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(6*R*,9*S*)-6′′-(4′′-Hydroxybenzoyl)-Roseoside, a New Megastigmane Derivative from *Ouratea polyantha*and its Effect on Hepatic Glucose-6-phosphatase

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A new megastigmane derivative, (6*R*,9*S*)-6′-(4′′-hydroxybenzoyl)-roseoside(**1**)and two known compounds, the biflavoneagathisflavone(**2**) and 4-hydroxybenzoic acid (**3**) were isolated and purified from leaves and stems of *Ouratea polyantha* Engl. Agathisflavone was isolated in a single high-speed counter-current chromatography run, while the megastigmane was purified in two steps, by using a combination of high-speed countercurrent chromatography and analytical column chromatography. All structures were elucidated on the basis of spectral evidence and comparison with literature data. Compound **1** was characterized by $[\alpha]_D^{20}$, UV-Vis, IR, MS, ^{1}H NMR, ^{13}C NMR, HMQC, HMBC, COSY and NOESY. Compounds **1** and **2** showed an inhibitory effect of 63.6 and 13.7% on the G-6-Pase intact microsomes, respectively.

Keywords: Agathisflavone,Countercurrent chromatography,Glucose-6-phosphatase, Megastigmanes, *Ouratea polyantha*, Terpenes, (6*R*,9*S*)-6′-(4′′-Hydroxybenzoyl)-roseoside.

Diabetes mellitus is the commonest endocrine disorder [1] and it is characterized by hyperglycemia resulting from insufficient insulin production and/or insulin resistance [2]. In Venezuela, a large number of plants have been empirically used for the treatment of diabetes and several studies have been undertaken to provide scientific proof and justify the medicinal use of these plants in the treatment of diabetes [3,4].

The genus *Ouratea* belongs to the Ochnaceae family. It is widely represented in tropical areas and comprises approximately 35 genera and 600 species [5]. Several studies of *Ouratea* species described the isolation of chloroisoflavonoids [6], biflavonoids [7,8], terpenes, like megastigmane [9], kauranes [10], and a depside [11], among other metabolites. Rodriguez *et al*. [12] reported that the MeOH extract from the aerial parts of *O. polyantha* Engl. strongly inhibited glucose-6-phosphatase, (G-6-Pase) EC 3.1.3.9.

The enzyme G-6-Pase is a multi-component system characterized by the catalytic subunit, and three carriers: G-6-P (T1), phosphate/pyrophosphate (T2), and glucose (T3) [13], and both enzyme and its transporters are potential targets for anti-diabetic therapy [14,15]. Fractions from the MeOH extract of *O. polyantha* showed inhibitory effects on hepatic microsomal G-6-Pase (unpublished results). Therefore, an effective separation and isolation technique is required for the identification of the bioactive fractions. Countercurrent chromatography (CCC), a technique used to separate mixtures into their individual components, was refined to High Speed Countercurrent chromatography (HSCCC) in the 1980s when it overshadowed other chromatography methods with its superior capacity to achieve rapid and efficient separation. This

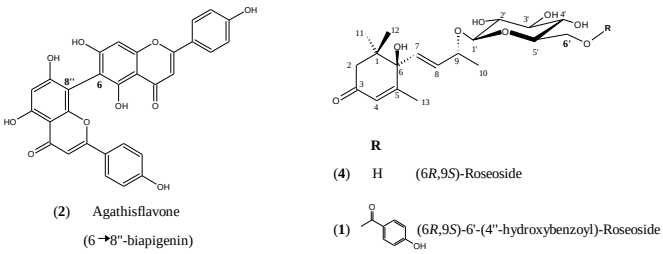

Figure 1. Structures of compounds **1**, **2** and **4**.

chromatographic system is now employed in a wide range of applications, most notably for extracting bioactive compounds from medicinal plants [16].

In the present paper, the isolation and structural elucidation of three compounds, (6*R*,9*S*)-6′-(4′′-hydroxybenzoyl)-roseoside**1**(Figure 1), agathisflavone(**2**) and 4-hydroxybenzoic acid (**3**),from *O. polyantha* aerial parts (leaves and stems) is reported.

(6*R*,9*S*)-6′-(4′′-Hydroxybenzoyl)-roseoside (**1**) was isolated as an amorphous solid ($[\alpha]_D^{20}$ – 57). The UV spectrum of **1** (λ_{max} 229 nm) and the IR absorption (ν_{max} 1652.6 cm-1) indicated the presence of anα,β-unsaturated ketone. The molecular formula of **1** was established as $C_{26}H_{34}O_{10}$ from the TSI-MS [*m/z* 507.88 $(M)^+$, 529.40 $(M+Na)^+$ and 1035.40 $(2M-H+Na)^+$] and NMR data. The mass fragmentation of the ion peak at 506.88 gave fragments for water loss at 488.88 (rel. int., 10), sugar fragmentations at 282.96 (97) and 264.96 (75), alkene fragmentations at 207.00 (92) and 149.04 (52), ester fragmentation at 120.96 (45) and ester

Table 1:[1]H (500 MHz) and [13]C NMR (125 MHz) chemical shifts of (6*R*,9*S*)-6′-(4″-hydroxybenzoyl)-roseoside (**1**).

H/C	δ_H (ppm)	Multiplicity (*J* in Hz)	δ_C (ppm)	DEPT[a]	HMQC	HMBC	COSY	NOESY
1			40.93	C		H-11; H-12		
2	2.44	d (16.9)	49.28	CH_2	H-2	H-11; H-12	H′-2	H′-2;H-11;H-7
	2.15				H′-2		H-2	H-2;H-11;H-12
3		d (16.9)	199.93	C				
4	5.85	s	125.70	CH	H-4	H-10	H-13	H-13
5			165.53	C		H-10		
6			78.46	C		H-10;H-11;H-12;H-13		
7	5.76	d (15.6)	132.44	CH	H-7			H-2; H-12
8	5.69	dd (15.6;6.8)	132.25	CH	H-8	H-10	H-9	H-2; H-10
9	4.39	dq (14.9;6.7)	73.49	CH	H-9	H-10; H-1′	H-8; H-10	H-10; H-2′
10	1.25	d (6.4)	20.78	CH_3	H-10	H-8	H-9	H-8; H-9
11	0.94	s	23.31	CH_3	H-11	C-1; C-2; C-6; C-12		H-2; H′-2
12	1.00	s	22.03	CH_3	H-12	C-1; C-2; C-6; C-11		H′-2; H-7
13	1.91	d (0.95)	18.21	CH_3	H-13	C-4; C-5; C-6	H-4	H-4
1′	4.29	d (7.8)	99.79	CH	H-1′	C-9; H-10	H-2′	H-5′
2′	3.22	dd (16.8;8.05)	73.49	CH	H-2′	C-4′	H-1′; H-3′	H-9; H-10
3′	3.29-3.32	m	76.81	CH	H-3′		H-2′	H-5′;H-3″;H-5″
4′	3.36	dd (9.5;8.75)	70.59	CH	H-4′	H-2′	H-5′	
5′	3.40-3.47	m	74.18	CH	H-5′	H′-6′	H-6′; H′-6′	H-3′
6′	4.61	s_{broad}	63.53	CH_2	H-6′	C-5′; C7″	H′-6′	H′-6′;H-5′
	4.37	dd (13.35;6.7)			H′-6′		H-5′; H-6′	H-6′
1″			120.73	C		H-3″; H-5″		
2″	7.87	d (8.75)	114.88	CH	H-2″	C-7″	H-3″	H-3″
3″	6.81	d (8.75)	131.42	CH	H-3″	C-1′	H-2″	H-3′;H-2″
4″			162.22	C				
5″	6.81	d (8.75)	131.42	CH	H-5″	C-7″	H-6″	H-3′;H-6″
6″	7.87	d (8.75)	114.88	CH	H-6″	C-1′	H-5″	H-5″
7″			166.58	C		H-2″; H-6′; H-6″		

a) at 270 MHz

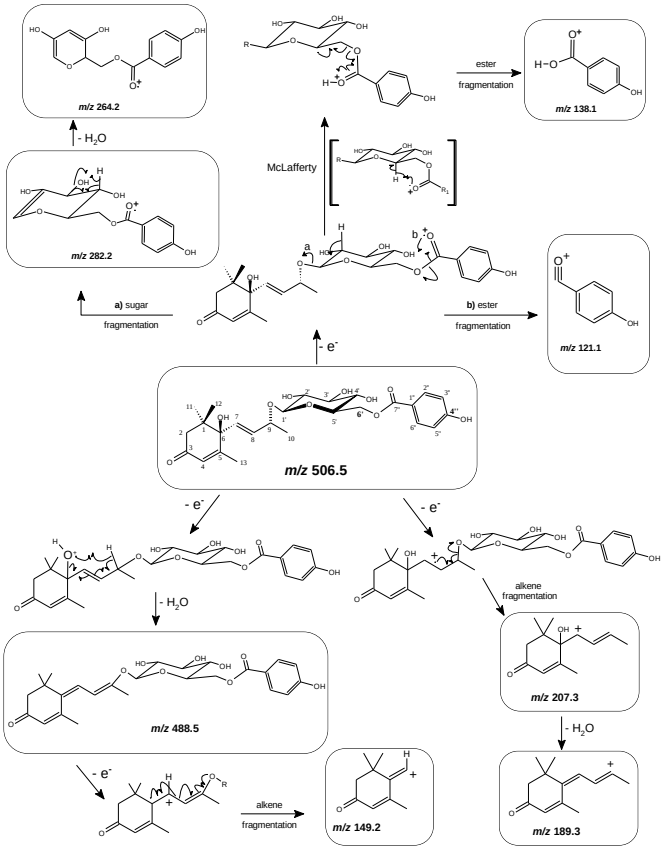

Figure 2: Proposed fragmentation mechanism of (6*R*,9*S*)-6′-(4″-hydroxybenzoyl)-roseoside.

fragmentation through McLafferty rearrangement (Figure 2). The IR spectrum showed the presence of a hydroxyl (broad band 3410.5 cm^{-1}), a conjugated ester (1692.0, 1277.5, 1067.6 cm^{-1}) and a *trans* olefin (1589.5 cm^{-1}). The ^1H and ^{13}C NMR spectra (Table 1), which were assigned by 2D experiments (COSY, HMQC, HMBC and NOESY) of **1** showed the presence of 4-hydroxybenzoyl and β-glucopyranosyl units and an aglycone moiety.

The ^1H NMR spectrum showed two doublet signals at δ 7.87 (2H, d,*J*=8.75, H-2″ and H-6″) and 6.82 (2H, d,*J*=8.75, H-3″ and H-5″), attributed to the AA′BB′ system in a 1,4- substituted benzene ring, assigned to the 4-hydroxybenzoyl group. In the HMBC spectrum, the proton signal at δ 7.87 showed a cross peak with the carbon signal at δ 166.58 (C-7″), which exhibited a long range coupling with the proton signal at δ 4.61 (1H, bs, H-6′) assignable to one proton of a methylene of a sugar unit. Thus, the location of the 4-hydroxybenzoyl group in the pyranosyl moiety was established at C-6′. In the ^1H NMR spectrum, a signal at δ 4.29 (1H, d, *J*=7.8 Hz, H-1′) with a large coupling constant (7.8 Hz) suggested an anomeric proton with a β-configuration. Furthermore, the presence of the COSY cross peak between H-6′ and the signal at δ 3.40-3.47 (1H, *m*, H-5′) and NOESY correlation between H-5′ and the signals at δ 4.29 (1H, *d*, *J*=7.8 Hz, H-1′) and δ 3.29-3.32 (1H, *m*, H-3′) demonstrated the presence of a glucopyranosyl moiety (Table 1).

The position of the glucosidic linkage was deduced from the ^{13}C chemical shift of C-9 (δ_C 73.49), and this was confirmed by long range correlation between H-1′ and C-9. In addition to the signals of the 4-hydroxybenzoyl and glucopyranosyl groups, the proton signals corresponding to the 13 remaining carbons were assignable to the aglycone. These included two *trans*-coupled olefinic protons at δ 5.76 (1H, d, *J*=15.6 Hz, H-7) and δ 5.69 (1H, dd, *J*= 15.6, 6.8 Hz, H-8), anolefinic proton at δ 5.85 (1H, s, H-4) located at the α-position of anα,β-unsaturated ketone, and four methyl groups at 0.94, 1.00, 1.25 and 1.91. The deshielded methyl signal at δ 1.91 (3H, d,*J*=0.95 Hz, H-13) and the olefinic proton H-4 indicated that the methyl was directly attached to an olefinic carbon. This was supported by the HMBC correlation between δ_H 1.91 (H-13) and δ_C 125.70 (C-4). The COSY and HMBC experiments showed a cross peak between a doublet at δ 1.25 (3H, d, *J*=6.4 Hz, H-10) and an oxygenated methine at δ 4.39 (1H, dd,*J*=14.9, 6.2 Hz, H-9). Furthermore, *gem*-dimethyl groups at δ 0.94 (3H, s, H-11) and δ 1.00 (3H, s, H-12) were observed. Geminally coupled signals at δ

2.44 (1H, d, *J*=16.8 Hz, H-2a) and δ 2.15 (1H, d, *J*=16.8 Hz, H-2b) suggested that a carbonyl group was linked to this methylene. Finally, the absolute configuration of C-6 proved to be *R* by the negative value of its optical rotation, as determined by Yamano and Ito in the total synthesis of the four stereoisomers of roseoside[17]. The stereochemistry of C-9 was determined by comparison with the chemical data for roseoside [**¡Error! Marcador no definido.**]. Therefore, the absolute configuration of C-9 (δ 73.49) in **1** was assigned as *S*. On the basis of these data, **1** turned out to be (6*R*,9*S*)-6′-(4′′-hydroxybenzoyl)-roseoside. To the best of our knowledge, this is the first report of the isolation and characterization of this compound from nature.

Agathisflavone (**2**) ($C_{30}H_{18}O_{10}$) was isolated as a yellow amorphous solid and exhibited a $[M-1]^+$ molecular ion peak at *m/z* 537.2813. The ^{13}C NMR spectrum displayed 30 carbons including two carbonyl carbons at δ 182.3 and 182.5, 16 quaternary sp^2 carbons with eight linked to an oxygen atom, and 12 tertiary sp^2 carbons. The ^1H NMR spectrum showed two singlets at δ 13.32 and 13.06 attributed to hydroxyl groups, two sets of A_2B_2-type doublets, one set at δ 8.01 and 6.97 and the other at δ 7.58 and 6.77 attributed to two AA′BB′ systems in two *para*- substituted aromatic rings, and four singlets at δ 6.88, 6.79, 6.38 and 6.37 attributed to four hydrogens attached to sp^2 carbon atoms. The complete assignment of the protons and carbons was made by comparison with literature data [18,19].

4-Hydroxybenzoic acid (**3**) was isolated as a white solid and was identified on the basis of its ^1H NMR and ^{13}C NMR data and comparative TLC with an authentic sample.

(6*R*,9*S*)-6′-(4′-Hydroxybenzoyl)-roseosideshowed a statistically significant (p<0.02) inhibition of 13.7% of the G-6-Pase in intact microsomes without affecting the enzyme activity of the disrupted system. The effect was lower than the 27.8% inhibition in intact microsomes obtained with phlorizin, a known inhibitor of the T1 transporter [20].These results suggest that **1** inhibits the G-6-P transporter (T1) without affecting either the catalytic subunit or phosphate/pyrophosphate transporter (T2) of the G-6-Pase system. The (6*R*,9*S*)-roseoside (**4**), isolated by Avila *et al.* [21], exerted an inhibition of 58.5% of the enzyme in intact microsomes, suggesting the 4-hydroxybenzoyl moiety in **1** had a negative effect on the biological activity.

Agathisflavonepresented a marked inhibition ofthe intact (63.6%) and disrupted (63.1%) microsomal enzyme system, suggesting that this molecule is capable of permeating through the microsomal membrane and inhibiting the catalytic subunit.

Experimental

General experimental procedures: High-speed countercurrent chromatography was performed using two chromatographs, an Ito Multi-Layer Coil Separator-Extractor (P.C. Inc. Potomac, MD, USA) with a single column of 325 mL and 1.6 mm internal diameter with a β between 0.5 and 0.85, and a CCC-1000 High-speed Counter-Current Chromatograph (Pharma-Tech Research, Baltimore, MD, USA) equipped with 3 coils connected in series (inner diameter of tubing 1.6 mm) with a β between 0.5 and 0.75. The total capacity of the column is 325 mL. CC was carried out using silica gel (230-400 mesh). TLC was performed using precoated silica gel F_{254} plates and detection was achieved at 254 and 366 nm, and by spraying with ceric sulfate in 10% H_2SO_4 and anisaldehyde in 50% H_2SO_4. NMR spectra were obtained using a JEOL spectrometer model Eclipse with an application camp of 270 MHz for ^1H and 67.5 MHz for ^{13}C, and a Bruker spectrometer with an application camp of 500 MHz for ^1H and 125 MHz for ^{13}C. Phlorizin, G-6-P and histones II-A were acquired from Sigma-Aldrich (Milwaukee, USA). All other chemicals used were analytical grade.

Plant material: Aerial parts (leaves and stems) of *O. polyantha* Engl. were collected in the Sipapo River, near to Cerro Pelota, southern Laja de Garza, between Autana and Guayapola Rivers in the Amazon forest, Amazon State, Venezuela in 1992, and identified by DrAnibal Castillo from the Biology School, Science Faculty, Venezuela Central University. A voucher specimen (N° 3308AC) was deposited in the Venezuela National Herbarium, (VEN).

Animals: Male Sprague–Dawley rats of 180–220 g were used after an overnight fast period.

Purification of microsomes and glucose-6-phosphatase assay: The microsomal fraction was purified following the method described Marcucci*et al.* [22]. In brief, the rat livers were homogenized in 3 volumes of 0.32 M sucrose and3 mM $MgCl_2$, centrifuged at 20,000*g* for 20 min. at 4ºC. The pellet was discarded and the supernatant centrifuged at 105,000*g* for 1 h at 4ºC; the pellet constituted the microsomal fraction. This was resuspended in 0.25 mM sucrose, 1 mM $MgCl_2$, and 5 mM HEPES (pH 6.5) to give a final protein concentration of 20 mg/mL and frozen at −80°C until use. Protein concentration was estimated using the Lowry method [23] modified by Markwell*et al.* [24].

G-6-Pase assays were performed by the method described by Burchell*et al.* [25] with intact and disrupted (histone treated) microsomes. In brief, the G-6-Pase hydrolyzed glucose-6-phosphate (G-6-P) to produce glucose and an inorganic phosphate ion. The latter formed a blue complex with ammonium heptamolybdate in acidic medium; this was measured at 820 nm using a Novaspec II spectrophotometer (Pharmacia).

Extraction and isolation: Dried and powdered vegetal material(leaves and stems; 944.04 g) was macerated and extracted with MeOH at room temperature to produce $MeOH_{rt}$. After that, the residual material was extracted with MeOH using a Soxhlet extractor to obtain the hot methanolic extract ($MeOH_{\Delta}$). Both extracts were concentrated *in vacuo* yielding 174.8 g (18.5%) and 8.14 g (0.86%) for $MeOH_{rt}$ and $MeOH_{\Delta}$, respectively. These were tested as G-6-Pase inhibitors and showed an inhibitory effect of 67.5 and 38.6% on intact microsomes, respectively.

Twenty gof the $MeOH_{rt}$ extract was suspended in a mixture of MeOH-water (1:1; v/v) yielding 2 fractions, soluble (Op-1) and insoluble (Op-2). Op-1 was concentrated *in vacuo*, while Op-2 was air-dried. Both fractions were treated with acetone separately, yielding 4 new fractions named OpA (5.19 g, 25.9%) and OpB (1.36 g, 6.80%) from Op-1 and OpC (5.96 g, 29.8%) and OpD (1.72 g, 8.60%) from Op-2. OpA and OpC were insoluble in acetone, while OpB and OpD were acetone soluble.

Fractions OpA, OpB and OpC were fractionated using a CCC-1000 HSCCC from Pharma-Tech Research (unpublished results), and OpD was analyzed using an HSCCC instrument from P.C. Inc.

OpB and OpD fractions had a common biflavonoidaglycone as the major compound, as determined by the iodine vapors. In order to obtain this biflavonoid as a pure compound the sample solution was

prepared by dissolving the crude fraction, OpD, in a solution composed of the upper and lower phases (1:1, v/v) of the solvent system used in HSCCC separation. The solvent system ($CHCl_3$-MeOH-H_2O, 4:3.5:2, v/v/v) was thoroughly equilibrated in a separation funnel by repeated vigorous shaking at room temperature. The 2 phases were separated shortly before use. The separation was initiated by filling the entire column with the stationary phase (upper phase) and this was immediately followed by sample injection dissolved in a mixture of stationary and mobile phases. The mobile phase was then eluted through the column at 2 mL/min, while the column was rotated at 800 rpm in the combined head to tail elution mode.

The fractions were monitored and combined by TLC similarity yielding 16 collective fractions. From OpD-Fr_5 (57.00 mg) a pure biflavonoid, agathisflavone was isolated. OpD-Fr_9 was purified by silica gel CC using the organic phase of the solvent system CH_2Cl_2-*n*-BuOH-MeOH-H_2O (4:0.1:1.5:2, v/v/v/v) as eluent to afford 4.91 mg of (6*R*,9*S*)-6′-(4″-hydroxybenzoyl)-roseoside, while OpD-$Fr_{10\text{-}11}$ was purified by silica gel CC using the mixture CH_2Cl_2-MeOH (94:6, v/v) as eluent to obtain 2.27 mg of 4-hydroxybenzoic acid.

(6*R*,9*S*)-6′-(4″-Hydroxybenzoyl)-roseoside (1)

Amorphous solid.

$[\alpha]_D^{20}$ – 57° (*c*=0.36, MeOH).

Rf value: 0.68 (AcOEt-MeOH-H_2O; 100:13.5:10; v/v/v).

UV-Vis (MeOH) λ_{max}: 229, 298 nm.

IR (films) υ_{max}: 3410.5, 2970.9, 2929.7, 1692.0, 1652.6, 1589.5, 1500.7, 1442.7, 1374.5, 1277.5, 1163.9, 1067.6, 854.0 cm^{-1}.

^1H NMR and ^{13}C NMR (CD_3OD):Table 1.

TSI-MS *m/z* (rel. Int.); 506.88 (100), 488.88 (10), 282.96 (97), 264.96 (75), 207.00 (92), 189.00 (34), 149.04 (52), 138.96 (75), 120.96 (45), figure 2.

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