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A new steroidal ester from *Tinospora cordifolia* Miers. stems

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ABSTRACT

Tinospora cordifoliya (Gioe, Gudduchi or Amrita) is very well known in Ayurvedic system of medicine and found its use in various ailments. The present paper describes the isolation and structure elucidation of a new steroidal ester along with aliphatic alcohols and fatty acid esters from the alcoholic extract of Tinospora cordifolia stems. To carry out phytochemical investigation of dried stems of Tinospora cordifolia Miers. (Menispermaceae) for the isolation of a new steroidal ester. Column was eluted with different concentrations of petroleum ether: chloroform and their isolation and characterization was done with spectroscopic techniques. ***Results:*** *Phytoconstituents characterized as stigmast-5-en-3β, 21-dio-3β-yl hexadecanoate (**5**) along with n-heptacosanol (**1**), n-octacosanol (**2**), n-nonacosanol (**3**), n-tetracontanol (**4**), n-triacontanyl palmitate (**6**) and n-hexacosanyl stearate (**7**). The structures of all these phytoconstituents have been established by means of chemical, spectral and chromatographic means.*

Keywords: *Tinospora cordifolia*, Menispermaceae, steroidal ester, aliphatic alcohols, fatty acid esters.

INTRODUCTION

Tinospora cordifolia Miers. (Menispermaceae), known as Giloe, is a large, glabrous and deciduous climbing shrub found throughout tropical Indian subcontinent and China, ascending to an altitude of 300 m [1]. It is used as a general tonic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-allergic, anti-diabetic, anti-leprotic, antimalarial, stomachic and diuretic; to stimulate bile secretion, to allay thirst, burning sensation and vomiting, to enrich the blood and to cure jaundice. The stem extract is beneficial to subside skin diseases [1-3]. The plant contained alkaloids [4], sesquiterpenoids [5-8], syringine [7], diterpenoid lactones [9], columbin [10], steroids [11] and phenylpropenes [12]. The present paper describes the isolation and structure elucidation of a new steroidal ester along with aliphatic alcohols and fatty acid esters from the alcoholic extract of *Tinospora cordifolia* stems.

MATERIALS AND METHODS

Melting points were determined on a Perfit melting point apparatus (Ambala, India) and are uncorrected. IR spectra were recorded on KBr discs, using a Bio-Rad FT-IR 5000 spectrometer (FTS 135, Hongkong, China). UV spectra were measured with a Lambda Bio 20 spectrophotometer (Perkin Elmer, Schwerzenbach, Switzerland) in methanol. ^{1}H and ^{13}C NMR spectra were scanned using Bruker Advance DRY 400 spectrospin and Bruker Advance DRY 100 spectrospin instruments (Germany), respectively, in DMSO-d_6 and TMS as an internal standard. FAB MS spectra were obtained using JEOL-JMS-DX 303 spectrometer (Bruker Daltonics, MA, USA). Column chromatography was

performed on silica gel 60-120 mesh. TLC was run on silica gel G (Qualigens, Mumbai, India). Spots were visualized by exposure to iodine vapours, UV radiation and by spraying reagents.

Plant Material

The stems of *Tinospora cordifolia* were procured from Bhowali region, Nainital, Uttra Khand, India and authenticated by Prof. M. P. Sharma, Department of Botany, Faculty of Science, Jamia Hamdard, and New Delhi. A voucher specimen No. SOP/SU/07/01 is deposited in the Department of Pharmacognosy and Phytochemistry, Sharda University, Greater Noida.

Extraction and Isolation

The air-dried and coarsely powdered plant material (3 kg) was exhaustively extracted with ethyl alcohol (95%) in a Soxhlet apparatus for 48 hrs. The combined extracts were dried under reduced pressure to obtain a dark brownish colored residue (298 g). The dried ethanolic extract was dissolved in minimum quantity of methanol and adsorbed on silica gel (60-120 mesh) to form slurry. The slurry was air dried and subjected to silica gel column chromatography loaded with petroleum ether. The column was eluted with petroleum ether and petroleum ether-chloroform to isolate the compounds.

***n*-Heptacosanol (1)**

Elution of the column with petroleum ether: chloroform (1:1) gave colorless crystals of **1**, yield 0.60 %, R_f 0.80 (petroleum ether: chloroform, 2:3), m.p. 65 - 66^0 C. FAB MS *m/z* (rel. int.) 396 $[M]^+$ ($C_{27}H_{56}O$) (65.8).

***n*-Octacosanol (2)**

Elution of the column with petroleum ether: chloroform (2:3) afforded colorless crystals of **2**, yield 0.55 %, R_f 0.69 (petroleum ether : chloroform : methanol, 3.8 : 6.1 : 0.1), m.p. 68 – 69^0 C. FAB MS *m/z* (rel. int.) 410 $[M]^+$ ($C_{28}H_{58}O$) (43.6).

***n*-Nonacosanol (3)**

Elution of the column with petroleum ether : chloroform (3:7) yielded colorless crystals of **3**, yield 0.65 %, R_f 0.72 (petroleum ether : chloroform : methanol, 3.5 : 6.4 : 0.1), m.p. 69 - 70^0 C. FAB MS *m/z* (rel. int.) 424 $[M]^+$ ($C_{29}H_{60}O$) (29.8).

***n*-Tetracontanol (4)**

Elution of the column with petroleum ether : chloroform (1:3) furnished colorless crystals of **4**, recrystallised from $CHCl_3$:CH_3OH (1:1), yield 0.50% w/w, R_f 0.76 (petroleum ether : chloroform : methanol,3 : 6.9 : 0.1), m.p. 81^0 - 82^0 C. IR ν_{max} (KBr): 3411, 803, 724 cm^{-1}. FAB MS *m/z* (rel. int.) 578 $[M]^+$ ($C_{40}H_{82}O$) (57.1). ^1H NMR ($CDCl_3$): δ 3.58 (1H, d, J=6.3 Hz, H_2-1_a), 3.54 (1H, d, J=6.30 Hz, H_2-1_b), 1.51 (2H, m, CH_2), 1.47 (2H, m, CH2), 1.18 (50H, brs, 25×CH_2), 0.87 (3H, t, J=6.5 Hz, Me-29). ^{13}C NMR ($CDCl_3$): δ 63.0 (C-1), 38.7 - 21.8 (38 X CH_2), 14.1 (Me-29).

***Tinosporasteryl palmitate* (5)**

Elution of the column with petroleum ether : chloroform (1:4) produced colorless crystals of **5**, recrystallized from $CHCl_3$:CH_3OH (1:3), yield 0.45% w/w, R_f 0.78 (petroleum ether : chloroform : methanol :: 2.8 : 7.1 : 0.1), m.p. 110-111° C. IR ν_{max} (KBr): 3415, 2922, 2851, 1740,1631, 1465, 1378, 1263, 1167, 1055, 802, 724 cm^{-1}. ^1H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) (Table 1); FAB MS *m/z* (rel. int.) 669 $[M+H]^+$ ($C_{45}H_{81}O_3$) (1.3), 429 (11.2), 413 (67.8), 399 (68.0), 384 (61.3), 382 (59.6), 381 (19.3), 351 (12.2), 338 (19.5), 272 (39.8), 257 (33.1), 256 (31.7), 255 (85.1), 242 (32.7), 241 (33.8), 239 (23.6), 227 (31.9), 213 (75.8), 211 (28.2), 201 (71.8), 199 (76.3), 198 (72.5), 185 (68.5), 183 (68.9), 169 (65.2), 157 (76.1), 155 (62.1), 143 (73.8), 141(62.7), 129 (83.5), 127 (81.2), 115 (71.6); HR-MS: 670.1391 $[M+H]^+$ (calcd for $C_{45}H_{81}O_{3,}$ 670.1398).

Alkaline hydrolysis of **5:** Compound **5** (50 mg) was dissolved in ethanol (5 ml), 1N NaOH solution (2 ml) added and reaction mixture was heated for 1 hour on a steam bath. It was dried under reduced pressure and dissolved in chloroform to separate 21-hydroxy-β- sitosterol (co-TLC comparable). The residue was dissolved in water, acidified with dil. HCl to pH 3 and extracted with chloroform to isolate palmitic acid, m.p. 63-64^0 C (co-TLC comparable).

***n*-Tricosanyl palmitate (6)**

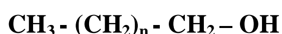
Elution of the column with petroleum ether: chloroform (1:9) yielded colorless crystals of **6**,

yield 0.60 %, R_f 0.65 (petroleum ether : chloroform : methanol, 2.5 : 7.3 : 0.2), m.p. 90^0 - 91^0 C. IR ν_{max} (KBr): 1731, 1633, 803, 722 cm^{-1}. FAB MS *m/z* (rel. int.) 578$[M]^+$ ($C_{39}H_{78}O_2$) (41.3).

***n-Hexacosanyl stearate* (7)**
Elution of the column with chloroform afforded colorless crystals of **7**, recrystallised from methanol, yield 0.50 %, $R_{f\,0.79}$ (petroleum ether: chloroform: methanol, 1: 8.8: 0.2), m.p. 72 - 73^0 C. IR ν_{max} (KBr): 1734, 804, 723 cm^{-1}. FAB MS *m/z* (rel. int.) 648$[M]^+$ ($C_{44}H_{88}O_2$) (33.8).

RESULTS

Compound **1**, **2**, **3**, **6** and **7** are the known phytoconstituents characterized as *n*-heptacosanol [13], *n*-octacosanol [14], *n*-nonacosanol [15], *n*-triacontanyl palmitate [16] *n*-hexacosanyl stearate [17], respectively.



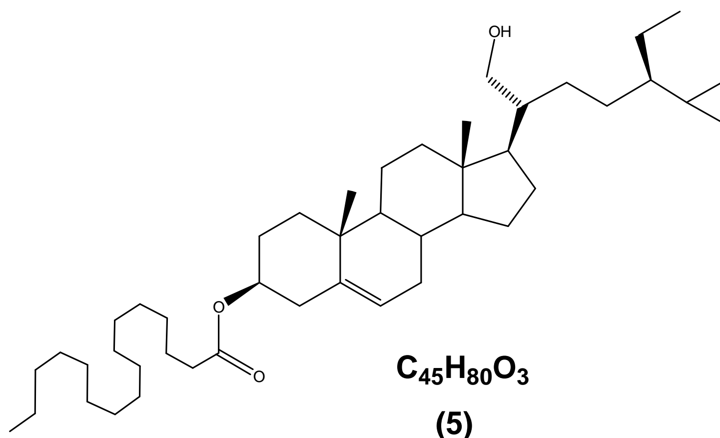
1. n = 25
(6)
2. n = 26

3. n = 27
(7)

Compound 4, an aliphatic alcohol, was obtained as colourless crystalline mass from petroleum ether–chloroform (1:3) eluents. It did not decolorize bromine water exhibited characteristic IR absorption bands for hydroxyl group (3417 cm^{-1}) and long aliphatic chain (803, 724 cm^{-1}). Its mass spectrum exhibited a molecular ion peak at *m/z* 578 consistent with the molecular formula of an aliphatic alcohol, $C_{40}H_{82}O$. The ^1H NMR spectrum displayed a two-proton broad signal at δ 3.63 assigned to hydroxymethylene H_2 -1 protons. A three-proton triplet at δ 0.85 (J = 6.5 Hz) was accounted to terminal C-40 primary methyl protons. The remaining methylene protons resonated between δ 1.58-1.01. The ^{13}C NMR spectrum showed signals for hydroxyl methylene carbon at δ 63.1 (C-1), methylene carbons between δ 32.8-22.6 and methyl carbon at δ 14.1 (C-40). The absence of any signal beyond δ 3.63 in the ^1H NMR spectrum and δ 63.1 in the ^{13}C NMR spectrum supported the saturated nature of the molecule. Based on these evidences the structure of compound **4** has been formulated as *n*-tetracontanol.

Compound 5, named Tinosporasteryl palmitate, was obtained as colourless crystalline mass from petroleum ether–chloroform (1:4) eluents. It responded positively to Liebermann-Burchardt test for steroids. Its IR spectrum exhibited characteristic absorption bands for hydroxyl group (3415 cm^{-1}), ester group (1740 cm^{-1}), unsaturation (1631 cm^{-1}) and long aliphatic chain (802, 724 cm^{-1}). On the basis of mass and ^{13}C NMR spectra the molecular ion peak of **5** was determined at *m/z* 669 $[M+H]^+$ consistent with a molecular formula of a steroidal ester, $C_{45}H_{81}O_3$. It indicated six double bond equivalents; four of them were adjusted in the steroidal skeleton and one each in the vinylic linkage and ester group. The prominent ion fragment generating at *m/z* 239 $[CO(CH_2)_{14}CH_3]^+$, 256 $[CH_3(CH_2)_{14}COOH]^+$ and 211 $[CH_3(CH_2)_{14}]^+$ indicated that palmitic acid was esterified with the steroidal component. The ion peaks arising at *m/z* 429 $[$ M-239$]^+$, 412 $[M-256]^+$, 397 $[412\text{-}Me]^+$, 382 $[397\text{-}Me]^+$, 351 $[382\text{-}CH_2OH]^+$ and 339 $[382\text{-}C_3H_7]^+$ supported the existence of two hydroxyl groups in the molecule and one of them was placed in the steroidal nucleus at C-3 on the basis of biogenetic consideration. The ion peaks formed at *m/z* 272 $[429\text{-}C_{10}H_{21}O\text{, side chain}]^+$, 257 $[272\text{-}Me]^+$, 242 $[257\text{-}Me]^+$, 201 $[242 \text{ - ring D}]^+$, 381 $[412 \text{ - } CH_2OH]^+$, 338 $[412 \text{ - } C_3H_7]^+$, 255 $[412 \text{ - } C_{10}H_{21}O]^+$, 213 $[255 \text{ - ring D fusion}]^+$ and 198 $[213 \text{ - Me}]^+$ suggested the saturated nature of the side chain containing one primary alcoholic function. The ^1H NMR spectrum of **5** displayed a one-proton deshielded doublet at δ 5.36 (J =4.8 Hz) assigned to vinylic H-6 protons. A one-proton broad multiplet at δ 4.15 with half width of 18.5 Hz was ascribed to α-oriented H-3 methine proton. Its shifting in the downfield region indicated the attachment of the ester group at C-3. Four one-proton doublets at δ 3.66 (J = 6.6 Hz), 3.61 (J = 6.6 Hz) and at *m/z* 2.80 (J = 4.8 Hz) and 2.77 (J = 5.4 Hz) were attributed to the hydroxyl methylene H_2-21 and the methylene H_2-2' protons adjacent to the ester function. Two three-proton broad signals at δ 0.70 and 1.03, two three-protons doublets at δ 0.84 (J=6.3 Hz) and 0.79 (J = 6.3 Hz) and two three-proton triplet at δ 0.82 (J = 6.3 Hz) and 0.88 (J = 4.8 Hz) were associated correspondingly with tertiary C-18 and C-19, secondary C-26 and C-27 and primary C-29 and C-16' methyl protons existed on the saturated carbons. The remaining methine and methylene protons appeared between δ 2.28-1.08. The ^{13}C NMR spectrum showed 29 carbon signals appeared for the steroidal nucleus and important signals appear for ester carbon at δ167.8 (C-1'), vinylic carbons at δ 140.7 (C-5) and 121.6 (C-6), carbinol carbon at δ 71.7 (C-3), hydroxymethylene carbon at δ 63.0 (C-21) and methyl carbons between δ 19.8-11.8. The ^1H NMR and

^{13}C NMR signals for the steroidal nucleus were compared with the reported values of similar steroidal compounds [18-20]. The ^{1}H-^{1}H COSY spectrum of **5** showed correlations of H-3 with H_2-2 and H_2-4; H-6 with H_2-4, H_2-7 and H-8; H_2-21 with H-20, H-17 and H_2-22; and H_2-2' with H_2-3'. The HMBC spectrum of **5** exhibited interactions of C-1' with H_2-2' and H-3; C-5 with H_2-4, H-6 and H_2-7; and C-21 with H-20, H_2-22 and H-17. The HSQC experiment showed key-correlations between the proton at δ 5.36 and the carbon signal at δ 121.69; between the proton at δ 4.15 and carbon signal at δ 71.79; between the proton at δ 3.66 and 3.61 and the carbon signal at δ 63.08. Alkaline hydrolysis of compound **5** yielded 21-hydroxy-*β*-sitosterol and palmitic acid. Based on these evidences the structure of compound **5** has been formulated as stigmast-5-ene-3*β*, 21-diol-3*β*-yl hexadecanoate. This is a new steroidal ester isolated from a natural source for the first time.



$C_{45}H_{80}O_3$

(5)

Table 1. ^{1}H NMR and ^{13}C NMR spectral data of Tinosporasteryl palmitate (5)

Position	^{1}H NMR		^{13}C NMR	Position	^{1}H NMR		^{13}C NMR
	Alpha	beta			Alpha	beta	
1	1.34 m	2.28 ddd (6.9, 7.5, 3.6)	37.2	24	1.68 m	-	45.8
2	1.87 m	1.83 m	32.8	25	1.56 m	-	29.1
3	4.15 brs ($w_{1/2}$ =18.5)	-	71.7	26	0.84 d (6.3)	-	19.8
4	2.03 d (7.0)	2.01 d (5.1)	42.2	27	0.79 d (6.3)	-	19.0
5	-	-	140.7	28	1.15 m	1.61 m	24.2
6	5.36 d (4.8)	-	121.6	29	0.82 t (6.3)	-	11.8
7	2.23 m	2.19 m	31.9	1’	-	-	167.8
8	-	1.65 m	31.6	2’	2.80 d (4.8)	2.77 d (5.4)	36.1
9	1.49 m	-	50.1	3’	1.25 brs	1.25 brs	33.7
10	-	-	36.4	4’	1.25 brs	1.25 brs	29.6
11	2.13 m	1.49 m	21.0	5’	1.25 brs	1.25 brs	29.6
12	1.08 m	1.79 m	39.7	6’	1.25 brs	1.25 brs	29.6
13	-	-	40.5	7’	1.25 brs	1.25 brs	29.8
14	1.15 m	-	56.7	8’	1.25 brs	1.25 brs	29.6
15	1.08 m	1.48 m	24.8	9’	1.25 brs	1.25 brs	29.6
16	1.61 m	1.44 m	28.2	10’	1.25 brs	1.25 brs	29.8
17	1.41 m	-	56.0	11’	1.25 brs	1.25 brs	29.4
18	0.70 brs	-	11.9	12’	1.25 brs	1.25 brs	29.4
19	1.03 brs	-	19.3	13’	1.25 brs	1.25 brs	29.3
20	-	1.97 m	36.4	14’	1.23 brs	1.23 brs	27.1
21	3.66 d (6.6)	3.61 d (6.6)	63.0	15’	1.22 brs	1.22 brs	22.6
22	1.56 m	1.07 m	33.9	16’	0.88 t (4.8)	-	14.1
23	1.27 m	1.79 m	25.7				

Coupling constants in Hertz are provided in parenthesis

Melting points were determined on a Perfit melting point apparatus (Ambala, India) and are uncorrected. IR spectra were recorded on KBr discs, using a Bio-Rad FT-IR 5000 spectrometer (FTS 135, Hongkong, China). UV spectra were measured with a Lambda Bio 20 spectrophotometer (Perkin Elmer, Schwerzenbach, Switzerland) in methanol. ^{1}H and ^{13}C NMR spectra were scanned using Bruker Advance DRY 400 spectrospin and Bruker Advance DRY 100 spectrospin instruments (Germany), respectively, in DMSO-d_6 and TMS as an internal standard. FAB MS spectra were obtained using JEOL-JMS-DX 303 spectrometer (Bruker Daltonics, MA, USA). Column chromatography was

performed on silica gel 60-120 mesh. TLC was run on silica gel G (Qualigens, Mumbai, India). Spots were visualized by exposure to iodine vapours, UV radiation and by spraying reagents.

CONCLUSION

Compound **1**, **2**, **3, 6** and **7** are the known phytoconstituents characterized as *n*-heptacosanol [13], *n*-octacosanol [14], *n*-nonacosanol [15], *n*-triacontanyl palmitate [16] *n*-hexacosanyl stearate [17], respectively.

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