# Phytoestrogens from *Aspalathus linearis*

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**From the leaves of *Aspalathus linearis*, 24 known compounds and a new one, aspalalinin (25), were isolated. The structures of the compounds were determined mainly based on spectral evidence. The absolute configuration of aspalalinin was presented on the basis of X-ray analysis. Each isolate was assessed for its estrogenic activity by an estrogen ELISA assay. Compounds 12, 15, and 24 showed the estrogenic activity.**

**Key words** *Aspalathus linearis*; Leguminosae; phytoestrogen; dihydrochalcone

The sex hormone estrogens play various roles in both the male and female body. Estrogens are used in hormone replacement therapy (HRT) to prevent hot flashes and osteoporosis in postmenopausal women.[1,2] Plant-derived phytoestrogens also demonstrate estrogenic activity, and are of interest as a dietary potential alternative to the estrogens in HRT.[3]

*Aspalathus linearis* (N. L. BURM.) R. DAHLGR. is a leguminous shrub and grows in a mountainous area of South Africa. The leaves are used for the production of rooibos tea, which has been consumed by the native population for a long time. The tea is a quaffable drink because it contains no caffeine and low levels of tannin,[4] and lately has attracted attention as a health drink. Recent research shows it has various activities, for example, antioxidant activity is well known.[5] Since we believed the plant may also have estrogenic activity, we attempted to identify any estrogenic activity.

## MATERIALS AND METHODS

**General Experimental Procedures** Melting point was determined on a Yanaco micromelting point apparatus MP-500 and is uncorrected. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. $^1$H- (400 MHz) and $^{13}$C-NMR (100 MHz) spectra were recorded on a JEOL JNM $\alpha$-400 FT-NMR spectrometer and chemical shifts are given in $\delta$ with TMS as an internal standard at 35 °C. Inverse-detected heteronuclear correlations were measured using HMQC (optimized for $^1J_{C-H}$=145 Hz) and HMBC (optimized for $^nJ_{C-H}$=8 Hz) pulse sequences with a pulse field gradient. FAB-MS and HR-FAB-MS data were obtained on a JEOL JMS 700 mass spectrometer, using a *m*-nitrobenzyl alcohol matrix. Preparative HPLC was performed on a JASCO system 800 instrument (Column, Nomura Chemical, Develosil ODS 10/20, 50 mm×50 cm×2). Gas chromatography was performed on a HITACHI G-3000.

**Plant Material** *Aspalathus linearis* was purchased in 2003 from Niiya in Shizuoka, Japan. The plant was identified by Prof. Akira Ueno, School of Pharmaceutical Sciences, University of Shizuoka. A voucher specimen (No. 98032) has been deposited in the herbarium of the University of Shizuoka.

**Extraction and Isolation** The dried leaves of *Aspalathus linearis* (5.0 kg) were extracted twice with MeOH under reflux. The extract was concentrated under reduced pressure to give the residue (384 g), which was dissolved in $H_2O$ and then extracted with ether. The ether layer (77 g) was suspended in 80% MeOH and partitioned with hexane–benzene (1 : 1). The 80% MeOH layer (33 g) was chromatographed on a silica gel (Fuji Silysia, PSQ 100B, 100 $\mu$m) column (9×22.5 cm) eluting with $CHCl_3$–MeOH (97 : 3→90 : 10) to afford 24 fractions. Each fraction was subjected to preparative HPLC to afford **1** (*p*-hydroxybenzoic acid)[6a] (43.0 mg), **2** (*p*-hydroxyphenylethanol)[6b] (12.9 mg), **3** (vanylglycol)[7] (7.1 mg), **4** (*p*-coumaric acid)[6c] (123.5 mg), **6** (esculetin)[6d] (21.1 mg), **11** (luteolin)[8] (25.8 mg), and **16** (quercetin)[8] (87.1 mg). The $H_2O$ layer was subjected to a porous polymer gel Mitsubishi Diaion HP-20 column (9×15 cm). The adsorbed material was eluted with 50% MeOH and MeOH, successively, after washing with $H_2O$. The 50% MeOH eluate (101 g) was chromatographed on a silica gel (Fuji Silysia, PSQ 100B, 100 $\mu$m) column (9×32 cm) eluting with $CHCl_3$–MeOH–$H_2O$ (83 : 15 : 1.5→71 : 25 : 4) to afford 21 fractions. Each fraction was subjected to preparative HPLC to afford **5** (syringin)[9] (8.6 mg), **7**[10] (15.2 mg), **12** (isovitexin)[11] (348.1 mg), **13** (luteolin-8-glucoside)[12] (622.8 mg), **14** (vitexin)[11] (143.8 mg), **17** (quercetin-3-robinobioside)[13] (775.4 mg), **18** (quercetin-3-galactoside)[8] (85.9 mg), **19** (quercetin-3-glucoside)[8] (29.2 mg), **20** (eriodictyol-6-glucoside)[14] (532.9 mg), **21** (eriodictyol-8-glucoside)[15] (671.8 mg), **22** (hemiphlorin)[16] (4.2 mg), **23** (aspalathin)[17] (474.9 mg), **24** (nothofagin)[18] (345.7 mg), and **25** (363.3 mg). The MeOH eluate (44 g) was chromatographed on a silica gel (Fuji Silysia, PSQ 100B, 100 $\mu$m) column (7×28 cm) eluting with $CHCl_3$–MeOH (97 : 3→80 : 20) to afford 15 fractions. Each fraction was subjected to preparative HPLC to afford **8** (secoisolariciresinol)[10] (140.1 mg), **9** (vladinol F)[19] (90.8 mg), **10**[20] (11.9 mg), **11** (luteolin)[8] (63.6 mg), **12** (isovitexin)[11] (29.4 mg), **13** (luteolin-8-glucoside)[12] (70.2 mg), **15** (luteolin-7-glucoside)[8] (138.7 mg), **16** (quercetin)[8] (73.6 mg), **17** (quercetin-3-robinobioside)[13] (99.6 mg), **18** (quercetin-3-galactoside)[8] (57.6 mg), **20** (eriodictyol-6-glucoside)[14] (23.7 mg), and **24** (nothofagin)[18] (33.8 mg).

Aspalalinin (**25**): Colorless plates from $H_2O$–MeOH; mp 219—221 °C; $[\alpha]_D^{23}$: +26.2° (*c*=1.0 MeOH); IR (KBr) cm$^{-1}$: 3370 (OH), 1610 (C=O), 1584 (aromatic); CD (MeOH, *c* 5.0×10$^{-3}$) $[\theta]_{235}$ −270200, $[\theta]_{280}$ −32400, $[\theta]_{315}$ +30600; FAB-MS: *m/z*: 451 $[M+H]^+$; HR-FAB-MS *m/z*: 451.1215 $[M+H]^+$ (Calcd 451.1240 for $[C_{21}H_{22}O_{11}+H]^+$); $^1$H-NMR

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($CD_3OD$): $\delta$ 3.02 (2H, t, $J$=7 Hz, $H_2$-$\alpha$), 3.38 (3H, overlapped, $H_2$-$\beta$ and H-5″), 3.45 (2H, overlapped, H-3″ and H-4″), 3.71 (1H, dd, $J$=12, 5 Hz, H-6″), 3.83 (1H, dd, $J$=12, 2.5 Hz, H-6″), 4.03 (1H, dd, $J$=10, 9 Hz, H-2″), 4.84 (1H, d, $J$=10, H-1″), 6.42 (1H, s, H-5′), 6.49 (1H, s, H-6), 6.61 (1H, s, H-3). $^{13}$C-NMR ($CD_3OD$): $\delta$ 29.5 (C-$\alpha$), 41.8 (C-$\beta$), 62.7 (C-6″), 71.6 (C-4″), 72.7 (C-2″), 75.5 (C-1″), 80.0 (C-3″), 82.4 (C-5″), 103.6 (C-5′), 109.3 (C-3′), 109.7 (C-1′), 110.9 (C-3), 117.1 (C-6), 122.2 (C-1), 144.1 (C-4), 145.4 (C-5), 151.7 (C-2), 165.4 (C-4′ and -6′), 165.7 (C-2′), 207.7 (C-$\gamma$). *Anal*. Calcd for $C_{21}H_{22}O_{11}\cdot 1/2H_2O$: C, 54.90; H, 5.05, Found: C, 54.68; H, 5.04.

**Sugar Identification** Aspalalinin (**25**) (12 mg) was refluxed with $FeCl_3$[21] (107 mg) in water (3 ml) for 5 h. After filtration the filtrate was passed through a column packed with Amberlite IRA-120–Amberlite IR-60E (1 : 1) (40 ml). The water eluate was concentrated under reduced pressure. The residue was derived to thiazolidine derivative[22] with D-cysteine methyl ester·HCl (3 mg) in pyridine (30 $\mu$l) at 65 °C for 60 min. To the reaction mixture, hexamethyldisilazane–trimethyl silyl chloride (9 : 1) (30 $\mu$l) was added. The supernatant was subjected to GC (Column, Supelco SPB$^{TM}$-1 0.25 mm×27 m; column temperature, 220 °C; carrier gas, $N_2$). D-Arabinose and D-glucose were detected as thiazolidine derivative at $t_R$ 8.9 min and 16.2 min, respectively. The $t_R$ values of standard thiazolidine derivatives from D-arabinose, L-arabinose, D-glucose and L-glucose were 8.9, 9.6, 16.2 and 15.4 min, respectively.

**X-Ray Analysis** Crystallographic data obtained by X-ray analysis are shown in Table 1. All measurements were made on a Rigaku AFC7R diffractometer with graphite monochromated Cu$K\alpha$ radiation and a rotating anode generator. The structure was solved by direct methods[23] and expanded using Fourier techniques.[24] Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were refined using the riding model.

**Estrogen ELISA Assay** ECOLOGIENA® Estrogen (E1/E2/E3) ELISA kit (96 wells) was purchased from Japan EnviroChemicals Ltd. (Osaka, Japan). Each sample (100 $\mu$g) was dissolved in 1 ml of 10% MeOH, and graded concentrations of 17$\beta$-estradiol (E2; 0, 0.05, 0.15, 0.5, 3.0 $\mu$g/l in 10% MeOH) were used as standards. The absorbance was measured at 450 nm using a micro plate reader MPR-A4i II (TOSOH Co., Tokyo), and generated a standard curve by 4-parameter logistic fitting. The estrogenic activity of the sample was calculated from the standard curve.

## RESULTS AND DISCUSSION

The MeOH extract of *Aspalathus linearis* was dissolved in water and extracted with ether. The ether layer was suspended in 80% MeOH and partitioned with hexane–benzene (1 : 1). The water layer was passed through a porous polymer gel Diaion HP-20 column, and water, 50% MeOH, and MeOH eluates obtained. Estrogenic activity was detected in the 80% MeOH layer, 50% MeOH eluate, and MeOH eluate in the estrogen ELISA assay as described below. These fractions were separated by preparative HPLC to afford 24 known compounds (**1**—**24**) and aspalalinin, (**25**), see Fig. 1.

The $^1$H- and $^{13}$C-NMR spectra of aspalalinin (**25**) were very similar to those of aspalathin (**23**). HR-FAB-MS gave a quasi molecular ion peak at *m*/*z*: 451.1215 $[M+H]^+$, consistent with a molecular formula $C_{21}H_{22}O_{11}$, suggesting that compound **25** had the molecular formula of aspalathin (**23**) with 2 protons less. The $^1$H-NMR spectrum of the aglycon moiety showed three aromatic proton signals as singlet [$\delta$ 6.42 (1H, H-5′), $\delta$ 6.49 (1H, H-6), $\delta$ 6.61 (1H, H-3)], and two methylene signals [$\delta$ 3.02 (2H, t, $J$=7 Hz, $H_2$-$\alpha$), $\delta$ 3.38 (2H, overlapped, $H_2$-$\beta$)]. In the ROE difference spectra, when the proton signals at $\delta$ 6.61 and $\delta$ 6.49 were irradiated, ROEs were observed at $\delta$ 6.42 and $\delta$ 3.02, respectively (See Fig. 2), suggesting that C-2 ($\delta$ 151.7) and C-6′ ($\delta$ 165.4) were linked through an ether bond. The position of the sugar moiety was defined to C-3′ by the HMBC spectrum. Cross-peaks between H-1″ [$\delta$ 4.84 (1H, d, $J$=10 Hz)] of glucose and C-2′ ($\delta$ 165.7), C-3′ ($\delta$ 109.3), and C-4′ ($\delta$ 165.4) were observed. The hydrolysis of this glycoside by ferric chloride gave D-glucose and D-arabinose, which were identified by gas chromatographic analysis of their thiazolidine derivatives.[21,22] The absolute structure of aspalalinin was determined on the basis of X-ray crystallographic analysis (Fig. 3.) These data led us to assign the structure of aspalalinin to be **25**.

Table 1. Crystallographic data of Compound **25**

| | |
|---|---|
| Molecular formula | $C_{21}H_{22}O_{11}$ |
| MW | 450.40 |
| Crystall system | monoclinic |
| Space group | *P*21(#4) |
| Cell dimensions | |
| *a* | 16.029(3) Å |
| *b* | 9.090(1) Å |
| *c* | 8.209(1) Å |
| Volume | 1176.4(3) Å$^3$ |
| *Z* | 2 |
| Density | 1.271 g/cm$^{-1}$ |
| Absorption coefficient | 8.95 cm$^{-1}$ |
| $F_{000}$ | 472.00 |
| Crystall dimensions (mm) | 0.35×0.30×0.20 |

Each fraction and compound were assessed for its estrogenic activity using an ECOLOGIENA® Estrogen (E1/E2/E3) ELISA kit (96 wells). Before separation in detail, 8 fractions were examined for their activity and the activity was shown in 50% MeOH eluate, MeOH eluate, and 80% MeOH layer (Table 2). The positive compounds (genistein and resveratrol) were used. Table 3 shows that compound **24** has high activity approximately equal to that of genistein. Although both dihydrochalcones, **24** (nothofagin) and **23** (aspalathin), are the major constituents of *Aspalathus linearis*,[14,25] the estrogenic activity of **23** was less than half that of **24**. This indicates that the hydroxyl group at C-3′ reduces its estrogenic activity. Aspalalinin (**25**) is a dihydrochalcone linked A- to B-ring through an ether bond, but it has low activity because its construction is changed from the normal type dihydrochalcone such as northofagin and phloretin (2′,4,4′,6′-tetrahydroxydihydrochalcone) which are known to have an estrogenic activity.[26] Compounds **12**, **15**, and **22** had moderate activity, and these flavones or flavanone are glycosylated in the A-ring. Compared with **11** or **14**, the glycosylation in the A-ring is not affected and an additional hydroxylation in the B-ring is more important to the estrogenic activity. Compounds **15**, **12**, **24** were presented in high levels in *Aspalathus linearis*, and were obtained from the 50% MeOH or MeOH eluate. Thus, these compounds were responsible

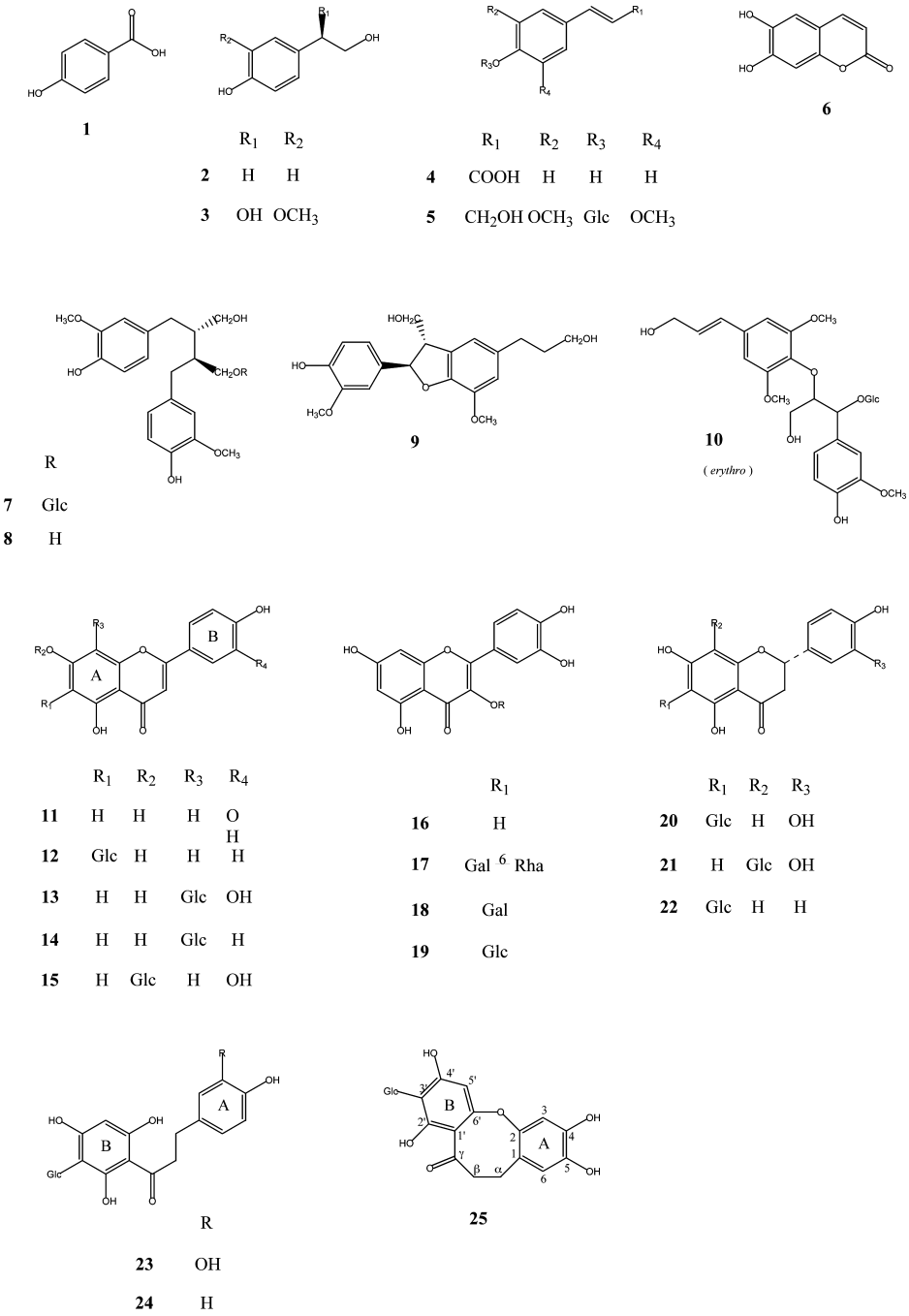


Fig. 1. Structue of Compounds **1**—**25**

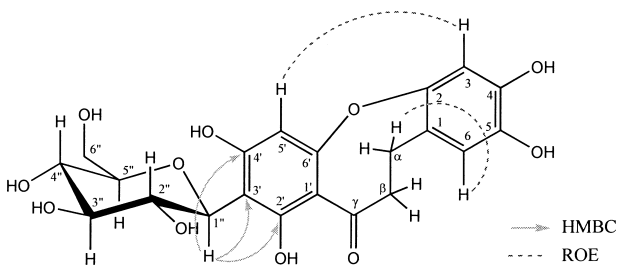


Fig. 2. Selected HMBC and ROE Correlations

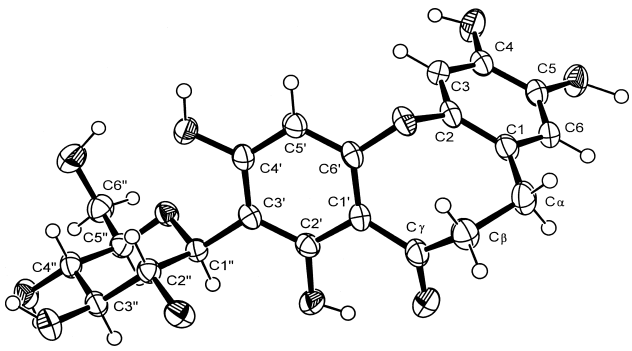

Fig. 3. ORTEP Drawing of Compound **25**

Table 2. The Estrogenic Activity of the Fractions

| Sample (100 $\mu$g/l) | E2 ($\mu$g/l) |
|---|---|
| MeOH ext. | 1.403 |
| $H_2O$ lay. | 1.497 |
| $H_2O$ elu. | n.d.[a] |
| 50% MeOH elu. | 1.395 |
| MeOH elu. | 1.839 |
| Ether lay. | 0.860 |
| 80% MeOH lay. | 1.352 |
| hexane–benzene (1 : 1) lay. | n.d. |
| Resveratrol | 2.930 |

*a*) n.d.: no activity could be detected.

Table 3. The Estrogenic Activity of Compounds **1**—**25**

| Sample (100 $\mu$g/l) | E2 ($\mu$g/l) |
|---|---|
| Genistein | 2.235 |
| Resveratrol | 2.930 |
| **1** | n.d.[a] |
| **2** | 0.088 |
| **3** | 0.037 |
| **4** | 0.088 |
| **5** | n.d. |
| **6** | 0.003 |
| **7** | 0.050 |
| **8** | 0.124 |
| **9** | 0.028 |
| **10** | 0.408 |
| **11** | 0.800 |
| **12** | 1.572 |
| **13** | 0.019 |
| **14** | 0.791 |
| **15** | 1.637 |
| **16** | 0.247 |
| **17** | 0.012 |
| **18** | 0.524 |
| **19** | 0.148 |
| **20** | 0.080 |
| **21** | 0.019 |
| **22** | 1.043 |
| **23** | 0.672 |
| **24** | 2.157 |
| **25** | 0.297 |

*a*) n.d.: no activity could be detected.

for the estrogenic activity in such fractions. The results suggest that *Aspalathus linearis* may has health benefits due to its mild estrogenic activity.

Bis(bibenzyl) type compounds, which are macrocyclic compounds having intermolecular ether linkages, were reported in liverwort.[27] To the best of our knowledge, aspalalinin is the first cyclic dihydrochalcone possessing an intramolecular ether linkage.

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