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THREE NEW STEROIDAL ALKALOIDS FROM THE BARK OF *HOLARRHENA ANTIDYSENTERICA**

K. K. BHUTANI,† M. ALI, S. R. SHARMA, R. M. VAID and D. K. GUPTA

Regional Research Laboratory (C.S.I.R.), Jammu Tawi 180001, India

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Abstract—Three new steroidal alkaloids, namely regholarrhenine A, B, and C, isolated from the stem bark of *Holarrhena antidysenterica* collected at flowering stage are described. Structural studies on the basis of ^{1}H NMR, ^{13}C NMR, ORD, CD and mass spectral and chemical evidences show that the first two alkaloids lack the C-3 amino function in the conenine structure but possess a dienone system in ring A. In addition, both contain a hydroxy group at C-11 but differ in the nature of the nitrogen in the heterocyclic ring (tertiary and secondary amino group, respectively). Regholarrhenine C is the partially demethylated enamine analogue of conessine where both the heterocyclic and C-3 nitrogens are secondary amines.

INTRODUCTION

The stem bark of *Holarrhena antidysenterica* commercially known as kurchi in the Indian subcontinent [2] has been extensively studied, and even today its alkaloids are the subject of great interest due to the traditional use of the plant for the treatment of dysentery [3]. An extensive bibliography on the work done on different aspects of this plant has been published [4] and the isolation of a number of steroidal alkaloid constituents have been reported. These include conanines [4], 3-aminoconanines [4, 5], 20-aminopregnanes [4], 3-aminopregnenes [4], 3,20-diaminopregnanes [4, 6] and their derivatives.

A study was initiated in our laboratory to isolate as many alkaloids as possible from *H. antidysenterica* collected from different locations and subjecting them empirically to *in vitro* amoebicidal tests. The comparative evaluation of the activity of the known alkaloids from this plant are reported elsewhere [7]. However, during the bulk isolation from plant material collected at flowering stage we encountered three new alkaloids, named as regholarrhenine A, B and C, whose structural elucidation is discussed in the present communication.

RESULTS AND DISCUSSION

The total alkaloid mixture (0.44% yield) isolated from the stem bark of the plant was chromatographed on basic alumina with petrol–ether. The initial fractions yielded conessine (0.081%). Regholarrhenine A (**1**), B (**2**) and C (**3**) were obtained from subsequent petrol–ether eluant fractions in 0.011, 0.017 and 0.007% yields, respectively.

Bases **1** and **2** had a close resemblance to holonamine [8], isolated earlier in minute amounts from the bark of *H. antidysenterica*, showing the presence of a dienone system in ring A and a hydroxyl group at C-11 but differing in the saturation of the heterocyclic ring. Base **3** resembled 3,4-didehydroconessine, obtained during the attempted total synthesis of racemic conessine [9], but differed in the degree of *N*-methylation at C-3 and in the nature of the heterocyclic ring.

Regholarrhenine A (**1**), mp 197–198°, $[M]^+$ at *m/z* 341 ($C_{22}H_{31}NO_2$), λ_{max} 240 nm, had properties characteristic of a conenine derivative and a steroidal dienone. Its IR spectrum showed absorptions at *ca* 3520 (OH), 1655 (α,β unsatd C=O), 1620 (C=C) and 1600 (C=C) cm^{-1}. In the mass spectrum, the appearance of the base peak at *m/z* 71 and another prominent peak at *m/z* 326 confirmed the presence of the 18,20-epimino group. These peaks resulted from the well documented fragmentation pattern of conanine derivatives [10], the former originating from the cleavage of the heterocyclic ring from the rest of the steroidal molecule and the latter from the α-cleavage of the C-20/C-21 bond with the formation of an $[M-15]^+$ species. In the ^{1}H NMR spectrum of **1**, the three deshielded olefinic protons at δ7.90 (*d*, J = 10 Hz), 6.14 (*dd*, J = 10 and 2 Hz) and 6.02 (*s*) confirmed the presence of a dienone system and on the basis of the chemical shifts and spin–spin coupling constants these signals were assigned to the C-1, C-2 and C-4 protons, respectively. The band at δ3.74 (*m*, 1H, $W_{1/2}$ 24 Hz) was assigned to the 11β-proton as the magnitude of the vicinal coupling constant measured in terms of band width at half-height indicated that it was axial. The C-11 axial proton was expected to be coupled with the vicinal C-9 axial, C-12 axial and C-12 equatorial protons and in turn provide a wide band (1H, $W_{1/2}$ 20–25 Hz). Thus, the hydroxyl group was placed at the C-11 α-equatorial position. The other positions where the hydroxyl group could be placed in the light of the above band in the ^{1}H NMR were at C-7 and C-15. However, these positions were discounted on the basis of ORD and IR spectra of **1a** and will be discussed later. On addition of D_2O, the contour of the wide band changed

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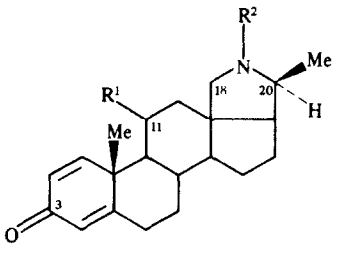
†Author to whom correspondence should be addressed.

from a multiplet to a triplet, the components of each being split into a doublet (J = 12 and 4 Hz). The appearance of a methine proton at δ3.00 as a doublet having a characteristic vicinal coupling constant (J = 10 Hz) for C-20 axial/C-17 axial protons was similar to the corresponding signal in conessine [11]. This further confirmed the conenine structure for **1**. Two tertiary methyl singlets (δ2.22 and 1.32) and one secondary methyl doublet (δ1.04, J = 8 Hz) protons were assigned to N–Me, C-19 and C-21, respectively. The C-19 signal, however, was found to be deshielded by δ0.34 in **1** from the corresponding signal in conessine which could possibly be ascribed to the spatial arrangement of ring A. Acetylation of base **1** gave a monoacetylated product, mp 101–103°, ν_{max}^{KBr} cm^{-1}: 1725 (C=O) and its ^{1}H NMR showed interesting chemical shifts. The N–Me, C-21 methyl and C-20 methine protons were deshielded by 0.60, 0.18 and 0.26 ppm, respectively, from the corresponding signals in **1**. However, the reverse was true for the olefinic proton signal of C-1 which shifted to δ6.94 from δ7.90 in the hydroxy compound **1**. The methyl protons of the acetyl function and the C-11 β-proton appeared at δ2.10 (*s*, 3H) and δ5.04 (*m*, 1H, $W_{1/2}$ 22 Hz), respectively.

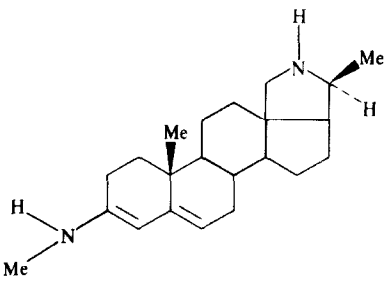
Jone's oxidation of (**1**) gave an oxo derivative (**1a**), mp 148–150°, ν_{max}^{KBr} cm^{-1}: 1700 (C=O), 1660 (α,β-unsatd C=O), 1620 (C=C) and 1598 (C=C). The ORD and CD curve shapes of **1a** measured in dioxan [12] were analogous to those obtained for 11-ketoprogesterone under similar conditions. The appearance of the six-membered cyclic carbonyl frequency (1700 cm^{-1}) in the IR of **1a** and the sensitivity of the resulting Cotton effect curve due to the combination of the characters of the optically active absorption bands of α,β-unsaturated ketone and saturated ketones as in **1a** and 11-ketoprogesterone conclusively ruled out the possibility of a hydroxyl group at either the C-15 or C-7 positions in base (**1**). Thus structure **1** has been assigned to the alkaloid named as regholarrhenine A.

Regholarrhenine B (**2**), mp 245–247°, $[M]^+$ at m/z 327 ($C_{21}H_{29}NO_2$), λ_{max} 237 nm, had properties similar to **1**. Its IR absorptions at *ca* 3400 (OH), 3220 (>NH), 1660 (α,β-unsatd C=O), 1625 (C=C), 1610 (C=C) and 1600 (NH bending) cm^{-1} suggested the presence of a 18,20-secondary epimino group instead of a tertiary one as in **1**. In the mass spectrum of **2**, the presence of an intense peak at m/z 55 (70%) further confirmed the secondary nature of the heterocyclic amine. The dienone ring A gave the base peak at m/z 91. As expected, ^{1}H NMR of **2** showed chemical shifts for C-1, C-2, and C-4 protons at δ7.95 (*d*, J = 9 Hz), 6.18 (*dd*, J = 10 and 2 Hz), and 6.04 (*s*), respectively, with the C-19 protons singlet at δ1.38. A wide band at δ4.00 (*m*, 2H, $W_{1/2}$ 24 Hz) from which a (NH) proton was exchanged on D_2O addition and likewise transformation of a multiplet to a triplet with splitting of individual components into a doublet (J = 12 and 4 Hz) as observed in **1** suggested a similar configuration at C-11. There was also another D_2O exchangeable proton at δ3.40 (*m*, OH). The secondary methyl doublet for C-21 was deshielded to δ1.42 (J = 8 Hz) as compared to its normal position at δ1.04 in **1** and conessine. On acetylation, **2** gave a diacetylated product, mp 224–226°, ν_{max}^{KBr} cm^{-1}: 1750 (OAc) and 1680 (N–Ac). The salient features of the ^{1}H NMR of this product were: δ2.04 (*br s*, 3H, N–Ac), 1.98 (*s*, 3H, OAc), 5.24 (*m*, $W_{1/2}$ 22 Hz, C-11 axial H) and shielding of the C-1 proton to δ6.64. The structure **2** assigned to the alkaloid regholarrhenine B was further confirmed by the conversion of **2** into **1** with a mixture of formic acid and formaldehyde (*N*-methylation).

Regholarrhenine C (**3**), mp 302–304°, $[M]^+$ at m/z 326 ($C_{22}H_{34}N_2$) indicated the presence of an enamine from its UV spectrum where the strong absorption at *ca* 275 nm could only be interpreted for such a chromophore. From the molecular formula, it was revealed that out of the two nitrogen atoms present in **3** only one had been methylated. From the mass spectrum, the position of the N–Me group was deduced. The base peak at m/z 257 was obtained by the loss of 69 (CH_2=CH-CH=N–Me) units from the $[M]^+$ peak. The important fragments at m/z 71 (99.5%) and 56 (15%) were generated from the exo- and endo-nitrogen species, respectively. The $[M-15]^+$ ion in **3** was short-lived as the small peak at m/z 311 (4%) was observed. In the ^{1}H NMR, the positions of the enamine and –NHMe protons were confirmed. The signals at δ7.60 (*br d*, 1H, J = 3.5 Hz) and 5.44 (*m*, 1H) were assigned to the C-4 and C-6 vinylic protons of the enamine system, respectively. The N–Me proton singlet was deshielded at δ2.65 confirming its connection with the secondary amino function at C-3. Deshielding was also observed in the C-21 methyl signal which appeared at δ1.38 (*d*, J = 8 Hz). Two D_2O exchangeable NH protons were discernible at δ4.12 and 7.20. Acetylation of **3** gave a diacetylated product, mp 190–192°, ν_{max}^{KBr}: 1645 cm^{-1} (N–Ac). *N*-Methylation of **3** with a mixture of formic acid–formaldehyde yielded 3,4-



1 R¹ = α-OH, R² = Me
1a R¹ = O, R² = Me
2 R¹ = α-OH, R² = H



3

didehydroconessine, mp 330–331°. 3,4-Didehydroconessine had been reported earlier in the literature [9]. Sodium borohydride reduction of 3,4-didehydroconessine in the presence of acetic acid [9] yielded conessine. Thus, structure **3** was assigned to the alkaloid named as regholarrhenine C.

Finally, ^{13}C NMR (Table 1) values for **1**, **1a** and **2** have been obtained which established the structures given. The signal assignments for the carbons of rings A, B and C were deduced by comparison with the chemical shifts reported for steroidal unsaturated ketones (dienone system) and hydroxysteroids [13, 14]. The assignments of the heterocyclic ring E and ring D carbons were based on comparison with those of conessine [15].

EXPERIMENTAL

Mps: uncorr. ^{1}H NMR were recorded at 60 MHz, ^{13}C FT-NMR at 22.49 MHz in $CDCl_3$ with TMS as an int std in 5 mm spinning tubes at room temp. Concns of compounds were *ca* 0.6–0.8 mmol/ml. FT-measurement conditions were: spectral width 5648 Hz; pulse flipping angle *ca* 45°; acquisition time 0.75 sec; number of data points 5000. ORD and CD curves were measured in MeOH soln except for **1a** in dioxan. CC was carried out using basic Al_2O_3. TLC was run on silica gel in C_6H_6–EtOAc–Et_2NH (6:3:1) and spots were developed by Dragendorff's reagent.

Plant material. Stem bark of *H. antidysenterica* (L.) Wall was collected from wild locations in district Kangra of Himachal Pradesh State during the flowering season (March to May 1985).

Isolation of alkaloids. Air-dried and powdered stem bark (50 kg) was extracted exhaustively by hot percolation with EtOH. The extract was dried under red. pres. and the 2 M HCl sol portion extracted with $CHCl_3$ (6 × 35 l) to remove neutral components. The aq. acidic layer was then made alkaline (pH 8.5) with NH_4OH (30%) soln and repeatedly extracted with $CHCl_3$ (12 × 50 l). The combined extracts were washed with H_2O, dried and evapd *in vacuo* to yield crude total alkaloids (220 g) as a dark brown solid. On TLC these showed four major and two minor spots. The crude extract was subjected to CC (5 kg) after the formation of a slurry. Fractions 1–5, eluted with petrol (60–80°) gave conessine (40.5 g, 0.081% yield) on crystallization from Me_2CO.

Table 1. ^{13}C NMR chemical shifts of compounds **1**, **1a** and **2** in $CDCl_3$

	Carbon							
	1	2	3	4	5	6	7	8
1	159.0	124.1	186.5	123.8	168.6	33.0	27.3	33.6
1a	154.2	126.8	185.5	124.5	165.7	31.3	25.8	33.2
2	159.2	125.0	186.6	124.5	168.7	33.8	29.2	34.7

	Carbon							
	9	10	11	12	13	14	15	16
1	59.5	44.2	68.6	49.3	50.0	52.8	24.4	35.7
1a	60.3	41.8	207.5	52.4	53.6	53.3	23.8	38.4
2	60.4	44.6	69.3	44.2	54.2	49.5	25.6	37.4

	Carbon					
	17	18	19	20	21	>N–Me
1	53.7	64.4	18.4	62.9	14.3	40.4
1a	55.2	63.7	18.3	61.3	13.5	40.1
2	54.2	69.3	19.3	66.8	17.7	—

Regholarrhenine A (**1**). Fractions 7–12 from CC, eluted with petrol on recrystallization from petrol gave **1** (5.5 g, 0.011% yield) as light yellow prisms, mp 197–198°, $[\alpha]_D^{33}$ − 105° (MeOH; *c* 0.2). UV λ_{max}^{MeOH} nm: 240 (log ε 5.31). IR ν_{max}^{KBr} cm^{-1}: 3520, 2940, 2920, 2860, 2780, 1655, 1620, 1600, 1460, 1440, 1405, 1380, 1360, 1240, 1200, 1160, 1090, 1020, 920 and 880. ^{1}H NMR: δ7.90 (*d*, 1H, *J* = 10 Hz, C-1), 6.14 (*dd*, 1H, *J* = 10 and 2 Hz, C-2), 6.02 (*s*, 1H, C-4), 3.74 (*m*, 1H, $W_{1/2}$, 24 Hz) on D_2O addition *m* becomes *t* and each component is split into a (*d*, *J* = 12 and 4 Hz), 3.00 (*d*, 1H, *J* = 10 Hz, C-20 axial), 2.80 (*s*, 1H, D_2O exchangeable, OH), 2.36 (*m*, 2H, C-18), 2.22 (*s*, 3H, N–Me), 1.32 (*s*, 3H, C-19) and 1.04 (*d*, 3H, *J* = 8 Hz, C-21). MS *m/z* (rel. int.): 341 $[M]^+$ ($C_{22}H_{31}NO_2$ 13.3%), 326 (79.8) and 71 (100). ORD (*c* 0.3): $[\phi]_{360}$ − 350°, $[\phi]_{340}$ − 450°, $[\phi]_{310}$ − 880°, $[\phi]_{290}$ − 1170° and $[\phi]_{280}$ − 390°; CD (*c* 0.3): $[\theta]_{400}$ − 80°, $[\theta]_{370}$ 0°, $[\theta]_{333}$ + 1170°, $[\theta]_{296}$ 0°, $[\theta]_{284}$ 780° and $[\theta]_{265}$ 0°. Acetylation of **1** with Ac_2O-pyridine at room temp for 24 hr and usual work-up gave a monoacetylated product, mp 101–103°. IR ν_{max}^{KBr} cm^{-1}: 2940, 1725, 1665, 1605, 1550, 1450, 1370, 1240, 1020. ^{1}H NMR: δ6.94 (*d*, 1H, *J* = 10 Hz, C-1), 6.16 (*dd*, 1H, *J* = 10 and 2 Hz, C-2), 6.10 (*s*, 1H, C-4), 5.04 (*m*, 1H, $W_{1/2}$ 22 Hz, C-11 axial), 3.26 (*d*, 1H, *J* = 10 Hz, C-20 axial), 2.82 (*s*, 3H, N–Me), 2.18 (*d*, 2H, *J* = 13 Hz, C-18), 2.10 (*s*, 3H, OAc), 1.50 (*d*, 3H, *J* = 7 Hz, C-21) and 1.24 (*s*, 3H, C-19). Jones oxidation of **1** and usual work-up gave an oxo derivative (**1a**) mp 148–150°; UV λ_{max}^{MeOH} nm: 240 (log ε 9.7). IR ν_{max}^{KBr} cm^{-1}: 2940, 1700, 1660, 1620, 1598 and 1440 cm^{-1}. ^{1}H NMR: δ7.62 (*d*, 1H, *J* = 10 Hz, C-1), 6.20 (*dd*, 1H, *J* = 10 and 2 Hz, C-2), 6.10 (*s*, 1H, C-4), 3.00 (*d*, 1H, *J* = 10 Hz, C-20 axial), 2.48 (*m*, 2H, C-18), 2.28 (*s*, 3H, N–Me), 1.42 (*s*, 3H, C-19) and 1.07 (*d*, 3H, *J* = 7 Hz, C-21). ORD (*c* 0.04): $[\phi]_{400}$ − 70°, $[\phi]_{390}$ 0°, $[\phi]_{385}$ + 50°, $[\phi]_{378}$ 0°, $[\phi]_{367}$ + 70°, $[\phi]_{362}$ 0°, $[\phi]_{358}$ − 40°, $[\phi]_{350}$ 0°, $[\phi]_{345}$ − 30°, $[\phi]_{342}$ 0°, $[\phi]_{327}$ + 340°, $[\phi]_{320}$ + 180°, $[\phi]_{316}$ + 160°, $[\phi]_{312}$ 0°, $[\phi]_{278}$ − 1310° and $[\phi]_{245}$ 0°; CD (*c* 0.04): $[\theta]_{400}$ − 80°, $[\theta]_{370}$ 0°, $[\theta]_{333}$ + 870°, $[\theta]_{296}$ 0°, $[\theta]_{284}$ − 780° and $[\theta]_{265}$ 0°.

Regholarrhenine B (**2**). CC fractions 15–28 eluted with the same solvent on crystallization from MeOH, gave **2** (8.3 g, 0.017% yield) as white needles, mp 245–247°. $[\alpha]_D^{33}$ − 83.5° (MeOH: *c* 0.3). UV λ_{max}^{MeOH} nm: 237 (log ε 5.5). IR ν_{max}^{KBr} cm^{-1}: 3400, 3220, 2980, 2940, 2865, 1660, 1625, 1610, 1600, 1440, 1405, 1300, 1242, 1165, 1060, 1040, 980 and 880. ^{1}H NMR: δ7.95 (*d* 1H, *J* = 9 HZ, C-1), 6.18 (*dd*, 1H, *J* = 10 and 2 Hz, C-2), 6.04 (*s*, 1H, C-4), 4.00 (*m*, 1H, $W_{1/2}$ 24 Hz, on D_2O addition *m* becomes *t* and each component is split into a (*d*, *J* = 12 and 4 Hz, C-11 axial), 3.40 (*m*, 1H, D_2O exchangeable, OH), 1.42 (*d*, 3H, *J* = 8 Hz, C-21) and 1.38 (*s*, 3H, C-19). MS *m/z* (rel. int.): 327 ($C_{21}H_{29}NO_2$, 1.5%), 204 (5.3), 91 (100) and 55 (70). ORD (*c* 0.3): $[\phi]_{360}$ − 440°, $[\phi]_{340}$ − 560°, $[\phi]_{310}$ − 1020°, $[\phi]_{290}$ − 1320°, $[\phi]_{280}$ − 350°, $[\phi]_{270}$ − 310°, $[\phi]_{260}$ − 550° and $[\phi]_{200}$ − 520°, CD (*c* 0.3): $[\theta]_{400}$ − 80°, $[\theta]_{370}$ 0°, $[\theta]_{350}$ + 680°, 680°, $[\theta]_{333}$ + 334°, $[\theta]_{320}$ + 760°, $[\theta]_{296}$ 0°, $[\theta]_{286}$ − 660° and $[\theta]_{270}$ − 10°. Acetylation of **2** with Ac_2O–pyridine at room temp for 24 hr and usual work-up gave a diacetylated product, mp 224–226°; IR ν_{max}^{KBr} cm^{-1}: 2950, 1750, 1680, 1630, 1600, 1430, 1370, 1230, 1040 and 880. ^{1}H NMR: δ6.64 (*d*, 1H, *J* = 10 Hz, C-1), 6.06 (*s*, 1H, C-4), 6.04 (*br d*, *J* = 10 Hz, C-2), 5.24 (*m*, $W_{1/2}$ 22 Hz; C-11 axial), 2.04 (*br s*, 3H, N–Ac), 1.98 (*s*, 3H, OAc), 1.36 (*d*, 3H, *J* = 8 Hz, C-21) and 1.28 (*s*, 3H, C-19). *N*-Methylation of **2** with HCO_2H–HCHO gave **1** (comparable mp, mmp, superimposable IR).

Regholarrhenine C (**3**). Further elution of the column with petrol yielded **3** (3.5 g, 0.007% yield) as a white powder in fractions 32–44 after crystallization from MeOH–Me_2CO (4:1); mp 302–304°. $[\alpha]_D^{33}$ − 80° (MeOH; *c* 0.2). UV λ_{max}^{MeOH} nm: 275, 240 and 232 sh (log ε 2.6 and 9.7). IR ν_{max}^{KBr}: 3400, 2950, 2700, 2420, 1660 sh, 1615, 1602 sh,

1465, 1445, 1380, 1100 and 1040. ^{1}H NMR: δ7.60 (*br d*, 1H, $J = 3.5$, C-4), 7.20 (*m*, 1H, D_2O exchangeable), 5.44 (*m*, 1H, C-6), 4.12 (*br m*, 1H, D_2O exchangeable), 2.65 (*s*, 3H, N–Me), 1.38 (*d*, 3H, $J = 8$ Hz, C-21) and 1.10 (*s*, 3*H*, *C*-19). *MS m/z* (rel. int.): 326 $[M]^+$ ($C_{22}H_{34}N_2$, 11.7%), 311 (4), 257 (100), 71 (99.5) and 56 (15). ORD (*c* 0.3): $[\phi]_{280} -630°$, $[\phi]_{260} -640°$, $[\phi]_{240} -680°$, $[\phi]_{220} -1210°$, $[\phi]_{214} -680°$ and $[\phi]_{210} -480°$; CD (*c* 0.3): $[\theta]_{274} -110°$, $[\theta]_{260} -190°$, $[\theta]_{251}$ 0°, $[\theta]_{231} +1170°$ and $[\theta]_{214}$ 0°. Acetylation of **3** with Ac_2O–pyridine at room temp for 24 hr and usual work-up gave a *N*-diacetylated product, mp 190–192°. IR ν_{max}^{KBr} cm^{-1}: 2930, 2850, 1645, 1610, 1600, 1440, 1420, 1350, 1330 and 1070. *N*-Methylation of **3** with HCO_2H–HCHO gave 3,4-didehydroconessine, mp 330–331°. UV λ_{max}^{MeOH} nm: 275 (log ε 5.3). A soln of 3,4-didehydroconessine (0.5 g) in anhydrous dioxan (5 ml) was stirred under N_2 with $NaBH_4$ (1 g). No decrease in *A* at 275 nm occurred even after 6 hr stirring. However, the addition of HOAc (2 ml) to the reaction mixt resulted in the immediate disappearance of A at 275 nm. At this stage, the soln was heated under reflux for 1 hr, the cooled soln then made basic with NH_4OH (30%) soln and extracted with $CHCl_3$ to obtain conessine in quantitative yield (comparable with authentic sample mp, mmp, superimposable IR).

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