BIOSYNTHESIS OF ANABASINE FROM DL-[4,5-$^{13}C_2$, 6-^{14}C]LYSINE IN *NICOTIANA GLAUCA* EXAMINED BY ^{13}C-NMR

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ABSTRACT.—DL-[4,5-$^{13}C_2$]Lysine was prepared by a 10-step synthetic sequence from the ethylene diamine complex of lithium [$^{13}C_2$]acetylide in a 12% overall yield. The presence of the highly enriched contiguous ^{13}C atoms in this lysine permitted unambiguous assignment of its ^{13}C-nmr chemical shifts which are quite sensitive to changes in pH of the solvent. This lysine was mixed with [6-^{14}C]lysine and administered to *Nicotiana glauca* plants, resulting in the formation of labeled anabasine (5.4% absolute incorporation, 0.24% specific incorporation). The ^{13}C-nmr spectrum of this anabasine revealed the presence of excess ^{13}C only at C-4' and C-5' of the piperidine ring. Degradation of the anabasine and assay of the radioactive fragments indicated that there is negligible (0.2%) incorporation of the lysine via a symmetrical intermediate such as cadaverine. These results confirm previous hypotheses on the biosynthesis of this alkaloid in *Nicotiana* species.

Lysine serves as a precursor of many alkaloids which contain a piperidine ring (1,2). In some cases the lysine is incorporated via a symmetrical intermediate, presumably cadaverine. The quinolizidine alkaloids of the lupin family are formed by this route. However, with most of the simple α-substituted piperidines, e.g., *N*-methylpelletierine (3,4), sedamine (5–7), and anabasine (8–12), the lysine is incorporated unsymmetrically. In general, C-2 of lysine becomes the point of attachment of the α-side chain. In other cases, due to difficulties in carrying out required degradation to determine the location of labeled atoms, the route of incorporation of lysine has not been established, e.g., mimosine (13,14). Such degradations can be circumvented if one uses precursors labeled with ^{13}C, especially with two contiguous ^{13}C atoms, since the location of the excess ^{13}C in the natural products derived from such labeled compounds can be determined by ^{13}C-nmr spectroscopy (15).

In the present article the synthesis of lysine labeled with contiguous ^{13}C atoms at C-4 and C-5 is described, and its utility has been established in a re-examination of the biosynthesis of anabasine in *Nicotiana glauca*. No synthesis of lysine labeled with two contiguous ^{13}C atoms has previously been described. There is nothing especially novel about our synthesis, illustrated in figure 1, which has utilized various methods and procedures previously described for the preparation of the intermediates labeled with ^{14}C (16). The ethylene diamine complex of lithium [$^{13}C_2$]acetylide (**1**) (>99% $^{13}C_2$) was decomposed with concentrated sulfuric acid, the evolved acetylene being condensed in ether and converted to its dilithium salt with excess butyl lithium. Reaction with carbon dioxide yielded acetylene dicarboxylic acid (**2**), which was hydrogenated to succinic acid (**5**). Its dimethyl ester (**4**) was reduced with lithium aluminum hydride to butane-1,4-diol (**3**). Hydrobromic acid converted this diol to 1,4-dibromobutane (**6**), which was reacted with one equivalent of potassium phthalimide in boiling acetone to yield *N*-(4-bromobutyl)phthalimide (**7**), along with some 1,4-diphthalimidobutane. This was then converted to [4,5-$^{13}C_2$]lysine monohydrochloride via compounds **8** and **10** by the method of Fields, *et al.* (17). Mass spectrometry on compound **10**, indicated the presence of ~99.7% of the $^{13}C_2$ species. Full details are provided

[1]Contribution No. 180 from this laboratory. Publication No. 46 in the series: Tobaceo Alkaloids and Related Compounds. A complete bibliography of previous papers in this series will be furnished on request to the author. The paper is dedicated to Ray F. Dawson, a pioneer in studies on the biosynthesis of the tobacco alkaloids, who celebrated his 70th birthday on February 11, 1981.

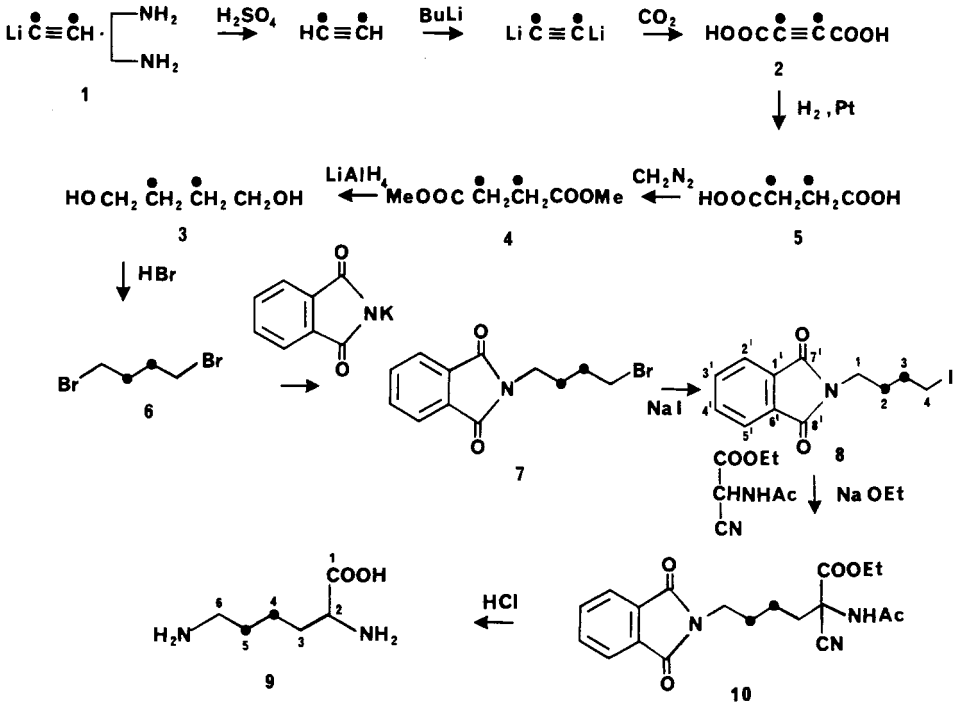

FIG. 1. Synthesis of [4,5-$^{13}C_2$]lysine.

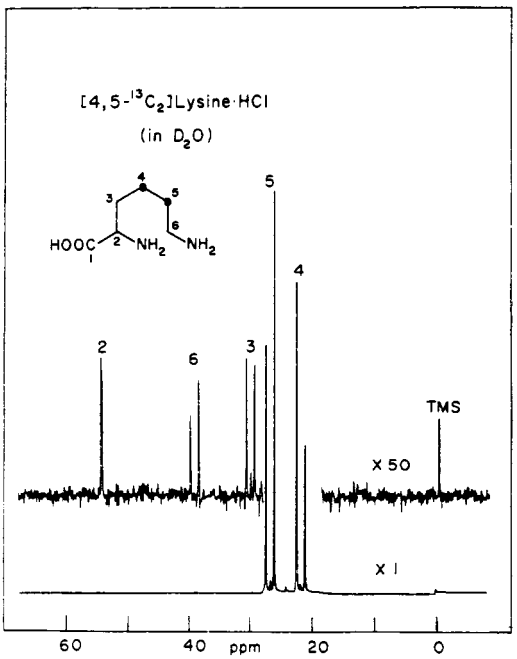

FIG. 2. Proton noise-decoupled FT ^{13}C-nmr spectrum of [4,5-^{13}C]lysine. HCl in D_2O (pH 7.0). The upper spectrum is enhanced 50 times in the vertical scale so that the natural abundance carbons can be observed.

in the Experimental section since it is anticipated that this labeled lysine will find wide use in metabolic studies in humans and for examination of the secondary structures of enzymes and proteins.

The ^{13}C-nmr spectrum of the [4,5-$^{13}C_2$]lysine·HCl in D_2O is illustrated in figure 2. It is to be noted that all the signals, except C–1 (which is a downfield singlet at 175.2 ppm), are split by coupling with the highly enriched positions. These coupling constants are in agreement with those previously reported for lysine uniformly enriched with 85% ^{13}C (18). The presence of the highly enriched C–4 and C–5 positions made assignments of the chemical shifts unequivocal. There is considerable confusion in the literature regarding the assignments of C–3 and C–5 and those recorded in recent compendia of ^{13}C-nmr spectra (19,20) and a text (21) are incorrect. The first publications on the ^{13}C-nmr spectrum of lysine also misassigned C–3 and C–5 (22,23). These assignments were based on the chemical shift parameters for substitution by amino and carboxylic functions on an alkyl chain. These positions were reassigned (correctly) by Christi and Roberts (24) based on the fact that the resonance at 27.0 ppm (at pH 6) assigned to C–5 was more affected by changes in pH than the resonance at 31.2 (C–3). Actually the difference in the change in chemical shifts for these two positions is hardly significant (25). As one goes from pH 0.5 to 13.85, the $\Delta\delta_c$ for C–3 is 5.23 ppm, and for C–5 is 5.40 ppm. All the resonances of lysine except C–4 and C–6 undergo a marked change with pH (25,26) and we have confirmed these changes (see table 1).

TABLE 1. Chemical shifts (ppm from Me_4Si) and coupling constants (in Hz) of [4,5-$^{13}C_2$]lysine (in D_2O).

Carbon No.:	1	2	3	4	5	6
pH 0.5	172.0	53.8	30.0	22.5	27.1	40.5
pH 7.0	175.5	55.4	30.9	22.4	27.3	40.2
pH 12.0	184.3	56.6	35.1	23.0	31.6	41.0
Coupling Constants		$^3J_{2,5}$	$^1J_{3,4}$	$^1J_{4,5}$	$^1J_{5,4}$	$^1J_{5,6}$
pH 0.5	—	4.1	33.7	34.4[a]	34.4[a]	35.4
pH 7.0	—	4.2	34.2	34.3	34.3	34.9
pH 12.0	—	3.4	34.5	34.4	34.4	34.8

[a]The average observed distance of the inner satellite to the central peaks of C–4 and C–5 at pH 0.5, 7.0, and 12.0 was 14.6, 14.5, and 15.4 Hz, respectively. The theoretical distances, calculated (27) from the formula:

$$\tfrac{1}{2}\,(V_{AB}+J_{AB}-\sqrt{V_{AB}^2+J_{AB}^2})$$

where V_{AB} = difference in chemical shifts of the coupled carbons, and J_{AB} = coupling constant, are 14.8, 14.8, and 15.9 Hz, respectively.

The ^{13}C-nmr spectrum of [2,3-$^{13}C_2$]-*N*-(4-iodobutyl)phthalimide (**8**) is also recorded in the experimental. In this compound, the enriched positions have chemical shifts very close together (V_{AB} = 37.1 Hz) resulting in very asymmetrically arranged satellites and peak heights (27). Carbons 1 and 4 appeared as doublets of doublets due to one and two bond couplings with the highly enriched positions. The chemical shifts were made by comparison with model compounds (28) and application of substituent parameters (21) (I– and –N <) to butane. However, the assignments to C–2 and C–3 are tentative since they are so close to each other.

In previous tracer experiments [2-^{14}C]lysine afforded anabasine (**14**) in which the bulk of the activity was found at the 2′ position. The degradation used to determine activity at this position involved oxidation to nicotinic acid, whose carboxyl group represents the activity at C–2′. However, because of typical errors (~5%) in radioactive counting, a small amount of activity could have been located at the C–6′ position. Thus, in our initial experiments (8), we found

~94% at C-2' and Griffith and Griffith (11) reported 93% at this position. Leistner and Spenser (29) proposed that lysine is converted to Δ^1-piperideine (**13a**), an established (30) precursor of the piperidine ring of anabasine, via cadaverine (**11**). However, in order to explain the unsymmetrical incorporation of lysine, it was suggested that no *free* cadaverine is present in this conversion. Their hypothesis involves the formation of an adduct of the α-amino group of lysine with pyridoxal phosphate which is decarboxylated to yield cadaverine covalently bound so that the C-1 and C-5 positions are not equilibrated. The bound amino group is dehydrogenated to an intermediate which yields 5-aminopentanal (**12a**) on hydrolysis (Route B in figure 3). In the present work the

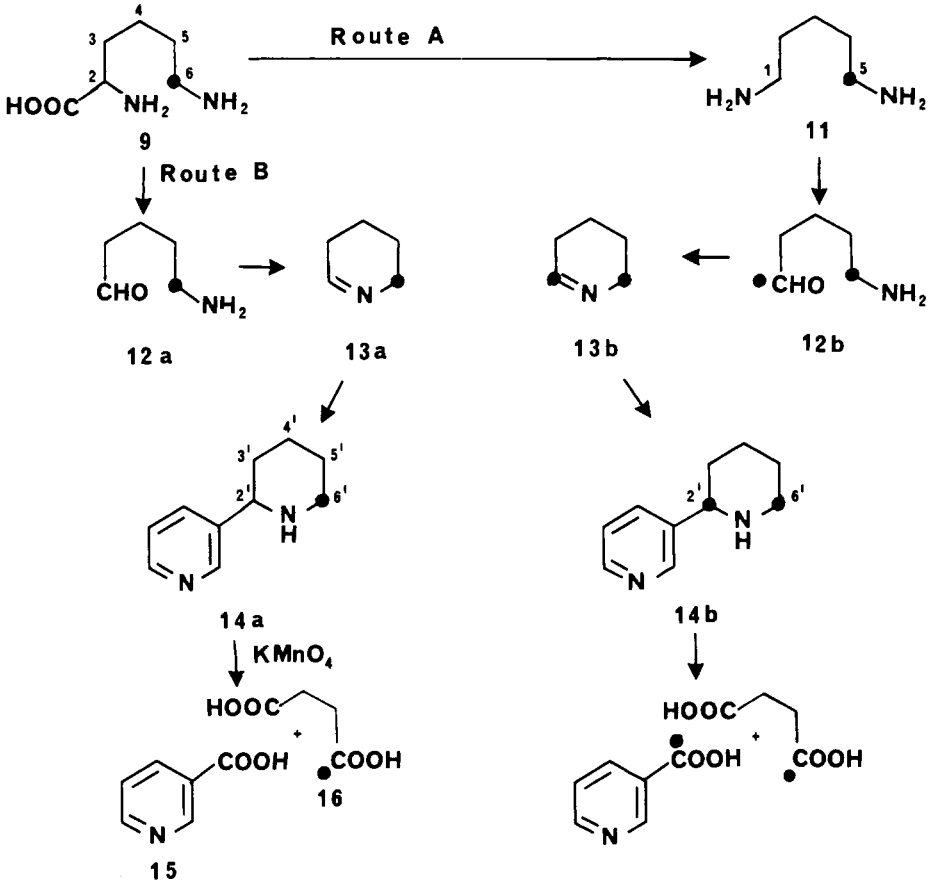

FIG. 3. Hypothetical biosynthesis of anabasine from [6-^{14}C]lysine with and without equilibration of the 2 and 6 positions of lysine.

DL-[4,5-$^{13}C_2$]lysine was mixed with DL-[6-^{14}C]lysine prior to feeding to *Nicotiana glauca* for two reasons. The presence of a ^{14}C-label in the lysine facilitates determination of the specific incorporation of the lysine into anabasine by radioactive assay. Secondly, the location of the ^{14}C at C-6 was decided upon so that any small incorporation of lysine via *free* cadaverine (Route A in figure 3) could be determined by the chemical degradation previously discussed. Thus, activity found on the carboxyl group of nicotinic acid from this degradation would represent the degree of participation, if any, of free cadaverine in this biosynthetic sequence.

The DL-[4,5-$^{13}C_2$, 6-^{14}C]lysine was fed to *N. glauca* plants by the wick method for 7 days. The absolute incorporation was remarkably high (5.7%). This percentage could actually be doubled since it has been shown that only L-lysine is utilized for the production of anabasine (31,32). The specific incorporation

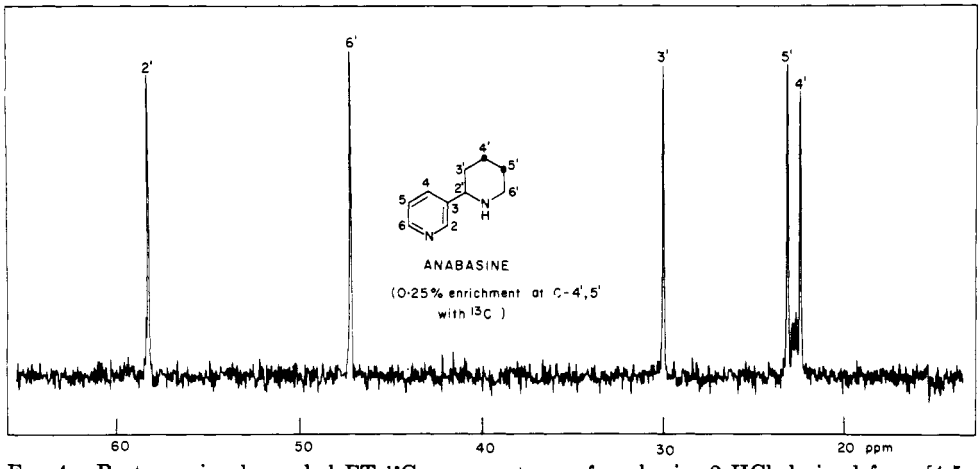

FIG. 4. Proton noise-decoupled FT ^{13}C-nmr spectrum of anabasine·2 HCl derived from [4,5-$^{13}C_2$]lysine in D_2O (aliphatic region).

tiguous ^{13}C atoms at C–4' and C–5'. The ^{13}C chemical shifts of the enriched anabasine in $CDCl_3$ and anabasine dihydrochloride in D_2O are recorded in table 2. Figure 4 illustrates the aliphatic region of anabasine, and details of the enriched carbons are presented in figure 5. Since the coupled carbons C–4' and C–5' have chemical shifts which are very close together, only the inner satellites could be observed above the background noise. The expected coupling constant between

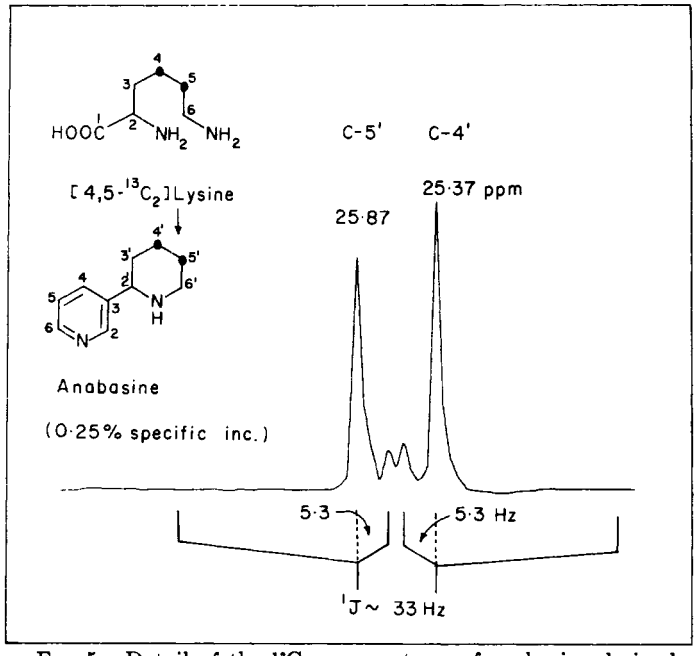

FIG. 5. Detail of the ^{13}C-nmr spectrum of anabasine derived from [4,5-$^{13}C_2$]lysine at the enriched C–4' and C–5' positions (in $CDCl_3$).

(^{14}C) was 0.24% and the ^{13}C-nmr of the anabasine revealed the presence of con- C–4' and C–5' of the piperidine ring of anabasine is 33.0 Hz (33). From this information and the observed separation of the inner satellites from the central natural abundance peaks, one can calculate (27) that the ratio of intensities of the inner satellites to the outer ones for anabasine in $CDCl_3$ (figure 5) should be 36:1. It is, thus, not surprising that the outer satellites could not be observed

TABLE 2. Chemical shifts (δ_c) (ppm from Me_4Si) of anabasine derived from [4,5-$^{13}C_2$]lysine.

Carbon No.	Solvent	
	$CDCl_3$ (free base)[a]	D_2O (·2 HCl salt)[b]
2	149.0	142.5[c]
3	141.3	138.2
4	134.3	148.1
5	123.6	129.9
6	148.8	143.7
2'	59.9	58.5
3'	35.2	30.2
4'	25.4	22.7
5'	25.9	23.4
6'	47.7	47.4
$\Delta\delta_c$ 4',5'(Hz)	12.6	17.5
Distance of inner satellite to central peak (Hz)[d]	Calc: 5.2 Found: 5.3	6.6 6.5

[a]Anabasine (248 mg) in $CDCl_3$ (0.3 ml) in a 5 mm tube, 82K transients, 1.43 sec acquisition time, 0.7 Hz/data point, sensitivity enhancement: 2.

[b]Anabasine·2 HCl (from 220 mg of the free base) in D_2O (0.4 ml) in a 5 mm tube; instrument parameters as above.

[c]The assignments of the aromatic carbons were made by comparison with the spectrum of anatabine·2 $HClO_4$ in D_2O (34). However, it should be noted that the assignments for C–2 and C–4 reported in this paper should be reversed (E. Leete and M. E. Mueller, unpublished work, and (35)).

[d]Calculated assuming a coupling constant of 33.0 Hz (33).

at this level of incorporation. The specific incorporation of the [4,5-$^{13}C_2$]lysine into anabasine, calculated from the intensities of these inner satellites to the central peaks, was 0.25% in excellent agreement with the value obtained by radioactive assay. As expected, no satellites were detected at the signal for C–3', however, a small amount of excess ^{13}C at C–3' would be undetectable at this level of incorporation.

The anabasine was oxidized with potassium permanganate to yield nicotinic acid (**15**). Previously no attempts were made to isolate other products from this oxidation, however, in the present work succinic acid (**16**) (from C–3', 4', 5' and 6' of anabasine) was obtained and characterized as its dianilide. The nicotinic acid was heated with calcium oxide to yield pyridine, assayed as its picrate. The activities of these degradation products are recorded in table 3. It is apparent that the incorporation of the lysine into the piperidine ring of anabasine has occurred almost completely via a non-symmetrical intermediate (Route B). The small amount of activity detected at C–2' (0.20%) is real. However, since some radioactivity was also detected in the pyridine ring (0.29%), it probably represents general catabolism of the labeled lysine to small radio-

TABLE 3. Activity of anabasine (derived from [4,5-$^{13}C_2$, 6-^{14}C]lysine and its degradation products.

	Specific activity (dpm/mM)	Relative specific activity
Anabasine·2 $HClO_4$	4.48×10^5	100
Nicotinic acid	2.11×10^3	0.47
Nicotinic acid·HCl	2.21×10^3	0.49
Pyridine picrate	1.28×10^3	0.29
[C–2']-by difference	0.88×10^3	0.20
Dianilide of Succinic acid	4.40×10^5	98

active fragments which then enter the general metabolic pools of primary metabolism. In support of this hypothesis, small but significant amounts of radioactivity were detected in the nicotine and nornicotine isolated from the plant. The failure of lysine to produce free cadaverine in this species was confirmed by adding non-radioactive cadaverine to the aqueous solution from the initial extraction of the plant with chloroform and concentrated ammonia. The reisolated cadaverine, characterized and purified as its dibenzoyl derivative, had an activity which corresponded to only 0.03% of the labeled lysine fed to the plant.

EXPERIMENTAL[2]

DL-[4,5-$^{13}C_2$]LYSINE (**9**).—Concentrated H_2SO_4 (20 ml) was added slowly to the ethylene diamine complex of lithium [$^{13}C_2$]acetylide[3] (1.8 g, 93% pure, 99.4% $^{13}C_2$, 17.8 mmol). The evolved acetylene was passed by a nitrogen stream into a stirred solution of butyl lithium (20 ml of a 2.6 M solution in hexane) in ether (100 ml) at −78°C. After 1 h the clear solution was poured into a slurry of dry ice and ether. When the excess CO_2 had evaporated, water (50 ml) was added. The brown aqueous layer was separated, acidified with 2 N H_2SO_4 and hydrogenated at 3 atmospheres pressure in the presence of Adams catalyst (0.2 g) for 6 h. The filtered solution (pale brown) was extracted with ether for 12 h. Evaporation of the ether (without drying) yielded a crystalline residue which was triturated with a little ether and filtered affording [2,3-$^{13}C_2$]succinic acid (939 mg, 44%).

This succinic acid (879 mg) was treated with a solution of diazomethane in ether (prepared from 8.0 g of *N*-methyl-*N*-nitroso-*p*-toluene sulfonamide, "Diazald"). After 1 h the solution was dried (Na_2SO_4) and added slowly to a solution of lithium aluminum hydride (1 g) in ether (100 ml) at 25°. After stirring for 2 h, the mixture was cooled to 0° and water (10 ml) and concentrated HCl (20 ml) added. The gray slurry was extracted with ether in a continuous extractor for 3 days. The ether was evaporated and the residue dissolved in water (10 ml) and the solution saturated with HBr gas at 0°. The solution was heated on a steam bath for 1 h, then cooled, extracted with ether which was then washed with 10% $NaHCO_3$, and dried over Na_2SO_4. Evaporation afforded [2,3-$^{13}C_2$]-1,4-dibromobutane (1.12 g, 75% from succinic acid).

Potassium phthalimide (1.0 g) was added to a solution of this dibromobutane (1.12 g) in acetone (100 ml), and the mixture was refluxed for 18 h. The reaction mixture was then filtered hot, and the filtrate was evaporated to dryness. The residue was refluxed with ethanol (40 ml) and filtered. The residue, (254 mg 13%) mp 238°, was 1,4-diphthalimidobutane, identical with an authentic specimen. The filtrate was evaporated to 10 ml and, on cooling, *N*-(4-bromobutyl)phthalimide separated as colorless plates (800 mg, 51%). This bromo compound was refluxed in acetone (100 ml) with sodium iodide (3 g) for 18 h. The residue obtained on evaporation of the acetone was suspended in water, filtered and the residue crystallized from ethanol to afford [2,3-$^{13}C_2$]-*N*-(4-iodobutyl)phthalimide (**8**) (879 mg, 91%) mp 88–89°; ^{13}C-nmr ($CDCl_3$) (ppm from Me_4Si): 168.3 (7',8'), 134.0 (3',4'), 132.1 (1',6'), 123.3 (2',5'), 36.8 (1), $^1J_{1,2}$ 28.8, $^2J_{1,3}$ 7.0 Hz, 30.6 (2), $^1J_{2,3}$ 34.6 Hz (distance of inner satellite to central peak: 9.2 Hz, Calc. 8.9 Hz), 29.6 (3), $^1J_{3,2}$ 34.5 Hz, 5.3 (3), $^1J_{3,4}$ 28.0 Hz, $^2J_{2,4}$ 6.2 Hz.

Ethyl acetamidocyanoacetate (433 mg) was added to a solution of sodium (69 mg) in absolute ethanol (5 ml) at 70°. A solution of **8** (855 mg) in boiling ethanol (10 ml) was then added, and the mixture was stirred in a N_2 atmosphere at 85° (oil bath temp) for 20 h. The reaction mixture was evaporated to 5 ml and cooled, and water (10 ml) added, when crude ethyl [4,5-$^{13}C_2$]-2-cyano-2-acetamido-6-phthalimidohexanoate (**10**) separated (992 mg). A mass spectrum of **10** (CI with butane as the carrier gas) gave a parent peak at 373.4 (100), 374.4 (21.3), 375.4 (3.2). The M-1 peak at 372.4 had an intensity of only 0.3 indicating that there was > 99.7% of the $^{13}C_2$ species in this intermediate. This material was refluxed in concentrated HCl (5 ml) for 18 h. The cooled reaction was diluted with water, phthalic acid was filtered off, and the filtrate was evaporated to dryness. The residue was dissolved in 95% ethanol and filtered, and pyridine (0.2 ml) was added to the filtrate, when DL-[4,5-$^{13}C_2$] lysine monohydrochloride separated (351 mg, 74% from **8**). Tlc on cellulose, developing with a mixture of 2-propanol-formic acid-water (80:10:4), and identifying the product with ninhydrin indicated that the lysine (R_f 0.2) was chromatographically pure. This was also evident from its ^{13}C-nmr spectrum (figure 2 and table 1).

DL-[4,5-$^{13}C_2$, 6-^{14}C]LYSINE.—This material was made by the addition of DL-[6-^{14}C]lysine HCl (0.34 mg, nominal activity 0.1 mCi) (Research Products International Corp., made by CEA, Gif-sur-Yvette, France) to the [4,5-$^{13}C_2$]lysine HCl (295 mg) and crystallizing from 95% ethanol. It had a specific activity of 1.88×10^8 dpm/mM.

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[2]The ^{13}C-nmr spectra were determined by Dr. Stephen B. Philson on a Varian XL-100-15 spectrometer (25.2 MHz) equipped with a VFT-100 Fourier-transform accessory. Mass spectra were determined by Dr. Roger Upham on an AEI-30 spectrometer. Radioactive materials were assayed in duplicate in a Nuclear Chicago Mark II liquid scintillation counter using dioxane-ethanol with the usual scintillators (36). Melting points are corrected.

[3]We are indebted to the Stable Isotope Resource at the Los Alamos Scientific Laboratory (supported by Grant RR-00962 from the Division of Research Resources, National Institutes of Health) for the lithium [$^{13}C_2$]acetylide.

ADMINISTRATION OF DL-[4,5-$^{13}C_2$, 6-^{14}C]LYSINE TO *Nicotiana glauca* AND ISOLATION OF THE ALKALOIDS.—Eight *Nicotiana glauca* plants[4] (5 months old) growing in a greenhouse were fed by the wick method DL-[4,5-$^{13}C_2$, 6-^{14}C]Lysine HCl (37.6 mg, total activity: 3.83 x 10^7 dpm). After 7 days the plants (fresh wt 1300 g) were macerated in a Waring blender with a mixture of chloroform (3 liters) and concentrated ammonium hydroxide (200 ml). The residual activity in the beakers used for the wick feeding was 0.01%. The alkaloids were isolated from the chloroform layer as previously described (37) affording anabasine (792 mg, 4.48 x 10^5 dpm/mM, 5.7% absolute inc. specific inc. 0.24%), purified as its diperchlorate, mp 153–4°, nicotine (4.8 mg, isolated as its dipicrate, 4.3 x 10^3 dpm/mM) and nornicotine (7.4 mg, isolated as its dipicrate, 7.2 x 10^3 dpm/mM).

The ammonical solution (450 ml) from the initial extraction of the plants had an activity of 6.44 x 10^6 dpm (16.8% of the amount fed). Cadaverine (272.6 mg) was added to an aliquot (100 ml) of this solution along with 30 ml of 10% NaOH. The solution was boiled for 15 min to remove ammonia, then cooled and shaken with benzoyl chloride (1 ml) for 1 h. The residue (453 mg) obtained on filtering this reaction mixture was dried, crystallized from a mixture of benzene, ethanol and hexane to yield dibenzoyl cadaverine, mp 132–3°, lit (38) mp 132°. This material was distilled (200°, 10^{-4} mm), and the product was crystallized from benzene. After several crystallizations, it had a constant activity of 2.9 dpm/mg, which represents 0.03% of the original [6-^{14}C]lysine fed to the plant.

DEGRADATION OF THE ANABASINE.—Anabasine (195 mg) was dissolved in 5% NaOH (20 ml) and stirred with potassium permanganate (1.0 g) at room temperature for 4 h. Additional (0.5 g) permanganate was then added and the mixture refluxed for 1 h. The reaction mixture was cooled and SO_2 passed in until a clear solution was obtained. The solution was extracted for 18 h with ether. The residue obtained on evaporation of the ether was sublimed (200°, 10^{-4} mm), and the sublimate crystallized from ethanol to yield nicotinic acid (108 mg). The mother liquors from the crystallization of the nicotinic acid were evaporated. The residue was dissolved in water (1 ml) to which was added aniline (1 drop) and 1-ethyl-3-(dimethylaminopropyl)carbodimide hydrochloride (50 mg). After a few min a precipitate (22 mg) separated which was crystallized from ethanol to yield fine colorless needles of the dianilide of succinic acid, mp 235–236°, identical with an authentic specimen.

Nicotinic acid (50 mg) was heated with calcium oxide (200 mg) *in vacuo* with a Bunsen burner. The evolved pyridine was condensed in a U-tube cooled in dry ice-acetone and converted to its picrate by adding an equivalent amount of picric acid.

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[4]Seeds of *Nicotiana glauca* were collected on the island of Tenerife, Spain, during the 12th IUPAC Symposium on the Chemistry of Natural Products, 21–27 September, 1980. A voucher specimen of the plant and seeds are deposited in the Herbarium of the Botany Department, University of Minnesota, St. Paul Campus.

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