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AN ECHINOCYSTIC ACID SAPONIN DERIVATIVE FROM *KALIMERIS SHIMADAE*

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Key Word Index—*Kalimeris shimadae*; Compositae; triterpene saponin; shimadoside A; echinocystic acid.

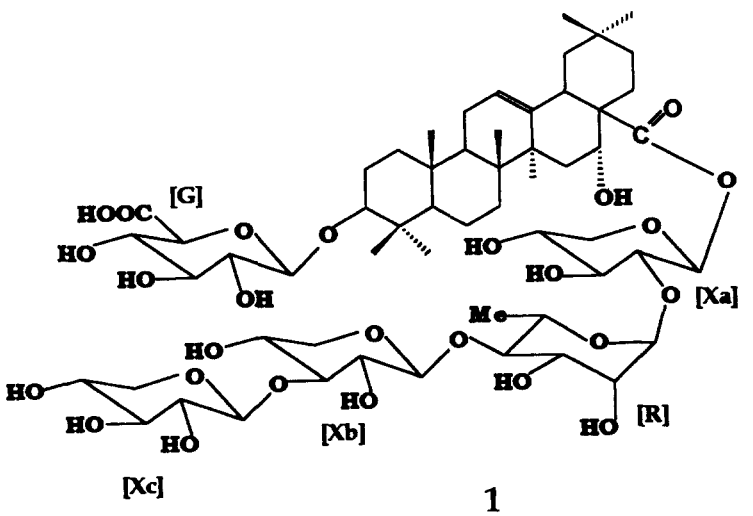
Abstract—A new triterpene saponin, shimadoside A, has been isolated from *Kalimeris shimadae* and its structure deduced as 3-*O*-β-D-glucopyranosiduronic acid–3β,16α-dihydroxyolean-12-en-28-oic acid–28-*O*-β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-xylopyranoside by means of spectral data, especially NMR, including COSY, HMQC, HOHAHA and ROESY techniques, and chemical degradation. Published by Elsevier Science Ltd

INTRODUCTION

Kalimeris shimadae Kitam is a perennial plant distributed widely in China. Its roots are used as anti-inflammatory and analgesic agents in Chinese traditional medicine [1]. The literature has revealed very little concerning its chemical constituents, except that it contains flavones and saponins. A previous biological investigation of an alcoholic extract of the whole plant exhibited antibacterial activity. Recently, in our preliminary screening, the crude saponin mixture from the roots of *K. shimadae* showed significant inhibitory effect on the DNA synthesis of human leukaemia HL-60 cells (IC_{50}, 30 μg ml^{-1}). This prompted us to undertake phytochemical studies on this plant. In this paper we present the isolation, structural elucidation and unambiguous NMR assignments of a new echinocystic acid glycoside shimadoside A (**1**) using ^{1}H NMR, ^{13}C NMR (BB and DEPT), COSY [2], HMQC [3], HOHAHA [4, 5] and ROESY [6–8] experiments.

RESULTS AND DISCUSSION

A methanol extract of the roots of *K. shimadae* was extracted successively with ethyl acetate and *n*-butanol. The butanol extract was subjected to Diaion HP-20 column chromatography and eluted with water and then



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methanol. The methanolic eluate was further chromatographed on silica gel and RP-8 Lobar columns to afford **1**.

Compound **1**, needles, mp 215–216°, $[\alpha]_D$ −29.4°, showed positive reactions in the Liebermann–Burchard and Molisch tests. The negative-ion FAB mass spectral data (*m/z* 1189 $[M-H^-]$), in combination with the ^{13}C NMR spectral data (Tables 1 and 2), indicated a molecular formula of $C_{57}H_{90}O_{26}$. The IR spectrum revealed the presence of a hydroxyl group (3400 cm^{-1}), ester group (1735 cm^{-1}), free carboxylic acid group (1709 cm^{-1}), double bond (1640 cm^{-1}) and glycosidic linkage (1000–1100 cm^{-1}). The ^1H NMR spectrum showed signals for seven singlet methyl groups at δ 0.81, 0.98, 1.00, 1.07, 1.09, 1.31 and 1.86, one trisubstituted olefinic proton at δ 5.62, five anomeric sugar protons at δ 5.18 (*d*, *J* = 8.0 Hz), 5.19 (*d*, *J* = 8.0 Hz), 5.05 (*d*, *J* = 8.0 Hz), 6.18 (*d*, *J* = 7.2 Hz) and 6.29 (*br s*) and one methyl group of a deoxy sugar unit at δ 1.76 (*d*, *J* = 6.0 Hz). The ^{13}C NMR spectrum exhibited the presence of six C–C bonded saturated quaternary carbons (δ 30.7, 36.8, 39.4, 39.8, 42.0 and 49.3), a pair of olefinic carbons (δ 122.4 and 144.2), a free carboxylic acid carbon (δ 172.7), an ester carbonyl carbon (δ 175.8), five anomeric carbons (δ 95.1, 101.2, 105.9, 106.0 and 107.1) and a sugar methyl carbon (δ 18.5). The number and chemical shifts of the tertiary methyl groups and quaternary carbons suggested that the aglycone of **1** was an oleanane-type triterpene and the ^1H NMR signal at δ 6.18 and ^{13}C NMR shift at δ 95.1 indicated the presence of an ester-linked sugar moiety [9].

Acidic hydrolysis of **1** yielded a sapogenin and glucuronic acid, xylose and rhamnose as sugar components. After treatment with diazomethane, the sapogenin was converted into its methyl ester (**1a**). The EI mass spectrum of **1a** showed a molecular ion peak at *m/z* 486, corresponding to $C_{31}H_{54}O_4$, and fragment ions at *m/z* 468, 278, 260, 219, 208 and 201. The fragment ions at *m/z* 278 derived from the D/E rings and at *m/z* 208 from the A/B rings were formed through the characteristic retro-Diels–Alder fragmentation of the C ring and indicated the presence of one hydroxyl group on the D and E rings and one hydroxyl group on the A and B rings [10]. The ^1H NMR spectrum of **1a** showed the signals of two hydroxy-

Table 1. NMR data for the aglycone parts of compounds **1**, **1b** and **1a**

	1		**1a**	**1b**	
Position	δ_H	δ_C	(δ_C)	(δ_C)	DEPT
1	0.90, 1.46	38.6	39.0	38.8	CH_2
2	1.86, 2.25	26.5	28.1	26.6	CH_2
3	3.42	88.9	78.1	89.0	CH
4	—	39.4	39.3	39.4	C
5	0.85	55.7	56.0	55.9	CH
6	1.76	18.5	18.8	18.4	CH_2
7	1.64	33.3	33.4	33.3	CH_2
8	—	39.8	40.1	39.9	C
9	1.79	46.9	47.2	47.1	CH
10	—	36.8	37.4	37.3	C
11	1.96	23.6	23.8	23.7	CH
12	5.62	122.4	122.8	122.6	CH
13	—	144.2	144.4	144.4	C
14	—	42.0	41.8	41.9	C
15	2.00, 2.23	36.0	36.0	35.9	CH_2
16	5.26	73.9	74.1	74.1	CH
17	—	49.3	49.2	49.0	C
18	3.45	41.3	41.4	41.3	CH
19	1.37, 2.78	47.2	47.0	46.9	CH_2
20	—	30.7	30 8	30.8	C
21	1.33, 2.45	35.9	36.0	35.9	CH_2
22	2.24, 2.41	32.0	32.3	32.4	CH_2
23	1.31	28.1	28.7	28.1	Me
24	0.98	16.8	16.8	16.9	Me
25	0.81	15.5	15.6	15.6	Me
26	1.09	17.3	17.3	17.2	Me
27	1.86	26.9	27.0	27.0	Me
28	—	175.8	177.7	177.6	C
29	1.00	33.0	33.1	33.1	Me
30	1.07	24.3	24.5	24.6	Me
OMe			51.7	51.7	Me

Pyridine-d_5, 500 MHz for δ_H of **1**; 125 MHz for δ_c of **1**; 100 MHz for δ_c of **1a** and **1b** (ppm).

Table 2. NMR data for the sugar moieties of compounds **1** and **1b***

	1		**1b**		
Position	δ_H	δ_C	δ_H	δ_C	DEPT
3-*O*-Sugar					
G-1	5.05 (*d*, 8.0)	107.1	4.98 (*d*, 8.0)	107.2	CH
2	4.14 (*d*, 8, 9)	75.4	4.07 (*dd*, 8, 9)	75.3	CH
3	4.32 (*dd*, 9, 9)	78.0	4.24 (*dd*, 9, 9)	77.9	CH
4	4.61 (*dd*, 9, 9)	73.3	4.61 (*dd*, 9, 10)	73.2	CH
5	4.69 (*d*, 9)	77.7	4.57 (*d*, 10)	77.2	CH
6		172.7		170.8	C
OMe			3.73 (*s*)	52.0	Me
28-*O*-Sugar†					
Xa-1	6.18 (*d*, 7.2)	95.1			CH
2	4.32	75.9			CH
3	4.25	77.0			CH
4	4.18	70.6			CH
5a	3.82 (*dd*, 10, 12)	66.9			CH_2
5b	4.34 (*dd*, 4, 12)				
R-1	6.29 (*br s*)	101.2			CH
2	4.77 (*br d*, 3)	71.5			CH
3	4.67 (*dd*, 3, 8)	72.4			CH
4	4.41 (*dd*, 8, 8)	83.4			CH
5	4.45 (*dq*, 8, 6.0)	68.3			CH
6	1.76 (*d*, 6.0)	18.5			Me
Xb-1	5.20 (*d*, 8.0)	105.9			CH
2	4.03	74.8			CH
3	4.04	87.3			CH
4	4.06	68.9			CH
5a	3.47 (*dd*, 10, 12)	66.7			CH_2
5b	4.21 (*dd*, 4, 12)				
Xc-1	5.18 (*d*, 8.0)	106.0			CH
2	4.02	75.0			CH
3	4.12	78.1			CH
4	4.13	70.7			CH
5a	3.66 (*dd*, 10, 12)	67.2			CH_2
5b	4.29 (dd, 4, 12)				

*Measured at 500 MHz for δ_H of **1**; 400 MHz for δ_H of **1b**; 125 MHz for δ_C of **1**; 100 MHz for δ_C of **1b** (ppm); *J* values (Hz) are shown in parentheses.

†Abbreviations: G, β-D-glucopyranoduronic acid; X, β-D-xylopyranosyl; R, α-L-rhamnopyranosyl.

methine groups at δ 3.45 (*dd*, $J = 10$, 4.5 Hz) and δ 5.04 (*br s*). These spectral features, and comparison of the ^{13}C NMR data for **1a** with literature data [11], suggested that **1a** was the methyl ester of 3β,16α-dihydroxyolean-12-en-28-oic acid (echinocystic acid). The correlation contours in the ROESY spectrum of **1**, showing the H-18 signal at δ 3.45 and the H-16 signal at δ 5.26, and the H-3 signal at δ 3.42 and the H-23 signal at δ 1.31, supported the α-configuration of a 16-hydroxyl group and the β-configuration of a 3-hydroxyl group, respectively.

Selective cleavage of the ester glycoside linkage of **1** by the method reported by Ohtani *et al.* [12], followed by treatment with diazomethane yielded a prosapogenin dimethyl ester (**1b**) and an anomeric mixture of a methyl tetraglycoside, which afforded rhamnose and xylose on acid hydrolysis. Compound **1b** showed a $[M + Na]^+$ ion peak at *m/z* 699 and gave D-glucuronic acid on acid hydrolysis. Comparison of the ^{13}C NMR data for **1b** with those of **1a** showed a significant shift for the C-3 signal (+10.9 ppm, from δ 78.1 to 86.2), demonstrating the C-3 position to be glycosylated. The anomeric proton signal at δ 4.98 (*d*, $J = 8.0$ Hz) and corresponding carbon signal at δ 107.2 of **1b** indicated the β-configuration of the glucuronic acid moiety [13]. The structure of **1b** was identified, therefore, as 3-*O*-β-D-glucopyranosiduronic acid–echinocystic acid dimethyl ester. Consequently, the remaining three molecules of xylose and one molecule of rhamnose in **1** must be present in the 28-*O*-sugar residue.

Elucidation of the structure of the 28-*O*-tetrasaccharide chain was performed as follows. In the first step, we proceeded to assign unambiguously the proton and carbon resonances of each monosaccharide unit by a combination of COSY, HOHAHA and HMQC experiments (Table 3). The anomeric proton resonances and some well-resolved resonances (i.e. Me-6 for the rhamnose and xylose methylene-5 for the xylose) were

used as starting points in the COSY and HOHAHA spectra, and allowed the assignment of the ^{1}H subspectra of various cabohydrate moieties. The assignment of the ^{13}C NMR data was then obtained from the HMQC spectrum. The complete assignments of the NMR data due to sugar units are listed in Table 2.

The next step was the determination of the linkage sites and sequence among the sugar residues in the 28-*O*-sugar chain. The ^{13}C chemical shifts of the sugar units were compared with those of methyl glycosides [13]. Glycosylation shifts were observed for the C-1 signal by −11 ppm and the C-2 by +1.3 ppm of one xylosyl group, for the C-4 signal of the rhamnosyl group by +9.8 ppm, and for the C-4 signal of another xylosyl group by +9.2 ppm. Thus, the presence of an inner 2-substituted xylose unit, a 4-substituted rhamnose unit, a 3-substituted xylose and a terminal xylose was revealed. The ROESY spectrum of **1** showed cross-peaks between the signals at δ 6.29 (H-1 of rhamnose) and 4.32 (H-2 of the 2-substituted xylose unit), 5.19 (H-1 of 3-substituted xylose) and 4.41 (H-4 of rhamnose unit), 5.19 (H-1 of terminal xylose unit) and 4.04 (H-3 of 3-substituted xylose unit). The above evidence allowed the establishment of a (1→28) linkage between the 2-substituted xylose unit and the aglycone unit, a (1→2) linkage between the rhamnose and 2-substituted xylose unit, a (1→4) linkage between the 3-substituted xylose unit and the rhamnose unit, and a (1→3) linkage between the terminal xylose unit and the 3-substituted xylose unit. Therefore, the structure of the 28-*O*-sugar chain could be deduced as xylosyl-(1→3)-xylosyl-(1→4)-rhamnosyl-(1→2)-xylosyl-(1→28)-aglycone.

The last step was the determination of the anomeric configuration of the sugar units. The large $J_{1,2}$ values of the three xylosyl groups (7.2, 8.0 and 8.0 Hz, respectively) indicated that their anomeric centres had β-configurations. The anomeric configuration of rhamnose could be identified from its ^{13}C NMR chemical shifts, with C-3 and C-5 of methyl α-L-rhamnopyranoside appearing at δ 72.5 and 69.4, and methyl β-L-rhamnopyranoside at δ 75.4 and 73.5, respectively [13]. The C-3 and C-5 signals of the rhamnosyl group in **1** appeared at δ 72.4 and 68.3; thus, the rhamnosyl group has the α-configuration.

On the basis of the above evidence, the structure of **1** was elucidated to be 3-*O*-β-D-glucopyranosiduronic acid-3β,16α-dihydroxyolean-12-en-28-oic acid–28 -

Table 3. Summary of 2D NMR data for the sugar moieties of compound **1**

Proton	COSY (H)	HMQC (C)	HOHAHA (H)	ROESY (H)
3-*O*-Sugar				
G-1	G-2	G-1	G-2, G-3	H-3, G-3, G-5
2	G-1, G-3	G-2	G-1, G-3, G-4	G-4
3	G-2, G-4	G-3	G-1, G-2, G-4, G-5	G-1, G-5
4	G-3, G-5	G-4	G-2, G-3, G-5	G-2
5	G-4	G-5	G-3, G-4	G-1, 4G-3
28-*O*-Sugar				
Xa-1	Xa-2	Xa-1	Xa-2, Xa-3	Xa-3, Xa-5a
2	Xa-1, Xa-3	Xa-2	Xa-1, Xa-3, Xa-4	Xa-4
3	Xa-2, Xa-4	Xa-3	Xa-1, Xa-2, Xa-4, Xa-5a	Xa-1, Xa-5a
4	Xa-3, Xa-5a, Xa-5b	Xa-4	Xa-2, Xa-3, Xa-5a, Xa-5b	Xa-2
5a	Xa-4, Xa-5b	Xa-5	Xa-3, Xa-4, Xa-5b	Xa-1, Xa-3
5b	Xa-4, Xa-5a	Xa-5	Xa-4, Xa-5a	Xa-5a
R-1	R-2	R-1	R-2	Xa-2, R-2
2	R-1, R-3	R-2	R-1, R-3, R-4	R-1
3	R-2, R-4	R-3	R-2, R-4, R-5	R-5
4	R-3, R-5	R-4	R-2, R-3, R-5, R-6	R-6
5	R-4, R-6	R-5	R-3, R-4, R-6	R-3, R-6
6	R-5	R-6	R-4, R-5	R-4, R-5
Xb-1	Xb-2	Xb-1	Xb-2, Xb-3	R-4, Xb-3, Xb-5a
2	Xb-1, Xb-3	Xb-2	Xb-1, Xb-3, Xb-4	Xb-4
3	Xb-2, Xb-4	Xb-3	Xb-1, Xb-2, Xb-4, Xb-5a	Xb-1, Xb-5a
4	Xb-3, Xa-5, Xb-5	Xb-4	Xb-2, Xb-3, Xb-5a, Xb-5b	Xb-4
5a	Xb-4, Xb-5b	Xb-5	Xb-3, Xb-4, Xb-5b	Xb-1, Xb-3
5b	Xb-4, Xb-5a	Xb-5	Xb-4, Xb-5a	Xb-5a
Xc-1	Xc-2	Xc-1	Xc-2, Xc-3	Xb-3, Xc-3, Xc-5a
Xc-2	Xc-1, Xc-3	Xc-2	Xc-1, Xc-3, Xc-4	Xc-4
Xc-3	Xc-2, Xc-4	Xc-3	Xc-1, Xc-2, Xc-4, Xc-5a	Xc-1, Xc-5a
Xc-4	Xc-3, Xc-5a, Xc-5b	Xc-4	Xc-2, Xc-3, Xc-5a, Xc-5b	Xc-2
Xc-5a	Xc-4, Xc-5b	Xc-5	Xc-3, Xc-4, Xc-5b	Xc-1, Xc-3
Xc-5b	Xc-4, Xc-5a	Xc-5	Xc-4, Xc-5a	Xc-5a

G = β-D-glucopyranoduronic acid; X = β-D-xylopyranosyl; R = α-L-rhamnopyranosyl.

O-*β*-D-xylopyranosyl-(1→3)-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranoside.

EXPERIMENTAL

General. Mps: uncorr.; $[\alpha]_D$ 28°. FAB-MS: direct inlet on a VG ZAB-HS mass spectrometer using glycerin as matrix; EIMS: MAT-95 mass spectrometer; ^1H and ^{13}C NMR: 500 and 400 MHz for δ_H, 125 and 100 MHz for δ_C. COSY, HMQC, HOHAHA and ROESY spectra were obtained on a GE OMEGA-500 spectrometer. PC of sugars were run on Whatman No. 1 paper using *n*-BuOH–HOAc–H_2O (4:1:5, upper layer) and *n*-BuOH–pyridine–H_2O (6:4:3), respectively, and detected with aniline phthalate.

Plant material. Roots of *K. shimadae* were collected in August 1994 from the Anhui Province of China. A voucher specimen was identified by Prof. K.M. Dai of Shanghai Medical School and is deposited in the Herbarium of Shanghai Institute of Materia Medica, Academia Sinica, Shanghai.

Extraction and separation. The dried roots (2.6 kg) of *K. shimadae* were percolated 5× with MeOH at room temp. After concn *in vacuo*, the residue (155 g) was suspended in H_2O and then extracted with EtOAc and *n*-BuOH successively. The *n*-BuOH extract (48 g) was subjected to CC over Diaion HP-20 (500 ml) and eluted with H_2O and MeOH, respectively. The MeOH eluate (43 g) was subjected to CC on silica gel (400 g, 170–230 mesh), eluted with a $CHCl_3$–MeOH–H_2O (80:10:1–10:10:1) gradient. The fr. eluted with $CHCl_3$–MeOH–H_2O (20:10:1) was further chromatographed on Sephadex LH-20 with MeOH and the fr. containing triterpene saponins was sepd over a Lichroprep RP-8 column eluted with a MeOH–H_2O (1:1–7:3) gradient to yield **1** (380 mg, 0.015%).

Shimadoside A (**1**). Needles, mp 215–6°, $[\alpha]_D$ −29.4° (MeOH, *c* 0.5). IR γ_{KBr} cm^{-1}: 3400, 1735, 1709, 1640, 1000–1100. Negative FAB-MS *m/z*: 1189 $[M-H]^-$, 1057 [M-xylose-H]$^-$, 1013 [M-glucuronic acid − H]$^-$, 925 [M-xylose-xylose-H]$^-$, 881 [M-glucuronic acid -xylose-H]$^-$, 779 [M-xylose-xylose-rhamnose-H]$^-$, 749 [M-glucuronic acid-xylose-xylose-H]$^-$ 647 [M-xylose-xylose-rhamnose-xylose-H]$^-$, 603 [M-glucuronic acid-xylose-xylose-rhamnose-H]$^-$, 471 [M-xylose-xylose-rhamnose-glucuronic acid-xylose-H]$^-$. ^1H and ^{13}C NMR: Tables 1 and 2.

Acid hydrolysis of compound **1**. A soln of **1** (80 mg) in 2 M HCl–MeOH (8 ml) was heated at 100° for 4 hr. after cooling to room temp, the reaction mixt. was neutralized with Ag_2CO_3 and filtered. The filtrate was evapd *in vacuo*, and the residue was dissolved in H_2O and extracted with Et_2O. From the aq. layer, xylose, rhamnose and glucuronic acid were identified by PC and TLC (direct comparison with authentic samples). The Et_2O soln was washed with H_2O and evapd to dryness. The residue was dissolved in MeOH and etherial CH_2N_2 was added. After removal of the MeOH, the residue was recrystallized from MeOH to afford the Me ester of **1a**. Compound **1a**: needles, $[\alpha]_D$ +25.3° (MeOH, *c* 0.34); EIMS *m/z*: 486, 278, 260, 219, 208; ^1H NMR (pyridine-d_5): δ 0.88, 0.90, 1.02, 1.04, 1.10, 1.23, 1.70 (3*H* each, *s*, *t*-Me × 7), 3.41 (1H, *dd*, J = 4.0, 13.8 Hz, H-18), 3.45 (1H, *dd*, J = 10.0, 4.5 Hz, H-3), 3.69 (3H, *s*, OMe), 5.04 (1H, *br s*, H-16), 5.59 (1H, *br s*, H-12). ^{13}C NMR: Table 1.

Selective cleavage of the ester glycoside linkage of **1**. Compound **1** (120 mg) and LiI (150 mg) was added to a mixt. of 2,6-lutidine (5 ml) and dry MeOH (3 ml), and the mixt. was heated in a sealed tube at 160–180° for 18 hr. H_2O (8 ml) was added to the reaction mixt. and passed through a column of Amberlite MB-3 (24 ml). The eluate was concd to dryness and the residue dissolved in MeOH and treated with CH_2N_2. The reaction product (108 mg) was subjected to CC on silica gel ($CHCl_3$–MeOH–H_2O, 14:6:1) to give a prosapogenin Me_2 ester (**1b**, 32 mg) and a Me glycoside fr. (27 mg). The latter fr. was hydrolysed in 2 M aq. HCl at 100° for 4 hr to show the presence of xylose and rhamnose by PC and TLC (direct comparison with authentic samples). **1b**: Amorphous powder. $[\alpha]_D$ −17.9° (MeOH, *c* 0.7). FAB-MS *m/z*: 699 $[M+Na]^+$. ^1H NMR (pyridine-d_5): aglycone moiety: δ 0.88, 0.90, 0.97, 1.01, 1.13, 1.29, 1.80 (each 3H, *s*, *t*-Me × 7), 3.40 (1H, H-3), 3.43 (1H, 18-H), 5.55 (1H, *br s* H-12), 5.03 (1H, *br s*, H-16); sugar moiety: Table 2. ^{13}C NMR: Tables 1 and 2).

Acid hydrolysis of **1b**. Compound **1b** (5 mg) was hydrolysed with 2 M aq. HCl at 100° for 4 hr. The reaction mixt was neutralized and then extracted with Et_2O. The aq. layer was examined by PC and TLC to show the presence of glucuronic acid.

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