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New oligospirostanoside from *Agave vera-cruz* Mill leaves

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ABSTRACT : The ethanolic extract of fresh leaves of *Agave vera-cruz* Mill. has been found to be a complex mixture of steroidal saponins, out of which one oligospirostanoside has been isolated and assigned the structure as: 3-O-[{ β-D-xylopyranosyl (1-4) }{ α-rhamnopyranosyl (1-2) }-β-D-glucopyranosyl (1-4)-β-D-glucopyranosyl] (25R)-5α-spirostan, 3β, ol (Veracruzonin-A) by chemical and spectral studies.

Keywords : *Agave vera-cruz,* amaryllidaceae, steroidal saponins, oligospirostanoside, Veracruzonin-A

I. INTRODUCTION

Agave sps. are reported to have diuretic, antiseptic, antisyphilitic, antifungal, anti-inflammatory, haemolytic and anticancer properties [1-5]. Recently, steroidal saponins [3, 4, 6-12] have been isolated and characterised from various species of *Agave*. *Agave vera-cruz* (Amaryllidaceae) commonly called 'Kuwarbuti' has been reported previously for the presence of furostans [13] and sapogenins [14]. Since no work has been done on this species for saponins, an attempt was made to isolate and characterise the saponin contents from the leaves.

II. EXPERIMENTAL

M.Pt. were determined in open capillaries in an electro thermal melting point apparatus in °C and are uncorrected. CC was carried out over silica gel (60-120 mesh, BDH) with $CHCl_3$: MeOH solvent system in the order of increasing polarity. Homogeneity of the fractions was tested by TLC (silica gel-G, BDH with binder) and spots were visualised by 8-10% H_2SO_4 and Ehrlich Reagent followed by heating. PC (descending) was carried out on Whatman Filter Paper No. 41 and spots were visualised by 'aniline hydrogen phthalate' reagent. IR, EIMS, FAB-MS and ^{13}C-NMR spectra were recorded on Perkin Elmer, Jeol D-300, Jeol SX-102/DA-6000 (6KV, 10 mA, Acc. Volt. 10 KV) and Bruker WM-400 (400 MHz) respectively. The solvent systems used were:

(A) $CHCl_3$: MeOH: H_2O (60 : 50 : 10)

(B) C_6H_6 : EtAc (8 : 2)

(C) C_6H_6 : Pet. Ether (1 : 1)

(D) n-BuOH : AcOH : H_2O (4 : 1 : 5)

(E) n-BuOH : AcOH : H_2O (4 : 1 : 5)

The leaves of *Agave vera-cruz* Mill. were collected from Hatwar, Dist. Bilaspur (HP), India.

Extraction and Isolation

The fresh leaves (3kg) of *Agave vera-cruz* were extracted with pet. ether (4 × 6 hrs.), EtAc (3 × 7 hrs.) and finally with EtOH (5 × 8 hrs.). The ethanolic extract was conc. under vac. and extracted with n-BuOH. n-BuOH extract was dried under vac. and dissolved in minimum quantity of MeOH, then precipitated drop wise in large volumes of acetone with constant shaking. The resulting residue was purified and separated by CC to get an oligospirostanosides [15], Veracruzonin- A (1).

Veracruzonin- A (1)

1 was crystallised from MeOH; mp 207-12° $[\alpha]_D^{20}$ – 58.5° (MeOH), R_f 0.88 (Solvent-A, 3.2g). It was +ve to +ve to Liebermann- Burchard test and -ve to Ehrlich Reagent test. Its IR spectrum showed well defined spiroketal absorption bands. FAB-MS showed molecular ion peak at 1019 $[M+H]^+$ and ^{13}C-NMR data as in Table 1.

Table 1: ^{13}C-NMR chemical shifts of sugar moieties in D_2O

Sugars	Carbon Nos. Chemical shifts (ppm)					
	1	2	3	4	5	6
Glucose	103.4	70.0	81.2	71.6	81.4	61.5
Glucose	104.8	74.4	81.4	72.2	81.2	61.1
Xylose	104.6	74.0	74.5	71.6	69.4	-
Rhamnose	102.2	72.2	72.6	74.0	69.3	18.3

Acidic Hydrolysis

Acidic hydrolysis of **1** (150 mg) with 8-10% H_2SO_4 (50 ml) was carried out by refluxing for 4 hrs. on a steam bath. The usual work up afforded an aglycone, crystallised as colourless needles from MeOH; mp 202-205°, $[\alpha]_D^{20}$ -65.5° ($CHCl_3$) [Tigogenin, Lit. mp 205-208°, $[\alpha]_D^{20}$ -67° ($CHCl_3$)], R_f 0.70 (Solvent- B). $IR_{\nu\ max}^{KBr}$ cm^{-1} 3400-3500 (OH), 984, 920, 902, 860 (902 > 920, 25R). EIMS -m/z 416$[M]^+$, 398, 357, 347, 344, 302, 287, 273, 139 (base peak) and 115. Its acetate was prepared in cold in usual manner and crystallised as colourless needles from MeOH; mp 203-6°, $[\alpha]_D^{20}$ – 72° ($CHCl_3$) [Tigogenin acetate, Lit. mp 206-8°, $[\alpha]_D^{20}$ – 74° ($CHCl_3$)], R_f 0.55 (Solvent – C)

The aq. hydrolysate was neutralised with $BaCO_3$, filtered and conc. under vac. PC studies (Solvent-D) revealed the

presence of D-glucose (R_f 0.18), D-xylose (R_f 0.28) and L- rhamnose (R_f 0.37)

Enzymatic Hydrolysis

1 (50 mg) was taken up in distilled water (25 ml) and β-glucosidase (10 mg) was added to it along with toluene (3 drops) to cover the aq. layer. The reaction mixture was kept at room temp. for 72 hrs. The PC (Solvent-D) did not show the presence of any sugar, where as TLC (Solvent-A) showed one spot corresponding to **1** (R_f 0.88).

Kiliani Hydrolysis

1 (50 gm) was kept with Kiliani mixture 25 ml, (AcOH : H2O : 35%HCl, 35 : 55 :10) at room temp. PC (Solvent -D) after 12 hrs. showed two spots corresponding to D-xylose (R_f 0.28) and L- rhamnose (R_f 0.37). PC after 36 hrs. and 60 hrs. showed one more spot corresponding to that of D-glucose (R_f 0.18) but its intensity was almost double after 60 hrs. There was no change on PC after 84 hrs. and even upon heating.

Permethylation

1 (250 mg) was permethylated by modified Hakomori's method (NaH, MeI, DMSO/N_2 atm.) to get permethyate (220 mg) which was purified by CC.{R_f 0.96 (Solvent -B)}

Methanolysis followed by hydrolysis

The above permethylate (200 mg) was refluxed with dry MeOH -1N HCl (50 ml) for 4 hrs. on a steam bath, MeOH evaporated, H_2O (25 ml) was added and hydrolysed. After usual work up the aq. neutralised hydrolysate on PC (Solvent-E) showed the presence of 2, 3, 6 tri-O-methyl-D-glucose (RG 0.51); 2, 3, 4 tri-O-methyl-D-xylose (RG 0.94); 2,3,4 tri-O-methyl-D-rhamnose (RG 1.01).

Partial hydrolysis

1 (1.5 g) was refluxed on a steam bath with 5% aq HCl- MeOH (50 ml, 1:1, 45 min.), neutralised (Ag_2CO_3) and filtered. The filtrate was dried under vac. and chromatographed to obtain an aglycone-Tigogenin (mp, mmp, Co-TLC) along with four prosaponins PS_1 to PS_4. Each prosaponin was acid hydrolysed and usual work up showed only one aglycone-Tigogenin. The aq. neutralised hydrolysates on PC (Solvent-D, with authentic samples) showed sugars as: PS_1 and PS_2-D-glucose (R_f 0.18); PS_3-D-glucose (R_f 0.18), L-rhamnose (R_f 0.37) and PS_4- D-glucose (R_f 0.18), D -xylose (R_f 0.28).

Each prosaponin was subjected to permethylation and methanolysis followed by hydrolysis. After usual work up PC (Solvent-E) of the neutral hydrolysate showed different sugars viz. PS_1 – 2, 3, 4, 6 tetra-O-methyl-D-glucose (R_G 1.00); PS_2 – 2, 3, 6 tri-O-methyl-D-glucose (R_G 0.83); 2, 3, 4, 6 tetra-O-methyl-D-glucose (R_G 1.00); PS_3 –2, 3, 6 tri-O-methyl-D-glucose (R_G 0.83); 3, 4, 6 tri-O-methyl-D-glucose (R_G 0.84); 2, 3, 4, tri-O-methyl-L-rhamnose (R_G 1.01) and PS_4 – 2, 3, 6 tri-O-methyl-D-glucose (2 moles, R_G 0.83); 2, 3, 4 tri-O-methyl-D-xylose (R_G 0.94).

III. RESULTS AND DISCUSSION

Veracruzonin –A (**1**) was separated by CC and crystallised from MeOH. Its IR spectrum showed characteristic spiroketal absorption bands [16-19], was +ve to Liebermann -Burchard test but -ve to Ehrlich Reagent test. Acid hydrolysis [20-22] of **1** afforded an aglycone-Tigogenin (mp, mmp, Co-TLC, EIMS, IR, its acetate) and the aq. neutralised hydrolysate contained D-glucose, D-xylose and L-rhamnose (R_f and Co-PC). Enzymatic hydrolysis [17, 23] of **1** with β-glucosidase revealed no β-D-glucose indicating that D-glucose is not the terminal sugar of the glycone moiety. In order to find out the sequence of the sugars, **1** was subjected to Kiliani hydrolysis [24]. The reaction mixture with the passage of time on PC showed that D-xylose, L-rhamnose emerging out first, must be the terminal sugars of the sugar chain, while two glucose molecules emerging out later are the inner sugars through which D-xylose, L-rhamnose are linked on one end and C_3 of aglycone at the other as there is no other hydroxyl group available in the aglycone -Tigogenin for the linkage. The configurations of the sugars were deduced as '?' by Klyne's Rule [25] as well as from ^{13}C-NMR data [26-27].

1 was permethylated by modified Hakomori's method [17, 28] to get a permethylate, which on methanolysis followed by hydrolysis furnished methylated sugars, identified by PC as 2, 3, 6 tri-O-methyl-D-glucose; 3, 6 di-O-methyl-D-glucose; 2, 3, 4 tri-O-methyl-D-xylose and 2, 3, 4 tri-O-methyl-L-rhamnose. These results again revealed that the D-xylose and L-rhamnose are the terminal sugars of sugar chain linked through two molecules of D-glucose attached with C_3 of aglycone.

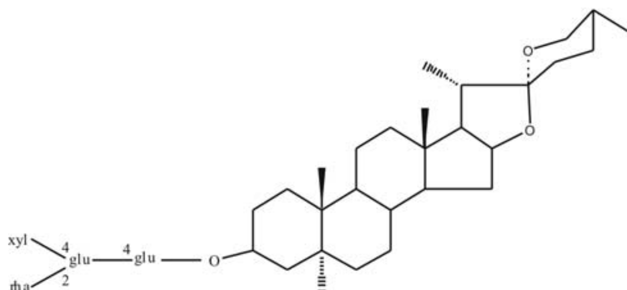
In order to establish the exact linkages of the sugars with each other, **1** was subject to partial hydrolysis [29-31] to get four prosaponins PS_1 to PS_4. Acid hydrolysis of these prosaponins furnished the same aglycone -Tigogenin but different sugars viz. D-glucose in PS_1 and PS_2; D-glucose, L-rhamnose in PS_3 and D-glucose with D-xylose in PS_4. Each prosaponin on permethylation followed by methanolysis and hydrolysis gave the following methylated sugars:

PS_1 – 2, 3, 4, 6 tetra-O-methyl-D-glucose ; PS_2 –2, 3, 6 tri-O-methyl-D-glucose ; 2, 3, 4, 6 tetra-O-methyl-D-glucose; PS_3 –2, 3, 6 tri-O-methyl-D-glucose; 3, 4, 6 tri-O-methyl-D-glucose; 2, 3, 4, tri-O-methyl-L-rhamnose and PS_4 –2, 3, 6 tri-O-methyl-D-glucose (2 moles); 2, 3, 4 tri-O- methyl-D-xylose.

Hence, PS_1 = Tigogenin + glucose (1-4); PS_2 = PS_1 + glucose (1-4); PS_3 = PS_2 + rhamnose (1-2) and PS_4 = PS_2 + xylose (1-4). These results confirmed a branching in the sugar chain at glucose (No. 2) with D-xylose (1-4) and L- rhamnose (1-2) linkage. FAB-MS of **1** showed molecular

ion peak at 1019 [M + H]$^+$, indicating an aglycone of molecular weight 416 (Tigogenin), two molecules of hexoses (glucose) and two molecules of pentoses (xylose and rhamnose). ^{13}C-NMR [26-27] data (Table 1) further confirmed these results, hence the structure of Veracruzonin A (**1**) was elucidated as:

3-O-[{β-D- xylopyranosyl (1-4)} {α-L-rhamnopyranosyl (1-2)}-β-D-glucopyranosyl (1-4)-β-D-glucopyranosyl] (25R)-5α-spirostan, 3β, ol.



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