Stereochemistry of *cis*- and *trans*-Hinokiresinol and Their Estrogen-like Activity

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Naturally occurring phenylpropanoids, hinokiresinol (*trans*-hinokiresinol) and nyasol (*cis*-hinokiresinol) were found to possess appreciable estrogen receptor binding activity. Strong differences in activity were observed between the geometrical isomers and enantiomers. Among these, (3*S*)-*cis*-hinokiresinol displayed the highest activity, one order of magnitude greater than the activity of genistein. Furthermore, *cis*- and *trans*-hinokiresinol stimulated the proliferation of estrogen-dependent T47D breast cancer cells, and their stimulatory effects were blocked by an estrogen antagonist, indicating that the compounds are estrogen agonists. In addition, the absolute configuration of C-3 in (+)-*cis*-hinokiresinol has been assigned as *S* by comparison with the circular dichroism spectra of the hydrogenated products prepared from *cis* and *trans* ((3*S*)-*trans*-hinokiresinol: previously assigned) isomers. These results incidentally provide us with an unambiguous answer to contradictory reports regarding the assignment of the full stereochemisry of *cis*- and *trans*-hinokiresinol that have existed in the literature for more than two decades.

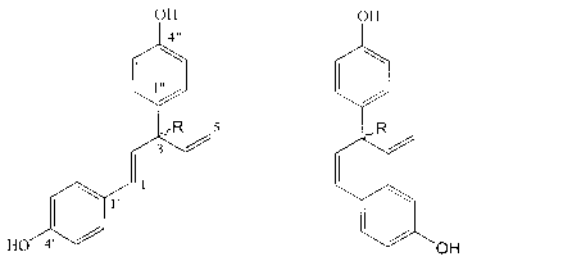
Key words phytoestrogen; hinokiresinol; stereochemistry; nyasol; *Chamaecyparis obtusa*, *Anemarrhena asphodeloides*

In the past few years there has been broad discussion on a wide variety of man-made chemicals that act as estrogen mimics present in the environment. A reduced fertility rate in addition to the impairment of reproductive function have been hypothesized to be linked to exposure to man-made estrogenic substances.[1] In contrast to the supposed risk of estrogenicity, mounting evidence suggests that estrogen may exert beneficial preventive effects in women against heart attacks and other cardiovascular problems, as well as osteoporosis and possibly Alzheimer's disease.[2] Although there are conflicting findings, phytoestrogens have been postulated to exert a tissue-dependent expression of estrogen agonist and antagonist activity and may promote a significant advancement in the prevention of diseases caused by estrogen deficiency in postmenopausal women compared to conventional hormone replacement therapy: the therapeutic efficacy of soybean phytoestrogens has been well reviewed.[3] Thus, the findings of phytoestrogen occurrence in traditional herbal medicines may open the door to considering the therapeutic value of these herbs.

Appreciable estrogen-like activity has been detected in a number of non-steroidal phytoestrogens such as isoflavones, coumestanes, and several phenolic compounds.[4] In addition, certain prenylflavones have been shown to have estrogen-like activity in *in vitro* and *in vivo* experiments.[5,6] The estrogenicity of phytoestrogens such as isoflavones can be understood in view of the superficial similarity between these compounds and the dihydroxystilbene structure. Previously, we reported that several homoisoflavones and retrodihydrochalcones were found to exhibit appreciable estrogen-like activity.[7] These compounds share two *p*-hydroxy benzene rings connected by a chain of three carbon atoms. On the basis of structural similarity, we examined the estrogen-like activity of hinokiresinol (*trans*-hinokiresinol) (**1**) and nyasol (*cis*-hinokiresinol) (**2**), which each consist of a diphenylpropanoid unit. Both *cis*- and *trans*-hinokiresinol were found to possess appreciable estrogen-like activity, and (3*S*)-*cis*-hinokiresinol (**2b**) displayed the highest activity among its geometrical isomers and enantiomers. In addition, the relative and absolute stereochemistry of these compounds were confirmed to provide an unambiguous answer to contradictory reports regarding the assignment of the full stereochemistry of *cis*- and *trans*-hinokiresinol that have existed in the literature for more than two decades. For the convenience of comparison and discussion, nyasol is referred to as *cis*-hinokiresinol (**2**) and hinokiresinol itself is referred to as *trans*-hinokiresinol (**1**) in this publication.

Results and Discussion

Chemistry In 1965, Hirose *et al.*[8] reported the isolation of hinokiresinol (**1**) from the heartwood of *Chamaecyparis obtusa*, and they proposed the structural formula of **1** as having *trans* geometry at the 1, 2 double bond. The absolute configuration at C-3 in **1** has been assigned as *S* based on the optical rotatory dispersion (ORD) spectrum of its degradation product,[9] and later on the enantioselective synthesis of its di-*O*-methylether.[10] The *cis* isomer (nyasol) (**2**) and



(**1**) *trans*-hinokiresinol (**2**) *cis*-hinokiresinol (nyasol)

(**1a**) 3(*R*): R = —H (**2a**) 3(*R*): R = —H

(**1b**) 3(*S*): R = —H (**2b**) 3(*S*): R = —H

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its glycosides have been isolated from various Liliaceous plants,[11—16] although the absolute configuration at C-3 is yet unknown. Subsequent studies on the synthesis[17,18] of **1** and **2** introduced ambiguities in the assignment of the geometry at the 1, 2 double bond. Messana *et al.*[14] pointed out in 1989 that the geometry of several synthetic hinokiresinols have wrongly been assigned and, therefore, erroneously identified.[17,18] In 1996 Tsui and Brown[15] reported that both hinokiresinols seemed likely to share the same *cis* geometry based on comparison with previously published data. Although Oketch-Rabah *et al.*[16] proposed in 1997 that the two isomers are naturally occurring, hinokiresinol is described as an identical compound to nyasol (*cis* geometry), together with a wrong absolute configuration in a widely used compilation of natural products.[19] Moreover, a very recent publication follows the same error.[20]

These ambiguities may have arisen from the limited spectroscopic data presented for natural hinokiresinol (**1**). Thus, we isolated **1** ($[\alpha]_D = -3°$) from a MeOH extract of the heartwood of *C. obtusa.* Assignments of ^{1}H- and ^{13}C-NMR spectra were deduced from the analysis of two dimensional (2D) chemical shift correlations (data not shown), and the results obtained were totally consistent with the structure of **1**. Previously, Nikaido *et al.*[12] reported the isolation of **2** from the Chinese medicinal drug Anemarrhenae Rhizoma (rhizome of *Anemarrhena asphodeloides* Bunge.). The authors did not document any evidence to assign the isolated compound as **2** nor any spectroscopic data to identify the compound. We obtained the compound ($[\alpha]_D = -67°$) from the same plant source and the compound was identified as **2** by comparisons of its ^{1}H- and ^{13}C-NMR data with those described in the literature.[11,15] Complete ^{1}H- and ^{13}C-NMR spectral assignments for **1** and **2** provided us with unambiguous evidence for the presence of these compounds in nature, and confirmed the geometry of the double bond at C-1,2 in the respective isomers: the *trans*-configured **1** exhibited a ^{1}H–^{1}H coupling constant of 16 Hz, while the *cis* isomer (**2**) showed a value of 12 Hz in its ^{1}H-NMR spectrum.

As was described earlier, the absolute configuration at C-3 in (−)-*trans*-hinokiresinol (**1**) has been assigned as *S*.[9,10] In order to establish the absolute configuration of **2**, we prepared tetrahydro derivatives of **1** and **2**. In the (3*S*)-configured tetrahydro derivative (**3b**) derived from **1**, a positive Cotton effect was observed in the region of 220—250 nm and a negative Cotton effect in the region of 270—290 nm. On the other hand, the tetrahydro derivative (**3a**) prepared from **2** exhibited a circular dichroism (CD) curve which was an antipode to that of **3b**, indicating that the absolute configuration at C-3 in **2** is *R*, as illustrated in the structure of **2a**.

However, the relatively low value of molecular ellipticity of **3a**, compared with that of the (3*S*)-isomer (**3b**), implied that **2** was obtained as a partially racemic mixture. The composition of **2** was then checked using a chiral HPLC column (Chiralpak OD(+)). Two peaks were clearly observed with an approximate ratio of 77 : 23. This composition is consistent with the ratio calculated from the amplitude of the CD spectrum of **3a**, assuming that **1** is optically pure. From the above data we concluded that **2** isolated from *A. asphodeloides* is as illustrated by the structure of **2a**, and is partially racemized at C-3. We then separated the respective enantiomers by preparative HPLC. Both enantiomers (**2a** and **2b**) were obtained and were characterized by their CD spectra: the CD spectrum of the major enantiomer (**2a**) appeared to be the mirror image of that observed for the minor enantiomer (**2b**). The results described here provide unequivocal proof of the structure of (−)-*trans*-hinokiresinol as **1b** and (+)- and (−)-*cis*-hinokiresinol as **2b** and **2a**, respectively.

Table 1. Binding Affinities of Isolates and Other Compounds for the Bovine Uterine Estrogen Receptor

Compound	IC_{50} (nM)		SEM[a]	n[b]
Estradiol	0.40	±	0.01	5
Genistein	480	±	80	5
1	2300	±	100	5
2	350	±	20	5
2a	400			2
2b	60			2

a) SEM: Standard error of mean. *b*) Number of experiments.

Biological Activity The compounds described above were then evaluated for their *in vitro* estrogenic activity by a competitive binding assay using [^{3}H]-17β-estradiol as the tracer and bovine uterine cytosol as the receptor source.[7] Genistein, the best known among naturally occurring non-steroidal estrogens, was used as the reference compound. The semi-logarithmic plot of bound radioactivity *vs.* molar concentration of the compounds exhibited curves parallel to those of 17β-estradiol, suggesting a common binding site for the compounds tested. Concentrations required for 50% displacement of specifically bound radioactivity (IC_{50} values) were calculated from the plotted data and are shown in Table 1. All compounds tested were proven to exhibit binding affinity for the estrogen receptor. (3*S*)-*cis*-Hinokiresinol (**2b**) displayed the highest activity among these compounds, having an IC_{50} of 60 nM. This value indicated that **2b** is one order of magnitude more efficient than genistein.

As far as the tested compounds were concerned, activity appeared to be related to the geometry of the 1,2-double bond in addition to the absolute configuration at C-3: the *cis* isomer (**2**) possesses an activity of about one order of magnitude greater than that of the *trans* isomer (**1**). This is in sharp contrast to previous reports stating that the ability of diethylstilbestrol to act as the functional equivalent of estradiol is due to the particular structural feature of its *trans* geometry. Previously, we reported that 8-isopentenylnaringenin (**4**) exerted estrogenic activity twice as high as that of genistein, as evaluated by a receptor binding assay, and that the (2*S*)- and (2*R*)-enantiomers of **4** did not show significantly different binding affinity for the estrogen receptor.[5] When we studied the receptor binding of the enantiomers of *cis*-hinokiresinol (**2**), we found a major difference between them: the (*S*)-enantiomer (**2b**) displayed activity of about one order of magnitude greater than that of the (*R*)-enantiomer (**2a**). This result was not unexpected because biomolecules are usually able to discriminate between optical isomers. Recently, Kuiper *et al.* discovered the existence of two estrogen receptor subtypes (ERα, ERβ).[21] Although the ligand binding affinity of ERα and ERβ is overall quite similar for the physiological ligands, several phytoestrogens, especially genistein, apigenin and kaempferol, have been reported to have significantly higher affinity for ERβ (20- to 30-fold more).[22] Our experiments have been carried out with a bovine uterine extract

which involves mixtures of ERα and ERβ protein. Additional studies using the respective subtypes of the receptors are critical to understanding the structure–activity relationship and to finding an explanation for the apparently contradictory phenomena described above.

Next, we tested the ability of **1** and **2** to stimulate the growth of estrogen-dependent T47D cells (derived from human breast cancer) in culture to examine if these compounds functioned as an estrogen receptor agonist or antagonist.[23] Test compounds were incubated for 4 d with cells which had been brought into log phase during a 2-d preculture period. The number of cells was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. As shown in Fig. 1, these compounds stimulated the cell proliferation in a concentration dependent manner and induced T47D proliferation to the same extent as estradiol. The calculated EC_{50} values (concentration of a compound required to increase the cell number to 50% of the plateau level) were 10^{-7} M for **2**, 10^{-6} M for **1** and 10^{-11} M for estradiol. The stimulatory effect was blocked by cotreatment with a pure/complete estrogen antagonist ICI 164 384[24] (Fig. 2). These results clearly indicate that **1** and **2** are estrogen agonists and that **2** possesses the activity of one order of magnitude greater than that of **1**.

cis-Hinokiresinol (**2**) has already been shown to potently inhibit the growth of *Leishmania major* promastigotes, to moderately inhibit *Plasmodium falciparum* schizonts,[16] and has been detected as a potent inhibitor of cAMP phosphodiesterase, having an IC_{50} value of 100 μM.[12] Compound **2** is also reported to be a LTB_4 receptor antagonist having an IC_{50} value of 5.24 μM.[20] We have shown here that **2** possesses noteworthy estrogen-like activity (an EC_{50} value of 100 nM as assessed by the stimulatory effect on the proliferation of estrogen-dependent human breast cancer cells). The rhizome of *A. asphodeloides* is a herbal drug recommended for the treatment of various ailments in Chinese traditional remedies, and **2** is known to be one of the major ingredients of this herbal medicine. Phytoestrogens are known to exert a variety of beneficial effects related to several chronic diseases including osteoporosis and heart diseases, and perhaps hormone-dependent cancer.[25] Although we are far from a satisfactory knowledge of the chemicals responsible for the potent effects of this medicinal plant, the occurrence of an estrogen agonist may contribute to the therapeutic value of this herb.

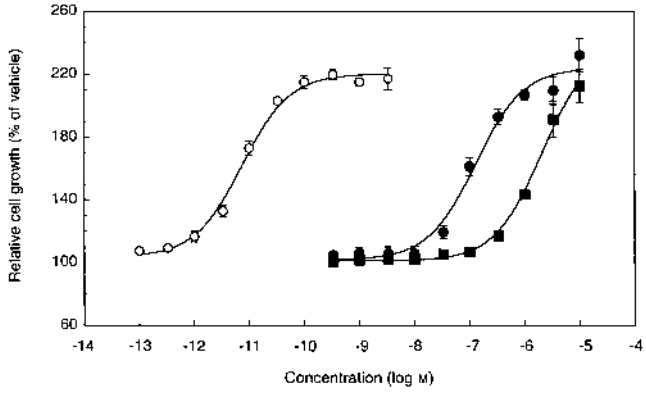

Fig. 1. The Dose-Response Effect of the Compounds on the Proliferation of T47D Cells

Points are mean values, and vertical bars represent standard errors of the mean (n=8). ○: 17β-estradiol, ■: *trans*-hinokiresinol (**1**), ●: *cis*-hinokiresinol (**2**).

Experimental

General Experimental Procedures The ^{1}H-NMR spectra were conducted on a Bruker AMX-360 at 360 and 90.8 MHz for ^{1}H- and ^{13}C-NMR spectra, respectively. Electron ionization mass spectrum (EI-MS) data was obtained on a JEOL JMS-D300 mass spectrometer at 70 eV. IR spectra were measured on a JASCO FT-IR 8300 spectrometer. UV spectra were measured using a Hitachi U-3500 spectrometer. CD measurements were carried out using a JASCO J-500 spectrometer to scan from 420 to 200 nm. Optical rotations were measured at room temperature on a Perkin-Elmer 241 polarimeter.

Plant Materials Powdered heartwood of *Chamaecyparis obtusa* ENDL. (Japanese name, Hinoki) (Cupressaceae) was a generous gift of Dr. M. Yatagai, Forestry and Forest Products Research Institute. The dried rhizome of *Anemarrhena asphodeloides* BUNGE (Japanese name, Chimo) (Liliaceae) was purchased from a Chinese crude drug store in Tokyo.

Origin of Individual Compounds *trans*-Hinokiresinol (**1**)[8]: Isolated from powdered heartwood of *C. obtusa*. Colorless oil, ^{1}H-NMR ($(CD_3)_2C=O$) δ: 4.12 (1H, t, J=6.9 Hz), 5.09 (1H, d, J=10.4 Hz), 5.09 (1H, d, J=17.2 Hz), 6.09 (1H, ddd, J=17.2, 10.4, 6.9 Hz), 6.26 (1H, dd, J=15.9, 6.9 Hz), 6.36 (1H, d, J=15.9 Hz), 6.79 (2H, d, J=8.4 Hz), 6.80 (2H, d, J=8.5 Hz), 7.10 (2H, d, J=8.5 Hz), 7.29 (2H, d, J=8.4 Hz). ^{13}C-NMR ($(CD_3)_2C=O$) δ: 52.7 (d), 114.9 (t), 116.2 (d), 116.3 (d), 128.3 (d), 129.8 (d), 130.2 (s), 130.3 (d), 130.6 (d), 134.9 (s), 142.3 (d), 156.9 (s), 157.8 (s). IR (liquid film) cm^{-1}: 3345, 1610, 1512, 1445, 1368, 1236, 1172, 969, 919, 833, 758. UV λ_{max} (EtOH) nm (ε): 191 (29900), 204 (32500), 265 (22000). EI-MS m/z: 252.1151 (M^+) (Calcd for $C_{17}H_{16}O_2$: 252.1150). $[\alpha]_D$ −3° (c=1.0, acetone). CD (EtOH): $[\theta]_{223}$ +11000, $[\theta]_{240}$ +6400, $[\theta]_{261}$ +4000, $[\theta]_{283}$ −4200. Compound **1** provided EI-MS consistent with previous published data.[26]

cis-Hinokiresinol (**2**): Isolated from the rhizome of *A. asphodeloides*.[12,20] Colorless oil, IR (liquid film) cm^{-1}: 3329, 1610, 1510, 1444, 1367, 1236, 1173, 920, 833, 756. UV λ_{max} (EtOH) nm (ε): 202 (37400), 231 (10700), 259 (15700). EI-MS m/z: 252.1154 (M^+) (Calcd for $C_{17}H_{16}O_2$: 252.1150).

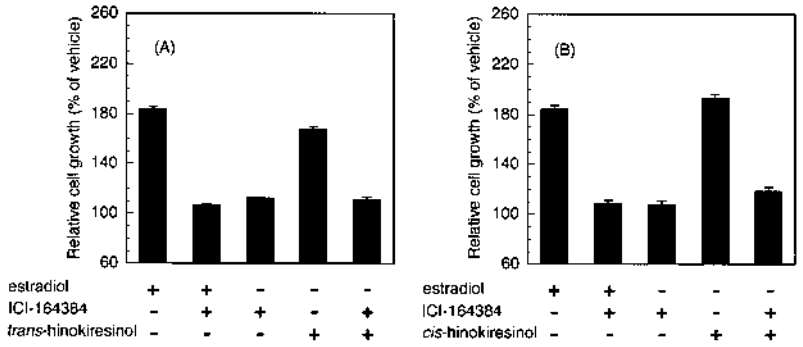

Fig. 2. The Effect of ICI 164 384 on the Ability of **1** and **2** to Stimulate the Growth of T47D Cells

Cells were grown in the presence (+) or absence (−) of (A) 100 pM of estradiol, 1 μM of ICI 164 384 or 10 μM of **1** and (B) 100 pM of estradiol, 1 μM of ICI 164 384 or 3 μM of **2**. Columns are mean values, and vertical bars represent standard errors of the mean (n=8).

$[\alpha]_D$ −67° (c=0.1, acetone). Lit.[11] −147° (c=1.0, acetone). CD (EtOH): $[\theta]_{230}$ +6500, $[\theta]_{254}$ −3300, $[\theta]_{287}$ +2000. ^{1}H- and ^{13}C-NMR data confirmed that the compound was identical to nyasol.[11,15]

Analysis and Separation of the Enantiomers of *cis*-Hinokiresinol, 2a and 2b Chiral HPLC analysis of *cis*-hinokiresinol was performed using a column packed with cellulose carbamate (4.6 mm×25 cm, 10 μm, OD; tris-3,5-dimethylphenylcarbamate, Chiralcel®, Daicel Chemical Industries, Japan). Two peaks were clearly observed at retention times of 26.9 and 29.9 min at an approximate ratio of 77 : 23 with *n*-hexane : 1-propanol (94 : 6) containing 1% trifluoroacetic acid as the mobile phase at a flow rate of 1.0 ml/min. The enantiomers of **2** were separated semi-preparatively by liquid chromatography on a cellulose carbamate column (2×25 cm, 10 μm, OD, Chiralcel®, Daicel Chemical Industries, Japan) using a mixed solvent of *n*-hexane : 1-propanol (94 : 6) +1% trifluoroacetic acid as an eluent (5 ml/min). The chromatogram showed an incomplete separation of the peaks for the two isomers (t_R=38.9 min and t_R=42.0 min). Repeated separation of the racemate (**2**, 20 mg) resulted in optically pure **2a** (2.5 mg) and **2b** (2.9 mg). CD **2a** (EtOH): $[\theta]_{228}$ +11000, $[\theta]_{254}$ −60000, $[\theta]_{287}$ +3000. **2b** (EtOH): $[\theta]_{228}$ −12000, $[\theta]_{254}$ +60000, $[\theta]_{287}$ −3000.

Tetrahydrohinokiresinol, 3a and 3b A mixture of **1** (30 mg), EtOH (5 ml) and PtO_2 (10 mg) was hydrogenated for 1 h. After filtration, the mixture was concentrated to dryness under reduced pressure, and dried to give a colorless solid. The residue was applied to HPLC (ODS column 2×25 cm, 5 μm, Senshu, Japan) eluted with CH_3CN–H_2O (1 : 1) at flow rate of 20 ml/min. The major peak (t_R=12 min) was collected. Evaporation of the solvent gave **3b** (18 mg) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.74 (3H, t, J=7.4 Hz), 1.52 (1H, m), 1.63 (1H, m), 1.78 (1H, m), 1.88 (1H, m), 2.36 (3H, m), 6.72 (2H, d, J=6.5 Hz), 6.78 (2H, d, J=6.5 Hz), 7.00 (2H, d, J=6.5 Hz), 7.01 (2H, d, J=6.5 Hz). IR (liquid film) cm^{-1}: 3324, 2928, 1613, 1513, 1452, 1375, 1233, 1174, 830, 758. UV λ_{max} (EtOH) nm (ε) 191 (35000), 202 (21000), 226 (15600), 279 (3400). CD (EtOH): $[\theta]_{203}$ +17000, $[\theta]_{229}$ +11500, $[\theta]_{286}$ −2000.

Compound **2** (72 mg) was hydrogenated in the same way, and its tetrahydro derivative was obtained (16 mg) as a colorless oil. Compound **3a** prepared from **2** was identified as **3b** by comparing ^{1}H- and ^{13}C-NMR spectra. CD (EtOH): $[\theta]_{203}$ −7700, $[\theta]_{229}$ −6200, $[\theta]_{286}$ +950.

Estradiol Receptor Binding Assay The competitive binding assay to measure affinity for the estrogen receptor follows the method described previously.[7]

Effect on T47D Cell Proliferation Hormone-sensitive human T47D breast cancer cells[27] were maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum. One day after seeding in a 96-well plate at a density of 2.5×10^3 cells/well, the media was changed to RPMI 1640 with 10% charcoal-treated fetal bovine serum. The media was refreshed again the next day. Then, the DMSO solution of the test compounds was added to the cell culture. The final concentrations of compounds were between 10^{-10} and 10^{-4} M. After 4 d in a humidified incubator at 37 °C with 5% CO_2, the number of cells was assessed by MTT assay, as modified by Carmichael *et al*.[28]

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