Chemical constituents of imported Rosae fructus

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Abstract Rosae fructus is a traditional Chinese crude drug, used for purgative purposes. It is included in the Japanese Pharmacopoeia XV, in which its origin is stated to be *Rosa multiflora* Thunberg. These days, some imported Rosae fructus are on the market and the pharmacological activity of the imported product is in question. The chemical constituents of Japanese Pharmacopoeial Rosae fructus, imported from the People's Republic of China and whose plant origin is expected to be *Rosa* aff. *multiflora*, were investigated to give 2-hydroxynaringin 5-*O*-β-D-glucopyranoside and L(*S*)-pyroglutaminic acid derivative, as new compounds. However, although we made every effort, major flavonoids in *R. multiflora*, multiflorins A and B, and multinoside A acetate could not be isolated.

Keywords Rosae fructus · *Rosa* aff. *multiflora* · Rosaceae · flavonoid

Introduction

In Rosae fructus, some flavonol glycosides, i.e., multiflorins A and B, multinoside A and its acetate, are major constituents and play an important role in its purgative effect [1, 2]. Although the market for Rosae fructus is not so large, there are some rumors that imported Rosae fructus does not possess sufficient pharmacological activity. In this study, the chemical constituents of Japanese Pharmacopoeial Rosae fructus, imported from the People's Republic of China, are investigated.

Results and Discussion

From Rosae fructus, imported from the People's Republic of China, two new compounds (**1** and **2**) (Fig. 1) along with a methyl ester of **2** (**3**), which must be formed during isolation, quercetin (**4**) [3], seven flavonoid glycosides, multinoside A (**5**) [3], isoquercitrin (**6**) [3], quercetin 3-*O*-β-D-glucuronide methyl ester (**7**) [4], hyperin (**8**) [3], isorhamnetin 3-*O*-β-D-glucoside (**9**) [5, 6], taxifolin 3-*O*-β-D-xylopyranoside (**10**) [7], heliocioside A (**11**) [8], syringin (**12**) [9, 10], (6*S*,9*R*)-roseoside (**13**) [11], and 9'-tetrahydroxy-3-methoxy-8-*O*-4'-neolignane 3'-*O*-β-D-glucopyranoside (**14**) [12] were isolated by means of various chromatographic techniques. The known compounds were identified by spectroscopical comparison with data reported in the literature.

2-Hydroxynaringin 5-*O*-β-D-glucopyranoside (**1**), $[\alpha]_D$ –61.7, was isolated as an

amorphous powder and its elemental composition was determined to be $C_{21}H_{22}O_{11}$ on negative-ion HR-FAB-MS. In the IR spectrum, absorption bands for hydroxyl groups (3344 cm^{-1}), a conjugated carbonyl group (1689 cm^{-1}), and aromatic rings (1618 and 1514 cm^{-1}) were observed. The UV absorption maxima indicated the phenolic feature of **1**. In the ^{13}C-NMR spectrum, some signals appeared as dual peaks in the ratio of nearly 1:1 (Table 1). However, essentially 21 signals were used to establish the structure. Six signals were assigned for β-glucopyranose, ten sp^2 signals, two of which exhibited double strength, for two benzene rings, one highly deshielded signal [δ_C 196.91 (196.82)] for a carbonyl carbon, and δ_C 42.12 (41.96) and 107.62 (107.57) signals for methylene and ketal carbons, respectively. In the ^{1}H-NMR spectrum, essentially two aromatic proton signals coupled in an AA'BB' system and two *meta* coupled aromatic protons were also observed. Based on this evidence, the fundamental structure of **1** was expected to have a flavanone skeleton and position 2 possessed a hydroxyl group to form a hemiketal functional group. Key HMBC correlations from δ_H 3.10 and 3.07 to δ_C 107.62 (107.57) and δ_C 196.91 (196.82) supported the structure (Fig. 2). The position of the sugar linkage was established to be on the hydroxyl group at C-5, based on the HMBC correlation from the anomeric proton [δ_H 4.87 (4.85)] to C-5 [δ_C 158.41 (158.29)]. This was further confirmed by the differential NOE experiment, in which

irradiation of the anomeric proton significantly enhanced the peak area of the H-6 signal [δ_H 6.06 (6.05)]. The mode of the sugar linkage was determined to be β from the coupling constants (8 Hz) of the anomeric proton and the absolute configuration of glucose was established to be of the D-series from the results of chirality analysis of the hydrolyzate of **1**. Therefore, the structure of **1** was elucidated to be 2-hydroxynaringin 5-*O*-β-D-glucopyranoside, as shown in Fig. 1. 2-Hydroxynaringin exists as an interconvertible epimeric mixture of hemiketal and 1,3-diketone forms [13–15]. However, based on the mass spectral and NMR data, the hemiketal form was dominant.

Compound **2**, $[\alpha]_D^{21}$ +30.3, was isolated as an amorphous powder, but its elemental composition could not be determined since no molecular ion peak was observed in the high-resolution electrospray time-of-flight mass spectrum (HR-ESI-TOF-MS) or on HR-FAB-MS. The IR spectrum showed strong absorption bands at 3395 cm^{-1} for a hydroxyl group, which tailed to around 2500 cm^{-1}, at 1651 cm^{-1} for an amide carbonyl group and at 1590 cm^{-1} for a carboxylate carbonyl group. From this evidence, compound **2** was implied to be present as a zwitterion. In the ^{13}C-NMR spectrum together with the ^{1}H-NMR spectrum, carbon signals for two carbonyl carbons, one monosubstituted benzene ring, three methylenes and two methines with electronegative substituents were observed. The methylene signal at δ_C 51.5 suggested that compound **2**

contained a nitrogen atom and the H-H COSY spectrum showed two distinct connectivities, H-2 to H_2-3 and then to H_2-4, and H-7' to H-8'. Based on the HMBC spectral data, although several correlations substantiated partial structures (Fig. 3), the planar structure could not be elucidated. Thus, compound **2** was reacted with *p*-bromophenacyl bromide to yield the *p*-bromophenacyl ester of **2** (**2a**), which gave a suitable crystal for X-ray crystallographic analysis. The structure of compound **2a** was confirmed to be *N*-7-hydroxyphenylethynyl L(*S*)-pyroglutamic acid, as shown by the ORTEP drawing in Fig. 4. Therefore, the structure of **2** was elucidated to be a free acid form of **2a**.

Compound **3**, $[\alpha]_D^{29}$ +17.8, was isolated as an amorphous powder and its elemental composition was determined to be $C_{14}H_{17}O_4N$ on HR-ESI-TOF-MS. The ^{1}H- and ^{13}C-NMR spectra showed that compound **3** was an analogous compound to **2**, except for the presence of a methoxy signal at δ_H 3.76 on δ_C 53.0. Thus, the structure of **2** was elucidated to be the methyl ester of **2**. This compound may be formed during extraction and isolation due to exposure to methanolic conditions.

Although the isolation work was extensive, only multinoside A (**5**) (0.01 %) was isolated, and multiflorins A and B, and multinoside A acetate have so far not been isolated. Until Japanese Pharmacopoeia XIV, the origin of Rosae fructus was stated to

be the fruit of *R. mutiflora* Thun. or a related species, *R. wichuraiana* Crépin. However, in the current Japanese Pharmacopoeia XV, *R. wichuraiana* is excluded according to the research finding by Seto *et al*. that no pharmacologically active principles are included in *R. wichuraiana* [3]. Thus, the imported Rosae fructus used in this study, although it was available as a Japanese Pharmacopoeially compatible material, must not to be *R. multiflora*, which is the only species in the current Japanese Pharmacopoeia. The results of qualitative analysis of flavonoid glycoside contents of Rosae fructus, obtained from several sources, and genetic investigation will appear elsewhere.

Experimental

General experimental procedures

Mps were measured with a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1030 polarimeter. IR and UV spectra were measured on Horiba FT-710 and JASCO V-520 UV/VIS spectrophotometers, respectively. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM α-400 spectrometer at 400 MHz and 100 MHz, respectively, with tetramethylsilane as the internal standard. Positive-ion HR-FAB-MS was performed with a JEOL SX-102

spectrometer and an Applied Biosystem QSTAR XL system ESI (Nano Spray)-TOF mass spectrometer.

A highly-porous synthetic resin (Diaion HP-20) was purchased from Mitsubishi Kagaku (Tokyo, Japan). Silica gel column chromatography (CC) and reversed-phase [octadecyl silica gel (ODS)] open CC were performed on silica gel 60 (Merck, Darmstadt, Germany) and Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan) [Φ = 50 mm, L = 25 cm, linear gradient: MeOH-H_2O (1:9, 1 l) $\rightarrow$ (1:1, 1 l), fractions of 10 g being collected], respectively. The droplet counter-current chromatograph (DCCC) (Tokyo Rikakikai, Tokyo, Japan) was equipped with 500 glass columns (Φ = 2 mm, L = 40 cm), and the lower and upper layers of a solvent mixture of $CHCl_3$-MeOH-H_2O-*n*-PrOH (9:12:8:2) were used as the stationary and mobile phases, respectively. Five-gram fractions were collected and numbered according to their order of elution with the mobile phase. HPLC was performed on an ODS-3 column (Inertsil; GL Science, Tokyo, Japan; Φ = 6 mm, L = 25 cm), and the eluate was monitored with a UV detector at 254 nm and a refractive index monitor.

Plant material

Rosae fructus, purchased from a crude drug store in Hiroshima was imported from the People's Republic of China by Kinokuniya Kan-yakkyoku (Tokyo, Japan).

Extraction and isolation

Rosae fructus (5.0 k) was crushed and extracted three times with MeOH (15 l) at 25 ºC for one week and then concentrated to 3 l *in vacuo*. The extract was washed with *n*-hexane (3 l, 35.4 g) and then the MeOH layer was concentrated to a gummy mass. The latter was suspended in water (3 l) and then extracted with EtOAc (3 l) to give 24.7 g of an EtOAc-soluble fraction. The aqueous layer was extracted with 1-BuOH (3 l) to give a 1-BuOH-soluble fraction (106 g), and the remaining water-layer was concentrated to furnish 475 g of a water-soluble fraction.

The 1-BuOH-soluble fraction was applied to a Diaion HP-20 column (Φ = 90 mm, *L* = 40 cm) using H_2O–MeOH (4:1, 6 l), (2:3, 6 l), (3:2, 6 l), and (1:4, 6 l), and MeOH (6 l), 1 l fractions being collected. The residue (13.7 g in fractions 10–16) of the 40%–60% MeOH eluent was subjected to silica gel (450 g) CC, with elution with $CHCl_3$ (3 l) and $CHCl_3$–MeOH [(99:1, 3 l), (97:3, 3 l), (19:1, 3 l), (37:3, 3 l), (9:1, 3 l), (7:1, 3 l), (17:3 3 l), (33:7, 3 l), (4:1, 3 l), (3:1, 3 l), and (7:3, 3 l)], 250 ml fractions being collected. An aliquot (57.1 mg) of fractions of 24–31 (135 mg) of the 1% MeOH eluate was separated by HPLC with H_2O–MeOH (7:3) to give 28.9 mg of **3** from the peak at 58 min. The residue (607 mg) of the 7.5% MeOH eluate in fractions 62–73 was separated by ODS open CC to afford 10.1 mg of crude **4**, which was then crystallized

from MeOH to give 3.6 mg of **4** as a crystalline state. The residue (2.00 g) of the 10% MeOH eluate in fractions 78–83 was applied to ODS open CC to give five fractions. From the first fraction (40.7 mg in fractions 80–88), 5.8 mg of **12** was obtained by DCCC in fractions 32–36. From the second fraction, 160mg of **13** was isolated. From the third fraction (85.7 mg in fractions 119–130), 8.6 mg of **10** was obtained by DCCC in fractions 20–26. The residue (111 mg in fractions 174–183) of the fourth fraction was crystallized from MeOH to give 51.9 mg of **7**. From the fifth fraction (75.1 mg in fractions 184–191), 6.2 mg of **9** was obtained by DCCC in fractions 61–64. From the residue (607 mg in fractions 62–73) of the 12.5% MeOH eluate, 104 mg of **8** was isolated as a precipitate and the residue of the mother liquid was subjected to ODS open CC to give three fractions. From the first fraction (159 mg in fractions 105–116), 51.6 mg of **1** was isolated by DCCC in fractions 15–19. From the second fraction (77.3 mg in fractions 117–127), 3.1 mg of **11** was isolated by DCCC in fractions 20–21. The residue (114 mg in fractions 176–180) of the third fraction was compound **7**. The residue (1.76 g) of the 20–25% MeOH eluate in fractions 109–126 was subjected to ODS open CC to give 104 mg of **2** in fractions 40–43, 12.9 mg of **14** in fractions 104–107 and 563 mg of **5** in fractions 160–186.

2-Hydroxynaringin 5-*O*-β-D-glucopyranoside (**1**)

Amorphous powder, $[\alpha]_D^{21}$ –61.7 (*c* 0.24, MeOH). IR ν_{max} (film) cm^{-1}: 3344, 2924, 1689, 1618, 1514, 1447, 1354, 1100, 1074, 832. UV λ_{max} nm (log ε): 290 (4.17), 225 (4.04). ^{1}H- and ^{13}C-NMR (400 MHz and 100 MHz, respectively, CD_3OD): see Table 1. HR-FAB-MS (negative-ion mode): *m*/*z* 449.1084 $[M - H]^-$ (calcd for $C_{21}H_{21}O_{11}$: 449.1071).

Compound **2**

Amorphous powder, Amorphous powder, $[\alpha]_D^{29}$ +30.3 (*c* 0.61, MeOH). IR ν_{max} (film) cm^{-1}: 3395, 1651, 1590, 1457, 1418, 1230, 1060. UV λ_{max} (MeOH) nm (log ε):280 sh (1.97), 259 (2.34), 229 (2.42). ^{1}H-NMR (400 MHz, CD_3OD) δ:7.37 (2H, d, *J* = 7 Hz, H-2' and 6'), 7.30 (2H, d, *J* = 7 Hz, H-3' and 5'), 7.23 (1H, t, *J* = 7 Hz, H-4'), 4.86 (1H, dd, *J* = 9, 3 Hz, H-7'), 4.30 (1H, m, H-2), 3.71 (1H, dd, *J* = 14, 3 Hz, H-8'a), 3.28 (1H, dd, *J* = 14, 9 Hz, H-8'b), 2.39 (1H, m, H-4a), 2.24 (2H, m, H-3a and 4b), 2.01 (1H, m, H-3b). ^{13}C-NMR (100 MHz, CD_3OD) δ:180.4 (C-1), 178.8 (C-5), 143.5 (C-1'), 129.3 (C-3' and C-5'), 128.6 (C-4'), 126.9 (C-2' and C-6'), 73.3 (C-7'), 65.3 (C-2), 51.5 (C-8'), 31.0 (C-4), 24.9 (C-3).

Compound **3**

Amorphous powder, $[\alpha]_D^{29}$ +17.8 (*c* 0.54, MeOH). IR ν_{max} (film) cm^{-1}: 3418, 1736, 1674, 1454, 1417, 1229, 1180. UV λ_{max} (MeOH) nm (log ε):278 sh (2.11), 247 (2.62). ^{1}H-NMR (400 MHz, CD_3OD) δ:7.36 (4H, m, H-2', 3' 5' and 6'), 7.26 (1H, tt, *J* = 7, 2 Hz, H-4'), 4.85 (1H, dd, *J* = 9, 4 Hz, H-7'), 4.61 (1H, m, H-2), 3.85 (1H, dd, *J* = 14, 4 Hz, H-8'a), 3.76 (3H, s, -OMe), 3.04 (1H, dd, *J* = 14, 9 Hz, H-8'b), 2.45 (1H, m, H-4a), 2.39 (1H, m, H-3a), 2.34 (1H, m, H-4b), 2.07 (1H, m, H-3b). ^{13}C-NMR (100 MHz, CD_3OD) δ:178.4 (C-5), 174.1 (C-1), 143.7 (C-1'), 129.5 (C-3' and C-5'), 128.7 (C-4'), 126.9 (C-2' and C-6'), 73.8 (C-7'), 63.0 (C-2), 53.0 (-OMe), 51.0 (C-8'), 30.4 (C-4), 24.0 (C-3). HR-ESI-TOF-MS (positive-ion mode) *m*/*z*:286.1063 $[M + Na]^+$ (Cacld for $C_{14}H_{17}O_4NNa$: 286.1049).

Acid hydrolysis of **1**

Compound **1** (520 μg) was hydrolyzed with 1N HCl (0.1 ml) at 100 ℃ for 2 h. The reaction mixture was partitioned with an equal amount of EtOAc (0.1 ml), and the water layer was analyzed with a chiral detector (JASCO OR-2090plus) on an amino column [Asahipak NH2P-50 4E, CH_3CN-H_2O (4:1), 1 ml/min]. The hydrolyzate of **1** gave a peak for D-glucose at the retention time of 15.0 min (positive optical rotation

sign). The peak was identified by co-chromatography with authentic D-glucose.

Preparation of *p*-bromophenacyl ester (**2a**)

Compound **2** (18.3 mg) was dissolved in 1.0 ml of EtOH and the solution was made slight basic by means of 0.1 N NaOH with a few drops of phenol phthalein solution as an indicator. After the addition of a small amount (2.0 mg) of **2**, *p*-bromophenacyl bromide (25.3 mg) in 1.0 ml of EtOH was added, followed by and refluxing for 9 h. After cooling, the solvent was evaporated off and the residue was purified by silica gel CC (Φ= 1.0 cm, *L*= 40cm) with $CHCl_3$ (50 ml) and then $CHCl_3$-MeOH (49:1, 150 ml) (1ml fractions being collected) to give crude crystals in fractions 138–175, which were then recrystallized from MeOH to give 10.3 mg of colorless plates (**2a**). *p*-Bromophenacyl ester (**2a**): colorless plates (MeOH), mp. 161-163 °C, $[\alpha]_D^{24}$ +6.8 (c 0.31, $CHCl_3$). IR ν_{max} (KBr) cm^{-1}: 3314, 2937, 1751, 1704, 1664, 1585, 1455, 1402, 1175, 1066, 970, 705. UV λ_{max} (EtOH) nm (log ε):257 (3.07), 210 (3.09). ^{1}H-NMR (400 MHz, CD_3OD) δ:7.75 (2H, d, *J* = 7 Hz, H-3" and 5"), 7.65 (2H, d, *J* = 7 Hz, H-2" and 6"), 7.41 (2H, dd, *J* = 7, 2 Hz, H-2' and 6'), 7.35 (2H, td, *J* = 7, 2 Hz, H-3' and 5'), 7.29 (1H, tt, *J* = 7, 2 Hz, H-4'), 5.41 (1H, d, *J* = 16 Hz, H-8"a), 5.33 (1H, d, J = 16 Hz, H-8"b), 5.02 (1H, dd, *J* = 8, 2 Hz, H-7'), 4.28 (1H, dd, *J* = 9, 4 Hz, H-2), 3.90 (1H, dd, *J*

= 14, 2 Hz, H-8'a), 3.39 (1H, dd, *J* = 14, 8 Hz, H-8'b), 2.59 (1H, m, H-4a), 2.42 (2H, m, H-3a and 4b), 2.34 (1H, m, H-3b). ^{13}C-NMR (100 MHz, $CDCl_3$) δ:190.4 (C-7''), 177.4 (C-5), 171.8 (C-1), 141.9 (C-1'), 132.5 (C-2'' and 6''), 129.6 (C-1''), 129.2 (C-3'' and 5''), 128.5 (C-3' and 5'), 127.5 (C-4'), 125.7 (C-2', 6' and 4''), 73.8 (C-7'), 66.4 (C-8''), 62.0 (C-2), 51.3 (C-8'), 29.4 (C-4), 23.7 (C-3). HR-ESI-TOF-MS (positive-ion mode) *m*/*z*:468.0402 and 470.0402 $[M + Na]^+$ (Calcd for $C_{21}H_{20}O_5N^{79}BrNa$ and $C_{21}H_{20}O_5N^{81}BrNa$: 468.0417 and 470.0392, respectively), .

Single-crystal X-ray strucutre analysis of **2a**

A suitable crystal (0.43 mm × 0.30 mm × 0.05 mm) was used for analysis. The data were measured using a Bruker SMART 1000 CCD diffractometer, using Mo Kα graphite-monochromated radiation (λ = 0.71073 Å). The structure was solved by a direct method using the program SHELXTL-97 [16]. The refinement and all further calculations were carried out using SHELXTL-97 [16]. The absorption correction was carried out utilizing the SADABS routine [17]. The H atoms were included at calculated positions and treated as riding atoms using the SHELXTL default parameters. The non-H atoms were refined anisotropically, using weighted full-matrix least-squares on F^2. Fig. 4 was drawn with ORTEP32 [18]. Crystal Data: $C_{21}H_{20}BrNO_5$, *M* = 446.29 g

mol^{-1}, monoclinic, $P2_1$, a = 6.3415(14) Å, b = 9.367(2) Å, c = 16.499(4) Å, β = 90.214(3)°, V = 980.1(4) $Å^3$, T = 90 K, Z = 2, D_c = 1.512 g cm^{-3}, μ(Mo Kα) = 2.130 mm^{-1}, F(000) = 456, 3296 reflections were measured, 2805 were unique (R_{int} = 0.0468) and used in all calculations. Final goodness-of-fit = 1.017, R_1 = 0.0502, wR_2 = 0.1175 based on $I > 2\sigma(I)$. The absolute parameter was 0.014(13) [19]. CCDC deposit contains the supplementary crystallographic data. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223 336 033; or e-mail: deposit@ccdc.cam.ac.uk.

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Table 1. NMR spectroscopic data for **1** (100 MHz for C and 400 MHz for H, CD_3OD)

	1			
	C		H	
2	107.62	107.57	-	
3	42.12	41.96	3.10 1H, d, 16	
			3.07 1H, d, 16	
4	196.91	196.82	-	
5	158.41	158.29	-	
6	97.74	97.40	6.06 ½H, d, 2	6.05 ½H, d, 2
7	171.46		-	
8	93.38	93.28	5.94 1H, d, 2	
9	174.50		-	
10	103.69	103.50	-	
1'	125.51	125.60	-	
2', 6'	132.51		6.99 2H, d, 8	
3', 5'	115.83	115.80	6.58 1H, d, 8	5.57 1H, d, 8
4'	157.20	157.17	-	
1"	101.73		4.87 ½H, d, 8	4.85 ½H, d, 8
2"	74.07	74.03	a	
3"	78.34	78.27	a	
4"	71.20		a	
5"	77.38	77.32	a	
6"	62.39		3.86 1H, dd, 12, 5	
			3.67 ½H, dd, 12, 2	3.68 ½H dd, 12, 2

a Overlapped between 3.40–3.57

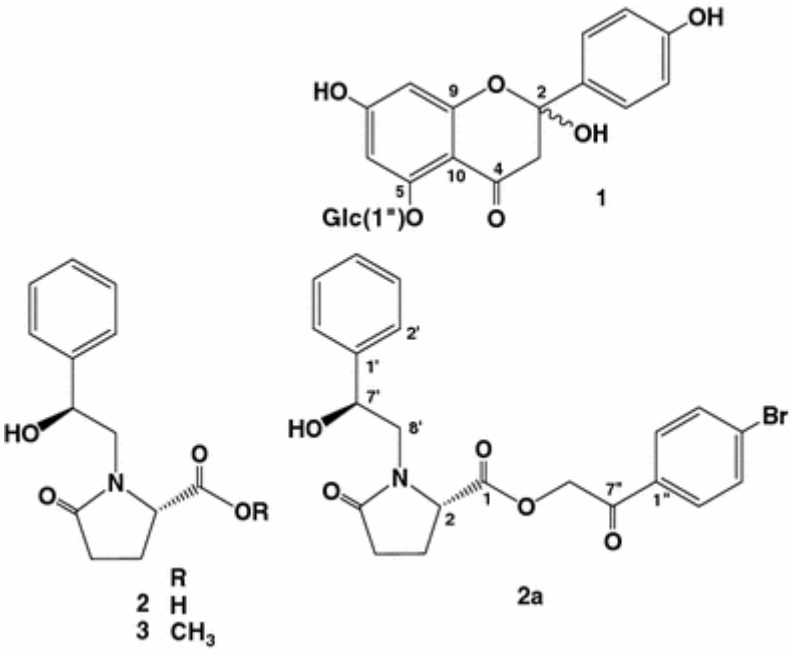

Fig. 1 Structures of new compounds, 1, 2 and 3, and *p*-bromophenacyl ester of 2 (**2a**)

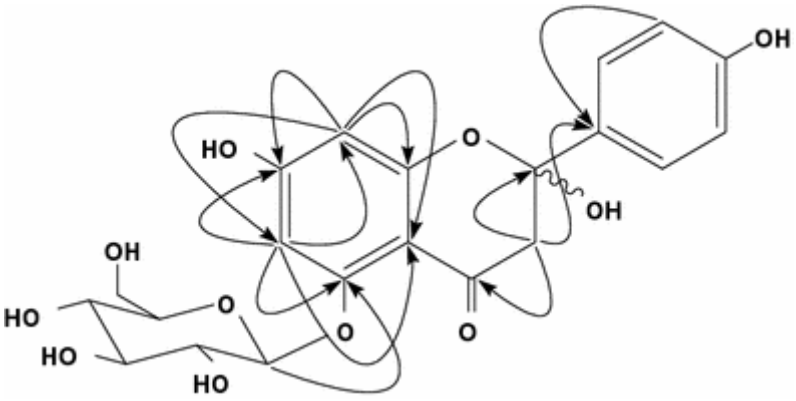

Fig. 2 Diagnostic HMBC correlations of **1**. *Arrowheads* denote carbons and *arrowtails* protons

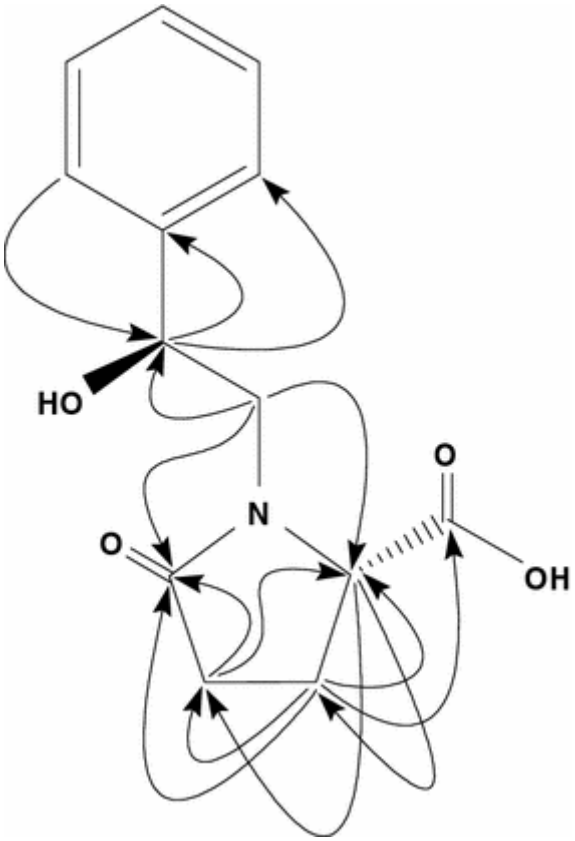

Fig. 3 Supportive HMBC correlations of **2**. *Arrowheads* denote carbons and *arrowtails* protons

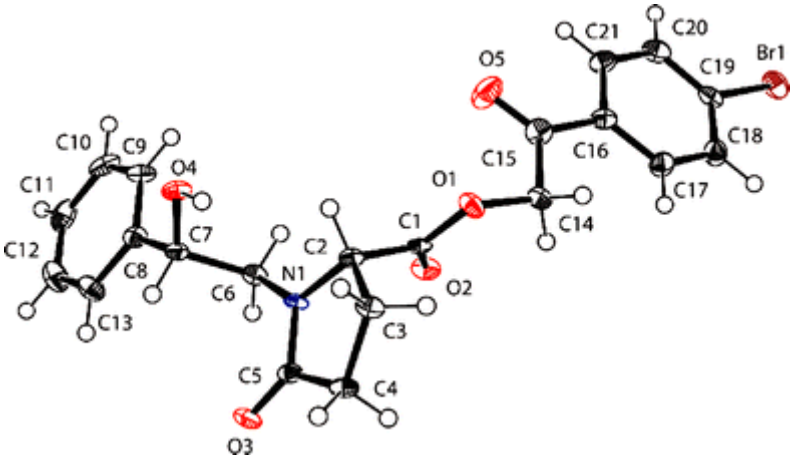

Fig. 4 ORTEP drawing of **2a**. The structure has X-ray crystallographic numbering