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Structure Elucidation of a Novel Compound Ichnoside isolated from *Ichnocarpus frutiscens*

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Abstract: A novel pregnane glycoside, Ichnoside, was isolated from the chloroform extract of the areal parts of the plant *Ichnocarpus frutiscens* (family: Apocynaceae). The structure of the isolated glycoside, Ichnoside, was elucidated by the acid hydrolysis and chemical transformation / degradation and physico-chemical techniques like ^{1}H, ^{13}C, 2D NMR, and FABMS techniques. The structure of the compound was deduced as 3β, 14β, 20β-trihydroxy-pregnane-3-O-α (6-O-methoxy phenyl) galactopyranoside.

Keywords: *Ichnocarpus frutiscens*, Apocyanaceae, Pregnane glycoside, 6-O-methoxy phenyl galactopyranose.

Introduction

Cardiac and pregnane glycosides [1] are medicinally and biologically important classes of compounds, which are known to possess anti-tumour, anti-cancer, cardiotonic [1-3], anti inflammatory [4], immunostimulant [5], and hypoglycaemic [6] activities. These are also found to inhibit cell to cell infection of HIV [7]. The Asclepiadaceae and Apocyanaceae families are found to be rich sources of naturally occurring cardiac and pregnane glycosides. The plants belonging to these families have been used in many traditional and ethnomedicinal systems across the globe. The stems of the plant *Stelmatocrypton khasianum* (Fam: Asclepiadaceae), found in China, are used in Chinese folk medicine for the treatment of colds, tracheitis, stomach aches, and rheumatic aches [8]. The roots of the plant *Asclepias curassavica* (Asclepiadaceae) have anodyne properties, and along with the rhizomes of the plant, are used in treatment of asthma, and typhus fever. The plant is also considered as diaphoretic, antihelminthic, and purgative in ayurvedic medicine and is used to improve blood circulation and to control bleeding in Chinese folk medicine [9]. Pregnane glycosides isolated from the plant *Hemidesmus indicus* were found to possess anti-oxidant and anti-dyslipidemic properties [10].

In an attempt to find more novel glycosides occurring in nature, the plant *I. frutiscens* (Family: Apocyanaceae) was selected. Commonly known as the black creeper, *I. frutiscens* is a large, evergreen, laticiferous, climbing shrub, native to China, India, Southeast Asia, and northern Australia. In India, it is widely distributed in the tropical and sub-tropical regions. It has numerous traditional medicinal uses as blood purifier and in the treatment for cholera and leucoderma [11]. Earlier phytochemical investigations have revealed the presence of polyphenols, terpenoids, alkaloids, phytosterols, carbohydrates, coumarins, glycosides, flavonoids, etc. [12-14]. The chloroform extract of the plant showed antimicrobial and antifungal activities against *E. coli* and *Aspergillus flavus*, respectively [15]. The methanol extract was found to have wound healing activity on rats [16]. Another study showed the prophylactic and hepatoprotective effect of *Ichnocarpus* against carbon tetrachloride and tamoxifen induced liver damage in rats [17]. The polyphenol extract of the plant effectively inhibited the in vitro proliferation of U-937 and K-562 cell lines, which showed that the extract displayed strong anti-tumor activity [18]. For this study, the plant was collected in bulk from Gonda District, UP, India. A specimen of the plant (BSIP 11940) was submitted at Herbarium Section, Birbal Sahni Institute of Paleobotany, Lucknow. In this paper, we have discussed the isolation and structural elucidation of a novel pregnane glycoside, Ichnoside (**1**), which was isolated from the chloroform soluble extract of the plant.

Materials and Methods

General experimental procedures

All solvents used were of analytical grade and were purified and dried according to standard procedures prior to their use. All melting points were recorded on Boetius, micro melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 digital polarimeter in 1 cm tube. ^{1}H NMR, ^{13}C NMR and 2D spectra were recorded with AVANCE DRX 300MHz, 200 MHz Bruker spectrometers in $CDCl_3$, d_5 and d_6 using TMS as internal standard. FAB mass spectra were recorded with JEOL mass spectrometer model JMS-SX-102 with DA-600 Data system and JEOL Mass spectrometer D-300 with IMA-2000 Data system respectively. Pure compounds were visualized on TLC with 50% aq H_2SO_4 reagent and on PC with vanillin-perchloric acid Partridge reagents. The absorbent for TLC was silica gel G (SRL) and for CC, silica gel for column (SRL 60-120 and 120-200 mesh) developed by Duncan's method, PC was performed on whatman No.-1 filter paper using solvent system C_6H_5-CH_3 : BuOH (4:1) saturated with water.

Plant extraction, Fractionantion and Isolation

Shade dried, powdered material (12 kg) was soaked in water and kept overnight at room temperature. Soaked plant material was then exhaustively extracted and percolated with 50-95% EtOH (200 litres). The combined ethanolic extract was concentrated under reduced pressure. Tannins were removed by the addition of equal volume of 95% ethanol into concentrated extract which precipitated them. The filtrate was reconcentrated under reduced pressure and extracted successively with hexane, chloroform, chloroform: ethanol (4:1), chloroform: ethanol (3:2) for getting their respective extracts. All the extracts exhibited diagnostic colours in Liebermann Buchardt test and Feigl test suggesting the presence of steroids and sugars in them. Ichnoside (**1**) (68.7 mg) was isolated from the chloroform extract as reddish brown powder, m.p. 128-131°C, $[\alpha]_D$ -10° (c, 0.001, MeOH). For the elemental analysis, this compound was dried over phosphorus pentoxide at 100°C for 4 hours. It gave a brown colour spot on TLC when spread with 50% aq. H_2SO_4 and heated to 100°C. It responded positively to Feigl test for normal sugar.

		% C	% H
$C_{34}H_{52}O_9$	Calculated	67.54	8.60
	Found	67.52	8.57

Mannich Hydrolysis of 1 with acid

To a solution of 1 (20 mg) in acetone (5 ml), conc. HCl (0.05 ml) was added. The solution was kept under CO_2 in a dark room at room temperature. After two days, the reaction mixture exhibited two new spots, along with the unreacted 1. Hydrolysis was complete in 5 days showing two spots on the TLC. The less polar was found to be identical with dihydrocalogenin (2) on comparison with an authentic sample (TLC, PC, mmp), while the polar spot was presumably a derivatised monosaccharide of normal sugar. The aq. portion was repeatedly extracted with $CHCl_3$ and the organic layer was washed in turn with H_2O, Na_2CO_3, and H_2O, and dried over Na_2SO_4, filtered and evaporated to dryness to afford dihydrocalogenin (2) (4.5 mg) m.p. 200-203°C, $[\alpha]_D$ +25° (c. 0.002, MeOH). The aq. hydrolysate was neutralized with freshly prepared Ag_2CO_3 and filtered. The filtrate was cooled in ice, H_2S was passed (to remove Ag^+ as Ag_2S) and the solution was again filtered. The aq. filtrate was concentrated under reduced pressure to afford pure sugar (3) (2.4 mg), $[\alpha]_D$ - 37.6 (c, 0.002, MeOH) identified as 6-O-methoxy phenyl galactose and was reduced by H_2/Pd to form Galactose and Anisole as identified by comparison with authentic samples (PC, TLC, $[\alpha]_D$).

Acetylation of 1

Reddish powder (30 mg) was acetylated with Ac_2O (1 ml) in pyridine (1 ml) at 100°C for 1 hour. Pyridine and excess of Ac_2O was then removed under reduced pressure. The viscous residue taken in $CHCl_3$ (5 ml) was washed in sequence with 2 N HCl (1x2 ml), ice-cold 2 N $NaHCO_3$ (2X2 ml) and finally with H_2O (2x2 ml). $CHCl_3$ layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding amorphous tetra-O-acetate ichnoside (6).

Ichnoside (1)

Amorphous powder, m.p. 128-131°C, $[\alpha]_D$ -10° (c, 0.001, MeOH). ^{1}H NMR (300 MHz, $CDCl_3$): δ7.72 (1H, m), 7.53 (1H, m), 6.58 (2H, s), 4.73 (1H, d, J = 3.9 Hz, H-1', s_1), 4.28 (2H, m, H-3', H-5'), 3.90 (3H, s, Aromatic OMe), 3.91 (5H, m, H-4', H-6', H-3, H-20), 3.10 (1H, m, H-2'), 2.16 (1H, m, H-17), 0.99 (3H, d, J = 6.6 Hz, H-21), 0.88 (3H, s, 19 Me), 0.86 (3H, s, 18 Me). ^{13}C NMR (75MHz, $CDCl_3$): δ147.5 (C-1''), 146.7 (C-4''), 134.2 (C-2''), 132.5 (C-6''), 131.7 (C-3''), 129.1 (C-5''), 102.3 (C-1', s_1), 85.8 (C-14), 71.8 (C-5'), 70.6 (C-3'), 70.2 (C-4'), 69.2 (C-2'), 62.2 (C-6'), 56.4 (-OMe), 54.3 (C-17), 54.0 (C-13), 51.3 (C-9), 47.1 (C-5), 39.3 (C-1), 34.5 (C-10), 31.8 (C-8), 31.3 (C-4, C-12), 29.6 (C-7), 29.4 (C-15), 29.3 (C-6), 29.2 (C-2), 29.0 (C-16), 27.7 (C-11), 22.6 (C-21), 19.1 (C-19), 14.0 (C-18). FAB-MS: m/z 627 $[M+Na]^+$, 604 $[M]^+$, 559 $[M - CH_3CHOH]^+$, 336 $[M - S_1]^+$, 318 $[genin - H_2O]^+$, 273 $[318 - CH_3CHOH]^+$, 269 $[S_1 - OH]^+$, 251 $[S_1 - OH - H_2O]^+$, 249 $[336 - OHCH_2CH_2CH_2CHCH]^+$, 246 $[318 - CH_3CHOH - CH_2CH]^+$, 233 $[251 - H_2O]^+$, 166 $[251 - OHCHCHCOHCH]^+$, 108 $[OHCHCHCOHCH + Na]^+$.

Acetylated Ichnoside (6)

^{1}H NMR (300 MHz, $CDCl_3$): δ4.76 (1H, d, J = 3.9 Hz, H-1', s_1), 5.03 (1H, m, H-3'), 4.12 (1H, m, H-4'), 4.66 (1H, m, H-20), 3.94 (1H, m, H-3), 3.93 (3H, s, -OMe), 3.91 (1H, m, H-2'), 2.33 (12H, s, 4 x OAc), 1.26 (3H, d, J = 6.6 Hz, H-21), 0.88 (3H, s, 19 Me), 0.85 (3H, s, 18 Me).

Result and Discussion

Ichnoside (**1**) was isolated by repeated column chromatography of chloroform extract of the *Ichnocarpus frutescens* (Family: Apocynaceae),

$C_{34}H_{52}O_9$, positive ion FAB-MS: m/z 627 $[M+Na]^+$ and 604 $[M]^+$, mp. 128-131°C, $[\alpha]_D$-10° (c, 0.001, MeOH). It gave positive Libermann – Buchardt [19] test for steroids and positive Feigl test [20] for normal sugars, showing it to be a steroidal glycoside of normal sugar. The ^{13}C NMR spectrum of **1** showed the presence of one anomeric carbon signal at δ102.3 along with a signal for one secondary methyl carbon at δ22.6, while the ^1H NMR spectrum of **1** at 300 MHz showed one anomeric proton signal at δ4.73 (1H) along with one secondary methyl group doublets at δ0.99, suggesting the monoglycoside nature of **1**.

The identification of the aglycon and the monosaccharide units of **1** were performed by hydrolyzing it under mild acid conditions by the method of Mannich and Siewert [21]. The hydrolysis was monitored on TLC and PC. After two days, the reaction mixture showed two new spots, besides the unreacted **1**. The less polar spot was identical in mobility with dihydrocalogenin (TLC) (**2**), while the polar one was presumably the glycon (**3**). Hydrolysis was completed in 4 days showing only 2 spots, which on column chromatography afforded dihydrocalogenin (**2**), m.p. 200-203°C, $[\alpha]_D$ +25.3° (c. 0.002, MeOH), that was identified by comparison with the authentic samples (TLC, PC, $[\alpha]_D$), and the glycon (**3**) $[\alpha]_D$ - 36.7° (c, 0.02, MeOH). It gave positive Feigl test and underwent $NaIO_4$ oxidation [22], but did not undergo sodium methoxide hydrolysis [23, 24]. It was treated with H_2/Pd, which gave compound (**4**) and (**5**) that was identical in mobility with D-galactose and Anisole, suggesting the presence of derivitized galactose in Ichnoside [25]. The ^1H NMR spectrum of **1** showed the signal for anomeric proton at δ4.73 (1H, J = 3.9 Hz), and also contained the aromatic proton signals at δ7.72 (1H, m), 7.53 (1H, m), and 6.58 (2H, s), along with a signal for the methoxy group substituent in the glycon part of the glycoside at δ 3.90 (3H, s). It also contained signals for ring protons of the glycon unit, showing signals for H-2' at δ3.10 (1H, m), H-3' at 4.28 (1H, m), H-4' at 3.91 (1H, m), H-5' at 4.28 (1H, m), and H-6' at 3.91 (2H, m) respectively. The 2D ^1H-^1H COSY spectrum contained the cross peaks for the glycon part as well as the aglycon part of the glycoside.

The ^1H NMR spectrum of **1** at 300 MHz was also instrumental in ascertaining the glycosidic linkage. The one proton doublet at δ4.73 (J = 3.9 Hz) was assigned to the one anomeric proton of one sugar unit i.e. 6-O-methoxy phenyl galactose. The smaller coupling constant (J = 3.9 Hz) of anomeric proton was typical of an equatorial configuration, suggesting the presence of the sugar in $^4C_1$ (D) conformation joined through α-glycosidic linkage [26]. Besides this, the ^1H NMR spectrum of **1** also contained a three protons singlet at δ3.90 for methoxy group, which was attached to the phenyl ring in glycon part. It also contained a doublet of three protons at δ0.99 (J = 6.6 Hz), which was assigned to the secondary methyl group of genin (21-CH_3). It also showed two tertiary methyl resonances at δ0.86 (3H, s) and 0.88 (3H, s) for C-18 and C-19, respectively. The ^1H NMR of acetylated Ichnoside (**6**) also confirmed the position of glycosidic linkage between the sugar and the aglycon. The position of the H-20 of the aglycon was present at δ3.91 in the ^1H NMR spectrum of Ichnoside (**1**), which shifted to δ4.66 in the ^1H NMR spectrum of acetylated Ichnosoide (**6**), while, the position of H-3 was present at δ3.91 in the ^1H spectrum of Ichnoside (**1**) and at δ3.94 in the ^1H spectrum of acetylated Ichnoside (**6**). The downfield shifted H-20 proton at δ4.66 confirmed that the H-20 hydroxyl group of the aglycon underwent acetylation and therefore, was not involved in the glycosidic linkage. However, the chemical shift of H-3 proton of the aglycon at δ3.94 confirmed that the H-3 of dihydrocalogenin (**2**) was involved in the glycosidic linkage.

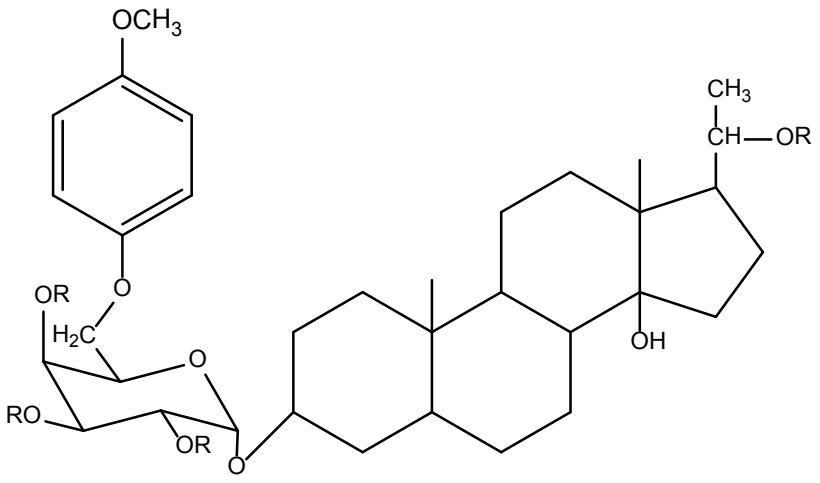
The ^{13}C NMR data of **1** was also in agreement with the results deduced from the ^1H NMR

spectrum. Besides, the anomeric carbon signal at δ102.3, it also contained the signals for the other carbons of the glycon part at δ69.2, 70.6, 70.2, 71.8, and 62.2, for C-2', C-3', C-4', C-5', and C-6', respectively. Besides these signals, the other carbon chemical shifts are given in the table 1.

All the ^{1}H NMR assignment for the ring protons of monosaccharide unit and glycon moiety of Ichnoside (**1**) were confirmed by 2D HOMOCOSY experiment. The H-1' anomeric proton of sugar S_1 with a peak at δ4.73 (1H, d, J = 3.9 Hz) showed a cross peak with the H-2' of the glycon ring at δ3.10 (1H, m). The H-2' proton, in turn, showed a cross peak with the H-3' proton at δ4.28 (1H, m). A broad cross peak was present at the intersection of δ4.28 and 3.91, showing that H-3' (δ4.28, 1H, m) was connected with the H-4' (δ3.91, 1H, m) and that the H-5' (δ4.28, 1H, m) was connected with the H-6' (δ3.91, 2H, m). The presence of these cross peaks suggested that the sugar present in the glycoside was 6-O-methoxyphenyl galactose. In addition to the aforementioned peaks, the 2D spectrum also contained the cross peaks for the aglycon part of the glycoside. The H-21 methyl protons at δ0.99 showed a cross peak with the H-20 proton at δ3.91. The H-20 proton also showed a cross peak with H-17 at δ2.16.

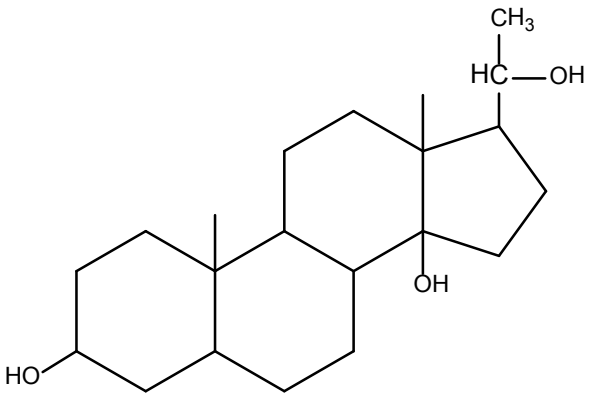
Table 1: ^{13}C NMR shifts of Ichnoside and Ichnoside acetate

Carbon	Chemical shifts of Ichnoside (δ)	Chemical shifts of Ichnoside acetate (δ)	Carbon	Chemical shifts of Ichnoside (δ)	Chemical shifts of Ichnoside acetate (δ)
1	39.3		**Sugar 1:**		
2	29.2		1'	102.3	
3	75.3		2'	69.2	76.6
4	31.3		3'	70.6	77.4
5	47.1		4'	70.2	77.0
6	29.3		5'	71.8	
7	29.6		6'	62.2	
8	31.8		OMe	56.4	
9	51.3		**Phenyl group:**		
10	34.3		1"	147.5	
11	27.7		2"	134.2	
12	31.3		3"	131.7	
13	54.0		4"	146.7	
14	85.8	85.8	5"	129.1	
15	29.4		6"	132.5	
16	29.0		**Carbonyl Carbon of Acetate (4 x CO)**		168.8
17	54.3				
18	14.0				
19	19.1				
20	75.8	85.8			
21	22.6				



S_1

1. R = H
6. R = OAc, Tetra-O-acet$_y$l Ichnoside



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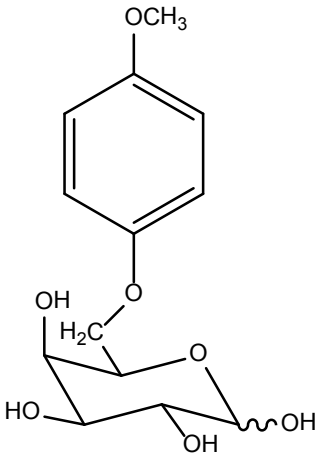

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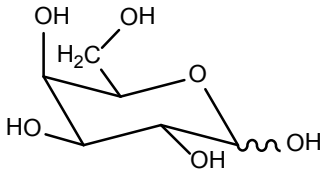

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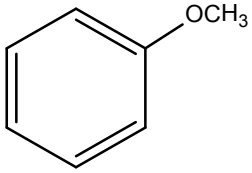

5

Figure – 1

Compound **1** on acetylation with Ac_2O in pyridine at 100°C yielded tetra-O-acetyl Ichnoside (**6**). The ^1H NMR spectrum confirmed the tetra-O-acetyl nature of Ichnoside at 300 MHz, which showed the signal of 12 protons as a singlet at δ2.33, and thus, accounted for three free hydroxyl groups present in the sugar and one acetylable hydroxyl group in the aglycon. The downfield shifting of the H-20 methine proton of the genin to δ4.66 (1H, m) with respect to its parent precursor at δ3.91 (1H, m), showed that the sugar chain was glycosidically linked to the C-3 of aglycon. Besides this, all the ^1H NMR signals of acetylated ring protons were shifted downfield in comparison to the corresponding protons of **1** at δ3.91 (1H, m) for H-2', 5.03 (1H, m) for H-3', and 4.12 (1H, dd, J = 6.6 and 2 Hz) for H-4', respectively.

The FAB mass spectrum showed the highest mass ion peak at m/z 627, which was assigned for $[M+Na]^+$. The molecular ion by the loss of C-17 side chain (CH_3CHOH) gave the mass ion fragment at m/z 559. This fragment confirmed that the sugar unit was linked through C-3 of the aglycon. Loss of 6-O-methoxy phenyl galactose from the molecular ion gave fragment at m/z 336 (genin) which was complemented by the mass ion peak m/z 269 (monosaccharide-OH [27]). The mass ion at m/z 336 further fragmented to give mass ion fragment at m/z 318 due to the loss of a water molecule, which due to further loss of CH_3CHOH at C-17, gave the mass ion peak at m/z 273. The sugar unit, due to the loss of a hydroxyl group, gave a mass ion peak at m/z 269, which further by the loss of a water molecule gave a mass ion peak at m/z 251. Another subsequent loss of water molecule from the peak at m/z 251 gave rise to a mass ion peak at m/z 233. The mass ion peak of the genin at m/z 336 further fragmented to give mass ion fragments at m/z 249 $[336 - OHCH_2CH_2CH_2CHCH]^+$ and 246 $[318 - CH_3CHOH - CH_2CH]^+$. A double bond was introduced between C2-C3 of the normal hexose from fragment ion m/z 251 leading to retro-DielsAlder rearrangement and subsequent loss of C_6H_4 (OCH_3)-O-CH_2-CHO giving mass ion fragment at m/z 108 [28].

Conclusion

In the light of foregoing spectral and chromatographical evidences along with the diagnostic colour reactions, the structure of Ichnoside (**1**) was confirmed as **3β, 14β, 20β-trihydroxy-pregnane-3-O-α (6-O-methoxy phenyl) galactopyranoside.**

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