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ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · FEBRUARY 2006

Impact Factor: 3.8 · DOI: 10.1021/np050341w · Source: PubMed

Isoflavonoids with Antiestrogenic Activity from *Millettia pachycarpa*[1]

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Received September 11, 2005

Three new isoflavonoids, named millewanins G (**1**) and H (**2**) and furowanin B (**3**), were isolated from the leaves of *Millettia pachycarpa*. Their structures were elucidated on the basis of spectroscopic analyses. The antiestrogenic activity in the yeast two-hybrid assay of these isoflavonoids was examined and shown to be comparable with that of 4-hydroxytamoxifen.

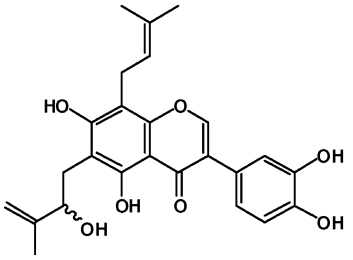
Isoflavonoids have a very limited distribution in the plant kingdom, and plants of the family Leguminosae are the major source of these compounds.[2] Some isoflavonoids show activity against several kinds of cancer cells.[3] Continuing our search for biologically active compounds from plant sources, we examined the constituents of stems of *Millettia pachycarpa* Benth.[4] (Leguminosae) cultivated in Japan. In a previous paper, we reported the antitumor-promoting effects of some isoflavonoids isolated from this plant on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein−Barr virus early antigen (EBV-EA) activation (in vitro) and in two-stage mouse skin carcinogenesis (in vivo).[5,6]

Some isoflavonoids are known to show both estrogen-like and antiestrogen actions.[7] Nishihara et al. developed an assay for endocrine disruptors using the yeast two-hybrid system and tested the estrogenic activity of more than 500 chemicals including natural isoflavonoids.[8] The phytoestrogen genistein has demonstrated the potential to act as a protective agent against hormone-dependent tumors such as breast cancer.[9] Recently, Ahn et al. reported that some prenylated isoflavonoids isolated from *Moghania philippinensis* (Leguminosae) showed antiestrogenic activity in the yeast two-hybrid assay.[10] These antiestrogenic compounds are expected to inhibit estrogen-dependent breast cancer proliferation.

This paper describes the isolation and structural elucidation of three new prenylated isoflavonoids, millewanins G (**1**) and H (**2**) and furowanin B (**3**), from the leaves of *M. pachycarpa* cultivated in Japan. In addition, we carried out primary screening of these new prenylated isoflavonoids by examining their antiestrogenic effect based on the inhibition of β-galactosidase activity induced by 17β-estradiol in the yeast two-hybrid assay.

The acetone extract of leaves of *M. pachycarpa* was fractionated by silica gel column chromatography and preparative TLC to obtain three new and five known isoflavones.

Millewanin G (**1**) was obtained as a colorless oil, $[\alpha]_D^{24}$ +7.0 (MeOH), having the molecular formula $C_{25}H_{26}O_7$. The IR spectrum exhibited bands at ν_{max} 3599, 3536, 3227br, and 1645 cm^{-1} due to hydroxy and carbonyl groups, respectively. The UV spectrum was similar to that of 3-(3′,4′-dihydroxyphenyl)-5,7-dihydroxy-6,8-bis-(3-methyl-2-butenyl)-4*H*-1-benzopyran-4-one (6,8-di-γ,γ-dimethylallylorobol),[11,12] which co-occurred with **1**. In the ^{1}H NMR spectrum (Table 1), a downfield signal at δ = 13.51 was assigned to a hydrogen-bonded hydroxyl group at C-5. The ^{1}H and ^{13}C NMR signals at δ_H = 8.22 and δ_C = 154.3, respectively, were assignable to H-2 and C-2, characteristic of the isoflavone nucleus. In the aromatic proton region, the appearance of only one set of ABC-type signals at δ = 7.15, 6.94, and 6.86 assignable to a 1,3,4-trisubstituted aromatic ring, indicated the presence of a fully substituted A ring in the isoflavone skeleton. The ^{1}H NMR spectrum (Table 1) also showed signals due to prenyl and 2-hydroxy-3-methylbut-3-enyl moieties. The observation of a characteristic MS fragment ion at *m*/*z* = 367, arising from the loss of [•C_4H_7O] from the molecular ion, supported the presence of a 2-hydroxy-3-methylbut-3-enyl moiety. To determine the locations of the substituents on the A ring of the isoflavone skeleton, an HMBC experiment (Figure 2) was carried out. The C−H long-range correlations from both C-5 and C-7 to H-1″ and from both C-8a and C-7 to H-1‴ suggested the location of the 2-hydroxy-3-methylbut-3-enyl moiety at C-6 and the prenyl moiety at C-8.



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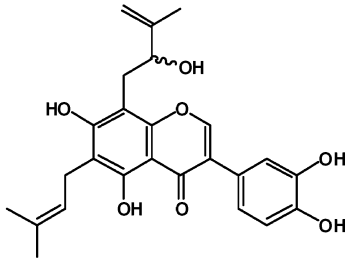

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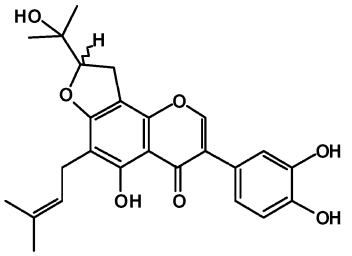

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Figure 1. Structures of isoflavonids from *Millettia pachycarpa*.

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10.1021/np050341w CCC: $33.50
Published on Web 12/22/2005

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of Millewanin G (**1**), Millewanin H (**2**), and Furowanin B (**3**)[a]

	millewanin G (**1**)[b]		millewanin H (**2**)[b]		furowanin B (**3**)	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	8.22 (s)	154.3	8.20 (s)	154.0	7.77 (s)	152.5
3		123.6		123.6		123.5
4		182.0		182.1		181.1
4a		105.9		106.0		105.8
5		158.8		159.1		160.0
5-OH	13.51 (s)		13.37 (s)		13.03 (s)	
6		110.1		113.2		107.7
7		162.0		162.1		164.7
8		107.8		104.6		102.8
8a		154.7		154.3		151.0
1′		123.9		123.9		123.0
2′	7.15 (d, 2.2)	117.3	7.14 (d, 2.2)	117.3	6.97 (br s)	116.3
3′		145.6		145.6		143.7
4′		146.2		146.2		144.4
5′	6.86 (d, 8.4)	115.9	6.86 (d, 8.4)	115.9	6.76 (d, 8.1)	115.3
6′	6.94 (dd, 8.4, 2.2)	121.5	6.93 (dd, 8.4, 2.2)	121.4	6.80 (br d, 8.1)	121.4
1″	2.90[c]	29.8	3.37 (2H, d, 7.0)	22.4	3.33 (2H, m)	21.9
	3.13 (dd, 15.7, 2.2)					
2″	4.40 (br d, 8.1)	77.3	5.26 (m)	123.4	5.27 (m)	121.4
3″		147.8		131.4		132.3
4″	4.81 (br s)	110.5	1.77 (3H, s)	17.9	1.78 (3H, s)	17.8
	5.01 (br s)					
5″	1.83 (3H, s)	18.6	1.63 (3H, s)	25.9	1.68 (3H, s)	25.8
1‴	3.45 (2H, d, 7.3)	22.4	2.98 (dd, 15.0, 8.1)	29.8	3.23 (2H, m)	27.2
			3.19 (br d, 15.0)			
2‴	5.23 (m)	123.4	4.44 (br d, 8.1)	77.3	4.79 (t, 8.4)	91.1
3‴		131.8		147.7		72.1
4‴	1.80 (3H, s)	17.9	4.83 (br s)	110.9	1.38 (3H, s)	25.8
			5.01 (br s)			
5‴	1.64 (3H, s)	25.8	1.85 (3H, s)	18.5	1.25 (3H, s)	24.1
other	10.34 (br s, OH)		10.21 (br s, 7-OH)		6.38 (br s, OH)	
	8.00 (br s, OH)		8.07 (br s, OH)		5.89 (br s, OH)	
	6.20 (br s, OH)		8.03 (br s, OH)			
			6.10 (br s, OH)			

[a] Values in (δ_H and δ_C) ppm. All ^{1}H signals correspond to 1H, unless otherwise stated. Figures in parentheses are coupling constants (J) in Hz. [b] Spectra were taken in acetone-d_6. [c] Overlapped with HOD signal.

Further C−H correlations in the HMBC spectrum are shown in Figure 2. Next, we examined the optical purity of millewanin G (**1**), $[\alpha]_D^{24}$ +7.0 (MeOH), using a Chiralpak AD-H HPLC column. From the results of this analysis, millewanin G (**1**) was found to occur as an enantiomeric mixture in the ratio 10:11. The absolute configuration of the major enantiomer of **1** remained undetermined because of the small amount of millewanin G (**1**) isolated from the natural source. On the basis of these data, the structure of millewanin G is proposed as shown by formula **1** in Figure 1.

Millewanin H (**2**) was isolated as a colorless oil, $[\alpha]_D^{24}$ +8.8 (MeOH). Chiral HPLC analysis again showed a 10:11 ratio of enantiomers, the same as **1**. The molecular formula of **2** ($C_{25}H_{26}O_7$) was the same as that of **1** by HREIMS. The ^{1}H NMR spectrum (Table 1) showed signals due to a hydrogen-bonded hydroxyl, three hydroxyls, a characteristic H-2 singlet, a 1,3,4-trisubstituted aromatic ring, a 2-hydroxy-3-methylbut-3-enyl moiety, and a prenyl moiety on the isoflavone skeleton, the same as **1**. The signal pattern of the ^{1}H NMR spectrum of **2** resembled that of **1**, except for chemical shifts of the methine proton bearing a hydroxy group on the 2-hydroxy-3-methylbut-3-enyl moiety. On the basis of the results of the HMBC experiment (Figure 2), the locations of the prenyl moiety at C-6 and the 2-hydroxy-3-methylbut-3-enyl moiety at C-8 suggest a locational interchange compared with the same moieties in **1**, as well as the presence of a 5,7,3′,4′-tetrahydroxy isoflavone skeleton. On the basis of these data, the structure of millewanin H is proposed as shown by formula **2** in Figure 1.

Furowanin B (**3**) was obtained as a colorless oil, $[\alpha]_D^{24}$ +6.5 (MeOH), and also consisted of a mixture of each enantiomeric component in the ratio 11:10 by chiral HPLC analysis. It was determined to have the molecular formula $C_{25}H_{26}O_7$ by HREIMS. The UV spectrum was similar to those of **1** and **2**, suggesting the presence of a 5,7,3′,4′-tetraoxygenated isoflavone skeleton. The ^{1}H NMR spectrum (Table 1) indicated signals typical of a prenyl moiety, a 1,3,4-trisubstituted aromatic ring, two hydroxyl groups (3′,4′-OH), and a characteristic 1H singlet (H-2), along with a hydrogen-bonded hydroxyl group (5-OH). Furthermore, a dihydrofuran ring bearing a 1-hydroxy-1-methylethyl moiety at C-2‴ was suggested by the observation of signals assignable to two quaternary methyl groups (δ_H = 1.38 and 1.25; δ_C = 25.8 and 24.1) linked to an oxygenated carbon (δ_C = 72.1) and ABC-type protons due to an oxymethine (δ_H = 4.79; δ_C = 91.1) linked to a benzylic CH_2 group (δ_H = 3.23; δ_C = 27.2). The arrangement of these substituents on the isoflavone nucleus was revealed by HMBC correlations (Figure 2). The hydrogen-bonded 5-hydroxyl signal correlated with both 4a- and 6-quaternary carbons. H-1″ showed correlations both with C-5 and C-7. The correlations between C-7 and H-2‴ and between C-8 and H-1‴ revealed the orientation of the dihydrofuran ring. These results confirmed the proposed structure of **3**. On the basis of these data, the structure of furowanin B was concluded to be that shown in formula **3** (Figure 1).

The known isoflavones, warangalone,[13] isoerysenegalensein E,[14] 8-γ,γ-dimethylallylwighteone,[11] euchrenone b_{10},[15] and 6,8-di-γ,γ-dimethylallylorobol,[11] were isolated and identified by referring to published spectroscopic data.

Antiestrogenic Activity. The prenylated isoflavonoids (**1**, **2**, and **3**) were tested for their antiestrogenic activity based on the inhibition of β-galactosidase activity induced by 17β-estradiol (E2) in the yeast two-hybrid assay.[8,16] The antiestrogenic activity of these isoflavonoids was determined under the conditions at which these compounds did not inhibit the growth of cells due to cytotoxic effects. Isoflavonoids (**1**, **2**, and **3**) showed potent dose-dependent inhibitory effects on β-galactosidase activity induced by E2 (Figure 3). The IC_{50} values (a 50% inhibitory concentration of 1 nM E2 activity) of millewanin G (**1**), millewanin H (**2**), and furowanin B

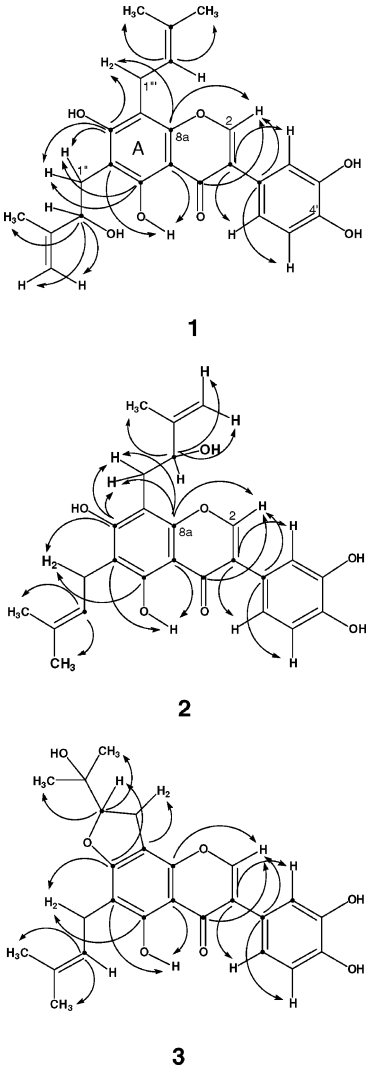

Figure 2. C−H long-range correlations in the HMBC spectrum of millewanin G (**1**), millewanin H (**2**), and furowanin B (**3**).

(**3**) were 29, 18, and 13 μM, respectively. These values were slightly higher than that of 4-hydroxytamoxifen (OHT, IC_{50} 4.4 μM), a typical ER antagonist.[17] Among these isoflavonoids, furowanin B (**3**) exhibited the most significant inhibition.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR, COSY, HMQC, HMBC (J = 8 Hz), and NOE were measured using a JNM A-400, an A-600, and/or an ECP-500 (JEOL) spectrometer. Chemical shifts are shown in δ (ppm) with TMS as an internal reference. All mass spectra were recorded under EI conditions, unless otherwise stated, using an HX-110 (JEOL) and/or a JMS-700 (JEOL) spectrometer having a direct inlet system. UV spectra were recorded on a UVIDEC-610C double-beam spectrophotometer (JASCO) in MeOH, and IR spectra on an IR-230 (JASCO) in $CHCl_3$. Preparative TLC was done on a Kieselgel 60 F_{254} (Merck).

Plant Material. The plant materials used in this study, *M. pachycarpa* cultivated at Higashiyama Zoo & Botanical Garden (Nagoya) in Japan, were collected in January 1998. A voucher specimen was deposited in Meijo University under number MUY0111.

Extraction and Separation. The dried leaves (461 g) of *M. pachycarpa* were extracted with acetone at room temperature, and the solvent was evaporated under reduced pressure to give the acetone extract (17.3 g). The acetone extract was subjected to silica gel column chromatography eluted with hexane−acetone (6:1, 4:1, 3:1, 2:1, 1:1, 1:4), acetone, and MeOH, successively, to separate eight fractions. Successive treatment of each fraction with the silica gel column and preparative TLC using appropriate combinations of solvents (hexane, EtOAc, $CHCl_3$, CH_2Cl_2, acetone, iPr_2O, benzene, and MeOH) as eluting or developing solvents gave the following compounds. Fraction 3 (hexane−acetone, 3:1) gave warangalone (53.1 mg); fraction 4 (hexane−acetone, 2:1) gave 8-γ,γ-dimethylallylwighteone (22.0 mg), isoerysenegalensein E (7.0 mg), 6,8-di-γ,γ-dimethylallylorobol (50.9 mg), euchrenone b_{10} (11.0 mg), millewanin G (**1**, 2.7 mg), millewanin H (**2**, 3.9 mg), and furowanin B (**3**, 4.7 mg).

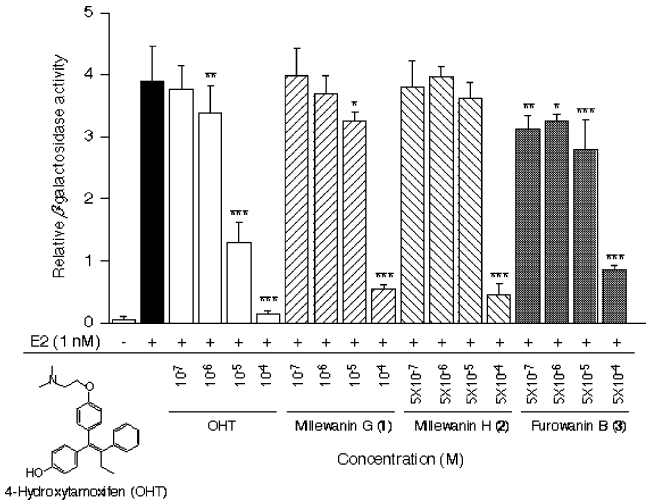

Figure 3. Inhibitory effects of the isoflavonoids (**1**, **2**, and **3**) and 4-hydroxytamoxifen (OHT) on β-galactosidase activity induced by 17β-estradiol (E2) in the yeast two-hybrid assay. The yeast strain was incubated with 1 nM E2 in the presence or absence of the test compounds. The bar at each point is the standard error of three independent experiments (n = 5). Significant differences in the inhibition of β-galactosidase activity of 1 nM E2 are shown at p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***).

Millewanin G (1): colorless oil; $[\alpha]_D^{24}$ +7.0 (c 0.108, MeOH); UV (MeOH) λ_{max} 204, 216sh, 272, 344 nm; IR ($CHCl_3$) ν_{max} 3599, 3536, 3227br, 1645, 1625 cm^{-1}; ^{1}H NMR (acetone-d_6, 600 MHz) and ^{13}C NMR (acetone-d_6, 150 MHz), see Table 1; EIMS m/z 438 (M^+, 11), 367 (39), 311 (100), 283 (8); HRMS m/z 438.1607 (calcd for $C_{25}H_{26}O_7$, 438.1616); HPLC Chiralcel AD-H (250 × 4.6 mm), hexane/2-propanol (90:10), flow rate 1.00 mL/min, UV detection 254 nm. Minor isomer of **1**: t_R = 3.4 min; major isomer of **1**: t_R = 6.0 min, ratio 10:11; CD (MeOH) (10:11 mixture of enantiomers) $[\theta]_{236}$ +1310, $[\theta]_{271}$ 0, $[\theta]_{300}$ −1438, $[\theta]_{346}$ 0, $[\theta]_{365}$ +609.

Millewanin H (2): colorless oil; $[\alpha]_D^{24}$ +8.8 (c 0.057, MeOH); UV (MeOH) λ_{max} 204, 216sh, 270, 348 nm; IR ($CHCl_3$) ν_{max} 3545, 3228br, 1647, 1618 cm^{-1}; ^{1}H NMR (acetone-d_6, 600 MHz) and ^{13}C NMR (acetone-d_6, 150 MHz), see Table 1; EIMS m/z 438 (M^+, 7), 422 (13), 368 (70), 311 (68), 239 (41), 216 (100); HRMS m/z 438.1694 (calcd for $C_{25}H_{26}O_7$, 438.1679); HPLC Chiralcel AD-H (250 × 4.6 mm), hexane/2-propanol (90:10), flow rate 1.00 mL/min, UV detection 254 nm. Minor isomer of **2**: t_R = 1.0 min; major isomer of **2**: t_R = 3.6 min, ratio 10:11; CD (MeOH) (10:11 mixture of enantiomers) $[\theta]_{237}$ +2042, $[\theta]_{265}$ 0, $[\theta]_{298}$ −2553, $[\theta]_{352}$ 0, $[\theta]_{369}$ 487.

Furowanin B (3): colorless oil; $[\alpha]_D^{24}$ +6.5 (c 0.093, MeOH); UV (MeOH) λ_{max} 206, 216sh, 272, 350 nm; IR ($CHCl_3$) ν_{max} 3545, 3264br, 1653, 1626 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz), see Table 1; EIMS m/z 438 (M^+, 93), 420 (100), 405 (49), 402 (59), 395 (77), 383 (99), 365 (92), 352 (42), 349 (87), 347 (43), 323 (79), 311 (61), 295 (43); HRMS m/z 438.1692 (calcd for $C_{25}H_{26}O_7$, 438.1679); HPLC Chiralcel AD-H (250 × 4.6 mm), hexane/2-propanol (3:1), flow rate 1.50 mL/min, UV detection 254 nm. Major isomer of **3**: t_R = 7.4 min; minor isomer of **3**: t_R = 12.0 min, ratio 11:10.

Antiestrogenic Activity Experiments. The yeast two-hybrid assay was carried out according to the method of Nishihara et al.[8] and Nishikawa et al.[16] Briefly, yeast cells expressing the estrogen receptor α (ERα) were grown overnight at 30 °C, with shaking in synthetic defined (SD) medium, lacking tryptophan and leucine. Test compounds, 4-hydroxytamoxifen (OHT, Aldrich Chemical Co., Milwaukee, WI) and 17β-estradiol (E2, Wako Pure Chemical Industries, Ltd., Osaka, Japan), were dissolved in DMSO, and the solution was diluted with SD medium to the appropriate concentrations to obtain the sample

solutions. The yeast cell suspension (90 μL, OD_{630} = 0.1) and the sample solution (5 μL, DMSO less than 10%) were added with the E2 solution (5 μL, 1 nM) to each well of a 96-well microtiter plate and incubated at 30 °C for 18 h. The growth of the yeast cells was monitored by measuring the optical density at 630 nm (OD_{630}) using a microplate reader (model 550, Bio-Rad Laboratories Inc., Hercules, CA). To disrupt the yeast cells, 4 × Z-buffer (33 μL) containing zymolyase-20T (4 mg/mL, Seikagaku Corp., Tokyo, Japan) was added to each well and incubated at 30 °C for 30 min. Then, 25 μL of 0.5 mg/mL chlorophenolred-β-D-galactopyranoside (CPRG, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added and incubated at 30 °C for 1 h. After incubation, 50 μL of 2 M Na_2CO_3 was added to stop the reaction. The absorbance of each well was measured at 540 and 630 nm (A_{540} and A_{630}). β-Galactosidase activity was calculated using the following formula:

$$\text{relative } \beta\text{-galactosidase activity} = (A_{540} - A_{630})/\mathrm{OD}_{630}$$

To examine antiestrogenic activity, the inhibition of β-galactosidase activity induced by 1 nM E2 was measured. The IC_{50} value of each compound was obtained from the concentration providing 50% activity of E2 only.

Acknowledgment. This work was supported in part by Grants-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science, High-Tech Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

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NP050341W