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ISOLATION AND CHARACTERIZATION OF A FUROSTANOL GLYCOSIDE FROM FENUGREEK

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Abstract—From the seed of fenugreek, a new glycoside has been isolated and shown to have the structure, (25*S*)-22-*O*-methyl-5α-furostan-3β,22,26-triol 3-*O*-α-rhamnopyranosyl(1→2)[-β-D-glucopyranosyl (1→3)]-β-D-glucopyranoside-26-*O*-β-D-glucopyranoside.

INTRODUCTION

The furostanol glycosides are known as precursors of spirostanol glycosides (steroidal saponins). Fenugreek seed is a potential source of raw material for the steroid industry. A furostanol glycoside, trigonelloside C, has been isolated from the seed [1]. This paper describes the isolation and the elucidation of the structure of another furostanol glycoside (**1**) also from the seed.

RESULTS AND DISCUSSION

The new glycoside was isolated by repeated column chromatography. It showed only one spot on TLC and with *p*-dimethylaminobenzaldehyde–HCl (Ehrlich's reagent) it gave a red colour [2]. Its IR showed no absorption of a spirostanol side chain [3]. Hydrolysis with β-glucosidase gave D-glucose and spirostanol glycoside identified by TLC and IR. These results indicate a furostanol glycoside. The ^{1}H NMR (C_5D_5N) exhibited a methoxy signal at δ 3.27 which disappeared on refluxing with H_2O, indicating a 22-OME becoming a 22-OH group. Analogous interconversion has been reported between 22-OMe and 22-OH furostanol glycosides [4, 5].

Acid hydrolysis afforded a sapogenin, D-glucose and L-rhamnose. The sapogenin, mp 200°, MS *m/e* 416 (M^+), IR ν_{max}^{KBr} 920 > 900 cm^{-1} (25*S*-configuration [3]), was shown to be (25*S*)-5α-spirostan-3β-ol (neotigogenin).

The permethylated glycoside was prepared by the Hakomori method [6]. Its MS (Fig. 1) gave as the highest identifiable peak *m/e* 1230 (**2**) and this may be assigned to the ion given by loss of MeOH from M^+. The projected M^+ suggests that it contains 3 mol of methylated glucose and 1 mol of methylated rhamnose. The base peak (*m/e* 189) and the ion at *m/e* 219 are due to the loss of terminal sugar residues, methylrhamnosyl and methylglucosyl ions, respectively. These losses are reflected by corresponding ions at *m/e* 1042 and 1012 and an ion arising from the loss of both residues at *m/e* 822. The permethylated glycoside was subjected to methanolysis. The products, three methylated sugars, were investigated by GLC and identified as methyl pyranoside of 2,3-4,6-tetra-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-L-rhamnose and 4,6-di-*O*-methyl-D-glucose; the latter gave a positive (red) result with TTC reagent on PC [7].

Based upon the above data, the new glycoside is believed to have a glucopyranose at the C-26 position and a trisaccharide of L-rhamnosyl (1→2)[-D-glucopyranosyl (1→3)]-D-glucopyranoside at the C-3 position of 22-methoxy-5α-furostan-3β, 26-diol.

The configuration at C-1 of the glycosidic residues was determined by ^{1}H NMR and the molecular rotation differences of the glycoside and its Ehrlich-positive hydrolysis products, EP-2 and EP-3 (Klyne's rule). In the ^{1}H NMR spectrum two proton doublets, at δ 4.82 (J = 7.3 Hz) and 5.07 (J = 7.0 Hz), suggested the β-configuration of the glucose moieties. According to the literature [8] these signals arise from two glucose units at the C-3 position of the aglycone. One broad singlet at δ 6.20 is assigned to the α-form of the rhamnosyl anomeric proton. The rotation contribution of the sugar components of EP-2, EP-3 and the original glycoside were obtained, and ^{1}H NMR data confirmed the results.

The structure of the new glycoside was further established by subjecting it to Marker's oxidative degradation using Tschesche's method [9]. The decomposition products were identified as 5α-pregn-16-en-3β-ol-20-one acetate, mp 163°, IR $\nu_{max}^{CCl_4}$ 1724, 1662 cm^{-1} (characteristic absorption of Δ^{16}-20-keto), MS *m/e* 358 (M^+) and δ-hydroxy-γ-methylvaleric acid-methyl ester glucoside tetraacetate, MS *m/e* 331 (tetra-*O*-acetylglucopyranosyl ion). The former product is corresponding to neotigogenin and the latter is corresponding to a glucose unit joined at C-26. The structure of the new glycoside is thus **1** (25*S*)-22-*O*-methyl-5α-furostan-3β, 22,26-triol-3-*O*-α-L-rhamnopyranosyl(1→2)[-β-D-glucopyranosyl(1→3)]-β-D-glucopyranoside-26-*O*-β-D-glucopyranoside.

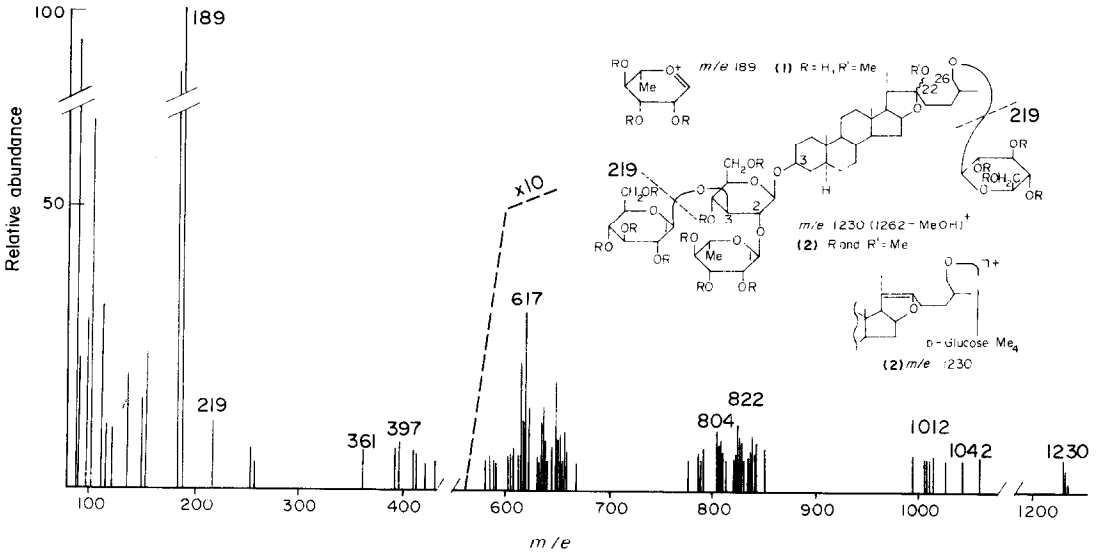

Fig. 1. MS (EI at 70 eV) of the permethylate (**2**, R and R′ = Me, MW 1262) of furostanol glycoside, (**1**, R = H, R′ = Me).

EXPERIMENTAL

Mps are uncorr. TMS was used as an internal standard in C_5D_5N for ^1H NMR (100 MHz). High resolution MS were obtained at 70 eV. Chromatography was on Si gel (Woelm), ion exchange resin (Dowex 50W-X8, H^+), acidic alumina (Merck) and Whatman No. 1 paper. The following solvents were employed: solvent A, $CHCl_3$–MeOH–H_2O (13:6:1); solvent B, *n*-BuOH–EtOH–H_2O (5:1:1); solvent C, *n*-hexane–EtOAc (9:1 and 3:1); solvent D, *n*-BuOH–HOAc–H_2O (4:1:2); solvent E, *n*-BuOH–EtOH–H_2O (4:1:1); solvent F, *n*-hexane–EtOAc (1:1). On TLC (Si gel) the glycosides were detected by Ehrlich's reagent [10] and by 10% H_2SO_4; sugars and methylated derivatives were located on PC (ascending) by ammoniacal $AgNO_3$ soln and TTC reagent [7]. GC of methylated sugars: dual FID; column 1, 1.5 m×2mm, 10% butanediol succinated polyester on Chromosorb W, N_2 (55 ml/min); column 2, 2 m×2 mm, 2.5% OV-17 on Chromosorb W, N_2 (40 ml/min), programmed from 140 to 260° at 20°/2 min [11].

Isolation. Powdered commercial Moroccan fenugreek seed (2 kg) was defatted with petrol in a Soxhlet, and solvent-free powder was likewise extracted with MeOH until the extractive became colourless. Removal of solvent from the extract gave a brown crude glycoside. Its soln in H_2O was washed with *n*-BuOH satd with H_2O. The aq. phase was evapd to obtain the crude **1** and this was purified by CC first on Si gel (system A) and then ion exchange resin eluted with MeOH. The pure **1** (2g) was obtained via an acidic alumina column (system B) and was recrystallized as an amorphous solid from MeOH–Me_2CO: mp 242–246°, IR γ_{max}^{KBr} cm^{-1}: 3600–3250; ^1H NMR (C_2D_5N): δ 3.27 (3H, *s*, C-22-OMe), 4.82 (1H, *d*, *J* = 7.3 Hz), 5.07 (1H, *d*, *J* = 7.0 Hz), 6.20 (1H, *s*). (Found: C, 58.29; H, 8.05. $C_{52}H_{88}O_{23}$ requires: C, 57.76; H, 8.20%).

22-Hydroxy and 22-methoxy derivatives. **1** (20 mg) was boiled with 10 ml toluene–H_2O (1:2) for 3 hr. On evapn an amorphous solid was obtained and was recrystallized from H_2O. Analysed by ^1H NMR it gave no methoxy signal. 22-Hydroxy **1**: $[\alpha]_D^{18}$ −81.8° (MeOH–EtOAc, *C* 1.1). Refluxing 22-hydroxy **1** (15 mg) with MeOH (10 ml) for 3 hr gave regenerated **1** (22-methoxy ^1H NMR signal).

Identification of sapogenin and sugars. **1** (40 mg) was hydrolysed by refluxing with 2 N HCl (1 ml) for 2 hr. The hydrolysate was filtered and the filtrate was neutralized with Ag_2CO_3 and subjected to PC (system D); the presence of D-glucose and L-rhamnose was revealed. The ppt. obtained above was purified by chromatography on Si gel (system C). Neotigogenin, needles, from MeOH; mp 199–201°, MS *m/e*: 416 (M^+) 139 (base peak). IR γ_{max}^{KBr} cm^{-1}: 3400–3500, 985, 952, 920, 900; with a larger intensity at 920 than at 900 indicating 25*S*-configuration.

Enzymatic hydrolysis. **1** (30 mg) was dissolved in 2 ml H_2O and 5 mg emulsin (BDH) was added. The mixture was incubated at 40° for 48 hr. A ppt. was collected by filtration and the filtrate was subjected to PC (system C). The presence of D-glucose was revealed. The ppt. obtained above was recrystallized from aq. MeOH, mp 259–263° IR γ_{max}^{KBr} cm^{-1}: 3500–3300, 924, 902 (intensity 924 > 890; 25*S*-configuration).

Methylation and identification of methylated sugars. **1** was methylated by Hakomori's method. The permethylate was purified by PLC (system F) and obtained as a syrup. IR $\gamma_{max}^{CCl_4}$ cm^{-1}: no OH absorption. MS, Fig. 1, *m/e*: 1230 (M^+ − MeOH), 189 (base peak). The permethylate (30 mg) was methanolysed with dry 2 N HCl–MeOH (3 ml) for 2 hr and the methylated sugars were analysed by GLC (column 1). Methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside (*RR*$_t$ 0.73), methyl 2,3,4,-tri-*O*-methyl-L-rhamnopyranoside (*RR*$_t$ 1.75) and methyl 4,6-di-*O*-methyl-D-glucopyranoside (*RR*$_t$) 11.36) were detected. Methylated sugars were hydrolysed by refluxing with 4% HCl for 4 hr. The reaction mixture was neutralized by Ag_2CO_3 and 4,6-di-*O*-methyl-D-glucopyranose was detected after PC as a red spot with triphenyltetrazolium chloride (TTC) reagent [7].

Partial hydrolysis. **1** (1 g) was partially hydrolysed by refluxing with N HCl for 25 min. When cool the ppt. was collected by filtration. The ppt. was subjected to chromatography on Si gel and this was eluted with $CHCl_3$–MeOH–H_2O

(100:2:0.2) by increasing MeOH–H_2O in a 3–4% step. Three Ehrlich-positive compounds (EP-1, EP-2 and EP-3) were obtained. EP-1: crystals (trace), mp 170–176°. EP-2: amorphous solid (45 mg), mp 236–238°, $[\alpha]_D^{19}$ – 79° (MeOH–EtOAc; *c* 0.8). (Found: C, 61.30; H, 8.45. $C_{39}H_{66}O_{14}$ requires C, 61.72; H, 8.77%). EP-3: amorphous solid (60 mg), mp 248–253°, $[\alpha]_D^{19}$ + 87° (MeOH–EtOAc, *c* 1.1). (Found: C, 59.80; H, 8.34%. $C_{45}H_{76}O_{18}$ requires C, 59.72; H, 8.46%). EP-2 (10.38 mg) was hydrolysed by heating with 2N H_2SO_4 for 6 hr at 90–100°. After cooling, the reaction mixture was concd and the ppt. was removed by filtration. Filtrate was neutralized with $BaCO_3$ and evapd to dryness '*in vacuo*'. To the well-dried residue was added 0.5 ml STOX Pierce Reagent (25 mg/ml hydroxylamine HCl and 6 mg/ml phenyl-β-D-glucopyranoside in C_5H_5N) and the mixture kept at 70–75° for 30 min in a sealed tube. The mixture was cooled to room temp. and trimethylsilylimidazole (0.5 ml) added, shaken for 30 sec and set aside for 30 min with occasional shaking. The mixture was concd, extracted with hexane (1.0 ml) and the extract was subjected to GLC (column 2). EP-3 was likewise treated. EP-2 gave D-glucose and the aglycone in the ratio 2:1 and EP-3 gave D-glucose, L-rhamnose and the aglycone in the ratio 2:1:1.

Oxidative decomposition. **1** acetate (1:1 g.) in Ac_2O (14 ml) was refluxed for 1 hr. After cooling, H_2O (5 ml) was added to the reaction mixture which was then evapd to dryness yielding a yellow syrup. To this was added HOAc (12 ml) and NaOAc (250 mg). The mixture was cooled to 15° and CrO_3 (400 mg) in 50% HOAc (4 ml) was added over a period of 15 min with continuous stirring which was continued for 1 hr at room temp. The mixture was then diluted with H_2O (30 ml) and shaken with Et_2O. The Et_2O extract was washed with H_2O before the extract was dried and then evapd. To the residue (1 g), in *t*-BuOH (20 ml), was added KOH (1.4 g) in H_2O (2 ml). The mixture was stirred under N_2 for 4 hr at 30°. Afterwards H_2O (20 ml) was added and the *t*-BuOH was removed. The aq. soln was extracted with *n*-BuOH.

5α-Pregn-16-en-3β-ol-20-one acetate. The *n*-BuOH extract was evapd. The crude 5α-pregn-16-en-3β-ol-20-one glycoside was purified by chromatography on Si gel eluted with $CHCl_3$–MeOH–H_2O (40:10:1). The pure glycoside was hydrolysed by refluxing with 2N HCl–toluene (20 ml) for 2 hr. After cooling, the toluene phase was separated and evapd and the acetate of the product was prepared in the usual manner. It gave, from aq. MeOH, crystals, mp 162–164°; IR $\gamma_{max}^{CCl_4}$ cm^{-1} 1724, 1662 (characteristic of Δ^{16}-20 ketone [11]; MS *m/e*: 358 (M^+).

δ-Hydroxy-γ-methylvaleric acid-methyl ester glucoside tetraacetate. The aq. phase was adjusted to pH 3 with 2 N HCl and washed with *n*-BuOH and $CHCl_3$ in turn. The aq. phase was then neutralized with 2 N NaOH and evapd. The residue (150 mg) was acetylated in a mixture of Ac_2O (2 ml) and C_5H_5N (3 ml) and poured into ice–H_2O to give ppt. Acetate was dissolved in MeOH (2 ml) and treated with CH_2N_2 soln (10 ml) (3% CH_2N_2 in Et_2O) for 10 min. The reaction mixture was evapd: a yellow syrup was obtained and was purified by chromatography on Si gel. This was eluted with hexane–EtOAc (18:1 and 16:1). A pure acetate was obtained as a colourless syrup. MS *m/e*: 331, 243, 242, 200, 169, 129, 115 in accord with the expected fragmentation [12].

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