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PLANT METABOLITES. NEW COMPOUNDS AND ANTI-INFLAMMATORY ACTIVITY OF *UNCARIA TOMENTOSA*

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ABSTRACT.—Bioassay-directed fractionation of the anti-inflammatory extracts of *Uncaria tomentosa*, using the carrageenan-induced edema in rat paw, has led to the isolation of a new quinovic acid glycoside **7** as one of the active principles. Furthermore, a new triterpene **8** was isolated as its methyl ester. The structures were elucidated by spectral and chemical studies.

Uncaria tomentosa (Willd.) DC. (Rubiaceae) is widely used in Peruvian traditional medicine as an anti-inflammatory, contraceptive, and cytostatic remedy (1).

Our previous studies on *U. tomentosa* resulted in the isolation and identification of six new quinovic acid glycosides having a C-3, a C-28, a C-3, -28 or a C-3, -27 glycosylation pattern and displaying moderate antiviral activity (1,2), from the $CHCl_3$-MeOH (9:1) extract. Recently, we also found three novel polyhydroxylated triterpenes in the $CHCl_3$ extract of the same species (3).

As part of our continuing search for new pharmacologically active metabolites from *U. tomentosa*, we describe the extraction and separation of the bark to identify the compounds responsible for the anti-inflammatory activity. The extracts and fractions were bioassayed by the carrageenan-induced edema test in rat paw (4).

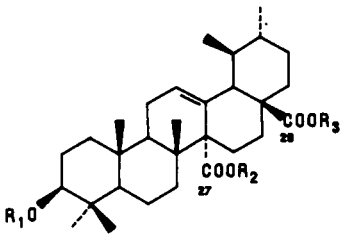
Subsequent bioassay-directed fractionation of the most active fractions has led to the isolation and characterization of a new quinovic acid glycoside **7**, with a C-3, -27 glycosylation pattern, as one of the active anti-inflammatory principles. A new triterpenoid **8**, a glycoside **9** recently isolated by us from *Uncaria guianensis* (5), the alkaloid 5α-carboxystrictosidine (6), oleanolic acid, and ursolic acid have also been isolated for the first time from *U. tomentosa*.

RESULTS AND DISCUSSION

The root bark of *U. tomentosa* was successively extracted with petroleum ether, $CHCl_3$, $CHCl_3$-MeOH (9:1), MeOH, and H_2O. Each extract was tested orally for anti-inflammatory activity using the carrageenan-induced edema in rat paw (4). The amounts administered corresponded to 2 g/kg of dry bark. The $CHCl_3$-MeOH (9:1) extract (50 mg/kg po) and the H_2O extract (84 mg/kg po) were the most active, displaying 69.2 and 41.2% inhibition of the maximum edema (3 h), respectively, while the anti-inflammatory effects of the $CHCl_3$ and the MeOH extracts were not significant.

The separation of the crude $CHCl_3$-MeOH (9:1) extract by Sephadex LH-20 column yielded five main fraction I–V, which were tested at doses equivalent to 2 g/kg of dry bark under the same experimental conditions. The most active fractions were I (4.2 mg/kg po) and III (2.3 mg/kg po), both inhibiting the edema by 46.8 and 37.4% (3 h), respectively, while the inhibitory rates at 3 h of fractions II (25.2 mg/kg), IV (5.9 mg/kg), and V (9.1 mg/kg) were 7.54, 2.56, and 26.8%, respectively.

By means of reversed-phase hplc, compounds **1–6** (1,2), **7**, and **9** together with 5α-carboxystrictosidine were isolated from fraction I; oleanolic acid, ursolic acid, compound **8**, and 3β,6β,19α-trihydroxyurs-12-en-28-oic acid together with its 23-oxo and 23-nor-24-esomethylene derivatives (3) were isolated from fraction III. All pure compounds, tested by the same procedure, were inactive. As the tested doses were very low (ranging from 0.04 mg/kg to 0.51 mg/kg), a further set of experiments was per-



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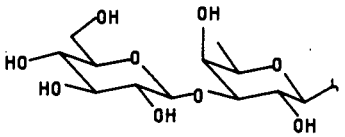
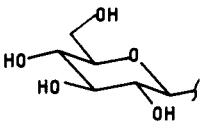
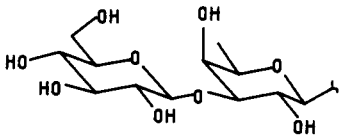
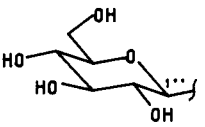
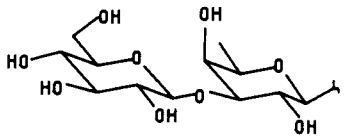
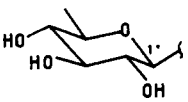
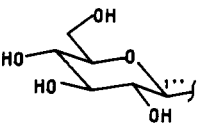
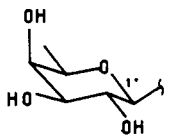
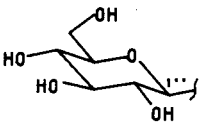
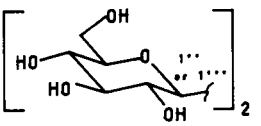
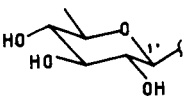
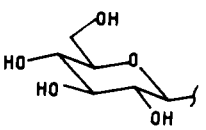
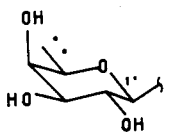
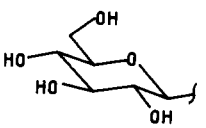
1			H
2		H	
3		H	H
4		H	
5		H	
6	H	H	
7			H
9			H

formed at higher doses (0.014 mmol of each compound/kg, equivalent to the ED_{50} of indomethacin); however, no significant inhibition of the edema was seen, while our control indomethacin (5 mg/kg po) was active.

In a further experiment, we tested compound **7**, oleanolic acid, and 3β,6β,19α-trihydroxyurs-12-en-28-oic acid (each 20 mg/kg po), at the highest doses available from the isolated samples, which in the previous screening had weak but not significant anti-inflammatory effects. As shown in Figure 1, only glycoside **7** caused 33% inhibition at 3 h of the inflammatory response, while oleanolic acid and the other triterpenic acid were inactive at the same doses. A more complete study of **7** and the other compounds (i.e., dose-effect or even higher doses) was not possible because of the low amounts isolated.

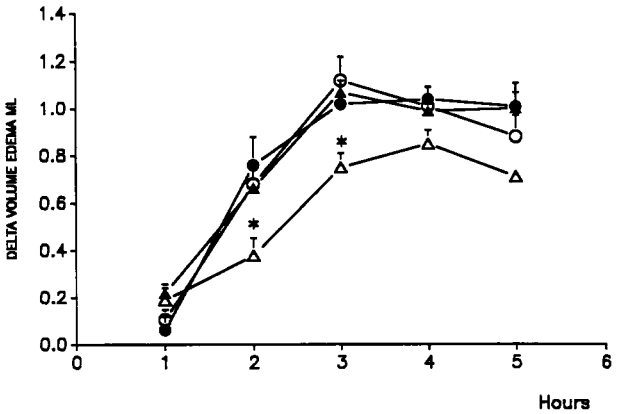


FIGURE 1. Effects of 3β,6β,19α-trihydroxyurs-12-en-28-oic acid (▲ − ▲), **7** (Δ − Δ), and oleanolic acid (● − ●) at doses of 20 mg/kg po on carrageenan edema. (○ − ○) Control. $*p < 0.05$.

On the basis of our results, we could hypothesize that the strong anti-inflammatory activity of the extracts and fractions may be due to the presence of a combination of compounds; it is possible that some compounds like **7** have an intrinsic anti-inflammatory effect while other compounds may act synergistically or as vehicles enhancing the biological activity. However, we cannot rule out that the activity of the extracts and fractions could be due to a very minor compound not isolated or to the isolated compounds but at higher doses. In fact, oleanolic acid is reported to have anti-inflammatory effects in the same test but at higher doses (40 mg/kg) and by ip or local administration (7).

The structural elucidation of the new glycoside **7** proceeded as follows. On acid methanolysis and glc analysis of the persylilated methyl sugars, methyl quinovoside and methyl glucoside were obtained in the ratio 1:1 from **7**, as had been obtained from glycoside **4** (2). The molecular formula $C_{42}H_{66}O_{14}$ was deduced by fabms and ^{13}C- and DEPT ^{13}C-nmr analysis. The fabms, in negative ion mode, showed a quasi-molecular anion at *m/z* 793 $[M - H]^-$ and the same fragmentation pattern (see Experimental) previously observed in the spectrum of **4**, indicating that **7** was isomeric to **4**. Major fragments were due to the loss of one deoxyhexose and one hexose unit.

An accurate inspection of the nmr spectra of **7** and comparison with those of related glycosides **1–6** (1,2) (Table 1 and Experimental) suggested a quinovic acid structure with a sugar unit (^1H nmr H-1′, δ 4.31; ^{13}C nmr C-1′, 106.4 ppm) linked at C-3 of the aglycone (90.7, CH by DEPT) and one other sugar unit linked at a carboxyl group (^1H nmr H-1″, δ 5.41; ^{13}C nmr C-1″, 95.7 ppm). The ^1H-nmr spectrum of **7** in particular resembled that of **4** (2). For the aglycone part a substantial difference was visible in the region of the olefinic proton chemical shifts; in fact, the spectrum of **7** showed the H-12 resonance as a broad multiplet at δ 5.64 whereas the spectrum of **4** exhibited the

TABLE 1. ^{13}C-nmr Spectral Data of Compounds **7** and **8** in ppm (CD_3OD).

Aglycone carbon	7	DEPT	Carbon	8	DEPT
C-1	40.0	CH_2	C-1	40.8	CH_2
C-2	27.1	CH_2	C-2	27.1	CH_2
C-3	90.7	CH	C-3	77.2	CH
C-4	40.3	C	C-4	54.0	C
C-5	57.0	CH	C-5	53.8	CH
C-6	19.3	CH_2	C-6	71.9	CH
C-7	38.0	CH_2	C-7	41.8	CH_2
C-8	41.0	C	C-8	39.5	C
C-9	48.1[a]	CH	C-9	49.0	CH
C-10	38.3	C	C-10	37.2	C
C-11	23.9	CH_2	C-11	24.6	CH_2
C-12	130.9	CH	C-12	129.7	CH
C-13	133.4	C	C-13	140.0	C
C-14	57.5	C	C-14	43.1	C
C-15	26.6	CH_2	C-15	29.5	CH_2
C-16	25.8	CH_2	C-16	27.2	CH_2
C-17	49.7[a]	C	C-17	48.0	C
C-18	55.4	CH	C-18	55.2	CH
C-19	40.1	CH	C-19	73.0	C
C-20	38.1	CH	C-20	43.1	CH
C-21	31.2	CH_2	C-21	26.6	CH_2
C-22	37.1	CH_2	C-22	38.7	CH_2
C-23	19.2	Me	C-23	179.5	C
C-24	28.6	Me	C-24	12.7	Me
C-25	16.9	Me	C-25	16.4	Me
C-26	18.1	Me	C-26	17.4	Me
C-27	178.0	C	C-27	24.9	Me
C-28	182.0	C	C-28	180.8	C
C-29	17.0	Me	C-29	27.2	Me
C-30	21.4	Me	C-30	16.5	Me
			-COOCH_3 × 2	51.5	Me
Sugar carbon					
Quinovose at C-3					
C-1′	106.4	CH			
C-2′	76.0	CH			
C-3′	78.1	CH			
C-4′	71.4	CH			
C-5′	77.2	CH			
C-6′	18.1	CH_3			
Glucose at C-27					
C-1″	95.7	CH			
C-2″	74.1	CH			
C-3″	78.6	CH			
C-4″	71.6	CH			
C-5″	78.4	CH			
C-6″	62.9	CH_2			

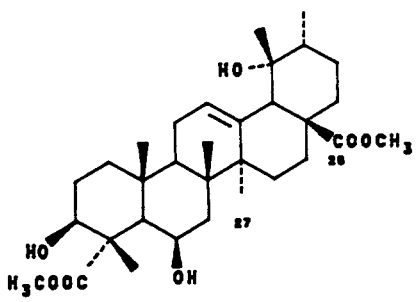
[a]Under CD_3OD signal.

H-12 resonance at δ 5.59. Moreover a downfield shift (+0.3 ppm) was recorded for C-26 methyl signal which in **7** appears at δ 0.92 (δ 0.89 in **4**). The ^{13}C-nmr signals of **7** (Table 1), assigned by the DEPT pulse sequence, also resembled those of **4**. Some diagnostic differences were noticed in the region of the sp^2 carbon resonances. In particular,

the signals resonating at 130.9 ppm (C-12) and at 133.4 ppm (C-13) were usually observed in a quinovic acid C-27 glycosyl ester like **1** (1), whereas in the spectrum of a C-28 glycosyl ester like **4**, C-12 and C-13 resonated at 130.1 and 134.4 ppm, respectively. The observed upfield shifts of C-14 and C-27 ($\Delta\delta$ −1.8 and 1.5 ppm, respectively) and the downfield shift of C-28 ($\Delta\delta$ +3.5 ppm) in the spectrum of **7**, with respect to **4**, confirmed the presence of a glycosyl ester linkage at C-27 (1).

For the sugar moiety the nmr data confirmed the presence of a β-D-glucopyranoside and a β-D-quinovopyranoside, also derived by fabms and by the results of methanolysis; so it only remained to establish the relative sugar position at C-3 and at C-27 of the aglycone. Alkaline hydrolysis of **7** yielded quinovic acid-3-β-*O*-β-quinovopyranoside (2), which had no ^{1}H- and ^{13}C-nmr signals ascribable to a glucose in an ester linkage but did for a β-D-quinovopyranose in an ether linkage, and clearly indicated that quinovose was linked at C-3 and glucose at C-27. The structure quinovic acid-3-β-*O*-(β-D-quinovopyranosyl)-(27↦1)-β-D-glucopyranosyl ester was therefore assigned to **7**.

Part of fraction III obtained from the Sephadex LH-20 column was methylated with CH_2N_2 and separated by rp hplc to yield the new methylester **8** ($C_{32}H_{50}O_7$ from ms and nmr data). The ^{1}H-nmr spectrum of the methylated compound indicated the presence of six methyls as five singlets and one doublet, suggesting an ursane derivative with one oxidized methyl. The eims showed major ions at *m/z* 278 $[C_{17}H_{26}O_3]^+$ and at *m/z* 268 $[C_{15}H_{24}O_4]^+$ for a retro-Diels-Alder cleavage of ring C, typical of an urs-12-ene and previously observed in similar compounds (3). The former ion and significant peaks at *m/z* 260 $[278-18]^+$ and 201 $[260-59]^+$ could be assigned to the ring D/E fragments which possess one hydroxyl and one carbomethoxyl groups. Other ions at *m/z* 250 $[268-18]^+$ and 191 $[250-59]^+$ must be derived from ring A/B fragments, which must also contain two hydroxyls and one carbomethoxyl (8).



8

Detailed analysis of nmr spectra by comparison with those of the triterpenes previously isolated (3) revealed characteristic features of a methyl 3,6,19-trihydroxyursolate. Two secondary hydroxyl groups were located at C-3β and C-6β positions from signals at δ 3.95 (1H, dd, J = 11.5, 4.0 Hz, H-3) and at δ 3.85 (1H, m, $W_{1/2}$ = 6 Hz, H-6) (3). Also the C-25 methyl (δ 1.34) and C-26 methyl (δ 1.00) resonances, shifted downfield by 1,3 diaxial interactions with respect to ursolic acid (3,9), confirmed the presence of a 6β-OH substituent.

Location of a tertiary hydroxyl group at C-19 was derived from a signal at δ 2.57 (1H, s, H-18) in the ^{1}H-nmr spectrum and a signal at 73.0 ppm (C-19, quaternary carbon) in the ^{13}C-nmr spectrum, both characteristic of a 19-*O*-substituted ursene (8,10). The nmr spectra of **8** also contained one fewer methyl singlet and one more carbomethoxyl signal than those of 3β,6β,19α-trihydroxyurs-12-en-28-oic acid (3),

suggesting one of the Me groups in **8** was replaced by a carbomethoxyl group. In addition, one of the Me signals (C-24) was shifted to δ 1.51 in the ^{1}H-nmr spectrum and to 12.7 ppm in the ^{13}C spectrum from δ 1.19 and 1.27 ppm, respectively, in 3β,6β,19α-trihydroxyurs-12-en-28-oic acid (3). Therefore, the -COOMe group would be located at the C-23 or C-24 position. Confirmation at the C-23 equatorial position was derived by the Δδ exhibited by C-4 (+13.7 ppm), C-6 (+2.9 ppm), C-3 (−3.0 ppm), C-5 (−3.4 ppm) and Me-24 (−4.6 ppm) with respect to the corresponding signals of 3β,6β,19α-trihydroxyurs-12-en-28-oic acid (3).

Substitution of Me for COOH was reported to induce similar shifts in the A and B ring carbons of gypsogenic acid which has a -COOH at the C-23 equatorial position with respect to methyl pomolate (8,11). Very different Δδ's were described for ilexgenin A methyl ester, which has a C-24 axial-COOMe with respect to methyl pomolate (8). The structure 3β,6β,19α-trihydroxyurs-12-ene-23,28-dimethyloate was therefore assigned to **8**.

EXPERIMENTAL

APPARATUS.—The following instruments were used: nmr, Bruker MW-250 Spectrospin; eims, AEI MS-30; fabms, Kratos MS 902 equipped with Kratos fab source; hplc, Waters hplc system with a refractive index detector; glc, Perkin-Elmer Sigma 115. The fabms and DEPT experiments were performed as described previously (1).

EXTRACTION AND ISOLATION.—The plant material was previously described (1). The bark of the air-dried liane (400 g) was extracted at room temperature with light petroleum ether, $CHCl_3$, $CHCl_3$-MeOH (9:1), MeOH, and H_2O in succession to give 3.50, 5.64, 10.16, 20.40, and 16.84 g of residues, respectively. The $CHCl_3$-MeOH (9:1) residue was chromatographed on a Sephadex LH-20 column (100 × 4 cm), with MeOH. Fractions (10 ml) were eluted, analyzed by tlc on SiO_2 in *n*-BuOH–HOAc–H_2O (60:15:25), and combined to give five main fractions I–V (0.840, 5.04, 0.46, 1.18, and 0.62 g, respectively). Fraction I was purified by hplc as described previously (1,2) to give **1** (46 mg), **2** (30 mg), **3** (26 mg), **4** (10 mg), **5** (8.2 mg), **6** (21 mg), **7** (60 mg), **9** (64 mg), and 5α-carboxystrictosidine (102 mg).

Purification of fraction III (360 mg) was achieved as before (3) to give 3β,6β,19α-trihydroxyurs-12-en-28-oic acid and its 23-oxo and 23-nor-24-esomethylene derivatives (3) (100.3, 78.6, and 34 mg, respectively), oleanolic acid (60 mg), and ursolic acid (40 mg). Part of fraction III (100 mg) was methylated with CH_2N_2 and then chromatographed on a C_{18} μ-Bondapak column (30 cm × 7.8 mm i.d., flow 3.0 ml/min) using MeOH-H_2O (95:5) to give **8** (3.2 mg, Rt = 11.2 min).

Compound **9** was identified as quinovic acid-3-β-*O*-(β-D-fucopyranosyl)-(27↦1)-β-D-glucopyranosyl ester by comparison with an authentic sample isolated by us from *U. guianensis* (5); 5α-carboxystrictosidine, oleanolic acid, and ursolic acid were identified by literature data and comparison with authentic samples (6,8,9).

Acid methanolysis of **7**.—Methanolysis of **7** (1 mg) and subsequent glc analysis of the persylylated methyl sugars were achieved in the usual manner (12).

Alkaline hydrolysis of **7**.—A 10 mg sample of **7** was 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-3-β-*O*-β-D-quinovopyranoside, identified by comparison with an authentic sample (5).

Compound **7**.—Negative fabms *m/z* $[M-H]^-$ 793, $[(M-H)-146]^-$ 647, $[(M-H)-162]^-$ 631, $[(M-H)-178]^-$ 615, $[647-44]^-$ 603, $[631-44]^-$ 587, $[615-44]^-$ 571, 441, 425; ^{1}H-nmr (CD_3OD at 250 MHz) δ 0.85 (3H, s, Me-23), 0.92 (3H, s, Me-26), 0.96 (6H, d, sharp, Me-29 and Me-30), 1.00 (3H, s, Me-25), 1.04 (3H, s, Me-24), 1.28 (3H, d, J = 6.0 Hz, Qui-Me), 4.31 (1H, d, J = 7.0 Hz, H-1′), 5.41 (1H, d, J = 7.5 Hz, H-1″), 5.64 (1H, m, H-12).

Compound **8**.—Hreims *m/z* $[M]^+$ 546.3564 (546.3556 calcd for $C_{32}H_{50}O_7$); eims *m/z* $[M]^+$ 546, $[M-H_2O]^+$ 528, $[M-COOMe]^+$ 486, $[486-H_2O]^+$ 468, $[M-2\times COOMe]^+$ 426, $[426-H_2O]^+$ 408, 414, 278, 268, $[278-H_2O]^+$ 260 base peak, $[268-H_2O]^+$ 250, $[268-15]^+$ 245, $[260-COOMe]^+$ 201, $[250-COOMe]^+$ 191, $[201-14]^+$ 187; ^{1}H-nmr (CD_3OD at 250 MHz) δ 0.95 (3H, d, J = 6.0 Hz, Me-30), 1.00 (3H, s, Me-26), 1.23 (3H, s, Me-27), 1.34 (6H, br s, Me-29 and Me-25), 1.51 (3H, s, Me-24), 2.58 (1H, s, H-18), 2.62 (1H, ddd, J = 13.5, 13.5, and 4.5 Hz, H-16ax), 3.60 (1H, s, -COOMe), 3.71 (3H, s, -COOMe), 3.85 (1H, m, W½ = 6.0 Hz, H-6eq), 3.95 (1H, dd, J = 11.5, 3.0 Hz, H-3ax), 5.26 (1H, m, H-12).

RAT PAW EDEMA ASSAY.—Male Wistar rats (Nossan) weighing 120–140 g were fasted for 12 h before the experiments. Group of at least 5 animals each received po 0.5 ml of tested extracts, fractions, or pure compound (at doses equivalent to 2 g of dry bark/kg unless otherwise stated) or indomethacin (5 mg/kg) (Sigma) suspended in 0.5% carboxymethylcellulose (Sigma), while the control group received vehicle only.

One hour after drug administration, rats were lightly anesthetized with Et_2O and paw edema was induced by a single subplantar injection of 0.1 ml carrageenan (Sigma) 1% (4). Paw volumes were measured using a water plethysmometer (Ugo Basile) immediately before the injection of carrageenan and at hourly intervals for 5 h thereafter. The volume of edema was expressed for each animal as the difference before and after the injection of carrageenan. The percent inhibition of edema was calculated for each group versus its vehicle-treated control group.

Data were analyzed using unpaired Student's *t*-test, and a $p<0.05$ was taken as significant.

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