Anti-estrogenic Activity of Mansorins and Mansonones from the Heartwood of *Mansonia gagei* DRUMM.

Ali Mahmoud EL-HALAWANY,[a] Mi Hwa CHUNG,[a] Chao-Mei MA,[a] Katsuko KOMATSU,[a] Tsutomu NISHIHARA,[b] and Masao HATTORI*,[a]

[a] *Institute of Natural Medicine, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan: and* [b] *Faculty of Pharmaceutical Sciences, Hyogo College of Medicine; 1–3–6 Minatojima, Chuo-ku, Kobe 650–0045, Japan.*
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Through an anti-estrogenic bioassay-guided fractionation of the methanol extract of *Mansonia gagei*, three new coumarins, called mansorins I (1), II (2) and III (3) and a new naphthoquinone, mansonone I (4), were isolated. Their structures were determined based on their NMR data and CD spectroscopy. The anti-estrogenic activity of the fractions and the isolated compounds were investigated using a yeast two-hybrid assay method expressing estrogen receptors α (ERα) and β (ERβ). In addition, an ERα competitor screening system (ligand binding screen) was used to verify the binding affinities of the isolated compounds to the estrogen receptor. 1,2-Naphthoquinones (mansonones) showed more binding affinities to ER in both assay systems. All the tested compounds showed higher binding affinities to ERβ than to ERα in the yeast two-hybrid assay. Mansonones F and S showed the most potent estrogen binding and estrogen antagonistic effects.

Key words *Mansonia gagei*; anti-estrogenic activity; estrogen receptor; sterculiaceae; mansonone; mansorin

Phytoestrogens are plant derived substances that structurally and/or functionally mimic mammalian estrogens. The diverse biological activities of phytoestrogens are due in part to their ability to act as estrogen agonists or as antagonists.[1] To date, two estrogen receptors (ER) have been identified (hERα and hERβ), and the physiological responses to estrogen are known to be almost entirely mediated through these two receptors.[2] There is a renewed interest in naturally occurring phytoestrogens as potential alternatives to synthetic hormonal replacement therapy. Among the known phytoestrogens, isoflavonoids, lignans and coumestans are the most well studied classes. Furthermore, other classes were also recently reported to possess estrogenic activities *viz.*, anthraquinones,[3] retrodihydrochalcones,[4] and prenylated flavonoids.[5] In our current search for new phytoestrogens effective in the prevention and treatment of estrogen-mediated diseases, several medicinal plants known in Thailand folk medicine were investigated. The methanol extract of the heartwood of *Mansonia gagei* showed potent anti-estrogenic activity. *Mansonia gagei* is a large tree belonging to the family Sterculiaceae and growing in the dry evergreen forests in Thailand.[6] *Mansonia gagei* heartwood has been used as a cardiac stimulant, anti-emetic, antidepressant and refreshing agent.[6] Several 1,2-naphthoquinones and coumarins called mansonones and mansorins, respectively, were reported to be extracted from this plant,[7—9] and a novel neolignan, mansoxetane, was also reported.[9] These compounds showed a wide variety of biological activities like larvicidal and antibacterial.[6,10] Mansonones E and F were reported to possess cytotoxic and antioxidant activities.[6,11,12]

The present study reports the biologically guided isolation of four new and ten known compounds from the chloroform-soluble fraction of the methanol extract of *Mansonia gagei* and their anti-estrogenic activities.

Results and Discussion

The methanol extract of the heartwood of *Mansonia gagei*, the $CHCl_3$-soluble and the remaining $CHCl_3$-insoluble fractions were evaluated for their estrogenic and anti-estrogenic activities using a yeast two-hybrid screening method expressing ERβ at concentrations of 10 and 100 μg/ml (Table 1). The chloroform-soluble fraction showed the most potent anti-estrogenic activity (*ca.* 49% and 21% reduction of 17β-estradiol activity in 100 and 10 μg/ml concentrations, respectively). None of the three fractions showed any estrogenic activities (data not shown). The chloroform-soluble fraction was further purified on a silica gel column to obtain 8 sub-fractions. Due to their relatively high anti-estrogenic activities, sub-fractions 1 and 3 were selected for further investigation. They were purified using normal and reversed phase silica gel columns to get four new compounds (**1**, **2**, **3**, **4**) (Fig. 1) along with 10 known compounds (Fig. 2). The known compounds were identified as mansorin A (**5**), mansorin C (**6**),[7] mansonone G (**7**),[7,13] mansonone O (**8**),[8] acetovanillone (**9**),[14] mansonone F (**10**),[15—17] mansonone H (**11**),[15,16] mansonone S (**12**),[9] mansonone C (**13**)[17] and mansonone N (**14**),[8] by comparison with the reported data. The structures of the new compounds were determined as follows:

Compound **1** was isolated as white needles and assigned a molecular formula of $C_{14}H_{14}O_4$ as estimated by high resolu-

Table 1. Inhibitory Effects of the Total Methanol Extract and Fractions of *Mansonia gagei* on Induction of β-Galactosidase Activity by 17β-Estradiol in Yeast Two-Hybrid Assay (ERβ)

Fraction	β-Galactosidase activity (*U*)	
	100 μg/ml	10 μg/ml
Total extract	58.8%±3.7**	77.5±1.5*
Chloroform-soluble fraction	51.7%±1.5***	79.2±6.4*
Chloroform-insoluble fraction	62.3%±1.8**	76.3±2.3*

β-Galactosidase activity (*U*) of 17β-estradiol was 774.0±42.1 at a concentration of 10^{-7} M (100%). β-Galactosidase activity (*U*) of the fractions is calculated as a percentage of the 17β-estradiol activity. Asterisks indicate significant differences from the control at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) ($n=6$).

* To whom correspondence should be addressed. e-mail: saibo421@inm.u-toyama.ac.jp

tion electron impact mass spectrometry (HR-EI-MS). The IR absorption of **1** revealed the presence of an α,β-unsaturated lactone at 1698 cm^{-1}, an aromatic moiety at 2924 and 1589 cm^{-1} and a hydroxyl group at 3423 cm^{-1}. The ^{1}H-NMR spectrum (Table 2) revealed the presence of an aromatic proton at δ_H 6.88 (1H, s, H-8). Two protons at δ_H 4.26 (1H, dd, J=10.8, 3.3 Hz, H-5$_a$) and δ_H 4.44 (1H, dd, J=10.8, 0.9 Hz, H-5$_b$) and a multiplet at δ_H 3.3 (1H, m, H-6) were assigned to a –CH–CH_2–O– moiety. Three methyls were observed at δ_H 1.26, 1.98 and 2.31. The methyl at δ_H 1.26 was positioned at –CH– of the above determined moiety as proved by ^{1}H–^{1}H correlation spectroscopy (COSY) and heteronuclear multiple-bond correlations (HMBC). The ^{1}H- and ^{13}C-NMR data showed similarity to those of mansorin C (**6**), previously isolated from the same plant with the presence of an extra oxygenated aromatic carbon at δ_C 150.2 (Fig. 2).[7] The skeleton of **1** and the position of the hydroxyl substituent were confirmed by HMBC correlations (Fig. 3). The HMBC correlations between the aromatic proton at δ_H 6.88 and the methyl group at δ_C 15.2 (CH_3-12) assigned the signal at δ_H 6.88 to H-8. Furthermore, the HMBC correlation between H-8 and δ_C 150.2 confirmed the position of the phenolic hydroxyl to be at C-7. The lack of diaxial coupling between the methylene protons at C-5 and the methine proton at C-6 suggested the axial orientation of the methyl group to be at C-6. The axial orientation of the methyl at C-6 was further confirmed by the presence of a nuclear Overhauser effect spectroscopy (NOESY) correlation between the methyl at C-6 and the equatorially oriented proton at C-5. It was reported that the pyran ring of the isochroman moiety of **1** is normally present in the half chair conformation either in P or M conformer (helicity) (Fig. 4).[18—20] The helicity of the non aromatic ring in the isochroman nucleus can be determined through the sign of cotton effect (CE) at the ^1L_b absorbance band of the benzene ring. The sign of the cotton effect in the ^1L_b absorbance band in isochroman was found to be not affected by the substitution pattern of both the benzene and pyran rings.[18] The circular dichroic (CD) spectrum of **1** showed a sharp negative CE at 286 nm ($\Delta\varepsilon$ −4.38) indicating the M conformation of **1**.[18,19] This configuration was further confirmed by comparing the CD spectrum of **1** with that of man-

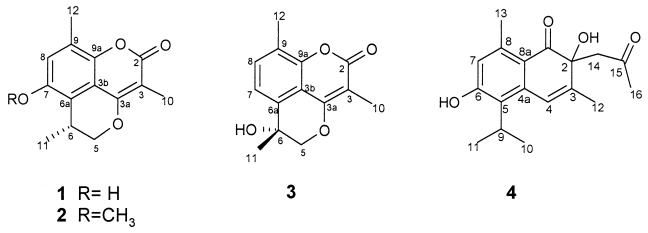

Fig. 1. Structures of the New Compounds **1**—**4** Isolated from *Mansonia gagei*

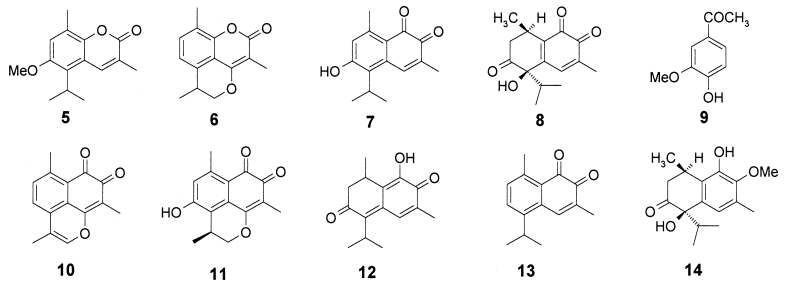

Fig. 2. Structures of Known Compounds **5**—**14** Isolated from *Mansonia gagei*

Table 2. ^{1}H- and ^{13}C-NMR Spectral Data of Compounds **1** (CD_3OD), **2** and **3** ($CDCl_3$)

Position	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		—		—		—
2		166.7		164.4		163.9
3		102.5		102.9		103.8
3a		161.8		158.9		158.5
3b		112.1		111.2		109.9
4		—		—		—
5	4.26 (1H, dd, J=10.8, 3.3 Hz) 4.44 (1H, dd, J=10.8, 0.9 Hz)	74.3	4.23 (1H, dd, J=10.8, 3.3 Hz) 4.39 (1H, dd, J=10.8, 0.9 Hz)	72.6	4.18 (1H, d, J=10.8 Hz) 4.25 (1H, d, J=10.8 Hz)	75.8
6	3.3 (1H, m)	28.0	3.3 (1H, m)	26.6		66.8
6a		120.4		120.9		135.1
7		150.2		150.4	7.27 (1H, d, J=8.0 Hz)	117.8
8	6.88 (1H, s)	121.6	6.9 (1H, s)	124.3	7.32 (1H, d, J=8.0 Hz)	132.9
9		125.4		115.4		125.8
9a		144.6		144.1		149.7
10	1.98 (3H, s)	8.6	2.07 (3H, s)	9.0	1.98 (3H, s)	8.9
11	1.26 (3H, d, J=7.2 Hz)	17.9	1.3 (3H, d, J=6.9 Hz)	17.9	1.56 (3H, s)	24.9
12	2.31 (3H, s)	15.2	2.43 (3H, s)	15.8	2.35 (3H, s)	15.5
OCH_3	—	—	3.87 (3H, s) (OCH_3)	55.6	—	—
OH	4.86 (1H, s)	—		—	2.30 (1H, s)	—

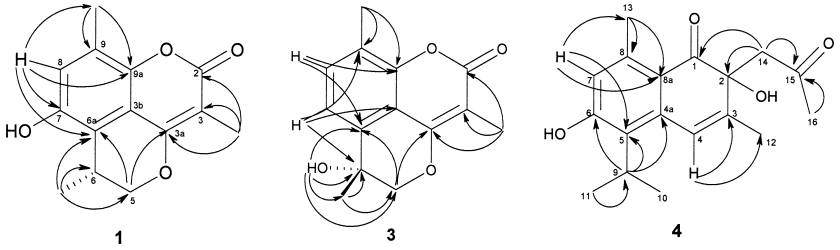

Fig. 3. ^{1}H–^{13}C Correlations in the HMBC Spectra of Compounds **1**, **3** and **4**

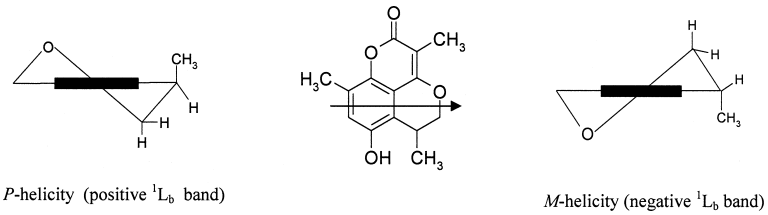

Fig. 4. The Half-Chair Configuration of Compound **1**
The arrow (black) indicates the direction of projection.

sonone H (**11**) isolated from the same plant and showed a positive CE at the $^{1}L_b$ band ($\Delta\varepsilon$ +2.75) [The P conformation was previously assigned for mansonone H through X-ray analysis].[21] Compound **1** which showed an opposite CE sign to that of mansonone H, must then be in the M conformation. Finally, based on the CD spectrum and NOESY correlations, the absolute configuration of **1** was suggested to be *R* at C-6. Compound **1** was therefore determined to be (−)(6*R*)-7-hydroxy-3,6,9-trimethyl-5,6-dihydropyrano[2,3,4-de]chromen-2-one, and called mansorin I.

Compound **2** was isolated as white needles having a molecular formula of $C_{15}H_{16}O_4$ as estimated by HR-EI-MS. The EI-MS spectrum of **2** showed a molecular ion peak at 260 (100%) and a fragment ion peak at 246 (90%) due to the loss of methyl group. The IR spectrum showed similarity to that of compound **1** with the absence of a hydroxyl group signal. The ^{1}H- and ^{13}C-NMR spectra of **2** were similar to those of **1**, except for the presence of an extra methoxyl group at δ_H 3.87 (3H, s) and δ_C 55.6. The HMBC spectrum showed a correlation between the methoxyl protons at δ_H 3.87 and the oxy-aromatic carbon at δ_C 150.4, indicating the presence of the methoxyl group at C-7. Similar to compound **1**, compound **2** showed a negative CE at 288 nm ($\Delta\varepsilon$ −4.27) indicative of its M conformation. Therefore, compound **2** was determined to be (−)(6*R*)-7-ethoxy-3,6,9-trimethyl-5,6-dihydropyrano[2,3,4-de]chromen-2-one, and termed mansorin II.

Compound **3** was isolated as white needles with the molecular formula of $C_{14}H_{14}O_4$. The IR spectrum showed similar signals to that of **1**. The ^{1}H-NMR spectrum revealed the presence of two *ortho*-coupled protons at δ_H 7.27 (1H, d, J=8.0 Hz, H-7), and δ_H 7.32 (1H, d, J=8.0 Hz, H-8), and two geminal protons at δ_H 4.18 (1H, d, J=10.8 Hz, H-5$_a$) and 4.25 (1H, d, J=10.8 Hz, H-5$_b$). The absence of the multiplet proton at C-6, the downfield shift of C-6 appearing at δ_C 66.8 and the presence of a singlet at δ_H 2.30 assigned to a hydroxyl proton, suggested the presence of a hydroxyl group at C-6. The position of this hydroxyl group was further confirmed through the HMBC correlations (Fig. 3) between the hydroxyl proton at δ_H 2.30 and the carbon signals at δ_C 24.9, 75.8 and 66.8, corresponding to CH_3-11, C-5 and C-6, respectively. The NOESY spectrum of **3** revealed a correlation between the methyl protons at δ_H 1.56 (CH_3-11) and both the methylene protons at δ_H 4.18 and 4.25, confirming the equatorial orientation of the methyl group at C-6. Similar to compound **1**, the negative CE at 291.0 nm ($\Delta\varepsilon$ −1.59) of compound **3** indicated the M helicity of its pyran ring. Based on the formerly mentioned CD and NOESY data, the absolute configuration of **3** at C-6 was suggested to be *R*. Compound **3** was determined to be (−)(6*R*)-6-hydroxy-3,6,9-trimethyl-5,6-dihydropyrano[2,3,4-de]chromen-2-one, called mansorin III.

Table 3. ^{1}H- and ^{13}C-NMR Spectral Data of Compound **4** ($CDCl_3$)

Position	**4**	
	δ_H	δ_C
1		204.4
2		78.9
3		142.8
4	6.54 (1H, s)	119.4
4a		137.1
5		127.9
6		159.9
7	6.38 (1H, s)	118.1
8		119.7
8a		140.3
9	3.4 (1H, m)	26.7
10	1.28 (3H, d, J=6.3 Hz)	20.9
11	1.31 (3H, d, J=6.3 Hz)	20.8
12	2.01 (3H, s)	17.9
13	2.37 (3H, s)	20.4
14	2.57 (1H, d, J=13.8 Hz)	52.4
	3.11 (1H, d, J=13.8 Hz)	
15		207.6
16	2.16 (3H, s)	32.3

Compound **4** was obtained as a yellowish amorphous powder, with the molecular formula $C_{18}H_{22}O_4$, based on HR-EI-MS. The IR spectrum of **4** showed the presence of two carbonyls at 1701 and 1663, a hydroxyl signal at 3423 and an

Table 4. Inhibitory Effects of the Isolated Compounds from *Mansonia gagei* on Induction of β-Galactosidase Activity by 17β-Estradiol in Yeast Two-Hybrid Assay (ERα and ERβ)

Compound	β-Galactosidase Activity (% of control)					
	ERα			ERβ		
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-4} M	10^{-5} M	10^{-6} M
1	96.9±2.3	85.3±0.1	103.7±12.3	87.9±4.0	88.4±5.7	103.7±12.3
2	100.9±0.9	109.5±3.8	113.5±4.7	77.8±3.8*	85.9±3.3	113.5±4.7
3	70.5±4.3*	96.2±3.0	88.3±1.9	71.5±2.8**	81.8±1.5*	96.2±1.9
4	80.5±1.3*	88.1±2.4	101.8±3.7	72.1±5.4*	82.7±2.2	101.8±3.7
5	72.7±2.3***	98.5±0.9	100.6±6.3	78.7±0.1*	80.8±0.1*	100.6±6.3
6	56.7±1.9***	102.5±1.8	113.4±6.6	67.7±6.6**	85.1±6.4	113.4±6.6
7	91.4±4.3*	100.9±2.9	116.5±4.1	70.6±0.6**	80.3±5.6*	116.5±4.2
8	79.9±3.9**	125.0±3.2	115.3±3.1	67.2±5.7**	93.2±4.2	115.3±3.1
9	113.5±1.7	113.6±6.2	112.8±3.5	103.3±4.2	94.8±6.5	112.8±3.5
10	40.4±2.4***	70.2±4.0***	92.1±1.2	35.6±3.5***	61.8±6.3**	90.1±3.8
11	109.9±7.3	106.2±3.0	117.1±18.0	83.5±2.8	85.1±5.8	117.1±18.1
12	—	71.4±4.2***	106.1±2.5	—	71.4±5.6*	106.1±2.5
13	—	64.9±2.2***	85.0±2.5**	—	74.9±2.5*	82.0±1.9**
14	70.5±4.3*	96.2±3.0	88.3±1.9	76.8±1.4*	85.8±10.0	88.3±1.9
Tamoxifen	28.2±1.5***	91.9±3.2	95.6±1.8	22.4±2.5***	98.8±10.9	103.3±7.7

β-Galactosidase activity (U) of 17β-estradiol was 641.19±22.5 and 2333.41±112.5 in yeast expressing ERα and ERβ, respectively, at a concentration of 10^{-7} M (100%). β-Galactosidase activity (U) of the tested compounds was calculated as a percentage of the 17β-estradiol activity. Asterisks indicate significant differences from the control at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) ($n=6$).

aromatic moiety at 1577. The ^{1}H-NMR spectrum of **4** showed the presence of two aromatic/olefinic protons at δ_H 6.38 (1H, s, H-7) and 6.54 (1H, s, H-4), three methyls at δ_H 2.01, 2.16 and 2.37 (each 3H, s), and two downfield shifted geminally coupled methylene protons at δ_H 2.57 and 3.11, each (1H, d, J=13.8 Hz, H-14). The ^{1}H–^{1}H COSY correlations between the methine proton at δ_H 3.40 (1H, m, H-9) and the two methyls at δ_H 1.28 and 1.31, each (3H, d, J=6.3 Hz) suggested the presence of an isopropyl moiety. This was further confirmed by ^{13}C-NMR and HMBC (Fig. 3). The ^{13}C-NMR of **4** revealed the presence of 2 methyls at δ_C 20.9 (CH_3-10) and 20.8 (CH_3-11) with a methine carbon at δ_C 26.7 (C-9), assigned as the isopropyl moiety. The HMBC spectrum showed a correlation between the two methyls at δ_H 1.31 and 1.28 and the methine carbon at δ_C 26.7, and between the methine proton at δ_H 3.40 and the oxyaromatic carbon at δ_C 159.9, confirming the presence of the isopropyl moiety at C-5. The HMBC correlations between the methylene protons at δ_H 2.57 and 3.11 with the carbonyl at δ_C 207.6 and the methyl at δ_C 32.3 suggested the presence of a 2-oxopropyl moiety in **4**. A correlation between the two methylene protons and both the oxygenated aliphatic carbon at δ_C 78.9 and the carbonyl at δ_C 204.4 revealed the presence of the 2-oxopropyl side chain and a hydroxyl group in the alpha position to the carbonyl at δ_C 204.4 (C-1). Based on the above evidence and other 2D-NMR correlations, compound **4** was determined to be (+)2,6-dihdroxy-5-isopropyl-3,8-dimethyl-2-(2-oxopropyl)naphthalene-1-one.

Anti-estrogenic Activity of the Isolated Compounds Using the yeast two-hybrid assay, all the isolated compounds were tested for their anti-estrogenic activities at various concentrations. Inhibition of 17β-estradiol (E2)-induced β-galactosidase activity by the isolated compounds was determined under the conditions at which these compounds did not inhibit the growth of the yeast cells. Tamoxifen, an estrogen receptor antagonist, was used as the positive control. Almost all the compounds under investigation showed a significant reduction in the E2-induced β-galactosidase activity (Table 4), which indicates possible estrogen antagonistic activity.

Yeast Two-Hybrid Screen Mansonone G (**7**), S (**12**), F (**10**) and C (**13**) showed the most potent concentration dependent estrogen antagonistic activities in the yeast two-hybrid assay (ERβ) (Table 4). Coumarins, mansorins A (**5**) and III (**3**), showed concentration dependent anti-estrogenic activity. On the other hand, mansorins C (**6**) and II (**2**), and mansonones I (**4**), O (**8**) and N (**14**) showed only significant activities at a 10^{-4} M concentration. In the yeast expressing ERα (Table 4) mansonones F (**10**), S (**12**), C (**13**) and I (**4**) showed concentration dependent antagonistic activities, while mansorins A (**5**), C (**6**) and III (**3**) together with mansonones G (**7**), O (**8**), and N (**14**) only showed activity at 10^{-4} M concentration.

Ligand Binding Screen All the isolated compounds were tested for their binding affinities to the ERα using the labeled estrogen mixture as a control (Table 5). Mansonone O (**8**), F (**10**) and S (**12**) showed the most potent binding affinity to ERα (56.3%, 45.1% and 32.7% of the control, respectively at 5×10^{-3} M). All the other compounds, except for mansorin I (**1**) and acetovanillone (**9**), showed moderate binding affinities.

According to the above data, mansonones were found to be generally more active than the corresponding coumarins (mansorins). Almost all the compounds showed more potent binding affinity toward ERβ than ERα, which is a characteristic of nearly all the reported phytoestrogens.[22] Mansonones F (**10**) and S (**12**) showed the most potent concentration dependent estrogen antagonistic effects in both yeast two-hybrid screen and ligand binding screen. In the case of mansonone O (**8**), significantly potent activity was observed at concentrations of 10^{-4} M and 5×10^{-3} M in the yeast two-hybrid and ligand binding screens, respectively. Many reports insist on the importance of the phenolic group in compounds exhibiting estrogenic and/or anti-estrogenic activities as it

Table 5. ERα Binding Affinity of the Isolated Compounds from *Mansonia gagei* as a Percentage of the Control (Labeled Estrogen Mixture)

Compound	% of the negative control		
	5×10^{-3} M	10^{-4} M	10^{-5} M
1	97.8±0.5	103.7±3.3	107.5±0.5
2	92.3±0.4*	98.0±1.9	110.0±1.1
3	81.2±3.8*	98.3±2.2	100.5±2.7
4	88.3±1.3*	97.5±0.6	100.8±0.9
5	83.1±0.7***	90.1±0.1*	98.7±1.4
6	89.3±1.9*	92.3±0.3	99.8±2.8
7	84.9±3.5*	100.2±1.4	108.7±1.0
8	56.3±1.0***	105.9±2.3	102.4±1.2
9	101.0±0.9	103.3±1.5	102.4±1.8
10	45.1±1.2***	85.8±1.0*	102.3±9.1
11	74.3±2.0*	100.5±0.7	102.0±2.3
12	32.7±2.2**	84.0±0.8*	107.9±5.1
13	72.4±3.7*	97.7±3.1	102.5±4.3
14	96.4±0.8*	98.2±1.1	99.2±1.1

The binding affinity of the positive control (17β-estradiol) was 47.52±1.76% at a concentration of 10^{-7} M. The negative control (labeled estrogen mixture) was 100±0.209%. Asterisks indicate significant difference from the negative control at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) ($n=4$).

mimics ring A of 17β-estradiol.[23] However, the presence of a bulky group in the *ortho*-position to the phenolic group was reported to interfere with the hydrogen bonding capability of such phenol leading to a decrease in ER binding affinity.[23] The previously mentioned factor could be a reason for the moderate or the complete inactivity of compounds with free phenolic groups like mansonone G (**7**), mansonone H (**11**) and mansorin I (**1**), which have a bulky substituent in the *ortho* position to the phenolic group (isopropyl or pyran ring). In addition, lacking a free phenolic group in the active compounds, mansonones O (**8**), F (**10**), C (**13**) and S (**12**), and mansorins A (**5**), C (**6**), II (**2**) and III (**3**) confirmed the low contribution of the phenolic group to the anti-estrogenic activity in such compounds. Moreover, the high activity of mansonone F (**10**) relative to that of mansonone H (**11**) indicates the importance of a planar pyran ring for binding to the estrogen receptor. Finally, to the best of our knowledge this is the first report about the estrogenic agonist and/or antagonist activities of these compounds. Further investigations are currently underway to clarify the structural activity requirements of these compounds through chemical modification using different assay systems.

Experimental

General Experimental Procedures Melting points were measured on a Yanagimoto microhot stage melting point apparatus. Optical rotations were measured with a DIP-360 automatic polarimeter (Jasco Co., Tokyo, Japan). UV spectra were measured with a UV-2200 UV–VIS recording spectrophotometer (Shimadzu Co., Kyoto, Japan). IR spectra were measured with an Jasco FT/IR-230 infrared spectrometer. ^{1}H- and ^{13}C-NMR were measured with a JHA-LAA 400 WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz; Jeol Co., Tokyo) spectrometer, the chemical shifts being represented as ppm with tetramethylsilane as an internal standard. HR-EI-MS and EI-MS were measured with a JMX-AX 505 HAD mass spectrometer (Jeol Co.) at an ionization voltage of 70 eV. The CD spectrum was recorded in MeOH on a Jasco J-805 spectrometer. TLC was carried out on pre-coated silica gel 60 F_{254} (0.25 mm, Merck) and RP-18 $F_{254}S$ (0.25 mm, Merck Co., Darmstadt). Column chromatography (CC) was carried out on BW-820MH silica gel, Wakosil C-300 silica gel (40—63 μm) (Wako Chem. Co., Osaka, Japan) and ODS DM 1020T (ODS, Fuji Silysia, Nagoya, Japan). Medium pressure liquid chromatography (MPLC) was performed on LiChroprep RP-18 and LiChroprep Si 60 (size A and B, Merck Co.).

Plant Material The heartwood of *Mansonia gagei* was bought from the herbal drugstore "Cho Krom Pur," Bangkok, Thailand, and identified by Dr. Katsuko Komatsu (Institute of Natural Medicine, University of Toyama). A voucher specimen has been kept in the herbarium of the Institute of Natural Medicine, University of Toyama, Japan.

Extraction and Isolation Dried pulverized heartwood of *M. gagei* Drumm (3.5 kg) was extracted with methanol on cold. The methanol extract (250 g) was suspended in water (500 ml) and partitioned with chloroform (1 l×3) and the pooled chloroform fractions were evaporated under vacuum. The combined chloroform-soluble fraction (100 g) was applied to a silica gel column (70 cm×8 cm) gradiently eluted with hexane–acetone (5—80% v/v) to obtain 8 sub-fractions. In the yeast two-hybrid system expressing ERβ, sub-fractions 1 and 3 showed the most potent anti-estrogenic activity. Fraction 1 (7 g) was applied to a silica gel column (40 cm×4 cm) eluted with hexane–ethyl acetate (9.5 : 0.5 v/v) to obtain compound **5** (4 g). The remaining portion of fraction 1 was purified using a MPLC silica gel 60 column (size A) (hexane–ethyl acetate, 9.5 : 0.5 v/v) to afford compounds **2** (16 mg), **6** (15 mg) and **14** (30 mg). Fraction 3 (5.2 g) was applied to a silica gel column (40 cm×4 cm) and eluted with hexane–ethyl acetate (9.5 : 0.5—9 : 1 v/v), then the eluate was pooled into three main sub-fractions. Sub-fraction 3-A was purified on a Wakosil C-300 silica gel column (20 cm×2.5 cm) using hexane–ethyl acetate (9.5 : 0.5 v/v) to obtain compounds **4** (40 mg), **12** (6 mg) and **13** (3 mg). Fraction 3-B was purified on a Wakosil C-300 column (20 cm×2.5 cm) eluted with hexane–ethyl acetate (9 : 1 v/v) to obtain compound **7** (2 g). The remaining portion of fraction 3-B was purified using an MPLC RP-18 column (size A) eluted with MeOH–H_2O (7 : 3 v/v) to get compounds **8** (14 mg) and **9** (5 mg). Fraction 3-C was purified on an MPLC RP-18 column (size B) eluted with MeOH–H_2O (7 : 3 v/v) to get compounds **1** (15 mg), **3** (7 mg), **10** (2 mg) and **11** (3 mg).

(−)(6*R*)-7-Hydroxy-3,6,9-trimethyl-5,6-dihydropyrano[2,3,4-de]chromen-2-one (**1**): White needles (MeOH), mp 242—244 °C. $[\alpha]_D^{25}$ −84.4° [c=0.8, MeOH]. CD (MeOH; c 0.002 g/100 ml): nm λ_{max} ($\Delta\varepsilon$) 286 (−4.38), 336 (+0.7), 245 (+3.2). UV ($CHCl_3$) nm λ_{max} (log ε): 330 (3.94), 300 (sh 4.35), 287 (4.44), 240 (4.09). IR (KBr) cm^{-1}: 3423, 2924, 2664, 2589, 1698, 1589, 1475, 1283, 1222, 1089. ^{1}H-NMR (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz) spectral data: (Table 1). EI-MS 70 eV *m/z* (rel. int.): 246 $[M]^+$ (100), 230 (90), 216 (58), 202 (44). HR-EI-MS *m/z*: 246.0860 (Calcd for $C_{14}H_{14}O_4$, 246.0892).

(−)(6*R*)-7-Methoxy-3,6,9-trimethyl-5,6-dihydropyrano[2,3,4-de]chromen-2-one (**2**): White needles (MeOH), mp 148—150 °C. $[\alpha]_D^{22}$ −57.08 [c=0.24, $CHCl_3$]. CD (MeOH; c 0.002 g/100 ml): nm λ_{max} ($\Delta\varepsilon$) 288 (−4.27), 244 (+5.75). UV ($CHCl_3$) nm λ_{max} (log ε): 352 (sh 3.44), 335 (370), 300 (sh 4.16), 287 (4.25), 276 (sh 4.12), 240 (3.94). IR (KBr) cm^{-1}: 2924, 1699, 1639, 1590, 1487, 1220. ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) spectral data: (Table 1). EI-MS 70 eV *m/z* (rel. int.): 260 $[M]^+$ (100), 246 (90), 230 (25), 217 (20), 189 (10), 84 (30). HR-EI-MS *m/z*: 260.1011 (Calcd for $C_{15}H_{16}O_4$, 260.1048).

(−)(6*R*)-6-Hydroxy-3,6,9-trimethyl-5,6-dihydropyrano[2,3,4-de]chromen-2-one (**3**): White needles ($CHCl_3$), mp 195—197 °C. $[\alpha]_D^{25}$ −2.86° [c=0.35, $CHCl_3$]. CD (MeOH; c 0.001 g/100 ml): nm λ_{max} ($\Delta\varepsilon$) 291 (−1.59), 240 (+5.06). UV ($CHCl_3$) nm λ_{max} (log ε): 327 (sh 3.99), 315 (sh 4.35), 293 (4.38), 286 (4.35), 240 (4.09); IR (KBr) cm^{-1}: 3422, 2923, 2854, 1685, 1606, 1459, 1376, 1203; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) spectral data: (Table 1). EI-MS 70 eV *m/z* (rel. int.): 246 $[M]^+$ (100), 230 (90), 216 (58), 202 (44); HR-EI-MS *m/z*: 246.0914 (Calcd for $C_{14}H_{14}O_4$, 246.0892).

2,6-Dihydroxy-5-isopropyl-3,8-dimethyl-2-(2-oxopropyl)naphthalene-1-one (**4**): Yellowish amorphous powder, $[\alpha]_D^{25}$ +3.2 [c=0.5, $CHCl_3$]. UV ($CHCl_3$) nm λ_{max} (log ε): 325 (3.56), 255 (4.34), 350 (sh 3.25). IR (KBr) cm^{-1}: 3423, 2926, 1701, 1663, 1577, 1268, 1121, 1078. ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) spectral data: (Table 2). EI-MS 70 eV *m/z* (rel. int.): 302 $[M]^+$ (20), 245 (12), 217 (100), 201 (85), 175 (20), 91 (30). HR-EI-MS *m/z*: 302.1505 (Calcd for $C_{18}H_{22}O_4$, 302.1518).

Anti-estrogenic Assay To examine the estrogen receptor (ER) antagonistic activity of the plant fractions and isolated compounds, the inhibition of β-galactosidase activity in the yeast two-hybrid assay expressing ERα and ERβ induced by 10^{-7} M 17β-estradiol was measured at various sample concentrations. In addition, the binding affinity of the isolated compounds to ERα was further tested with an Estrogen-R (α) competitor assay kit (Wako Japan Inc.).

Yeast Two-Hybrid Assay The yeast two-hybrid assay was carried out according to the method of Nishikawa and Kanayama.[24,25] Briefly, yeast cells expressing ERα and ERβ were separately grown overnight at 30 °C

with shaking in synthetic defined medium (SD) lacking tryptophan and leucine. Yeast cells were treated with 17β-estradiol and the isolated compounds for 4 h at 30 °C, and β-galactosidase activity was determined as follows. The growth of the yeast cells was monitored by measuring the turbidity at 600 nm. The treated yeast cells were collected by centrifugation (8000×***g***, 5 min) and re-suspended in 200 μl of *Z*-buffer (0.1 M sodium phosphate, pH 7.0, 10 mM KCl, and 1 mM $MgSO_4$) containing 1 mg/ml of zymolyase at 37 °C for 15 min. The reaction was started by the addition of 40 μl of 4 mg/ml *O*-nitrophenol β-D-galactopyranoside (ONPG) as a substrate. When yellow color developed (incubation time: *t*), 100 μl of 1 M Na_2CO_3 was added to stop the reaction. The absorbance of the solution (150 μl) was measured at 420 and 550 nm. The β-galactosidase activity was determined using the following formula:

$$U=1000\times(A_{420}-1.75\times A_{550})/(t\times 0.05\times A_{600})$$

The anti-estrogenic activity of the test compounds was measured as percentage inhibition of β-galactosidase induction, relative to that of the control, 17β-estradiol.

Ligand Binding Assay An estrogen-R (α) competitor assay kit was purchased from Wako Chemical Japan Inc. The assay determines the competition between the samples applied in different concentrations and the labeled estrogen mixture. The amount of the ligand that binds to the ERα coated on the microplate well is determined by the dynamic equilibrium among all the ligand concentrations in the mixture, the difference of their binding affinities to the receptor and incubation time. Therefore, the reduction in fluorescence intensities from the labeled estrogen retained is an indication of the affinity of the added compounds to the estrogen receptor. The isolated compounds were tested in 10^{-5}, 10^{-4} and 5×10^{-3} M concentrations. Estradiol was used as a positive control and the labeled estrogen mixture was used as a negative control. The results were calculated as percentages of the negative control.

Statistical Analysis Each set of experiments was repeated at least three times. Values are expressed as mean±S.E.M. Student's *t*-test was used using 95% confidence intervals.

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