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STEREOCHEMISTRY OF THE ENZYMIC PROCESSES INVOLVED IN THE BIOSYNTHESIS OF SCOPOLAMINE FROM HYOSCYAMINE[1]

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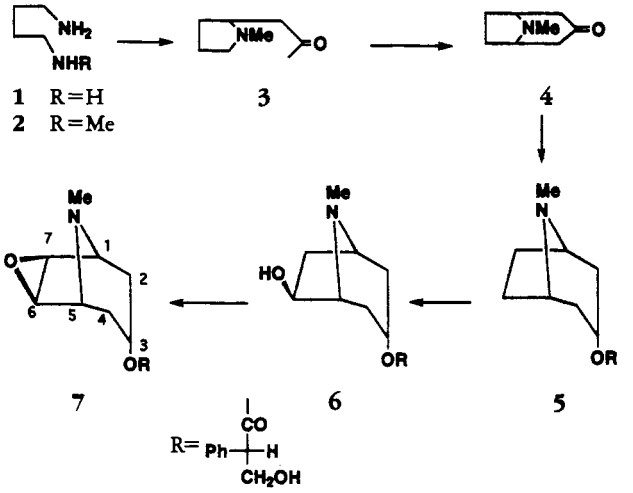
ABSTRACT.—An improved synthetic route to (*R*)- [**8**] and (*S*)-[2-^{2}H]putrescine [**9**] dihydrochloride has been established. These precursors were fed to transformed root cultures of a *Datura* hybrid which produce hyoscyamine [**5**] and scopolamine [**7**]. These alkaloids were separated, and labeling patterns in scopolamine were established by ^{2}H-nmr spectroscopy after detailed analysis of the ^{1}H- and ^{13}C-nmr spectra of scopolamine had been carried out. Feeding of (*R*)-[2-^{2}H]putrescine [**8**] dihydrochloride produced scopolamine [**12**] with ^{2}H present at H-6, whereas incorporation of the (*S*)-[2-^{2}H]putrescine [**9**] dihydrochloride resulted in scopolamine [**15**] labeled with ^{2}H at H-7. These labeling patterns confirm that conversion of hyoscyamine [**10**] and **13** into 6β-hydroxyhyoscyamine [**11**] and **14** occurs with retention of configuration, and that epoxide formation takes place with removal of the β hydrogen from H-7 to give scopolamine [**12**] and **15**. The protons at the 6α and 7α positions of hyoscyamine [**10**] and **13** are retained throughout the processes involved in epoxide formation.

The biosynthesis of the tropane ring system in hyoscyamine [**5**] is generally accepted to take place from L-ornithine or L-arginine via putrescine [**1**], *N*-methylputrescine [**2**], hygrine [**3**], and tropinone [**4**] (Scheme 1). The evidence in favor of this metabolic sequence was reviewed comprehensively by Leete in 1979 and 1990 (1,2). The importance of arginine as a metabolic precursor is supported by precursor-feeding experiments, inhibitor studies and enzymological data (3–5). Putrescine [**1**] appears to be involved either in its free state or in a bound form, since four of the carbon atoms of ornithine can be incorporated into tropane alkaloids either symmetrically or non-symmetrically depending upon the species (1,2,6). A very recent publication (7) has drawn attention to unpublished results from Leete which throw doubt upon the established intermediacy of hygrine [**3**] (8) in the formation of tropinone [**4**]. Furthermore, Hemscheidt and Spenser (7) have presented evidence that the formation of the tropane ring system in *Datura stramonium* arises by the stepwise incorporation of acetate and not by the incorporation of acetoacetate followed by decarboxylation, as previously supposed (1,2). This stepwise incorporation mechanism is essentially similar to that suggested by Leete and Kim (1,2,9) to occur in the biosynthesis of cocaine.

Conversion of hyoscyamine [**5**] into scopolamine [**7**] involves the overall formation of an epoxide requiring the net addition of one oxygen atom and the removal of two hydrogen atoms. Leete and Lucast (10) showed that most of the tritium was lost from [6β,7β-^{3}H]tropine (tropine is **5** with R=H) on formation of scopolamine [**7**] in *Datura innoxia* and *Datura meteloides* plants. They suggested that the conversion of hyoscyamine into scopolamine takes place by hydroxylation to give 6β-hydroxyhyoscyamine [**6**] followed by cis-dehydration and epoxide formation on the 6,7-dehydrohyoscyamine. Hashimoto and Yamada (11) isolated an enzyme from *Hyoscyamus niger* which catalyzes the conversion of hyoscyamine [**5**] into 6β-hydroxyhyoscyamine [**6**]. They used this

[1]This manuscript is dedicated to the memory of a world expert on alkaloid biosynthesis, Professor Edward Leete.



SCHEME 1. Biosynthesis of hyoscyamine [**5**] and scopolamine [**7**].

enzyme to prepare [6-^{18}O]-labeled **6** and showed that the ^{18}O was retained on conversion of [6-^{18}O]-**6** into scopolamine by *Duboisia myoporoides*. Thus, dehydrohyoscyamine cannot be an intermediate in the biosynthetic pathway (12). The same group also prepared 6β-hydroxy-[7β-^{2}H]hyoscyamine and showed that deuterium was lost from this compound on conversion into scopolamine by enzyme preparations from *H. niger* (13).

We have previously prepared (*R*)-**8** and (*S*)-[2-^{2}H]putrescine [**9**] in order to study the stereochemical course of some enzymic reactions involved in pyrrolizidine alkaloid biosynthesis (14,15). We have also developed transformed root cultures of a *Datura* hybrid that produce hyoscyamine [**5**] and scopolamine [**7**] in good quantity (16). We therefore decided to study the fate of hydrogen atoms at the 6 and 7 positions of **5** in the conversion into **7** using ^{2}H-labeled precursors in conjunction with ^{2}H-nmr spectroscopy to establish complete labeling patterns in the alkaloids.

RESULTS AND DISCUSSION

Transformed root cultures of *Datura candida*×*Datura aurea* hybrid plants were set up and maintained as described previously (16). These cultures produce hyoscyamine [**5**] and scopolamine [**7**] as major alkaloidal metabolites. Initial feeding experiments were carried out with [1,4-^{14}C]putrescine dihydrochloride in order to establish optimum conditions for producing scopolamine. The tracer was added 4 days after subculturing, and the cultures were allowed to grow for a further 10 days. The roots were then filtered off and the alkaloids were extracted. The total ^{14}C incorporation into the whole alkaloid mixture was 2.8% after feeding putrescine at 0.1 mM, 4.4% at 0.5 mM, 2.9% at 1.0 mM, and 1.2% at 2.0 mM. The amounts of scopolamine [**7**] present were determined in each alkaloidal extract by hplc analysis (16). The amount of scopolamine and its specific incorporation at each of these feeding concentrations (per 10 ml medium) were 317 μg, 0.4% (0.1 mM); 127 μg, 3.3% (0.5 mM); 20 μg, 11.5% (1.0 mM); 15 μg, 13.5% (2.0 mM). Accordingly, 0.5 mM was selected as a suitable concentration to produce sufficient scopolamine with a reasonable specific incorporation for studies with ^{2}H-labeled precursors.

The ^{1}H- and ^{13}C-nmr spectra of scopolamine [**7**] were assigned completely by 2D techniques and nOe experiments. The ^{1}H nmr assignments of Chazin and Colebrook (17)

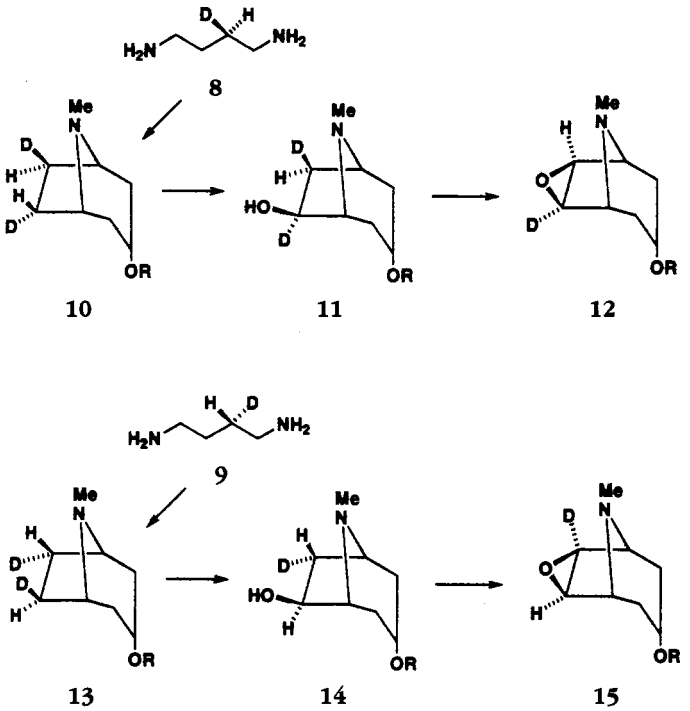
and the ^{13}C-nmr assignments of Feeney *et al.* (18) were confirmed. The crucial ^{1}H-nmr chemical shifts of H-6 and H-7 in scopolamine were found to be well separated at δ 3.31 and 2.66, respectively. An initial feeding experiment was carried out with [2,2,3,3-$^{2}H_4$]putrescine dihydrochloride, made by exchanging the protons in succinonitrile with ^{2}H, followed by reduction with borane-dimethyl sulfide complex, and isolation of the product as the dihydrochloride with a $^{2}H_4$ content of ca. 90% (^{1}H-nmr and eims data) (19). This compound was fed to the transformed *Datura* root cultures together with [1,4-^{14}C]putrescine dihydrochloride at 0.5 mM. Hyoscyamine and scopolamine were isolated and purified by preparative tlc. The purity of each alkaloid was checked by gc and hplc analysis (16). The ^{14}C specific incorporation for hyoscyamine was 6.1% and the ^{2}H (^{1}H) nmr spectrum contained a broad singlet at δ 1.53 corresponding to ^{2}H present at H-6 and H-7. The ^{2}H specific incorporation was estimated to be 7.8%. Scopolamine was obtained with a ^{14}C specific incorporation of 3.8%, and its ^{2}H-nmr spectrum showed two broad singlets at δ 2.63 and 3.36 in a 1:1 ratio corresponding to ^{2}H present at H-6 and H-7 in equal amounts. The estimated ^{2}H specific incorporation of ca. 1% suggested that there is a substantial ^{2}H isotope effect in operation in the formation of [6,7-$^{2}H_2$]scopolamine from [6,6,7,7-$^{2}H_4$]hyoscyamine. A ^{2}H isotope effect was observed during the conversion of [7β-^{2}H]6β-hydroxyhyoscyamine into scopolamine in *Duboisia* shoot cultures (20).

Samples of (*R*)- [**8**] and (*S*)-[2-^{2}H]putrescine [**9**] dihydrochloride were prepared according to the procedure of Kunec and Robins (14), except that the conversion of the [2-^{2}H]butane-1,4-diol into putrescine was carried out by an improved procedure, involving treatment of the diol with hydrazoic acid, di-isopropylazodicarboxylate, and triphenylphosphine, followed by hydrolysis (21). The ^{2}H content of the *R* and *S* isomers was estimated to be ca. 99% and ca. 98%, respectively, based on ^{1}H; ^{13}C; and ^{2}H-nmr data. Administration of (*R*)-[2-^{2}H]putrescine dihydrochloride together with [1,4-^{14}C]putrescine dihydrochloride to the transformed *Datura* root cultures gave a sample of scopolamine with a ^{14}C specific incorporation of 3.5% and a ^{2}H (^{1}H) nmr spectrum containing a singlet at δ 3.37 corresponding to scopolamine labeled with ^{2}H at H-6. The estimated ^{2}H specific incorporation was 2.4%. Feeding the (*S*)-[2-^{2}H]putrescine dihydrochloride in a similar fashion produced a sample of scopolamine with 3.2% ^{14}C specific incorporation. A singlet at δ 2.66 was observed in its ^{2}H (^{1}H) nmr spectrum corresponding to scopolamine labeled at H-7 with ^{2}H, and the ^{2}H specific incorporation was estimated to be 2.2%.

The labeling patterns from these feeding experiments are shown in Scheme 2, and the course of events is probably as follows. Hydroxylation of hyoscyamine [**10**] and **13** at C-6 occurs at the β-position to give 6β-hydroxyhyoscyamine [**11**] and **14** with loss of the 6β hydrogen and retention of the hydrogen at C-6α. Formation of the epoxide **12** and **15** from 6β-hydroxyhyoscyamine occurs by removal of the β hydrogen at C-7 and retention of the hydrogen at C-7α. In overall terms, two hydrogens are retained at the 6α and 7α positions and two hydrogens are lost from the 6β and 7β positions in hyoscyamine, and the epoxide is thus formed with overall retention of configuration. This work confirms and extends the previous results of Yamada and co-workers (11–13), which were obtained using *H. niger*.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded on a Bruker WP 200-SY spectrometer operating at 200 MHz (δ_H), 50 MHz (δ_C), and 30.7 MHz (δ_D) or on a Jeol GX400 spectrometer operating at 399.65 MHz (δ_H) and 100.4 MHz (δ_C). Unless otherwise stated, all spectra were recorded for solutions in $CDCl_3$ with TMS as internal standard. Mass spectra were recorded on AEI MS12 or MS902 spectrometers. Gc and hplc were carried out as described previously (16). Radiochemicals were purchased from Amersham International. Radioactivity was measured with a Philips PW 4700 Liquid Scintillation



SCHEME 2. Stereochemistry of epoxide formation in the conversion of hyoscyamine [**5**] into scopolamine [**7**] via 6β-hydroxyhyoscyamine [**6**] in transformed *Datura* root cultures.

Counter using toluene/MeOH solutions. Sufficient counts were accumulated to give a standard error of less than 1% for each determination. A Panax thin layer scanner RTLS-1A was used for the radioscanning of the tlc plates.

TRANSFORMED ROOT CULTURES.—These were set up from *Da. candida*×*Da. aurea* hybrid plants (16), and the fastest-growing culture DB5 was used for these experiments. (The hybrid is in cultivation in Norwich and live material can be supplied by Dr. N.J. Walton. No voucher is deposited.)

FEEDING METHODS.—Rapidly growing roots (0.2–0.5 g) were passed into 50 ml fresh Gamborg's medium containing 3% sucrose in 250-ml Erlenmeyer flasks (16). Solutions of the precursors in H_2O were filter-sterilized and added to the cultures (20 flasks) after 4 days to give a concentration of 0.5 mmol. Roots were harvested after 10 further days and macerated in MeOH. Each MeOH extract was concentrated, and the residue was taken up in 1 M HCl (100 ml). The acid solution was washed with CH_2Cl_2 (6×75 ml). The acid solution was basified with concentrated NH_3 and extracted with $CHCl_3$ (4×100 ml). The organic extracts were dried (Na_2SO_4), filtered, and concentrated to yield the alkaloid mixture. Tlc was carried out on Kieselgel G plates of 0.25 mm thickness, developed with EtOAc-iPrOH-25% NH_3 (45:35:10). Alkaloids were detected by Dragendorff's reagent (22). Hyoscyamine had R_f 0.40 and scopolamine, R_f 0.61. Hyoscyamine and scopolamine were separated by preparative tlc. Yields of 5–20 mg of each alkaloid were obtained in each experiment. Radioscans of tlc plates of each purified alkaloid showed one radioactive band coincident with authentic unlabeled hyoscyamine or scopolamine.

Hyoscyamine.—δ_H 1.12 (H-6eq), 1.39 (H-4eq), 1.60 (H-2eq and H-6ax), 1.66 (H-7eq), 1.78 (H-7ax), 1.94 (H-4ax), 2.02 (H-2ax), 2.11 (NMe), 2.83 (H-5), 2.94 (H-1), 3.69 (H-2′), 3.74 (H-3′), 4.09 (H-3′), 4.94 (H-3), 7.19–7.26 (ArH) (17).

Scopolamine.—δ_H 1.25 (H-4eq), 1.49 (H-2eq), 1.93 (H-4ax), 2.01 (H-2ax), 2.36 (NMe), 2.66 (H-7), 2.88 (H-1), 3.00 (H-5), 3.31 (H-6), 3.65 (H-2′), 3.71 (H-3′), 4.06 (H-3′), 4.92 (H-3), 7.15–7.27 (ArH) (18).

PREPARATION OF (*R*)- [**8**] AND (*S*)-[2-^{2}H]PUTRESCINE [**9**] DIHYDROCHLORIDE.—(*R*)-[2-^{2}H]Butane-1,4-diol (14) (137 mg, 1.5 mmol) in dry THF (1 ml) was treated with a solution of hydrazoic acid in C_6H_6

(0.8 M, 4.5 ml). A solution of di-isopropylazodicarboxylate (667 mg, 3.3 mmol) in dry THF (1 ml) was added with stirring. Triphenylphosphine (1.73 g, 6.6 mmol) in dry THF (10 ml) was added, maintaining the temperature of the mixture at ca. 40°. The mixture was stirred for 1 h at room temperature, then at 50° for 3 h. H_2O was added and the mixture was heated at 50° for 3 h. The solvent was removed under reduced pressure, and the residue was partitioned between 1 M HCl (10 ml) and CH_2Cl_2 (10 ml). The aqueous layer was concentrated under reduced pressure to yield a pale brown solid which was crystallized from aqueous EtOH to give (*R*)-[2-^{2}H]putrescine [**8**] dihydrochloride (58%): R_f 0.66 [cellulose; iPrOH-concentrated NH_3 (5:3); ninhydrin spray]; δ_H (D_2O) 1.63 (3H, m), 2.94 (4H, m); δ_C (D_2O) 24.4 (C-2, $J_{C\text{-}H}$=19.6 Hz), 24.6 (C-3), 39.67 (C-4), 39.70 (C-1); δ_D (H_2O) 1.58 (s).

(*S*)-[2-^{2}H]Putrescine [**9**] dihydrochloride was made in a similar fashion from (*S*)-[2-^{2}H]butane-1,4-diol with the same R_f value and spectroscopic data.

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