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THE ABERRANT FORMATION OF (−)-*N*-METHYLANABASINE FROM *N*-METHYL-Δ^1-PIPERIDEINIUM CHLORIDE IN *NICOTIANA TABACUM* AND *N. GLAUCA**

E. LEETE and M. R. CHEDEKEL

Natural Products Laboratory,† School of Chemistry, University of Minnesota, Minneapolis, MN 55455, U.S.A.

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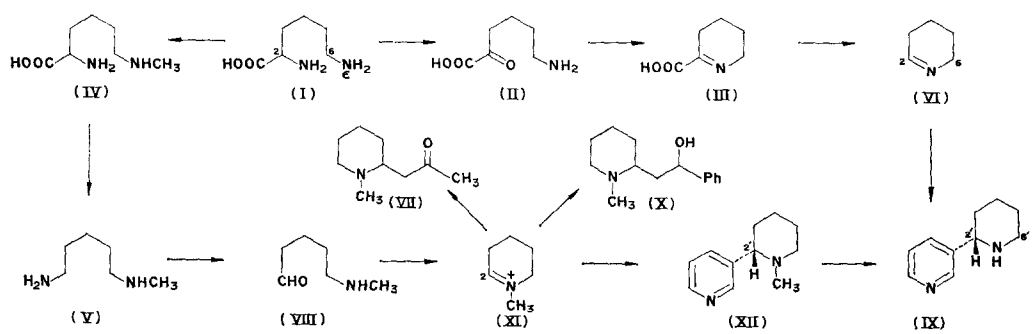
Key Word Index—*Nicotiana*; Solanaceae; *N*-methyl-Δ^1-piperideinium chloride; *N*-methylanabasine; alkaloid biosynthesis; anabasine biosynthesis.

Abstract—The administration of *N*-methyl-[2-^{14}C]-Δ^1-piperideinium chloride to *Nicotiana tabacum* and *N. glauca* plants led to the formation of (−)-[2′-^{14}C]-*N*-methylanabasine, having the same specific activity as the administered radioactive compound. Radioactive anabasine of lower specific activity was also isolated from these plants. When DL-[2-^{14}C]-lysine, a well established precursor of anabasine, was fed to these species, negligible activity was detected in *N*-methylanabasine, which had been added to the harvested plants in order to trap any activity which might have resided in this alkaloid. It is therefore concluded that *N*-methylanabasine is not an intermediate in the biosynthesis of anabasine from lysine, and its formation in the present work is an example of an aberrant biosynthesis.

INTRODUCTION

IT HAS been previously established that lysine (I) is a precursor of the piperidine ring of anabasine (IX), the major alkaloid of *Nicotiana glauca*. Until recently all the experiments carried out with labelled putative precursors have been consistent with the biosynthetic



SCHEME 1.

scheme illustrated in Scheme 1. Thus the administration of [2-^{14}C]-lysine to *N. glauca* yielded anabasine labelled solely at C-2′;[1] furthermore, only the ϵ-amino nitrogen of lysine

* Part II in the series "Aberrant Syntheses in Higher Plants". For Part I see E. LEETE, G. B. BODEM and M. F. MANUEL, *Phytochem.* **10**, 2687 (1971).

† Contribution No. 118 from this Laboratory.

[1] E. LEETE, *J. Am. Chem. Soc.* **78**, 3520 (1956).

is incorporated into the piperidine ring.[2] More recently[3] [6-^{14}C]-Δ^1-piperideine was incorporated affording [6'-^{14}C]-anabasine. However it has now been shown by Spenser[4] that [6-^{14}C, 2-^{3}H]-lysine is incorporated into anabasine, *N*-methylpelletierine (VII), and sedamine (X) with retention of most of the tritium relative to the C-14. This result makes the formation of Δ^1-piperideine from lysine via ϵ-amino-α-ketocaproic acid (II) untenable, since all the tritium would be lost from this intermediate. Spenser thus suggested[4] that [6-^{14}C]-lysine, which is incorporated into these piperidine alkaloids in a specific fashion, maintains its asymmetry by the formation of ϵ-*N*-methyllysine (IV). Decarboxylation of this amino acid yields *N*-methylcadaverine (V) which is oxidized at the primary amino group (originally the α-amino group of lysine) yielding 5-methylaminopentanal (VIII), whose cyclic derivative *N*-methyl-Δ^1-piperideinium ion (XI) serves as the immediate precursor of the piperidine rings of *N*-methylpelletierine and sedamine. Condensation of this compound with a suitably activated derivative of nicotinic acid[5] would yield *N*-methylanabasine (XII). Anabasine would then be formed by *N*-demethylation. This final step would be analogous to the formation of nornicotine from nicotine.[6–8] Dawson has, in fact, demonstrated that *N*-methylanabasine is demethylated in *N. glutinosa*[9] by an apparently non-specific dealkylating enzyme.

In order to test this hypothesis, *N*-methyl-[2-^{14}C]-Δ^1-piperideinium chloride was added to the nutrient solution in which the roots of intact *N. tabacum* and *glauca* plants were growing. This labelled compound was obtained by a synthesis analogous to the one used for the preparation of *N*-methyl-[2-^{14}C]-Δ^1-pyrrolinium chloride.[10] The final step in the synthesis (see Experimental) involves the partial reduction of *N*-methyl-2-piperidone with $LiAlH_4$.[11] The desired product was separated from unchanged piperidone and *N*-methylpiperidine by TLC on silica gel. The *N*-methyl-[2-^{14}C]-Δ^1-piperideinium chloride was obtained as a hygroscopic solid and it was not possible to measure its specific activity directly. However, the *N*-methylpiperidine resulting from complete reduction of the piperidone could be accurately assayed as its crystalline picrate. The piperideinium salt XI was not absorbed by the roots of the *Nicotiana* plants at a rapid rate, and appreciable quantities remained in the nutrient solutions at the termination of the feeding experiments (see Table 1). The alkaloids were extracted from the plants by established methods and then separated by GLC. The main alkaloids isolated from *N. tabacum* were nicotine, anabasine, and *N*-methylanabasine. The nicotine had very low activity, insufficient to carry out any degradations to determine the distribution of activity. The absolute incorporation of activity into anabasine (1·3%) and *N*-methylanabasine (2·2%) was significant. However, the most dramatic observation was that the *N*-methylanabasine had essentially the same specific activity as the administered *N*-methyl-[2-^{14}C]-Δ^1-piperideinium chloride. The *N*-methylanabasine was optically active and was identical (ORD, UV, TLC, GLC) with authentic (−)-*N*-methylanabasine prepared by the methylation of (−)-anabasine with formaldehyde and formic acid. In *N. glauca*, where anabasine is the main alkaloid, similar results were

[2] E. LEETE, E. G. GROS and T. J. GILBERTSON, *J. Am. Chem. Soc.* **86,** 3907 (1964).
[3] E. LEETE, *J. Am. Chem. Soc.* **91,** 1697 (1969).
[4] R. N. GUPTA and I. D. SPENSER, *Phytochem.* **9,** 2329 (1970), and private communication.
[5] E. LEETE, *Advances in Enzymol.* **32,** 373 (1969).
[6] W. L. ALWORTH and H. RAPOPORT, *Arch. Biochem. Biophys.* **112,** 45 (1965).
[7] E. LEETE, *Tetrahedron Letters* 4433 (1968).
[8] K. MOTHES and H. R. SCHÜTTE, in *Biosynthese der Alkaloide*, p. 240, VEB Deut. Verlag der Wissen. Berlin (1969).
[9] R. F. DAWSON, *J. Am. Chem. Soc.* **73,** 4218 (1951).
[10] E. LEETE, *J. Am. Chem. Soc.* **89,** 7081 (1967).
[11] F. GALINOVSKY, A. WAGNER and R. WEISER, *Monatsch. Chem.* **82,** 551 (1951).

TABLE 1

Compound fed	*Nicotiana tabacum*		*Nicotiana glauca*	
	N-Methyl-[2-^{14}C]-Δ^1-piperideinium chloride	DL-[2-^{14}C]-Lysine monohydrochloride*	*N*-Methyl-[2-^{14}C]-Δ^1-piperideinium chloride	DL-[2-^{14}C]-Lysine monohydrochloride
mmol	0·526	0·102	0·263	0·028
Specific act. (dpm/mmol)	$5{\cdot}6 \times 10^7$	$2{\cdot}19 \times 10^9$	$5{\cdot}6 \times 10^7$	$2{\cdot}12 \times 10^9$
Total act. (dpm)	$2{\cdot}95 \times 10^7$	$2{\cdot}16 \times 10^8$	$1{\cdot}47 \times 10^7$	$5{\cdot}93 \times 10^7$
Method of feeding	Hydroponics	Hydroponics	Hydroponics	Wick
Length of feeding (day)	5	5·5	5	8
Activity not absorbed by plant (%)	12·5	3·4	15·1	1·4
Fresh wt. of plants (g)	440	510	70	415
Activity of aqueous extract (% of amount absorbed)	30	16	14	14·8
Activity in crude alkaloids (%)	18·8	0·033	18·9	16·1
Anabasine				
wt (mg)	2·53	Not isolated	31·3	132
activity (dpm/mmol)	$2{\cdot}1 \times 10^7$		$7{\cdot}8 \times 10^5$	$4{\cdot}4 \times 10^6$
Absolute inc (%)	1·28		1·21	6·4
Specific inc (%)	38		1·4	0·21
N-Methylanabasine				
wt (mg)	1·79	32·7†	0·27	35·5†
activity (dpm/mmol)	$5{\cdot}7 \times 10^7$	$1{\cdot}9 \times 10^3$	$5{\cdot}8 \times 10^7$	$3{\cdot}1 \times 10^3$
Absolute inc (%)	2·25	0·0002	0·71	0·001
Specific inc (%)	102	—	104	—
Nicotine				
wt (mg)	90·8	77·7	Not isolated	Not isolated
activity (dpm/mmol)	$1{\cdot}7 \times 10^4$	$1{\cdot}3 \times 10^4$		
Absolute inc (%)	0·037	0·003		
Specific inc (%)	0·03	0·006		

* Purchased from Tracerlab, Waltham, Mass.

† This is the amount of cold (−)-*N*-methylanabasine added to the harvested plants at the time when they were macerated with $CHCl_3$, and the absolute incorporation is based on this weight.

obtained, the specific incorporation into *N*-methylanabasine again being 100%. Although *N*-methylanabasine has been reported in *N. tabacum* in minute quantities,[12] we have been unable to detect any by GLC in the crude alkaloids of *N. tabacum* or *N. glauca* which had not been fed the piperideinium salt XI. We thus consider that the formation of *N*-methylanabasine in the current feeding experiments represents an aberrant synthesis from an unnatural precursor. The 100% specific incorporation of XI into *N*-methylanabasine is consistent with this idea. The formation of (−)-*N*-methylanabasine, having the same

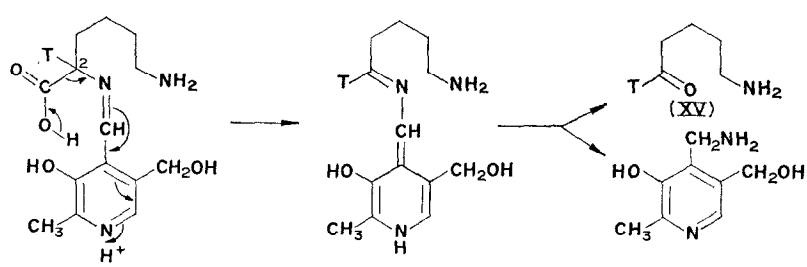
[12] E. SPÄTH and F. KESZTLER, *Chem. Ber.* **70**, 2450 (1937).

configuration at C-2′ as natural (−)-nicotine and (−)-anabasine,[13] strongly suggests that this aberrant synthesis is being controlled by the enzymes which catalyse the formation of nicotine and anabasine. Rapoport[14] has also discovered that *Nicotiana* plants are capable of aberrant syntheses. It was found that methyl derivatives of *N*-methyl-Δ^1-pyrrolinium chloride, an established precursor of the pyrrolidine ring of nicotine,[10,15,16] afforded 'unnatural' methylnicotines when fed to *N. glutinosa*.

The radioactive *N*-methylanabasine was expected to have all its activity located at C-2′, and degradations confirmed this. Oxidation of the alkaloid with nitric acid yielded nicotinic acid having the same specific activity. The pyridine obtained by decarboxylation of the nicotinic acid had negligible activity. The radioactive anabasine was similarly shown to have all its activity located at C-2′. The active anabasine obtained in the present experiments could be formed by the demethylation of the *N*-methylanabasine, or possibly from Δ^1-piperideine resulting from demethylation of the administered *N*-methyl-Δ^1-piperideinium salt.

The potential role of *N*-methylanabasine in the biosynthesis of anabasine was also investigated by feeding DL-[2-^{14}C]-lysine to both *N. tabacum* and *N. glauca*. When the alkaloids were being isolated, non-radioactive *N*-methylanabasine was added in order to trap any radioactive *N*-methylanabasine which might have been formed as an intermediate *en route* to anabasine. In both experiments the reisolated *N*-methylanabasine had negligible activity. The lysine was fed to the *N. glauca* plants by the wick method and the incorporation of activity into anabasine was surprisingly high (6·4%), compared with the incorporation obtained (0·046%) when lysine was administered to the roots of intact plants growing in a nutrient solution.[1] All the activity in the anabasine derived from the [2-^{14}C]-lysine was located at C-2′.

Our results thus do not substantiate Spenser's hypothesis for the mode of incorporation of [6-^{14}C, 2-^{3}H]-lysine into anabasine and other piperidine alkaloids.[17] We therefore suggest that [2-^{3}H]-lysine can be converted to Δ^1-piperideine with retention of the tritium



(XIII) (XIV) (XVI)

SCHEME 2.

[13] J. C. CRAIG and S. K. ROY, *Tetrahedron* **21**, 401 (1965).
[14] M. L. RUEPPEL and H. RAPOPORT, *J. Am. Chem. Soc.* **93**, 7021 (1971).
[15] T. KISAKI, S. MIZUSAKI and E. TAMAKI, *Arch. Biochem. Biophys.* **117**, 677 (1966).
[16] S. MIZUSAKI, T. KISAKI and E. TAMAKI, *Plant Physiol.* **43**, 93 (1968).
[17] SPENSER has also abandoned the idea that ϵ-*N*-methyllysine is an intermediate between lysine and piperidine alkaloids such as sedamine. ϵ-*N*-Methyllysine was detected in *Sedum acre* plants (E. LEISTNER, R. N. GUPTA and I. D. SPENSER, *Abstr. 10th Ann. Symp. Phytochem. Soc. N. Am.*, and private communication). However, when [*methyl*-^{14}C]-methionine was fed to *S. acre* plants along with [^{3}H]-lysine the resultant labelled ϵ-*N*-methyllysine had a ^{3}H/^{14}C ratio quite different from the sedamine, indicating that the ϵ-*N*-methyllysine could not be serving as a precursor of the alkaloid.

by the mechanism illustrated in Scheme 2. The Schiff base XIII formed from lysine and pyridoxal could undergo a decarboxylation yielding XIV, which on hydrolysis affords 5-aminopentanal (XV), the open chain form of Δ^1-piperideine, and pyridoxamine (XVI). By such a mechanism the tritium originally at C-2 in lysine, is retained in the aldehyde XV.

EXPERIMENTAL

General methods. Radioactive compounds were assayed in duplicate in a Nuclear Chicago Mark II Liquid Scintillation Counter, using as solvents either dioxane–ethanol or toluene, with the usual scintillators.[18] Microanalyses were determined by the Clark Microanalytical Laboratories, Urbana, Illinois.

N-*Methyl*-[2-^{14}C]-Δ^1-*piperideinium chloride* (XI). Potassium [^{14}C]-cyanide (Amersham/Searle) (39·5 mg, 0·5 mCi) was dissolved in H_2O (0·2 ml) and added to a solution of 1-bromo-4-chlorobutane (206 mg) in EtOH (0·5 ml), and the mixture refluxed for 4 hr. H_2O (2 ml) was then added and the mixture extracted with $CHCl_3$. The dried ($CaCl_2$) extract was evaporated (below 20°) and the residue distilled (100°, 0·1 mm). The distillate (169 mg) was dissolved in EtOH (3 ml) which had previously been saturated with HCl gas. H_2O (0·05 ml) was added and the mixture refluxed overnight. Excess H_2O was removed by azeotroping off with benzene. The residual oil obtained on evaporation of the solvent was distilled (90°, 0·01 mm) affording ethyl-5-chlorovalerate (64 mg). This ester was heated in a sealed tube with benzene (1 ml) and $MeNH_2$ (0·3 ml) at 120° for 18 hr. Non-radioactive *N*-methyl-2-piperidone (237 mg) was added to the reaction mixture, which was filtered, dried ($MgSO_4$), and distilled yielding *N*-methyl-2-piperidone (267 mg) as a colorless oil. This compound (2·36 mmol) was dissolved in dry ether (10 ml) and an ether solution of $LiAlH_4$ (0·12 M, 0·5 ml = 0·6 mmol) added, and the mixture refluxed for 1 hr. Aqueous 10% NaOH (10 ml) was added and the ether layer extracted with N HCl (5 × 10 ml). This acidic aqueous layer (2·2 × 10^8 dpm) was evaporated and the residue dissolved in a little ethanol and chromatographed on a 20 × 20 × 0·2 cm plate of Silica gel P254 (Merck), developing with $CHCl_3$–MeOH–conc. NH_4OH(150:30:2). Three zones were detected (with I_2), corresponding to *N*-methylpiperidine (R_f 0·48), *N*-methyl-Δ^1-piperideinium salt (0·75), and *N*-methyl-2-piperidone (0·90). Radioactivity was distributed between these compounds in the ratio of 15:64:21, respectively. The middle zone was scraped off and extracted several times with cold EtOH. The combined extracts were acidified with HCl and evaporated to dryness yielding *N*-methyl-[2-^{14}C]-Δ^1-piperideinium chloride (1·1 × 10^8 dpm) as a colorless hygroscopic solid. The top zone of the TLC plate which contained *N*-methyl-2-piperidone was extracted with ether and the solution reduced with excess $LiAlH_4$. After addition of water, the ether layer was extracted with HCl. This acid extract was evaporated to dryness, made basic with NaOH and extracted with ether. The dried ($MgSO_4$) extract was evaporated and the residue treated with an ethanolic solution of picric acid, when yellow needles of the picrate of *N*-methylpiperidine separated, m.p. 224–225°, (lit.[19] m.p. 227°), having an activity of 5·6 × 10^7 dpm/mmol.

(−)-N-*Methylanabasine.* (−)-Anabasine (Fluka AG, Buchs) (2·0 g) was heated on a steam bath with 90% HCO_2H (5 ml) and 40% HCHO (15 ml) for 1 hr. The solution was evaporated to small bulk and made alkaline with KOH solution. The mixture was extracted with ether, dried ($MgSO_4$), evaporated and distilled (110°, 0·01 mm) affording (−)-*N*-methylanabasine as a colorless oil (1·9 g). TLC on Silica gel G in CCl_4–*n*-PrOH–EtOH–conc. NH_4OH (100:25:1:1) indicated that the product was free of anabasine. R_fs were *N*-methylanabasine (0·82), nicotine (0·81), anabasine (0·60), nornicotine (0·28). The alkaloids were detected with I_2 vapor. (−)-*N*-Methylanabasine diperchlorate was obtained as colorless needles from EtOH–EtOAc, m.p. 228–229°. *Anal.* Calc. for $C_{11}H_{16}N_2.2HClO_4$: C, 35·03; H, 4·81; N, 7·48. Found: C, 34·98; H, 5·10; N, 7·37%. The ORD curve was determined on a Cary Model 60 spectropolarimeter using 2 cm cells at 25°: (*c*, 0·0094 g/100 ml 95% EtOH) $[\alpha]_{300}$ −1040°, $[\alpha]_{274}$ −3080° (trough), $[\alpha]_{269}$ +210° (peak), $[\alpha]_{266}$ −430° (trough), $[\alpha]_{262}$ +3190° (peak), $[\alpha]_{259}$ +1600° (trough), $[\alpha]_{256}$ +2440° (peak), $[\alpha]_{247}$ 0°. This ORD curve is very similar to that of (−)-nicotine.[13]

Administration of the labelled compounds to Nicotiana *and isolation of the alkaloids.* The *N. tabacum* and *glauca* plants were 2–3 months old at the time of feeding. The hydroponic nutrient solution in which the roots of the plants were growing was aerated.[1] In experiment 4 where the [2-^{14}C]-lysine was administered by the wick method, small (5 ml) beakers were attached to the stems of the plants by means of adhesive tape, and cotton wicks inserted through the stems just above the beakers. The harvested plants were macerated in a Waring Blendor with $CHCl_3$-conc. NH_3. The mixture was filtered and the activity of the aqueous layer determined (see Table 1). The $CHCl_3$ layer was evaporated in the presence of 2 N HCl. This acid solution was then filtered through Celite to remove tarry material, and then made basic with NaOH and extracted several times with CH_2Cl_2. Evaporation of the dried (Na_2SO_4) extract yielded the crude alkaloids, their activity being recorded in Table 1. The alkaloids were separated by GLC in a Varian Aerograph, Model A90P with a thermal conductivity detector. A 300 × 0·64 cm stainless steel column packed with 10% Carbowax 20 M on 60–80 Chromosorb W (KOH washed) was used with a He flow rate of 50 ml/min at a temperature of

[18] A. R. FRIEDMAN and E. LEETE, *J. Am. Chem. Soc.* **85**, 2141 (1963).
[19] R. LUKES, *Coll. Czech Chem. Commun.* **12**, 71 (1947).

179°. The following retention times (min) were obtained: nicotine (8·0), *N*-methylanabasine (11·3), nornicotine (19·3), anabasine (20·9). The alkaloids were collected in cooled U-tubes, and their weights determined by UV spectroscopy, comparing with known standards. After suitable dilutions, if necessary, solid derivatives (dipicrates, diperchlorates) were prepared and crystallized to constant activity. These activities are recorded in Table 1. The *N*-methylanabasine isolated in experiments 1 and 3 was found to have the same ORD curve as authentic (−)-*N*-methylanabasine. A portion of the *N*-methylanabasine from experiment 1 was diluted to afford *N*-methylanabasine diperchlorate with a specific activity of $1{\cdot}50 \times 10^5$ dpm/mmol.

Degradation of the alkaloids. (−)-*N*-Methylanabasine diperchlorate (641 mg, $1{\cdot}50 \times 10^5$ dpm/mmol) was heated on a steam bath with conc. HNO_3 (10 ml) and a few drops of EtOH for 18 hr. The solution was evaporated to dryness, redissolved in H_2O, and the pH adjusted to 5·0. Cupric acetate solution was added and the resultant copper nicotinate separated. This salt was suspended in H_2O and decomposed with H_2S. The filtered solution was evaporated to yield nicotinic acid, purified by sublimation and crystallization from EtOH (98 mg, $1{\cdot}45 \times 10^5$ dpm/mmol). The nicotinic acid was decarboxylated by heating with calcium oxide, the resultant pyridine being collected and assayed as its oxalate ($<0{\cdot}01 \times 10^5$ dpm/mmol). The anabasine isolated from experiment 4 ($3{\cdot}6 \times 10^5$ dpm/mmol) was similarly oxidized with HNO_3 affording nicotinic acid ($3{\cdot}8 \times 10^5$ dpm/mmol) from which pyridine oxalate ($<0{\cdot}04 \times 10^5$ dpm/mmol) was obtained.

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