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"Kwao Keur", *Pueraria mirifica*. The
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**JOURNAL OF
NATURAL
PRODUCTS®**

Reprinted from
Volume 63, Number 2, Pages 173-175

JOURNAL OF NATURAL PRODUCTS

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Volume 63, Number 2

February 2000

Rapid Communications

Identification of Deoxymiroestrol as the Actual Rejuvenating Principle of "Kwao Keur", *Pueraria mirifica*. The Known Miroestrol May Be an Artifact

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Received November 1, 1999

Abstract: Miroestrol (**1**) has been isolated previously as an active principle from "Kwao Keur" (*Pueraria mirifica*), a rejuvenating folk medicine from Thailand. Reinvestigation using bioassay-guided purification has resulted in the isolation of a new potent phytoestrogen, deoxymiroestrol (**2**). The facile aerial oxidation of **2** into **1** suggests the possibility that **1** may be an artifact.

In Thailand, the plant "Kwao Keur" is considered to be a rejuvenating drug and has a fascinating history in folk medicine.¹ Although the name "Kwao Keur" had been applied to several species of plants having tuberous roots, it was definitively identified as *Pueraria mirifica* in 1952,² from which an unusual estrogenic phenol miroestrol^{1,3} (**1**) was isolated as an active principle eight years later.⁴ Although the possible presence of an alternative active component was suggested,^{4c} there has been no isolation of any other powerful phytoestrogens, with further studies leading instead to the isolation of isoflavones and

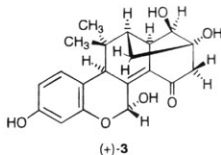
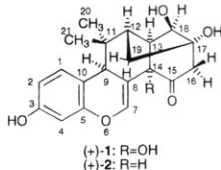


Figure 1. Structures of (+)-miroestrol (**1**), (+)-deoxymiroestrol (**2**), and (+)-isomiroestrol (**3**).

coumestans.⁵ Therefore, **1** has been considered to be the actual phytoestrogen of *P. mirifica* and shows the highest activity of all estrogens of plant origin.

In the present communication the bioassay-guided separation has resulted in the successful isolation of a new phytoestrogen, deoxymiroestrol (**2**), together with **1** and isomiroestrol³ (**3**). The growth-promoting effect of phytoestrogens on MCF-7 human breast cancer cells showed the strongest activity with **2**. Furthermore, **2** was easily converted into **1** and **3** by aerial oxidation. In our investigation we have identified deoxymiroestrol (**2**) as the actual phytoestrogen of *P. mirifica*.

Separation of the active components from the ethyl acetate extract of the roots of *P. mirifica* by chromatographic techniques resulted in the isolation of three compounds, among which the middle polarity component was identified as the known (+)-miroestrol (**1**) ($2.0 \times 10^{-3}\%$ yield from the dried roots) by comparison with an authentic sample.^{1,3,6} The absolute configuration of (+)-**1** has been

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Table 1. NMR Data^a of Miroestrol (1), Deoxymiroestrol (2), and Isomiroestrol (3)

carbon	1	2	3
1	6.99 (d, $J = 8.4$) [131.2]	6.96 (d, $J = 8.4$) [131.6]	6.99 (d, $J = 8.4$) [131.7]
2	6.50 (dd, $J = 8.4, 2.5$) [112.0]	6.44 (dd, $J = 8.4, 2.6$) [111.2]	6.46 (dd, $J = 8.4, 2.5$) [110.2]
3	— [158.3]	— [158.1]	— [158.5]
4	6.30 (d, $J = 2.5$) [103.7]	6.27 (d, $J = 2.6$) [103.3]	6.37 (d, $J = 2.5$) [106.9]
5	— [153.8]	— [154.0]	— [154.9]
6	— [—]	— [—]	— [—]
7	6.29 (s) [139.7]	6.45 (s) [138.2]	5.46 (s) [90.9]
8	— [116.5]	— [111.1]	— [146.4]
9	3.31 (s) [40.8]	3.23 [43.2]	3.07 [47.0]
10	— [113.5]	— [112.5]	— [116.1]
11	— [38.0]	— [38.0]	— [30.7]
12	2.74 (ddd, $J = 12, 9.5, 5.5$) [45.6]	2.75 (dd, $J = 12, 5.8$) [47.1]	3.02 (m) [42.2]
13	2.43 (d, $J = 5.5$) [51.2]	2.61 (d, $J = 7.7$) [42.4]	2.70 (d, $J = 5.1$) [46.8]
14	— [79.7]	3.27 (s) [51.3]	— [138.9]
15	— [210.5]	— [210.8]	— [199.8]
16	2.58 (1H, dd, $J = 18.5, 2$) 2.90 (1H, d, $J = 18.5$) [55.3]	2.58 (1H, m) 2.81 (1H, d, $J = 18.4$) [56.2]	2.53 (2H, s) [54.3]
17	— [78.0]	— [77.8]	— [78.3]
18	3.69 (s) [79.3]	3.79 (s) [80.4]	3.94 (s) [78.5]
19	1.88 (1H, dd, $J = 14, 8.6$) 1.99 (1H, br t, $J = 12.1$) [40.7]	1.85 (1H, dd, $J = 14, 8.1$) 2.05 (1H, br t, $J = 13$) [38.9]	1.60 (1H, dd, $J = 14.2, 8.4$) 1.92 (1H, dd, $J = 13.5, 10.6$) [39.3]
20	0.56 (s) [22.5]	0.55 (s) [22.0]	0.40 (s) [22.9]
21	1.23 (s) [32.5]	1.15 (s) [32.7]	1.19 (s) [31.7]

^a ¹H NMR (600 MHz in CD₃OD) are reported downfield from internal TMS at 0.00 ppm, and coupling constants are quoted in hertz (Hz). ¹³C NMR assignments are related to internal CD₃Cl at 77.00 ppm, and the data are given in square brackets. ¹H and ¹³C NMR assignments are based on decoupling, DEPT, differential NOE, C-H COSY or HMQC, and HMBC experiments.

recently determined as shown in Figure 1 by its enantioselective total synthesis.⁶

The least polar component, **2**, [α]_D²⁵ +217° (MeOH), was obtained as colorless prisms, mp 213–216 °C (dec), in 2.0 × 10⁻³% yield. The molecular formula was deduced to be C₂₀H₂₂O₅ from the HRFABMS, indicating the absence of one oxygen atom compared to **1** (C₂₀H₂₂O₆). A signal pattern in the proton (¹H: 600 MHz) and carbon (¹³C: 150 MHz) NMR spectra of **2** analogous to those of **1** (Table 1) suggested that the two components have a common skeleton with the same stereochemistry. The ¹³C NMR spectrum showed that the new component **2** was composed of eleven sp³ and nine sp² carbons in common with **1**. An oxygen-bonded sp³ quaternary carbon (C-14) resonating at δ 79.7 ppm in **1** was shifted upfield to δ 51.3 ppm, appearing as a tertiary carbon in the ¹³C NMR spectrum of **2**. The corresponding methine proton was observed at δ 3.27 ppm as a singlet in the ¹H NMR spectrum. NOE enhancements between this singlet and H-18 (δ 3.79) and H-13 (δ 2.61), respectively, indicated the same stereochemical orientation of the two components. Thus, the new compound was deduced to be (+)-deoxymiroestrol (**2**), in which the C-14 hydroxyl group in **1** has been replaced by a hydrogen atom with retention of configuration.

The most polar component, **3**, [α]_D²⁵ +78° (MeOH), was obtained as colorless prisms, mp 158–161 °C (dec), in 1.9 × 10⁻³% yield. The HRFABMS (C₂₀H₂₂O₆) of **3** indicated it to be an isomeric product of **1**. The ¹H and ¹³C NMR spectra of **3** (Table 1) showed not only the change of an sp² carbon corresponding to C-7 [δ _H 6.29 (s); δ _C 139.7] in **1** into an sp³ carbon with a hemiacetal group (δ _H 5.46; δ _C 90.9) but also the presence of a tetrasubstituted α,β -unsaturated ketone functionality [δ _C 138.9 (C α) 146.4 (C β), and 199.8 (C=O)], suggesting that **3** possesses a structure³ resulting from acid-catalyzed isomerization of **1**, although the stereochemistry at C-7 has not been determined definitively. Irradiation of H-9 (δ 3.07) caused NOE enhancements of both H-12 (δ 3.02) and H-13 (δ 2.70), but not H-7 (δ 5.46), lending circumstantial support to a distal relationship between H-7 and H-9. Thus, (+)-isomiroestrol (**3**) could formally result from hydroxyl group migration of **1** from C-14 to C-7 with

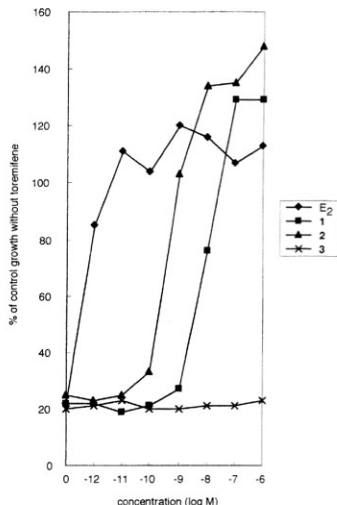
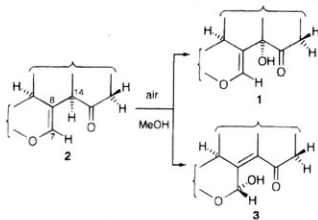


Figure 2. Growth-promoting effect of miroestrol (**1**), deoxymiroestrol (**2**), isomiroestrol (**3**), and 17 β -estradiol (E₂) on MCF-7 human breast cancer cells in the presence of toremifene, an estrogen antagonist.

retention of configuration concomitant with isomerization of the double bond at $\Delta^{7,8}$ to $\Delta^{8,14}$.

The estrogenic activity of miroestrol (**1**) was previously estimated to be about 2.5 × 10⁻¹ times that of 17 β -estradiol (E₂) in the rat vaginal cornification test,^{4b} and **1** was considered to be the compound with the highest estrogenic

Scheme 1. Aerial Oxidation of Deoxymiroestrol (2), Producing Two Isomeric Products, Miroestrol (1) and Isomiroestrol (3)



potency among the known phytoestrogens. The activities of miroestrol (1), deoxymiroestrol (2), and isomiroestrol (3) were estimated in the present investigation in terms of their growth-promoting effects on MCF-7 human breast cancer cells in the presence of toremifene, an estrogen antagonist (Figure 2). Strong activity was observed for 1 and 2, but the latter was found to be about 10-fold more potent. Interestingly, these phytoestrogens exhibited stronger growth than E_2 at the higher concentrations (> 10 nM) tested.

The α position of carbonyl functions is easily oxygenated with molecular oxygen in air under various conditions to afford the corresponding α -hydroxy compounds.⁷ This chemical property of carbonyl compounds suggests that miroestrol (1) and isomiroestrol (3) are artifacts derived from deoxymiroestrol (2) during isolation work. Hence, the facile aerial oxidation of 2 was observed under mild conditions, simply by keeping a solution of 2 in methanol at room temperature (Scheme 1). After 1 week, examination of the reaction mixture by HPLC and ^1H NMR spectroscopy indicated the production of both anticipated oxidation products. Therefore, we conclude that 1 and 3 are artifacts produced by the air oxidation of 2 during isolation work and that deoxymiroestrol (2) is the actual phytoestrogen of *P. mirifica*.

Experimental Section

General Experimental Procedures. ORD spectra were measured on a JASCO J-20 spectrometer. ^1H (600 MHz) and ^{13}C NMR (150 MHz) spectra were measured on a JEOL JNM LA600 (TMS as an internal standard). HRFABMS were obtained by a JEOL JMS-HX110 spectrometer using *p*-nitrobenzyl alcohol as a matrix.

Plant Material. The tuberous roots of *P. mirifica* Airy Shaw and Suvatibandhu (Leguminosae) were collected in the Lumpang province in northern Thailand in April 1996. Voucher

herbarium specimens (No. 23028) of the plant were identified and deposited at the Forest Herbarium (BKF), Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangken, Bangkok, Thailand.

Isolation of Deoxymiroestrol (2). The dried and powdered plant material (1.31 kg) was successively extracted for 10 h by Soxhlet apparatus using hexane (5 L \times 2), EtOAc (5 L \times 2), and EtOH (5 L \times 2). The combined EtOAc extracts (23.37 g) were fractionated by column chromatography (Si gel: 1:1 hexane- CHCl_3 , CHCl_3 , 1:1 CHCl_3 -EtOAc, EtOAc, 1:1 EtOAc-MeOH). The fraction of 1:1 CHCl_3 -EtOAc (1.2 g) was washed with MeOH (30 mL \times 2) in order to remove insoluble materials, and the residue obtained after evaporation of the extracts was purified by medium-pressure column chromatography (ODS-Si gel: 30%, 40%, 50%, 60%, and 70% aqueous MeOH). The eluent with 60% aqueous MeOH afforded 2 (0.0284 g, $2 \times 10^{-3}\%$ from the root) as colorless crystals, mp 213-216 $^\circ\text{C}$, which were recrystallized from EtOAc.

Bioassay. MCF-7 human breast cancer cells (ATCC HTB-22) were plated in 96-well microtiter plates (Sumilon, Tokyo, Japan) at a density of 1000 cells/well suspended in GIT medium (Japan Pharmaceutical Co., Ltd., Tokyo, Japan). Following overnight preincubation, 5 μM toremifene (Nippon Kayaku Co., Ltd., Tokyo, Japan) and the test samples were added at various concentrations to each well in triplicate, and the cells were further incubated for 7 days. After incubation, the cell number was determined by the colorimetric methylene blue assay. The absorbance was measured with a microplate reader using a 660 nm test filter (Dyex, Chantilly, VA). The growth rates were calculated by comparison with control experiments, which were carried out with toremifene.

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Acknowledgment. One of us (T.I.) gives special thanks to Shiratori Pharmaceutical Co., Ltd., Chiba, Japan, for the supply of the plant materials. We also thank Dr. J. L. Ingham of Reading University, Reading, U.K., for the gift of authentic miroestrol.

NP990547V