Phytochemistry, 1974, Vol. 13, pp. 2247 to 2251. Pergamon Press. Printed in England.

FENUGREEKINE, A NEW STEROIDAL SAPOGENIN-PEPTIDE ESTER OF *TRIGONELLA FOENUM-GRAECUM**

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(*Revised received* 19 *February* 1974)

Key Word Index—*Trigonella foenum-graecum*; fenugreek; Leguminosae; C_{27}-steroidal sapogenin-peptide ester; 4′-hydroxyisoleucyl-4-hydroxyisoleucine lactone; C_{14}-dipeptide; (2S,3R,4R)-4-hydroxyisoleucine lactone; biological activities.

Abstract—A new C_{27}-steroidal sapogenin-peptide ester, fenugreekine, has been isolated from seeds of *Trigonella foenum-graecum*. On acid hydrolysis, it afforded diosgenin, yamogenin, (25R)-spirosta-3,5-diene, a mixture of three isomeric (2S,3R,4R-, 2S,3R,4S-, 2S,3S,4R-)-4-hydroxyisoleucine lactones, 4′-hydroxyisoleucyl-4-hydroxyisoleucine lactone, and a C_{14}-dipeptide which was partially characterized. On the basis of this chemical transformation and spectral (UV, IR, PMR, MS) evidence of fenugreekine and its transformation products, the steroidal sapogenin-peptide ester is assigned structure (**1**). The two dipeptides also have not been encountered before in nature or prepared synthetically. The compound shows a number of interesting pharmacological and virological activities.

INTRODUCTION

SOMETIME ago we studied[1] the nitrogenous constituents of seeds of *Trigonella foenum-graecum* but delayed publication because insufficient information was available regarding the structure of the complex molecules. A recent report by Fowden *et al.*[2] on the same problem prompts us to report our work at this time. Fowden and coworkers reported the isolation of (2S,3R,4R)-4-hydroxyisoleucine as the principal free amino acid of fenugreek. We have isolated, in addition to this compound, a complex mixture of steroidal sapogenin-peptide esters from seeds of this plant by using conventional methods of alkaloid isolation.[3,4] Crystallization and PLC of this complex mixture afforded a homogeneous steroidal sapogenin-peptide ester, which we named fenugreekine. The isolation of the component C_{27}-steroidal sapogenins (six in number), from the acidic hydrolysates of the complex steroidal sapogenin-peptide ester, has been reported earlier.[5] In this paper, we set out evidence for the partial formulation of fenugreekine as (**1**). Additional information about the properties of (2S,3R,4R)-4-hydroxyisoleucine lactone[2] has been reported. Preliminary biological screening of fenugreekine seemed to indicate that the therapeutic properties ascribed to

* Part I in the projected series, "Extractives of *Trigonella*".

[1] CHATTERJEE, D. C. (1970) M. Pharm. Thesis, Banaras Hindu University.
[2] FOWDEN, L., PRATT, H. M. and SMITH, A. (1973) *Phytochemistry* **12**, 1707.
[3] GHOSAL, S. and MUKHERJEE, B. (1966) *J. Org. Chem.* **31**, 2284.
[4] GHOSAL, S., BANERJEE, P. K. and BANERJEE, S. K. (1970) *Phytochemistry* **9**, 429.
[5] GHOSAL, S., CHATTERJEE, D. C. and DUTTA, S. K. (1971) *Proc. Indian Sci. Congress* **III**, 196.

seed extracts of *T. foenum-graecum*, in the Indian system of medicine,[6] are essentially due to this and related entities contained in the plant.

RESULTS AND DISCUSSION

EtOH extract of seed meal of *T. foenum-graecum* when processed according to a previously described procedure for $CHCl_3$-soluble bases,[3] gave a basic gum consisting of at least five major constituents (PC, TLC). Crystallization of this mixture from Me_2CO–MeOH afforded (2S,3R,4R)-4-hydroxyisoleucine as the lactone. The aqueous mother liquor, after separation of the $CHCl_3$-soluble nitrogenous constituents, was extracted with BuOH. The BuOH extract, after the usual work up, gave appreciable quantity of a complex mixture of steroidal sapogenin-peptide esters. Crystallization and PLC of this complex mixture afforded two pure basic compounds, R_f 0·66 (major) and 0·78 (minor); the major component is named fenugreekine. A further crop of fenugreekine was obtained from the aqueous mother liquor through the reineckate salts.[4]

Fenugreekine exhibited IR absorption bands characteristic of a C_{27}-steroidal sapogenin-peptide ester. The intensity of the band at ν 905 cm^{-1} is about twice as that at ν 925 cm^{-1} indicating that the steroidal sapogenin moiety belongs to the *iso*-series[7] (25R). The ester band appeared at ν 1765 cm^{-1} presumably due to the CO and *N*-groups being in the β-position.[8] Fenugreekine did not exhibit any M^+ peak in its MS but significant fragment ion peaks appeared at *m/e* 396, 324, 282 and 139 which are characteristic of the C_{27}-steroidal sapogenins of the diosgenin-type.[9] The fragment ion peaks were formed by the expulsion of the peptide moiety.

Hydrolysis of fenugreekine afforded diosgenin, yamogenin (formed by epimerization of diosgenin), (25R)-spirosta-3,5-diene (major component), two dipeptides (A and B) and a mixture of the three isomeric (2S,3R,4R-, 2S,3R,4S-, 2S,3S,4R-) hydroxy amino acids[2,10] as their lactones (**3**). The acetylated products of the neutral fraction from the hydrolysis, yielded only (25R)-spirosta-3,5-diene, and acetates of diosgenin and yamogenin could be detected by co-TLC and MS. The results of acid hydrolysis, particularly the facile formation of the spirostadiene[11] (the ease of the diene formation remained essentially unaltered on refluxing with 2 N HCl for 2 hr), and the typical spirostan IR spectrum of fenugreekine suggested that the attachment of the peptide moiety is only at O(3), and not at O(26), of the steroidal sapogenin moiety. On the basis of these observations and conclusions, fenugreekine is assigned the partial Structure (**1**).

The sequence of amino acids in (**1**) is currently being investigated. The identity of the steroidal sapogenins and the spirostadiene, obtained from fenugreekine by acid hydrolysis, was established by direct comparison with reference materials. The hydroxyamino acid lactones and the dipeptides were characterized as described below.

(2*S*,3*R*,4*R*)-4-*hydroxyisoleucine lactone*

The lactone was directly obtained from the EtOH extract of fenugreek seeds using established methods.[3] The compound furnished an isomeric mixture of the three lactones

[6] CHOPRA, R. N., NAYAR, S. L. and CHOPRA, I. C. (1956) *Glossary of Indian Medicinal Plants*, p. 248. C.S.I.R., New Delhi.

[7] WALL, M. E., EDDY, C. R., MCCLENNAN, M. L. and KLUMPP, M. E. (1952) *Analty Chem.* **24**, 1337.

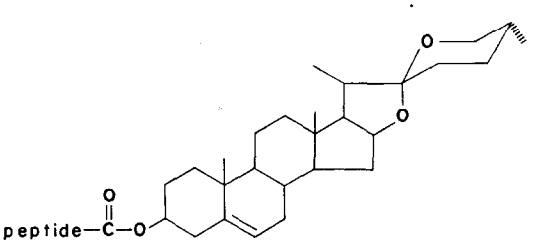
[8] NAKANISHI, K. (1962) *Infrared Absorption Spectroscopy*, p. 198. Holden-Day, San Francisco.

[9] BUDZIKIEWICZ, H., DJERASSI, C. and WILLIAMS, D. H. (1964) *Structure Elucidation of Natural Products by Mass Spectrometry*, Vol. II, p. 113. Holden-Day, San Francisco.

[10] WIELAND, TH., HASAN, M. and PFLEIDERER, P. (1968) *Ann. Chem.* **717**, 205.

[11] HARDMAN, R., WOOD, C. N. and BRAIN, K. R. (1972) *Phytochemistry* **11**, 2027.

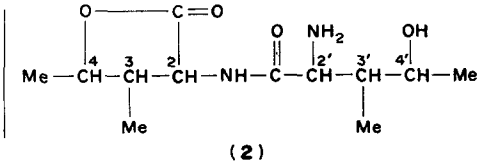
(2S,3R,4R-, 2S,3R,4S- and 2S,3S,4R-) (**3**) when heated with 6 N HCl. In the PMR spectrum of the corresponding Na salt, two small methyl doublets (C_3-, C_4-) at δ 0·72 and 1·5 were observed, caused by the overlapping of the methyl signals belonging to the three isomeric 4-hydroxyisoleucine lactones (**3**). The ratios of intensities of the methyl protons indicated that the three isomeric (2S,3R,4R-, 2S,3R,4S-, 2S,3S,4R-) lactones were present in the ratio of about 25:20:55, respectively.



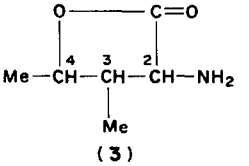
(**1**)

Dipeptide A

The dipeptide, $C_{12}H_{22}N_2O_4$, showed IR absorption bands characteristic of –NH, –OH, –CONH and γ-lactone CO. The PMR spectrum, exhibited seven groups of peaks: doublets at δ 0·95 and 1·22, multiplets at δ 2·22 and 3·75, and broad singlets at δ 3·1, 4·8, and 7·2, with the ratios of intensities of 6:6:2:4:1:2:1. The two doublets are attributed to four methyl groups associated with two –CH–Me and two –O–CH–Me functions; the multiplets are attributed to methine protons, and the broad singlets are presumably due to –OH, –NH and $-NH_2$ protons since on addition of CF_3COOD these signals disappeared. The MS of the dipeptide showed, apart from the M^+ at *m/e* 258, significant fragment ion peaks arising from the loss of Me, H_2O and CO_2 from the M^+. On acid hydrolysis the dipeptide gave, albeit in small yield, a mixture of the three isomeric 4-hydroxyisoleucine lactones (**3**). On the basis of these findings, dipeptide A is assigned 4′-hydroxyisoleucyl-4-hydroxyisoleucine lactone structure (**2**). 4-Hydroxyisoleucine appears to be the building unit of this dipeptide. The dipeptide has not been encountered before in nature or prepared synthetically. The chirality of the dipeptide and its potential as a metabolic inhibitor are currently being investigated.



(**2**)



(**3**)

Dipeptide B

Empirical analysis and MW determination established its molecular formula as $C_{14}H_{22}N_2O_5$. The UV and IR spectral data are similar to those of dipeptide A (**2**). Some striking similarities are also discernible in the mass fragmentation patterns of the two dipeptides (loss of 15, 33, 44, 45, 73, 146 a.m.u. are common in both). The facile loss of 15 a.m.u. (base peak), in dipeptide B together with the significant loss of 33 a.m.u. suggests the presence of a *tert*-Me associated with an hydroxyl group. The loss of 29 a.m.u. from

the M^+, could be from the loss of a –CHO group which is supported by the strong band at ν 1740 cm^{-1} in the IR spectrum and a DNP-positive spot on TLC. An insufficient quantity of the dipeptide was available for PMR. However, on the basis of the limited data and from biogenetic considerations of non-protein amino acids[12] it seems possible that the C_{14}-dipeptide is derived from two hydroxyhomoisoleucines (or equivalents).

Fenugreekine exhibited a number of significant biological activities, the detials of which will be reported elsewhere. It showed about 80% inhibition of vaccinia virus replication when administered at 0·2 mg/ml along with the virus in CAM cultures. This observation is consistent with the reported uses of the seed extracts as a prophylactic for chickenpox and smallpox.[6] The significant pharmacological activities of fenugreekine include marked cardiotonic, hypoglycaemic, diuretic, antiphlogestic and anti-hypertensive actions, which would account for the reported therapeutic uses of the seed extracts of *T. foenum-graecum* in the Indian system of medicine.

EXPERIMENTAL

M.ps are uncorr. UV spectra were recorded in EtOH; IR spectra were determined in mineral oil and only the major bands are quoted. PMR spectra were measured in $CDCl_3$–DMSO-d_6 (using TMS as an internal standard) or in D_2O using a 60 MHz instrument. MS were recorded at 70 eV. Separation by column chromatography was carried out on Brockmann neutral alumina (activity grade *ca* **3**) and TLC on silica gel G. Whatman no. 1 was used for PC. For the nitrogenous constituents, BuOH–AcOH–H_2O (4:1:2) was used and for the steroidal sapogenins and the spirostadiene 2 solvent systems $CHCl_3$–EtOH–AcOH (94:5:1) and CH_2Cl_2–Et_2O (97:3). I_2 vapour or satd. $SbCl_3$ in conc HCl was used for detection. Modified Dragendorff and ninhydrin reagents were used for detecting the nitrogenous constituents.

Extraction of seeds. Trigonella foenum-graecum L. seeds were purchased from Varanasi and were properly identified. The powdered seeds (2·8 kg) were successively extracted, in a Soxhlet, with petrol (60–80°) and 95% EtOH (16 hr each). The EtOH extract was conc. under red. pres. to give a viscous brown mass (148 g). A portion of this extractive (15 g) was triturated with aq. AcOH (4%, 200 ml) and the mixture was kept at 20° for 16 hr. The precipitated solid was collected by filtration. It consisted of a mixture of *n*-alkanes (C_{27}–C_{33}), *n*-alkanols (C_{26}–C_{32}), triterpenes, sitosterols and C_{27}-steroidal sapogenins.[5] The acidic aq. soln was extracted with $CHCl_3$ (3 100 ml portions) to remove any residual non-nitrogenous compound. The clarified acid soln was cooled, made alkaline (NH_4OH) and the liberated bases extracted with $CHCl_3$. The $CHCl_3$ extract was worked up in the usual way to give a brown basic gum (0·8 g) which showed a number of Dragendorff-, ninhydrin- and I_2-positive spots on PC and TLC.

Isolation of (2S,3R,4R)-4-hydroxyisoleucine lactone. The brown basic gum, on repeated crystallizations from Me_2CO–MeOH, afforded (2S,3R,4R)-4-hydroxyisoleucine lactone[2,10] as colourless needles (0·112 g), m.p. 217–218°; $[\alpha]_D^{25}$ +5° (*c* 0·468, AcOH); UV: λ_{max} 202 (0·588), 280 nm (0·062); IR: ν_{max} 3120, 1630, 1580, 1512, 1022, 1010 cm^{-1}; PMR (D_2O): δ 1·04 (3H, *d*, *J* 6·5 Hz, C_3–Me), 1·34 (3H, *d*, *J* 6·5 Hz, C_4–Me), 2·2 (1H, *br m*, C_3–H), 4·0 (2H, *m*, C_2– and C_4–H); MS: *m/e* 129 (M^+, 12%), 114 (M^+–Me, 4), 102 (M^+–C_2H_3, 34), 85 (M^+–C_2H_4O, 26), 74 (M^+–C_4H_7, 68), 70 (M^+–C_2H_4O–Me, 100), 58 (82), 56 (72). (Found: C, 55·49; H, 8·70; N, 11·20. Calc. for $C_6H_{11}NO_3$: C, 55·81; H, 8·52; N, 10·85).

Isolation of fenugreekine (**1**). The aq. mother liquor, after separation of the $CHCl_3$-soluble bases, was extracted with *n*-BuOH (5 100 ml portions). After the usual work up, the BuOH extract afforded a brown basic gum which on trituration with Me_2CO gave a straw coloured solid (0·65 g). It showed two major spots on TLC, R_f 0·66 and 0·78. These were separated by PLC. The R_f-zone 0·65 (major zone) was removed and eluted with MeOH. The solvent was removed from the MeOH soln when colourless micro crystals, m.p. 202–208°, were obtained; $[\alpha]_D^{25}$ +43° (*c* 0·528, AcOH); pK_a ~8; UV: λ_{max} 200 (0·647), 280 (plateau, 0·052) nm; IR: ν_{max} 3400, 3240, 3120, 1780, 1765, 1680, 1615, 1258, 1062, 988, 925, 905, 874 cm^{-1}; MS: significant fragment ion peaks at *m/e* 396 (12%), 326 (7), 324 (10), 295 (22), 282 (11), 279 (25), 186 (14), 183 (100), 139 (28), 102 (12), 98 (8). The aq. mother liquor, after the BuOH extraction, was further processed for residual nitrogenous constituents by treatment with ammonium reineckate according to a previously described procedure.[4] A further crop of fenugreekine (98 mg), two non-protein amino acids, m.p. 178–181° and 221–213°, and a dipeptide, m.p. 272°, were obtained from this fraction.

Hydrolysis of fenugreekine. Fenugreekine (0·48 g) was heated with 6 N HCl at 100° for 4 hr. The steroidal sapogeneins, separated in the hydrolysate as a brown amorphous powder, were extracted with $CHCl_3$. The $CHCl_3$ extract was worked up in the usual way when a colourless solid (0·232 g), consisting of a mixture of 3 components

[12] FOWDEN, L. (1972) *Abh. Dtsch. Akad. wiss. Berlin, Kl. Chem. Geol. Biol.* p. 75.

(TLC), was obtained. The aq. acidic soln was cooled, made alkaline and extracted with $CHCl_3$. The $CHCl_3$ extract on evaporation gave a brown gum (0·128 g) consisting of a mixture of the three isomeric 4-hydroxyisoleucine lactones, dipeptide A and dipeptide B.

Diosgenin, yamogenin and (25R)-spirosta-3,5-diene. The presence of diosgenin, yamogenin and (25R)-spirosta-3,5-diene in the neutral fraction from the acidic hydrolysates was determined by TLC. Subsequently, these were separated by PLC and identified. From a mixture (0·11 g) of the steroidal sapogenins, diosgenin (37 mg, m.p., m.m.p., co-TLC, IR, acetate, m.p., m.m.p.), yamogenin (8 mg, m.p., m.m.p., co-TLC, IR), and (25R)-spirosta-3,5-diene (52 mg, m.p., m.m.p., co-TLC, UV) were obtained. Comparison of the absorbance data in the region 800–1000 cm^{-1}, with standard calibration IR curves, established the purity of diosgenin and yamogenin. Another portion (0·096 g) of the mixture of steroidal sapogenins, from the acidic hydrolysate, was acetylated with Ac_2O and pyridine, at 100° for 2 hr. The product, m.p. 168–172°, was subjected to analytical TLC using reference materials, and MS. The presence of the acetates of diosgenin, yamogenin, and (25R)-spirosta-3,5-diene was established; MS: *m/e* 456 (4%), 396 (100), 324 (62), 282 (68), 139 (28).

Isolation of dipeptides A and B. The brown basic gum (0·128 g) was triturated with Me_2CO when a light brown solid, m.p. 188–191°, separated. It showed three ninhydrin-positive (purple–rose–red) spots on TLC, R_f 0·32, 0·45, 0·57. Repeated crystallization of the solid from EtOH afforded a mixture of the three isomeric 4-hydroxyisoleucine lactones (42 mg), m.p. 212–214°; R_f 0·32; as the sparingly soluble component. The EtOH mother liquor, on column chromatography gave the two dipeptides (A and B) as pure entities. The $CHCl_3$–MeOH (99:1) eluates gave dipeptide B (18 mg) which crystallized from Me_2CO–MeOH as colourless needles, m.p. 210–212°; R_f 0·57; $[\alpha]_D^{25}+59°$ (*c* 0·38, pyridine); UV: λ_{max} 212 (0·48), 280 nm (plateau, 0·03); IR: ν_{max} 3260, 3200, 1780, 1740, 1680, 1530, 1405, 1242, 1198, 1050, 914 cm^{-1}; MS: *m/e* 298 (M^+, 36%), 283 (M^+–Me, 100), 269 (M^+–CHO, 2), 265 (M^+–Me–H_2O, 14), 255 (M^+–Me–CO, 14), 254 (M^+–CO_2, 9), 253 (28), 239 (2), 327 (3), 225 (42), 211 (8), 209 (7), 170 (28), 154 (18), 152 (7), 142 (34), 98 (92), 84 (32), 82 (11), 69 (30), 58 (29). (Found: C, 56·01; H, 7·46; N, 9·77. $C_{14}H_{22}N_2O_5$ requires: C, 56·37; H, 7·38; N, 9·39). The MeOH eluate from the column gave dipeptide A which crystallized from MeOH as colourless needles (37 mg), m.p. 245°; R_f 0·45; $[\alpha]_D^{25}+143·8°$ (*c* 0·754, AcOH); UV: λ_{max} 202 (0·952), 280 nm (0·004); IR: ν_{max} 3385, 3358, 3252, 3218, 1782, 1682, 1670, 1595 cm^{-1}; PMR ($CDCl_3$–DMSO-d_6): δ 0·95 (6H, *d*, *J* 6 Hz, C_3–, C'_3–Me), 1·22 (6H, *d*, *J* 6 Hz, C_4–, C'_4–Me), 2·22 (2H, *m*, C_3–, C'_3–H), 3·75 (4H, *br m*, C_2–, C'_2–, C_4–, C'_4–H), 3·1, 4·8, 7·2 (*br s*, *ca* 1H, 2H, 1H); MS: *m/e* 258 (M^+, 14%), 243 (M^+–Me, 4), 240 (M^+–H_2O, 5), 255 (M^+–Me–H_2O, 3), 214 (M^+–CO_2, 9), 213 (14), 196 (3), 195 (7), 186 (16), 185 (4), 112 (6), 102 (32), 85 (22), 70 (100), 58 (92). (Found: C, 56·27; H, 8·14; N, 10·95. $C_{12}H_{22}N_2O_4$ requires: C, 55·81; H, 8·53; N, 10·53).

Hydrolysis of dipeptide A. A suspension of dipeptide A (16 mg) in 4 N H_2SO_4 (20 ml), was heated in a sealed tube at 120° for 4 hr. After cooling and neutralization, the product was extracted with $CHCl_3$. The solid from the $CHCl_3$ extract crystallized from EtOH as light brown crystals (4 mg), m.p. 210–214°; R_f 0·32; *m/e* 129 (M^+); IR spectrum was indistinguishable from that of a mixture of the three isomeric 4-hydroxyisoleucine lactones, prepared from (2S,3R,4R)-4-hydroxyisoleucine lactone in the usual way.[2,10]

Acknowledgements—The authors are grateful to Professor G. B. Singh, Department of Chemistry, Banaras Hindu University, Dr. F. W. Wehrli, Research Laboratory, Varian AG, Zug, Switzerland, Dr. S. C. Pakrashi, Indian Institute of Experimental Medicine, Calcutta and Dr. B. C. Das, CNRS, Gif-Sur-Yvette, France, for the spectral and optical rotation data. Research fellowships awarded to R.S.S., D.C. and S.K.D., by the Council of Scientific and Industrial Research and the University Grants Commission, New Delhi, during the tenure of this work are thankfully acknowledged.