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ISOLATION OF TWO NEW SAPONINS, GUAIANIN D AND E FROM THE BARK OF GUAIACUM OFFICINALE.

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Abstract - Two new saponins guaianin D (**1**) and E (**2**), obtained from the bark of Guaiacum officinale have been characterised by ^{13}C-nmr spectroscopy and chemical reactions. The complete structure of these saponins have been determined as 3-O[α-L-rhamnopyranosyl (1→3) [α-L-rhamnopyranosyl] (1→2) α-L-arabinopyranosyl (3→1)-[β-D-glucopyranosyl]-30-norolean-12,20 (29) dien-28-oic acid (**1**) and 3-O-[α-L-rhamnopyranosyl] (1→3)-[α-L-rhamnopyranosyl] (1→2)-[α-L-arabinopyranosyl (3→1)-[β-D-glucopyranosyl] 30-norolean-12,20(29)-dien-28-O-[β-D-glycopyranosyl] ester (**2**).

Introduction: In the course of our search for new saponins from the bark of G.officinale (Zygophyllaceae) we have already reported three new saponins (**1-3**). In the present communication we wish to describe further two new saponins. The aglycone of both saponins is the same as 3 β-hydroxy-30-norolean-12,20(29)-dien-28-oic acid which has been established by ^{1}H and ^{13}C-nmr spectra of the intact saponins (Table 1). The oligosaccharide chain is attached at C-3 of the terpenoide in both saponins while one additional glucose moiety is attached to C-28 in guaianin E. The inter-glucoside linkages of the sugars in guaianin E has been determined through the chemical reactions and ^{13}C-nmr spectrum while the structure of guaianin D has been established with direct comparison of ^{13}C-nmr spectrum with that of guaianin E.

Table 1: ^{13}C-NMR Chemical Shifts of **1** and **2** (Aglycone moieties).

Carbon	1[a]	2[b]	Carbon	1[a]	2[b]
C-1	40.12	39.21	C-16	24.52	23.86
C-2	27.16	26.67	C-17	-	47.45
C-3	89.71	88.40	C-18	-	47.76
C-4	40.37	39.65	C-19	42.80	41.85
C-5	57.21	56.16	C-20	149.90	148.56
C-6	19.35	-	C-21	39.20	37.73
C-7	34.03	33.28	C-22	31.05	30.25
C-8	40.65	40.06	C-23	28.68	28.30
C-9	-	48.15	C-24	17.78	17.57
C-10	37.97	37.15	C-25	10.07	15.75
C-11	24.34	23.86	C-26	17.29	17.11
C-12	123.92	123.47	C-27	26.45	26.20
C-13	144.75	143.57	C-28	-	175.91
C-14	42.92	42.23	C-29	107.03	107.51
C-15	28.94	-			

a, The spectra were recorded in CD_3OD. b, The spectra was recorded in C_5D_5N.

Results and Discussion:

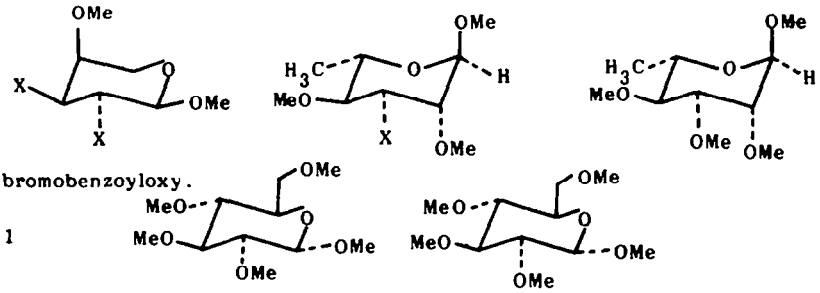
Guaianin E **2** is the most polar saponin isolated from the stem bark of Guaiacum officinale so far. Methanolysis of **2** with 2N HCl in dry methanol furnished methyl arabinoside, methyl glucoside and methyl rhamnoside (GLC) in the ratio 1:2:2. The ^{13}C-nmr spectrum of **2** exhibited five anomeric signals at (ppm)104.54, 101.68, 102.81, 104.16 and 95.89 which also indicated the presence of five sugar moieties in **2** (see Table 2).

Table-2. ^{13}C-NMR Chemical shifts of **1** and **2** (sugar moieties).

Carbon	1[1]	2[b]	Carbon	1[a]	2[b]
Arabinose			Glucose (terminal)		
C-1	105.00	104.54	C-1	104.02	104.16
C-2	75.32	75.05	C-2	75.32	74.50
C-3	84.42	83.65	C-3	77.93	78.37
C-4	68.36	67.84	C-4	70.24	70.09
C-5	64.46	64.44	C-5	77.93	78.37
			C-6	62.37	62.43
			28-Glucose		
			C-1		95.89
			C-2		74.08
			C-3		78.23
			C-4		71.29
Rhamnose(inner)			C-5		78.81
C-1	101.60	101.68	C-6		62.43
C-2	72.14	72.30			
C-3	82.02	81.59			
C-4	73.95	72.67			
C-5	70.12	69.65			
C-6	17.89	18.65			
Rhamnose (terminal)					
C-1	102.76	102.81			
C-2	72.26	72.42			
C-3	72.32	72.58			
C-4	73.82	73.92			
C-5	69.84	69.98			
C-6	17.99	18.65			

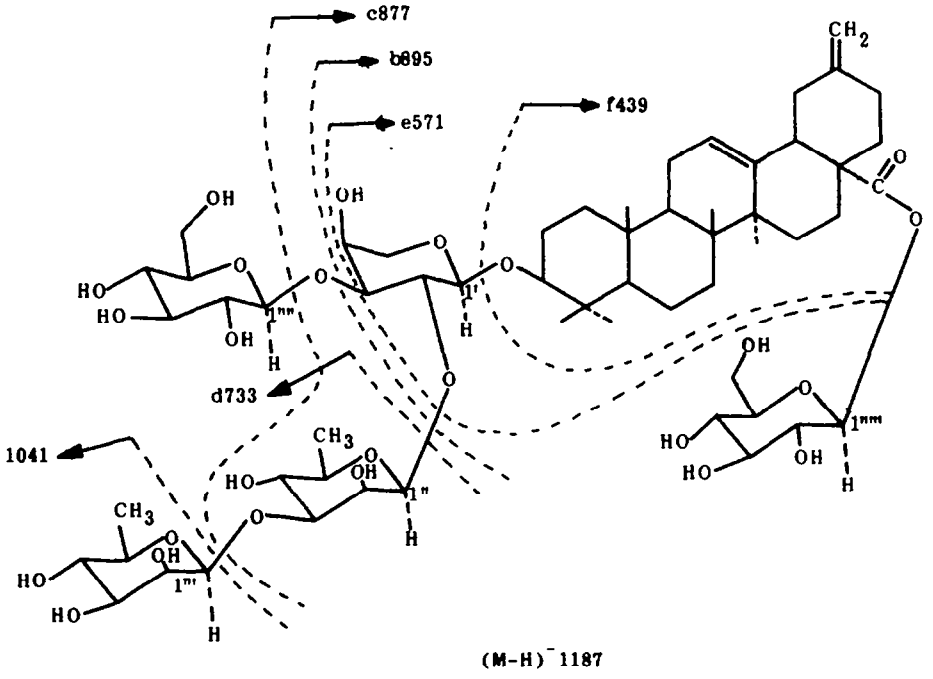
a, The spectra were recorded in CD_3OD. b, The spectra was recorded in C_5D_5N.

The interglycosidic linkages of these sugars were determined by permethylation of **2** with methyl iodide and dimsyl carbanion [4] and methanolysis with 2N HCl in dry methanol, followed by the preparation of p-bromobenzoyl derivative. The mixture of p-bromobenzoyl derivatives was separated by h.p.l.c. to give axial anomers as major and equatorial anomers as minor products. The two major products were characterised as methyl 2,3-di-O-(p-bromobenzoyl)-4-O-methyl-α-L-arabinopyranoside and methyl 3-O-(p-bromobenzoyl)-2,4-di-O-methyl-α-L-rhamnopyranoside on the basis of ^{1}H-nmr spectroscopy [5] (see chart 1).



The presence of three terminal sugars is consistent with the branching in saccharide chain. Since one sugar moiety is present at C-28 in the form of ester linkage, the remaining two sugars are supposed to be attached at rhamnose and arabinose. The linkage point in arabinose (established by di-O-(p-bromobenzoyl derivative) are C-2 and C-3 while in rhamnose there is only one linkage point at C-3. This indicates the presence of rhamnose on one side of arabinose while the other two terminal sugars are linked to arabinose and rhamnose. The comparison of ^{13}C-nmr spectrum of guaianin E with the spectral data for C-2 saponin reported by Kimura et al. [6] showed the linkage of glucose with arabinose at C-3 while the two rhamnose at C-2 otherwise it would give downfield shift of C-2 and upfield shift of C-3 in ^{13}C-nmr spectrum.

The sequence of the sugars has been established through FAB-mass spectrum (negative mode) which exhibited molecular ion peak at m/z 1187 $(M-H)^-$. The fragments at m/z 1041 (a) and 895 (b) showed the gradual loss of two rhamnose moieties while the fragments at m/z 879 (c) and 733 (d) corresponds to the loss of glucose + rhamnose and two rhamnose + glucose respectively. This clearly indicates the attachment of arabinose with glucose on one side and with the rhamnose on the other side. The attachment of arabinose directly with the aglycone was indicated through the fragment at 571 (e) formed due to the loss of two rhamnose + two glucose moieties and further the fragment at 439 (f) consistent with the loss of arabinose from fragment (e) (see chart 2).



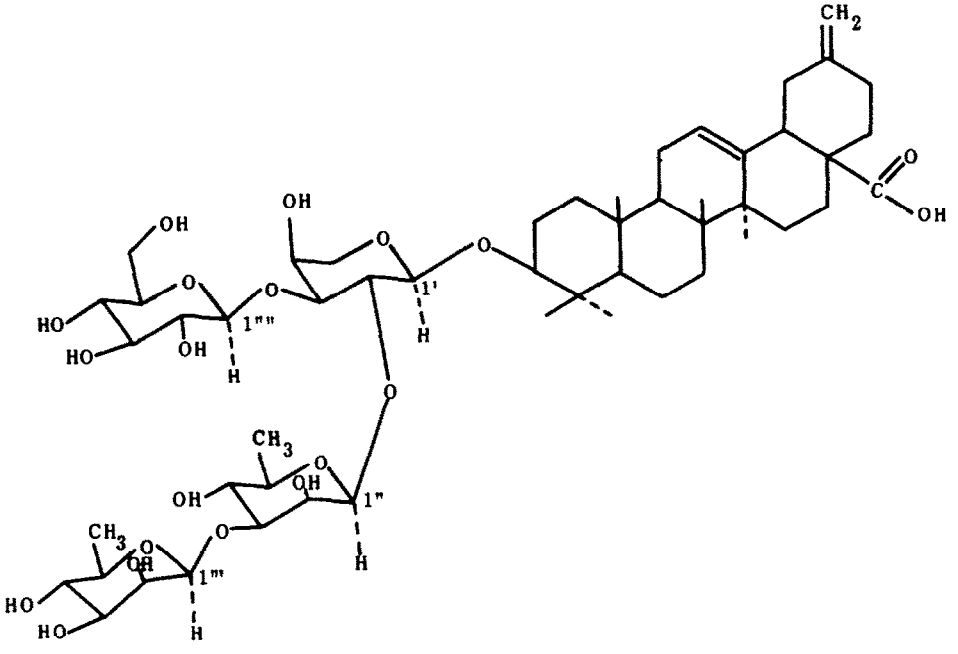
Guaianin E (2) Chart (2)

The anomeric configuration of the sugars were determined from the ^{1}H-nmr spectrum. The signals of the anomeric protons appeared at δ 4.52 (d,$J_{1,2}$ = 7.2Hz, H-1' and H-1''''), 5.20 (d,$J_{1,2}$ = 1.5Hz, H-1''), 5.21 (d,$J_{1,2}$ = 1.5Hz, H-1''') and 5.40 (d,J = 7.6Hz, H-1''''). These results showed that the first two and the last one sugars have 1,2-diequatorial, and the third and fourth sugars have 1,2-diaxial relationship. This is only consistent with the α-L-arabinopyranosyl, β-D-glucopyranosyl and α-L-rhamnopyranosyl configuration.

From the above results the structure of guaianin E (2) was concluded to be 3-O-[α-L-rhamnopyranosyl (1→3)-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranosyl (3→1)-β-D-glucopyranosyl]-30-

norolean-12,20(29)-dien-28-O-β-glucopyranosyl ester.

Guaianin D **1** was purified through preparative HPLC, (See exp.). The ^{13}C spectrum of **1** exhibited only four anomeric signals at 105.00, 101.60, 102.76 and 104.02 ppm which are similar as present in compound **2** except the anomeric signal at 95.89 ppm of glucose moiety attached as ester to C-28 of the genin. The other signals of sugars are also identical with that of compound **2**. The FAB mass spectrum (positive mode) of **1** exhibited molecular ion peak at m/z 1027 $(M+Na)^+$. This also indicated that compound **1** has the same structure as **2** except the lack of one glucose moiety. The sugars were analysed on TLC after acid hydrolysis of 1 and were identified as arabinose, glucose, and rhamnose. Based on the above mentioned evidence, the structure of Guaianin D was determined to be 3-O[-L-rhamnopyranosyl (1- 3)-α-L-rhamnopyranosyl (1→ 2)-α-L-arabinopyranosyl (3→ 1)-β-D-glucopyranosyl-30-norolean-12,20(29)-dien-28-oic acid (see chart 3).



Guaianin D (1) Chart (3)

Experimental

Optical rotation was taken on a Schmidt + Haensch polartronic meter. ^1H-nmr and ^{13}C-nmr spectra were taken on Bruker AM-300 or Bruker WM-250 spectrometer. HPLC was performed with a model 6000 pump equipped with U6K injector and a Model 401 differential refractometer detector.

Extraction and isolation of Saponins:

The stem bark of Guaiacum officinale was collected from the campus of University of Karachi. The powdered stem bark was extracted three times with MeOH. The combined MeOH extract were evaporated at reduced pressure to afford a gummy residue (600 gm). 100gm of this MeOH extract was chromatographed on a silica gel column using a gradient of MeOH in $CHCl_3$ as eluent. The fraction eluted with $CHCl_3$:MeOH (7:3) (70mg) was a mixture of two major and one minor saponins. This mixture was purified on semipreparative hplc (μ-bondapack C-18 column, MeOH:H_2O, 7:3). It afforded guaianin D (18mg) in pure form. Further elution with the same solvent furnished guaianin E (120mg) $[\alpha]_D^{20}$ + 3.84 (c,0.26, methanol) purified by repeated flash column chromatography using silica gel 60 (PF254).

Guaianin D 1: ^{1}H-NMR (CD_3OD): 0.82 (s, $C\underline{H}_3$), 0.85 (s, $C\underline{H}_3$), 0.95 (s, $C\underline{H}_3$), 1.02 (s, $C\underline{H}_3$), 1.19 (s, $C\underline{H}_3$), 1.22 (d, J = 6.4Hz, H-6")*, 1.26 (d, J = 6.5Hz, H-6"'))*, 4.51 (d, J = 7.4Hz, H-1')**, 4.50 (d, overlaped by H-1 signal, H-1"")**, 4.60 (brs, H-29), 5.14 (d, J = 2Hz, H-1")***, 5.18 (d, J = 1.5Hz, H-1"')***, 5.30 (t, H-12).

*, **, ***, assignments may be reversed.

Acid hydrolysis of 1: 5 mg of compound 1 was refluxed with methanolic HCl (9 ml MeOH, 1 ml H_2O, 1.5 ml HCl) for 3 hr. The reaction mixture was concentrated under reduced pressure to remove methanol. It was then diluted with water, extracted with ethyl acetate. The aqueous layer was concentrated at reduced pressure and the residue obtained was compared with standard sugars on TLC (silica gel, H_2O:MeOH:AcOH:EtOAc, 15:15:20:65). Spots were detected by spraying with sugar reagent (orcinol + H_2SO_4 + $FeCl_3$) which showed that the sugars were arabinose, glucose and rhamnose in guaianin D.

Guaianin E (2): ^{1}H-NMR (CD_3OD): δ 0.83 (s, $C\underline{H}_3$), 0.89 (s, $C\underline{H}_3$), 0.97 (s, $C\underline{H}_3$), 1.05 (s, $C\underline{H}_3$), 1.22 (s, $C\underline{H}_3$), 1.24 (d, J = 6.5Hz, H-6")*, 1.27 (d, J = 6.5Hz, H-6"')*, 4.52 (d, J = 7.2Hz, H-1' and H-1""), 4.65 (brs, H-29), 5.20 (d, J = 1.5Hz, H-1")**, 5.21 (d, J = 1.5Hz, H-1"')**, 5.30 (t, H-12), 5.40 (d, J = 7.6Hz, H-1"").

*, **, assignment may be reversed.

Alkaline Hydrolysis of 2: A solution of 2 (5mg) in 5% NaOH (10 ml) was heated under reflux for 2hr. The reaction mixture was worked up in the usual manner. The aqueous layer showed presence of glucose on silica gel TLC.

Methanolysis of 2: 2 mg of compound 2 was dissolved in anhydrous 2N methanolic HCl (1.5 ml) and the solution was heated at 80°C in a stoppered reaction vial for 8h. It was neutralised with Ag_2CO_3, centrifuged and evaporated to dryness under reduced pressure. The residue obtained was analysed by Glc in the form of TMS derivative.

Methylation of 2: 25 mg of compound 2 dissolved in DMF (1ml) was slowly added under nitrogen to a stirred mixture of NaH (100mg) in dry DMF (1ml) cooled in an ice bath. The mixture was stirred for 10 min and then 0.5 ml of methyl iodide was added in two portions, and the mixture was kept for another 10 min in ice bath and then for 4h. at room temp. The excess of NaH was destroyed by a few drops of methanol, and after addition of water, the mixture was extracted with chloroform. The chloroform layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford 22 mg of permethylated saponin (2a).

Methanolysis of 2a with HCl: A solution of 2a (22mg) in anhydrous 2N methanolic HCl (1-2 ml) was heated at 80°C in a stoppered reaction vial for 8h. The reaction mixture was worked up as described for the methanolysis of 2.

p-Bromobenzoylation and Separation of Benzoates by HPLC: The residue obtained (20mg) after the methanolysis of 2a was dissolved in dry pyridine (1ml). It was treated with p-bromobenzoyl chloride (100mg) and a few mg of 4-dimethylamino pyridine. The mixture was stirred overnight at 60°C under nitrogen, and chilled water was added to the reaction mixture and extracted with chloroform. The chloroform layer was washed with saturated aqueous $NaHCO_3$ and water and then evaporated under reduced pressure to afford a dark brown residue of benzoate mixture, which was separated by h.p.l.c. using Whatman Partisil column, diethyl ether:hexane (2:8), 260 nm detection. From hplc following 2 major sugars were obtained.

Methyl 2,3-di-O (p-bromobenzoyl)-4-methyl- -L-arabinopyranoside: ^{1}H-NMR ($CDCl_3$): 1.35 (d, J = 6.5Hz, H-6), 3.40 (s, $OC\underline{H}_3$), 3.44 (s, $OC\underline{H}_3$), 3.48 (s, $OC\underline{H}_3$), 3.38 (m overlaped by OCH_3 signals, H-4), 3.73 (m, H-2 and H-5), 5.34 (dd, J = 10.2, 3Hz, H-3), 7.62 (d, J = 10Hz, ArH), 7.92 (d, J = 9Hz, ArH).

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