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Phytochemistry 67 (2006) 579–583
PHYTOCHEMISTRY
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C-prolinylquercetins from the yellow cocoon shell of the silkworm, *Bombyx mori*

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Received 6 September 2005; accepted 26 November 2005
Available online 23 January 2006

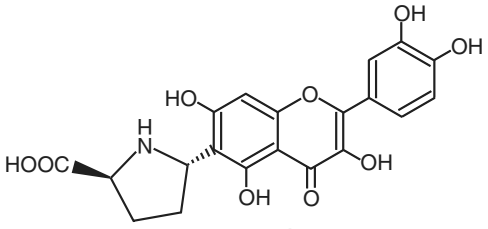
Abstract

Two flavonoids containing the L-proline moiety, 6-*C*-[(2*S*,5*S*)-prolin-5-yl] quercetin (prolinalin A) and 6-*C*-[(2*S*,5*R*)-prolin-5-yl] quercetin (prolinalin B), were isolated from the cocoon shell of the silkworm, *Bombyx mori*. Their structural elucidation was achieved by application of acid hydrolysis and spectroscopic methods. These compounds were not found in the leaves of mulberry (*Morus alba* L.), the host plant of the silkworm, suggesting that the flavonoids are metabolites of the insect. This is the first time that flavonoids with an amino acid moiety have been found as naturally occurring compounds.

Keywords: Silkworm; *Bombyx mori*; Mulberry; *Morus alba*; Moraceae; Flavonoid; Quercetin; L-Proline

1. Introduction

It has been reported that cocoon shells of some strains of the silkworm, *Bombyx mori*, contain flavonoid-like pigments (Oku, 1934; Hayashiya et al., 1959). Recently, Tamura et al. (2002) isolated quercetin 5-glucoside, quercetin 5,4′-diglucoside, and quercetin 5,7,4′-triglucoside from the yellow-green cocoon shell of a race Multi-Bi. These flavonoids were not found to be present in leaves of its host plant, mulberry tree (*Morus alba*), in which quercetin-3-glycosides (rutin, isoquercitrin) and quercetin aglycon are naturally occurring (Zhishen et al., 1999). This suggests that flavonoids from the diet are modified within the silkworm by a glucosyltransferase that can transfer a glucose residue to the C-5 hydroxy position of quercetin. Such an enzyme has not been reported in animal tissues. Flavonoids modified by *B. mori* may be useful as medicinal or cosmetic materials because the ethanolic extracts of yellow-green colored cocoon shells of a range of strains of *B. mori* have potent antibacterial activity (Kurioka et al., 1999) and strong antioxidant activity (Yamazaki et al., 1999). In a continuing search among silkworm strains for novel flavonoids with possible biological activity, we found that the aqueous MeOH extract of the yellow cocoon shell of a Chinese race of Daizo contain novel flavonoids with an amino acid moiety. In the present study, we describe the isolation and structural elucidation of two new compounds, designated as prolinalin A **(1)** and prolinalin B **(2)**.



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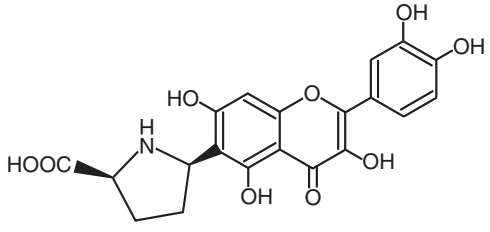

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0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.11.030

2. Results and discussion

The aqueous MeOH extract of the cocoon shell of the silkworm, *B. mori*, was purified as described in the experimental section to yield two new compounds. Prolinalin A (**1**) was obtained as a yellow amorphous solid. The UV absorption data of the compound in MeOH were similar to those of quercetin. The UV spectral changes with various shift reagents were indicative of the presence of a flavonol skeleton with free hydroxyl groups at C-3, C-5, C-7, and C-4′ positions, and the presence of a free *ortho*-dihydroxyl group (Markham, 1982). The compound gave a red-purple pigment by reaction with ninhydrin reagent, indicating that it contains nitrogen. The compound was resistant to hydrolysis by 0.1 N HCl at 100 °C, but afforded δ-pyroline-5-carboxylic acid in 6 N HCl at 110 °C. The D/L configuration of δ-pyroline-5-carboxylic acid was determined to be L by the chemical correlation to L-proline. The high-resolution FT-ICR-MS displayed a protonated ion at *m/z* 416 (observed 416.0973, calculated 416.0976 for $C_{20}H_{18}NO_9$), and product ions from $[M+H]^+$ were observed at *m/z* 303 and 370 by MS/MS experiments. From these results, we considered **1** to be a quercetin derivative with an L-proline moiety. Finally, the structural elucidation was established by ^1H and ^{13}C NMR spectroscopic analysis.

The ^1H and ^{13}C NMR spectroscopic data (Table 1) were similar to those of quercetin (Markham and Geiger, 1994; Markham et al., 1978). From the ^1H NMR data, a 3′,4′-dihydroxy group in ring-B was evidenced by three aromatic resonances located at δ 6.88 (1H, *d*, *J* = 8.5 Hz), δ 7.62 (1H, *dd*, *J* = 2.1 and 8.5 Hz), and δ 7.73 (1H, *d*, *J* = 2.1 Hz), which were assigned to H-5′, H-6′, and H-2′, respectively. A one-proton singlet at δ 6.45 suggested the presence of a substitution at C-6 or C-8. The ^{13}C NMR spectrum displayed 15 distinct carbon signals, which were assigned to the quercetin moiety, including a carbonyl carbon (δ 177.3) and five oxygenated aromatic carbons (δ 146.3, 149.0, 157.9, 160.2, 165.1). The absence of a methine carbon signal in ring-A, but the presence of a quaternary carbon signal at δ 105.3, indicated a ring-A substitution.

Residual ^1H and ^{13}C resonances of **1** were observed in the relatively up-field region of the spectra. The DQF-COSY, HSQC, and HMBC spectra showed that the residual moiety contained the fragment –CO–CH–CH_2–CH_2–CH–. Signals at δ4.30 (*dd*, *J* = 7.7, 9.6) and δ 5.27 (*dd*, *J* = 7.3, 10.8) were assigned to H-2″ and H-5″, respectively. Furthermore, the signals at δ 2.12 (*dddd*, *J* = 7.1, 9.6, 11.1, 13.1) and δ 2.62 (*dddd*, *J* = 2.1, 7.4, 7.7, 13.1) were attributable to H-3″, while the signals at δ 2.30 (*dddd*, *J* = 2.1, 7.1, 7.3, 12.8) and δ 2.40 (*dddd*, *J* = 7.4, 10.8, 11.1, 12.8) could be assigned to H-4″. In addition, the five carbon resonances were assigned, respectively, to δ 174.3 to the carboxyl carbon (C-6″), and δ 63.4, 31.6, 31.5, and 56.1 to the alicyclic carbons (C-2″, C-3″, C-4″, and C-5″). The ^1H and ^{13} C NMR spectroscopic data for the moiety were consistent with those of C-5-substituted proline derivatives (Severino et al., 2003). These NMR observations and the acidolysis results indicated the presence of an L-prolin-5-yl substituent in **1**.

Table 1
^{13}C NMR (125 MHz) and ^1H NMR (500 MHz) Spectroscopic data for compounds **1** and **2** (δ ppm, in CD_3OD)

Position	**1**		**2**	
	δ C	δ H	δ C	δ H
2	148.3		147.8	
3	137.4		137.1	
4	177.3		177.0	
5	160.2		160.1	
6	105.3		104.3	
7	165.1		165.0	
8	94.9	6.45*s*	95.7	6.38*s*
9	157.9		158.3	
10	103.9		103.0	
1′	124.0		124.2	
2′	116.1	7.73d (2.1)[a]	116.0	7.74*br*, *s*
3′	146.3		146.3	
4′	149.0		148.8	
5′	116.3	6.88*d* (8.5)	116.3	6.88*br*, *s*
6′	121.7	7.62*dd* (2.1, 8.5)	121.7	7.62*br*, *s*
2″	63.4	4.30*dd* (7.7, 9.6)	61.8	4.08*dd* (5.1, 10.4)
3″	31.6	*a*: 2.12*dddd* (7.1, 9.6, 11.1, 13.1) *b*: 2.62*dddd* (2.1, 7.4, 7.7, 13.1)	30.0	*a*: 2.33*m* *b*: 2.51*m*
4″	31.5	*a*: 2.30*dddd* (2.1, 7.1, 7.3, 12.8) *b*: 2.40*dddd* (7.4, 10.8, 11.1, 12.8)	29.6	*a*: 2.24*m* *b*: 2.27*m*
5″	56.1	5.27*dd* (7.3, 10.8)	57.2	5.05*t* (7.9)
6″	174.3		174.6	

[a] *J* values in parentheses are recorded in Hz.

Comparison of the carbon shifts of **1** with those of published data for quercetin (Markham et al., 1978) revealed a down-field shift of C-6 ($\Delta\delta$ 7.0 ppm), which indicated that the proline substitution occurred at C-6. The linkage of the L-prolin-5-yl group at C-6 was also deduced from an HMBC experiment, which showed correlations of the resonance at δ 5.27 (H-5″) to carbon signals at δ 105.3 (C-6), 160.2 (C-5), and 165.1 (C-7), while the resonance at δ 6.45 (H-8) showed correlations to the signals at δ 103.9 (C-10),δ 157.9 (C-9) and δ 165.1 (C-7) (Fig. 1). On the basis of these observations, the structure **1** was determined as 6-*C*-(prolin-5-yl) quercetin.

The stereochemistry of **1** was determined by the 1,2-diaxial relationships between H-2″ and Ha-3″ ($J = 9.6$), Ha-3″ and Hb-4″ ($J = 11.1$), and Hb-4″ and H-5″ ($J = 10.8$), as shown in Fig. 1. These large coupling constants (9.6 ∼ 11.1) suggested that H-2″, Ha-3″, Hb-4″, and H-5″, respectively, occupy pseudo-axial positions, with respect to the five-membered ring. This configuration should be observed when the five-membered ring adopts an envelope conformation, in which C-2″, C-4″, C-5″, and N atoms of the ring are coplanar, with C-3″ displaced above that plane. In this case, the carboxyl group at C-2″ and the quercetin at C-5″ should be *trans* on the pyrrolidine ring. Based on the L-configuration of the δ-pyroline-5-carboxylic acid, acidolysis product of **1**, the absolute configuration of pyrrolidine ring was determined to be 2″*S*, 5″*S*. Thus, prolinalin A (**1**) was characterized as 6-*C*-[(2*S*,5*S*)-prolin-5-yl] quercetin.

Prolinalin B (**2**) was observed to be a yellow amorphous powder as prolinalin A. The high-resolution FT-ICR-MS measurement of **2** revealed the elementary composition of protonated ion to be $C_{20}H_{18}NO_9$ ($[M + H]^+$), which is the same as that of **1**. The ^{1}H and ^{13}C NMR (Table 1) and other spectroscopic data of **2** were very similar to those of **1**, suggesting that the overall structures of **1** and **2** were the same. Upon acid hydrolysis, **2** also yielded L-δ -pyroline-5-carboxylic acid, indicative of a 2″*S* configuration. Therefore, **2** was identified as the diastereoisomer of **1** at the C-5″ position of the proline moiety. In the ^{1} H NMR spectrum, the H-5″ signal of **2** was shifted 0.22 ppm upfield compared with that of **1**, thus suggesting a 5″*R* configuration of **2** (Manfré and Pulicani, 1994; Severino et al., 2003). In association with the 2″*S*, 5″*R* configuration of **2**, it is noteworthy that its lower solubility in MeOH and broadened NMR signals compared to **1** were possibly due to the presence of intra-molecular hydrogen bonds between the carboxyl group of the proline moiety and free hydroxyl groups at C-5 and C-7 of the quercetin moiety. Such intra-molecular interactions were not present in **1**. From these observations, **2** was determined to be 6-*C*-[(2*S*,5*R*)-prolin-5-yl] quercetin.

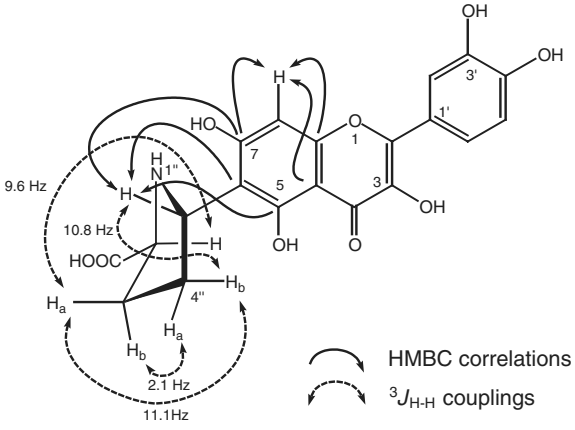

Fig. 1. Selected HMBC correlations and $^3J_{H-H}$ couplings of **1**.

Flavonoids containing nitrogen are very rare natural products. So far, only eight flavonoidal alkaloids have been reported: ficine and isoficine from *Ficus pantoniana* (Johns et al., 1965), phyllospadine from *Phyllosphadix iwatensis* (Takagi et al., 1980), vochsine from *Vochysia guaianensis* (Baudouin et al., 1983), lilaline from *Lilium candidum* (Masterova et al., 1987), aquiledine and isoaquiledine from *Aquilegia ecalcarata* (Chen et al., 2001), and lotthanongine from *Trigonostemon reidioides* (Kanchanapoom et al., 2002). To our knowledge, prolinalin A **(1)** and B **(2)** represent the first examples of a flavonoid conjugated with an amino acid.

Using an LC mass spectrometer, we attempted to determine whether the novel flavonoids isolated from the silkworm naturally occur in its host plant, *M. alba*, but found no evidence of these compounds in the plant. In contrast, these flavonoids were found in the cocoon shells of the silkworm reared on a semi-synthetic diet containing quercetin (data not shown), indicating that prolinalin A **(1)** and B **(2)** are produced from dietary quercetin within the insect. A number of lepidopteran insects and some grasshoppers are known to sequester dietary flavonoids, and some of these insects have been shown to be able to glycosylate flavonoid aglycones (Harborne and Grayer, 1994). In larvae of several races of *B. mori*, in addition to glycosylation reactions, the dehydration reaction with L-5-hydroxyproline may be a major pathway of sequestered flavonoids, however, the physiological roles for the pathway remain to be studied.

3. Experimental

3.1. General experimental procedure

UV spectra were recorded on a Shimadzu UV-2500 PC spectrophotometer. High-resolution ESI mass spectra were obtained by a Bruker Apex II 70e Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer. The MS/MS experiment was carried out with the FT-ICR mass spectrometer coupled with an infrared multiphoton dissociation (IRMPD) system. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE 500 spectrometer (500 MHz for ^{1}H; 125 MHz for ^{13}C) or a Bruker AVANCE 800 spectrometer (800 MHz for ^{1}H; 200 MHz for ^{13}C) in CD_3OD, with TMS as an internal standard. The preparative and analytical HPLC system consisted of a Shimadzu

LC-7A pump, a Shimadzu SPD-7AV UV–Visible detector, and a Shimadzu CTO-10A column oven.

3.2. Biological material

Daizo, a Chinese race of *B. mori* is stocked in the National Institute of Agrobiological Sciences. The larvae of Daizo were reared on fresh leaves of mulberry *M. alba* L. cv. Shin-ichinose planted at the institute. Cocoon shells produced by the larvae were collected after pupation, then cut into small pieces.

3.3. Extraction and isolation

Yellow pigments were extracted from the cocoon shell sample (160 g) by MeOH–H_2O (2:1, v/v) at 60 °C for 2 h. The extract was filtered and concentrated to a small volume under reduced pressure, after which H_2O was added. The aqueous solution was applied to a solid-phase extraction cartridge (Oasis HLB, 35 ml, Waters). After washing with MeOH–H_2O (50:50, V/V), the column was eluted with MeOH–H_2O (80:20, V/V). The eluate was concentrated by evaporation and loaded on a column of Toyoperl HW-40F (TOSOH). The column was eluted at room temperature with a linear gradient from 50% to 80% solvent B (MeOH containing 0.1% formic acid) in solvent A (0.1% formic acid in H_2O). Fractions containing flavonoids were concentrated in vacuo and further purified by reversed-phase HPLC using a Nova-Pak C18 column (19 × 300 mm, Waters) with a flow rate of 10 ml/min at 40 °C. A 90-min gradient, from 12.5% to 25% solvent B (solvent A, 0.2% formic acid; solvent B, 0.2% formic acid in MeCN), was used to afford compounds **1** (t_R = 55.2 min, 3.2 mg) and **2** (t_R = 51.3 min, 1.5 mg).

3.4. Absolute configuration of amino acid derived from *1* and *2*

Each purified compound (0.5 mg) was dissolved in DMSO (50 μl) and mixed with 6 M HCl (1 ml). After deaeration, the tube was sealed with a cap and the contents were heated at 110 °C for 3 h. The hydrolysate was brought to dryness in vacuo to remove HCl, and the residue was dissolved in distilled water. The acid hydrolysis of the compounds gave δ-pyrolline 5-carboxylic acid, which was confirmed by the reaction with *o*-amino benzaldehyde and by the analysis using an automatic amino acid analyzer (Hitachi 8500A). Since the absolute configuration of this amino acid could not be directly elucidated, δ-pyrolline 5-carboxylic acid was reduced to proline by $NaBH_4$. The D-, L-configurations of proline derived by reduction of δ-pyrolline 5-carboxylic acid were identified by an HPLC method using a chiral derivatizing reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA; Marfey's reagent, Pierce, Co.), according to the literature (Marfey, 1984). The HPLC analysis was carried out using a reversed-phase column, Nova-Pak C-18 (3.9 × 150 mm, Waters). FDAA derivatives were eluted from the column with a linear gradient of MeCN in 50 mM triethylamine–phosphate (pH 3.5), from 10% to 40%, for 45 min at a flow rate of 1 ml/min at 25 °C. The FDAA-derivatized L-proline was clearly distinguishable from the D-isomer, and its retention time exactly corresponded to those for the FDAA derivatives of amino acids derived from **1** and **2**.

3.5. Prolinalin A (*1*)

Yellow amorphous solid. UV (MeOH) λ_{max} nm: 267, 385; 283, 471 (+$AlCl_3$); 279, 440 (+$AlCl_3$ + HCl); 285, 332, 397 (+NaOAc); 275, 402 (+NaOAc + H_3BO_3). HR-FT-ICR-MS: 416.0973 (calculated 416.0976). For ^1H, and ^{13}C NMR spectroscopic data, see Table 1.

3.6. Prolinalin B (*2*)

Yellow amorphous solid. UV (MeOH) λ_{max} nm: 266, 384; 283, 470 (+$AlCl_3$); 279, 444 (+$AlCl_3$ + HCl); 285, 332, 397 (+NaOAc); 275, 401 (+NaOAc + H_3BO_3). HR-FT-ICR-MS: 416.0962 (calculated 416.0976). For ^1H, and ^{13}C NMR spectroscopic data, see Table 1.

Acknowledgements

We thank Ms. Ikuko Maeda, Dr. Takashi Murata (Instrumental Analysis Center for Food Chemistry) for their technical help with the NMR and MS measurements. We also thank Ms. Mayumi Hazeyama (National Institute of Agrobiological Sciences) for her technical assistance.

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