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SPERMIDINE: AN INDIRECT PRECURSOR OF THE PYRROLIDINE RINGS OF NICOTINE AND NORNICOTINE IN *NICOTIANA GLUTINOSA**

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Key Word Index—*Nicotiana glutinosa*, Solanaceae, biosynthesis, nicotine, nornicotine, spermidine, putrescine

Abstract—Spermidine, which was labeled asymmetrically in its four-carbon moiety ([6-^{14}C]-1,5,10-triazadecane), was administered to *Nicotiana glutinosa* plants After 7 days the plants were harvested, yielding radioactive nicotine (0 43 % incorporation) and nornicotine (0 07 % inc) A systematic degradation of the alkaloids indicated that they were labelled equally at C-2′ and C-5′ of their pyrrolidine rings These results are consistent with the hypothesis that spermidine is degraded to putrescine prior to its incorporation into the pyrrolidine rings of nicotine and nornicotine

INTRODUCTION

Putrescine (**2**), spermidine (**3**) and spermine (**1**) have been detected in many different species of plants [1, 2] including *Nicotiana* [3, 4], and it is now recognized that the polyamines function in plants and animals as cations, stabilizing and regulating the nucleic acids which are involved in cell division [5] Putrescine is a biosynthetic precursor of the four-carbon unit found in spermidine and spermine [5] It is also a well-established precursor of the pyrrolidine ring of nicotine (**9**) [6–8], the generally accepted route to nicotine being via *N*-methylputrescine (**4**), 4-methylaminobutanal (**6**) and the *N*-methyl-Δ^1-pyrrolinium salt (**8**) Extensive work with isotopically labeled precursors and the isolation of enzymes which catalyse these steps favor this pathway [9] However, since nicotine is mainly produced in the rapidly developing root cells [10], I considered that spermidine and nicotine formation in *Nicotiana* might be intimately related Such a hypothesis is illustrated in Fig 1 It is proposed that spermidine is methylated to the *N*-methylspermidine (**5**) An oxidation then affords the Schiff base (**7**) which on hydrolysis yields 4-methylaminobutanal, the established precursor of the iminium salt (**8**) This hypothesis has now been tested by feeding spermidine labeled with ^{14}C at C-6 to *Nicotiana glutinosa* The incorporation of this labeled spermidine by the metabolic pathway in Fig. 1 would yield nicotine and nornicotine (**10**) labeled at their C-2′ positions

RESULTS AND DISCUSSION

The [6-^{14}C]spermidine (the isotopic numbering is based on the systematic naming of spermidine as 1,5,10-triazadecane) was prepared from commercially available [1-^{14}C]-4-aminobutanoic acid (**11**) by the route illustrated in Fig 2 The last two steps in this sequence are the same as these used by Robins [11] for the synthesis of [2-^{14}C]spermidine (1,5,10-triazadecane numbering) The *N*-benzyloxycarbonyl derivative of 4-aminobutanoic acid (**12**) was condensed with 3-aminopropionitrile in the presence of a water-soluble carbodiimide to yield the amide (**13**) which was obtained as a crystalline solid This compound was previously described as a thick oil [11] A brief hydrogenation in the presence of palladium on charcoal removed the benzyloxycarbonyl group, affording (**14**) which was reduced with borane in tetrahydrofuran to yield [6-^{14}C]spermidine (**3**) isolated as its trihydrochloride salt

This labeled spermidine was fed to *Nicotiana glutinosa* plants by the wick method After 7 days, radioactive nicotine and nornicotine were isolated from the plants The nornicotine had a lower specific activity than the nicotine, a result which is consistent with previous work, in which it has been shown that the main route to nornicotine is by the demethylation of nicotine [12–14] Degradation of the labeled nicotine, obtained from the plants which had been fed the radioactive spermidine, yielded products whose activity was consistent with equal activity at the C-2′ and C-5′ positions of the pyrrolidine ring The lower specific activity of the nornicotine did not permit a complete degradation, however, half of its activity was found at its C-2′ position, a result consistent with equal activity at the C-2′ and C-5′ positions

These results indicate that the hypothetical formation of nicotine from putrescine via spermidine is untenable The most reasonable explanation of these results is that the [6-^{14}C]spermidine is metabolized to [1-^{14}C]putrescine Since this molecule is symmetrical, the iminium salt (**8**) derived from this labeled compound via *N*-methylputrescine will be labeled equally at the C-2 and C-5 positions. There is some evidence that spermidine can be metabolized to putrescine in higher plants When spermidine was fed to barley seedlings, an increase in the putrescine level was observed [15], although it was suggested that this result was due to a mass action effect,

*Part 49 in the series "Tobacco Alkaloids and Related Compounds" For Part 48, see Leete, E (1984) *Beitr Tabakforsch Int* **12**, 113

†Contribution No 192 from this laboratory This paper is dedicated to Michael Edward Leete who was born on the same day that the spermidine was fed to the tobacco

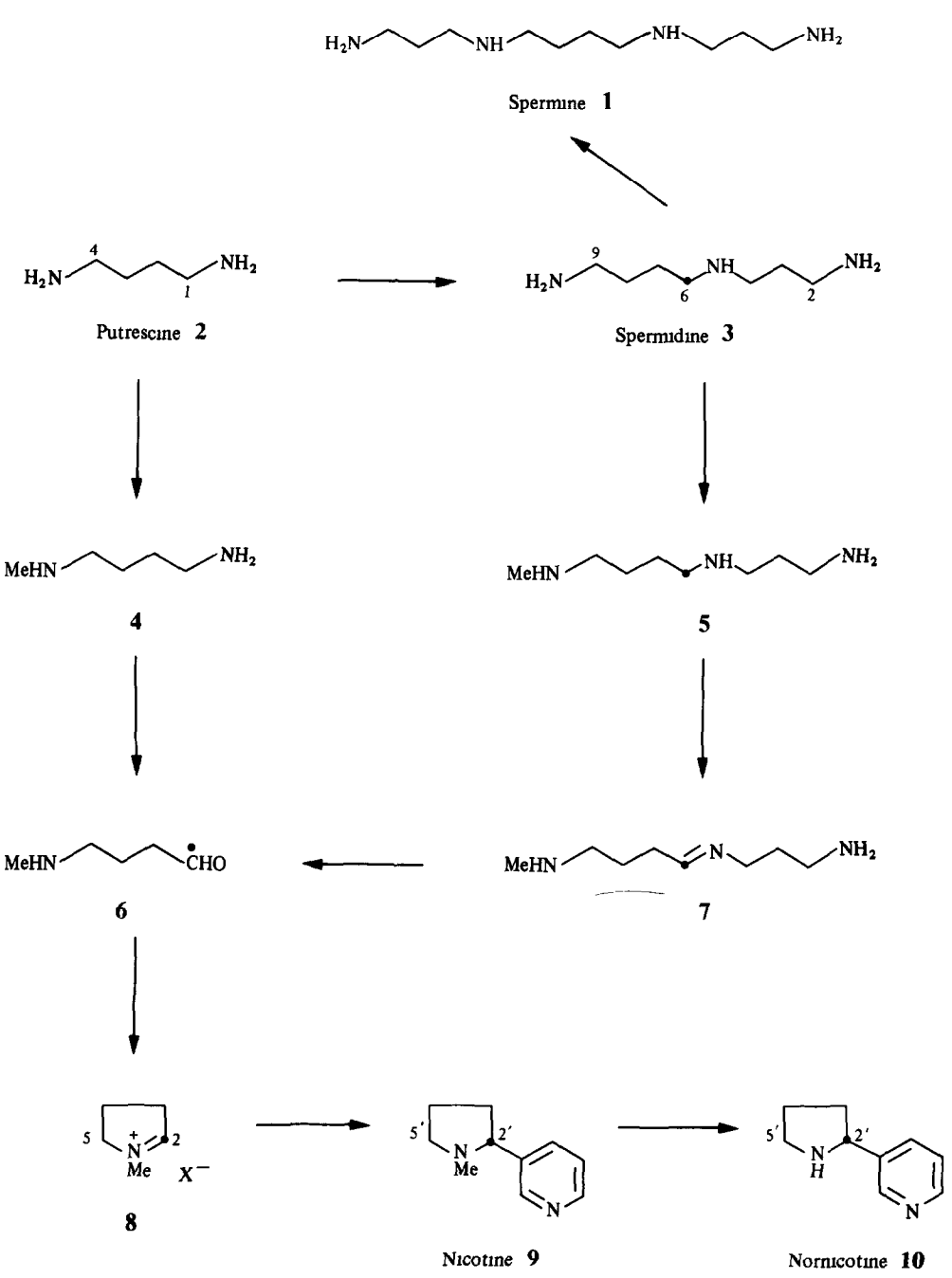

Fig 1 Hypothetical formation of the pyrrolidine ring of nicotine via spermidine

the synthesis of spermidine from putrescine being inhibited by the excess spermidine Putrescine is a well-established precursor of the pyrrolizidine base, retronecine, in *Senecio* species, but it was discovered that [6,9-^{14}C]spermidine is also an excellent precursor of retronecine [11, 16] This result is also consistent with the labeled spermidine being metabolized to [1,4-^{14}C]putrescine prior to incorporation into retronecine

EXPERIMENTAL

General Mps are corr MS were determined by Dr Edmund Larka on an AE1-30 spectrometer ^{13}C NMR were determined on a Nicolet 300 spectrometer in $CDCl_3$ with TMS as an int standard by Dr Stephen B Philson Radioactive materials were assayed in a Tracor Analytic Mark III liquid scintillation counter using dioxane–EtOH as a solvent with the usual scintillators [17] Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ Seeds of *Nicotiana glutinosa* were obtained from Dr Walter Gannon of the Philip Morris Research Center, Richmond, VA

[6-^{14}C]*Spermidine* (3) [1-^{14}C]-4-Aminobutanoic acid (515 mg, 5 mmol, nominal activity 0 1 mCi) (Research Products International Corp) was dissolved in a mixture of H_2O (2 ml),

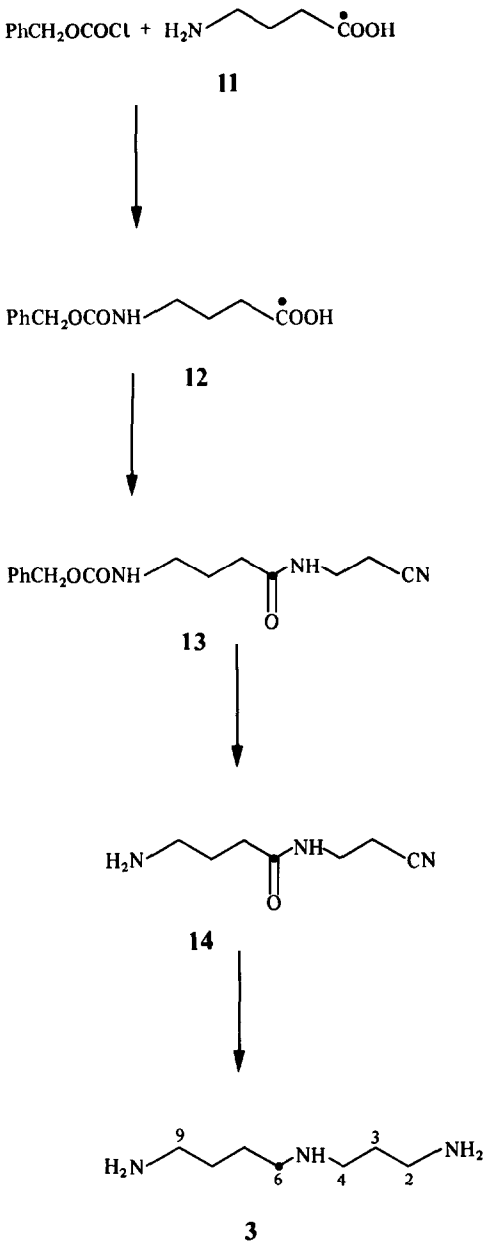

Fig 2 Synthesis of [6-^{14}C]spermidine (^{14}C •)

Me_2CO (2 ml) and 10%NaOH (3 ml) and cooled to 0° Benzyl chloroformate (1 35 g, 7 9 mmol) dissolved in Me_2CO (2 ml) was slowly added during 1 hr to the stirred reaction mixture along with 10% NaOH so that a pH of 10–11 was maintained The mixture was then stirred at room temp for 16 hr Water (20 ml) was then added to the reaction mixture, which was then partially evapd on a rotary evaporator to remove Me_2CO The residual aq soln was extracted with Et_2O which was discarded The clear aq soln was then acidified with HCl when *N*-benzyloxycarbonyl-4-aminobutanoic acid separated (0 720 g, 61 %), mp 64–65 (lit [18] mp 65 5–67°) 3-Aminopropionitrile was obtained by a continuous Et_2O extraction of an aq soln of the fumarate salt of 3-aminopropionitrile (Aldrich Chem Co) which had been made basic with NaOH Evaporation of the Et_2O extract (without drying) yielded 3-aminopropionitrile as a colorless mobile liquid This wet nitrile (0 6 ml, about 7 mmol) was added to a suspension of *N*-benzyloxycarbonyl-4-aminobutanoic acid (0 72 g, 3 0 mmol) in H_2O (10 ml) 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1 0 g, 5 2 mmol) was added to the clear soln, which after standing for 1 hr deposited crystals of 4-[(benzyloxycarbonyl)-amino]-*N*-cyanoethylbutanamide (**13**) (0 450 g, 52%) Crystallization from C_6H_6 afforded colorless rhombic plates, mp 104–105° IR (KBr pellet) 2255 (CN), 1690 (–OCONH–), 1650 cm^{-1} (–CONH–) MS (20 eV) *m/z* (rel abundance) 290 (0 4) M + 1, 246 (0 4), 182 (2 8), 154 (3 8), 108 (7 1), 91 (100) ^{13}C NMR δ 173 4 (–NHCO–), 156 9 (–OCONH–), 136 4, 128 5, 128 1, 127 9, 118 4 (CN), 66 6, 40 2, 35 5, 33 2, 26 0, 18 2 ppm Anal Calc for $C_{15}H_{19}N_3O_3$ C, 62 27, H, 6 62, N, 14 52 Found C, 62 03, H, 6 61, N, 14 59

The nitrile (**13**) (0 35 g, 1 21 mmol) was dissolved in MeOH (10 ml) and hydrogenated at 2 atm pres in the presence of 10% Pd–C (50 mg) for 1 hr The filtered soln was evapd to afford **14** as a colorless oil (170 mg) This material in THF (20 ml) was refluxed with stirring with M BH_3 in THF (9 ml) for 18 hr The clear soln was evapd, the residue dissolved in EtOH (10 ml) and HCl gas passed into the soln when [6-^{14}C]spermidine trihydrochloride separated (165 mg, 54%) It was purified by sublimation (240°, 10^{-4} mm) affording material with a sp act 4 69 × 10^7 dpm/mmol TLC on SiO_2, developing with a mixture of 1-butanol, pyridine, water, and 40% HCHO (70 30 20 10) [19], indicated that all the radioactivity was coincident with authentic spermidine (R_f 0 5)

Administration of [6-^{14}C]*spermidine to* Nicotiana glutinosa *plants and isolation of the alkaloids* [6-^{14}C]Spermidine 3HCl (124 4 mg, 0 49 mmol, 2 30 × 10^7 dpm) dissolved in H_2O (20 ml) was divided equally between 10 *N. glutinosa* plants (3 months old, 15–20 cm tall) growing in soil in a greenhouse (23 March 1984) The feeding was carried out by the wick method Two days after the initial feeding, some of the leaves, near the site of insertion of the cotton wicks, wilted However, most of the plants remained healthy and they were harvested 7 days after the initial feeding (fr wt 310 g) (residual activity not absorbed by the plants 0 02%) The plants were chopped up in a Waring Blender with a mixture of $CHCl_3$ (2 l) and conc NH_3 (200 ml) The aq ammoniacal layer contained 20 5% of the activity fed The crude alkaloids (3 10 × 10^6 dpm, 13 5% incorporation) were separated by TLC as previously described [20] Radioactivity was present at positions coincident with nicotine and nornicotine, but the bulk of the activity was near to the origin where spermidine is located in the TLC system used (SiO_2 with $CHCl_3$–EtOH–conc NH_3 (90 10 1) as the developing solvent) Preparative TLC afforded nicotine (161 2 mg, 9 85 × 10^4 dpm/mmol, 0 43% absolute inc) and nornicotine (74 mg, 3 26 × 10^4 dpm/mmol, 0 07% inc) The alkaloids were crystallized to constant activity as their dipicrates

Degradations of the alkaloids Oxidation of the nicotine (9 85 × 10^4 dpm/mmol) with nitric acid as previously described [21] afforded a mixture of nicotinic acid (4 59 × 10^4 dmp/mmol, 47%) and 3-nitro-5-(3'-pyridyl)pyrazole (4 68 × 10^4 dpm/mmol, 48%) The nicotinic acid was heated with CaO affording pyridine, assayed as its picrate (< 0 1 × 10^4 dpm/mmol) The nornicotine (3 26 × 10^4 dpm/mmol) was oxidized with $KMnO_4$ to afford nicotinic acid (1 59 × 10^4 dpm/mmol, 49%) This nicotinic acid afforded essentially inactive pyridine on pyrolysis with CaO

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