQC, HMBC, and NOESY correlations (Figures 2 and 3). The ECD data of **2** indicated the 7*R*,8*S*,7′*R*,8′*S* absolute configuration for the 7,9′-7′,9-diepoxylignan moiety. The only difference between **1** and **2** was observed in the NOESY spectra, where the cross-peak between H-7″ (δ_H 4.80) and H-9″ (δ_H 4.36) was absent. Therefore, the configuration at C-7″ and C-9″ in **2** was proposed to be *R*,*S* or *S*,*R* (Figure 4, *anti* configuration).[33] Compound **2** was named cuscutaresinol B.

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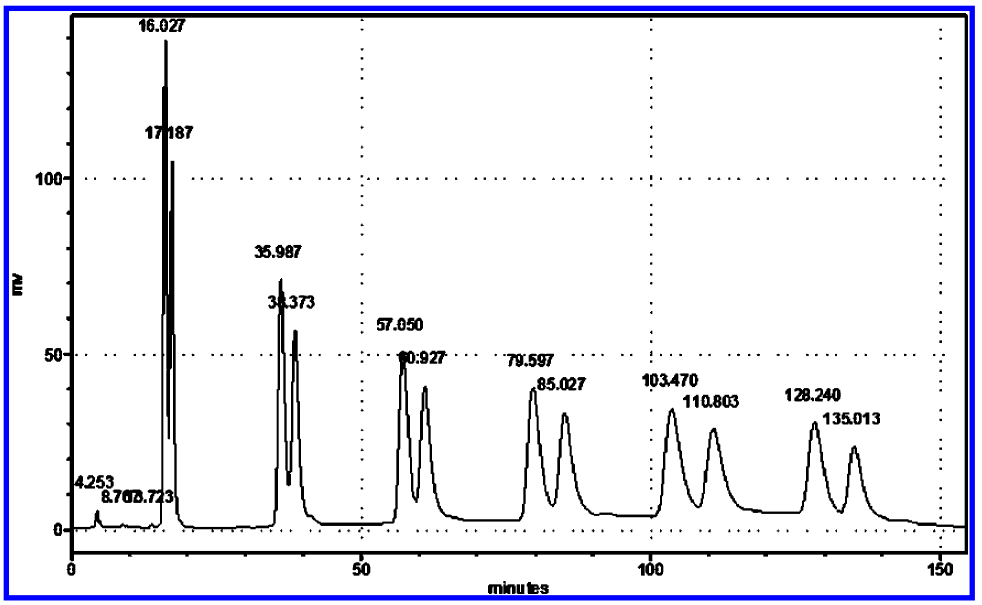

Figure 1. Isolation of compounds **1** and **2** by recycle HPLC (retention time (t_R): **1**, 16.03 min; **2**, 17.19 min in the first run).

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectroscopic Data for Compounds 1−3 in Methanol-d_4 (δ in ppm, J in Hz)

	1		2		3	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	133.7, C		133.8, C		135.9, C	
2	111.0, CH	6.95, d (1.8)	111.0, CH	6.95, d (1.8)	112.1, CH	6.91, d (3.0)[a]
3	149.1, C		149.1, C		145.5, C	
4	147.3, C		147.4, C		130.2, C[a]	
5	116.1, CH	6.77, d (8.4)	116.1, CH	6.77, d (8.4)	149.0, C	
6	120.1, CH	6.81, dd (8.4, 1.8)	120.1, CH	6.80, dd (8.4, 1.8)[a]	116.1, CH	6.91, d (3.0)[a]
7	87.5, CH	4.71, d (4.2)	87.5, CH	4.71, d (4.2)	87.5, CH	4.72, d (4.2)
8	55.5, CH	3.14, m	55.5, CH	3.12, m	55.6, CH	3.17, m
9	72.6, CH_2	α 4.25, m	72.6, CH_2	α 4.23, m	72.7, CH_2	α 4.25, m
		β 3.86, m		β 3.85, m		β 3.84, m
1′	136.9, C		136.7, C		133.8, C	
2′	111.5, CH	7.01, d (1.8)	111.4, CH	6.94, d (2.4)	111.0, CH	6.95, d (1.8)
3′	148.8, C		152.0, C		147.6, C	
4′	149.2, C		148.8, C		130.2, C[a]	
5′	118.9, CH	7.03, d (8.4)	118.9, CH	6.89, d (8.4)	116.0, CH	6.77, d (8.4)
6′	119.9, CH	6.87, dd (8.4, 1.8)	119.7, CH	6.82, dd (8.4, 2.4)[a]	121.1, CH	6.82, dd (8.4, 1.8)[a]
7′	87.2, CH	4.75, d (4.2)	87.2, CH	4.72, d (4.2)	87.7, CH	4.74, d (7.2)
8′	55.4, CH	3.14, m	55.3, CH	3.12, m	55.4, CH	3.17, m
9′	72.7, CH_2	α 4.25, m	72.7, CH_2	α 4.23, m	72.6, CH_2	α 4.25, m
		β 3.86, m		β 3.85, m		β 3.84, m
1″	133.8, C		134.1, C		134.6, C	
2″	111.7, CH	7.02, d (1.8)	111.8, CH	6.98, d (1.8)	110.5, CH	6.94, d (1.8)
3″	151.8, C		148.6, C		149.1, C	
4″	147.2, C		147.0, C		147.3, C	
5″	115.8, CH	6.74, d (8.4)	115.6, CH	6.70, d (8.4)	115.9, CH	6.76, d (8.4)
6″	120.7, CH	6.84, dd (8.4, 1.8)	121.1, CH	6.81, d (8.4, 1.8)[a]	119.7, CH	6.81, dd (8.4, 1.8)[a]
7″	74.0, CH	4.87, m	74.1, CH	4.80, m	55.5, CH	3.50, m
8″	61.9, CH_2	a 3.73, dd (11.4, 4.2)	62.4, CH_2	a 3.85, m	89.2, CH	5.53, d (6.0)
		b 3.48, m		b 3.80, m		
9″	87.3 CH	4.29, m	86.3, CH	4.36, m	64.9, CH_2	α 3.78, m
						β 3.84, m
1‴	56.4 CH_3	3.85, s (3H)	56.5, CH_3	3.86, s (3H)	56.8, CH_3	3.88, s (3H)
2‴	56.3 CH_3	3.81, s (3H)	56.4, CH_3	3.79, s (3H)	56.3, CH_3	3.82, s (3H)
3‴	56.6 CH_3	3.88, s (3H)	56.3, CH_3	3.78, s (3H)	56.4, CH_3	3.86, s (3H)

[a]Assignments may be interchanged.

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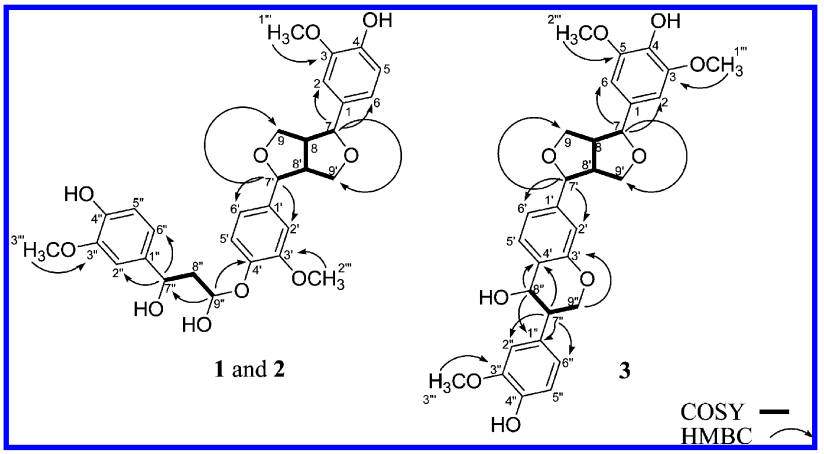

Figure 2. Key COSY (bold lines) and HMBC correlations (H→C) of compounds **1**–**3**.

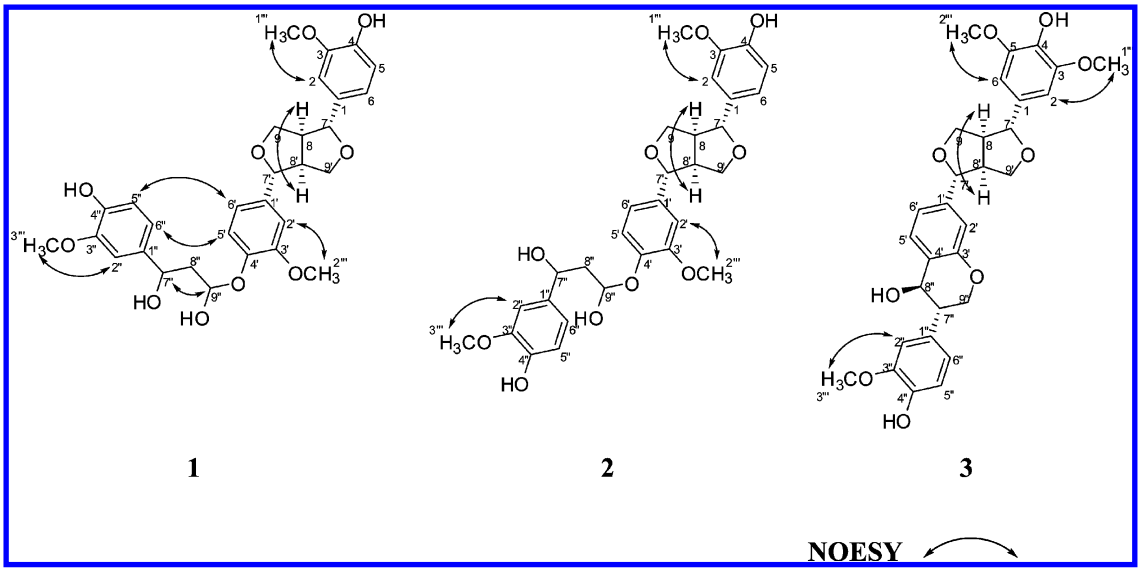

Figure 3. Key NOESY correlations of compounds **1**–**3**.

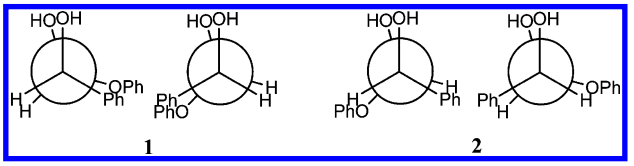

Figure 4. Newman projection showing the relative configuration of **1** and **2**.

The molecular formula of **3** was calculated as $C_{30}H_{32}O_9$ by analysis of its HRESIMS data (*m*/*z* 559.1941 $[M + Na]^+$, calcd for 559.1944), corresponding to 15 degrees of unsaturation. The IR spectrum indicated the presence of hydroxy (3409 cm^{-1}) and aromatic (1604, 1516 cm^{-1}) functionalities. Three methoxy groups and three methylene, 14 methine (six sp^3 carbons and eight sp^2 carbons), and 10 quaternary aromatic carbons were observed in the ^{13}C NMR and DEPT spectra. A hexahydrofuro[3,4-*c*]furan system connected to two phenolic rings was proposed by comparison of COSY and HMBC data of **3** with those of **1** (Figure 2), suggesting a 7,9′-7′,9-diepoxylignan moiety.[27] One methine (δ_H 3.50, CH-7″), one oxymethine (δ_H 5.53, CH-8″), and one oxymethylene (δ_H 3.78 and 3.84, CH_2-9″) were connected on the basis of COSY correlations. The HMBC correlations of H-7″/C-1″, C-2″, and C-6″ (δ_H 3.50/δ_C 134.6, 110.5, and 119.7) indicated the aromatic ring connection at C-7″. The cross-peaks from H-7″ (δ_H 3.50) to C-4′ (δ_C 130.2), H-8″ (δ_H 5.53) to C-4′ (δ_C 130.2), and H_2-9″ (δ_H 3.78 and 3.84) to C-3′ (δ_C 147.6) implied a cyclic structure involving C-3′ and C-4′ to form a 4-chromanol moiety.[34] Furthermore, three methoxy groups were assigned at C-3, C-5, and C-3″ using HMBC and NOESY correlations (Figures 2 and 3). The configuration of the 7,9′-7′,9-diepoxylignan skeleton in **3** was assigned as 7*R*,8*S*,7′*R*,8′*S* by comparing with the ECD data of (+)-pinoresinol.[31,32] The NOESY correlation between H-7″ (δ_H 3.50) and H-8″ (δ_H 5.53) was absent. Additionally, comparing the ^1H NMR data and coupling constants of *cis*- and *trans*-isoflavan-4-ols, the $J_{7'',8''}$ value of 6.0 Hz of the 4-chromanol moiety suggested a *trans* configuration.[35] Compound **3** exhibited a significant positive Cotton effect at 282 nm, suggesting the absolute configuration of the 4-chromanol moiety to be 7″*S*,8″*R*.[35] Compound **3** was named cuscutaresinol C.

In addition to the three new 7,9′-7′,9-diepoxylignans (**1**–**3**), 16 known compounds, (+)-sesamin (**4**),[36,37] (+)-xanthoxylol (**5**),[38] 9-hydroxysesamin (**6**),[39,40] (+)-pinoresinol (**7**),[38,41] kaempferol (**8**),[42] isorhamnetin (**9**),[15] (+)-aptosimon,[15,26] (+)-5′-hydroxylpinoresinol,[43] (4-dimethylaminophenyl)(4′-methylaminophenyl)methanone,[44,45] bis(4-

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dimethylaminophenyl)methanone,[44,46] methyl 4-hydroxybenzoate,[47] methyl *p*-hydroxybenzoylformate,[48] 4-methoxybenzoic acid,[47] vanillin,[49] and a mixture of *β*-sitosteryl-*β*-D-glucoside and stigmasteryl-*β*-D-glucoside,[50] were also isolated from *C. chinensis*. This is the first report of diepoxylignan derivatives [(+)-xanthoxylol, 9-hydroxysesamin, (+)-aptosimon, and (+)-5′-hydroxylpinoresinol], diphenylmethanones [(4-dimethylaminophenyl)(4'- methylaminophenyl)methanone and bis(4-dimethylaminophenyl)methanone], and a benzoylformate [methyl *p*-hydroxybenzoylformate] isolated from *Cuscuta* genus.

The estrogenic activity of the isolates was assessed in the pER8:GUS reporter assay system.[11,12,25] The reference compound, 17*β*-estradiol, was used as a positive control for estrogenic activity tests. Compounds **1**−**9** exhibited estrogenic activity, and their results are presented in Table 2. Kaempferol

Table 2. Estrogenic Activity of Compounds 1−9 in the *Arabidopsis* pER8:GUS Reporter Assay System

compound	MAC[a]
1	≥200.0
2	≥200.0
3	≥200.0
4	≥200.0
5	≥200.0
6	≥200.0
7	100.0
8	37.5
9	9.4
17*β*-estradiol[b]	1.0

[a]MAC: minimum active concentration (*μ*g/mL). [b]Positive control, MAC in nM.

(**8**) and isorhamnetin (**9**) showed estrogenic activity with MAC values of 37.5 and 9.4 *μ*g/mL, respectively. Kaempferol (**8**) (2.43%) and isorhamnetin (**9**) (0.09%) represented the major components of the 75% aqueous MeOH layer. The presence of these components can be considered as biomarkers for *C. chinensis* estrogenic activity, emphasizing the potential use of this herb as a source of phytoestrogens. Interestingly, (+)-pinoresinol (**7**) displayed estrogenic activity at higher concentrations (≥100 *μ*g/mL). The estrogenic activity of **7** supported previous reports that mammalian lignans (enterodiol and enterolactone), well-known phytoestrogens, are formed from plant-lignan glycoside precursors, pinoresinol, lariciresinol, syringaresinol, 7-hydroxymatairesinol, and arctigenin, by the activity of the gut microflora in the proximal colon.[9]

In conclusion, three new 7,9′-7′,9-diepoxylignans together with 16 known compounds were identified from *C. chinensis* seeds, and their estrogenic activities were tested. These findings represent an addition to the ongoing research on phytoestrogens, which should continue to clarify their actual health benefits.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 digital polarimeter (JASCO Inc., Tokyo, Japan). The IR spectra were measured on a Mattson Genesis II TM FT-IR spectrophotometer (Mattson Instruments, Madison, WI, USA). ^{1}H and ^{13}C NMR spectra were recorded on Varian VNMRS 600 MHz FT-NMR, Varian Unity-plus 400 MHz FT-NMR, or Varian Gemini-2000 200 MHz FT-NMR spectrometers (Varian Inc., Palo Alto, CA, USA). Chemical shifts are reported in parts per million (*δ*), and coupling constants (*J*) are expressed in hertz. LRESIMS was measured on a Finnigan POLARISQ mass spectrometer (Thermo Finnigan, Austin, TX, USA). HRESIMS were measured on a Bruker Daltonics APEX II 30e mass spectrometer (Bruker Instruments, Billerica, MA, USA). Silica gel (Kieselgel 60, 70−230 and 230−400 mesh, Merck KGaA, Darmstadt, Germany) and Sephadex LH-20 gel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were used for column chromatography. TLC was carried out using Si gel (Kieselgel 60, F_{254}, Merck KGaA, Darmstadt, Germany) and RP-18 (F_{254s}, Merck KGaA, Darmstadt, Germany) precoated plates, and compounds were detected with 50% H_2SO_4 followed by heating on a hot plate. HPLC analyses were performed with a Shimadzu LC-10AT (Shimadzu Inc., Kyoto, Japan) pump interface equipped with a Shimadzu SPD-10A UV/vis detector using ODS (Thermo Hypersil, 250 × 4 mm; Thermo Hypersil, 250 × 10 mm, Thermo Fisher Scientific Inc., Rockford, IL, USA) columns.

Plant Material. The dried seeds of *Cuscuta chinensis* Lam. were collected from Taichung County, Taiwan, in July 2008, and were identified by a specialist in Chinese herbal medicine, Dr. Ming-Hong Yen. A voucher specimen (CC0001) was deposited at the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Isolation. The dried and powdered seeds of *C. chinensis* (5.4 kg) were extracted with 95% EtOH (4 × 10 L) at room temperature for 24 h. The solvent was removed under reduced pressure to yield a dried EtOH extract (339.2 g). This residue was partitioned between EtOAc and H_2O. The EtOAc layer was further partitioned into *n*-hexane and 75% aqueous MeOH. The aqueous MeOH layer (59.9 g) was subjected to CC using Si gel as the stationary phase and eluted successively with *n*-hexane/CH_2Cl_2 (90:10 to 0:100) and CH_2Cl_2/MeOH (100:0 to 80:20) to afford 21 fractions. Fraction 9 (2587 mg) was subjected to Si gel CC (70−230 mesh) and eluted with CH_2Cl_2/MeOH (97.5:2.5) to yield eight fractions. Fraction 9-2 (88 mg) was purified on Si gel and reversed-phase open-column chromatography to yield (+)-aptosimon (8 mg). Fraction 9-3 (149 mg) was chromatographed on Si gel (230−400 mesh) to give **4** (12 mg). Fraction 9-5 (988 mg) was subjected to CC in the following order: Si gel, Sephadex LH-20, and HPLC to afford **5** (12 mg), **6** (72 mg), **7** (152 mg), (4-dimethylaminophenyl)(4′-methylaminophenyl)methanone (3 mg), bis(4-dimethylaminophenyl)-methanone (19 mg), and vanillin (1 mg). Fraction 11 (217 mg) was subjected to Si gel CC (230−400 mesh) and eluted with CH_2Cl_2/MeOH (97:3) to yield five fractions. Fraction 11-3 (69 mg) was chromatographed on Si gel (230−400 mesh) and HPLC (Thermo Hypersil, 250 × 10 mm, MeOH/H_2O = 60:40, detector *λ*: 254 nm) to give 4-methoxybenzoic acid (2 mg). Fraction 12 (338 mg) was subjected to Si gel CC (230−400 mesh) and eluted with CH_2Cl_2/MeOH (96.5:3.5) to yield five fractions and a precipitate, **9** (52 mg). Fraction 12-2 (90 mg) was chromatographed on Sephadex LH-20 and HPLC (Thermo Hypersil, 250 × 10 mm, MeOH/H_2O = 65:35, detector *λ*: 254 nm) to afford **3** (2 mg) and (+)-5′-hydroxylpinoresinol (2 mg). Fractions 15−19 were subjected separately to Si gel CC (230−400 mesh) and eluted with CH_2Cl_2/MeOH (95:5), yielding compound **8** (total weight collected 1458 mg). Fraction 15-2 (59 mg) was chromatographed on reversed-phase open-column chromatography and HPLC (Thermo Hypersil, 250 × 10 mm, MeCN/H_2O = 50:50, detector *λ*: 254 nm) to afford methyl 4-hydroxybenzoate (5 mg) and methyl *p*-hydroxybenzoylformate (7 mg). Fraction 15-4 (475 mg) was subjected to CC in the following order: Si gel, Sephadex LH-20, and recycle HPLC (Thermo Hypersil, 250 × 10 mm, MeOH/H_2O = 50:50, HPLC-Jasco PU-1580 pump, detector *λ*: 254 nm) to give **1** (2 mg) and **2** (3 mg). A mixture of *β*-sitosteryl-*β*-D-glucoside and stigmasteryl-*β*-D-glucoside (51 mg) was collected as a precipitate from fraction 20 (480 mg).

Cuscutaresinol A (1): white, amorphous powder; $[\alpha]_D^{25}$ +24.3 (*c* 0.22, MeOH); UV (MeOH) λ_{max} (log *ε*) 231 (4.39), 279 (3.87) nm; CD (MeOH) $[\theta]_{214}$ −1.00, $[\theta]_{229}$ −3.84, $[\theta]_{248}$ −1.71, $[\theta]_{269}$ +1.69, $[\theta]_{281}$ −0.40, $[\theta]_{289}$ −0.96; IR (neat) ν_{max} 3417, 2936, 2851, 1603, 1514 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 577 [M +

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Na]$^+$; HRESIMS m/z 577.2047 [M + Na]$^+$ (calcd for $C_{30}H_{34}O_{10}Na$, 577.2050).

Cuscutaresinol B (2): white, amorphous powder; $[\alpha]_D^{25}$ +40.0 (c 0.31, MeOH); UV (MeOH) λ_{max} (log ε) 231 (4.30), 279 (3.82) nm; CD (MeOH) $[\theta]_{213}$ −0.77, $[\theta]_{227}$ −1.04, $[\theta]_{250}$ −4.72, $[\theta]_{266}$ +0.55, $[\theta]_{285}$ +0.42, $[\theta]_{299}$ −2.81; IR (neat) ν_{max} 3417, 2939, 2865, 1604, 1515 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS m/z 577 [M + Na]$^+$; HRESIMS m/z 577.2048 [M + Na]$^+$ (calcd for $C_{30}H_{34}O_{10}Na$, 577.2050).

Cuscutaresinol C (3): white, amorphous powder; $[\alpha]_D^{25}$ +8.9 (c 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 234 (4.16), 280 (3.68) nm; CD (MeOH) $[\theta]_{217}$ −2.15, $[\theta]_{236}$ −0.78, $[\theta]_{246}$ −1.09, $[\theta]_{252}$ +0.57, $[\theta]_{282}$ +1.99; IR (neat) ν_{max} 3409, 2929, 2875, 1604, 1516 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS m/z 559 [M + Na]$^+$; HRESIMS m/z 559.1941 [M + Na]$^+$ (calcd for $C_{30}H_{34}O_{10}$ Na, 559.1944).

Estrogenic Activity Assay. The *Arabidopsis* pER8:GUS reporter assay system was originally developed by Brand et al.[12] The seeds of *Arabidopsis* pER8:GUS were sown and grown on MS solid medium (3% sucrose, 0.9% agar) in the dark for 24 to 36 h at 4 °C for vernalization and then left at 24 °C for three days under continuous light. The seedlings were transferred into 24-well microtiter plates containing MS liquid medium with or without the tested compounds in each well and incubated at 24 °C for 48 h. The culture medium was removed; then staining buffer [50 mM Na_3PO_4 buffer (pH 7.0), 10 mM EDTA (pH 8.0), 0.2 mM X-Gluc, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 0.1% Triton X-100, Sigma-Aldrich Corp., St. Louis, MO, USA] was added for GUS reaction at 37 °C for 3 h.[24] The reference compound, 17β-estradiol (Wako Inc., Tokyo, Japan), was used as a positive control (0.31−10 nM). A ZEISS Axiovert 200 inverted microscope (Carl Zeiss, Oberkochen, Germany) was used to examine GUS staining, and images were captured with the microscope digital camera.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR, COSY, HSQC, HMBC, and NOESY spectra of **1**−**3** are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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