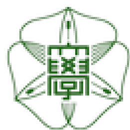
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ANTIFUNGAL ISOFLAVONE LUTEONE AND ITS METHYL ETHERS

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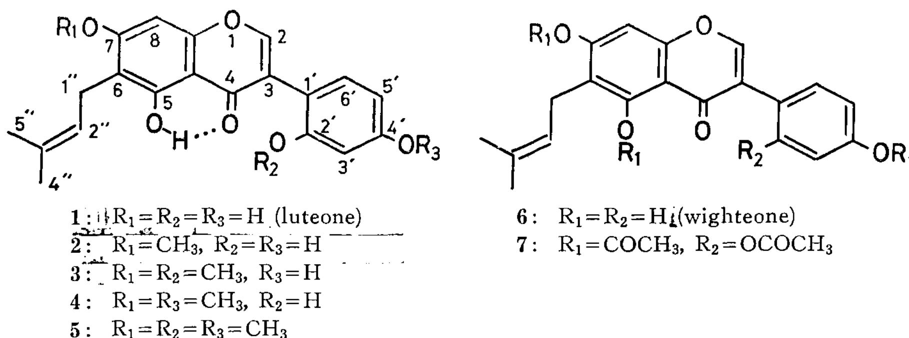
Introduction

An antifungal isoflavone luteone [**1**, 5,7,2',4'-tetrahydroxy-6-(3,3-dimethylallyl)isoflavone] was first isolated from the young fruits of *Lupinus luteus* (yellow lupin) and found to be strongly antifungal.[1] Considerable quantities of **1** and the related fungitoxin 5,7,4'-trihydroxy-6-(3,3-dimethylallyl)-isoflavone (wighteone, **6**) also occur on the surface of lupin leaves,[2,5] and they may also offer some degree of protection against potential fungal pathogens as pre-infectional antifungal agent (prohibitin).

Some derivatives of **1**, tetra-acetate (**7**), acid-catalyzed cyclization products (α- and β-isoluteone, **8** and **9**), and 7,2',4'-tri-*O*-methyl-luteone (**5**) have been prepared and the antifungal activity of β-isoluteone has revealed to be far less active than **1**.[1] In our earlier paper, we described that the fungal metabolites of luteone (**1**), for example, luteone hydrate (**10**), luteone glycol (**11**), dihydrofurano-isoflavone (**12**) and dihydrohydroxypyrano-isoflavone (**13**) are much less toxic than luteone (**1**) itself against *Cladosporium herbarum*.[12,13]

We have recently prepared four *O*-methyl derivatives of **1** and subjected them to antifungal bioassay to know some relationships between the structure



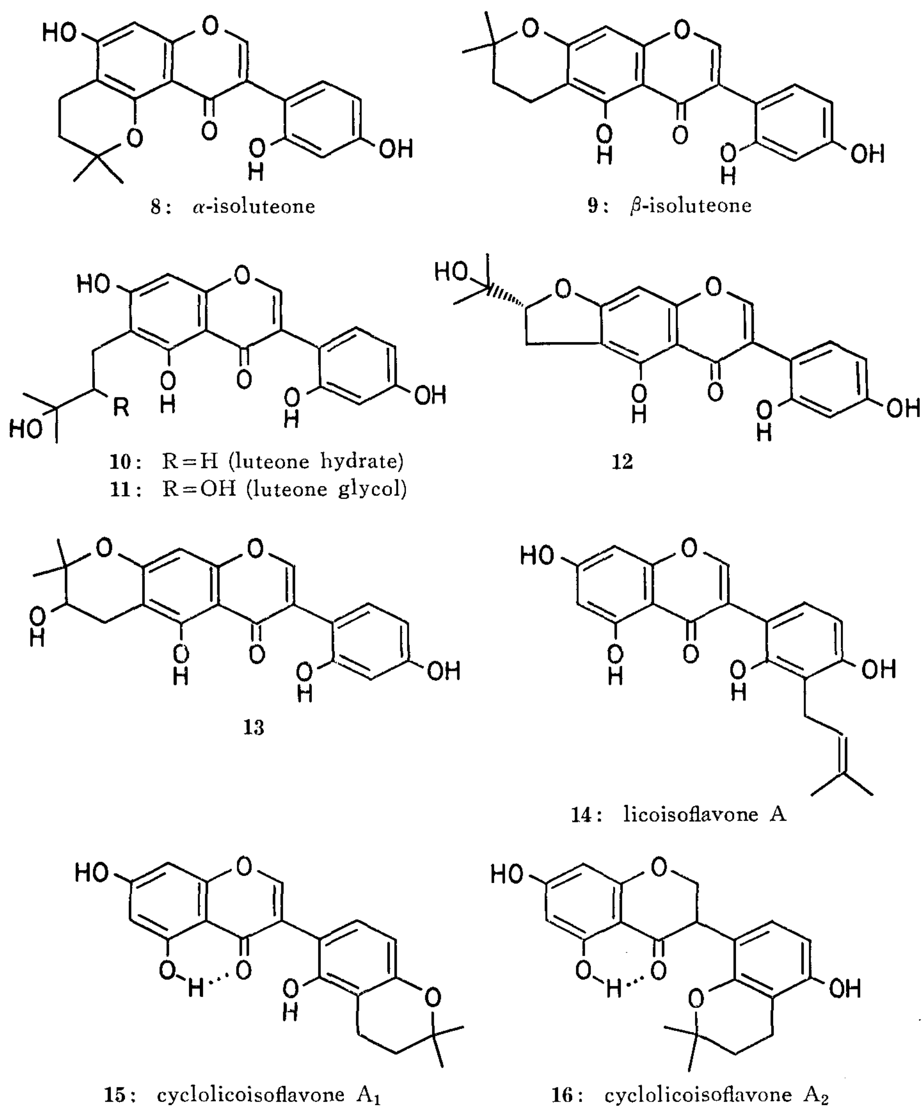


Fig. 1. Luteone and its derivatives, and related isoflavones mentioned in the text.

and antifungal activity of luteone derivatives. In this paper we present physico-chemical data for three new luteone methyl ethers (7-*O*-methyl-luteone **2**, 7, 2′-di-*O*-methyl-luteone **3**, and 7, 4′-di-*O*-methyl-luteone **4**) in addition to those of 7, 2′, 4′-tri-*O*-methyl-luteone (**5**)[1] and the results of bioassays on their antifungal activities comparing with that of luteone (**1**).

Results and Discussion

Luteone (**1**, 322 mg) isolated from the yellow lupin roots (*L. luteus* cv. Barpine) as reported previously[3] was partly methylated in a mixture of methanol and dichloromethane containing excess amounts of diazomethane. The methylation gave four methyl ethers detectable on TLC plates [*Rf* 0.34 (**2**), 0.43 (**3**), 0.73 (**4**) and 0.80 (**5**) developed in EtOAc/benzene=1 : 4]. The mixture of these methyl ethers was initialy subjected to silica gel column chromatography and the constituents (**2**~**5**) were eventually isolated from each other by preparative TLC (PTLC) (for details refer to Experimental section). The physico-chemical properties of four methyl ethers thus isolated were determined and the exact structure of each *O*-methyl derivative of **1** was inferred as follows.

There are some reliable methods for differentiation if one of the four phenolic OH's in **1** is derivatized or not.

[I] By the addition of NaOAc, UVλ_{max}^{MeOH} around 250~270 nm is shifted bathochromically (+3~10 nm) when the isoflavone has C-7-OH, but unchanged by a blocked C-7-OH (for example, by *O*-alkylation).[8]

[II] By the addition of $AlCl_3$, UVλ_{max}^{MeOH} around 250~270 nm is shifted bathochromically (+5~14 nm) when the isoflavone has C-5-OH, but unchanged by a blocked C-5-OH.[8]

[III] ^{1}H-NMR absorption signal for C-5-OH in an isoflavone is observed in the remarkably lower field (12~14 ppm) because of the strong H-bond formation with the C-4 carbonyl oxygen atom.[7]

[IV] Discriminative Gibbs test response (see ref. 6 and 11) :

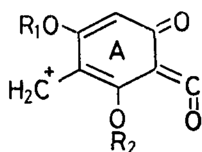
(i) Response to the reagent

- a) C-7-OH and C-4′-OH ; negative OH's because of *para*-substitution
- b) C-2′-OH (with 5′-unsubstituted), positive and rapid response
- c) C-5-OH (with 8-unsubstituted), positive and slow response

(ii) Colors formed with the reagent

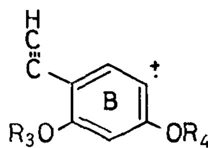
- a) C-5-, C-7-, C-2′- and C-4′-OH free : rapid and a purple-blue color [for example, **1** and licoisoflavone A (**14**)]
- b) C-5-, C-7- and C-2′-OH free and C-4′-OH blocked : rapid and a clear blue color [for example, cyclolicoisoflavone A_1 (**15**)]
- c) C-5-, C-7- and C-4′-OH free and C-2′-OH blocked : slow and a blue-green~dull blue color [for example, cyclolicoisoflavone A_2 (**16**)]

[V] MS fragments caused by the retro-Diels-Alder fission : Examples due to luteone derivatives ;[9]



a

$R_1=R_2=H$, *m/z* 165
$R_1=CH_3$, $R_2=H$, *m/z* 179
A-ring fragments



b

$R_3=R_4=H$, *m/z* 134
$R_3,R_4=(CH_3,H)$, *m/z* 148
$R_3=R_4=CH_3$, *m/z* 162
B-ring fragments

Detection of these fragments (or other fragments closely related to these fragments also) sometime gives clues to determine where the *O*-methylation has taken place (on A-ring and/or on B-ring).

Compound 2. From the molecular ion detected by MS at *m/z* 368 (that of luteone **1**+14 mass units) indicated **2** to be a mono-*O*-methyl ether [^{1}H-NMR signal for OCH_3, δ 4.00 (3H, s) (Table 2)]. The structure of 7-*O*-methyl-luteone for **2** was immediately concluded from two lines of evidence. First, two retro-Diels-Alder mass fragments *m/z* 179 [28%, mono-*O*-methylated A-ring fragment, **a** : (R_1, R_2)=H and CH_3] and *m/z* 134 (10%, unmethylated B-ring fragment, **b**: $R_3=R_4=H$) were clearly detected and secondly λ_{max}^{MeOH} at 266 nm was bathochromically shifted (10 nm) by the addition of $AlCl_3$ (C-5-OH free), but unchanged by the addition of NaOAc (C-7-OH blocked) (Table 1). ^{1}H-NMR detection of an H-bonded OH proton at δ 12.97 also supported the presence of underivatized C-5-OH (Table 2). The mono-methyl-luteone can thus be represented by structure **2** in Fig. 1.

Compounds 3 and 4. From the MS (both M^+ 382) and ^{1}H-NMR data [**3**: $2\times OCH_3$ δ 3.98 and 3.75 (both 3 H, two s); **4**: $2\times OCH_3$, δ 4.00 and 3.80 (both 3H, two s)], these two isolates were apparent to be isomeric dimethyl-luteones. As with compound **2** (7-*O*-methyl-luteone), their UVλ_{max}^{MeOH} at 265 nm or 266 nm showed no shift by the addition of NaOAc, but both showed bathochromic shifts by $AlCl_3$. Therefore the A-ring part structure for **3** or **4** must be the same to that of **2**. From the above evidence, it is clear that **3** and **4** are structural isomers in which the second methylation took place at 2′-OH in one compound and at 4′-OH in the other. The methylated position can in fact be easily and unambiguously determined by means of Gibbs test.[6,11] As reported earlier, the Gibbs test clearly discriminates cyclolicoisoflavone A_1 (**15**) from cyclolicoisoflavone A_2 (**16**) in terms of both the color and rate of color development. Thus **15** and **16** are Gibbs-

TABLE 1. UV Spectral Data for Luteone (**1**) and Its Methyl Ethers (**2**~**5**)

λ_{max} nm	Luteone* (**1**)	7-*O*-Methyl-luteone (**2**)	7,2′-Di-*O*-methyl-luteone (**3**)	7,4′-Di-*O*-methyl-luteone (**4**)	7,2′,4′-Tri-*O*-methyl-luteone (**5**)
in MeOH	218	230sh	230sh	230sh	230sh
	265.5	266	265	266	260sh
	292sh	287sh	287sh	287sh	265.5
		330sh	330sh	330sh	286sh
					330sh
+NaOMe	227sh	230sh	230sh	232sh	265.5
	279	270 (br.)	270 (br.)	264	287sh
	337	289sh	290sh	296	
+$AlCl_3$	215	233sh	235sh	234sh	234sh
	275	276	269	277	270
	316	310sh	277sh	312	277sh
	370	374 (br.)	310sh	373	310sh
			362		364
+NaOAc	272.5	266.5	265.5	266	265
	343	287sh	287sh	287sh	287sh
		330sh	330sh		

* Data in ref. 12.

TABLE 2. ^{1}H–NMR Data for Luteone (**1**) and Its Methyl Ethers (**2**~**5**) δ ppm in acetone-d_6 at 100 MHz and J in Hz

Proton	Luteone (1)*	7-*O*-Methyl-luteone (**2**)	7,2′-Di-*O*-methyl-luteone (**3**)	7,4′-Di-*O*-methyl-luteone (**4**)	7,2′,4′-Tri-*O*-methyl-luteone (**5**)
2-H	8.14 s	8.22 s	8.06 s	8.24 s	8.09 s
5-OH	13.05 s	12.97 s	13.24 s	12.95 s	13.21 s
8-H	6.53 s	6.69 s	6.63 s	6.70 s	6.64 s
3′-H	6.48 d incompl.	6.50 d incompl.	6.57 d $J=2.2$	6.55 d $J=2.4$	6.65 d $J=2.3$
5′-H	6.44 dd $J=8.9, 2.4$	6.45 dd $J=8.6, 2.4$	6.49 dd $J=8.2, 2.2$	6.54 dd $J=9.1, 2.4$	6.58 dd $J=8.1, 2.3$
6′-H	7.12 d $J=8.9$	7.14 d $J=8.6$	7.14 d $J=8.2$	7.23 d $J=9.1$	7.24 d $J=8.1$
1″-H	3.37 br. d $J=7.3$	3.35 br. d $J=7.1$	3.34 br. d $J=7.3$	3.35 br. d $J=7.1$	3.34 br. d $J=7.1$
2″-H	5.28 br. t $J=7.3$	5.33 br. t $J=7.1$	5.21 br. t $J=7.3$	5.21 br. t $J=7.1$	5.21 br. t $J=7.1$
4″-H	1.78 br. s	1.78 br. s	1.77 br. s	1.77 br. s	1.77 br. s
5″-H	1.65 br. s	1.65 br. s	1.64 d $J=1.0$	1.64 br. s	1.64 d $J=0.7$
7-OCH_3		4.00 s	3.98 s	4.00 s	3.98 s
2′-OCH_3			3.75 s		3.79 s
4′-OCH_3				3.80 s	3.85 s

* Data in ref. 12.

positive, but whilst on TLC plates the former compound rapidly afforded a clear blue color, the latter reacted much more slowly to give a blue-green product. The difference between **15** and **16** refrects the fact that **16** possesses only an H-bonded C-5-OH group capable of reacting with Gibbs reagent, whereas **15** additionally contains an unchelated hydroxyl group at C-2′. Non-substitution at a *para* position to a phenolic hydroxyl group is also an essential requirement if the Gibbs test is to be successful, and for this reason OH substituents at C-7 and C-4′ in isoflavones are not involved in the reaction. It is the 2′-OH substituent which allows **15** to react immediately with Gibbs reagent and thereby form an intense clear blue indophenol derivative.

When tested together with **15** and **16**, compound **4** rapidly gave a clear blue color as with **15** indicative of 2′-hydroxylation (and therefore methylation at 4′-OH). In contrast, **3** responded slowly to yield a blue-green product (turned reddish purple after several hours), and in this respect **3** resembled compound **16**. On the basis of these results, **3** and **4** were assigned to be 7,2′-di-*O*-methyl-luteone (**3**) and 7,4′-di-*O*-methyl-luteone (**4**), respectively. The MS fragments, *m/z* 191 (**c**) from **3**, and *m/z* 179 [**a** : (R_1, R_2)=H and CH_3]

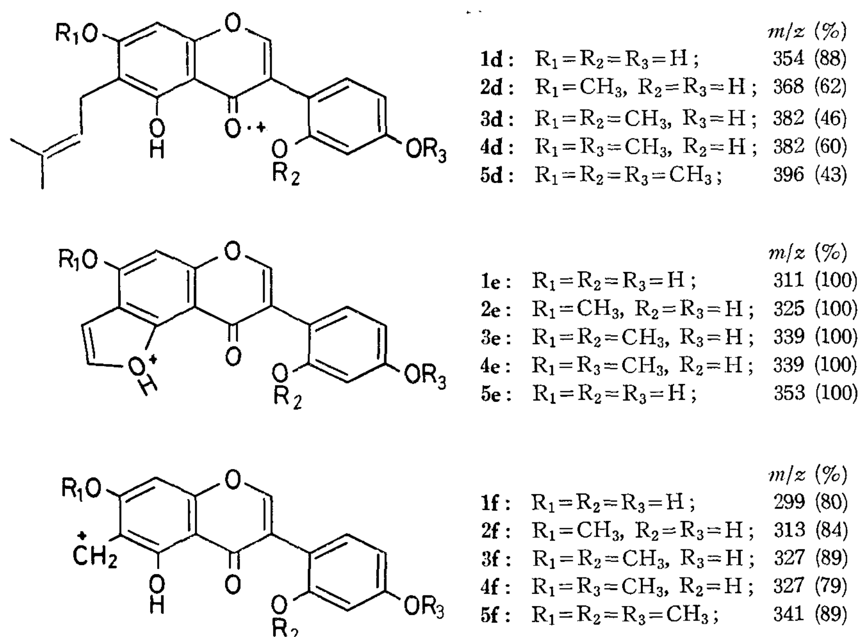
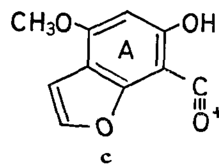


Fig. 2. Mass fragments (M^+, M^+-C_3H_7 and M^+-C_4H_7) for luteone (**1**) and luteone methyl ethers (**2**~**5**).

and 148 [**b**: (R_3, R_4)=H and CH_3] from 4 are well compatible with their proposed structures.



m/z 191 (A-ring fragment)

Compound 5. The forth methyl-luteone (**5**) was confirmed to be a trimethylated derivative (MS, M^+ 396). The melting point and spectroscopic properties (^{1}H-NMR, UV and MS) of **5** were reasonably identical to those of previously prepared 7, 2′, 4′-tri-*O*-methyl-luteone (**5** in Fig. 1).

The structures for partly methylated four derivatives of luteone (**1**) were thus confirmed and the results of UV spectroscopy, the assignments for ^{1}H-NMR signals and the characteristic MS fragments are respectively summarized in Table 1 (UV), Table 2 (^{1}H-NMR) and Fig. 2 (MS).

The antifungal properties of luteone methyl ethers (**2**~**5**) were determined together with luteone (**1**) as a standard compound, against the growth of *Cladosporium herbarum* using the TLC plate bioassay technique.[4,12] As shown in Table 3, less than 2 μg of **1** was sufficient to adversely affect fungal growth, and it gave prominent inhibition zones at applied levels of 25, 50 and 100 μg. However it was proved that not only 7-*O*-methylation, but also 7- and 2′- or 7- and 4′-di-*O*-methylation remarkably reduced the fungal toxicity of **1**. Trimethyl-luteone (**5**) was practically non-toxic against the test fungus.

TABLE 3. Relative Fungitoxic Activity of Luteone (**1**) and Its Methyl Ethers (**2**~**5**)

	Growth of *Cladosporium herbarum* as affected by the amount of isoflavone (μg) applied to each 14 mm dia. test zone*							
	100	50	25	12.5	6.25	3.1	1.6	0
Luteone (**1**)	+++	+++	+++	++	+	+	+	−
7-*O*-Methyl-luteone (**2**)	+++	++	++	+	+	−	−	−
7, 2′-Di-*O*-methyl-luteone (**3**)	++	++	+	−	−	−	−	−
7, 4′-Di-O-methyl-luteone (**4**)	++	+	−	−	−	−	−	−
7, 2′, 4′-Tri-*O*-methyl-luteone (**5**)	−	−	−	−	−	−	−	−

Each test compound was applied in acetone solution to give a 14 mm diameter zone on a Silica Gel 60 plate (Merck, F-254; layer thickness 0.25 mm). The plates were then sprayed with a spore suspension of *Cladosporium herbarum* and incubated under warm (25°C), moist conditions until fungal growth was apparent (2~3 days).

* +++ = complete inhibition; ++ = a small inhibition zone; + = slight growth retardation or partial inhibition; − = no-effect.

Experimental

General methods. Melting points (mp) were determined by the micro hot-plate method and are uncorrected. *Instrumentation*: Mass spectra (MS) were obtained on a JEOL JMS-DX300 instrument (direct inlet system, 70 eV ionization potential), and ^{1}H-NMR spectra on a JEOL FX-100 spectrometer. UV spectra were recorded according to the procedure presented by MABRY *et al.*[8] by using a Hitachi EPS-3T instrument. *TLC*: Analytical TLC and PTLC separations were carried out on Merck pre-coated Silica Gel 60 plates (F_{254}; layer thickness, 0.25 mm or 0.5 mm). Isoflavone derivatives were detected by inspecting developed TLC/PTLC plates under long (365 nm) and short (254 nm) wavelength UV light and by the characteristic colors formed with Gibbs reagent.[6] Compounds of interest were eluted from the silica gel with EtOAc. *Gibbs test*: See ref. 6, 10, 11 and 13 for details.

Bioassays. The antifungal activity of each methyl-luteone against *Cladosporium herbarum* was compared with that of luteone (**1**) using the TLC plate bioassay method[4] outlined in our earlier paper.[12]

Preparation and separation of luteone methyl ethers. Luteone (**1**, 322 mg) was dissolved in 5 ml MeOH and to which was added excess amounts of diazomethane in CH_2Cl_2. The mixture was maintained at 4°C for 1 hr, and the solvent and remaining diazomethane were evaporated. The remainder consisting of four products (compound **2** *Rf* 0.34, compound **3** *Rf* 0.43, compound **4** *Rf* 0.73 and compound **5** *Rf* 0.80 on TLC plates developed in EtOAc/benzene=1 : 4) was subjected to column chromatography over Wakogel C-200 (25 g). The charged constituents were eluted as follows: F-1, eluate with 50 ml of benzene; F-2, 50 ml of 3% EtOAc in benzene; F-3, 90 ml of 6% EtOAc in benzene; F-4, 25 ml of 6% EtOAc in benzene successively eluted; F-5, 50 ml of 10% EtOAc in benzene; F-6, 25 ml of 15% EtOAc in benzene; F-7, 25 ml of 15% EtOAc in benzene successively eluted, and F-8, 50 ml of 20% EtOAc in benzene. Compound **2** was found in F-8, compound **3** in F-7 and F-8, and compounds **4** and **5** in F-3, respectively. The concentrate of F-3 gave pale yellow needles of compound **4** (39.8 mg) and the mother liquor was subjected to PTLC in EtOAc/benzene= 1 : 5 to yield 57.5 mg of compound **5** and 38 mg of compound **4**. Compound **2** was isolated from F-7 and F-8 being combined and concentrated, by PTLC in EtOAc/benzene=1 : 4 and finally purified by re-PTLC in diethyl ether/benzene=4 : 6. The crude isolate of compound **3** obtained by the first PTLC of F-7+F-8 was recrystallized from acetone (29 mg) and the mother liquor and washings were concentrated and combined with the con-

centrate of F-6. The mixture was subjected to PTLC in EtOAc/benzene= 1 : 4 to yield 13 mg of pure **3**.

Properties of luteone methyl ethers. The UV and ^{1}H-NMR data for the following luteone methyl ethers are given in Tables 1 and 2, respectively.

7-O-Methyl-luteone (*Compound **2***) : Colorless fine rods from EtOAc/benzene, consisting of **2** and EtOAc (*ca.* 1 : 1 on ^{1}H-NMR). Mp : EtOAc complex, 65～67°C ; EtOAc free crystals, 179～180°C. $UV_{365\,nm}$ fluorescence : dark purple. Gibbs test response : (+), rapid ; purple-blue. MS *m/z* (%, at 250°C) : 369 (M^++1, 15), 368 (M^+, 62), 353 (M^+-CH_3, 19), 326 (21), 325 (M^+-C_3H_7, 100), 314 (17), 313 (M^+-C_4H_7, 84), 312 (15), 283 (9.7), 219 (9.4), 191 (8.2), 179 (28), 134 (10), 69 (8.8).

7, 2′-Di-O-methyl-luteone (*Compound **3***) : Pale brown rods, mp 196～197°C. UV_{365nm} fluorescence : dark purple. Gibbs test response : (+), slow ; blue-green → reddish purple. MS *m/z* (%, at 280°C) : 383 (M^++1, 12), 382 (M^+, 46), 367 (M^+-CH_3, 14), 340 (22), 339 (M^+-C_3H_7, 100), 328 (19), 327 (M^+-C_4H_7, 89), 191 (8.9), 176 (9.6), 147 (7.1).

7, 4′-Di-O-methyl-luteone (*Compound **4***) : Pale yellow needles, mp 151～152°C. UV_{365nm} fluorescence : dark purple. Gibbs test response : (+), rapid ; clear blue. MS *m/z* (%, at 270°C) : 383 (M^++1, 15), 382 (M^+, 60), 367 (M^+-CH_3, 16), 340 (22), 339 (M^+-C_3H_7, 100), 328 (17), 327 (M^+-C_4H_7, 79), 326 (15), 219 (11), 191 (12), 179 (32), 149 (12), 148 (16).

7, 2′, 4′-Tri-O-methyl-luteone (*Compound **5***) : Colorless fine needles, mp 134.5～135.5°C (lit.,[1] 136～137°C). UV_{365nm} fluorescence : dark purple. Gibbs test response : (+), slow, blue-green. MS *m/z* (%, at 200°C) : 397 (M^++1, 11), 396 (M^+, 43), 381 (M^+-CH_3, 12), 354 (23), 353 (M^+-C_3H_7, 100), 342 (19), 341 (M^+-C_4H_7, 89), 161 (8.1), 148 (15).

Summary

An antifungal isoflavone luteone [5, 7, 2′, 4′-tetrahydroxy-6-(3, 3-dimethylallyl)isoflavone, **1**] was methylated under mild conditions to give some partly methylated luteone derivatives (**2**～**5**). Four products, 7-*O*-methyl-, 7, 2′-di-*O*-methyl-, 7, 4′-di-*O*-methyl- and 7, 2′, 4′-tri-*O*-methyl-luteones were isolated and their chemical structures were unambiguously determined on the basis of their spectroscopic and chemical properties. The antifungal activity of **1** proved to be remarkably reduced by monomethylation at C-7-OH, or C-7-OH and C-4′-OH or C-2′-OH di-*O*-methylation.

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