Studies on the Constituents of *Lupinus mutabilis* (Fabaceae). Isolation and Characterization of Two New Isoflavonoid Derivatives

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Two new flavonoid derivatives, mutabilin (**1**) and mutabilein (**2**), were isolated from the seeds of *Lupinus mutabilis*. Their structures were elucidated on the basis of spectral and chemical data to be 3′-methoxy-5-hydroxy-7-*O*-*β*-D-glucosylisoflavone and 3′-methoxy-5,7-dihydroxyisoflavone.

Keywords: *Lupinus mutabilis; Fabaceae; tarwi; flavonoid glycosides; 3′-methoxy-5-hydroxy-7-O-β-D-glucosylisoflavone, mutabilein; 3′- methoxy-5,7-dihydroxyisoflavone, mutabilin; South American crop; food source;* ^{1}H *and* ^{13}C *NMR;* $^{1}H-^{1}H$ *2D COSY correlated H;* $^{13}C-^{1}H$ *2D HMQC correlated C;* $^{13}C-^{1}H$ *2D HMBC correlated C; FABMS*

INTRODUCTION

Among >300 *Lupinus* species found in the natural world, there are 5 species cultivated worldwide (*L. albus, L. angustifolius, L. luteus, L. mutabilis*, and *L. cosentenii*), in climates ranging from northern Europe and Russia to the arid Australian plains and the Andean highlands. Cultivated lupines are cool-season grain legumes or forage crops (Putnam, 1991). Agricultural production of lupine represents only a fraction of the grain legumes grown worldwide, largely because of the bitter taste of its seeds (Williams, 1986). However, lupine is one of the few grain legumes that comes close to soybean in protein content of the seed (Hymowitz, 1990), is a high N-fixer, has value in crop rotation, and has an upright, nonshattering habit. The large seed and lack of antinutritional factors, such as trypsin inhibitors, make lupine a potential crop for many animal feed formulations, for direct feeding, and as a human food.

Tarwi, *Lupinus mutabilis*, is a grain from the Inca empire. The Inca developed agricultural systems and plants that could grow in very different habitats, and they were able to grow enough food to feed 15 million people and also have a 3−7 year surplus despite the fact that their technology may be nowadays considered "primitive". Several of their crops were introduced to the rest of the world: potatoes, lima beans, chile peppers, and tomatoes. However, the majority of Incan crops, including seeds, tubers, fruits, and vegetables, are generally unknown outside the New World, because the Spanish conquistadors forced the native people to switch to the use of "Western" plants such as wheat, barley, carrots, and broad beans. Several plants neglected by people of European and Indian descent as "poor people food", have begun to be exploited now, in a renewed interest for their nutritive contents and for aptitude to overcome many types of environmental difficulties, such as poor soil and primitive technology. Furthermore, new foods are sought as alternatives to the usual, nutritionally complete, foods, particularly for personal needs or for vegetarians.

The tarwi contains isoflavones. These natural compounds, like others present in trace amounts only in particular alimentary plants, such as polyphenols, carotenoids, and glucosinolates, because of their important nutritional properties, can be essential to human health. Studies have shown that isoflavones inhibit the growth of many types of cancer cells, inhibit tumor development in laboratory animals, stimulate bone formation (thus reducing the risk of osteoporosis), lower cholesterol levels, relieve the symptoms of menopause, and help reduce the risk of heart disease. The discovery of the benefits of isoflavones started with the recognition of the lower incidence of certain degenerative diseases in countries where soy is traditionally eaten in high quantities (Brandi, 1997).

The mechanism through which isoflavones may exert the above-mentioned effects seems to depend, at least in part, on their mixed estrogen agonist−antagonist properties. An alternative hypothetical mechanism could derive from other biochemical actions of isoflavones such as inhibition of enzymatic activity, in particular protein kinases, or activation of an "orphan" receptor distinct from the estrogen type I receptor. Genistein, a member of the isoflavonoids and a potent protein tyrosine kinase (PTK) inhibitor, is present in soy food products. The PTK inhibitors are implicated in much reported disease (Akiyama et al., 1987) and have potential for drug development (Levitzki and Gazit, 1995). In addition, genistein stimulates the body's production of its own antioxidant enzymes, including glutathione peroxidase, catalase, and superoxide dismutase (Cai and Wei, 1996; Ruiz-Larrea et al., 1997).

This paper deals with the elucidation of the structures of two new isoflavonoid derivatives, the only ones found in our tarwi specimen, named by us mutabilin (**1**) (3′-methoxy-5-hydroxy-7-*O*-*β*-D-glucosylisoflavone) and mutabilein (**2**) (3′-methoxy-5,7-dihydroxyisoflavone), as a continuation of our chemical studies on the constituents of alimentary underexploited plants from South America (De Simone et al., 1990; Dini et al., 1991, 1994; Rastrelli et al. 1995a,b, 1996).

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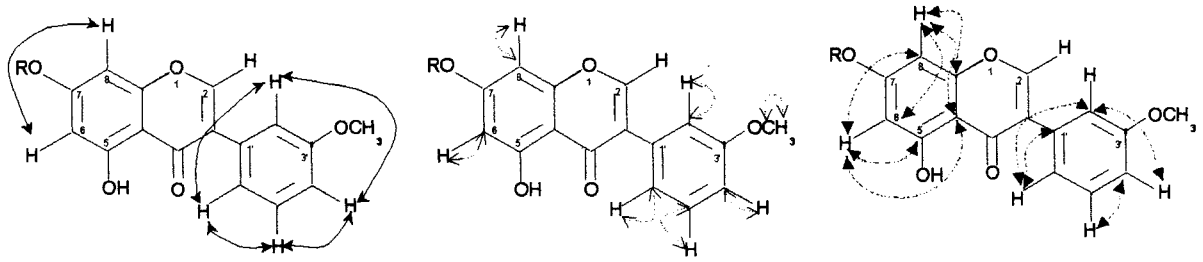

Figure 1. COSY, HMQC, and HMBC correlations of *L. mutabilis* isoflavones: (left) COSY; (middle) ^{1}H detected HMQC; (right) ^{1}H detected HMBC. R = β-D-glucose, mutabilin (**1**); R = H, mutabilein (**2**).

MATERIALS AND METHODS

Materials. The plant materials were supplied by Ce Pe Ser (Central Peruviane de Servicios) collected in Ayakawa, Peru, in 1990. A voucher specimen is deposited in the Herbario de Museo de Historia Natural "J. Prado" Un. H. S. Lima (Peru).

Apparatus. The FABMS spectra, in positive ion mode, were obtained by dissolving the samples in a glycerol·thyoglicerol matrix and placing them on a copper probe tip prior to bombardment with Ar atoms of 2−6 kV energy in a Kratos MS 902 spectrometer equipped with a Kratos FAB source.

GC/MS were run using a Hewlett-Packard 5890 apparatus, a gas chromatograph fitted with an HP 5970B mass detector, and a HP 59970 MS Chemstation, equipped with an HP-5 column (25 m × 0.2 mm i.d.; 0.33 μm film).

HPLC separations were performed on a Hewlett-Packard HP 1050 series pumping system with a Varian RI-4 refractive index detector equipped with a Whatman Partisil M9 10/50 10 ODS-2 column.

The NMR spectra were obtained in CD_3OD using a Bruker AMX-500 spectrometer, using the solvent shift as reference (δ 3.34 for ^{1}H and δ 49.0 for ^{13}C). Distortionless enhancement by polarization transfer (DEPT) experiments were performed using a transfer pulse of 135° to obtain positive signals for CH and CH_3 and negative ones for CH_2. Polarization transfer delays were adjusted to an average CH coupling of 135 Hz. Two-dimensional homonuclear proton chemical shift correlation (COSY) experiments were measured by employing the conventional pulse sequence. The COSY spectrum was obtained using a data set ($t_1 \times t_2$) of 1024 × 1024 points for a spectral width of 1165 Hz (relaxation delay = 1 s). The data matrix was processed using an unshifted sine bell window function, followed by transformation to give a magnitude spectrum with symmetrization (digital resolution in both F_2 and F_1 dimensions is 1.13 Hz for point). ^{1}H detected heteronuclear multiple quantum coherence (HMQC) experiments were performed according to the procedure of Bax and Subramanian (1986), using an initial BIRD pulse to suppress ^{1}H resonances not coupled to ^{13}C and GARP sequence for ^{13}C decoupling during data acquisition. The spectral width in ^{1}H dimension was 2994.05 Hz; 256 experiments of 240 scans each (relaxation delay = 1.5 s, delay after BIRD pulse = 0.4 s, fixed delay t_1 = 3.3 ms) were acquired in 1K points. A sine square function was applied in t_2 dimension (TM, 0.03 Hz TM2, and 0.6 Hz) before Fourier transformation (digital resolution in F_2 dimension = 2.994 Hz/point). ^{1}H detected heteronuclear multiple bound correlation (HMBC) spectroscopy was performed according to the method of Bax (Bax and Summers, 1986; Bax et al., 1986).

Extraction and Isolation. The meal from the seeds (277 g) was defatted with ether and $CHCl_3$ and then extracted with MeOH and after with MeOH/H_2O 10%. The combined extracts give 44.02 g of residue. The residue was partitioned between *n*-BuOH and H_2O to afford an *n*-BuOH-soluble portion (13.7 g). Part of the BuOH extract (6.12 g) was chromatographed on a Sephadex LH-20 column (100 × 5 cm) with MeOH as eluent. Fractions (10 mL) were collected and checked by TLC [silica gel plates in *n*-BuOH/HOAc/H_2O (60:15:25)]. Fractions 57−71 (21 mg) and 72−90 (16 mg) containing the crudecompound mixture were submitted to HPLC using MeOH/H_2O (70:30) (flow rate = 2.5 mL/min) as eluent to give pure compounds **1** (12.2 mg, equivalent to 44.08 mg/kg of meal, t_R = 9.0 min) and **2** (2.6 mg, equivalent to 9.40 mg/kg of meal, t_R = 20 min).

Methanolysis of Compounds 1. Each fraction (1.0 mg) was heated in a vial for 24 h at 80 °C in MeOH/HCl 2% (2 mL). After MeOH and HCl distillation in an N_2 stream, Ag_2CO_3 and MeOH were added until CO_2 production stopped. The centrifugate was dried over P_2O_5. The resulting monosaccharide was treated with TRISIL-Z (Pierce) and analyzed by GC/MS. Retention times were identical to those of the authentic Trisil sugar.

RESULTS AND DISCUSSION

The whole flour from *L. mutabilis* was successively extracted with petroleum ether, $CHCl_3$, MeOH, and MeOH/H_2O 10%. The combined methanolic extracts were then partitioned into a mixture of *n*-BuOH and H_2O to afford the *n*-BuOH-soluble portion, which was subjected to Sephadex LH-20.

The fractions containing the isoflavonoid mixture, checked by thin layer chromatography, were further purified by high-performance liquid chromatography to obtain the two compounds **1** and **2** (Figure 1). The structures and molecular formulas (see Materials and Methods) were determined by positive ion FABMS spectra and ^{1}H, ^{13}C, and ^{13}C DEPT NMR data.

Compound **1**, 44.08 mg/kg of meal, showed an $[M + H]^+$ ion at *m/z* 447 and prominent fragments at *m/z* 285 $[(M + H)^+162]$ and *m/z* 269 $[(M + H)^+ - 178]$, which were interpreted as the cleavage of a glucose moiety without and with the glycosidic oxygen. The ^{13}C and ^{13}C DEPT NMR spectra showed 16 signals (Table 1), of which 6 were assigned to the saccharide portion and 10 to an isoflavonoidic moiety. From the mass and ^{13}C and ^{13}C DEPT NMR data, a $C_{22}H_{22}O_{10}$ molecular formula was deduced. On acid hydrolysis, it gave compound **2**, identified by direct comparison by UV and ^{1}H NMR spectra. Inspection of the proton and carbon-13 nuclear magnetic resonance spectra indicated that glycoside **1** was the glucoside of compound **2**. On acidic hydrolysis **1** afforded **2**, identified by ^{1}H, ^{13}C, and ^{13}C DEPT NMR (Table 2) spectra as a 3′-*O*-methoxy-5,7-dihydroxyisoflavone.

The aromatic side of the ^{1}H NMR spectrum of **1** (Table 1) exhibited the pattern of meta-coupled protons of ring A at δ 6.53 (d, J = 2 Hz, H-6) and at δ 6.71 (d, J = 2 Hz, H-8) and the characteristic pattern for meta-substituted phenyl ring (ring B) with proton signals at δ 7.2 (d, J = 2.2 Hz, H-2′), 6.99 (dd, J = 2.2 and 8.0 Hz, H-4′), 6.88 (dd, J = 8.0 and 8.0 Hz, H-5′), and 7.41(d, J = 8.3 Hz, H-6′). These B-ring signals, more like those

Table 1. NMR Data for Compound 1 (in CD_3OD)

C	^1H	*J* (Hz)	^{13}C	DEPT	$^1H-^1H$ 2D COSY correlated H	$^{13}C-^1H$ 2D HMQC correlated C	$^{13}C-^1H$ 2D HMBC correlated C
2	8.2	(s)	155.6	CH		155.6	
3			123.6	C			
4			182.5	C			
5			163.6	C			
6	6.53	(d, 2)	101.2	CH	H 8 (6.71)	101.2	C8 (96.0) C5 (163.6) C10 (108.1)
7			164.8	C			
8	6.71	(d, 2)	96.0	CH	H6 (6.53)	96.0	C9 (159.3) C10 (108.1) C6 (101.2)
9			159.3	C			
10			108.1	C			
1′			125.1	C			
2′	7.2	(d, 2.2)	114.1	CH	H4′ (6.99) H6′ (7.41)	114.1	
3′			155.4	C			
4′	6.99	(dd, 2.2, 8)	123.0	CH	H2′ (7.2) H5′ (6.88)	123.6	C2′ (114.1)
5′	6.88	(dd, 8, 8)	116.4	CH	H4′ (6.99) H6′ (7.41)	116.4	C4′ (123.0)
6′	7.41	(d,8.3)	131.4	CH	H5′ (6.88) H2′ (7.2)	131.4	C1′ (125.1) C2′ (114.1)
1″	5.07	(d, 6.7)	101.9	CH		101.9	
2″	3.53	(d, 2.8)	74.8	CH	3.74 3.45	74.8	C3″ (77.9)
3″			77.9	CH		77.9	
4″	3.45	(d, 8.3)	71.3	CH	3.53	3.45	C3″ (77.9)
5″	3.52	(d, 2.8)	78.4	CH	3.74	3.52	
6″	3.74	(dd, 5.5, 5.5)	62.5	CH_2	3.52 3.92	3.74	
OCH_3	3.91		56.64	CH		56.64	

Table 2. NMR Data for Compound 2 (in CD_3OD)

C	^1H	*J* (Hz)	^{13}C	DEPT	$^1H-^1H$ 2D COSY correlated H	$^{13}C-^1H$ 2D HMQC correlated C	$^{13}C-^1H$ 2D HMBC correlated C
2	8.2	(s)	155.4	CH		155.6	
3			123.8	C			
4			182.5	C			
5			163.6	C			
6	6.26	(d, 2)	100.2	CH	H8 (6.38)	100.2	C8 (94.8) C5 (163.6) C10 (108.6)
7			166.0	C			
8	6.38	(d, 2)	94.8	CH	H6 (6.26)	94.8	C9 (159.2) C10 (108.6) C6 (100.2)
9			159.2	C			
10			108.6	C			
1′			125.0	C			
2′	7.19	(d, 2.2)	114.1	CH	H 4′ (6.99) H 6′ (7.41)	114.1	
3′			155.4	C			
4′	6.99	(dd, 2.2, 8.3)	122.9	CH	H 2′ (7.19) H 5′ (6.88)	122.9	C2′ (114.1)
5′	6.88	(dd, 8.3, 8.6)	116.3	CH	H 4′ (6.99) H 6′ (7.41)	116.3	C1′ (125.0)
6′	7.41	(d, 8.6)	131.4	CH	H 5′ (6.88) H 2′ (7.19)	131.4	C1′ (125.0) C2′ (114.1)
OCH_3	3.91		56.64	CH		56.64	

of flavanones rather than flavones, are not conjugated-with the carbonyl group (the 2′ and 6′ signals are found at ~0.5 ppm higher field than those of the corresponding flavones). Moreover, the ^1H NMR spectrum of **1** shows the signal at δ 8.2 characteristic for H-2 in isoflavone compounds. The singlet at δ 3.91 showed the presence of an aromatic methoxy group; its 3′ position was established for the 30 ppm downfield shift of the C-4′ peak accompanied by 16 ppm upfield shifts of C-3′ and C-5′ signals, in comparison with the corresponding peaks of genistin. Therefore, the compound must be a 3′-methoxy derivative. The signal at δ 5.07 signal (1H, d, J = 6.7 Hz) in ^1H NMR and the data from ^{13}C and ^{13}C DEPT NMR and from gas chromatography of the product after hydrolysis indicated the presence of a glucosyl moiety in β anomeric form. The 7-position for the O- glucosidation was established from comparison of compounds **1** and **2** in ^{13}C NMR (Markham et al., 1978): a 1.2 ppm upfield shift for the C-7 in glucosidated compound (164.8 and 166.0, respectively) and a downfield shift, ~1 ppm in C-6 and C-8 (94.8, 96.0 and 101.2, 100.2, respectively).

From all of these data the structure of **1** was concluded to be 3′- methoxy-5-hydroxy-7-*O*-β-D-glucosylisoflavone.

Compound **2**, 9.40 mg/kg of meal, showed an $[M + H]^+$ ion at *m/z* 283. The ^{13}C and ^{13}C DEPT NMR spectra showed 16 signals (Table 2), of which 6 were assigned to the glycoside and 10 to an isoflavonoidic moiety.

From the mass and ^{13}C and ^{13}C DEPT NMR data, a $C_{16}H_{12}O_5$ molecular formula was deduced. The 1,3 disubstitution in ring B is deduced from the characteristic pattern in the aromatic moiety of the ^1H NMR spectrum (Table 2) with signal at δ 7.19 (dd, $J = 2.2$ Hz, H-2′) coupled with the signal at δ 6.99 (dd, $J = 2.2$ and 8.3 Hz, H-4′) and the signal δ 7.41 (ddd, $J = 2$, 8.3, and 8.6 H, H-6′). The signal at δ 6.99 is also coupled with the signal at δ 6.88 (dd, $J = 8.3$ and 8.6 H, H-5′) and the signal at δ 7.41 (ddd, $J = 2$, 8.3, and 8.6 Hz, H-6′). These data and the above consideration of compound **1** led to the formulation of **2** as 3′-methoxy-5,7-dihydroxyisoflavone.

Moreover, the ^{13}C DEPT spectrum shows two quaternary carbons at δ 125.0 (C-1′) and at δ 154.8 (C-3′) and seven CH signals at δ 155.4 (C-2), 100.2 (C-6), 94.8 (C-8), 114.1 (C-2′), 122.9 (C-4′), 116.3 (C-5′), and 131.4 (C-6′), which were established also by means of $^1H-^1H$ 2D COSY correlated H, $^{13}C-^1H$ 2D HMQC correlated C, and $^{13}C-^1H$ 2D HMBC correlated C experiments (Figure 1).

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