$CH_3C{=}O$), 4.78 (m, 1, *CH), 6.58 (s, 2, 2CH=C), 7.06-7.28 (m, aromatic), 8.09 (d, 1, NHC*), 8.33, 8.62 (2 s, 1, 2NHC=C).

Anal. Calcd: C, 74.15; H, 6.01; N, 9.26. Found: C, 74.90; H, 6.06; N, 9.00.

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References and Notes

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Use of Carbon-13 Nuclear Magnetic Resonance to Establish That the Biosynthesis of Tropic Acid Involves an Intramolecular Rearrangement of Phenylalanine

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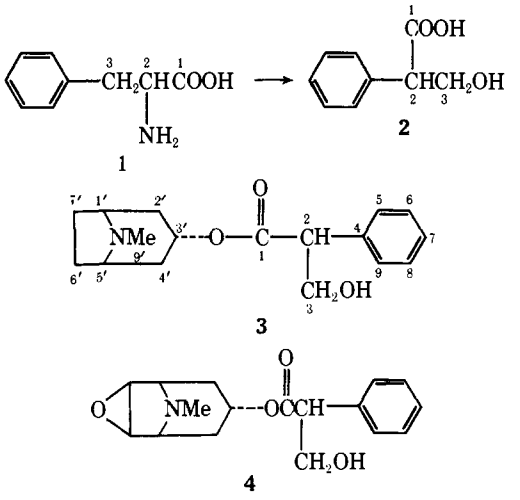
Abstract: The administration of DL-[1-^{14}C,1,3-^{13}C]phenylalanine (containing 81% of the $^{13}C_2$ species) to *Datura innoxia* plants yielded labeled hyoscyamine and scopolamine. Proton noise decoupled ^{13}C NMR spectra of these enriched alkaloids revealed the presence of satellite peaks, due to ^{13}C–^{13}C spin–spin coupling, symmetrically located about the singlet peaks arising from C-1 and C-2 of the tropic acid moiety of these alkaloids. This result indicates that the rearrangement of phenylalanine to tropic acid involves an *intra*molecular migration of the carboxyl group. Hyoscyamine, scopolamine, and phenylalanine isolated from *Datura stramonium* plants which had been fed [1-^{14}C]phenylacetic acid had negligible activity, indicating that phenylacetic acid is not involved in the biosynthesis of tropic acid.

Tropic acid (**2**) is found in Nature as the acid moiety of the ester alkaloids hyoscyamine (**3**) and scopolamine (**4**). In 1960 it was discovered[2] that the administration of [3-^{14}C]phenylalanine (**1**) to intact *Datura stramonium* plants yielded labeled tropic acid having essentially all its activity located at C-2. Later workers confirmed this result in *D. stramonium* (intact plants)[3] and *D. metel* (root cultures).[4] It was then established that the other carbons of the phenylalanine side chain were utilized for the formation of tropic acid.[5,6] The tropic acid formed from [1,3-^{14}C]phenylalanine had the same ratio of activity at C-1 to C-2 as C-1 to C-3 in the administered phenylalanine.[7,8] These results suggested that a molecular rearrangement of the side chain of phenylalanine was occurring. Despite extensive subsequent tracer work[9,10] the mechanism of this rearrangement is unknown. This reaction may be related to the conversion of succinyl coenzyme A to methylmalonyl coenzyme A which is catalyzed by a transcarboxylase from propionic acid bacteria.[11]

It has been reported[3,12] that phenylacetic acid is a precursor of tropic acid. The activities of degradation products of tropic acid derived from [1-^{14}C]phenylacetic acid were consistent with specific labeling of the tropic acid at C-3. It was suggested[3] that phenylpyruvic acid (**5**) formed from phenylalanine by transamination undergoes an oxidative decarboxylation yielding phenylacetyl coenzyme A (**7**) and carbon dioxide (possibly bound to a coenzyme such as biotin). Carboxylation of **7** then affords phenylmalonyl coenzyme A (**6**) which on reduction yields tropic acid, as illustrated in Scheme I. Another plausible route to tropic acid is shown in Scheme II. It has been established that polyporic acid (**8**) and related 2,5-diphenylbenzoquinones are formed by the condensation of two molecules of phenylpyruvic acid derived from phenylalanine.[13] A cleavage of the polyporic acid (indicated by a dotted line) would result in the formation of two C_6–C_3 units having the skeleton of tropic acid and a distribution of the side-chain carbon atoms consistent with the previously described tracer experiments.



We considered that it would be possible to determine whether the migration of the carboxyl group is *intra-* or *inter*molecular by carrying out a feeding experiment with [1,3-$^{13}C_2$]phenylalanine in which the majority of the labeled molecules would contain two ^{13}C atoms. If the rearrangement of the side chain is intramolecular the resultant tropic acid will contain two contiguous ^{13}C atoms which would afford satellite peaks in the ^{13}C NMR, due to spin-spin coupling, symmetrically located about the corresponding singlet peaks arising from the isolated ^{13}C atoms.[14,15] An intermolecular migration of the carboxyl group (as in Schemes I and II) would afford no such satellite peaks (assuming low specific incorporation of the administered [1,3-$^{13}C_2$]phenylalanine) but would show enrichment of the appropriate singlet peaks if the specific incorporation was high enough.[16]

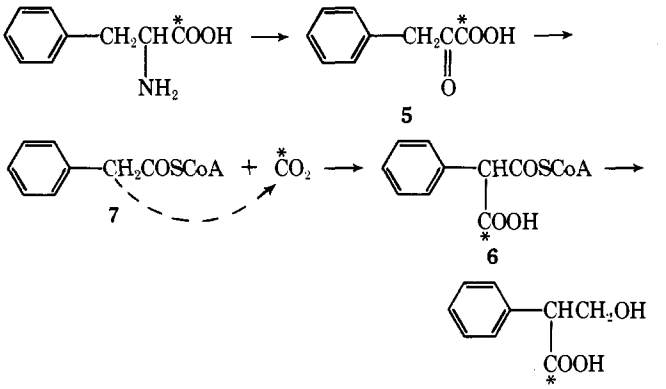
DL-[1,3-$^{13}C_2$]Phenylalanine was prepared by the route illustrated in Scheme III in 32% overall yield from barium [^{13}C]carbonate and potassium [^{13}C]cyanide. The proton NMR spectra of the ^{13}C-enriched intermediates in this synthetic sequence were of interest, showing expected[17] ^{13}C-^1H coupling constants in the range of 120–152 Hz (see Experimental Section). The mass spectrum of the *N*-acetyl derivative of the [1,3-$^{13}C_2$]phenylalanine had a parent peak at *m/e* 209, corresponding to 81.0% of the doubly labeled species. An independent estimation of the isotope enrichment of the phenylalanine was obtained by an examination of its ^{13}C NMR spectrum.

Recently geminal ($^2J_{C-C}$) and vicinal ($^3J_{C-C}$) coupling constants have been shown to exist[18,19] and have been observed in ^{13}C-enriched amino acids.[20] Since it has been shown that $^3J_{C-C}$ is 0.5 Hz greater than $^2J_{C-C}$ in substituted toluenes,[21] it was possible to make unique assignments to the aromatic carbons of phenylalanine on the basis of their coupling with the benzylic carbon (90% enriched) despite their near degeneracy (Table I). $^2J_{C-C}$ is also typically less than $^3J_{C-C}$ in aliphatic systems.[18] Thus in phenylalanine C_1–C_3 coupling was not observed (<0.5 Hz), whereas $^3J_{1-1'}$ was 2.5 Hz.

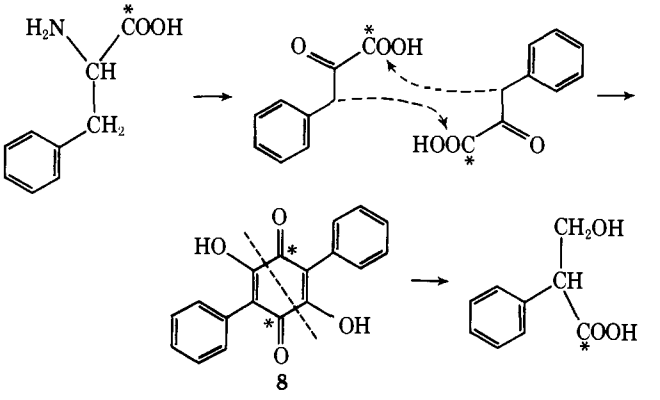
The [1,3-$^{13}C_2$]phenylalanine was mixed with a small amount of [1-^{14}C]phenylalanine so that the specific incorporation of the phenylalanine into tropic acid could be estimated by means of a radioactive assay. This material was fed to *Datura innoxia* plants by the wick method. After 7 days the plants were harvested and the alkaloids separated affording radioactive hyoscyamine and scopolamine having specific incorporations (^{14}C) of 0.32 and 0.25%, respectively. Meteloidine and other alkaloids in this species not containing a tropic acid moiety had negligible activity. The proton noise decoupled ^{13}C NMR spectra of hyoscyamine and scopolamine were determined and the assignment of chemical shifts was made by comparison with appropriate model compounds and by continuous wave off-resonance decoupling. The chemical shifts are recorded in Table II and are in essential agreement with values reported by Wenkert et al.[22] Doublets, with splittings up to 0.5 ppm, were observed for the chemically equivalent carbons of the tropine moiety of the alkaloids. This splitting is due to the asymmetric tropic acid substituent. The maximum splitting was observed for C-6′ and C-7′. This result is consistent with the tropic acid moiety being folded under the tropine nucleus, the chiral center C-2 being closest to C-6′ and C-7′ as illustrated in structural formula **9**.

The ^{13}C NMR spectrum of the radioactive scopolamine isolated from the plant is illustrated in Figure 1. Satellites of C-1 and C-2 are clearly seen, having ^{13}C–^{13}C coupling

Scheme I. Hypothetical Formation of Tropic Acid via Phenylacetyl Coenzyme A



Scheme II. Hypothetical Formation of Tropic Acid via Polyporic Acid



Scheme III. Synthesis of DL-[1,3-$^{13}C_2$]Phenylalanine

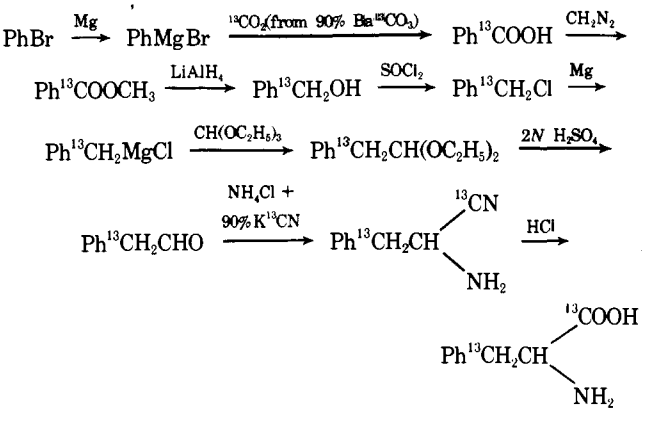
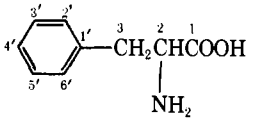


Table I. ^{13}C NMR Spectra of DL-[1,3-^{13}C]Phenylalanine (90% Enriched with ^{13}C at C-1 and C-3)

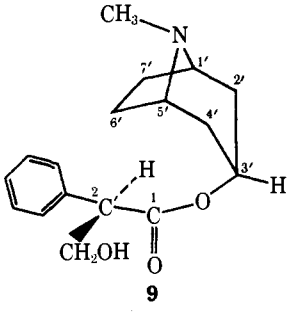


Carbon atom	Chemical shift (ppm from Me_4Si)	Multiplicity	$^{13}C-^{13}C$ coupling, Hz
1	181.1	Singlet	$^2J_{1-3} < 0.5$
2	58.0	AB quartet	$^1J_{1-2} = 53$, $^1J_{2-3} = 33$
3	40.8	Singlet	$^2J_{1-3} < 1$
1′	138.6	AB quartet	$^1J_{1'-3} = 43.6$, $^3J_{1'-1} = 2.5$
2′ = 6′	130.3	Doublet	$^2J_{2'-3} = 2.5$
3′ = 5′	129.5	Doublet	$^3J_{3'-3} = 3.5$
4′	127.7	Singlet	$^4J_{4'-3} < 1$

Table II. Chemical Shifts of Hyoscyamine and Scopolamine

Carbon atom	Hyoscyamine, δ_C from Me_4Si	Scopolamine, δ_C from Me_4Si
1	172.0	171.3 (171.7)[a]
2	54.5	54.1 (54.5)
3	63.8	63.5 (63.7)
4	135.7	135.5 (135.9)
5,9	128.6[b]	128.5[b] (128.5)
6,8	128.0[b]	127.7[b] (127.9)
7	127.4	127.5 (127.4)
1′,5′	59.3, 59.4 (59.3)[a]	57.3, 57.4 (58.2)
2′,4′	35.9, 36.1 (35.7)	30.3, 30.4 (31.7)
3′	67.8 (67.6)	66.4 (66.6)
6′,7′	25.2, 24.7 (24.8)	55.6, 56.0 (55.9)
9′	40.0 (39.7)	41.6 (43.4)

[a] Values reported by Wenkert et al.[22] [b] These assignments may be interchanged.



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constants of 56 ± 1 Hz. Similar satellites were observed in the ^{13}C NMR spectrum of the radioactive hyoscyamine at C-1 (172.0 ppm) and C-2 (54.5 ppm) having coupling constants of 57 ± 1 Hz. The specific incorporation of phenylalanine into the alkaloids, determined from the intensity of the satellite peaks relative to the naturally occurring singlet,[14e] was 0.33% for hyoscyamine and 0.25% for scopolamine, in excellent agreement with the incorporations determined from radioactive assay.

The intramolecular nature of the phenylalanine → tropic acid conversion has thus been clearly established, and the identity of specific incorporations determined by radioactive assay and by examination of the ^{13}C NMR spectra indicates that any intermolecular migration of the carboxyl group is negligible. Biogenetic Schemes I and II are thus invalid.

We have previously[7,9] suggested that the incorporation of phenylacetic acid could be rationalized by assuming that it undergoes a carboxylation affording phenylpyruvic acid and thence to phenylalanine by transamination. This conversion has been observed by Allison in ruminal bacteria[23] and photosynthetic anaerobic bacteria.[24] He showed that [1-^{14}C]phenylacetic acid yielded [2-^{14}C]phenylalanine.[24] In order to determine whether this carboxylation occurs in *Datura* species we have now fed [1-^{14}C]phenylacetic acid to *D. stramonium* plants. Carrier amounts of L-phenylalanine and the alkaloids were added to the plants on harvesting 5 days later. The reisolated phenylalanine had very low activity (0.0022% incorporation). The crude alkaloids were radioactive, and initially the separated hyoscyamine had significant activity. However, on repeated crystallization of derivatives and chromatography, the activity dropped to a negligible amount (0.005% incorporation). The scopolamine after purification also had insignificant activity (0.0013% incorporation). We thus conclude that the previous reports[3,12] in which phenylacetic acid was claimed to be a precursor of tropic acid are incorrect, and we suggest that the alkaloids which were isolated were contaminated with radioactive impurities. The source of the radioactive contaminants was not determined. We considered that the administration of phenylacetic acid may have led to the formation of phenylacetyltropine.[25] However, activity was not retained by this compound when it was added as a carrier to the crude radioactive alkaloids from the plant.

Experimental Section[26]

[1-^{13}C]Benzyl Chloride. Phenylmagnesium bromide was prepared from bromobenzene (3.5 ml, 33 mmol) and magnesium (0.8 g, 33 mmol) in ether (45 ml) under N_2. Carboxylation was carried out at −20° with $^{13}CO_2$ generated from barium [^{13}C]carbonate (90% ^{13}C, Monsanto Chemical Co.) by means of concentrated sulfuric acid. After stirring for 1.5 hr and warming to room temperature, the reaction mixture was acidified with dilute HCl. The ether layer and ether washings of the aqueous layer were extracted with dilute Na_2CO_3. The carbonate washings were acidified with HCl, and the precipitated benzoic acid was extracted with ether. Diazomethane in ether was added to this solution until a yellow color persisted. This solution of methyl [1-^{13}C]benzoate was evaporated to 10 ml and added dropwise with stirring to a suspension of $LiAlH_4$ (1.5 g) in ether (50 ml). The mixture was refluxed for 2 hr and cooled, and excess hydride decomposed by the successive addition of H_2O (1 ml), 15% NaOH (1.5 ml), and H_2O (4 ml). Evaporation of the filtered mixture afforded [1-^{13}C]benzyl alcohol which was added slowly to thionyl chloride (10 ml) stirred at 0°. After 2 hr at 0° the mixture was refluxed for 2 hr and the excess thionyl chloride then removed in vacuo. The residue was distilled (bp 88°, 20 mm) affording [1-^{13}C]benzyl chloride (2.93 g, 77% yield based on $Ba^{13}CO_3$): proton NMR ($CDCl_3$) δ 7.33 (5 aromatic H), 4.57 (2 benzylic H, d, $J_{^{13}C-H}$ = 152 Hz). The ratio of the area under the methylene doublet (H-^{13}C-H) to that under the singlet (H-^{12}C-H) was consistent with 90% ^{13}C enrichment at the benzylic position.

[2-^{13}C]Phenylacetaldehyde Diethylacetal.[29] [1-^{13}C]Benzyl chloride (2.0 ml, 17.4 mmol) was added slowly during 1.5 hr to a stirred suspension of magnesium (0.42 g, 17.4 mmol) in ether (5 ml) under N_2. When the reaction started, additional ether (15 ml) was added to suppress the formation of dibenzyl. After stirring for 20 min, ethyl orthoformate (2.87 ml, 17.4 mmol) in ether (5 ml) was added and the mixture refluxed for 6 hr. The white solid obtained on evaporation of the ether was stirred with saturated NH_4Cl solution (40 ml) at 90° under N_2 overnight. The cooled mixture was extracted with ether, dried ($MgSO_4$), and evaporated to afford [2-^{13}C]phenylacetaldehyde diethylacetal (3.24 g, 95%): proton NMR ($CDCl_3$) δ 7.25 (5 aromatic H, d, J = 2 Hz), 4.63 (1 H, t, J = 6 Hz), 3.57 (4 H, m), 2.93 (2 benzylic H, doublet of doublets, J = 6 Hz, $J_{^{13}C-H}$ = 130 Hz), 1.17 (6 H, t, J = 7 Hz). A small amount of [$^{13}C_2$]dibenzyl was also obtained ($J_{^{13}C-H}$ = 125 Hz).

[2-^{13}C]Phenylacetaldehyde. The acetal (3.24 g) from the previous experiment was refluxed with 2 *N* sulfuric acid (50 ml) and then steam distilled in N_2 atmosphere. The aqueous distillate was extracted with CH_2Cl_2 and ether. Evaporation of the combined extracts afforded [2-^{13}C]phenylacetaldehyde (2.14 g) which was used in the subsequent step without further purification: proton

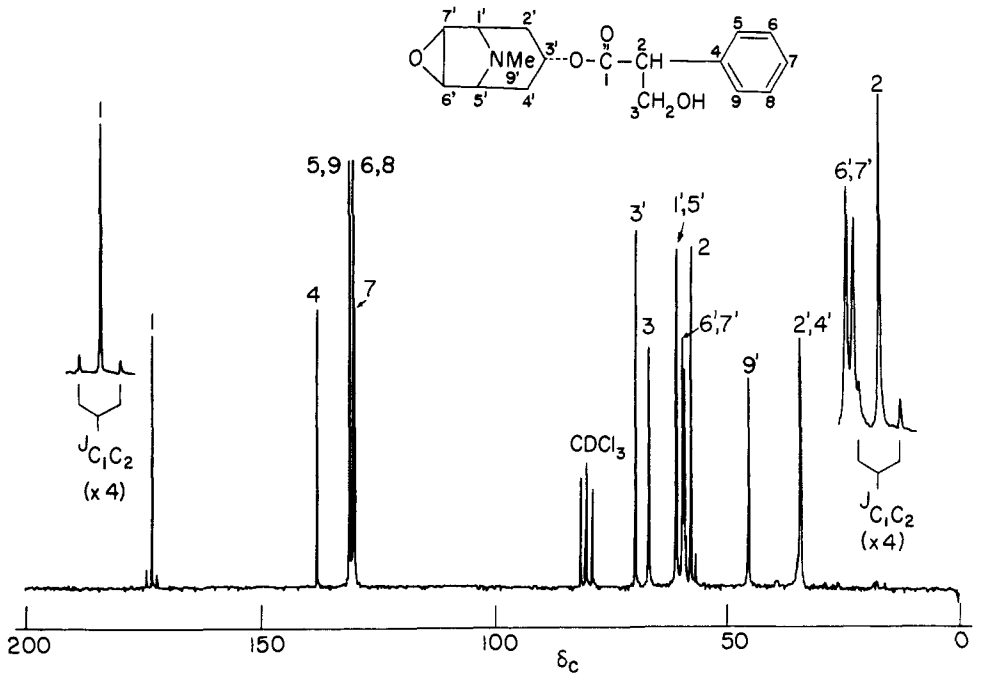

Figure 1. Proton noise decoupled Fourier transform ^{13}C NMR spectrum of the enriched scopolamine derived from [1,3-$^{13}C_2$]phenylalanine. The triplets at C-1 and C-3 are also shown expanded fourfold.

NMR ($CDCl_3$) δ 9.65 (1 aldehyde H, t, J = 2.5 Hz, $^2J_{^{13}C-C-H}$ = 26 Hz), 7.23 (5 aromatic H, m), 3.57 (2 benzylic H, d, d, J = 2.5 Hz, $J_{^{13}C-H}$ = 128 Hz).

DL-[1,3-$^{13}C_2$]Phenylalanine. The [2-^{13}C]phenylacetaldehyde (2.14 g) was added to a cooled (0°), stirred mixture of potassium [^{13}C]cyanide (90% ^{13}C, Prochem Ltd.) (1 g) and NH_4Cl (2.0 g) in 50% aqueous methanol (12 ml). The reaction mixture was slowly allowed to warm to room temperature, and after 9 hr the odor of phenylacetaldehyde could no longer be detected. Concentrated NH_3 (1 ml) was then added and the mixture heated at 60° for 7 hr. The cooled mixture was then added to concentrated HCl (50 ml). After standing overnight the solution was refluxed for 1 hr and then evaporated in vacuo to a small volume. Some tar (about 200 mg) was removed and the filtrate chromatographed on Bio-Rad 50W X4 (100–200 mesh) ion-exchange resin (H^+ form). DL-[1,3-$^{13}C_2$]Phenylalanine (1.05 g, 42%) was eluted with 0.7 N NH_3 and crystallized from aqueous ethanol. The product was identical in all respects with an authentic specimen of DL-phenylalanine (TLC, mp and mmp 237° dec, ir (KBr), mass spectrum).

DL-[1-^{14}C,1,3-$^{13}C_2$]Phenylalanine. DL-[1-^{14}C]Phenylalanine (nominal activity 250 μCi, 0.7 mg, Amersham-Searle) was dissolved in H_2O and added to a hot ethanol solution of the DL-[1,3-$^{13}C_2$]phenylalanine (1.05 g) and the mixture crystallized to constant radioactivity (923 mg), 8.77×10^7 dpm/mmol. The mass spectrum of the derived N-acetyl[1-^{14}C,1,3-$^{13}C_2$]phenylalanine had a parent peak at m/e 209 (corresponding to 81.0% ^{13}C enrichment) and a base peak at m/e 92 (corresponding to 90.4% ^{13}C enrichment) in the benzyl portion of the molecule. Independent determination of the isotopic enrichment of the [1-^{14}C,1,3-$^{13}C_2$]phenylalanine was obtained from its ^{13}C NMR spectrum. The ratios of the integrated areas of the benzylic and carbonyl carbons relative to the aromatic carbons in the enriched and normal abundance phenylalanine, run under identical spectrometer conditions in 5-mm tubes, indicated 85.2 ± 5% at C-1 and 88.3 ± 5% at C-3.

Administration of DL-[1-^{14}C,1,3-$^{13}C_2$]Phenylalanine to *Datura innoxia* and Isolation of the Alkaloids. DL-[1-^{14}C,1,3-$^{13}C_2$]Phenylalanine (100.6 mg, 8.77×10^7 dpm/mmol) dissolved in H_2O was fed to eight *Datura innoxia* plants, varying in age from 2 to 4 months, by the wick method.[30] Seven days later the plants (fresh wt 788 g) were harvested and macerated in a Waring blendor with a mixture of $CHCl_3$, ether, and concentrated NH_3 as previously described.[31] The crude mixture of alkaloids (3.18×10^5 dpm, 0.6% absolute incorporation) was separated by preparative TLC on silica gel PF-254 (Merck) yielding hyoscyamine (73 mg, 2.80×10^5 dpm/mmol, 0.32% sp inc.), scopolamine (180 mg, 2.19×10^5 dpm/mmol, 0.25% sp inc.), and meteloidine (23 mg, 1.37×10^3 dpm/mmol, 0.0015% sp inc.). The isolated hyoscyamine and scopolamine were identical with authentic specimens and were crystallized to constant activity as their hydrochloride or hydrobromide salts. The ORD curve of the labeled scopolamine hydrochloride was determined on a Cary Model 60 spectropolarimeter using 0.5-cm cells at 20° (c 0.031 g/100 ml, 0.1 N HCl) $[\alpha]_{233}$ −4300° (trough). A commercial sample measured under the same conditions had $[\alpha]_{233}$ −4200°.

Administration of [1-^{14}C]Phenylacetic Acid to *Datura stramonium* and Isolation of the Alkaloids and Phenylalanine. [1-^{14}C]Phenylacetic acid (0.76 mg, 7.2×10^8 dpm, Amersham-Searle) dissolved in dilute NH_3 was fed by the wick method to six *D. stramonium* plants (3–4 months old) growing in soil in a greenhouse. After 5 days the plants were harvested (fresh wt 570 g) and macerated in a Waring blendor with 50% aqueous ethanol (2 l.), inactive hyoscyamine (1 mM), scopolamine (1 mM), and L-phenylalanine (1 mM) being added at this time as carriers. After standing overnight the mixture was filtered and the filtrate evaporated under reduced pressure until all the ethanol had been removed. The solution was then made basic with NH_3 and extracted with a mixture of $CHCl_3$ and ether. This extract (8.7×10^7 dpm) was evaporated; the residue was dissolved in ether (100 ml) and extracted with 0.5 N HCl. This aqueous extract was made basic with Na_2CO_3 and extracted with a mixture of $CHCl_3$ and ether. The dried ($MgSO_4$) extract was evaporated and the residual alkaloids were separated on a Celite column[2] yielding hyoscyamine hydrochloride (96 mg) and scopolamine hydrochloride (245 mg). Initially the hyoscyamine hydrochloride had appreciable activity (3.4×10^6 dpm/mmol). However, after four recrystallizations, purification by TLC on silica gel, conversion to its picrate, and three more recrystallizations, this activity had fallen to an insignificant level (3.6×10^4 dpm/mmol). The scopolamine hydrochloride, initially having an activity of 4.2×10^5 dpm/mmol, after three recrystallizations had negligible activity (9.5×10^3 dpm/mmol). The ammoniacal aqueous solution from which the alkaloids had been extracted was acidified with HCl and extracted in a continuous extractor for 3 days. This ether extract (7.8×10^7 dpm) presumably contained unmetabolized [1-^{14}C]phenylacetic acid. The aqueous solution (3.54×10^6) was adjusted to pH 6.9 with NH_3 and placed on a Dowex 50 X8 (H^+ form) ion-exchange resin. After washing with H_2O, the amino acids were eluted with 1 N NH_3. This mix-

ture of amino acids was then subjected to chromatography on several thick sheets of filter paper, developing with *n*-BuOH–HOAc–H_2O (8:2:1). The zone corresponding to phenylalanine was extracted with hot H_2O. Evaporation and crystallization of the residue from aqueous ethanol afforded phenylalanine (58 mg) having an activity of only 1.6×10^4 dpm/mmol.

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Studies of Peptide Conformation. Evidence for β Structures in Solutions of Linear Tetrapeptides Containing Proline[1]

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Abstract: Carbobenzyloxy tetrapeptide *tert*-butoxycarbonyl hydrazides containing proline were investigated by study of their proton magnetic resonances in methanol and chloroform and their infrared absorption in chloroform. The infrared spectra indicate that internally hydrogen bonded structures are important. Studies of the resonances of nitrogen-bound protons included observation of differential line broadening effects produced by an added nitroxyl, used as an indication of solvent exposure, and determination of the concentration dependence of chemical shift (in chloroform), used as an indication of participation in intermolecular hydrogen bonding. For derivatives with the sequence Gly-L-Pro-D-Xxx-Gly the data support a major contribution, in both solvents, of a β structure with two intramolecular hydrogen bonds and a type II turn at Pro-Xxx. For the sequence D-Val-L-Pro-Gly-Xxx, evidence is less convincing, but a structure with a type I turn at Pro-Gly provides an explanation of the observations.

Nuclear magnetic resonance studies of oligopeptide conformation have dealt largely with cyclic peptides, because the information contained in the NMR spectra relates to only a fraction of the conformational variables. The cyclic constraint reduces the field of conformational possibilities for the molecule itself and limits the number of models that must be tested against the observable data. The conformation space available to linear, noncyclic peptides is much greater, and it is also less likely that there will be small regions of minimum conformational energy separated by bar-