Phytochemistry, 1979, Vol. 18, pp. 75–78. ©Pergamon Press Ltd. Printed in England. 0031-9422/79/0101-0075$02.00/0

THE METABOLISM OF ANATABINE TO α,β-DIPYRIDYL IN *NICOTIANA* SPECIES

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(*Received* 8 *May* 1978)

Key Word Index—*Nicotiana glauca*; *N. glutinosa*; *N. tabacum*; Solanaceae; anatabine; α,β-dipyridyl; ^{13}C NMR spectroscopy; metabolism.

Abstract—α,β-Dipyridyl isolated from *Nicotiana tabacum* plants which had been fed anatabine-[2′-^{14}C, ^{13}C], and then allowed to dry in air for 20 days was radioactive (82% specific incorporation). An examination of its ^{13}C NMR spectra established that it was enriched only at C-2, indicative of its direct formation from anatabine. The labelled anatabine was also fed to *N. glauca* and *N. glutinosa* plants, which were extracted immediately after harvesting. In these experiments no radioactive α,β-dipyridyl was detected, suggesting that α,β-dipyridyl is an artifact produced by the oxidation of anatabine in the drying leaves of tobacco. Anabasine isolated from the *Nicotiana* species which had been fed anatabine-[2′-^{14}C, ^{13}C] was unlabelled, indicating that none of this alkaloid is formed by the reduction of anatabine.

INTRODUCTION

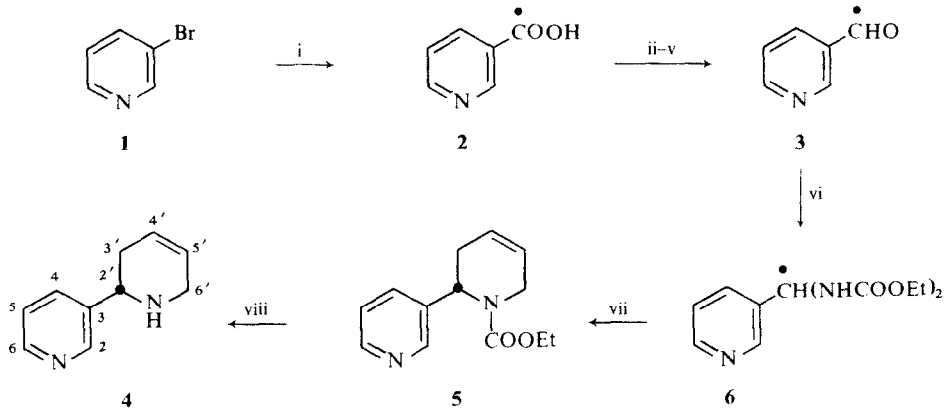
We have previously [1] shown that α,β-dipyridyl (**7**) is formed in tobacco from nicotinic acid, and preliminary work indicated that both rings are formed from this precursor. Actually there was very little incorporation of labelled nicotinic acid into α,β-dipyridyl in fresh plants. A significant incorporation was only observed when the tobacco which had been fed labelled nicotinic acid, was allowed to dry in air for several weeks before the alkaloids were extracted. We suggested that the α,β-dipyridyl was formed by the oxidation of anatabine. This process could be catalysed by enzymes, or be a non-enzymic aerial oxidation. Another plausible route to α,β-dipyridyl would be its direct formation from two molecules of nicotinic acid, not involving anatabine as an intermediate.

RESULTS AND DISCUSSION

We have tested the first hypothesis by feeding anatabine-[2′-^{14}C,^{13}C] to various species of tobacco (*N. tabacum*, *N. glauca*, and *N. glutinosa*). The anatabine was labelled with ^{14}C to facilitate the detection (by radioactive assay) of any α,β-dipyridyl which might be formed from the anatabine. Carbon-13 was also introduced at C-2′ so that specific labelling of the α,β-dipyridyl, indicative of a direct transformation, could be established by examination of its ^{13}C NMR spectrum. There are no simple degradations of α,β-dipyridyl available which could be used to establish the incorporation of anatabine labelled at a specific position with ^{14}C. The anatabine-[2′-^{14}C,^{13}C] (**4**) was prepared by the route illustrated in Scheme 1, and is based on a previously described [2] synthesis, modified for work on a small scale. Carbon-14 was introduced at C-2′ by dilution of the nicotinic-[7-^{13}C] acid (**2**) with commercially available nicotinic-[7-^{14}C] acid.

The ^{13}C NMR spectrum of α,β-dipyridyl has not been previously recorded. The chemical shifts (Table 1) were unambiguously assigned as follows. C-2 and C-3′, being quaternary carbons, were of low intensity. Tentative assignments of the carbons in ring B were made by comparison with the ^{13}C NMR spectrum of anabasine (**8**) [3]. The chemical shifts of C-5, C-5′ and C-6, C-6′ were, as expected, very close. However they were differentiated by examination of the spectrum of α,β-dipyridyl-[5′,6′-$^{13}C_2$] (Fig. 1) which was prepared by the silver acetate dehydrogenation [4] of anabasine-[5,6-$^{13}C_2$] previously obtained biosynthetically from nicotinic-[5,6-$^{13}C_2$] acid [3]. The contiguous ^{13}C atoms in this α,β-dipyridyl afford satellite peaks, due to spin-spin coupling, on either side of the resonances of C-5′ and C-6′. C-4 was differentiated from C-4′ by examination of the ^{13}C NMR spectra (in $CDCl_3$) of α,α-dipyridyl (**9**) and β,β-dipyridyl (**10**), which are recorded on the formulas of these compounds. It is noted that C-4 in (**9**) where the bonding between the pyridine rings is *meta* to C-4, is downfield of C-4 in (**10**), where the bonding is *ortho* to C-4. We thus deduce that C-4 in α,β-dipyridyl is downfield of C-4′. The ^{13}C NMR spectrum of α,β-dipyridyl diperchlorate in D_2O was considerably different from the spectrum of the free base in $CDCl_3$ (see Table 1), the observed changes in the chemical shifts being in accord with those previously observed on protonation of pyridine [5]. Unfortunately the resonance for C-2 was not observed in acidic solution, presumably being obscured under one of the more intense peaks. Resonances were again unambiguously assigned by examination of the spectrum of α,β-dipyridyl-[5′,6′-^{13}C] in acidic D_2O. The coupling constant between C-5′ and C-6′ was significantly higher in acidic solution. From a practical point of view (see Experimental) it was found desirable

† Contribution No. 157 from this laboratory.



Scheme 1. Synthesis of Anatabine-[2′-^{14}C, ^{13}C].

The labelled atom is indicated with a heavy dot. i BuLi, $^{13}CO_2$, ii $SOCl_2$, iii MeOH, iv $LiAlH_4$, v $Pb(OAc)_4$, vi NH_2COOEt, C_6H_6, H^+, vii BF_3.2HOAc + 1,3-butadiene, viii KOH in 50% EtOH.

Table 1 ^{13}C NMR spectra of α,β-dipyridyl

Carbon no.	$CDCl_3$*	Chemical shift (ppm from Me_4Si) in CD_3OD + NaOD*	D_2O†	Normalized peak intensities§ Unenriched α,β-dipyridyl	Enriched α,β-dipyridyl	% Difference enriched
2	154.5	155.1	‡	1.0	3.2	+220
3	120.8	122.6	128.1	9.9	10.4	+ 5
4	137.3	139.3	144.7	9.5	9.2	− 3
5	123.1	124.7	128.7	10.5	11.8	+ 12
6	148.1	148.3	147.0	8.3	7.7	− 7
2′	150.8	150.7	148.1	9.3	10.1	+ 9
3′	135.0	135.9	132.7	1.8	1.4	− 22
4′	134.7	136.3	142.1	8.9	10.1	+ 13
5′¶	123.9	125.4	129.2	9.6	7.1	− 26
6′¶	149.7	150.2	144.5	9.6	10.1	+ 6

* Free base in these solvents. † α,β-Dipyridyl.2$HClO_4$ in this solvent. ‡ Not observed. § The peak intensities are normalized so that the sum of all the peak intensities (except C-2) in the unenriched and enriched α,β-dipyridyl are made equal. ¶ The following coupling constants (Hz) were observed in α,β-dipyridyl-[5′,6′-$^{13}C_2$]: $^1J_{5',6'}$ = 54.9 ($CDCl_3$), 54.8 (CD_3OD + NaOD), 58.7 (D_2O + H^+).

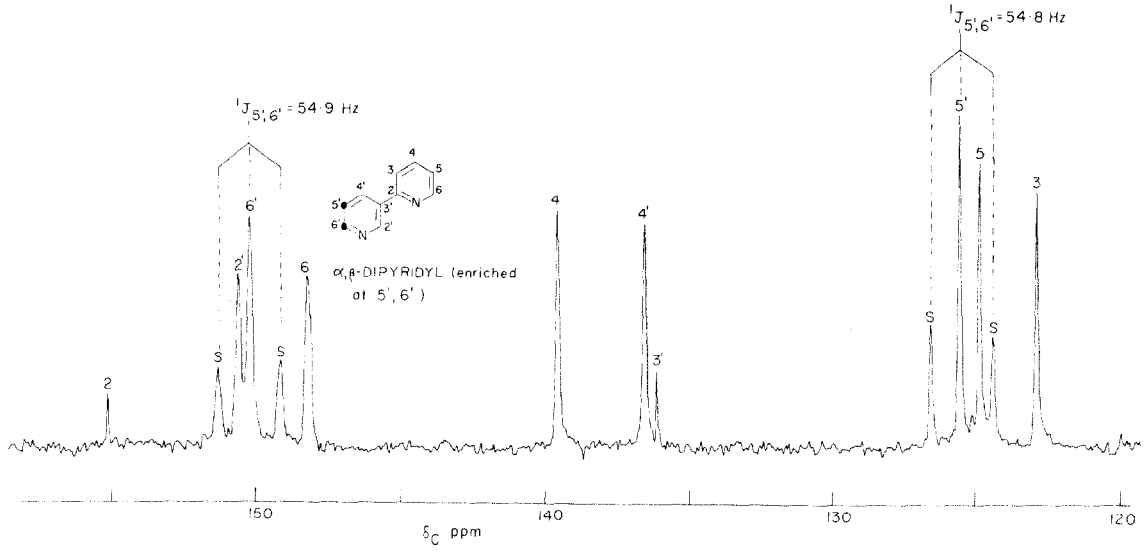
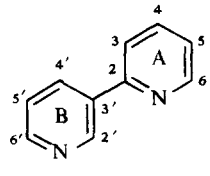


Fig. 1. Proton noise decoupled ^{13}C NMR spectrum of α,β-dipyridyl-[5′,6′-$^{13}C_2$] in $CDCl_3$. Satellite peaks indicated with S.



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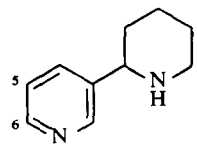

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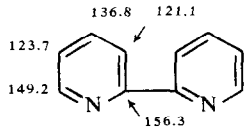

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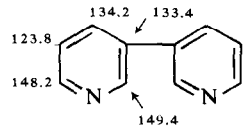

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to determine the ^{13}C NMR spectrum of α,β-dipyridyl in CD_3OD, which had been made alkaline by the addition of NaOD in D_2O. The chemical shifts in this solvent were close to those observed in $CDCl_3$. It was easier to obtain an instrument lock on the larger amount of deuterium in the CD_3OD.

The anatabine-[2′-^{14}C,^{13}C] was fed to *N. glauca* and *N. glutinosa* by the wick feeding method. After 7 days the alkaloids were isolated and separated as previously described [1]. Almost all the activity of the alkaloids was present in anatabine. No α,β-dipyridyl was detected in these plant extracts. In *N. glauca* the major alkaloid is anabasine, and this was found to be unlabelled. This result is in accord with our previous work [1] in which we showed that this alkaloid and anatabine are formed by two quite different biosynthetic routes. Our present work eliminates the possibility that anabasine is formed to a minor extent by the reduction of anatabine.

The anatabine-[2′-^{14}C,^{13}C] was also fed to *N. tabacum* plants. After 7 days the plants were harvested and allowed to dry in air at room temperature for 20 days. During this time the leaves became brown. Chromatography of the crude alkaloids resulted in the isolation of α,β-dipyridyl, having a characteristic absorption in the UV. The amount isolated was quite small (0.19 mg), however its ^{14}C specific activity was 82% that of the administered anatabine. In view of this high specific incorporation it was possible to dilute this labelled material with inactive α,β-dipyridyl and determine its ^{13}C NMR spectrum. This spectrum is illustrated in Fig. 2. Measurement of the intensities of the peaks and comparison with unenriched α,β-dipyridyl indicated that only C-2 of the α,β-dipyridyl isolated from the dried tobacco was enriched (see Table 1). The enhancement of the C-2 peak, taking into account the dilution of the sample, corresponds to a 71% enrichment of this position and a 78% specific incorporation of the anatabine-[2′-^{14}C,^{13}C] (90% ^{13}C), in excellent agreement with the observed specific incorporation of the ^{14}C. This result indicates that a direct conversion of the anatabine to α,β-dipyridyl has taken place, and we assume that this transformation takes place in the drying leaves.

EXPERIMENTAL

General methods. Radioactive materials were assayed in duplicate in a liquid scintillation counter, using dioxane–EtOH, with the usual scintillators [6]. ^{13}C NMR spectra were obtained on a Varian XL-100-15 spectrometer (25.2 MHz) equipped with a VFT-100 Fourier transform accessory. The spectrum of the enriched α,β-dipyridyl (Fig. 2) (4 mg in 40 μl of CD_3OD + NaOD) was determined in a 2 mm tube, with an acquisition time of 0.8 sec (1.25 Hz/data point) for 92000 transients. We thank Lenas Hedlund for fabrication of the 2 mm tube sample holder and for determination of some of the spectra. Peak intensities were determined by expanding the spectra in the horizontal scale and measuring the areas of the peaks by cutting them out and weighing. ^1H NMR spectra were determined on a Varian HFT-80 spectrometer. Preparative TLC was carried out on Si gel PF-254 (Merck), developing with $CHCl_3$–EtOH–conc NH_4OH (100:20:1) (System I).

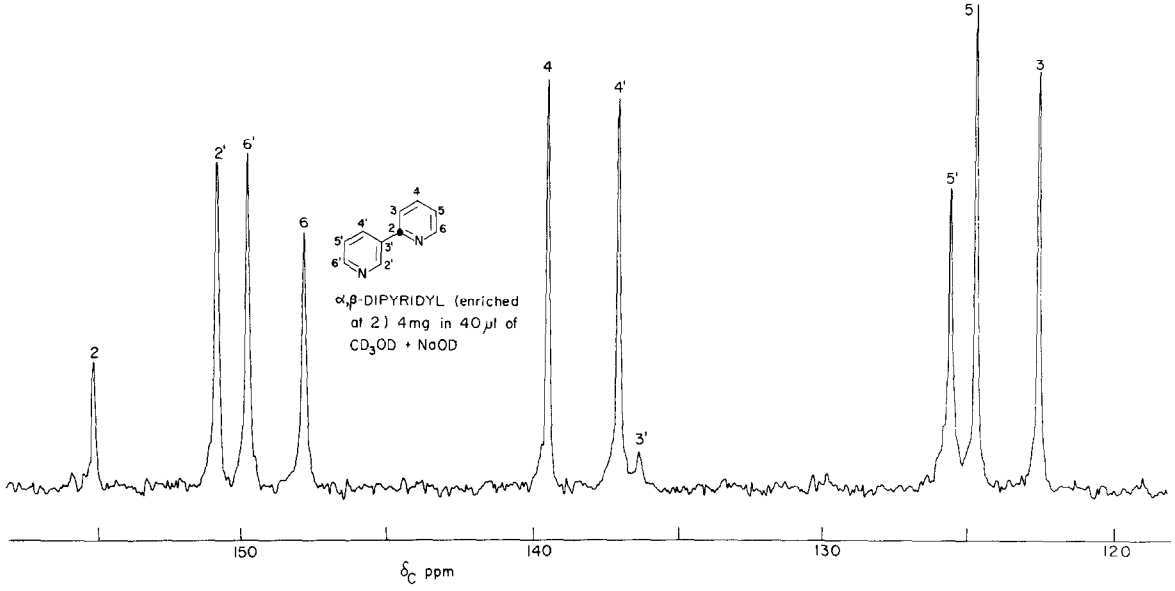

Fig. 2. Proton noise decoupled ^{13}C NMR spectrum of α,β-dipyridyl (enriched at C-2).

Anatabine-[2′-^{14}C,^{13}C]. Nicotinic-[7-^{13}C] acid was prepared by carboxylation of 3-lithio pyridine with $^{13}CO_2$ (90% enriched, obtained from the Stable Isotope Resource—see Acknowledgements), using essentially the same procedure described for nicotinic-[7-^{14}C] acid [7]. The following coupling constants were observed in its ^{13}C NMR spectrum (Na salt in D_2O): $^1J_{3,7} = 65.7$, $^2J_{2,7} = 3.7$, $^2J_{4,7} = 2.4$, $^3J_{5,7} = 3.6$ Hz. This nicotinic-[7-^{13}C] acid (1.05 g) was mixed with nicotinic-[7-^{14}C] acid (5 mg, nominal activity 0.1 mCi, Mallinckrodt Co.) and converted to pyridine-3-aldehyde-[7-^{14}C,^{13}C] (**3**) via methyl nicotinate and 3-hydroxymethylpyridine. This aldehyde (0.97 g, 9.1 mmol) was added to a stirred soln of ethyl carbamate (1.62 g, 18.2 mmol) and *p*-toluenesulfonic acid (40 mg) in C_6H_6 (30 ml). The mixture was refluxed for 5 days, H_2O being removed in a Dean-Stark trap. Evapn of the C_6H_6 and cooling resulted in the separation of diethyl *N,N′*-(3-pyridylmethylene) *bis*-carbamate (**6**) (1.21 g) as fine colorless needles, mp 164°. This compound (1.0 g) was dissolved in HOAc (10 ml) and the *bis*-HOAc complex of BF_3 (15 g) added slowly. 1,3-Butadiene was bubbled slowly through this soln which was heated to 75°, in a flask having a dry ice–Me_2CO cooled condenser. After 16 hr the reaction miture was cooled and added cautiously to Na_2CO_3 (30 g) in H_2O (150 ml) and the mixture extracted with Et_2O (5 × 25 ml). The Et_2O extract was extracted with 10% HCl (3 × 50 ml). This acid extract was made basic with NaOH and extracted with $CHCl_3$. The dried (K_2CO_3) extract was evapd and the dark brown residual oil subjected to prep TLC (System I). Extraction of the main zone (R_f 0.68) with 15% MeOH in $CHCl_3$ afforded 1′-ethoxycarbonyl-anatabine-[2′-^{14}C,^{13}C] (**5**) as a pale yellow oil (310 mg). This compound (310 mg) was refluxed with a soln of KOH (8 g) in 50% aq. EtOH (40 ml) for 3 days. The cooled reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The residue obtained on evapn of the dried (K_2CO_3) extract was subjected to prep TLC (System I). The zone corresponding to anatabine (R_f 0.3) was extracted with MeOH, to afford after evapn a pale yellow oil which was distilled (140°, 10^{-3} mm) yielding *RS*-anatabine-[2′-^{14}C,^{13}C] (88 mg), having a specific activity of 2.56×10^7 dpm/mM. ^{1}H NMR ($CDCl_3$) δ 8.52 (*d*, 2-PyH), 8.44 (*dd*, 6-PyH), 7.64 (*dt*, 4-PyH), 7.17 (*qd*, 5-PyH), 5.75 (*s*, 5′-H), 5.73 (*s*, 4′-H), 3.80 (*tt*, $^1J_{^{13}CH} = 137$ Hz, 2′-H), 3.49 (*d*, 6′-H), 2.54 (*s*, NH), 2.15 (*dd*, 3′-H). Its ^{13}C NMR ($D_2O + H^+$) was identical with that previously described [3] except that C-2′ was enriched and a coupling of C-3′ to C-2′ was observed, $J = 37$ Hz. It afforded a dipicrate, mp 200–201° (lit. [8] mp 201–201.5°.)

Administration of anatabine-[2′-^{14}C,^{13}C] *to* Nicotiana *species and isolation of the alkaloids.* The labelled anatabine (20 mg, 3.2×10^6 dpm) dissolved in H_2O, was fed by the wick method to 10 *N. glutinosa* plants (3 months old) growing in soil in a greenhouse. After 7 days the whole plants (fr. wt 634 g) were extracted [1] to afford the crude alkaloids (1.93×10^6 dpm, 60% incorporation). TLC (System I) yielded non-radioactive nornicotine (9 mg), anabasine (1 mg) and nicotine (20.4 mg). The reisolated anatabine (20 mg) had an activity of 8.45×10^6 dpm/mM (33% specific incorporation). The enhancement of the C-2′ peak in its ^{13}C NMR corresponded to a 34% incorporation of the anatabine-[2′-^{13}C], in excellent agreement with the ^{14}C. No radioactivity was detected in the zone where α,β-dipyridyl would be expected to appear, however some radioactivity (4%) was present in a compound of unknown constitution having R_f 0.85.

Anatabine-[2′-^{14}C,^{13}C] (20 mg, 3.2×10^6 dpm) was fed to 6 *N. glauca* plants (4 months old) by the wick method for 7 days. The plants (fr. wt 780 g) afforded crude alkaloids (1.96×10^6 dpm, 61% incorporation) which on separation yielded inactive nornicotine (5 mg), anabasine (208 mg) and nicotine (11 mg). The reisolated anatabine (19 mg) had an activity of 6.9×10^6 dpm/mM (27% specific incorporation). No α,β-dipyridyl was detected in the crude alkaloids.

Anatabine-[2′-^{14}C,^{13}C] (10 mg, 1.6×10^6 dpm) was fed to 2 *N. tabacum* plants (3 months old) growing in hydroponics [9], by addition to the nutrient soln in which the roots were growing. Uptake of radioactivity from this soln was rapid, at the time of harvesting (7 days after the initial feeding) only 5% of the initial activity remained in the soln. The plants were allowed to dry in air at room temp. in the sun in a greenhouse for 20 days. The dried material (24 g) was extracted as before yielding the crude alkaloids (2.26×10^5 dpm, 14% incorporation). TLC (System I) revealed the presence of α,β-dipyridyl (10% of the total activity of the crude alkaloids). This zone was extracted with MeOH, and assayed by UV spectroscopy, λ_{max} at 240 and 274 nm. Its specific activity was 2.11×10^7 dpm/mM (82% specific incorporation). A soln of this enriched α,β-dipyridyl (0.19 mg) in 95% EtOH was acidified with HCl, and evapd in the presence of enenriched α,β-dipyridyl (4 mg), final lyopholization being carried out in a 2 mm NMR capillary tube. The residue was dissolved in CD_3OD (40 μl) and the free base liberated by the addition of a drop of NaOD in D_2O.

Acknowledgements—This investigation was supported by a research grant GM-13246 from the U.S. Public Health Service. We thank the Stable Isotope Resource (SIR) at the Los Alamos Scientific Laboratory (supported) by NIH grant RR-00962, Division of Research Resources) for supplying the $BaCO_3$-[^{13}C] used in the present investigation.

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