Steroids 76 (2011) 358–364

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

A unique immuno-stimulant steroidal sapogenin acid from the roots of *Asparagus racemosus*

Punita Sharma[a], Prashant S. Chauhan[b], Prabhu Dutt[a], Musarat Amina[a], Krishan A. Suri[a], Bishan D. Gupta[a], Om P. Suri[a], Kanaya L. Dhar[a], Deepak Sharma[c], Vivek Gupta[c], Naresh K. Satti[a,*]

[a] *Natural Product Chemistry (Plants) Division, Indian Institute of Integrative Medicine, Canal Road, Jammu 180001 (J&K), CSIR, India*
[b] *Cell Biology Lab., Pharmacology Division, Indian Institute of Integrative Medicine, Canal Road, Jammu 180001 (J&K), CSIR, India*
[c] *Post-Graduate Department of Physics, University of Jammu, Jammu 180006 (J&K), India*

ARTICLE INFO

Article history:
Received 9 August 2010
Received in revised form 9 December 2010
Accepted 9 December 2010
Available online 21 December 2010

Keywords:
Asparagus racemosus
Steroidal sapogenin
X-ray crystallography
Restraint stress
Immunostimulation
Cyclosporin-A

ABSTRACT

A new steroidal sapogenin molecule **1** having unique characteristics, 21-nor and unusual C19 carboxylic acid has been isolated from the roots of *Asparagus racemosus*. On the basis of chemical evidence, extensive spectroscopic analysis including two dimensional (2D) NMR and X-ray studies of single crystal, the structure of **1** was determined as (1*S*,2*R*,3*S*,8*S*,9*S*,10*S*,13*S*,14*S*,16*S*,17*R*,22*R*,25*R*)-21-nor-18β,27α-dimethyl-1β,2β,3β-trihydroxy-25-spirost-4-en-19β-oic acid. **1** crystallizes in monoclinic space group $P2_1$ with $a = 9.295(2)$, $b = 11.238(2)$, $c = 11.376(2)$ Å; $\beta = 91.993(4)^\circ$, $Z = 2$, $D_{cal} = 1.344$ Mg/m^3. The structure was solved by direct methods and refined by full-matrix least-squares procedure to a final *R*-value of 0.0561 for 4064 observed reflections. **1** was tested against the type of immune responses generated during treatment in normal and immune-suppressed animals and detailed biological activity evaluation suggests it to be a potent immunostimulator.

1. Introduction

Steroidal sapogenins and steroidal saponins are major secondary metabolites present in *Asparagus racemosus* Willd. (Asparagaceae), an important medicinal plant of tropical and subtropical India [1]. Its medicinal uses have been reported in the Indian and British Pharmacopoeias and also in Indian traditional systems of medicine such as Ayurveda, Unani and Siddha. Several therapeutic uses have been reported in the literature for this plant. To mention a few, it is used as galactagogue, aphrodisiac, anodyne, diuretic, antispasmodic and nervine tonic [2]. The plant finds use in about 64 Ayurvedic formulations which include traditional formulations such as 'Shatavari kalpa', 'Phalaghrita'and 'Vishnu taila' [3]. Earlier our group has reported the immunomodulatory activity of root extracts and the isolation of two steroidal saponins *viz*., Shatavarin-IV and immunoside from *A. racemosus* [4,5]. As part of our systematic study on steroidal constituents with biological activities from the rhizomes of *A. racemosus*, we report in the present communication the isolation, characterization and bioactivity of unique polyhydroxylated steroidal sapogenin acid (**1**) (Fig. 1). The paper provides detailed spectral evidence as well as X-ray analysis which is consistent with the structural assignment of **1** as (1*S*,2*R*,3*S*,8*S*,9*S*,10*S*,13*S*,14*S*,16*S*,17*R*,22*R*,25*R*)-21-nor-18β,27α-dimethyl-1β,2β,3β-trihydroxy-25-spirost-4-en-19β-oic acid.

Immunodeficiency disorders are a group of disorders in which the body's defence system is compromised, causing it to be less effective against foreign invaders. Therefore, the body's ability to fight infections is impaired. As a result, the person with an immunodeficiency disorder will have frequent infections that are generally more severe and last longer than usual. Therefore, today there is a great need of an immune system booster which serves as prophylactic or promotive agent in healthy conditions and as a therapeutic agent in immune compromised conditions. In our efforts to discover and develop effective immune system booster, we studied the effect of **1** on immune system of normal and cyclosporine-A induced immune-suppressed animals and found that **1** is a potent immune system stimulator and can be developed as a novel immunostimulator for the treatment of immune compromised individuals.

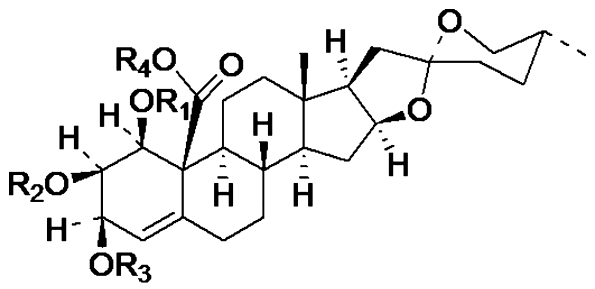
2. Experimental

2.1. General methods

Melting points were determined on a Buchi melting point apparatus Model B-545 and are uncorrected. Optical rotations were measured using a Perkin-Elmer model 241 polarimeter. IR spectra was recorded on a Hitachi 270-30 spectrophotometer in KBr.

* Corresponding author. Tel.: +91 9419207181; fax: +91 1912569019.
E-mail address: nksatti@rediffmail.com (N.K. Satti).

0039-128X/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2010.12.006



1 = R_1,R_2,R_3,R_4=H
1 a = R_1,R_2,R_3,=$COCH_3$; R_4=H
1b = R_1,R_2,R_3,=$COCH_3$; R_4=CH_3

Fig. 1. Structure of **1**, **1a** and **1b**.

^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H–^{1}H COSY, HSQC, and HMBC spectra were recorded at 400 MHz, 500 MHz for ^{1}H and at 125 MHz for ^{13}C with a Bruker Avance 400 MHz/500 MHz spectrometer in pyridine-d_5/$CDCl_3$solution, using TMS as internal standard. FAB-MS was recorded on a JEOL SX 102/DA-6000 mass spectrometer. Elemental analytical data was recorded on Elementar, Vario EL III elemental analyzer. Column chromatography was carried out using silica gel (100–200 mesh, Merck). Spots on TLC were visualized by spraying with 2% cerric ammonium sulphate in 30% aqueous H_2SO_4, followed by heating the plate at 105 °C for 15 min. All the organic solvents used are of LR grade (Merck).

2.2. Plant material

The roots of *A. racemosus* were obtained from Chatha farm of Indian Institute of Integrative Medicine (IIIM), located at Jammu, India. A voucher specimen (RJM/0001) was identified by Dr. S.N. Sharma, Botany Division, IIIM Jammu and deposited in the herbarium of IIIM, Jammu.

2.3. Extraction and Isolation of compounds

Air-dried plant material (4 kg) was ground to a coarse powder and extracted with ethyl acetate in a Soxhlet apparatus for 48 h. The extract was freed of solvent on a wiped film evaporator at 50 ± 5 °C to get EtOAc extract residue (12 g). The EtOAc extract 10 g was subjected to column chromatography. It was dissolved in minimum quantity of MeOH and adsorbed on silica gel (100–200 mesh, 25 g). The solvent was completely removed to get free flowing material. A glass column of 1 in. diameter was packed with 100 g silica gel (100–200 mesh) in $CHCl_3$. The adsorbed extract was charged into the column. The column was eluted successively with $CHCl_3$ (3 L), MeOH/$CHCl_3$ (1:99, 3 L), MeOH/$CHCl_3$ (1:19, 5 L), MeOH/$CHCl_3$ (1:9, 3 L) and then with MeOH. The pooled MeOH/$CHCl_3$ (1:19) fraction (1.6 g) was subjected to rechromatography using 100–200 mesh silica gel column (1:50 ratio) and eluted with $CHCl_3$:MeOH mixtures of increasing polarity. In all, 110 fractions of 50 mL each were collected. Fractions 48–64 eluted in $CHCl_3$/MeOH (9:1) on concentration under reduced pressure and repeated crystallizations from EtOAc yielded compound **1** (164 mg).

1 was crystallized as colorless crystals, m.p. 199 °C, $[\alpha]_D^{27}$ −90° (ca. 0.50 pyridine); FABMS *m/z*: 485.47 $[M+Na]^+$ ion and 463.27 $[M+H]^+$, (calcd. for $C_{26}H_{38}O_7$), in combination with its elemental analysis. IR ν_{max} (KBr pallet) cm^{-1}: 3549–3410 (OH); 1727 (acid carbonyl) and characteristic absorption bands for 25(*R*)-spiroketal at 925 and 883 cm^{-1}. ^{1}H and ^{13}C NMR (Table 1).

2.4. Acetylation of *1*

Compound **1** (50 mg) was dissolved in pyridine (2 mL) and acetic anhydride (3 mL) was added to the solution. The reaction mixture was heated on a steam bath for 2 h under dry conditions. Usual workup followed by crystallization yielded triacetate **1a**, m.p. 158–159 °C. The $[M]^+$ in MS at *m/z* 588 and elemental analysis established its molecular composition to be $C_{32}H_{44}O_{10}$. In ^{1}H NMR ($CDCl_3$) signals at δ 5.00, 4.56, 5.06 due to protons at C1, C2 and C3 got shifted at δ 5.38, 5.02, 5.61 respectively. Signals due to the three methyl groups of the acetyl groups at C1, C2 and C3 were recorded at δ 2.03, 2.06 and 2.07 (values interchangeable). ^{1}H and ^{13}C NMR (Table 1).

2.5. Esterification of *1a*

Compound **1a** (20 mg) was dissolved in ethereal solution of diazomethane and allowed to stand at low temperature. The progress of the reaction was monitored on TLC. After the completion of the reaction, the solvent was removed and the residue on crystallization from MeOH gave **1b**, m.p. 117–118 °C. The $[M]^+$ in MS at *m/z* 602 and elemental analysis established its molecular formula as $C_{33}H_{46}O_{10}$. ^{1}H NMR (Table 1).

2.6. X-rays crystal studies of *1*

2.6.1. Crystal data

$C_{26}H_{38}O_7 + H_2O$, monoclinic space group $P2_1$ with a = 9.295(2), b = 11.238(2), c = 11.376(2) Å; β = 91.993(4)°, Z = 2, D_{cal} = 1.344 Mg/m^3. The structure was solved by direct methods and refined by full-matrix least-squares procedures to a final *R*-value of 0.0561 for 4064 observed reflections. The crystal structure data have been deposited at the Cambridge data Centre (CCDC-781670). The crystallographic data are summarized in Table 2.

2.6.2. Structure analysis

The crystal structure was solved by direct methods and refined on F^2 by full-matrix least-squares procedures using *SHELX97* software [6]. The positions of the hydroxy and the water H atoms were determined from a difference Fourier map and refined freely along with their isotropic displacement parameters. All remaining H atoms were geometrically fixed and allowed to ride on the corresponding non-H atoms with C–H = 0.96–0.98 Å and $U_{iso}(H) = 1.5U_{eq}(C)$ of the attached C atom for methyl H atoms and $1.2U_{eq}$ for other H atoms. The final refinement cycles converged $R = 0.0561$ and $wR(F^2) = 0.1314$ for the observed data. Residual electron densities ranged from −0.256 to 0.302 $eÅ^{-3}$. Atomic scattering factors were taken from International Tables for X-ray Crystallography [7]. An ORTEP-3 drawing of the molecule [8] is shown in Fig. 2. The bond distances and bond angles of the title steroid are in good agreement with the corresponding values obtained in the case of related molecules [9–11]. The mean bond lengths are: $C(sp^3)$–$C(sp^3)$ = 1.532(4); $C(sp^3)$–$C(sp^2)$ = 1.515(4); $C(sp^3)$–O = 1.434(3) Å. The C1–C2 and C2–C3 bond distances are significantly shorter than the expected value of 1.533 Å for a C–C bond length. This feature has also been observed in steroids with hydroxyl moieties, similarly positioned. The C22–O1 bond with a length of 1.420(3) Å is shorter than the C16–O1 bond with a length of 1.451(3) Å, probably due to the presence of two electronegative atoms O1 and O2, with non-bonded electrons adjacent to C22. Similar bond-length variations have been observed in related structures [9–11]. The bond angles (C12–C13–C17, C14–C13–C17, C8–C14–C15, C8–C14–C13, C13–C14–C15) show significant deviations from the ideal tetrahedral value of 109.4°. Deviations of this