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New estrogenic compounds isolated from *Broussonetia kazinoki*

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ABSTRACT

Two new and two known compounds were identified as estrogenic constituents from *Broussonetia kazinoki*. Their structures were elucidated as broussonin A (**1**), tupichinol C (**2**), kazinol U (**3**), and (+)-(2*R*) kazinol I (**4**). They showed estrogenic activity with ligand-binding activity of estrogen receptor, transcriptional activity of estrogen-responsive element-luciferase reporter genes. They also control the cellular gene expression levels of estrogen-responsive genes. Phytoestrogens from *B. kazinoki* may have beneficial effects in the treatment of menopausal symptoms.

Estrogens have diverse biological actions including proliferation and differentiation of a number of cells.[1] Biological activities of estrogen are mediated through two distinct and functional estrogen receptors (ERs), ER-α and ER-β, which are members of a large superfamily of nuclear receptors.[2] The mechanism of ER action involves estrogen binding to intracellular ERs followed by dimerization of receptor and binding to specific estrogen response elements (EREs) located in the promoters of target genes. Two ERs exhibit distinct tissue distribution patterns, different ligand-binding ability and transactivational properties. This difference could contribute to the selective action of ER agonists and antagonists in different tissues.[3] ER has been a pharmaceutical target for hormonal therapy (HT) in menopausal women and for chemotherapeutic drugs against reproductive cancers. HT can restore estrogen levels but increases the women's risk of heart disease, dementia and breast cancer.[4] ER-α has been known to cause proliferation of breast cancer cells, while ER-β has been demonstrated to be a tumor suppressor.[5]

Among the compounds that can bind to ERs, selective ER modulators (SERMs) have the ability to act as agonists or antagonists depending on the cellular context and ER isoforms involved.[6] The well known SERMs, raloxifene and tamoxifen are used clinically in the treatment of osteoporosis and estrogen dependent breast cancer. Phytoestrogens are plant derived compounds that can mimic or modulate the actions of endogenously produced estrogens by binding to ERs.[7] These groups of compounds include flavonoids, stilbenes and lignans. The structural features of them confer ability to bind ERs and can exert estrogenic or anti-estrogenic activity. Most of the phytoestrogens used in HT activate both subtypes of ERs. Phytoestrogens that selectively activate ER-α or ER-β might exert some of the beneficial effects, avoiding the adverse side effects. The approach to discover ER subtype selective estrogens from plant extracts would be a good strategy for finding good HT regimens. The purpose of this study was to find new phytoestrogen from medicinal plants and evaluate their subtype selectivity of ERs.

We have screened estrogenic activity of plant extracts and identified four phytoestrogenic substances from *Broussonetia kazinoki* Sieb (Moraceae). In Chinese folk medicine *B. kazinoki* has been used as diuretic, tonic, and suppressant for edema. Several prenylated flavonols, flavanes and diphenyl propanes were reported, and their cytotoxic, antioxidative, anti-inflammatory, and tyrosinase inhibitory activities were evaluated.[8–11] The ethyl acetate soluble fractions of *B. kazinoki* were repeatedly subjected to open column chromatography using silica gel and reverse phase MPLC. Final purifications were performed by reverse phase semi-preparative HPLC to give active compounds **1**–**4**.[12] Compounds **1** and **2** were identified as broussonin A and tupichinol by the analysis of their spectroscopic data and comparison with literature values.[13,14] Compounds **3** and **4** were identified as new isoprenylated flavans.

Compound **3** was obtained as oily substance and the HREIMS showed the $[M]^+$ ion at *m/z* 326.1516 (calcd 326.1518) that corresponded to a molecular formula $C_{20}H_{22}O_4$. The infrared spectrum of **3** suggested the presence of hydroxyl group (3393 cm^{-1}) and aromatic ring (1618 and 1457 cm^{-1}). Its ^1H NMR spectrum showed typical signals of flavan skeleton at δ 1.80–2.10 (2H, m, H-3), 2.50–2.90 (2H, m, H-4), 5.00 (1H, dd, J = 10.4, 2.0 Hz, H-2) and five aromatic protons. One ABX spin system [δ 6.25 (d, J = 2.4, H-8), 6.32

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(dd, J = 8.4, 2.4 Hz, H-6), 6.84 (d, J = 8.4 Hz, H-5)] in ring A and one AB spin system [δ 6.69 (d, J = 8.4 Hz, H-5′), 6.79 (d, J = 8.4 Hz, H-6′)] in ring B were found in ^{1}H-^{1}H COSY. The ^{1}H NMR spectrum of **3** also showed the presence of one isoprenyl group [δ 1.64 and 1.68 (3H each, s, H_3-4″ and H_3-5″), δ 3.43 (2H, m, H_3-1″), 5.13 (1H, br t, H-2″)]. The position of isoprenyl group was assigned to C-2′ from the correlation between 2-H (δ 5.00) and C-2′ (δ 127.5), H-2″ (δ 5.13) and C-2′ (δ 127.5) in HMBC. Two coupled aromatic protons [δ 6.69, 6.79] and two methine carbons [δ 113.5, δ 118.3] in ring B were identified from HSQC spectrum. The correlation between H-2 and C-6′, H-6′ and C-2′ in HMBC also confirmed the structure of **3**. The stereochemistry of C-2 was identified as (*R*) according to the literature that deduced the (*R*)-configuration from the sign of positive [α] value.[14,15] Compound **3** was confirmed as a novel structure named kazinol U.

Compound **4** was purified from the ethyl acetate soluble fraction of *B. kazinoki* as oily substance. The molecular formular $C_{25}H_{30}O_4$ was deduced from HREIMS $[M]^+$ at *m/z* 394.2142 (calcd 394.2144). From the overall pattern of NMR, we assumed **4** as an analogue structure of compound **3** with one more isoprenyl group in ring B. The two isoprenyl groups were located at C-5′ and C-6′ from the analysis of HMBC spectrum (Fig. 1B HMBC correlations). In the HMBC spectrum, the signal of H-2 (δ 4.83) correlated with three aromatic carbon signals of C-2′ (δ 111.7), C-6′ (δ 131.0) and C-1′ (δ 132.1). A singlet aromatic proton at δ 6.63 (H-2′) correlated with C-2 (δ 76.3), 4′ (δ 143.9) and C-6′ (δ 131.0). An allylic methylene signal of H_2-1‴ (δ 3.15) correlated with C-1′ (δ 132.1) and C-5′ (δ 128.3). In the same way, H_2-1″ correlated with C-4′ and C-6′. These correlations indicated the presence of isoprenyl groups at C-5′ and C-6′. From the positive value of [α], the structure of compound **4** were determined as a new structure, (+)-(2*R*)-kazinol I. The C-2 epimer of **4**, (−)-(2*S*)-kazinol I was reported obtainable from isoprenylated 1,3-diphenylpropan (kazinol F) by treatment of 2,3-dichloro-5,6-dicyanobenzoquinone through the ring closure.[16]

For the activity-guided purification of ER ligands from plant extracts, we evaluated the ligand activity of fractions with a competitive binding assay (Invitrogen Corp., Carlsbad, CA) using 3 nM [^{3}H]17β-estradiol (E_2) and human recombinant full-length hER-α and hER-β.[17] As summarized in Table 1, four compounds from *B. kazinoki* showed moderate binding affinity to hER-α and hER-β. The relative binding affinity (RBA) for hER-α and hER-β was calculated as the relative affinity to E_2. Compounds **1**–**4** from *B. kazinoki* showed higher affinity to hER-β than hER-α. The IC_{50} values of the most potent **1** was 8.1 and 1.8 μM for hER-α and hER-β, respectively. Compound **1** displayed 5.71-fold selectivity for ER-β over ER-α. We also evaluated transactivation potential of compounds by using luciferase reporter gene assay by transfection of CV-1 cells with plasmids containing ERs expression vector and ERE-driven reporter.[18] Compound **1** showed dose dependent transcriptional activity of ERE with EC_{50} value of 1.59 and 0.53 μM for ER-α and ER-β, respectively (Fig. 2). The transactivation activity of **1** was suppressed by ER antagonist, ICI 182780 indicating that the estrogenic activity of **1** is ER dependent. 4,4′,4″(4-Prophy-[1*H*]-pyrazole-1,3,5-triyl) trisphenol (PPT) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DNP) were used as control ligands those are specific for the respective ER-α and ER-β (Fig. 3).

The most potent compound **1** is obtainable from the ring opening of compound 2 and the compounds **2**–**4** have (*R*) configuration at C-2. At this moment, we do not infer the structural requirement for the ER ligand activity with very limited structures those were obtained from the activity-guided purification procedures. The structure–activity relationship of isoflavanones and flavonols for the ER binding was discussed with respect to the position of phenol ring and hydroxy groups, the substitution of hydroxy or methoxy groups, the opening of the phenol ring.[19,20] But the reports on the estrogenic activity of flavans were very limited. When we have structurally diverse flavans, we can discuss and optimize the type selectivity of ER ligands.

For the further characterization of compounds **1**–**4** as functional ER ligands, their effects on the ER protein levels were examined in MCF-7 breast cancer cells.[21] It is well known that E_2 induces the down-regulation of ER as early as 30 min of treatment in E_2-responsive cells. The treatment of compounds **1**–**4** (10 μM) down-regulated the level of ER-α protein as compared with control. To characterize the effects of **1**–**4** at the cellular gene expression level, their effects on known E_2-regulated ER, PR (progesterone receptor) and pS2 genes were examined. After treatment of MCF-7 cells with the compounds for 24 h, steady-state mRNA levels were measured by qPCR. Constitutively expressed human β-actin mRNA was used as an internal control. Compounds **1**–**4** (10 μM) increased mRNA levels of the PR and pS2 genes and decreased ER-α mRNA level as compared with control. These data indicate that compounds **1**–**4** can regulate the E_2-responsive genes as functional ER ligands like E_2 in ER-sensitive MCF-7 cells.

The new ER ligands from *B. kazinoki* can be developed as potential drugs to treat menopausal symptoms. Their selective binding affinity to ER-β over ER-α might have a safer profile than the estrogens currently used in HT that activate both ERs. Future studies are

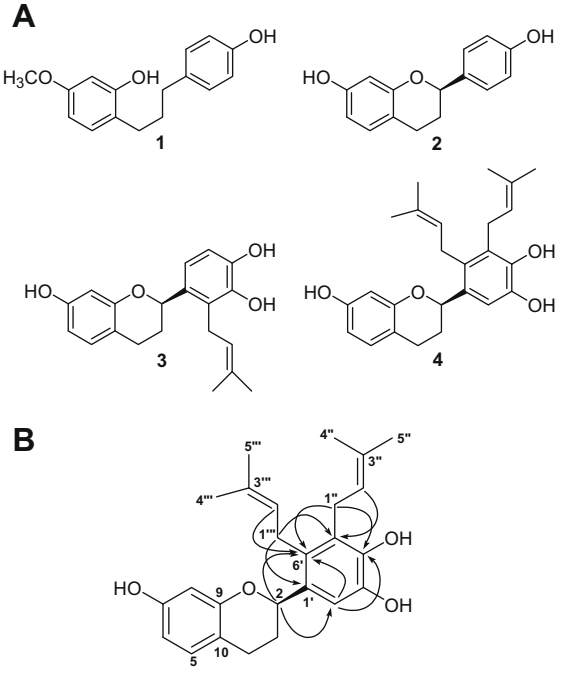

Figure 1. Chemical structures of estrogen receptor ligands from *B. kazinoki* (A) and the selected correlations in HMBC spectra of **4** (B).

Table 1
Ligand-binding affinity and ER selectivity of compounds **1**–**4** for recombinant human ER-α and ER-β

Compds	IC_{50}[a] (μM)		RBA[b] (%)		Selectivity (β/α)
	hER-α	hER-β	hER-α	hER-β	
1	8.1 (±0.8)	1.8 (±0.3)	0.069	0.394	5.71
2	26.2 (±0.3)	14.7 (±0.4)	0.021	0.048	2.29
3	57.2 (±2.3)	41.7 (±2.7)	0.010	0.017	1.70
4	151.1 (±11.1)	96.0 (±10.5)	0.004	0.007	1.75

[a] Values are means of three experiments, standard deviation is given in parentheses. The IC_{50} values of E_2 for ER-α and ER-β are 5.6 and 7.1 nM, respectively.

[b] RBA is expressed as relative binding affinity to that of E_2 and was calculated as follows: 100 × $IC_{50}(E_2)/IC_{50}$ (compds).

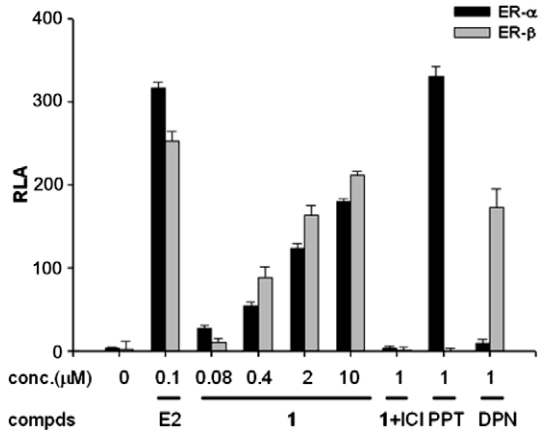

Figure 2. Effects of compound **1** on ER-α and ER-β mediated transcription in CV-1 cells. Cells were transfected by ERE-Luc reporter construct with the expression vector for hER-α or hER-β. After 24 h incubation with compounds, cells were lysed and luciferase expression was determined. Values represent the mean ± SD (n = 3).

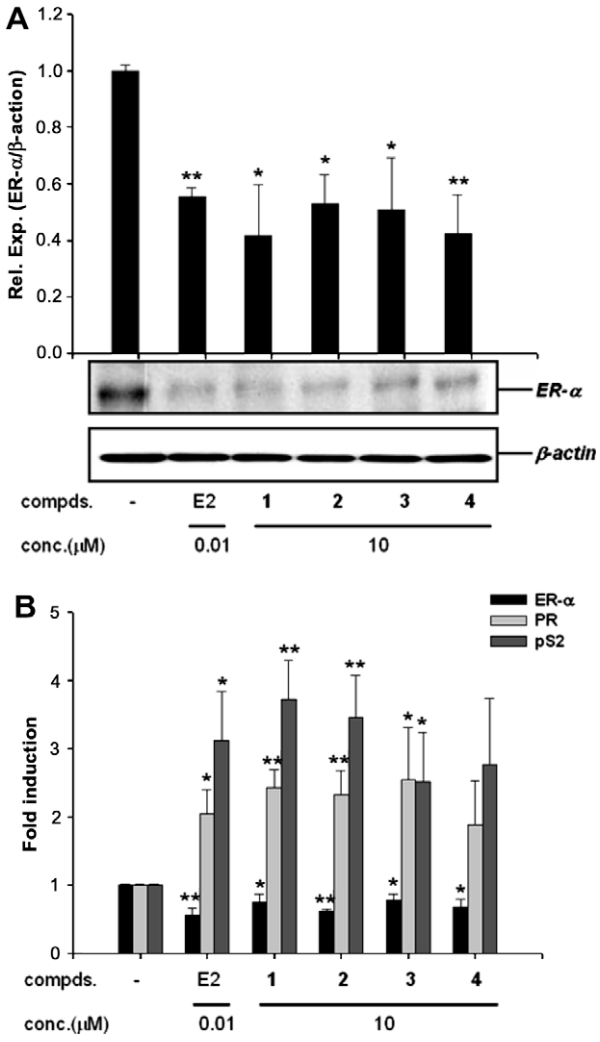

Figure 3. The effects of compounds **1**–**4** on the expression of ER-related genes. MCF-7 cells were treated for 24 h with 10 nM E_2 or 10 μM compounds. After the incubation, the cells were lysed and total protein extracts were resolved by SDS–PAGE and immunoblotted using an anti-ER-α antibody or an anti-β-actin antibody. The blot is the representative one of three experiments (A). Total RNA was analyzed for ER-α, pS2, PR and β-actin mRNA expression by real-time PCR and normalized by β-actin expression (n = 3) (B). $^{*}p$ <0.05; $^{**}p$ <0.01.

required to determine whether the compounds from *B. kazinoki* are effective to menopausal symptoms or osteoporosis.

Acknowledgments

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12. *Extraction and purification*: The air dried root bark of *Broussonetia kazinoki* collected from Kyunggi province, Korea in July 2007 (voucher specimen no. SPH 07002) (0.6 kg) was extracted for 24 h at room temperature with 2000 mL of ethanol. The extract (51 g) was suspended in water and successively partitioned with *n*-hexane, EtOAc, $CHCl_3$ and BuOH. The EtOAc fraction (31 g) was subjected to silica gel column chromatography eluting with *n*-hexane/acetone gradient system (20:1 → 1:10) and 11 fractions were collected. Fraction 7 was further chromatographed with a silica gel column with $CHCl_3$/MeOH gradient system (100:1 → 1:1) to afford six sub-fractions. The fraction 7-3 was chromatographed on a RP-C18 column with a gradient elution of MeOH (40% → 100%) to yield compound **1** (17 mg) and compound **2** (7 mg). And then fraction 7-4 purified by a silica gel column chromatography with a gradient elution of *n*-hexane/EtOAc (40:1 → 2:1) to yield compound **4** (14 mg). Fraction 8 was chromatographed on a RP-C18 column with a gradient elution of MeOH (30% → 100%) to afford compound **3** (139 mg).
Spectroscopic data for compound **3**: $C_{20}H_{22}O_4$, oily substance (brown). UV λ_{max} (MeOH) nm (log ε): 233 (3.76), 286 (3.69). $[\alpha]_D^{24}$ +4.3° (*c* 3.0, MeOH). IR (NaCl) ν_{max} (NaCl): 3502, 3446, 2360, 2340, 1699, 1558, 1043, 668 cm^{-1}. ^1H NMR (CD_3OD, 400 MHz): δ 1.64 (3H, H_3-4″), 1.68 (3H, s, H_3-5″), 1.80–2.10 (2H, m, H-3), 2.50–2.90 (2H, m, H-4), 3.43 (2H, m, H-1″), 5.00 (1H, dd, *J* = 10.4, 2.0 Hz, H-2), 5.13 (1H, br t, *J* = 6.4 Hz, H-2″), 6.25 (1H, d, *J* = 2.4 Hz, H-8), 6.32 (1H, dd, *J* = 8.4, 2.4 Hz, H-6), 6.69 (1H, d, *J* = 8.4 Hz, H-5′), 6.79 (1H, d, *J* = 8.4 Hz, H-6′), 6.84 (1H, d, *J* = 8.4 Hz, H-5). ^{13}C NMR (CD_3OD, 100 MHz): δ 18.0 (C-4″), 25.5 (C-1″), 25.8 (C-5″), 26.1 (C-4), 30.7 (C-3), 76.2 (C-2), 104.0 (C-8), 109.0 (C-6), 113.5 (C-5′), 114.3 (C-10), 118.3 (C-6′), 124.7 (C-2″), 127.5 (C-2′), 130.9 (C-5), 131.4 (C-3″), 132.7 (C-1′), 144.0 (C-3′), 145.4 (C-4′), 157.2 (C-9), 157.4 (C-7). EIMS (70 eV) *m*/*z*: 326 ($[M]^+$), 270, 203, 189, 161, 143, 123. HREIMS *m*/*z*: 326.1516 (calcd for $C_{20}H_{22}O_4$, 326.1518)
Spectroscopic data for compound **4**: $C_{25}H_{30}O_4$, oily substance, UV λ_{max} (MeOH) nm (log ε): 227 (3.28), 243 (3.40), 283 (3.59), 306 (3.10). $[\alpha]_D^{28}$ +2.5°(*c* 3.05, $CHCl_3$). IR (NaCl) ν_{max} (NaCl): 3463, 3364, 2920, 1617, 1507 cm^{-1}. ^1H NMR (CD_3OD, 400 MHz): δ 1.43 (9H, s, H_3-4″, H_3-4‴, H_3-5″), 1.51 (3H, s, H-5‴), 1.66–1.90 (2H, m, H_2-3), 2.40–2.70 (2H, m, H_2-4), 3.01 (1H, dd, *J* = 16.2, 5.0 Hz, H_a-1″), 3.10–3.17 (3H, m, H_b-1″, H_2-1‴), 4.78 (1H, br t, *J* = 5.6 Hz, H-2‴), 4.83 (1H, dd, *J* = 10.6, 2.0 Hz, H-2), 4.87 (1H, br t, *J* = 6.4, Hz, H-2″), 6.09 (1H, d, *J* = 2.4 Hz, H-8), 6.14 (1H, dd, *J* = 8.4, 2.4 Hz, H-6), 6.63 (1H, s, H-2′), 6.66 (1H, d, *J* = 8.4 Hz, H-5). ^{13}C NMR (CD_3OD, 100 MHz): δ 18.0 (C-4″), 18.1 (C-4‴), 25.8 (C-5‴), 25.9 (C-5″), 26.2 (C-4), 26.3 (C-1‴), 28.0 (C-1″), 31.1 (C-3), 76.3 (C-2), 104.1 (C-8), 109.0 (C-6), 111.7 (C-2′), 114.3 (C-10), 124.9 (C-2″), 125.8 (C-2‴), 128.3 (C-5′), 130.3 (C-5), 131.0 (C-6′), 131.4 (C-3″), 131.6 (C-3‴), 132.1 (C-1′), 143.9 (C-4′), 144.1 (C-3′), 157.4 (C-7), 157.5 (C-9). EIMS (70 eV) *m*/*z*: 394 ($[M]^+$), 377, 338, 295, 272, 257, 229, 215, 201, 173, 137, 123. HREIMS *m*/*z*: 394.2142 (calcd for $C_{25}H_{30}O_4$, 394.2144)
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