Journal of Natural Products
Vol. 52, No. 4, pp. 679-685, Jul-Aug 1989

PLANT METABOLITES. STRUCTURE AND IN VITRO ANTIVIRAL ACTIVITY OF QUINOVIC ACID GLYCOSIDES FROM *UNCARIA TOMENTOSA* AND *GUETTARDA PLATYPODA*

R. AQUINO, F. DE SIMONE, C. PIZZA,*

Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli, via Domenico Montesano, 49, I-80131 Napoli, Italy

C. CONTI,

Istituto di Microbiologia, Facoltà di Medicina

and M.L. STEIN

Dipartimento di Studi Farmaceutici, Università degli Studi "La Sapienza," P. le Aldo Moro 5, 00185 Roma, Italy

ABSTRACT.—A reinvestigation of the bark of *Uncaria tomentosa* afforded, in addition to the major quinovic acid glycosides **1–3**, three further glycosides **4–6**. The structures were elucidated by spectral and chemical studies. Furthermore, a series of antiviral tests were performed on all these glycosides and on the related glycosides **7–9**, previously isolated from *Guettarda platypoda*.

Uncaria tomentosa (Willd.) DC., commonly known as "una de gato," is used in traditional Peruvian medicine for the treatment of arthritis, gastritis, cancer, and certain epidermic diseases, in the form of an aqueous extract of the bark. The aqueous or EtOH extract of this plant was shown to have cytostatic, contraceptive, and anti-inflammatory activity (1). A number of alkaloids displaying a pronounced enhancement of phagocytosis were isolated (2), while no phytochemical or pharmacological work has been done on the nonalkaloidal constituents.

As part of a series of studies on Rubiaceae of South American flora used in local medicine (3–6), we recently have isolated three new quinovic acid glycosides **1–3** from *U. tomentosa* (5) and seven quinovic acid glycosides from *Guettarda platypoda* DC. (4,6). All these glycosides showed varied structures among which four groups can be discerned, including glycosides having a C-3, a C-28, a C-3,28, or a C-3,27 glycosylation pattern. The last group of compounds has not previously been reported in nature.

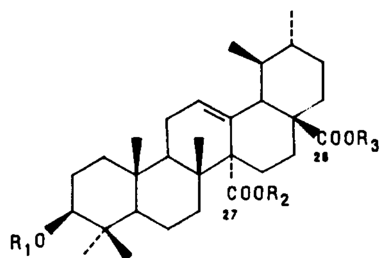
Various biological activities have been described for triterpenoid saponins (7). However, little is known regarding antiviral activity, which was reported for the glycoside glycyrrhizin (8), glycyrrhetinic acid (9), and some other triterpenoids (10) that were found to inhibit the multiplication of some DNA viruses.

In this paper we report a reexamination of a much larger sample of *U. tomentosa* that afforded, in addition to the major compounds **1–3**, three minor compounds **4–6**. We also conducted an investigation on the possible antiviral activity of the above compounds against two RNA viruses: a minus-strand RNA virus (vesicular stomatitis virus, VSV) and a plus-strand RNA virus (rhinovirus type 1B, HRV1B). The antiviral tests have been performed also on the related glycosides **7–9**, previously isolated from *G. platypoda*.

RESULTS AND DISCUSSION

The $CHCl_3$-MeOH (9:1) extract of the bark of *U. tomentosa* afforded, in addition to the major glycosides **1–3** (5), three other quinovic acid glycosides **4**, **5**, and **6**, the last one occurring in very minor amount.

On acid methanolysis compound **4** liberated methylquinovoside and methylgluco-



	R_1	R_2	R_3
3		H	H
1			H
2		H	
5		H	
4		H	
6	H	H	
7		H	H
8			H
9		H	

side in the ratio 1:1, and compound **5** gave methylfucoside and methylglucoside in the ratio 1:1. The fabms, in negative ion mode, of **4** and **5** showed the same fragmentation pattern (Table 1). Both compounds gave a quasi-molecular anion at *m/z* 793 [also observed in the spectrum of **3** (3)], a fragment at *m/z* 647 $[(M-H)-146]^-$ suggesting the loss of a deoxyhexose without the glycosidic oxygen, a fragment at *m/z* 615 $[(M-H)-178]^-$ resulting from the cleavage of a glucose with the glycosidic oxygen, and a fragment at *m/z* 631 $[(M-H)-162]^-$ corresponding to the loss of a glucose without the glycosidic oxygen or to the loss of a deoxyhexose with the glycosidic oxygen. Starting from the 647, 631, and 615 peaks, the facile loss of a carboxyl group, observed also in the fabms spectra of other quinovic acid glycosides (**4**–**6**), led to peaks at *m/z* 604, 587 and 571, respectively. Other fragments at *m/z* 441 and 425 were ascribable to the decarboxylated aglycone and were also present in the spectra of **1**–**3** (5).

TABLE 1. Fabms Spectral Data for Compounds **4**, **5**, and **6**.[a]

m/z	**4** and **5**	*m/z*	**6**
793	$[M-H]^-$	809	$[M-H]^-$
647	$[(M-H)-146]^-$	647	$[(M-H)-162]^-$
631	$[(M-H)-162]^-$	631	$[(M-H)-178]^-$
615	$[(M-H)-178]^-$	603	$[(M-H)-(44+162)]^-$
603	$[(M-H)-(44+146)]^-$	587	$[(M-H)-(44+178)]^-$
587	$[(M-H)-(44+162)]^-$	441	$[(M-H)-(2\times 162)]^-$
571	$[(M-H)-(44+178)]^-$	425	$[(M-H)-(162+178)]^-$
441	603 − 162 or 587 − 146		
425	603 − 178 or 587 − 162		

[a]The mass unit lost corresponded to fragments indicated in the text.

The molecular formula $C_{42}H_{66}O_{14}$ for **4** and **5** was deduced by fabms and DEPT ^{13}C nmr. The quinovic acid structure of the aglycone and the substitution pattern of **4** and **5** followed from the nmr data. All aglycone carbon and proton signals could be assigned by comparison with the spectra of the related quinovic acid glycosides **1**–**3** (5). Particularly the aglycone spectral data for **4** and **5** (Table 2 and 3) closely resembled those of **2**. The presence in **4** and **5** of a sugar unit linked at a carboxyl group (C-27 or C-28) of the aglycone was derived by the ^1H-nmr signal at δ 5.41 (1H, d, $J=7.5$ Hz) (anomeric hydrogen) and by the ^{13}C-nmr signal at 95.8 ppm (anomeric carbon) (4,5,11).

The glycosyl ester linkage was proposed to be at C-28 of quinovic acid on the basis of the resonances of C-12, C-13, and C-14 (130.1, 134.4, 59.3 ppm, respectively) and of C-27 (179.5 ppm) and C-28 (178.5 ppm) carboxyl groups. All these values matched well with those found in a C-28 glycosyl ester like **2**, while in a C-27 glycosyl ester like **1** the C-12, C-13, and C-14 resonated at 130.8, 133.7, and 57.7 ppm, respectively, and C-27 and C-28 were shifted at 178.1 and 182.0 ppm, respectively (5). Also in agreement with a C-28 glycosylated quinovic acid structure, the ^1H-nmr signals of H-12 (δ 5.59, 1H, m) and of Me-26 (δ 0.89, 3H, s) were virtually unshifted with respect to **2**, whereas in **1** the H-12 and Me-26 signals appeared at δ 5.62 (1H, m) and at δ 0.92 (3H, s), respectively (5). C-3 of quinovic acid as the ether glycosidation site was derived from the ^{13}C-nmr signal at 90.7 ppm (CH by DEPT) (5,12).

For the sugar moiety the ^1H- and ^{13}C-nmr spectra confirmed the presence of a glucose and a fucose in **5** and of a glucose and a quinovose in **4**, also derived by fabms spectra and by the results of methanolysis; they proved that all the sugars were occurring in the β-D-pyranosyl form (13). It remained, therefore, to establish the relative

TABLE 2. [1]H-nmr Data for Compounds **4**, **5**, and **6** in δ (CD_3OD).

Aglycone proton	Compound		
	4	**5**	**6**
Me-23 (3H, s)	0.85	0.85	0.80
Me-26 (3H, s)	0.89	0.89	0.89
Me-29 and 30 (6H, d, sharp)	0.96	0.96	0.94
Me-25 (3H, s)	1.00	1.00	0.99
Me-24 (3H, s)	1.04	1.04	submerged by Me-25 signal
H-12 (1H, m)	5.59	5.59	5.58
Sugar proton[a]			
Fu[b]-Me (3H, d, $J=6.0$ Hz)	—	1.28	—
Qui[c]-Me (3H, d, $J=6.0$ Hz)	1.30	—	—
H-1′ (1H, d, $J=7.0$ Hz)	4.32	4.25	—
H-1″ (1H, d, $J=7.5$ Hz)	5.42	5.42	5.42
H-1‴ (1H, d, $J=7.5$ Hz)	—	—	4.62

[a]Other signals were overlapped in the region δ 3.0–4.0.
[b]Fucose
[c]Quinovose

sugar position in **4** and **5**. The alkaline hydrolysis of **4** and **5** yielded quinovic acid-3β-*O*-β-D-quinovopyranoside and quinovic acid-3β-*O*-β-D-fucopyranoside, respectively (see Experimental), whose [1]H- and ^{13}C-nmr spectra showed no signals ascribable to a glucose in an ester linkage but exhibited the signals ascribable to a β-D-quinovopyranose and to a β-D-fucopyranose, respectively, virtually unshifted with respect to **4** and **5**. This finding clearly confirmed that the deoxyhexose unit was linked at C-3 and the glucose unit at C-28 of the aglycone. From all these data the structure quinovic acid-3β-*O*-(β-D-fucopyranosyl)-(28↦1)-β-D-glucopyranosyl ester was assigned to **5**, and the structure quinovic acid-3β-*O*-(β-D-quinovopyranosyl)-(28↦1)-β-D-glucopyranosyl ester was assigned to **4**.

On acid methanolysis and glc analysis, compound **6** gave a gc pattern characteristic of glucose. The fabms spectrum of **6** showed a quasi-molecular anion at *m/z* 809, corresponding to the molecular formula $C_{42}H_{66}O_{15}$, and a fragmentation pattern due to the subsequent loss of two hexose units (Table 1).

Comparison of fabms and [1]H-nmr spectral data of **6** with those of **4** and **5** (Tables 1–3) indicated a quinovic acid structure for the aglycone. The chemical shifts of the aglycone protons were superimposable on those of **4** and **5** except for the signals of Me-23 (δ 0.80, 3H, s) and Me-24 (δ 0.99, 3H, s) which were shifted upfield by 0.05 ppm relative to Me-23 (δ 0.85, 3H, s) and Me-24 (δ 1.04, 3H, s) in **4** and **5**.

These shifts were indicative of a quinovic acid derivative with a free 3β-hydroxyl group and with a C-28 glycosylated carboxyl group such as we have observed in a previous study on *G. platypoda* (6). Moreover, for the sugar moiety, the signal at δ 5.42 (1H, d, $J=7.5$ Hz, anomeric hydrogen) was ascribable to a hexose (glucose by glc) linked at C-28 through an ester linkage, and the signal at δ 4.62 (1H, d, $J=7.5$ Hz, anomeric hydrogen) was due to another hexose (glucose by glc) unit.

On the other hand, comparison of the spectral data of **6** with those of quinovic acid-3β-*O*-(β-D-glucopyranosyl)-(28↦1)-β-D-glucopyranosyl ester indicated that the molecular formula, the fabms fragmentation pattern, and the results of the

TABLE 3. ^{13}C-nmr Spectral Data for Compounds **4** and **5** in ppm (CD_3OD).

Aglycone carbon	Compound **4** and **5**	DEPT	Sugar carbon	Compound **4**	Compound **5**	DEPT
1	39.9	CH_2		Quinovose at C-3	Fucose at C-3	
2	27.1	CH_2				
3	90.7	CH	1′	106.6	107.0	CH
4	40.4	C	2′	76.0	73.1	CH
5	56.8	CH	3′	78.1	75.4	CH
6	19.3	CH_2	4′	71.4	73.0	CH
7	37.9	CH_2	5′	77.1	71.5	CH
8	40.8	C	6′	18.0	17.1	Me
9	48.0[a]	CH				
10	37.9	C				
11	24.1	CH_2				
12	130.1	CH				
13	134.4	C				
14	59.3	C		Glucose at C-28		
15	26.9	CH_2				
16	26.1	CH_2	1″	95.7		CH
17	48.0[a]	C	2″	74.1		CH
18	55.6	CH	3″	78.6		CH
19	40.2	CH	4″	71.6		CH
20	38.2	CH	5″	78.4		CH
21	31.3	CH_2	6″	62.8		CH_2
22	37.3	CH_2				
23	19.3	Me				
24	28.6	Me				
25	16.9	Me				
26	18.4	Me				
27	179.5	C				
28	178.5	C				
29	17.1	Me				
30	21.4	Me				

[a]Under CD_3OD signal.

methanolysis were the same for both compounds, while the ^{1}H-nmr spectra showed main differences for the Me-23 and Me-24 resonances (4).

The amount of compound **6** was insufficient for further investigation and the position of the interglycosidic linkage remained undefined. From the above findings the structure quinovic acid-(28↦1)-β-D-glucopyranosyl β-D-glucopyranosyl ester was assigned to **6**.

An inhibitory effect against VSV infection was evident for all the nine compounds tested, although at relatively high concentrations with respect to the toxic dose (tox c_{50}) for CER cell morphology and growth (Table 4). No relationship can be noted between the number of the sugar residues and the antiviral activity. The presence of the free C-27 carboxyl group seems to be important; also the nature of the sugar moiety affects the activity, e.g., quinovose (in **4**) is better than fucose (in **5**), when all the other characters in the structure are the same. The most active compound is **3**, with both carboxyl groups free.

Almost all these quinovic acid glycosides were inactive against rhinovirus type 1B infection in HeLa cells; only **6** and **9**, both containing two glucose units and the free C-27 carboxyl group, reduced the viral cytopathic effect by 50% at 30 and 20 μg/ml, respectively. The maximum nontoxic concentration for HeLa cells of compound **6** was 60 μg/ml and of compound **9** was 100 μg/ml.

TABLE 4. In vitro anti-VSV Activity and Cytotoxicity of Glycosides **1–6** from *Uncaria tomentosa* and Glycosides **7–9** from *Guettarda platypoda* in CER Cells.

Compound	MIC_{50}		tox c_{50}
	μg/ml	mmol	μg/ml
1	60.0	0.063	100
2	40.0	0.042	80
3	20.0	0.025	100
4	22.4	0.028	80
5	31.6	0.040	80
6	31.0	0.039	100
7	33.1	0.051	150
8	70.8	0.087	150
9	33.1	0.040	150

EXPERIMENTAL

APPARATUS.—^{1}H- and ^{13}C-nmr spectra were obtained on a Bruker MW-250 Spectrospin spectrometer; the fabms spectra in negative ion mode and the DEPT experiments were performed as described previously (14). Hplc was carried out on a Waters hplc system with a refractive index detector; glc on a Perkin-Elmer Sigma 115 instrument; optical rotation on a Perkin-Elmer 241 polarimeter.

PLANT MATERIAL.—The bark of *U. tomentosa* was collected at Lima, Peru, and identified by Edmundo Szeliga of the Instituto Peruano de Investigation Fitoterapica Andina. A voucher sample of the plant is deposited at the Herbarium of this Institute.

EXTRACTION AND ISOLATION.—The extraction procedure has been described in a previous paper (5). The $CHCl_3$-MeOH (9:1) dried extract (10 g) was chromatographed on a Sephadex LH-20 column (100 × 4 cm), using MeOH as eluent. Fractions (8 ml) were eluted, analyzed by tlc on SiO_2 in *n*-BuOH–HOAc–H_2O (60:15:25), and similar fractions were combined. Fractions 25–27 (680 mg) were submitted to hplc on a C-18 μ-Bondapack column (30 cm × 7.8 mm i.d.) using MeOH-H_2O (65:35) (flow rate 3.5 ml/min) as eluent to give four main fractions. The fraction collected after 10.4 min from injection contained glycoside **4** (20 mg), $[\alpha]^{25}$ +17° (c = 1, MeOH). The fraction collected after 16.1 min contained glycoside **5** (16 mg), $[\alpha]^{25}$ +15° (c = 1, MeOH). The fraction collected after 23.5 min contained glucoside **3** (45 mg) (5). The fraction collected after 25.6 min contained glycoside **6** (2 mg). The fabms, ^{1}H- and ^{13}C-nmr spectra of **4–6** are in Tables 1–3.

ACIDIC METHANOLYSIS OF COMPOUNDS **4–6**.—Methanolysis of each glycoside (0.5–1 mg) was achieved in the usual manner (14).

ALKALINE HYDROLYSIS OF **4** AND **5**.—Compounds **4** (10 mg) and **5** (8 mg) were separately heated in 0.5 M aqueous KOH (1 ml) at 110° in a stoppered reaction vial for 2 h. The reaction mixture was adjusted to pH 7 with HCl and then extracted with *n*-BuOH (5 ml). The organic phase was evaporated to dryness to give quinovic acid-3β-*O*-β-D-quinovopyranoside from **4** and quinovic acid-3β-*O*-β-D-fucopyranoside from **5**; these compounds were identified by comparison with literature data (15, 16) and by ^{13}C-nmr data (CD_3OD); all aglycone signals of these compounds were superimposable on those of glycoside **3** (5). Sugar signals of quinovic acid-3β-*O*-β-D-quinovopyranoside: C-1′ to C-6′ δ = 106.5 (CH), 76.0 (CH), 78.0 (CH), 71.3 (CH), 77.1 (CH), 18.0 (Me); sugar signals of quinovic acid-3β-*O*-β-D-fucopyranoside: C-1′ to C-6′ δ = 106.9 (CH), 73.0 (CH), 75.4 (CH), 73.0 (CH), 71.5 (CH), 17.2 (Me).

CELL AND VIRUSES.—M-HeLa (17) and CER (18) cells were grown at 37° in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (fcs) and 6% newborn calf serum, respectively. Rhinovirus type 1B was grown as described (19) and titrated in HeLa cells to estimate its tissue culture infectivity dose ($tcid_{50}$). VSV, Indiana serotype, was cultivated in CER cells and titrated by plaque assay as reported previously (20).

ESTIMATION OF CYTOTOXIC ACTIVITY IN UNINFECTED CELLS.—HeLa (1×10^4 cells/well) and CER (3.5×10^3 cells/well) cells in 96-well tissue culture plates were incubated in the presence of increasing concentrations of each compound for 2 days at 37°. Cytotoxicity was monitored by two different assays:

by a daily microscope examination of cell morphology and by counting the cell number in untreated and drug treated cells on the second day of incubation. The viability of cells was checked by neutral red uptake.

ANTI-RHINOVIRUS 1B BIOASSAY.—In 24-well plates 500 μl of twofold dilutions of each compound in maintenance medium (MEM 2% fcs) were added with 100 μl of virus dilution containing 100 $tcid_{50}$ and 400 μl of HeLa cells suspension (7.5×10^5 cells/ml). Plates were shaken and incubated at 33° in a 5% CO_2 atmosphere; they were checked daily by light microscopy and read when virus controls (no drug) showed 100% cytopathic effect (cpe).

The antiviral activity of the compounds was defined as the minimal inhibitory concentration (MIC_{50}) required to inhibit virus-induced cpe by 50%, when 100% of cells were destroyed by the virus in the control infected cultures.

ANTI-VESICULAR STOMATITIS VIRUS BIOASSAY.—Confluent monolayers of CER cells in 6-well plates were infected with 200 μl of a virus dilution containing approximately 100 plaque forming units (pfu). Following a 1 h adsorption period at 37°, the cells were added with an overlay medium containing doubling concentrations of compounds and incubated until plaques developed (36–48 h at 37°).

The antiviral activity of the compounds was expressed as the minimal inhibitory concentration (MIC_{50}) required to reduce the plaque number by 50% with respect to control infected cells. The MIC_{50} value was calculated by plotting the percentage of plaque counts in drug-treated cells versus the logarithm of the compound concentration (μg/ml).

ACKNOWLEDGMENTS

The authors are grateful to Dr. P. Iaccarino for the supply of the plant. They also thank the staff of the "Servizio di Spettrometria di massa del C.N.R. e dell'Università di Napoli" for fabms spectra. The antiviral study in this work was supported by the Istituto Pasteur, "Fondazione Cenci Bolognetti," Università di Roma.

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Received 29 June 1988