Two novel steroidal derivatives from chloroform-soluble extract of *Hoya longifolia*

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The structures of two novel steroidal derivatives (1 and 4) were elucidated.

Steroidal derivatives namely fologenin (1) and hoyagenin (4) have been isolated from chloroform-soluble extract of the whole plant of *Hoya longifolia* (family: Asclepiadaceae), and their structures were determined by using $^1$H NMR, $^{13}$C NMR, $^1$H–$^1$H COSY and FAB-MS techniques as well as chemical degradation and derivatisation.

**Keywords:** Asclepiadaceae; *Hoya longifolia*; steroid; pregnane; fologenin; hoyagenin

1. Introduction

Plant family Asclepiadaceae has been a rich source of biologically active steroids and their derivatives are mostly pregnane (Deepak et al. 1989; Ketwaru et al. 1993; Khare et al. 1986, 1987), cardenolides (Reichstein 1967) and glycosides (Deepak et al. 1997; Chen et al. 1999). Plants of this family have been traditionally used for their anti-inflammatory, anti-tumours (Chopra et al. 1956; Ahmad, Usmaghani, et al. 1983, 1988; Ahmad, Noorwala, et al. 1993; Masoodi et al. 2008; Mahmood et al. 2010), immunostimulant (Pan et al. 2003, 2013), anti-diabetic (Noor et al. 2013), anti-oxidant, antimicrobial, anti-complementary, antiepileptic and antineoplastic activities (Piacente et al. 1998; Sethi et al. 2008, 2012, 2013; MacLeod et al. 1997). In the search of these novel compounds, various species of the genus *Hoya* have been examined chemically and a number of biologically active steroids and their glycosides have been isolated (Mahmood et al. 2012; Dembitsky et al. 2004).

2. Results and discussion

Compound 1 named fologenin and compound 4 named hoyagenin were isolated by repeated column chromatography (CC) of CHCl$_3$-soluble extract of *Hoya longifolia*.
2.1. Fologenin (1)

Fologenin (1), $[\alpha]_D + 20^\circ$, C$_{37}$H$_{50}$O$_7$, m/z 606 [M]$^+$, showed positive Libermann–Burchard test (Abisch et al. 1960) and tetra-nitro methane test (Ostromisslensky 1910) suggesting 1 to be a steroidal moiety containing a double bond. It did not give positive alkaline hydrolysis test suggesting the absence of an ester group in 1. The basic skeleton of pregnane is known to possess 21 carbons but the FAB mass spectrum of compound 1 showed the highest mass ion peak at m/z 606, which was in agreement with the molecular formula C$_{37}$H$_{50}$O$_7$. Compound 1 on treatment with ceric ammonium nitrate (Fukuyama et al. 1985) resulted in compound 3. Compound 3 was found to be cissogenin (Singhal et al. 1980) on comparison with the authentic sample (m.p., m.m.p., [α]$_D$, TLC).

The difference of C$_{16}$H$_{16}$O$_2$ atoms between the C-21 pregnane moiety and molecular formula of 1 (C$_{37}$H$_{50}$O$_7$) and on the basis of ceric ammonium test along with its $^1$H and $^{13}$C NMR spectra, suggested the presence of at least two additional para methoxy benzyl ether groups attached to the pregnane moiety in 1. The presence of two aromatic benzyl ether groups in the compound is evident by the presence of peaks at δ 7.08–6.69 for eight aromatic protons as multiplet in the $^1$H NMR spectrum which was also confirmed by the peaks for 12 aromatic carbons in the $^{13}$C NMR spectrum of 1. The presence of vicinal diol group in 1, indicated by positive NaIO$_4$ oxidation (Sawlewiez et al. 1954) in 1, was evident by a doublet ($J = 8$ Hz) at δ 3.80 for H-12 and a triplet ($J = 8$ Hz) at δ 4.05 of H-11 methine protons in the $^1$H NMR spectrum of 1. Therefore, it was evident that the para methoxy benzyl ether groups could only be present at C-3 and C-20, which was also supplemented by signals at δ 75.8 (C-11) and 72.8 (C-12) in the $^{13}$C NMR spectrum. The position of hydroxyl groups at C-11 and C-12 was also confirmed by its COSY spectrum. The nature of mono substitution in the benzyl ether group was shown by the presence of one singlet of six protons at δ 3.86 for the two aromatic methoxy groups in addition to a sharp signal at δ 55.9 for two carbons of methoxy groups in the $^{13}$C NMR spectrum.

The absence of two carbon keto methyl side chains at C-17 of pregnane moiety was indicative in the $^1$H NMR spectrum of 1 as well as in the negative NaBH$_4$ test shown by it. Instead a secondary methyl group doublet ($J = 7.2$ Hz) for three protons at δ 0.90 of C-21 methyl group as well as a multiplet for two protons at δ 2.87 confirmed the presence of methine protons at C-3 and C-20, which was also supplemented by signals at δ 75.8 (C-11) and 72.8 (C-12) in the $^{13}$C NMR spectrum. The position of hydroxyl groups at C-11 and C-12 was also confirmed by its COSY spectrum. The presence of vicinal diol group in 1, indicated by positive NaIO$_4$ oxidation (Sawlewiez et al. 1954) in 1, was evident by a doublet ($J = 8$ Hz) at δ 3.80 for H-12 and a triplet ($J = 8$ Hz) at δ 4.05 of H-11 methine protons in the $^1$H NMR spectrum of 1. Therefore, it was evident that the para methoxy benzyl ether groups could only be present at C-3 and C-20, which was also supplemented by signals at δ 75.8 (C-11) and 72.8 (C-12) in the $^{13}$C NMR spectrum. The position of hydroxyl groups at C-11 and C-12 was also confirmed by its COSY spectrum. The nature of mono substitution in the benzyl ether group was shown by the presence of one singlet of six protons at δ 3.86 for the two aromatic methoxy groups in addition to a sharp signal at δ 55.9 for two carbons of methoxy groups in the $^{13}$C NMR spectrum.

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by the loss of H$_2$O molecule from $m/z$ 606 which further fragmented to give the fragment peaks at $m/z$ 575 [M$^+ - $ OCH$_3$], 523 [540-OH] and 193 [300-C$_7$H$_7$O]. Other mass peaks at $m/z$ 326, 262, 258, 165, 197 and 97 were obtained by the retro-Diels–Alder (RDA) fission (Ichihara 1987) in 1 (Schemes S1 and S2).

In the light of the foregoing evidences, the structure of compound was established as 3, 20-O-di para methoxy benzyl pregn-5-ene-11α,12β,14β-triol.

2.2. Hoyagenin (4)

Hoyagenin (4), [α]$_D$ + 2.4°, C$_{21}$H$_{28}$O$_5$, $m/z$ 360 [M$^+$], showed positive Libermann–Burchard test (Abisch et al. 1960), tetra-nitro methane test (Ostromisslensky 1910) and also underwent NaIO$_4$ oxidation (Sawlewicz et al. 1954), suggesting 4 to be the steroidal moiety with a double bond and vicinal diol system. It did not give positive alkaline hydrolysis test suggesting the absence of an ester group. The plants of the Asclepiadaceae family are found to be rich source of pregnanes (C-21) and their derivatives. Pregnanes are C-21 steroidal compounds having usual perhydro-1,2-cyclopentano phenanthrene ring system with β-oriented angular methyl groups at C-10 and C-13, bearing either a two-carbon side chain of hydroxyl ethyl or acetyl groups at C-17. Usually pregnane derivatives possess a β-oriented hydroxyl group at C-14. The FAB mass spectrum of compound 4 showed the highest mass ion peak at $m/z$ 360, which was in agreement with the molecular formula C$_{21}$H$_{28}$O$_5$. Owing to the number of carbon atoms in compound 4, it suggested the presence of C-21 pregnane derivative. The lesser number of hydrogen atoms present in the molecular formula of 4 as compared with the number of hydrogen atoms present in C-21 pregnane derivatives (Srivastava et al. 2007) indicated the presence of the dehydrogenated ring system in compound 4. The dehydrogenated ring system in the form of aromatic ring in 4 was evident by the presence of peak at δ 6.82–6.32 for three protons in the $^1$H NMR spectrum which was also supplemented by the carbon peaks at δ 159.3, 137.0, 133.1, 122.3, 114.3 and 110.1 for six aromatic carbons in its $^{13}$C NMR spectrum. The characteristic signals of compound 4 show only C-18 angular methyl group singlet at δ 1.25 for three protons, which was further supported by a signal at δ 20.0 in the $^{13}$C NMR spectrum of 4 and the absence of a characteristic signal of angular methyl group usually present at C-10 in the pregnane moiety in their $^1$H and $^{13}$C NMR spectra, suggesting compound 4 to be a 19-nor pregnane derivative (Kittakoop et al. 1999; Higgs et al. 1977; Ono et al. 1986). This was further supported by the presence of two doublets ($J = 2.8$ Hz) of one proton each at δ 6.82 and 6.56 for H-1 and H-2, respectively, in addition to a singlet of one proton at δ 6.32 for H-4 in the $^1$H NMR spectrum of 4. A singlet of three protons at δ 3.80 in the $^1$H NMR spectrum of 4 was attributed to the aromatic methoxy group which could be present in the aromatic ring ‘A’ presumably at the C-3 position which was also confirmed by the signal at δ 56.0 in the $^{13}$C NMR spectrum of 4 which is also supported by the $^1$H–$^1$H COSY experiment of 4.

The presence of vicinal diol group in 4, as evident by positive NaIO$_4$ oxidation, was shown by a doublet ($J = 8.0$ Hz) of one proton at δ 3.50 for H-12 and a triplet ($J = 8.0$ Hz) of one proton at δ 3.70 for H-11 methine protons in the $^1$H NMR spectrum of 4, which was also supported by signals at δ 66.4 (C-11) and 63.0 (C-12) in its $^{13}$C NMR spectrum. The $^1$H NMR spectrum did not show the presence of either keto methyl or hydroxyl ethyl side chain usually present at C-17 in pregnane derivatives. Instead, a two-carbon chain of vinyl group was presumably present at C-17 position because it contains two broad singlets at δ 5.24 and 5.23 for methylene protons of H-20 and a multiplet at δ 5.60 (H-19) for methine proton. It was further confirmed by sharp signals at δ 115.6 (C-21), 146.7 (C-20) and 76.4 (C-17) in the $^{13}$C NMR spectrum of 4. The loss of 27 a.m.u. (CH$_2 = $ CH–) also confirmed the presence of a vinyl group at C-17 in the FAB mass spectrum of 4.
To ascertain the number of free acylable hydroxyl groups in 4, on acetylation, it gave an amorphous product, 5, \([\alpha]_D^25 + 3.5^\circ\). The \(^1\)H NMR spectrum of compound 5 contained two singlets of three protons at \(\delta 2.02\) and \(2.29\), indicating the presence of two acylable hydroxyl groups in the 19-nor pregnane moiety of 4. The position of acetyl groups in substance 5 at C-11 was inferred by the presence of a triplet \((J = 8.0 \text{ Hz})\) of one proton at \(\delta 4.08\) along with a doublet \((J = 8.0 \text{ Hz})\) of one proton at \(\delta 4.16\) assigned to methine protons present at C-11 and C-12, which was shifted downfield due to the presence of ester groups in the \(^1\)H NMR spectrum of 5. The characteristic signals of compound 5 show C-18 angular methyl group singlet at \(\delta 1.25\) for three protons. The presence of the C-19 angular methyl group was not observed, it again confirmed that compound 5 to be 19-nor pregnane derivatives.

The FAB mass spectrum of 4 showed highest mass ion peak at \(m/z\) 360. This in turn further fragmented to give the important mass ion fragment. The mass ion at \(m/z\) 342 was obtained by the loss of H\(_2\)O molecule from \(m/z\) 360 which further fragmented to give the fragment ion at \(m/z\) 128 [159-OCH\(_3\)], 107 [124-OH] and 97 [124-C\(_2\)H\(_3\)]. Another mass ion peak at \(m/z\) 326 was obtained by the RDA fission (Ichihara 1987) in 4 (Scheme S3).

In the light of the foregoing evidences, the structure of compound was established as 19-Norpregna 1(10), 2,4,20-tetraen-3-methoxy-11\(a\),12\(b\),14\(b\),17-tetraol.

3. Experimental

3.1. General experimental procedures

The procedure was the same as those reported earlier (Kumar et al. 1999). \(^1\)H NMR, \(^{13}\)C NMR and 2D spectra were recorded with Bruker Spectrometer (Model: Avance DRX 300 MHz) in CDCl\(_3\) using TMS as internal standard. FAB mass spectra were recorded with a Jeol Mass Spectrometer (Model: D-300) with IMA-2000 data system, respectively. Optical rotations were measured with an automatic polarimeter, Optical Activity (Model: AA-5 series). TLC was performed on silica gel G (Qualigens) and CC was done over silica gel 60–120 mesh (Qualigens, Fisher Scientific).

3.2. Plant extraction

The whole plant H. longifolia was collected in bulk (30 kg) from the foot hill of Himalaya of North India and was identified by Dr D.C. Saini, Botanist, Birb"al Sahni Institute of Paleobotany (BSIP), Lucknow. A specimen (No. BSIP 12027) of the plant has been deposited in the herbarium of BSIP, Lucknow. Shade-dried chopped plants (10 kg) were extracted by the method used for pregnane glycosides (Khare et al. 1984) using 50–95% ethanol. The combined ethanolic extract was concentrated under reduced pressure and the concentrate was exhaustively extracted successively with hexane (3.05 g), CHCl\(_3\) (9.50 g) and CHCl\(_3\)–EtOH (3:2) (3.00 g).

Fologenin (1) (114 mg) and hoyagenin (4) (65 mg) were isolated by the repeated CC of the CHCl\(_3\)-soluble extract.

3.3. Fologenin (1)

Amorphous substance; \([\text{ALPHA}]_D^{25} + 20\) \((c = 1.0, \text{CHCl}_3)\); \(^1\)H NMR (CDCl\(_3\)): \(\delta 7.08–6.69\) (8H, m, aromatic H), 5.34 (1H, m, H-6), 4.05 (1H, t, \(J = 8 \text{ Hz, H-11})\), 3.86 (6H, s, 2 \(\times\) OCH\(_3\)), 3.80 (1H, d, \(J = 8 \text{ Hz, H-12})\), 2.87 (2H, m, H-3, H-20), 1.25 (6H, s, 19-CH\(_3\), 18-CH\(_3\)), 0.90 (3H, d, \(J = 7.2 \text{ Hz, 21-CH}_3\)), \(^{13}\)C NMR (CDCl\(_3\)): \(\delta 30.30\) (C-1), 29.6 (C-2), 82.8 (C-3), 38.7 (C-4), 143.9 (C-5), 121.1 (C-6), 23.7 (C-7), 33.2 (C-8), 42.3 (C-9), 32.2 (C-10), 75.8 (C-11), 72.8 (C-12), 84.5 (C-13), 78.8 (C-14), 38.7 (C-15), 14.1 (C-16), 52.5 (C-17), 8.1 (C-18), 22.9 (C-19), 76.2 (C-20), 23.7 (C-21), 55.9, 73.1 (\(p\)-methoxy benzyl-CH\(_2\)), 145.0, 111.2, 154.5, 115.4, 132.2, 118.8,
143.9, 108.3, 146.6, 114.2, 130.0, 113.4 (aromatic carbons). FAB-MS: m/z 606 [M^+]^+, 588 [606-H_2O]^+, 575 [606-OCH_3]^+, 558 [575-OH]^+, 549 [575-CH=CH]^+, 540 [558-H_2O]^+, 522 [540-H_2O]^+, 326 [588-C_16H_22O_3]^+, 317 [348-OCH_3]^+, 300 [317-OH]^+, 262 [588-C_21H_26O_3]^+, 193 [300-C_19H_28O]+, 176 [193-OH]^+, 165 [262-C_6H_9O]^+, 97 [262-C_10H_13O_2]^+, 95 [121-C_2H_2]^+.

(calcld for C_{37}H_{50}O_7: C, 73.24; H, 8.31. found: C, 73.16; H, 8.29%)

3.3.1. Di-O-acetyl fologenin (2)
Compound 1 (5 mg) was acetylated with A_2O (0.4 mL) in pyridine (0.4 mL) and the mixture was kept for 24 h at 60°C yielding acetate 2, (4.2 mg) [ALPHA]_D^{25} + 14° (c = 0.25, CHCl_3); ^1H NMR (CDCl_3): δ 2.00 (6H, s, 2×OAc), 7.08–6.69 (8H, m, aromatic H), 5.34 (1H, m, H-6), 3.86 and 3.87 (6H, 2s, 2×OCH_3), 2.87 (2H, m, H-3, H-20), 1.25 (6H, s, 19-CH_3, 18-CH_3), 0.90 (3H, d, J = 7.2 Hz, 21-CH_3).

3.3.2. Reaction with ceric ammonium nitrate
Compound 1 (20 mg) was on treatment with ceric ammonium nitrate in aqueous acetonitrile at 0°C resulted in compound 3, (9.5 mg) m.p. 211–224°C, [ALPHA]_D^{25} –2.31° (c = 0.5, MeOH).
Compound 3 was identified as cissogenin, m.p. 211–224°C, [ALPHA]_D^{25} –2.34° (c = 0.5, MeOH) in comparison with the authentic sample (m.p., m.m.p., TLC and Paper Chromatography).

3.3.3. Hoyagenin (4)
Amorphous substance; [ALPHA]_D^{25} +2.4° (c = 1.0, CHCl_3); ^1H NMR (CDCl_3): δ 6.82 (1H, d, J = 2.8 Hz, H-1), 6.56 (1H, d, J = 2.8 Hz, H-2), 6.32 (1H, s, H-4), 5.60 (1H, m, H-19), 5.24 (1H, s, H-20), 5.23 (1H, s, H-20), 3.80 (3H, s, OCH_3), 3.70 (1H, t, J = 8.0 Hz, H-11), 3.50 (1H, d, J = 8 Hz, H-12), 1.25 (3H, s, 18-CH_3).
^13C NMR (CDCl_3): δ 122.3 (C-1), 110.1 (C-2), 159.3 (C-3), 114.3 (C-4), 137.0 (C-5), 33.3 (C-6), 29.7 (C-7), 40.1 (C-8), 24.8 (C-9), 133.1 (C-10), 77.4 (C-11), 66.4 (C-12), 63.0 (C-13), 75.0 (C-14), 24.8 (C-15), 27.0 (C-16), 76.4 (C-17), 20.0 (C-18), 146.7 (C-19), 115.6 (C-20), 56.0 (C-3'). FAB-MS: m/z 360 [M^+]^+, 342 [M^+H_2O]^+, 333 [360-CH=CH_2]^+, 329 [360-OCH_3]^+, 325 [342-OH]^+, 315 [333-H_2O]^+, 311 [329-H_2O]^+, 307 [325-H_2O]^+, 297 [315-H_2O]^+, 293 [311-H_2O]^+, 289 [307-H_2O]^+, 279 [297-H_2O]^+, 275 [293-H_2O]^+, 262 [289-CH=CH_2]^+, 261 [279-H_2O]^+, 248 [275-CH=CH_2]^+, 230 [261-OCH_3]^+, 125 [159-OCH_3]^+, 123 [140-OH]^+. (calcld for C_{21}H_{28}O_5: C, 69.98; H, 7.83. found: C, 69.90; H, 7.80%).

3.3.4. Di-O-acetyl hoyagenin (5)
Substance 4 (5 mg) was acetylated with A_2O (0.4 mL) in pyridine (0.4 mL) and the mixture was kept for 24 h at 60°C yielding acetate 5, (4.9 mg), [ALPHA]_D^{25} +3.5° (c = 0.25 CHCl_3); ^1H NMR (CDCl_3): δ 2.02 (3H, s, 11-OAc), 2.29 (3H, s, 12-OAc), 1.25 (3H, s, 18-CH_3), 3.80 (3H, s, OCH_3), 4.08 (1H, t, J = 8 Hz), 4.20 (1H, d, J = 8 Hz), 2.29 (3H, s, OAc).

Supplementary material
Experimental details relating to this paper are available online at http://dx.doi.org/10.1080/14786419.2015.1052066.
Disclosure statement
No potential conflict of interest was reported by the authors.

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