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FORMATION OF *N'*-ETHYL-*S*-NORNICOTINE BY TRANSFORMED ROOT CULTURES OF *NICOTIANA RUSTICA*

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IN HONOUR OF PROFESSOR JEFFREY HARBORNE'S SIXTY-FIFTH BIRTHDAY

Key Word Index—*Nicotiana rustica*; Solanaceae; analogue biosynthesis; alkaloid; *N'*-ethyl-*S*-nornicotine; nicotine.

Abstract—*N*-Ethylputrescine dihydrochloride has been synthesized by an improved procedure and it is converted by transformed root cultures of *Nicotiana rustica* into the nicotine analogue, *N'*-ethyl-*S*-nornicotine, preferentially in the optically active *S*-form, with an efficiency similar to that of the corresponding natural process.

INTRODUCTION

Natural products such as nicotine with useful biological activity are often more easily obtained from natural sources than by total synthesis. *S*-Nicotine is biosynthesized from nicotinic acid (**1**) and *N*-methylputrescine (**2**) and is a feeding deterrent for many animals, and an insecticide[1]. The conversion of *S*-nicotine into analogues for biological evaluation is limited by the functional groups present in the compound. A different approach is to study the formation of nicotine analogues from unnatural precursors. For this strategy to be successful, the precursor analogue should be easy to prepare and the biosynthesis should be efficient in a system that is easy and reliable to use. We report the biosynthesis of a nicotine analogue which fulfils these criteria.

Root cultures of *Nicotiana rustica* transformed with *Agrobacterium rhizogenes* have been shown to produce mainly *S*-nicotine (**5**), with smaller quantities of anabasine (**9**), nornicotine (**6**) and anatabine (**8**). Addition of cadaverine (**4**) to these transformed root cultures stimulates the formation of anabasine (**9**) [2]. We have used cadaverines stereospecifically labelled with deuterium to establish the stereochemistry of the oxidation of cadaverine which leads to 1-piperideine and to show that equal amounts of (*R*)- and (*S*)-anabasine are produced on coupling with nicotinic acid (**1**) [3]. Leete has shown that feeding 5-fluoronicotinic acid to different *Nicotiana* plant species results in the formation of small quantities of 5-fluoroanabasine [4] and 5-fluoronicotine [5]. We now find that addition of *N*-ethylputrescine (**3**) dihydrochloride to *Nicotiana rustica* root cultures stimulates the formation of a new metabolite, *N'*-ethyl-*S*-nornicotine (**7**), preferentially in the optically active *S*-form, in reasonable yield.

RESULTS AND DISCUSSION

N-Ethylputrescine (**3**) dihydrochloride was prepared by an improved [6] procedure (Scheme 1). The amino group of 4-aminobutanoic acid was protected as the *N*-t-butoxycarbonyl derivative (**10**), then the acid was converted into the *N*-ethylamide via the mixed anhydride. Careful acid hydrolysis selectively removed the *N*-t-butoxycarbonyl protecting group to afford the amine hydrochloride (**12**). Reduction of the amide (**12**) followed by acidification gave *N*-ethylputrescine (**3**) dihydrochloride. This was shown to be a substrate for pea seedling diamine oxidase in earlier work [6]. When *N*-ethylputrescine dihydrochloride was fed to transformed root cultures of *Nicotiana rustica* at 1 mM concentration, a new alkaloid analogue was produced in 6% yield (i.e. *ca* 30 mg was obtained from the roots present in 3 l of culture medium). This compound was separated by preparative TLC or HPLC from *S*-nicotine (**5**) and accurate mass data, together with ^{1}H and ^{13}C NMR spectroscopic evidence, and comparison with literature spectroscopic data confirmed the formation of *N'*-ethylnornicotine (**7**). The material was optically active, $[\alpha]_D^{13} - 104.6°$ (cf. *S*-nicotine (**5**) $[\alpha]_D^{25} - 158.0°$). Furthermore, the CD curve of *N'*-ethylnornicotine (**7**) was almost superimposable on that of natural nicotine indicating that both compounds have the same absolute configuration (*S*). Since *N*-ethylputrescine (**3**) is known to be a reasonable substrate for pea seedling diamine oxidase, it is assumed that it competes with the normal metabolite, *N*-methylputrescine, for the enzyme *N*-methylputrescine oxidase which is involved in

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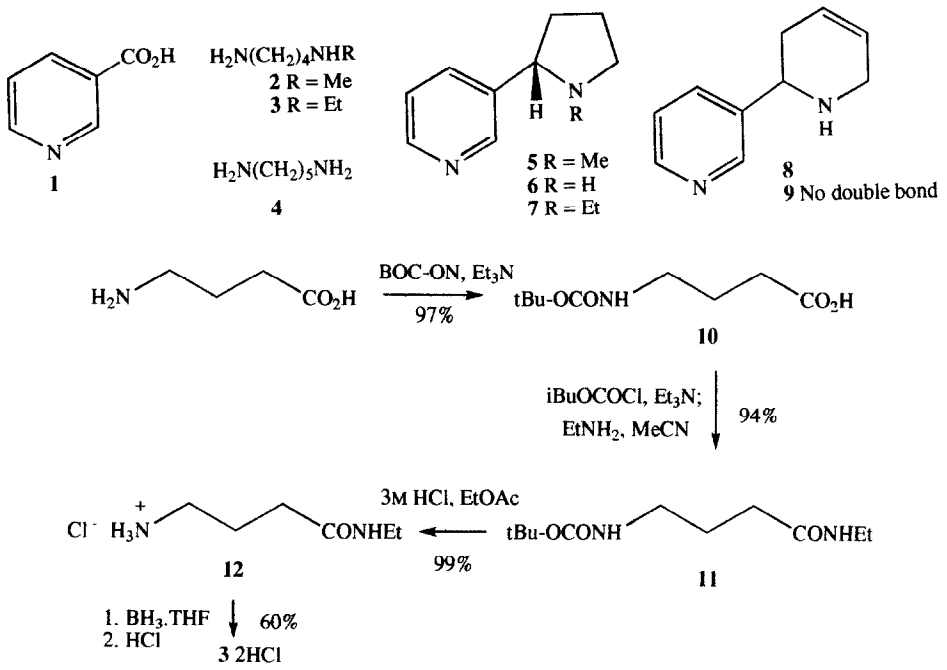

Scheme 1. Synthesis of *N*-ethylputrescine dihydrochloride.

nicotine biosynthesis [7]. It appears that the new metabolite is formed in response to the addition of *N*-ethylputrescine (**3**). No detectable amounts of *N'*-ethyl-*S*-nornicotine (**7**) were found when the cultures were grown on normal culture media. *N'*-Ethylnornicotine (**7**) has only been detected previously in tobacco [8] and tobacco smoke [9, 10]. The ability of transformed root cultures of *Nicotiana rustica* to effect the chemically demanding steps in the synthesis of other analogues of nicotine from different analogues of putrescine is currently under investigation.

EXPERIMENTAL

General. Mp: uncorr. ^{1}H NMR (90 and 200 MHz) and ^{13}C NMR spectra (50.3 MHz) were recorded in $CDCl_3$ (with TMS as int. standard) or D_2O solns. EIMS were obtained with direct inlet 70 eV. TLC was carried out on Merck Kieselgel 60 F_{254} precoated plates. Spots were detected by the modified Dragendorff reagent [11].

4-t-Butoxycarbonylaminobutanoic acid (**10**). A soln of BOCON (9.257 g, 37.6 mmol) in 1,4-dioxane (30 ml) and de-ionized H_2O (30 ml) was added to a stirred soln of 4-aminobutanoic acid (3.198 g, 31 mmol) in distilled Et_3N (9.395 g, 93 mmol). The reaction mixt. rapidly became homogenous. Stirring was continued at room temp. for 2 hr, H_2O (40 ml) was added, then EtOAc (50 ml). The aq. layer was sepd and washed with EtOAc (2 × 50 ml). The aq. layer was acidified with 5% citric acid soln then extracted with EtOAc (5 × 50 ml). The organic extracts were combined, dried (Na_2SO_4), filtered and the solvent removed *in vacuo* to give 4-t-butoxycarbonylaminobutanoic acid (**10**, 6.125 g, 30.2 mmol, 97%, mp 56–57°). IR ν_{max}^{KBr} cm^{-1}: 3360, 1710, 1680. ^{1}H NMR (200 MHz, $CDCl_3$):δ1.45 (9H, *s*, 3 × Me), 1.82 (2H, *m*, H-3), 2.39 (2H, *t*, J = 7.1 Hz, H-2), 3.18 (2H, *m*, H-4), 9.97 (1H, *s*, CO_2H). ^{13}C NMR (50 MHz): δ 25.1 (C-3), 28.4 (3 × Me), 31.3 (C-2), 39.8 (C-4), 79.5 (CMe_3), 156.3 (CONH), 178.2 (CO_2H). MS *m/z* (rel. int.):147 (16), 130 (9), 102 (10), 57 (100).

N-Ethyl-4-t-Butoxycarbonylaminobutanamide (**11**). 4-t-Butoxycarbonylaminobutanoic acid (**10**, 1.302 g, 6.41 mmol) and Et_3N (0.753 g, 7.46 mmol) were cooled to −5° (ice–MeOH bath) in acetonitrile (50 ml). i-Butylchloroformate (1.045 g, 7.68 mmol) was added dropwise with stirring and the mixt. was left for 3 min, then $EtNH_2$ (1.174 g, 26 mmol) was added dropwise. The resulting soln was left at 0° for 2 hr. The solvent was removed and the resulting solid was partitioned between EtOAc (25 ml) and H_2O (25 ml). The organic extracts were combined, dried (Na_2SO_4), filtered and the solvent was removed *in vacuo* to yield a yellow solid which was recrystallized from EtOAc to yield white *N*-ethyl-4-t-butoxycarbonylaminobutanamide (**11**, 1.388 g, 6.03 mmol, 94%), mp 94°. IR ν_{max}^{KBr} cm^{-1}: 3320, 3310, 1695, 1650. ^{1}H NMR (200 MHz, $CDCl_3$): δ1.14 (3H, *q*, J = 7.26 Hz, MeCH_2), 1.44 (9H, *s*, 3 × Me), 1.81 (2H, *m*, H-3), 2.22 (2H, *t*, J = 7.00 Hz, H-2), 3.25 (4H, *m*, CH_2Me and H-4), 5.13 (1H, *s*, OCONH) 6.12 (1H, *s*, CONH). ^{13}C NMR (50 MHz):δ14.9 (MeCH_2), 25.5 (C-3), 28.5 (3 × Me), 33.8 (C-2), 34.5 (CH_2Me), 39.9 (C-4), 79.3 (CMe_3), 156.8 (OCONH), 172.9 (CONH). MS *m/z* (rel. int.): 230 (0.2) [M]$^+$, 174 (10), 101 (18), 87 (100), 72 (50), 57 (66), 44 (55). HRMS *m/z* 230.1609 [M]$^+$ ($C_{11}H_{22}N_2O_3$) requires: 230.1630.

N-Ethyl-4-aminobutanamide hydrochloride (**12**). The ethylamide (**11**, 0.865 g, 3.7 mmol) was dissolved in 3 M HCl–EtOAc (1:1, 8 ml). After 30 min the solvents were removed *in vacuo* to produce a clear oil, which on trituration with diethylether yielded a white solid of *N*-

ethyl-4-aminobutanamide hydrochloride (**12**) (0.589 g, 3.54 mmol, 96%). IR ν_{max}^{KBr} cm^{-1}: 3300, 1640, 1550. ^1H NMR (200 MHz, D_2O):δ0.88 (3H, *t*, *J* = 7.33 Hz, Me), 1.72 (2H, *m*, H-3), 2.13 (2H, *t*, *J* = 7.56 Hz, H-2), 2.80 (2H, *t*, *J* = 7.37 Hz, H-4), 2.99 (2H, *q*, *J* = 7.31 Hz, $C\underline{H}_2Me$). ^{13}C NMR (50 MHz): δ14.3 (Me), 23.9 (C-3), 33.4 (C-2), 35.4 (C-4), 39.7 ($\underline{C}H_2Me$), 175.3 (C-1). MS *m/z* (rel. int.):131 $[MH]^+$ (1), 130 (1), 87 (56), 72 (90), 44 (100). HRMS *m/z* 130.1112 $[M]^+$ ($C_6H_{14}N_2O$ requires: 130.1106).

N-*Ethylputrescine* (**3**) *dihydrochloride*. To a solution of (**11**, 1.06 g, 6.37 mmol), in THF (35 ml) was added 1 M BH_3–THF (25.5ml) over 15 min at 0° under N_2. The suspension was heated at reflux for 24 hr. The flask was allowed to cool to room temp. HCl (6 M) was added slowly to the soln until no more H_2 was evolved. THF was removed by distillation at atm. pres. and H_2O was removed under red. pres. MeOH (10 ml) and conc HCl (2 drops) were added to the yellow solid in order to remove the boric acid as the borate ester *in vacuo*. The crude product was then recrystallized from MeOH to yield *N*-ethylputrescine (**3**) dihydrochloride (0.72 g, 3.81 mmol, 60%), mp 217–219° (ref. [12] 218–220°). IR ν_{max}^{KBr} cm^{-1}: 3400, 3000, 1600. ^1H NMR (200 MHz, D_2O); δ0.94 (3H, *t*, *J* = 7.32 Hz, Me), 1.40 (4H, *m*, H-2 and H-3), 2.74 (6H, *m*, H-1, H-4 and $C\underline{H}_2Me$). ^{13}C NMR (50 MHz); δ11.5 (Me), 23.7 (C-3), 24.8 (C-2), 39.7 ($\underline{C}H_2Me$), 43.8 (C-1), 47.2 (C-4). MS *m/z* (rel. int.): 116 $[M]^+$ (2), 58 (83), 30 (100). HRMS *m/z* 116.1315 $[M]^+$ (calcd for $C_6H_{16}N_2$: 116.1313).

Root cultures. Hairy roots from *N. rustica*, transformed with *Agrobacterium rhizogenes*, were set up and maintained as described [2].

Feeding experiments. *N*-Ethylputrescine hydrochloride (378 mg, 2 mmol) was fed at 1 mM concns to flasks of *N. rustica* transformed root cultures, 4 days after subculture. The alkaloids were then harvested 10 days after feeding.

Extraction of alkaloids. *Nicotiana rustica* transformed root cultures (195.44 g, dry wt) were chopped finely and extracted with MeOH at ambient temp. The MeOH soln was concd under red. pres. The residue (5.21 g) was taken up in CH_2Cl_2 (20 ml) and extracted with 1 M HCl (2 × 20 ml). The acid layers were combined and washed with CH_2Cl_2 (6 × 20 ml). The aq. layer was basified with conc NH_3 and extracted with CH_2Cl_2 (4 × 30 ml). The organic extrcts were dried (Na_2SO_4), filtered and concd under red. pres. to yield alkaloidal extract (78 mg).

Detection and isolation of N'-*ethyl*-S-*nornicotine*. *N'*-Ethyl-*S*-nornicotine was detected by both TLC (toluene–Me_2CO–MeOH–25% aq. NH_3, 8:9:4:1) R_f 0.64 (cf. nicotine R_f 0.58) and HPLC (UV detection, analytical reverse-phase C_{18} 5 μm Hypersil column 150 × 4.6 mm, 40% aq. MeOH containing 0.2% phosphoric acid buffered to pH 7.25 with Et_3N) R_t 5.94 min (cf. nicotine R_t 4.5). The isolation of *N'*-ethyl-*S*-nornicotine (20.7 mg) was carried out via prep. TLC on 0.25 mm plates, solvent system as above. IR ν_{max}^{KBr} cm^{-1}: 2980, 1580, 1030, 720. ^1H NMR (200 MHz, $CDCl_3$); δ1.02 (3H, *t*, *J* = 7.2 Hz, Me), 1.6–2.3 (6H, *m*, $C\underline{H}_2Me$ + H-4' + H-3'), 2.52 (1H, *m*, H-5'), 3.2–3.4 (2H, *m*, H-2' + H-5'), 7.25 (1H, *m*, H-5), 7.71 (1H, *d*, *J* = 7.9 Hz, H-4), 8.52 (2H, *m*, H-2 + H-6). ^{13}C NMR (50 MHz, $CDCl_3$); δ13.8 ($\underline{Me}CH_2$), 22.4 (C-4'), 35.0 (C-3'), 48.2 ($\underline{C}H_2Me$), 53.2 (C-5'), 67.5 (C-2'), 123.5 (C-5), 134.9 (C-4), 139.4 (C-3), 148.5 (C-6), 149.5 (C-2), IR, NMR and MS were similar to those reported [8]. HRMS *m/z* 176.1310 $[M]^+$ (calcd for $C_{11}H_{16}N_2$ 176.1313). $[\alpha]_D^{13}$ −104.6°($CHCl_3$, *c* 2) (cf. *S*-nicotine $[\alpha]_D^{25}$ −158.0°). CD (MeCN) $\Delta\varepsilon_{248}$ +0.65, $\Delta\varepsilon_{270}$ −1.0 [cf. *S*-nicotine CD (MeCN) $\Delta\varepsilon_{246}$ +0.70, $\Delta\varepsilon_{270}$ −1.1].

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