Pergamon S0031-9422(96)00016-7 *Phytochemistry*, Vol. 42, No. 2, pp. 505–508, 1996

0031-9422/96 $15.00 + 0.00

UNSATURATED E-RING TRITERPENES FROM *RUBUS PINFAENSIS*

DAVID G. DURHAM, IAIN X. LIU and R. MICHAEL E. RICHARDS

School of Pharmacy, The Robert Gordon University, Aberdeen AB9 1FR, U.K.

(*Received in revised form* 27 *November* 1995)

Key Word Index—*Rubus pinfaensis*; Rosaceae; pentacyclic triterpene; E-ring unsaturation; pinfaenosides.

Abstract—Two new isomeric unsaturated E-ring pentacyclic triterpenoids have been isolated from the roots of *Rubus pinfaensis*. These triterpenes, glucosides of 2α,3β,23-trihydroxyurs-12-en-28-oic acids with additional Δ^{18} or Δ^{19} unsaturation, were hydrolysed to the previously unreported pinfaenoic and isopinfaenoic acids, which, on acetylation and methylation, were converted into methyl triacetoxy-esters (methyl 2α,3β,23-triacetoxyursa-12,18- or -12,19-dien-28-oates), allowing purification and characterization by ^{13}C and ^{1}H NMR techniques. The possibility of these triterpenoids arising as artefacts of the isolation process has been addressed.

INTRODUCTION

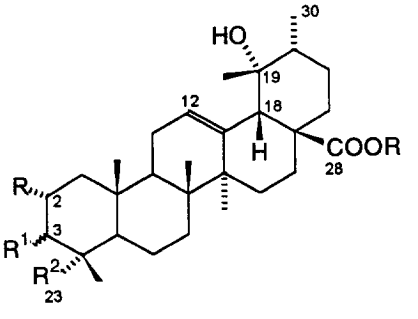
Rubus pinfaensis Levl. et Vant is a herb indigenous to southwest China, growing in mountainous areas at an altitude of 1000 m. An extract of the root (*Lao xuongbao*) has been used in traditional Chinese medicine to promote the healing of burns [1]. Studies on the antibacterial properties of the constituents suggest that they inhibit the *in vitro* growth of a number of infective organisms and that the elimination of secondary infection may be the basis of their wound healing action [2, 3].

Chemical examination of the root constituents have revealed the presence of ursene triterpenoids both unique to this species (**1**) [4] and of more widespread occurrence in Rosaceae and other families (**2**–**5**) [5, 6]. The presence in root extracts of isomeric triterpenoid glycosides (**6**, **7**) shown to be 2α,3β,23-trihydroxyurs-12-en-28-oic acid glucosides, with further Δ^{18} or Δ^{19} unsaturation, is now reported. The possibility that **6** and **7** arose as chromatographic artefacts is discounted.

RESULTS AND DISCUSSION

After extraction, mixture **A** was obtained by chromatographic elution from silica. A positive Liebermann–Burchard test indicated the presence of triterpenoids, which were confirmed by comparison of the ^{13}C NMR spectra with those for literature compounds [7]. Although mixture **A** gave a single spot on both normal phase and RP TLC with a number of solvent systems, the multiple ^{13}C NMR signals between δ 123.6 and 138.8 suggested the presence of two polyunsaturated ursene compounds. In addition, signals in the range δ 62.2–95.8 were attributable to two β-glucopyranosyl ester units [8]. Mixture **A** was hydrolysed enzymically to mixture **B** using hesperidinase [9], with the appearance of glucose in the hydrolysate, confirming the presence of glycosides. Mixture **B** was separated partially on RP TLC into two spots (R_f 0.27 and 0.31) using methanol–H_2O (11:9). To characterize fully these components, mixture **B** was methylated with diazomethane to give esters **10** and **11**, which were separated by chromatograph and then acetylated to give **12** and **13**, respectively.

The ^{13}C NMR signals at δ 125.2 and 138.5 indicated that **10** was an ursene triterpenoid [10], while signals at



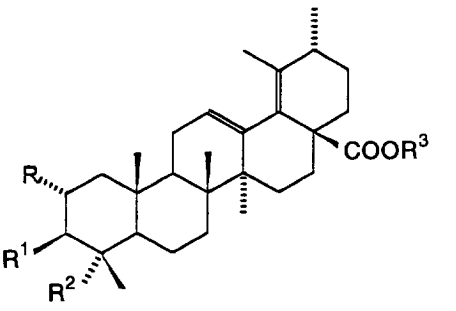
	R	R^1	R^2	R^3
1	α-OH	β-OH	CHO	Glu
2	α-OH	β-OH	CH_2OH	Glu
3	H	β-OH	Me	COOH
4	α-OH	α-OH	Me	COOH
5	α-OH	β-OH	Me	Glu

δ 132.6 and 135.9 suggested the presence of a second double bond. Further signals at δ 68.7, 79.4 and 69.1 indicated three hydroxyl-bearing carbons. After acetylation of **10** to give **12** these signals were shifted to δ 69.9, 74.7 and 65.2 and were accompanied by the expected acetoxy signals. The triacetylation of **10** seemed to preclude the presence of a 19α-OH group, which acylates only with difficulty [7]. The assignment of the substitution pattern as that of a 2α,3β, 23-trihydroxyursene was consistent with ^{13}C NMR data for reported triterpenoids [11, 12]. The ^{1}H NMR signals at δ 5.20 and 5.11 were assigned to 2β and 3α axial protons, while the C-23 primary alcohol group gave rise to a quartet at δ 3.59 and 3.88 [13].

The additional double bond was confirmed by high resolution FAB mass spectrometry. Compound **10** gave a molecular ion (m/z 523.3406, $[M+Na]^+$) for the molecular formula $C_{31}H_{48}O_5$, while the triacetoxy derivative **12** gave data consistent with the formula $C_{37}H_{54}O_8$. The mass fragmentation patterns of both **10** and **12** showed peaks at m/z 247, arising from ursene D/E rings by retro-Diels–Alder fragmentations [14], in contrast to the peak at m/z 249, from the saturated rings of methyl 2α,3β,23-trihydroxyurs-12-ene-28-oate (**14**). The UV absorbance at λ 226 nm suggested the presence of a heteroannular diene system [15], with the second double bond in conjugation at C-18/C-19. This observation is similar to that seen in other triterpene cisoid Δ^{18} systems [16, 17] and is supported by the absence of ^{13}C NMR signals at δ 53.1 or 39.3, assigned in the saturated analogue **14** to C-18 and C-19 [11]. The ^{1}H NMR spectrum of **10** showed a vinylic methyl group (δ 1.73, C-29), and a single olefinic proton (δ 5.38, C-12), while lacking the 18β-H signal observed at δ 2.25 for **14**. A crystallographic study, which unequivocally establishes the structure of **12** as a 12,18-diene [18], has confirmed these results.

The spectral evidence establishes the structure of **10** as methyl 2α,3β,23-trihydroxyursa-12,18-dien-28-oate, while **12** is the 2α,3β,23-triacetoxy analogue. The parent acid (**8**) from which they derive is 2α,3β,23-trihydroxyursa-12,18-dien-28-oic acid. This acid has been named pinfaenoic acid and occurs naturally in *R. pinfaensis* as the glycoside D-glucosyl 2α,3β,23-trihydroxyursa-12,18-dien-28-oate (**6**).

The FAB mass spectrum for **11** also gave a molecular ion corresponding to a molecular formula $C_{31}H_{48}O_5$, with agreeing data for the formula of the triacetoxy derivative **13**. The mass fragmentation patterns of **11** and **13** showed evidence of unsaturation in the D/E rings from the peak at m/z 247. Compounds **11** and **13**, in contrast to **10** and **12**, did not show UV absorbance above 213 nm, suggesting that the additional double bond was not conjugated with the Δ^{12} bond. While the ^{13}C NMR data for **11** and **13** allowed assignment of signals for C-1 to C-16 by comparison with those of **10** and **12**, the signals for the E-ring carbons (C-17 to C-22) showed some significant differences in chemical shifts (Table 1). The ^{1}H NMR spectrum for **13** (Table 2) showed vinylic methyl

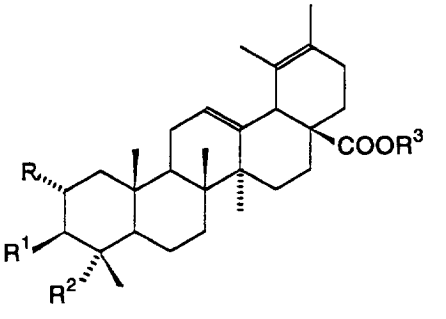


	R	R^1	R^2	R^3
6	α-OH	β-OH	CH_2OH	Glc
8	α-OH	β-OH	CH_2OH	H
10	α-OH	β-OH	CH_2OH	Me
12	α-OAc	β-OAc	CH_2OAc	Me

singlets (δ 1.65 and 1.54, C-29 and C-30), suggesting a tetrasubstituted double bond located at C-19/C-20.

This evidence leads to the structural assignment of **13** as methyl 2α,3β,23-triacetoxyursa-12,19-dien-28-oate. Consequently, **9** must be a 12,19-dienoic acid (isopinfaenoic acid), while the naturally occurring glycoside (**7**) is the 28-glucosyl ester.

Unsaturated E-ring ursene derivatives (tormentosolic and vanguerolic acids [16], cordepressenic acid [19] and randialic acid B [17]) have been reported previously. The occurrence of related glycosides [9] has been ascribed to artefacts formed during isolation by dehydration of 19α-hydroxyl derivatives. In contrast, the mild hydrolytic and transformation conditions used in



	R	R^1	R^2	R^3
7	α-OH	β-OH	CH_2OH	Glc
9	α-OH	β-OH	CH_2OH	H
11	α-OH	β-OH	CH_2OH	Me
13	α-OAc	β-OAc	CH_2OAc	Me

Table 1. ^{13}C NMR data for compounds **10–13** (62.5 MHz; $CDCl_3$)

C	2* [4]	10	12	11	13
1	47.9	46.6	44.4	46.8	43.9
2	68.9	68.7	69.9	68.7	69.8
3	78.2	79.4	74.7	79.9	74.7
4	43.6	42.5	41.7	42.4	41.7
5	48.5	48.6	47.6	48.9	47.6
6	18.7	18.5	18.5	18.1	17.7
7	33.1	34.0	33.9	33.2	33.1
8	40.6	38.8	38.8	39.1	39.1
9	47.9	47.5	47.8	47.5	47.7
10	38.3	37.9	37.6	38.0	37.7
11	24.1	31.0	31.0	23.1	23.3
12	128.3	125.2	125.0	126.6	126.5
13	139.2	138.5	138.6	137.5	137.6
14	42.1	34.8	34.8	43.2	43.2
15	29.1	28.3	28.4	28.2	28.2
16	26.0	21.7	21.4	21.8	21.6
17	48.5	49.3	49.3	46.4	46.8
18	54.3	135.9	136.0	49.8	49.8
19	72.6	132.6	132.6	128.0	127.9
20	42.1	44.5	44.0	123.6	123.8
21	26.6	26.3	26.4	32.5	32.5
22	37.7	34.1	34.1	27.8	27.8
23	66.5	69.1	65.2	69.8	65.1
24	14.3	12.9	13.8	12.8	13.9
25	17.5	18.1	17.5	17.4	17.3
26	17.5	17.7	17.7	16.8	16.8
27	24.5	23.1	23.1	23.3	23.3
28	177.0	177.0	176.8	177.9	177.8
29	27.0	19.2	19.1	20.2	20.1
30	16.7	17.7	17.7	17.5	17.3
28-OMe	†	51.5	51.5	51.5	51.5
OAc 1			170.7		170.7
2			20.7		20.7
OAc 1			170.3		170.3
2			20.7		20.7
OAc 1			170.3		170.3
2			20.1		20.2

*Run in pyridine-d_5.
†Glucose: 95.5, 73.7, 78.6, 70.8, 79.0 and 61.9.

Table 2. ^1H NMR chemical shifts for compounds **12** and **13** (250 MHz, $CDCl_3$, TMS internal standard)

H	12*	13†
2	5.20 (1H, *m*)	5.17 (1H, *dd*)
3	5.11 (1H, *d*)	5.10 (1H, *d*)
12	5.38 (1H, *m*)	5.50 (1H, *s*)
23_A	3.59 (1H, *d*)	3.58 (1H, *d*)
23_B	3.88 (1H, *d*)	3.88 (1H, *d*)
24	0.89 (3H, *s*)	0.84 (3H, *s*)
25	0.98 (3H, *s*)	0.98 (3H, *s*)
26	0.91 (3H, *s*)	0.90 (3H, *s*)
27	1.17 (3H, *s*)	1.15 (3H, *s*)
29	1.73 (3H, *s*)	1.65 (3H, *s*)
30	1.08 (3H, *d*)	1.54 (3H, *s*)
Acetoxy-CH_3	2.00 (3H, *s*)	2.00 (3H, *s*)
	2.05 (3H, *s*)	2.04 (3H, *s*)
	2.10 (3H, *s*)	2.09 (3H, *s*)
Methoxy-CH_3	3.06 (3H, *s*)	3.65 (3H, *s*)

*Shift of H-20 signal not assigned.
†Shift of H-18 signal not assigned.
s = singlet, *d* = doublet, *m* = multiplet.

this study suggest that **6** and **7** occur naturally as metabolites of *R. pinfaensis* along with 19α-hydroxyl derivatives.

In support of this claim, the presence in the root extract of 'native' unsaturated glycoside **6** has been demonstrated by RP- HPLC and -TLC. A spot from the original extract co-chromatographed on TLC in a similar fashion to the purified sample **A** (a mixture of **6** and **7**) (Table 3). A chromatographic peak for the original putative diene showed similar retention on HPLC to sample **A** as well as showing similar UV absorption characteristics.

To eliminate the possibility that **6** and **7** are artefacts of purification formed under mildly acidic chromatographic conditions, the stability of **2** as a model 19α-hydroxylated glycosidic substrate was examined. Treatment of **2** either at room temperature or 60° with silica gel in chloroform–methanol yielded unchanged starting material with no detectable 19α-hydroxyl dehydration product (Table 3). This evidence suggests that 19α-hydroxylated triterpenoids, while difficult to acylate, do not dehydrate under mildly acidic conditions. Hence, **6** and **7** occur naturally in *R. pinfaensis* and are not products of the purification procedures.

EXPERIMENTAL

General. NMR:250 MHz (^1H) and 62.5 MHz (^{13}C), $CDCl_3$, TMS as int. standard; FAB-MS: from Na^+ matrix.

Plant material. Roots of *R. pinfaensis* were collected from plants growing in Sichuan Province, P. R. China, and compared with authentic samples deposited in the herbarium of Chendu College of Traditional Medicine, P. R. China.

Extraction and isolation. The dried powdered roots were extracted and chromatographed as described in ref. [6]. Silica gel column elution with $CHCl_3$–MeOH—H_2O (10:3:1) afforded a fr., which was further purified by prep. RP-TLC (RP-18, Alltech) using MeOH–H_2O (3:2) to give **A**, a mixture of **6** and **7**.

HPLC. Jasco PU-980 HPLC pump with LC3 UV detector. Hypersyl (5 μm) ODS column (100 × 3 mm). Solvent: MeOH–H_2O (9:11), flow rate 0.8 ml min^{-1}. Detection at λ 207 nm.

TLC. Polygram sil G/UV 254. Solvent: $CHCl_3$–MeOH (5:1). Detection as purple spots with H_2SO_4/anisaldehyde.

Hydrolysis. Mixture **A** (110 mg) in McIlvain buffer was hydrolysed with hesperidinase (Sigma) (450 mg) at room temp. over 48 hr to give **B** (56 mg), a mixt. of acids **8** and **9**. The appearance of glucose in the hydrolysate was confirmed by PC against an authentic sample.

Methyl 2α,3β,23-trihydroxyursa-12,18-dien-28-oate (methyl pinfaenoate) (**10**) *and methyl 2α,3β,23-trihydro-*

Table 3. Chromatographic data for 19α-hydroxyl compound (**2**) and unsaturated triterpenoid mixture **A** (**6** and **7**)

	HPLC* (R_t, min^{-1})		TLC† (*Rf* values)	
	2	**A**	**2**	**A**
Initial root extract	2.8	12.5	0.32	0.40
Stability test (room temp.)	2.8	—	0.32	—
Stability test (60°)	2.8	—	0.32	—

*,†For chromatographic conditions, see Experimental.

xyursa - 12,19 - *dien* - 28 - *oate* (*methyl isopinfaenoate*) (**11**). Mixture **B** (56 mg) was methylated with CH_2N_2 in Et_2O at 0° for 1 hr. RP-CC using an *in situ* liquid paraffin coated silica column on elution with MeOH–H_2O (13:7) gave **10** (22 mg), needles mp 210–213° (aq. MeOH); FAB-MS (Na^+) *m/z*: 523.3406 $[M+23]^+$, for $C_{31}H_{48}O_5Na$ calc. 523.3399; ^{13}C NMR: Table 1; UV: λ_{max}^{EtOH} 226 nm. Further column elution gave **11** (15 mg), needles mp 183–184° (aq. MeOH); FAB-MS (Na^+) *m/z*: 523.3389 $[M+23]^+$, for $C_{31}H_{48}O_5Na$ calc. 523.3399; ^{13}C NMR: Table 1; UV: no **A** above 217 nm.

Methyl 2α,3β,23-*triacetoxyursa*-12,18-*dien*-28-*oate* (**12**). Compound **10** (17 mg) in pyridine (0.5 ml) was acetylated with ethanoic anhydride (0.5 ml) at room temp. for 24 hr. After removal of solvent, chromatography on a RP-silica gel column eluted with aq. MeOH (65%) gave **12** (15 mg), mp 215–217° (aq. MeOH); FAB-MS (Na^+) *m/z*: 649.3717 $[M+23]^+$, for $C_{37}H_{54}O_8Na$ calc. 649.3716; ^{13}C NMR: Table 1; ^1H NMR: Table 2.

Methyl 2α,3β,23-*triacetoxyursa*-12,19-*dien*-28-*oate* (**13**). Compound **11** (12 mg) was acetylated as before to yield **13** (8 mg), needles mp 118–120° (aq. MeOH); FAB-MS (Na^+) *m/z*: 649.3929 $[M+23]^+$, for $C_{37}H_{54}O_8Na$ calc. 649.3716; ^{13}C NMR: Table 1; ^1H NMR: Table 2.

Stability tests. Compound **2** (10 mg) was treated with silica gel (Merck 60 mesh, 500 mg) and solvent ($CHCl_3$–MeOH–H_2O, 7:3:1) at either room temp. or 60° for 24 hr. Chromatographic examination showed only unreacted starting material (Table 3).

Acknowledgement—We are grateful to Dr K. Welham (School of Pharmacy, University of London, U.K.) for recording the mass spectra.

REFERENCES

1. Jiangsu Xing Yi Xue Yuan (1977) *Dictionary of Chinese Herbal Medicine*, p. 841. Shanghai People's Publishing House, Shanghai.
2. Liu, I. X. (1994) Ph.D. Thesis. The Robert Gordon University, Aberdeen, U.K.
3. Richards, R. M. E., Durham, D. G. and Liu, X. (1994) *Planta Med.* **60**, 471.
4. Durham, D. G., Liu, X. and Richards, R. M. E. (1994) *Phytochemistry* **36**, 1469.
5. Liu, X., Durham, D. G. and Richards, R. M. E. (1992) *J. Pharm. Pharmacol.* **44** (suppl.), 1080.
6. Durham, D. G., Liu, X. and Richards, R. M. E. (1993) *J. Pharm. Pharmacol.* **45** (suppl. 2), 1152.
7. Seto, T., Tanaka, T., Tanaka, O. and Naruhasi, N. (1984) *Phytochemistry* **23**, 2829.
8. Yamasaki, K., Kohda, H., Kobyashi, T., Kasai, R. and Tanaka, O. (1976) *Tetrahedron Letters* 1005.
9. Higuchi, R., Kawasaki, T., Biswas, M., Pandey, V. D. and Dasgupta, B. (1982) *Phytochemistry* **21**, 907.
10. Doddrell, D. M., Khong, P. W. and Lewis, K. G. (1974) *Tetrahedron Letters* 2381.
11. Kojima, H. and Ogura, H. (1986) *Phytochemistry* **25**, 729.
12. Kojima, H. and Ogura, H. (1989) *Phytochemistry* **28**, 1703.
13. Cox, P. J., Durham, D. G., Liu, X. and Richards, R. M. E. (1993) *J. Spectrosc. Res.* **23**, 779.
14. Kikuchi, T., Matsuda, S., Sakai, Y., Nanba, T., Watenabe, K. and Dissanayake, M. R. B. (1984) *Chem. Pharm. Bull.* **32**, 3906.
15. Scott, A. I. (1964) *Interpretation of the Ultraviolet Spectra of Natural Products*, p. 45. Pergamon Press, London.
16. Barton, D. H. R., Cheung, H. T., Daniels, P. J., Lewis, K. G. and McGhie, J. F. (1962) *J. Chem. Soc. IV*, 5163.
17. Tandon, J.S., Agarwal, K. P. and Dhar, M. M. (1966) *Indian J. Chem.* **4**, 483.
18. Cox, P. J., Durham, D. G., Liu, I. X. and Richards, R. M. E. (1995) *Acta Cryst.* **C51**, 2135.
19. Khan, S. Y. M., Javed, K., Khan, M. H., Shamsii, M. A. and Siddiqui, A. A. (1991) *Phytochemistry* **30**, 1989.