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ALKALOIDS OF *HAEMANTHUS KALBREYERI**

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Key Word Index—*Haemanthus kalbreyeri*; Amaryllidaceae; alkaloids; haemanthamine; haemanthidine; hippadine; kalbretorine; lycorine; narciclasine; pratorimine; glucosyloxy alkaloid; kalbreclasine; anti-tumour activity; immuno stimulant.

Abstract—The roots of *Haemanthus kalbreyeri* contain a new phenanthridone alkaloid, kalbretorine, and a new glucosyloxy alkaloid, kalbreclasine. Additionally, six known alkaloids, viz. haemanthamine, haemanthidine, hippadine, lycorine, narciclasine and pratorimine, previously reported from other Amaryllidaceous plants have now been isolated also from this species. Kalbretorine produced marked inhibition of growth and viability of S-180 tumour cells. Kalbreclasine caused significant mitogenic activation of splenic lymphocytes characteristic of immuno stimulants.

INTRODUCTION

Haemanthus kalbreyeri (Amaryllidaceae) is cultivated in the upper Gangetic plains in India as a medicinal plant and a garden flower. Extracts of its roots, bulbs and flowers are used in popular medicine in the treatment of common cold, cough, asthma and in healing wounds. A number of alkaloids were earlier reported [1, 2] from *H. kalbreyeri* (synonymous with *H. multiflorus* Martyn.) of European origin. However, this is the first report on the phytochemical investigation of the species naturalized in India. The study was warranted in view of the medicinal property of the plant and the commonly observed variations in the alkaloidal constituents of Amaryllidaceae species due to ecological variation. We report here the isolation and characterization of alkaloids of the roots of the flowering plants. Additionally, the findings on the initial immunobiological testing of two new alkaloids are appraised.

RESULTS AND DISCUSSION

Extensive CC and TLC of the petrol, EtOAc and *n*-BuOH–soluble basic fractions of the roots of *H. kalbreyeri* afforded hippadine [3], pratorimine [4], lycorine [3, 4], haemanthamine [5], haemanthidine [5], narciclasine (= lycoricidinol) [6, 7] and two new alkaloids, named kalbretorine and kalbreclasine. Complete characterization of the two new alkaloids only is described here.

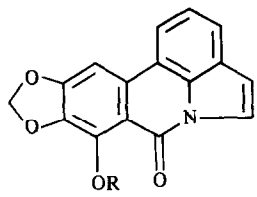
Kalbretorine

This compound, $C_{16}H_{19}NO_4$ ($[M]^+$ and elemental analyses), mp 245–248°, was soluble in alkali and gave a reddish ferric test for chelated phenols. Its UV maxima were similar to those of 8,9-dioxygenated pyrrolophenanthridones [3, 4]. The longer-wavelength maxima (Band I) exhibited bathochromic shifts on addition of NaOMe or anhydrous $AlCl_3$ indicating the presence of a hydroxyl group *peri* to a carbonyl function. The IR spectrum exhibited bands characteristic of chelated hydroxyl, lactam carbonyl and methylenedioxy functions. The ^1H NMR spectrum showed signals for four aromatic and two indolic protons, ascribable to H-1, H-2, H-3, H-4, H-5 and H-10 of a pyrrolo-6-phenanthridone, a methylenedioxy and a hydroxyl group (exchangeable with D_2O). The mass fragmentation pattern of the compound was also characteristic of pyrrolophenanthridone alkaloids [3, 4]. Methylation of kalbretorine with an excess of CH_2N_2–Et_2O gave a monomethyl ether, $C_{17}H_{11}NO_4$, mp 258–260°, which was found to be identical with a synthetic sample of 4,5-etheno-7-methoxy-8,9-methylenedioxy-6-phenanthridone, prepared according to earlier described procedures [3, 8]. Demethylation of the synthetic phenanthridone (**2**), with HBr–HOAc, gave kalbretorine. Thus, kalbretorine is assigned the structure 4,5-etheno-7-hydroxy-8,9-methylenedioxy-6-phenanthridone (**1**).

Kalbreclasine

This compound, $C_{20}H_{23}NO_{12}$, obtained as a hygroscopic solid, was optically active. It responded to Dragendorff, ferric and benzidine–metaperiodate tests for glycoalkaloids. In the EI mass spectrum, the alkaloid fragmented before exhibiting any $[M]^+$. However, identifiable fragment ion peaks appeared due to the aglucone (m/z 307) and glucose (m/z 163 $[M-17]^+$) moieties. On enzymatic hydrolysis with emulsin, it gave narciclasine and D-glucose. Kalbreclasine did not give a consistent combustion analysis due to irregular solvation of the molecule. Attempts to prepare a crystalline derivative were also unsuccessful because of its facile aromatization in the presence of acidic or basic reagents. Kalbreclasine, however, formed a hexaacetate on acetylation with Ac_2O–Et_3N at room temperature. The hexaacetate derivative exhibited a small but identifiable $[M]^+$ peak in its EI mass spectrum. Its ^1H NMR spectrum, in $CDCl_3$, suggested that all the acetyl groups were attached to the

*Part 13 in the series "Chemical Constituents of Amaryllidaceae". For part 12 see ref. [14]



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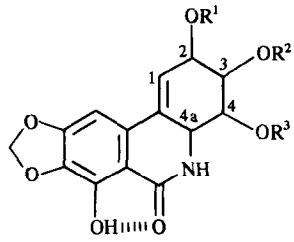
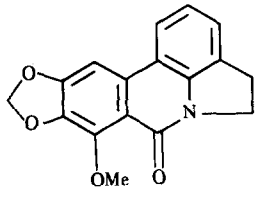
2 R = Me



3 $R^1 = \beta$-D-Glc, $R^2 = R^3 = H$
4 $R^1 = \beta$-D-Glc(Ac)$_4$, $R^2 = R^3 = Ac$



5

alcoholic hydroxyl functions. Apart from the aromatic and the methylenedioxy proton signals, the molecule exhibited one olefinic and four methine (excluding those of the glucosyl moiety) proton resonances which suggested the substitution pattern as in **4**. Irradiation of the H-1 signal at δ6.28 caused collapse of the double-doublet at δ5.18 (H-2) to a doublet (J = 9 Hz) with *ca* 15% enhancement (NOE) of its intensity. There was also a concomitant attenuation of the complex multiplicity at δ5.1 (associated with H-1′, H-3′ and H-4′ glucosyl protons). Conversely, irradiation of the H-2 resonance caused simplification and sharpening of the H-1 as well as that of the glucosyl proton signals. On the basis of these observations, the glucosyl moiety was located at the C-2 hydroxyl and Kalbreclasine is assigned the structure narciclasine-2-*O*-β-D-glucopyranoside (**3**).

Kalbretorine markedly inhibited the growth of S-180 tumour cells (transplantable ascites tumour in mice) and their viability (see Experimental). Kalbreclasine, in doses of 20 μg and above, produced extensive proliferation of the splenic lymphocytes in healthy adult male mice. The mitogenic activation produced by kalbreclasine was comparable to that of the known mitogen, concanavalin A (Con A).

EXPERIMENTAL

The general procedures were those reported recently [9].

Extraction. In a typical expt, fresh roots of *H. kalbreyeri* Bak.* (9.8 kg) of the flowering bulbs were macerated in MeOH (8 l.) and the macerate kept for 3 days at room temp. It was filtered and the filtrate concd under red. pres. to give a thick brown residue. The residue was extracted in succession with petrol (fraction A), EtOAc (fraction B) and *n*-BuOH (fraction C).

Treatment of fraction A. The brown gummy material (4.4 g) from this fraction on analytical TLC (solvent 1, plant 1) [9] showed two major and several minor Dragendorff-positive spots. A portion (0.43 g) was dissolved in C_6H_6 and chromatographed over a column of silica gel (24 × 2 cm). Elution was carried out with petrol (0.7 l.), petrol–C_6H_6 (1:1, 1.8 l.), C_6H_6 (1.5 l.), C_6H_6–$CHCl_3$ (1:1, 1 l.), and $CHCl_3$ (0.5 l.). Fractions (100 ml) were collected and monitored by analytical TLC.

Hippadine. Fractions 14–22 were combined and concd when hippadine was obtained as colourless flakes (34 mg), mp 208–210°. The identity of the alkaloid was established by direct comparison (mp, mmp, co-TLC, IR) with a ref. sample [3].

Pratorimine. Fractions 40–47 were combined and evapd and the residue crystallized from $CHCl_3$–EtOH as light brown needles (7 mg), mp 263–265°. The physical and spectral properties of this alkaloid were identical with those of pratorimine [4].

Treatment of Fraction B. The brown residue (1.8 g) obtained from this fraction was triturated in succession with hot petrol (fraction B_1), C_6H_6 (fraction B_2) and Me_2CO (fraction B_3). The Me_2CO-insoluble solid was designated (fraction B_4). CC of fraction B_1, as before, gave further quantities of hippadine (11 mg) and pratorimine (2 mg).

Treatment of fraction B_2. The soln was evapd and the residue repeatedly crystallized from $CHCl_3$–EtOH to give kalbretorine as light brown microcrystals (2.5 mg). The operation was repeated × 10 to collect *ca* 22 mg of alkaloid.

Kalbretorine (**1**). Mp 245–248° (dec.); R_f 0.23 (solvent 1, plate 1); UV λ_{max}^{MeOH} nm (log ε): 225 (4.32), 234 (4.02), 248 (4.31), 255 (4.11), 292 (3.78), 335 (3.24), 355 (3.48); $\lambda_{max}^{MeOH-0.1N\ NaOMe}$ 222 (4.27), 250 (4.29), 260 (4.20), 298 (3.66), 348 (3.33), 375 (3.40); $\lambda_{max}^{MeOH-AlCl_3}$ 222, 232 sh, 258, 300 sh, 332, 347 sh, 390; IR ν_{max}^{nujol} cm^{-1}: 3300 (br, chelated OH), 1672 (>NCO), 1618 (*vic*-trioxygenated benzene ring), 1088, 1032, 935 (OCH_2O); MS *m/z* (rel. int.): 279 $[M]^+$ (100%), 251 (7.5), 250 (8), 223 (6), 222 (5),

*The plant species, cultivated in Varanasi, was identified by Professor S. K. Roy, Department of Botany, Banaras Hindu University. The plant materials were collected in two consecutive years during the flowering time (May–June).

139.5; ^{1}H NMR [$CDCl_3$–$(CD_3)_2SO$]: δ13.18 (1H, exchangeable with D_2O, C-7 OH), 8.01 (1H, *d*, J = 3.6 Hz, H-5), 7.98 (1H, *dd*, J = 1.0, 7.6 Hz, line broadening due to long range coupling with H-10, H-1), 7.81 (1H, *dd*, J = 1.0, 7.5 Hz, H-3), 7.62 (1H, *s*, line broadening, H-10), 7.50 (1H, *dd*, J = 7.6, 7.5 Hz, H-2), 6.84 (1H, *d*, J = 3.6 Hz, H-4), 6.15 (2H, *s*, OCH_2O). (Found: C, 68.55; H, 3.08; N, 4.98. $C_{16}H_9NO_4$ requires: C, 68.81; H, 3.24; N, 5.01%.)

O-Methylkalbretorine (2). Kalbretorine (4 mg) was dissolved in MeOH (2 ml) and a large excess of anhyd Et_2O–CH_2N_2 added. After 24 hr, the soln was evapd and the residue crystallized from petrol–Me_2CO to give brown microcrystals (4 mg), mp 258–260°; R_f 0.3 (solvent 1, plate 1); MS m/z (rel. int.): 293 $[M]^+$ (100%), 276 $[M-17]^+$ (12), 265 (7), 264 (6), 263 (4), 248 (6), 236 (3), 219 (3).

Synthesis of kalbretorine (1). 4,5-Ethano-7-methoxy-8,9-methylenedioxy-6-phenanthridone (5), mp 266–268°, was prepared according to ref. [8]. The ethanophenanthridone (5, 14 mg) and DDQ (52 mg) were dissolved in dry C_6H_6 (50 ml) and the mixture refluxed (10 hr). The soln was evapd and the residue, in $CHCl_3$–MeOH (1:1, 2 ml), subjected to prep. TLC (solvent 2, plate 1). Work-up of the silica gel around R_f 0.4 afforded 7-*O*-methylkalbretorine (2, 9 mg), mp and mmp 258–260° (co-TLC, UV, MS). The above compound (5 mg) was heated with HBr in HOAc (2 ml, 45% w/v) for 15 min. The soln was evapd *in vacuo*, dil with H_2O (20 ml), basified and the liberated base processed in the usual way to give kalbretorine (1) as a straw coloured solid (3 mg), mp and mmp 245–248° (dec.) (co-TLC, UV, IR).

Treatment of fraction B_3. This fraction was concd and kept at room temp when a colourless solid separated. It showed two major Dragendorff-positive spots on analytical TLC.

Haemanthidine. Crystallization of the above solid from Me_2CO–petrol gave haemanthidine as colourless prisms (7 mg), mp 189–190°. The physical, spectral and optical properties (^{1}H NMR, MS, $[\alpha]_D$) of this compound were indistinguishable from those reported for haemanthidine [10, 11].

Haemanthamine. The Me_2CO–petrol mother liquor was filtered through a short column of Florisil to give haemanthamine as colourless needles (15 mg), mp 200°. Direct comparison (mp, mmp, co-TLC) with a reference sample [5] established that they were identical.

Treatment of fraction B_4. This fraction was triturated with a large vol of hot $CHCl_3$ and the $CHCl_3$ soluble and insoluble components separated.

Lycorine. The $CHCl_3$-insoluble solid crystallized from EtOH as shining rods (74 mg), mp 255–257°. Direct comparison (co-TLC, IR, $[\alpha]_D$) with a reference sample of lycorine [3, 4] established that they were identical.

Treatment of fraction C. This fraction was evapd under red. pres. to give a thick brown slurry (11 g). A portion (1.2 g) was dissolved in a small vol. of MeOH–dioxane, filtered and the filtrate kept at room temp when a brown solid separated (0.28 g). It was a mixture of two major compounds both of which responded to the benzidine–metaperiodate test for polyols. The solid was redissolved in MeOH, combined with silica gel (25 g), carefully dried under vacuum and charged on a chromatographic column (32 × 3 cm). Elution was carried out with EtOAc (2 l.), EtOAc–MeOH (99:1, 3 l.), and MeOH (0.5 l.). Fractions were collected (100 ml) and monitored by analytical TLC.

Narciclasine. Fractions 7–15 were combined and evapd to give a cream coloured residue which crystallized from Me_2CO–MeOH as fine needles (44 mg), mp 250–252° (dec); $[\alpha]_D^{22}$ + 142.8° (*c* 0.7, MeOH); λ_{max}^{MeOH} nm (log ε): 252 (4.38), 303 (3.80), 330 sh (3.63); IR ν_{max}^{KBr} cm^{-1}: 3400 (br), 1670, 928; ^{1}H NMR [$(CD_3)_2SO$]: δ13.23 (1H, *s*, exchangeable with D_2O), 7.8 (1H, *br*, NH), 6.84 (1H, *s*, H-10), 6.1 (1H, *br m*, H-1), 6.05 (2H, *s*, OCH_2O), 5.3–4.5 (3H, *m*, exchangeable with D_2O, OH), 4.2–3.5 (4H, *m*, H-2, H-3, H-4, H-4a); MS m/z (rel. int.): 307 $[M]^+$ (22%), 247 (100), 60 (18).

Kalbreclasine. The later EtOAc and EtOAc–MeOH eluates, which showed a streak on TLC, were combined and evapd under red. pres. The residue was repeatedly dissolved in MeOH and ppted with Et_2O when a straw coloured hygroscopic solid (78 mg) was obtained; R_f streak from 0.2–0.4 (solvent 3, plate 1); $[\alpha]_D^{22}$ + 78.14° (*c* 0.7, MeOH). The elemental analyses of the compound were unsatisfactory due to solvation.

Kalbreclasine hexaacetate (4). A mixture of kalbreclasine (30 mg), Ac_2O (1 ml) and Et_3N (1 ml) was kept at room temp for 48 hr under anhydrous conditions. The solvent was removed *in vacuo* and the solid crystallized from Me_2CO as colourless microcrystals, mp 198–201°; IR ν_{max}^{Nujol} cm^{-1}: 1730, 1718, 1612, 1595, 930; ^{1}H NMR ($CDCl_3$): δ12.25 (1H, *s*, exchangeable with D_2O, C-7 OH), 7.05 (1H, *s*, H-10), 6.28 (1H, *m*, H-1), 6.10 (2H, *s*, OCH_2O), 5.50 (1H, *m*, H-4), 5.40 (1H, *m*, H-3), 5.18 (1H, *dd*, J = 9, 4 Hz, H-2), 5.1–5.08 (3H, H-1′, H-3′ and H-4′), 4.6 (1H, *m*, H-4a), 4.21 (4H, *m*, H-2′, H-5′ and H-6′), 2.05–1.98 (18H, OAc); MS m/z (rel. int.): 721 $[M]^+$ (1.2%), 391 (5), 331 (24), 271 (12), 169 (100). (Found: C, 53.0; H, 5.1; N, 1.7. $C_{32}H_{35}NO_{12}$ requires C, 53.2; H, 4.8; N, 1.9%.)

Enzymatic hydrolysis of kalbreclasine. Kalbreclasine (18 mg) was dissolved in pH 5 buffer (5 ml) and emulsin (10 mg) was added. The mixture was kept at room temp overnight (32 ± 2°). It was filtered and the filtrate extracted with EtOAc. The EtOAc extract on work up afforded narciclasine (8 mg), mp and mmp 248–250° (co-TLC, MS). The presence of only D-glucose in the aq. hydrolysate was detected as before [12].

Anti-tumour activity of kalbretorine. S-180 transplantable ascites tumour of mice was used. The tumour was maintained in Swiss mice by serial passage of 1×10^6 tumour cells inoculated intraperitoneally. A stalk soln of kalbretorine (1×10^{-2} M) was prepared in phosphate buffered saline (PBS), pH 7.2, adding 1–2 drops of 7% aq. HOAc. The S-180 tumour cells proliferated at a high rate between days 2 to 10 post-inoculation. The *in vitro* growths of tumour cells in the no-treatment and vehicle-treatment control [70 ± 23.4 (mean ± s.d.) × 10^7] and in the test compound-treated [(13.0 ± 2.6) × 10^7] expts were determined on day 10. Following a 3 hr-incubation, there was no detectable change in the viability of the tumour cells in the no-treatment control (75%), vehicle-treated control (75%), but a marked reduction in viability in the kalbretorine-treated group (32.5%).

Mitogenic activation of splenic lymphocytes by kalbreclasine. Kalbreclasine was dissolved in H_2O and the soln filtered through a 0.45 μ membrane and stored, in small aliquots, at 0°. Concanavalin A (Con A) was dissolved in PBS (0.15 M), at the desired concn, membrane filtered and stored in small aliquots at 0°. The mitogenic activation of splenic lymphocytes of healthy adult male DBA/2 mice was determined according to ref. [13]. The stimulation index (SI) of kalbreclasine, in doses of 20 μg, was 3.1 ± 0.94 (s.d.). Con A, in doses of 5 μg, showed a stimulation index of 3.0.

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