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4,5-Dehydro-14-β-Hydroxy Scilladienolide-3-O-β-D-Glucopyranoside (AC-3) from the Stems of *Milletia ovalifolia*

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The plant *Millettia ovalifolia* is commonly known as Gauj and belongs to the natural order Leguminosae. The stems of this plant are reported to be poisonous. The ethyl acetate soluble part of the concentrated alcoholic extract of stem when worked up by column chromatography yielded a novel cardenolide which was identified by chemical degradations, colour reactions and spectroscopic studies as 4,5-dehydro-14-β-hydroxy scilladienolide-3-O-β-D-glucopyranoside (AC-3).

Key words: 4,5-Dehydro-14-β-hydroxy scilladienolide-3-O-β-D-glucopyranoside, Stems, *Milletia ovalifolia*

INTRODUCTION

The plant *Millettia ovalifolia*[1] is commonly known as Gauj in Hindi and belongs to the natural order Leguminoseae. It is cultivated in the outer Himalayas in Sikkim. It is also found in the forest of Dehradun. The stems of this plant are reported to be poisonous. In view of its poisonous nature, the plant has been under phytochemical investigations by earlier workers who have already reported the presence of important biologically active compounds in it[2-7].

Its stems were therefore subjected to further phytochemical investigations and the ethyl acetate soluble part of the concentrated alcoholic extract of stem when worked up by column chromatography yielded a novel cardenolide which was identified by chemical degradations, colour reactions and spectroscopic studies as 4,5-dehydro-14-β-hydroxy scilladienolide-3-O-β-D-glucopyranoside (AC-3).

EXPERIMENTAL

The stems of the plant *Millettia ovalifolia* were collected with the help of the Forest Department from Dehradun and herbarium specimen has been preserved also.

Extraction of stem: The stems of *Millettia ovalifolia* were chipped, dried and powdered and defatted. The defatted stems were then taken out and extracted with rectified spirit. The rectified spirit was filtered while hot and concentrated under reduced pressure to get brown viscous mass.

The viscous mass was extracted with various solvents of increasing polarity and studied separately. On examination, the chloroform-ethyl acetate soluble part was found to respond positive to test of cardenolide (Kedde's, Keller Kiliani and Legal's test). Therefore, it was concentrated under reduced pressure to a dark yellow viscous mass. It was subjected to TLC examination, using chloroform : ethyl acetate (6 : 4) and Kedde's reagent (2% 3,5-dinitrobenzoic acid followed by KOH in methanol) as visualizing agent, when it showed a single spot, thereby confirming its homogeneous character.

The fraction was therefore subjected to purification over silica gel column (60–120 mesh) and eluted with chloroform : ethyl acetate (1 : 1). The fractions were collected and removal of the solvent yielded a colourless solid (yield 0.04%) (AC-3).

The compound AC-3 was analyzed for m.f. $C_{30}H_{42}O_9$. m.p. 179– 181°C, M^+ = 546 (by EIMS) and had $[\alpha]_D$ = 35.6 (in $CHCl_3$). UV λ_{max} 302 nm. IR ν_{max} 1085, 1160, 1180, 1240, 1320, 1376, 1386, 1445 cm^{-1}. Found: C = 65.78, H = 7.43%; calculated for $C_{30}H_{42}O_9$: C = 65.57, H = 7.45%.

AC-3 was found to be soluble in ethyl acetate, methanol, ethanol and solvent ether. It responded to a positive Keller-Kiliani reaction which is characteristic of the cardiac nature of this compound[8, 9].

Hydrolysis of AC-3: AC-3 (700 mg) was taken with 50 mL of 7% ethanolic sulphuric acid in 250-mL B-14 ground joint quick-fit round-bottomed flask having a reflux condenser attached to it. The reaction mixture was heated for 6 h on a water bath. The solution was neutralized with a dilute solution of NaOH. Subsequently the solution was concentrated under reduced pressure. The cardiogenin was separated out in well-defined crystals, which were dried at 80°C in vacuum (yield 530 mg).

Study of cardiogenin, AC-3a: AC-3a was soluble in ethyl acetate, methanol and absolute alcohol, had m.p. 230–240°C and $[\alpha]_D$ = 13 (in $CHCl_3$) and responded to the following characteristic colour reaction of cardiogenin, *i.e.*, Liebermann-Burchard reaction, Salkowski's reaction and Tschugaeve reaction. M.f. $C_{24}H_{32}O_4$. Found: C = 74.87, H = 8.28%; calculated for $C_{24}H_{32}O_4$: C = 75.00, H = 8.33%; $[\alpha]_D$ = 13 (in $CHCl_3$); M^+ = 384 (by mass spectroscopy); UV λ_{max} 302 nm (MeOH). IR ν_{max} 1080, 1163, 1184, 1246, 1320, 1355, 1392, 1456, 1650, 1736, 1794, 2945, 3487 cm^{-1}.

Preparation of acetyl derivative of cardiogenin: About 50 mg of the cardiogenin AC-3a was taken in a 100 mL B-14 ground-joint quick-fit round-bottomed flask and acetic anhydride (25 mL) was added and a reflux condenser was attached to it. The mixture was refluxed on a water bath and cooled, when a crystalline acetyl derivative was obtained which was separated by filtration. It crystallized from ethanol in light yellow needles (60 mg). m.p. 210–211°C. It was analyzed for m.f. $C_{26}H_{34}O_5$; m.w. 426 (by mass spectroscopy). Found: C = 73.17, H = 7.48, acetyl group = 9.83%; calculated for $C_{26}H_{34}O_5$: C = 73.23, H = 7.98, acetyl group = 9.85%. Significant signals observed in ^1H NMR spectrum are: δ = 1.40–2.10 (polymethylene), 2.11 (C_3-OAc), 2.85 (C_{17}-H), 3.55 (C_{14}-OH), 5.87 (C_4-CH), 4.40 (C_3-H), 5.58 [C_{23}-H (—CH=)], 5.78 [C_{22}-H (—CH=)], 5.86 [C_{21}-H (—CH=)], 0.90 (C_{19}-CH_3), 0.71 (C_{18}-CH_3).

Preparation of anhydro cardiogenin: About 60 mg of the cardiogenin was taken in a 100 mL B-14 ground-joint quick-fit round-bottomed flask and mixed with 50% alcohol (10 mL) containing 0.7 N hydrochloric acid (0.5 mL). The solution was boiled with 10 mL of water and alcohol removed by evaporation under reduced pressure. After standing for some time the semi-crystalline deposit (73 mg) was separated and crystallized from the mixture of acetone : ethyl acetate when the anhydro cardiogenin was obtained as brilliant prisms. M.f. $C_{24}H_{28}O_2$ and m.p. 198–200°C. Found: C = 81.84, H = 9.86%; calculated for $C_{24}H_{28}O_2$: C = 81.81, H = 7.95%, M^+ = 348.

Preparation of hexahydro cardiogenin: About 60 mg of the cardiogenin AC-3a was dissolved in 10 mL of 90% alcohol and shaken with Pd-black and hydrogen until no more gas was absorbed. Reduction proceeded slowly for 2 h and was completed after 12 h. After removal of the catalyst the solution was concentrated until a solid separated out (65 mg) which was crystallized from acetone. The compound was analyzed for m.f. $C_{24}H_{38}O_4$ and melted at 208°C. Found: C = 73.80, H = 9.75%; calculated for $C_{24}H_{38}O_4$: C = 73.84, H = 9.74%.

Preparation of iso-cardiogenin: About 60 mg of the cardiogenin AC-3a was dissolved in 10% solution of KOH in EtOH (5 mL) and kept for 30 min and then the solution was diluted with water (12 mL) and acidified with 20% HCl (10 mL). After standing for 30 min, the solution was further diluted and then concentrated under reduced pressure. The solid which separated was filtered off and purified by crystallization and the compound obtained had m.f. $C_{24}H_{32}O_4$, m.p. 280°C, M^+ = 384. Found: C = 74.86, H = 8.26%; calculated for $C_{24}H_{32}O_4$: C = 72.00, H = 8.33%.

Preparation of etianic acid: 70 mg of the cardiogenin was taken in a 100-mL conical flask and 10 mL of acetic anhydride and 0.5 mL of pyridine were added to it. The contents were allowed to reflux and cooled to get a product. To it $KMnO_4$ (15 mg) in acetone (12 mL) was added and the contents hydrolysed by 7% H_2SO_4 when it yielded an amorphous mass which was crystallized from methanol; m.f. $C_{20}H_{30}O_4$, m.p. 186–188°C. (60 mg). Found: C = 72.26, H = 8.41%; calculated for $C_{20}H_{30}O_4$: C = 72.28, H = 8.43%.

Identification of sugars after hydrolysis of AC-3: The aqueous hydrolysate obtained after hydrolysis of AC-3 was neutralized with barium carbonate and barium sulphate was filtered off. The filtrate was concentrated to a yellow viscous mass and subjected to paper chromatography on Whatmann No. 1 filter paper using different solvent system and aniline hydrogen phthalate as spraying reagent.

Permethylation and hydrolysis of AC-3: The permethylation and hydrolysis of AC-3 (100 mg) was done when it showed the presence of 4,5-dehydro-3β,14β-scilladienolide (45 mg) as a cardiogenin and the methylated sugar was identified as 2,3,4,6-tetra-O-methyl-D-glucose (by co-PC and co-TLC).

Enzymatic hydrolysis of cardiogenin AC-3: The cardiac glycoside AC-3 (50 mg) was dissolved in 20-mL ethanol and mixed with almond emulsion (30 mL) in a conical flask (100-mL) fitted with stopper. The reaction mixture was left for 72 h at room temperature and then filtered. The procardiogenin and hydrolysate were examined separately. The hydrolysate after concentration was examined for sugar moiety by paper chromatography using Whatmann No. 1 filter

paper and BAW (4 : 1 : 5) solvent system. The sugar was identified as D-glucose (R_f 0.17).

RESULTS AND DISCUSSION

Presence of the methyl group in AC-3: Characteristic peak at 2952 cm^{-1} in the IR spectrum of the AC-3 confirmed the presence of —CH_3 group(s), which when estimated by Zeisel method (4.67%) confirmed the presence of two methyl groups in the compound AC-3.

Presence of the —OH group in the compound AC-3: The significant peak at ν_{max}(KBr) 3485 cm^{-1} in the IR spectrum of AC-3 indicated the presence of —OH group(s) in AC-3. The number of the —OH group(s) in it were estimated by its acetylation with Ac_2O/pyridine to get an acetylated product. The percentage of acetyl group in the acetylated product (m.p. 253–255°C), m.f. $C_{38}H_{50}O_{13}$ and $M^+ = 714$ was determined by the method of Wiesenberger[10] as described by Belcher and Codbert[11] which indicated the presence of four —OH groups in AC-3.

Presence of the lactone ring in AC-3: Presence of another peak at 1788 cm^{-1} and 1730 cm^{-1} in the IR spectrum of AC-3 confirmed the presence of >C=O group in the lactone ring. Their number was estimated by the hydrolysis of AC-3 and subsequent titration, which showed the presence of only one lactone ring in AC-3. Thus it is clear that six oxygen atoms have been accounted for: four as —OH groups (which were acetylated) and two in the lactone ring in AC-3. Thus the nature of the remaining three oxygen atoms was established by the hydrolysis of the compound AC-3 which gave a cardiogenin AC-3a and sugar(s) which were separated and studied separately.

Study of the cardiogenin AC-3a: The cardiogenin AC-3a responded to characteristic colour reactions of steroids. It was soluble in ethyl acetate, methanol and absolute alcohol. AC-3a analyzed for m.f. $C_{24}H_{32}O_4$; m.p. 238–240°C, M^+ = 384 (by mass spectroscopy) and had $[\alpha]_D = 13$ (in $CHCl_3$).

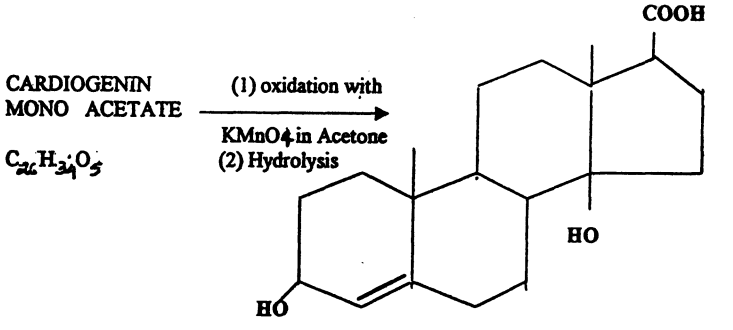
AC-3a yielded Diels' hydrocarbon on dehydrogenation with selenium at 360°C.

Presence of the lactone ring in AC-3a: The characteristic band at ν_{max}(KBr) 1794 cm^{-1} and 1736 cm^{-1} in the IR spectrum of the cardiogenin indicated the presence of a lactone ring[12] in the cardiogenin AC-3a which was estimated by hydrolysis and titration when it showed the presence of one lactone ring in AC-3a.

Size of the lactone ring in AC-3a: The lactone AC-3a did not respond to the Legal test but with methanolic potassium hydroxide yielded enol ester. Ozonolysis of the lactone ring afforded formic acid, oxalic acid and glyoxalic acid together with 3β,14β dihydroxy 4,5-dehydro etianic acid. Thus these points indicated that the side chain is a six-membered α : β : γ : δ unsaturated lactone ring[12, 13] in AC-3a.

Position of the lactone ring in AC-3a: The position of the lactone ring in the cardiogenin AC-3a was established at C-17, since cardiogenin monoacetate

AC-3a$_1$, on oxidation with $KMnO_4$ in acetone followed by hydrolysis gave a compound, m.f. $C_{20}H_{30}O_4$, which was found to be 3β:14β-dihydroxy-4,5-dehydro etianic acid (confirmed mmp with authentic sample).



The presence of double band(s) in AC-3a: The presence of band at ν_{max}(KBr) 1650 cm^{-1} in the IR spectrum of AC-3a indicated the presence of unsaturation in it, which was further supported by the fact that cardiogenin AC-3a on catalytic hydrogenation yielded a hexahydro derivative, m.f. $C_{24}H_{38}O_4$, m.p. 215–217°C and M^+ = 370, thereby confirming the presence of three double bonds in AC-3a.

$$\text{Cardiogenin (Ac-3a) } (C_{24}H_{32}O_4) \xrightarrow{6H} \text{Hexahydrocardiogenin } (C_{24}H_{38}O_4)$$

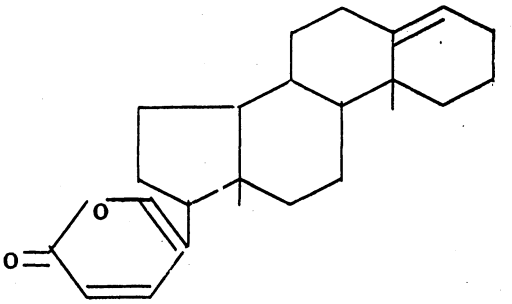
Position of double bond(s) in AC-3a: In the cardiogenin AC-3a, the position of one double bond was fixed in the steroidal nucleus, since the cardiogenin AC-3a responded to positive TNM test. It was also confirmed by the oxidation of cardiogenin monoacetate with $KMnO_4$ in acetone followed by hydrolysis to get 3β,14β-dihydroxy-4,5-etianic acid. Thus on the basis of the above fact the position of double bond in cardiogenin AC-3a was fixed at C_4–C_5 in AC-3a.

The lactone ring on ozonolysis gave formic acid and glyoxalic acid together with 3β:14β-dihydroxy-4,5-dehydro etianic acid. Also on the other hand this lactone did not give the Legal test but with methanolic potassium hydroxide, it yielded enol ester which afforded 14β,21β-epoxy esters (iso compound). This fact shows that the side chain was a six-membered α : β : γ : δ lactone ring and a double bond in the lactone ring was at C_{20-21}, and C_{22-23} in AC-3a.

Presence of —OH group(s) in AC-3a: The cardiogenin AC-3a did not give positive test for —COOH group but showed a peak in the IR spectrum at 3487 cm^{-1} and also indicated the presence of —OH group(s) in it. It was found to form a monoacetyl derivative, m.f. $C_{26}H_{34}O_5$, m.p. 210–211°C and M^+ = 426, thereby indicating the presence of any one acetylable OH group, but when treated with HCl, formed a dianhydro cardiogenin, m.f. $C_{24}H_{28}O_2$, m.p. 196–198°C thereby confirming the presence of two OH group(s) in the cardiogenin out of which one

OH group was acetylated and so was either primary or secondary and the remaining —OH group had escaped acetylation and hence must be tertiary in AC-3a.

Therefore the cardiogenin AC-3 could be represented as a dihydroxy α : β : γ : δ unsaturated steroidal lactone as shown below.



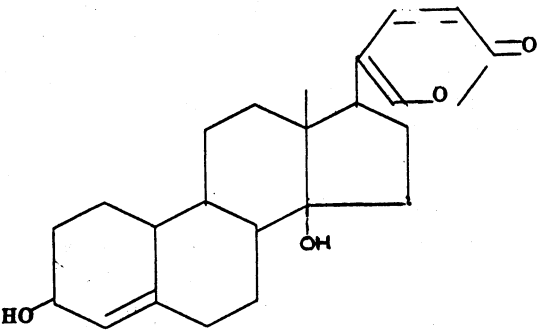
Position of —OH group in AC-3a

Presence of C-3 OH group: The chromium trioxide/pyridine oxidation of the cardiogenin yielded a ketone, m.f. $C_{24}H_{30}O_4$, m.p. 206°C and $M^+ = 382$. This ketone showed positive Zimmermann test for C-3 keto group, thereby confirming the presence of one —OH group at C-3[14] and further indicated its nature as secondary in AC-3a.

Presence of C-14 OH group: The cardiogenin AC-3a on reaction with alcoholic KOH formed an iso-cardiogenin, m.f. $C_{24}H_{32}O_4$, m.p. 280°C and $M^+ = 384$ and its formation was accounted by placing the tertiary—OH group at C-14[15].

$$\text{Cardiogenin (Ac-3a) } (C_{24}H_{32}O_4) \xrightarrow{\text{Alcoholic KOH}} \text{Isocardiogenin } (C_{24}H_{32}O_4)$$

Thus on the basis of the above observations, finally it was concluded that the cardiogenin under examination was 3β:14β-dihydroxy-4,5-dehydro scilladienolide, and was assigned the structure as below:



Sugar in AC-3: AC-3 on hydrolysis with the enzyme emulsion for one day at room temperature liberated D-glucose showing that it must be attached to the cardiogenin AC-3a by β-linkage.

Quantitative estimation of sugar: Quantitative estimation of sugar was done by the procedure of Mishra and Rao[15] which revealed that both sugar and the aglycone were present in equimolecular ratio (1 : 1). From the above results it was concluded that one molecule of the cardiac glycoside was made up of one molecule of the cardiogenin AC-3(a) and one molecule of D-glucose.

Periodate oxidation of AC-3: The sodium meta-periodate oxidation[16] of the AC-3 consumed 2.03 moles of periodate and liberated 1.09 mole of formic acid, thereby showing that one molecule of AC-3 was made up of one molecule of cardiogenin AC-3a, which also confirmed that the sugar glucose was present in the pyranose form.

Permethylation and hydrolysis of AC-3: The permethylation of AC-3 was carried out by the procedure of Kuhn *et al.*[17] The permethylated product on hydrolysis yielded cardiogenin AC-3a which was identified as 3β,14β-dihydroxy 4:5-dehydro scilladienolide.

Position of attachment of sugar: The position of attachment of sugar residue to cardiogenin was fixed at C-3, because the aglycone AC-3a (cardiogenin) responded to positive Zimmermann test, while the glycoside AC-3 did not respond to this test, thus confirming that the sugar was attached to C_3-OH group in the cardinolide glycoside. The point of attachment of the aglycone to sugar was confirmed by methylation of the glycoside followed by hydrolysis when the sugar was obtained, which was identified as 2:3:4:6-tetra-O-methyl-D-glucose thereby confirming that C-1 of the sugar was involved in glycosilation.

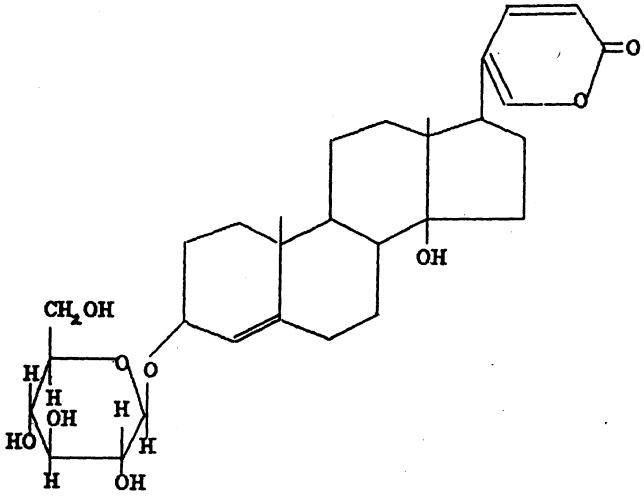
Permethylation and hydrolysis of AC-3: The permethylation and hydrolysis of AC-3 (100 mg) was done, when it showed the presence of 4:5-dehydro 3β,14β-scilladienolide (45 mg) as a cardiogenin and the methylated sugar was identified as a 2:3:4:6-tetra-O-methyl-D-glucose (by co-PC and co-TLC).

Enzymatic hydrolysis of cardiogenin AC-3: Cardiac glycoside AC-3 (50 mg) was dissolved in 20 mL ethanol and mixed with almond emulsion (30 mL) in a conical flask (100 mL) fitted with stopper. The reaction mixture was left for 72 h at room temperature and then filtered. The pro-cardiogenin and hydrolysate were examined separately. The hydrolysate after concentration was examined for sugar moiety by paper chromatography using Whatmann No.1 filter paper and BAW (4 : 1 : 5) solvent system. The sugar was identified as D-glucose (R_f 0.17). AC-3 when hydrolysed by enzyme almonds emulsion[18] yielded D-glucose thereby indicating that the D-glucose was linked via β-linkage to the cardiogenin. Thus the following structure was assigned to AC-3: 4,5-dehydro-14β-hydroxy scilladienolide-3-O-β-D-glucopyranoside.

Mass spectrum of AC-3: Several sinificant fragmentation patterns observed in the electron impact mass spectrum of AC-3 are described below:

m/e (%) [M^+]: 692(30), 546(2), 384(35), 366(22), 348(23), 316(41), 298(100), 290(10), 272(38), 254(68), 202(36), 160(12), 146(19) and 131(26).

Various species formed during fragmentation were found to be in complete accord with the structure (I) assigned to AC-3 which also satisfactorily explained all the reactions of this cardiac glycoside.



AC-3

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