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RUINE: A GLUCOSIDIC β-CARBOLINE FROM *PEGANUM HARMALA*

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Abstract—A new alkaloid, *ruine*, found in seedlings and undifferentiated callus of *Peganum harmala* has been identified as 8-hydroxyharmine-β-D-glucoside. Feeding experiments have shown that it is biosynthesized from harmine.

INTRODUCTION

SINCE Robinson and Perkin elucidated the structures of the alkaloids harmine and harmaline, *Peganum harmala*, the wild rue, has been the subject of a large number of chemical studies. In addition to the chemistry of the carboline and quinazoline alkaloids, their biosynthesis has been the subject of preliminary experiments.[1,2] In the course of investigating whether harmine is further metabolized in the plant we have discovered a new alkaloid, ruine. Although we found ruine in seedlings of *Peganum harmala*, for convenience it was isolated and characterized from tissue culture from which it can be readily obtained as a transformation product of harmine (I).

RESULTS AND DISCUSSION

Structure of Ruine

The u.v. spectrum of ruine is typical of a β-carboline without a phenolic or *pyr*-N-substituent.[3] The mass spectrum, which is consistent with ruine being a hexoside,[4] shows a very weak parent at *m/e* 390 (0·2%), with the primary fission yielding the base peak fragment at 228 (mass-matched as $C_{13}H_{12}N_2O_2$). The subsequent degradation is analogous to that of harmine, except that comparable fragments occur 16 mass units higher, showing loss of CHO (199, 3%) from the methoxyl group and also the serial loss of CH_3 (213, 62%), $C{\equiv}O$ (from the methoxy group), and CH_2N (157, 3%) (from the pyridine ring). The glucose oxonium ion is not present, but some fragments attributable to its further degradation occur at *m/e* 116 (0·1%), 98 (3), 89 (0·6), 73 (13) and 60 (31).

The degradations of the sugar moiety in the mass spectrum of ruine tetraacetate are more

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[1] D. GROGER and H. SIMON, *Abhand. Deut. Akad. Wiss. Berlin, Kl. Chem. Geol. Biol.* 343 (1963).

[2] D. R. LILJGREN, *Phytochem.* 7, 1299 (1968).

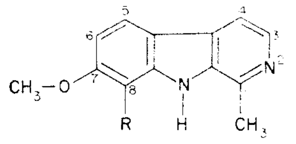
[3] A. I. SCOTT, *Interpretation of the Ultra Violet Spectra of Natural Products*, p. 297, Pergamon Press, Oxford (1960).

[4] I. A. PEARL and S. F. DARLING, *Phytochem.* 7, 836 (1968).

prominent than those of the β-carboline, particularly the A series[5] where the primary fission is of the glycosidic bond,

Fragments from other series which are present are C_3 at 184 (2·9), F_1 at 157 (2·9), F_2 at 115 (3·2), and E_4 at 139 (6·2). Primary fission of the $C_{1'}$—$C_{2'}$ bond leads to the C and F series, while primary fission of the $C_{5'}$—$C_{6'}$ bond leads to the E series. The base peak was the acetyl ion, *m/e* 43. This fragmentation is diagnostic of a hexopyranoside.[5,6]

The NMR spectra of the aglycone portions of harmine, ruine and ruine acetate are compared in Table 1; ruine must be 1-methyl-β-carboline (harman) ortho disubstituted in the benzene ring. Considering the biosynthetic evidence given below on the derivation of ruine from harmine, it is clear that ruine is harmine substituted in the 8-position. The best resolution of the sugar protons was found in the spectrum of ruine tetraacetate. This was compared with the published spectra of acetylated[7] and methylated[8] hexosides in assigning peaks. $H_{6'}^{\text{axial}}$ gives rise to a quartet at δ 4·32, $H_{6'}^{\text{equatorial}}$ a quartet at δ 4·10 and $H_{5'}$ a broad multiplet centered on δ 3·74. $H_{6'}^{\text{equatorial}}$, $H_{6'}^{\text{axial}}$ and $H_{5'}$ form an ABX system where J_{AB} = 23c/s, J_{AX} = 5·2 c/s, J_{BX} = 2·5 c/s. Thus the sugar tetraacetate has restricted rotation about the $C_{5'}$–$C_{6'}$ bond and $H_{5'}$ is axial. A sharp doublet at low field (δ 4·87, J = 7 c/s) in the ruine spectrum corresponds to a doublet at δ 5·01, J = 7·5 c/s in the tetraacetate spectrum which collapses to a singlet on irradiation at δ 5·33—the tentative position of the 2′ proton; these doublets are assigned to the anomeric proton. The coupling constant of the anomeric doublet, in conjunction with the clear separation of the $H_{5'}$ from the $H_{6'}$ protons, indicates that the sugar has the β-configuration.



(I) R = H
(II) R = O-β-D-glu

Ruine could be hydrolyzed by acid; the sugar released was identified as glucose by reaction with the specific enzyme D-glucose oxidase and by chromatography. Although ruine is resistant to β-glucosidase at 30°, other examples of β-glucosides not hydrolyzed by this enzyme are known.[9] Thus ruine has formula II.

In vivo *Conversion of Harmine to Ruine*

Feeding harmine to callus which did not contain alkaloids resulted after 2–3 days in the appearance of ruine. The turnover was essentially complete after 3 weeks. This conversion of harmine to ruine was verified by feeding harmine hydrochloride (methyl-1-^{14}C) to callus.

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[5] N. K. KOCHETKOV and O. S. CHIZHOV, *Advan. Carbohydrate Chem.* **21**, 39 (1966).

[6] K. BIEMANN, D. C. DE JONGH and H. K. SCHNOES, *J. Am. Chem. Soc.* **85**, 1763 (1963).

[7] R. U. LEMIEUX and J. D. STEVENS, *Can. J. Chem.* **43**, 2059 (1965).

[8] A. P. TULLOCH and A. HILL, *Can. J. Chem.* **46**, 2485 (1968).

[9] T. YOKOTA, N. TAKAHASHI, N. MUROFUSHI and S. TAMURA, *Tetrahedron Letters* 2081 (1969).

TABLE 1. NMR SPECTRA OF HARMINE, RUINE AND RUINE TETRAACETATE*

Proton δ	Harmine (DMSO)	Ruine (DMSO)	Ruine Tetraacetate ($CDCl_3$)
3-H d	8·19	7·78 e	8·31
4-H d	7·79	7·62 e	7·69
5-H d	8·04	7·70	7·84
6-H	6·88 q	7·04 d	6·93 d
8-H	7·07 d	—	—
ind-N-H	11·43 br	10·56 s	8·62 s
1-methyl s	2·77	2·75	2·82
7-MeO s	3·88	3·91	3·96
$J_{3,4}$ (c/s)	5·5	5–6	6
$J_{5,6}$	9	9	9
$J_{6,8}$	2	—	—

* Numbering as in (I). s = Singlet, d = doublet, q = quartet, br = broad, e = overlapping other signals.

After 13 days 78 per cent of the radioactivity was taken up by the callus, and, of this 49 per cent was in ruine. This high incorporation indicated the conversion was by direct hydroxylation and glycosylation. Thus the callus appears capable of hydroxylating harmine; this is one of the first demonstrations of a plant system capable of introducing oxygen into a pre-formed indole alkaloid, and may be the sequence of reactions operating in the normal biosynthesis of ruine. This is interesting as the stage at which oxygen is introduced into the benzene ring has been demonstrated in the biosynthesis of only one indole alkaloid.[10]

EXPERIMENTAL

M.ps. were determined on a Kofler hot block and are uncorrected. Steam sterilization of apparatus and media was carried out by autoclaving at 15 p.s.i. and 120° for 15 min. ^{14}C was determined in a liquid scintillation spectrometer. NMR spectra were determined at a frequency of 100 MHz using tetramethylsilane as the internal standard.

Production and Maintenance of Callus

The basic medium used contained the mineral salts of Heller[11] with the following variations: KI 0·1 mg/l., $MnSO_4$ 2·0 mg/l., $Na_2Mo_2O_7$ 0·01 mg/l., $FeSO_4$ 28 mg/l., Na_2EDTA 37 mg/l., glycine 3·0 mg/l., thiamine HCl 0·5 mg/l., calcium pantothenate 2·5 mg/l., nicotinamide 0·5 mg/l., pyridoxine 0·1 mg/l., sucrose 20 g/l. and agar–agar 10 g/l.: omissions, nickel and aluminium chlorides. This was supplemented with one or more of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) (6 mg/l.), coconut milk (15 %) or Difco yeast extract (0·2 %) as a source of growth factors.

Seeds of *P. harmala* were surface sterilized by shaking for 4 min in 15 per cent Calsol, then washing with sterile H_2O. They germinated after 3–4 days in the dark at 30° on sterile moist filter paper. After 7–8 days they were transferred to the basic medium +2,4-D and allowed to dedifferentiate for 3 weeks in the dark at 30° when the root and hypocotyl portion of each callused plantlet was transferred to the basic medium +2,4-D+coconut milk. Callus growth was well established after 6–8 weeks; after this, it was sub-cultured onto fresh medium every 4–6 weeks and grown at 30° in the dark for at least 4 months before being used for transformation work.

Conversion of harmine into ruine. Harmine (300 mg) and the basic medium plus 2,4-D and yeast extract (3 l.) were autoclaved. Callus was transferred onto this and grown for 6 weeks. The callus was then harvested, lyophilized and exhaustively extracted with MeOH. The extract was diluted to 9:1 (MeOH–H_2O) and the bases exchanged on cellulose phosphate, from which they were eluted with acid MeOH (90 %; 0·5 M HCl). This eluate was neutralized with KOH, evaporated to small vol., filtered to remove KCl and fractionated by

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[11] R. HELLER, *Ann. Sci. Natl. Biol. Veg. Ser.* **11**, 1 (1953).

TLC on silica gel with $CHCl_3$–MeOH (2:1). The major blue-green fluorescent band under u.v. (R_f 0·5) was eluted with MeOH to give a brown gum (200 mg) from which colourless crystals of *ruine* (20 mg), m.p. 227–229° were obtained by recrystallization from MeOH; u.v. spectrum (MeOH) λ_{max} 246 (log ϵ 4·68), 299 (4·15), 328 nm (3·68); in acid, λ_{max} 256, 330 nm; *m/e* 390 (M^+, 0·2%), 228 (100) 213 (62), 199 (3), 185 (13), 157 (3), 98 (3), 73 (13), 60 (31). From the mother liquors, 60 mg of an unstable picrate, m.p. 234–235°, was isolated.

A sample (800 g) of the exhausted culture medium was lyophilized, exhaustively extracted with MeOH, and the ruine extracted more efficiently by the following procedure. After making the solution 9:1 (MeOH–H_2O) by the addition of H_2O it was passed through DEAE-Sephadex (10 g) in the OH^- form and the non-exchanged eluates were neutralized with HCl. The MeOH was removed and the aqueous solution remaining lyophilized. The MeOH soluble portion was fractionated as before on preparative TLC to give ruine as a yellow gum (26 mg). Total recovery of ruine from callus and medium—75 mg.

Ruine Tetraacetate

Crude ruine was acetylated (Ac_2O/pyridine) for 2 days at room temp. After removal of the solvent *in vacuo*, the residue was chromatographed on alumina in benzene–$CHCl_3$ (3:1). The *ruine tetraacetate* was recrystallized as colourless prisms (benzene), m.p. 195·5–196·5 (Found: C, 58·3; H, 5·4: N, 4·7. $C_{27}H_{30}N_2O_{11}$ requires C, 58·1; H, 5·4; N, 5·0%). In mass spectrum, M^+ was mass-matched as 558·1848: $C_{27}H_{30}N_2O_{11}$ requires 558·1849.

Hydrolysis of Ruine

Ruine was hydrolyzed with 1 N HCl at 90° for 1 hr. DEAE Sephadex was used to neutralize the reaction and to remove the highly unstable aglycone. The non-exchanged fraction was chromatographed in 2 dimensions on Kieselgel G impregnated with 0·1 N boric acid, doubly developed in each dimension with isopropanol–EtOAc–H_2O (2:6:1) or acetone–H_2O (9:1). A single sugar, co-chromatographing with glucose, was detected with diphenylamine reagent spray.[12] This fraction was also oxidized by D-glucose oxidase, as shown by the peroxidase-mediated oxidation of tyramine HCl to a fluorescent biphenyl.[13]

Conversion of Harmine HCl (methyl-1-^{14}C) into Ruine

Callus was grown on the basic medium plus 2,4-D and yeast extract containing ^{14}C-harmine HCl (0·1 mg; $2·88 \times 10^3$ dis/min) for 13 days. The callus, (2·45 g) was homogenized in MeOH (8 ml) and the MeOH-soluble fraction chromatographed on preparative TLC–Kieselgel G was developed with $CHCl_3$–MeOH (4:1). The ruine band (R_f 0·4), detected by its fluorescence in u.v. light, was scraped off and divided into 2 portions. One was counted directly as a suspension in toluene scintillant containing 0·5 g Cab-o-Sil thixotropic gel/15 ml; the second was eluted with MeOH, then chromatographed on TLC in BuOH–HOAc–H_2O (8:3:2). The ruine band was scraped off and counted as before. Regions on the plates above and below the fluorescent bands were used as blanks. The percentage of incorporation of activity into ruine was 50 and 48 respectively in the two solvent systems used.

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