A New Isoflavone with Antifungal Activity from Immature Fruits of *Lupinus luteus*

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A new isoflavone having antifungal activity was isolated from immature fruits of *Lupinus luteus* (Leguminosae), and named luteone. The structure was shown to be 5,7,2',4'-tetrahydroxy-6-(3,3-dimethylallyl)-isoflavone by degradative and spectroscopic studies.

Previously, we had isolated three new C₂₀-gibberellins, A₁₈,¹⁾ A₂₃,²⁾ and A₂₈,³⁾ abscisic acid⁴⁾ and its glucosyl ester⁵⁾ from the methanol extract of immature fruits of *Lupinus luteus* (Leguminosae). In the course of a continuing search for other biologically active substances from higher plants, we found that the benzene extract obtained from the titled fruits showed significant inhibitory activity against the conidia of *Cochliobolus miyabeanus* (*Helminthosporium oryzae*). An active component isolated was a new isoflavone, and named luteone. This paper presents the structure and biological activities of luteone.

The methanol extract obtained from immature fruits (60 kg) of *Lupinus luteus* was concentrated under reduced pressure. The aqueous concentrate, after adjustment to pH 3 with 6 N HCl, was extracted with benzene. The benzene extract was purified by chromatography successively over silicic acid (benzene-ethyl acetate) and over Florisil (benzene-ethyl acetate), affording a crude active material. Recrystallization from methanol-water gave luteone as pale yellow plates (550 mg), mp 225~227°C.

Luteone, from elemental analysis and mass spectrometric measurement, had the molecular formula C₂₀H₁₈O₆. The UV spectrum showed absorption maxima at 266 and 290 sh. m μ (ϵ 28300 and 14100), and the IR spectrum the absorption at 1655 cm⁻¹ assignable to

carbonyl function. These spectroscopic properties immediately suggested that luteone had an isoflavone skeleton.^{6~8)}

Treatment of luteone with dimethyl sulfate in acetone in the presence of anhydrous potassium carbonate gave a trimethyl ether (Ib), which was also prepared by treatment of luteone with ethereal diazomethane. The PMR spectrum (CCl₄) of the methyl ether (Ib) showed signals attributable to aromatic protons at δ 6.29(1H, s.), δ 6.35~7.22(3H, ABX system) and δ 7.72(1H, s.). The last characteristic low-field singlet was assignable to an isoflavonoid C₂-H.⁹⁾ Signals at δ 1.65 and 1.78(3H each, br.s.), δ 3.28(2H, br.d., $J=8$ Hz) and δ 5.15 (1H, br.t., $J=8$ Hz) indicated the presence of a 3,3-dimethylallyl group. In addition, the spectrum showed the signals assignable to three methoxy-groups (δ 3.78, 3.80 and 3.89, 3H each, s.) and one strongly chelated hydroxy-group (δ 12.91, 1H, s.), which was attributable to C₆-OH and failed to undergo methylation by the reagents described above. Acetylation of luteone with acetic anhydride-pyridine under reflux gave a tetraacetate (Ic), whose PMR spectrum exhibited the signals due to four acetoxy-groups at δ 2.04, 2.18, 2.22 and 2.31. These data indicated that luteone was a tetrahydroxyisoflavone having a 3,3-dimethylallyl substituent.

In the UV spectra (Table I) of luteone, the

TABLE I. THE UV ABSORPTION MAXIMA* ($m\mu$) OF LUTEONE

	MeOH	NaOAc ^{a)}	AlCl ₃ ^{b)}	AlCl ₃ /HCl ^{c)}	NaOAc ^{d)}	NaOAc/H ₃ BO ₃ ^{e)}
Luteone	266 290sh.	223 279 337	276 316sh.	277 313sh.	271 340sh.	267 290sh.

* All the spectra were obtained according to the procedure presented by T. J. Marbry *et al.* (cf. ref. 10).

a) The NaOMe spectrum was measured immediately after the addition of three drops of 2.5% NaOMe in methanol to a methanolic solution of luteone.

b) The AlCl₃ spectrum was measured immediately after the addition of six drops of 5% AlCl₃ in methanol to a methanolic solution of luteone.

c) The AlCl₃/HCl spectrum was recorded immediately after the addition of three drops of 2 N HCl to the cuvette containing the AlCl₃ (from step b).

d) The NaOAc spectrum was determined immediately after the addition of excess anhyd. NaOAc (powder) to a methanolic solution of luteone.

e) The NaOAc/H₃BO₃ spectrum was determined immediately after the addition of sufficient anhyd. H₃BO₃ to the cuvette which contained the NaOAc (from step d).

Ia : R = R' = H (luteone)

Ib : R = Me, R' = H

Ic : R = R' = Ac

IIa : R = H (α -isoluteone)

IIb : R = Me

IIIa : R = H (β -isoluteone)

IIIb : R = Me

AlCl₃ spectrum did not exhibit a bathochromic shift with respect to the AlCl₃/HCl spectrum, and a bathochromic shift was not detectable also on the addition of NaOAc/H₃BO₃ to a methanolic solution of luteone. These suggested that luteone neither contained *o*-dihydroxyl groups at C-6,7 nor at C-7,8.¹⁰⁾ On the other hand, a bathochromic shift (10 $m\mu$) was observed on the addition of AlCl₃ to the methanolic solution, and also NaOAc reagent caused the absorption maximum in methanol to shift 5 $m\mu$ bathochromically. These data indicated the presence of phenolic hydroxy-groups at C-5 and C-7.¹⁰⁾ The remaining two phenolic hydroxy-groups have to be associated with the B-ring.

The pattern of ABX system in the PMR spectra of Ia, Ib and Ic suggested the presence

of a 2,4-dihydroxyphenyl residue in luteone. This was established by the degradation of luteone methyl ether (Ib) with alkaline hydrogen peroxide;^{9, 11)} this afforded 2,4-dimethoxybenzoic acid. Thus, luteone was found to be a 5,7,2',4'-tetrahydroxy-isoflavone having a 3,3-dimethylallyl group. On the basis of evidence discussed above, the position of the 3,3-dimethylallyl group was restricted to either positions 6 or 8.

Luteone was treated with a small amount of conc. sulfuric acid in acetic acid to yield two isomers called α - and β -isoluteone (IIa and IIIa, respectively). Both PMR spectra (pyridine-d₅) of the isomers showed signals assignable to 2,2-dimethylchroman^{9, 12)} (α -isoluteone: 6H, s. at δ 1.47, 2H, t., $J=7$ Hz at δ 1.80 and 2H, t., $J=7$ Hz at δ 2.92, β -isoluteone: 6H, s. at δ 1.30, 2H, t., $J=7$ Hz at δ 1.71 and 2H, t., $J=7$ Hz at δ 2.73). These data indicated that α - and β -isoluteone were produced by an acid-catalized interaction of the 3,3-dimethylallyl group and two appositely placed hydroxygroup (at C-5 or C-7). The decision between two possible structures for α - and β -isoluteone followed from the recognition that the PMR spectrum of the α -isomer (IIa) showed no signal due to the chelated phenolic hydroxy-group, while that of the β -isomer (IIIa) exhibited the signal due to a hydroxy-group at C-5. Methylation of α - and β -isomers with ethereal diazomethane afforded a trimethyl ether (IIb) and a dimethyl ether

(IIIb) respectively, supporting the above assignment. Thus, the 3,3-dimethylallyl group was shown to be attached at C-6, and the structure Ia was assigned to luteone.

It is worthy of note that isoflavones having 2,4-dihydroxyphenyl residue as the B-ring are only auriculatin¹³⁾ and luteone among naturally-occurring isoflavones.

Some isoflavones have been known to have oestrogenic, insecticidal, piscicidal and antifungal activities.^{6, 14)} The antifungal substances in red clover have been investigated and formonetin was characterized as the agent active against *Sclerotinia trifoliorum* and *Fusarium nivale*.¹⁵⁾

TABLE II. THE INHIBITORY EFFECT OF LUTEONE AND β -ISOLUTEONE AGAINST THE CONIDIA OF *Cochliobolus miyabeanus*

Concentration ($\mu\text{g/ml}$)	The conidial germination (%)	
	Luteone	β -Isoluteone
1000	0	0
500	0	63.6
250	0	91.8
125	0	97.2
62.5	2.0	90.9
31.25	3.5	96.5
15.63	11.9	94.7
7.81	59.3	95.0
3.90	93.8	96.8
1.95	95.0	95.8
0	97.3	

The germination inhibitory effect of luteone against the conidia of *Cochliobolus miyabeanus* is shown in Table II. The half inhibition was obtained at a concentration of approximately 8 $\mu\text{g/ml}$. β -Isoluteone had about one-fiftieth inhibitory activity of luteone. α -Isoluteone was insoluble in solvents appropriate for biological studies.

The antimicrobial spectrum with 14 kinds of microorganisms is summarized in Table III. Luteone showed the activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Trichophyton mentagrophytes* and *T. rubrum*. The piscicidal activity (TLm, 24 hr) of luteone was demonstrated against killifish (*Oryzias latipes*) at a concentration of 3 $\mu\text{g/ml}$. β -Isoluteone was inactive or less active than luteone in all bioassays tested.

EXPERIMENTAL

Melting points were determined on a hot stage, and are uncorrected. IR spectra were recorded on Hitachi EPI-G3 and were calibrated with the bands of polystyrene at 3027.1, 1601.4 and 906.7 cm^{-1} . UV spectra were recorded with a Hitachi Perkin-Elmer EPS-3T spectrometer. Mass spectra were obtained with a Hitachi RMU-6D mass spectrometer (direct inlet system, 80 eV) and PMR spectra (60 MHz) on a Hitachi R-20 spectrometer. Chemical shifts in the PMR spectra are expressed in ppm from tetramethylsilane as an internal standard and coupling constants in

TABLE III. ANTIMICROBIAL SPECTRUM OF LUTEONE AND β -ISOLUTEONE

Test organism	MIC ($\mu\text{g/ml}$)		Method	Medium
	Luteone	β -Isoluteone		
<i>Aspergillus oryzae</i>	> 200	> 200	Agar dilution method	Potato-sucrose
<i>Cochliobolus miyabeanus</i>	> 200	> 200	"	"
<i>Fusarium oxysporum</i>	> 200	> 200	"	"
<i>Neurospora crassa</i>	200	200	"	"
<i>Rhizoctonia solani</i>	> 200	> 200	"	"
<i>Trametes sanguinea</i>	200	> 200	"	"
<i>Saccharomyces cerevisiae</i>	200	200	"	"
<i>Bacillus subtilis</i>	100	> 200	"	Bouillon
<i>Escherichia coli</i>	> 200	> 200	"	"
<i>Staphylococcus aureus</i>	$\leq$ 25	> 200	"	"
<i>Chlorella ellipsoidea</i>	200	> 200	"	Potato-sucrose
<i>Trichophyton rubrum</i>	10	—	Dilution method	Sabouraud's
" <i>mentagrophytes</i>	10	—	"	"
<i>Candida albicans</i>	> 10	—	"	"

Hz. Singlet, doublet, triplet and multiplet are abbreviated to s., d., t., and m., respectively. Merck silica gel GF₂₅₄ was used for thin-layer chromatography.

Bioassay: *conidial germination test of Cochliobolus miyabeanus.* The conidia of *C. miyabeanus* cultured on Czapek's medium at 28°C for 10~14 days were used. The test was performed according to the procedure reported by H. Asuyama *et al.*¹⁶⁾

Antimicrobial spectrum. Fourteen microorganisms listed in Table III were used for the test. Agar dilution method and dilution method were performed according to the procedure presented by R. Yamamoto.¹⁷⁾

Piscicidal test. The piscicidal test was carried out according to the procedure reported by K. Kawazu *et al.*¹⁸⁾

Isolation of luteone. The isolation procedure was controlled by the inhibitory effect against the conidia of *C. miyabeanus*. Methanol extract obtained from the immature fruits (60 kg) of *Lupinus luteus* was concentrated under reduced pressure. The aqueous concentrate, after adjustment to pH 3 with 6 N HCl, was shaken with benzene. The benzene extract (120 g) was chromatographed over silicic acid-Celite (1:2) by stepwise elution with benzene containing an increasing ratio (5%) of ethyl acetate. Elution with 30% ethyl acetate in benzene gave a crude active material (33 g). Further chromatography on Florisil column by stepwise elution with benzene containing an increasing ratio (5%) of ethyl acetate afforded a partially crystalline gum (830 mg, 15% ethyl acetate). Recrystallization from methanol-water gave luteone (550 mg) as pale yellow plates, mp 225~227°C, (Found: C, 67.61; H, 5.34. Calcd. for C₂₀H₁₈O₆: C, 67.79; H, 5.12%), and recrystallization from methanol-ethyl acetate-water as pale yellow rods, mp 111~113 and 225~226°C, (Found: C, 65.04; H, 6.02. Calcd. for C₂₀H₁₈O₆·CH₃COOC₂H₅: C, 65.15; H, 5.92%), TLC (ethyl acetate: benzene, 1:2): a characteristic reddish purple spot (*R*_f 0.51) after spraying with 5% vanillin in conc. sulfuric acid, PMR δ_{TMS} (DMSO-d₆): 1.63 (3H, br.s.), 1.73 (3H, br.s.), 3.24 (2H, br.d., *J*=8), 5.18 (1H, br.t., *J*=8), 6.18~7.02 (3H, ABX system), 6.35 (1H, s.), 8.08 (1H, s.), 9.20 (1H, s.), 9.31 (1H, s.), 10.78 (1H, s.) and 13.21 (1H, s.); (pyridine-d₅): 1.68 (3H, br.s.), 1.87 (3H, br.s.), 3.76 (2H, br.d., *J*=8), 5.73 (1H, br.t., *J*=8), 6.64 (1H, s.), 6.71~7.57 (3H, ABX system), 8.22 (1H, s.) and 13.88 (1H, br. s.), IR ν_{max} (Nujol) cm⁻¹: 3425, 3300, 3100 br., 1655, 1620, 1592, 1557, 1220, 1063 and 820, Mass *m/e*: 354 (M⁺), 339, 311 (base peak), 299, 281, 269, 241, 219, 205, 177 and 165.

Luteone trimethyl ether (Ib). A mixture of luteone (70 mg), anhyd. potassium carbonate (700 mg), dimethyl sulfate (0.8 ml) and acetone (30 ml) was heated under reflux for 36 hr. The bulk of the acetone was removed by distillation under reduced pressure and the residue was poured into water (100 ml). Extraction with benzene and evaporation yielded an oil (80 mg) which was crystallized from benzene-hexane, giving luteone trimethyl ether (Ib) (50 mg) as colorless needles, mp 136~137°C, (Found: C, 69.68; H, 6.14. Calcd. for C₂₃H₂₄O₆: C, 69.68; H, 6.10%), UV λ_{max} *m* μ : 265 and 286 sh. (MeOH, ϵ 25200 and 13200), 269 and 280 sh. (AlCl₃), 270 and 281 sh. (AlCl₃/HCl), PMR δ_{TMS} (CCl₄): 1.65 (3H, br.s.), 1.78 (3H, br.s.), 3.28 (2H, br.d., *J*=8), 3.78 (3H, s.), 3.80 (3H, s.), 3.89 (3H, s.), 5.15 (1H, br.t., *J*=8), 6.29 (1H, s.), 6.35~7.22 (3H, ABX system), 7.72 (1H, s.) and 12.91 (1H, s.), IR ν_{max} (Nujol) cm⁻¹: 3100, 1660, 1655, 1620, 1580 and 1510, Mass *m/e*: 396 (M⁺), 381, 365, 353 (base peak), and 341.

Methylation of luteone with ethereal diazomethane yielded the same trimethyl ether (Ib).

Luteone tetraacetate (Ic). A mixture of luteone (20 mg), pyridine (1 ml) and acetic anhydride (2 ml) was heated (5 hr) under reflux, cooled, poured into water (40 ml), stirred (2 hr) at room temp., and extracted with benzene. The benzene extract was washed with water, dried over anhyd. sodium sulfate and the solvent was removed to give an oil (31 mg), which was purified by chromatography on silicic acid. Elution with benzene-ethyl acetate (9:1) gave luteone tetraacetate (Ic) as an intractable gum (25 mg), UV λ_{max} *m* μ : 247 and 309 (MeOH, ϵ 32000 and 5300), PMR δ_{TMS} (CCl₄): 1.70 (3H, br.s.), 1.72 (3H, br.s.), 2.04 (3H, s.), 2.18 (3H, s.), 2.22 (3H, s.), 2.31 (3H, s.), 3.21 (2H, br.d., *J*=8), 4.96 (1H, br.t., *J*=8), 6.95~7.20 (3H, ABX system), 6.98 (1H, s.) and 7.68 (1H, s.), IR ν_{max} (CCl₄) cm⁻¹: 1780, 1667, 1620 and 1190, Mass *m/e* 522 (M⁺), 481, 439, 426, 395 (base peak), 383 and 353.

Oxidation of luteone trimethyl ether (Ib): formation of 2,4-dimethoxybenzoic acid. Hydrogen peroxide (30%, w/v) was added dropwise in four portions (1, 1.5, 1.5 and 0.5 ml) at intervals of 1 hr to a stirred solution of luteone trimethyl ether (Ib) (40 mg) in aqueous potassium hydroxide (15%, 5 ml) and ethanol (7 ml) at room temp. After 18 hr, the solution was concentrated under reduced pressure, acidified with 2 N HCl, and extracted with ether. The extract was shaken with aqueous sodium hydrogen carbonate (10%), and the aqueous layer, after acidification, was re-extracted with ether to give acidic materials (10 mg). Crystallization from ether gave an acid, mp 107~108°C, which was identified as 2,4-dimethoxybenzoic acid by comparison (mixed mp and IR spectra) with an authentic

sample. Alkaline hydrogen peroxide oxidation of luteone failed to afford 2,4-dihydroxybenzoic acid.

Acid-catalyzed transformation of luteone: formation of α - and β -isoluteone (IIa and IIIa). A mixture of luteone (80 mg), glacial acetic acid (1 ml) and conc. sulfuric acid (0.1 ml) was heated at 100°C until solution occurred (30 sec.). The solution was left at room temp. (36 hr), poured into water (80 ml) and extracted with ethyl acetate. The ethyl acetate extract was chromatographed on silicic acid-Celite column by stepwise elution with benzene containing an increasing ratio (5%) of ethyl acetate.

The eluate with 15% ethyl acetate was crystallized for benzene-hexane, giving β -isoluteone (IIIa) (19 mg) as pale yellow rods, mp 248~250°C, (Found: C, 68.01; H, 5.03. Calcd. for $C_{20}H_{18}O_6$: C, 67.79; H, 5.12%), UV λ_{max} m μ : 264 and 288 sh. (MeOH, ϵ 29000 and 15100), 273 and 318 (AlCl₃), 275 and 316 (AlCl₃/HCl), 264 and 289 sh. (NaOAc), and 264 and 289 sh. (NaOAc/H₃BO₃), PMR δ_{TMS} (pyridine-d₅): 1.30 (6H, s.), 1.71 (2H, t., $J=7$), 2.73 (2H, t., $J=7$), 6.51 (1H, s.), 6.81~7.65 (3H, ABX system), 8.28 (1H, s.) and 13.88 (1H, br.s.), IR ν_{max} (Nujol) cm⁻¹: 3520, 3390, 3080, 1660, 1625, 1602, 1575 and 1520.

The eluate with 35% ethyl acetate was crystallized from acetone-water, giving α -isoluteone (IIa) (24 mg) as pale yellow prisms, mp >300°C, (Found: C, 67.78; H, 5.15. Calcd. for $C_{20}H_{18}O_6$: C, 67.79; H, 5.12%), UV λ_{max} m μ : 262 and 288 sh. (MeOH, ϵ 27500 and 14400), 263 and 289 sh. (AlCl₃), 263 and 287 sh. (AlCl₃/HCl), 271 and 330 sh. (NaOAc), and 262 and 288 sh. (NaOAc/H₃BO₃), PMR δ_{TMS} (pyridine-d₅): 1.47 (6H, s.), 1.80 (2H, t., $J=7$), 2.92 (2H, t., $J=7$), 6.68 (1H, s.), 6.78~7.45 (3H, ABX system) and 8.08 (1H, s.), IR ν_{max} (Nujol) cm⁻¹: 3280, 3180, 1625 and 1550.

α -Isoluteone trimethyl ether (IIb). Treatment of α -isoluteone (IIa) (10 mg) with ethereal diazomethane gave a gum, which was crystallized from ether to yield α -isoluteone trimethyl ether (IIb) (10 mg) as colorless plates, mp 186~187°C, UV λ_{max} m μ : 254 sh., 260 and 283 sh. (MeOH, ϵ 22600, 23800 and 10700), 254 sh., 261 and 283 sh. (AlCl₃), and 254 sh., 261 and 283 sh. (AlCl₃/HCl), PMR δ_{TMS} (CCl₄): 1.40 (6H, s.), 1.76 (2H, t., $J=7$), 2.60 (2H, t., $J=7$), 3.77 (3H, s.), 3.79 (3H, s.), 3.86 (3H, s.), 6.23 (1H, s.), 6.32~7.21 (3H, ABX system) and 7.56 (1H, s.), IR ν_{max} (Nujol) cm⁻¹: 1655, 1635, 1610 br. and 1580, Mass m/e : 396 (M⁺) and 365.

β -Isoluteone dimethyl ether (IIIb). β -Isoluteone (IIIa) (15 mg) was methylated with ethereal diazomethane. Recrystallization from ether-hexane gave β -isoluteone dimethyl ether (IIIb) as pale yellow prisms, mp 151~152°C, UV λ_{max} m μ : 263 and 292 sh. (MeOH,

ϵ 24400 and 10300), 263 and 300 sh. (AlCl₃), and 266 and 275 sh. (AlCl₃/HCl), PMR δ_{TMS} (CCl₄): 1.35 (6H, s.), 1.80 (2H, t., $J=7$), 2.69 (2H, t., $J=7$), 3.77 (3H, s.), 3.80 (3H, s.), 6.20 (1H, s.), 6.35~7.22 (3H, ABX system), 7.69 (1H, s.) and 13.10 (1H, s.), IR ν_{max} (Nujol) cm⁻¹: 3070, 1665~1655, 1620, 1610 and 1590, Mass m/e : 382 (M⁺) and 351.

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