with ether. The combined ether extracts were shaken with three portions of water, each aqueous wash being in turn back-extracted with a single 20-ml volume of ether. Shaking of the ethereal solution with saturated sodium chloride followed by drying over sodium sulfate and evaporation of the solvent gave 0.027 g of allyl tosylhydrazide **19**: nmr (CCl_4) δ 0.80 (s, CH_3, 3 H), 1.60, 1.63 (s, CH_3), 2.40 (s, aromatic CH_3) and 1.2–2.6 (m) (total 19 H), 3.5 (m, CH_2N, 2 H), 5.1 (m, olefinic, 1 H), 5.35 (m, olefinic, 1 H), 7.20 (d, J = 8 Hz, aromatic, 2 H), 7.65 (d, J = 8 Hz, aromatic, 2 H); ir $\lambda_{max}^{CCl_4}$ 6.23 μ (aromatic).

β-*trans*-Bergamotene (4). Allyl tosylhydrazide **19** (0.027 g, 0.07 mmol) and 0.2 g of sodium acetate trihydrate in 1 ml of acetic acid were stirred for 1.25 hr at 60°. The solution was diluted with 25 ml of water and extracted with four 10-ml volumes of pentane. The combined pentane extracts were shaken with saturated potassium bicarbonate and saturated sodium chloride, then dried over sodium sulfate and concentrated to *ca.* 5 ml. This solution was passed through 2 g of basic alumina (II) (Woelm), and the eluent was concentrated under vacuum to yield 0.010 g of oil which was further purified by tlc (silica gel; pentane) to afford 0.007 g of β-*trans*-bergamotene (**4**), homogeneous by tlc (R_f 0.5, pentane) and vpc (6 ft × 0.125 in., Carbowax 20M on 80–100 H.P. Chrom W; 180°; R_t 15.6 min): nmr (CCl_4) δ 0.72 (s, CH_3, 3 H), 1.60, 1.68 (s, CH_3) and 1.1–1.27 (m) (total 18 H), 4.55 (m, exo methylene, 2 H), 5.1 (m, olefinic, 1 H); ir $\lambda_{max}^{CCl_4}$ 3.4 (C–H), 6.06 (C=C), 6.9, 7.28, 11.32 μ (CH_2=C). An exact mass determination gave m/e 204.1874 (calcd for $C_{15}H_{24}$: 204.1878).

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Aberrant Alkaloid Biosynthesis. Formation of Nicotine Analogs from Unnatural Precursors in *Nicotiana glutinosa*[1]

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Abstract: Several methyl derivatives of nicotine have been biosynthesized using *Nicotiana glutinosa* plants and the corresponding substituted pyrrolinium precursors. The syntheses of the ^{14}C-labeled pyrrolinium precursors, which were utilized in the biosynthetic experiments, are also described. For chromatographic and spectral comparisons, authentic samples of some of the substituted nicotines were also synthesized. The stereochemistry and absolute configuration of the biosynthesized nicotine analogs have been determined. Incorporation results with 2- and 3-methyl-substituted pyrrolinium precursors allow some speculation on the specificity and steric requirements of the enzyme(s) involved in the latter stages of nicotine biosynthesis.

The biosynthetic pathway of formation of nicotine (**1**) in *Nicotiana* has been subject to a great deal of study. A multitude of experiments have been carried out by means of precursor feedings and short-term biosyntheses with CO_2-^{14}C; however, the precise biosynthetic pathway has yet to be completely elucidated.[2] In conjunction with other biosynthetic experiments with *Nicotiana glutinosa*, we became interested in the possibility of biosynthesizing unnatural nicotine analogs by using substituted precursors instead of the normal, natural precursor.

The possibility of biosynthesizing unnatural nicotine analogs using substituted natural precursors was interesting for several reasons. First, the incorporation of an unnatural precursor (*i.e.*, a substituted natural precursor) into an unnatural product (*i.e.*, nicotine analog) had not been previously reported in plants. Second, experiments with a series of substituted precursors might define the specificity of the enzyme system which catalyzes the biosynthesis of nicotine from a 1-methyl-1-pyrrolinium salt **2** and a nicotinic acid derivative. Third, the formation of unnatural alkaloids *in vivo* should be useful in the preparation of analogs of biologically active natural products. Finally, since the unnatural products possess a structural label in addition to the usual radioactivity label, they should also be of great utility in the study of metabolism and interrelationships among the various alkaloids and other natural products in a given plant.

In a preliminary communication,[3] we have reported the incorporation of 1,3-dimethyl-1-pyrrolinium-*3-methyl*-^{14}C chloride (**4**), into the nicotine analog, 3′-methylnicotine (**3**). We now report the full details for this preliminary communication and additional related experiments concerning the formation of the nicotine analogs **6** and **7** from **9b** and **10b**, respectively.

Precursor Synthesis. In the present work, only derivatives of the natural pyrrolidine ring precursor, 1-methyl-1-pyrrolinium salt (**2**), have been examined as potential unnatural precursors for analogs of nicotine. This choice was based on the fact that **2** has been reported to be a highly efficient precursor of the pyrrolidine ring of nicotine.[4,5] *A priori*, derivatives of the pyridine ring precursor, nicotinic acid, could also have been examined; however, nicotinic acid is a less efficient precursor, probably due to its more wide-

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(2) For complete reviews of the present status of nicotine biosynthesis see: M. L. Rueppel, B. P. Mundy, and H. Rapoport, submitted for publication; E. Leete, *Advan. Enzymol.*, 32, 373 (1969).

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1, $R_1 = R_2 = R_3 = H$
3, $R_1 = R_3 = H; R_2 = CH_3$
5, $R_1 = R_2 = H; R_3 = CH_3$
6, $R_1 = CH_3; R_2 = R_3 = H$
7, $R_1 = H; R_2 = R_3 = CH_3$
8, $R_1 = D; R_2 = R_3 = CH_3$

2, $R_1 = R_2 = R_3 = H$
4, $R_1 = R_2 = H; R_3 = {}^{14}CH_3; X = Cl^-$
9a, $R_1 = {}^{14}CH_3; R_2 = R_3 = H; X = ClO_4^-$
b, $R_1 = {}^{14}CH_3; R_2 = R_3 = H; X = Cl^-$
10a, $R_1 = H; R_2 = R_3 = {}^{14}CH_3; X = ClO_4^-$
b, $R_1 = H; R_2 = R_3 = {}^{14}CH_3; X = Cl^-$

spread metabolic functions.[6] Accordingly, it seemed reasonable to first concentrate on analogs of **2**.

The first candidate unnatural precursor examined was 1,3-dimethyl-2-pyrrolinium-*3-methyl-*^{14}C chloride (**4**). It was synthesized by condensation of 1-methyl-2-pyrrolidinone (**11**) with diethyl carbonate using sodium hydride as the base to give ester **12**. 1,3-Dimethyl-3-carbethoxy-2-pyrrolidinone-*3-methyl-*^{14}C (**13**)[7] was obtained by alkylating the sodium enolate of **12** with methyl-^{14}C iodide.[8] The procedure utilized in the isolation of 3-methyl derivative **13** precluded the presence of unalkylated compound **12**; this was verified by glpc analysis. Hydrolysis of the alkylated ester **13** (specific activity 2.71 × 10^7 dpm/mmol) quantitatively gave the acid **14** (specific activity 2.68 × 10^7 dpm/mmol) which on decarboxylation gave 1,3-dimethyl-2-pyrrolidinone (**15**).[9] Stoichiometrically controlled reduction of **15** with lithium aluminum hydride gave in 92% yield a mixture of pyrrolinium salt **4** (63%) and the pyrrolidine hydrochloride **16** (37%). Chromatography on silica gel followed by ion exchange gave pure **4** in 40% overall yield from **12**. The precursor **4** was characterized spectrally, chromatographically, microanalytically, and by conversion to the hygrine derivative **17**.

11 → 12 → 13, $R = C_2H_5$; 14, $R = H$ → 15 → 16 + 4

The second candidate unnatural precursor (**9b**), also labeled with carbon-14, was synthesized as follows. Methyl-^{14}C iodide[8] was converted to the corresponding Grignard reagent and subsequent addition of 1-methyl-2-pyrrolidinone (**11**) gave a mixture of 1,2-dimethyl-1-pyrrolinium-*2-methyl-*^{14}C chloride (**9b**) and 1,2,2-trimethylpyrrolidine-*2,2-dimethyl-*^{14}C hydrochloride.[10-12] Conversion of the mixture to the corresponding free bases followed by the addition of 70% perchloric acid gave the pure iminium perchlorate **9a** (specific activity 9.57 × 10^6 dpm/mmol) in an overall yield of 27%. Immediately prior to the actual feeding experiments, the perchlorate **9a** was converted into the chloride **9b** by ion exchange.

17 18

Synthesis of the third candidate unnatural precursor (**10b**) was also initiated with 1-methyl-2-pyrrolidinone (**11**). Alkylation of **11** with methyl-^{14}C iodide[8] in diethyl ether at −78° with lithium diisopropylamide as the base gave 1,3,3-trimethyl-2-pyrrolidinone-*3,3-dimethyl-*^{14}C (**18**)[9,13] in 78% yield. Controlled lithium aluminum hydride reduction of **18** (specific activity 8.63 × 10^6 dpm/mmol) gave a mixture of **10b** (66%) and 1,3,3-trimethylpyrrolidine-*3,3-dimethyl-*^{14}C hydrochloride (**19**; 34%). The pure perchlorate (specific activity 8.93 × 10^6 dpm/mmol) was obtained by the addition of perchloric acid to the corresponding free base of **10b** and **19**. Prior to the feeding experiments **10a** was reconverted into the chloride **10b**.

Biosynthesis and Isolation. Each biosynthetic experiment (Tables I and II) was carried out using four *Nicotiana glutinosa* plants which were growing in an aerated hydroponic solution.[14] The plants had been grown prior to the feeding experiment as previously described.[15] Appropriate precursor was added daily in portions to the aerated hydroponic solution; the rate of addition, age of plants, and the initial and final weights of the plants are given in the footnotes to Table I. The rate and amount of uptake of the precursors in experiments 1–4 were monitored continually by liquid scintillation counting of aliquots of the hydroponic nutrient solution. Particularly in experiments 3 and 4 in which an excess of the appropriate precursor was constantly maintained in the hydroponic solution, the rate and amount of precursor uptake were directly proportional to the mass of the plants; that is, as the mass of the plants increased by normal growth, a corresponding increase in the rate and amount of precursor uptake was observed. The rate and amount of uptake were apparently independent of the amount of precursor available in the hydroponic solution. Finally, no harmful effects were noted in plant growth although up to 40 mg of precursor was incorporated daily.

Since the plants failed to completely absorb all the radioactivity associated with each precursor from the hydroponic solution, the residual nutrient solution

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Table I. Distribution of Activity in Various Fractions of *Nicotiana glutinosa* after Feeding Unnatural Pyrrolinium Precursors **4**, **9b**, and **10b**

		Activity, dpm × 10^{-6}, in fractions				
Expt	Precursor	Total plant[a]	Marc[b]	Acidic and neutral	Alkaloidal	Residual aqueous
1[c]	**4**	6.58	*d*	*d*	1.85	2.15
2[e]	**4**	31.40	2.52	4.00	14.30	9.95
3[f]	**9b**	21.60	0.76	1.32	15.10	4.85
4[g]	**10b**	12.30	0.16	0.21	10.90	0.59

[a] Based on total activity fed minus activity remaining in nutrient solution. [b] The activity present in the marc was determined by combusting an aliquot using a modification of the method of F. Kalberer and J. Rutschman (*Helv. Chim. Acta*, **44**, 1956 (1961)). [c] Administered in portions over a period of 5 days with 1 day additional for growth. Total weight of the four plants was 261 g at the start and finish; their initial age was 66 days. [d] Not determined. [e] Administered in increasing amounts over a period of 8 days to 59-day-old plants. Total weight of the four plants was 54 and 139 g at the start and finish, respectively. [f] Administered in increasing amounts over a period of 13 days to 43-day-old plants. Total weight of the four plants was 13.7 and 53.3 g at the start and finish, respectively. [g] Administered in increasing amounts over a period of 8 days to 55-day-old plants. Total weight of the four plants was 27.2 and 54.5 g at the start and finish, respectively.

Table II. Administration of Pyrrolinium Precursors **4**, **9b**, and **10b** to *Nicotiana glutinosa*[a] and Incorporation into Nicotine Analogs **3**, **6**, and **7**

		Incorporation			
Expt	Precursor fed	Wt, mg	Act., dpm	Nicotine analog formed	Yield of analog, dpm (%)
1	**4**	33	6.58×10^6	**3**	4.22×10^5 (6.5)
2	**4**	159	31.4×10^6	**3**	4.34×10^6 (13.8)[b]
3	**9b**	304	21.6×10^6	**6**	8.26×10^3 (0.04)
4	**10b**	202	12.3×10^6	**7**	9.50×10^4 (0.77)[c]

[a] For the preparation of the plants, see ref 15. [b] Using nicotine (**1**) as the standard, glpc analysis of the crude alkaloid fraction indicated the presence of 56.0 mg of **1** and 21.6 mg (10.5%) of 3′-methylnicotine (**3**). [c] Glpc analysis of the crude alkaloid fraction indicated the presence of 17.0 mg of **1** and 2.2 mg (0.8%) of 3′,3′-dimethylnicotine (**7**).

was examined in order to assess the stability of each precursor administered. In experiments 3 and 4, greater than 95% of the radioactivity in the residual nutrient was shown by nmr and tlc to be due to the presence of **9b** and **10b**, respectively. In experiment 1, 90% of the total precursor administered each day was absorbed into the plants in 24 hr. The remaining 10% of the activity was due to chemical or biological change of **4** and accumulated as the experiment proceeded. Since these plants, and plants added to the nutrient solution after removal of the previous plants, were incapable of absorbing the transformation products of **4**, the observed incorporation most probably is due to the uptake of 1,3-dimethyl-1-pyrrolinium-*3-methyl-*^{14}C chloride (**4**). Further support for the role of intact **4** as the actual precursor is provided by the excellent agreement in specific activities (see following) between **4** and the biosynthesized 3′-methylnicotine (**3**).[16]

After each feeding experiment had proceeded for several days and the desired amount of precursor had been incorporated, the plants were fractionated as described previously[15] to give the four fractions indicated in Table I. The distribution of activity found in these four fractions is of interest for several reasons. First, essentially all (100 ± 3%) of the activity incorporated into the plants in each experiment (with the exception of expt 1 where the activities of two fractions were not determined) has been accounted for by these four fractions. Clearly no loss of activity has occurred by metabolism of the administered precursors to respired CO_2-^{14}C. Second, significant differences in metabolism of each of the three precursors are indicated in the activity distributions. The compilation of activity distributions of the type given in Table I should prove valuable in metabolism and precursor feeding experiments. Although incorporations of the range of 30 to 0.003% have been reported in biosynthetic experiments, generally no attempt has been made to ascertain the fate of the majority of the precursor administered. Undoubtedly, significant metabolic and biosynthetic information could be obtained in many precursor feeding experiments from such a treatment.

The crude alkaloid fraction was analyzed by preparative glpc on either a 15 ft × 0.25 in. column (expt 1, 2, and 4) or a 5 ft × 0.25 in. column (expt 3) of 10% KOH, 10% polybutylene glycol on 60–80 firebrick.[17] In the latter case (expt 3) no fractionation of nicotine (**1**) and 2′-methylnicotine (**6**) was attempted due to the presence of only approximately 150 μg of **6** in a total of 19.6 mg of **1**. The yields of the three analogs (**3**, **6**, and **7**) of nicotine are shown in Table II. Relative incorporations of 1,3-dimethyl- (**4**), 1,2-dimethyl- (**9b**), and 1,3,3-trimethyl-1-methylpyrrolinium chloride (**10b**) into the corresponding nicotine analogs (**3**, **6**, and **7**, respectively) are in an approximate ratio of 360:1:20. The differences in incorporation appear to be consistent with the relative amount of steric hindrance expected in each case in joining the substituted precursor with the hypothesized 1,6-dihydronicotinic acid derivative (**20**)[18] to give the intermediate **21a**, **b**, or **c**, respectively.

(16) The possible incorporation of **4** as a chemical or biological transformation product cannot be completely excluded. However, such an hypothesis would require rapid and total absorption of the transformation product and would be extremely difficult to subject to experimental test. It would also require reconversion in the plant to a form capable of incorporation into the observed substituted nicotine, a most improbable event.

(17) See footnote *c* in Table III for the conditions utilized with the 15 ft × 0.25 in. column. The 5 ft × 0.25 in. column at a temperature of 162° and flow rate of 100 ml/min gave a retention time of 9.3 min for nicotine (**1**).

(18) R. F. Dawson, D. R. Christman, A. D'Adamo, M. L. Solt, and A. P. Wolf, *J. Amer. Chem. Soc.*, **82**, 2628 (1960).

Oxidation–decarboxylation of **21** and subsequent loss of R afford the appropriate nicotine analog.

4, 9b or 10 + [dihydropyridine carboxylate, R] → [**21**]

21a, $R_1 = R_3 = H; R_2 = CH_3$
b, $R_1 = CH_3; R_2 = R_3 = H$
c, $R_1 = H; R_2 = R_3 = CH_3$

In Vitro **Synthesis and Characterization of Nicotine Analogs.** To aid in the characterization of the biosynthesized nicotine analogs **3** and **7**, authentic samples of **3** and **7** were synthesized along with *cis*-3′-methylnicotine (**5**) and 3,3′-dimethylnicotine-*2′-d* (**8**). Adapting a method previously utilized for synthesizing nicotine (**1**),[19] 1,3,3-trimethyl-2-pyrrolidinone was added to an ethereal solution of 3-pyridyllithium at −78°; isolation gave the iminium salt **22a** which was not purified or characterized. The sample was divided into two portions, and reduction with $NaBH_4$ in one case and $NaBD_4$ in the other gave **7** and **8** in 17 and 16% overall yield, respectively. Complete characterization

[structure **22**, Cl^-]

22a, $R_1 = R_2 = CH_3$
b, $R_1 = H; R_2 = CH_3$

of **7** and **8** is given in Table III and the Experimental Section; however, the following points need emphasis for utilization in further discussion. In the nmr of **7** and **8** important resonances occur at δ 0.64 (s, 3 H) and 1.08 (s, 3 H). The assignment of the singlet at δ 0.64 to the *cis*-methyl in **7** and **8** follows from an examination of molecular models which indicate that the *cis*-methyl, in the most stable confirmation, is in the shielding cone of the pyridine ring; the *trans*-methyl in **7** and **8** is in the deshielding cone and occurs at significantly lower field, δ 1.08.

In an analogous manner, *trans*-3′-methylnicotine (**3**) and *cis*-3′-methylnicotine (**5**) were synthesized by sodium borohydride reduction of the iminium salt **22b** in 10 and 4% overall yield, respectively. By means of preparative glpc, **3** and **5** were separated and characterized as summarized in Table III and the Experimental Section. The most significant features arise from an examination of the nmr spectra of **3** and **5** along with that of nicotine (**1**) and nicotine-*5′,5′-d_2* (**23**).[20]

trans-3′-Methylnicotine (**3**) was assigned trans stereochemistry with respect to the methyl group and the pyridine ring on the basis of the methyl doublet occurring at δ 0.97 in analogy with the assignment for methyl groups in **7** and **8**. In a similar manner, *cis*-3′-methylnicotine (**5**) was assigned cis stereochemistry since its methyl resonance was centered at δ 0.55 as a doublet.

On the basis of the nmr spectra of **1**, **7**, **8**, and **23**, a very interesting difference in the shift of the C-2′ hydrogen in **3** and **5** can also be noted. The nmr of nicotine (**1**) has a multiplet integrating for two hydrogens centered at δ 3.2. Since **23** also shows a multiplet at 3.2 but integrating for only one H, this resonance can be assigned in **1** to the C-2′ hydrogen and to one of the C-5′ hydrogens. The large difference (>0.8) in the shifts of the two hydrogens is no doubt due to deshielding by the lone-pair electrons of the pyrrolidine ring nitrogen. In the nmr spectrum of **7**, the multiplet at δ 3.21 integrates for one H, corresponding to one of the C-5′ hydrogens. The C-2′ hydrogen has been shifted upfield to δ 2.88 as confirmed by the absence of this singlet in the spectrum of 3′,3′-dimethylnicotine-*2′-d* (**8**). The nmr spectrum of *cis*-3′-methylnicotine (**5**) shows a multiplet at δ 3.2 integrating for two H's, assigned to the C-2′ hydrogen and one of the C-5′ hydrogens in analogy to the spectrum of **1**. In marked contrast, the nmr spectrum of *trans*-3′-methylnicotine (**3**) has a multiplet at δ 3.2 which integrates for only one H while a doublet (J = 7.5 Hz, 1 H) is present at δ 2.54 and is assigned to the C-2′ hydrogen in analogy with the spectrum of **7**.

With reference to the spectra of **5**, **7**, and **8**, the large shift of the C-2′ hydrogen in **3** is attributed primarily to shielding by the trans methyl group rather than confirmational influences. Finally, it should be noted that the coupling constant (J = 7.5 Hz) observed for the C-2′ hydrogen in **3** is consistent with the assigned trans stereochemistry.[21]

Characterization of the Biosynthetic Nicotine Analogs. The characterization of the biosynthetic product obtained (6.4–13.8% yield) from the administration of 1,3-dimethyl-1-pyrrolinium-*3-methyl-^{14}C* chloride as *trans*-3′-methylnicotine (**3**) has been established. High-resolution mass spectroscopy established the molecular formula as $C_{11}H_{16}N_2$, *m/e* 176 (calcd: 176.1313; found: 176.1313) and $C_6H_{12}N$, *m/e* 98 (calcd: 98.0970; found: 98.0974) for the 1,3-dimethyl-1-pyrrolinium fragment formed by α cleavage. The nmr, mass spectrum, and glpc retention time of biosynthetic **3** are identical with those of synthetic **3** (see Table III); these comparisons eliminate alternative structures such as 4′-methylnicotine from consideration. The specific activity of biosynthetic **3** was determined by a combination of uv absorption and liquid scintillation counting to be 2.76 × 10^7 dpm/mmole, in excellent agreement with its precursor **4** (sp act. 2.74 × 10^7 dpm/mmole). The ultraviolet spectrum of biosynthetic **4** showed $\lambda_{max}^{C_2H_5OH}$ 261 nm as expected for a derivative of nicotine.[22]

Biogenetically, *trans*-3′-methylnicotine (**3**) would be expected to have the same absolute configuration at C-2′ as nicotine (**1**) which has been assigned the *S* configuration with reference to L-proline,[23] L-serine,[24] and

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Table III. Nmr, Mass Spectral, and Gas Chromatographic Data for Nicotine and Its Pyrrolidine Ring Analogs

Compound, 3-pyridyl-	Nmr, δ[a]	Assignment of nmr resonances	Mass spectrum, m/e[b] (rel abund)	Glpc[c] retention time, min
1[d]	1.6–2.6 (m, 5 H)	C-3′ (2 H)	162 (36)	20.6
		C-4′ (2 H)	134 (9)	
		C-5′ (1 H)	133 (60)	
	2.17 (s, 3 H)	N-CH_3	119 (19)	
	3.2 (m, 2 H)	C-2′ (1 H), C-5′ (1 H)	84 (100)	
	7.21 (m, 1 H)	C-5		
	7.68 (m, 1 H)	C-4		
	8.43 (m, 1 H)	C-2, C-6		
3	0.97 (d, J = 6.3 Hz, 3 H)	C-3′-CH_3	176 (37)	22.4
			134 (30)	
	1.4–2.4 (m, 4 H)	C-3′ (1 H),	133 (100)	
		C-4′ (2 H),	119 (9)	
		C-5′ (1 H)	98 (73)	
	2.10 (s, 3 H)	N-CH_3		
	2.54 (d, J = 7.5 Hz, 1 H)	C-2′		
	3.2 (m, 1 H)	C-5′ (1 H)		
	7.17 (m, 1 H)	C-5		
	7.60 (m, 1 H)	C-4		
	8.40 (m, 2 H)	C-2, C-6		
5	0.55 (d, J = 6.3 Hz, 3 H)	C-3′-CH_3	176 (33)	24.3
			134 (29)	
	1.4–2.4 (m, 4 H)	C-3′ (1 H),	133 (100)	
		C-4′ (2 H),	119 (12)	
		C-5′ (1 H)	98 (64)	
	2.14 (s, 3 H)	N-CH_3		
	3.2 (m, 2 H)	C-2′ (1 H), C-5′ (1 H)		
	7.15 (m, 1 H)	C-5		
	7.58 (m, 1 H)	C-4		
	8.38 (m, 2 H)	C-2, C-6		
7	0.64 (s, 3 H)	C-3′-CH_3 (cis)	190 (15)	
	1.08 (s, 3 H)	C-3′-CH_3 (trans)	134 (48)	26.2
	1.64 (m, 2 H)	C-4′	133 (100)	
	2.13 (s, 3 H)	N-CH_3	119 (6)	
	2.43 (m, 1 H)	C-5′	112 (7)	
	2.88 (s, 1 H)	C-2′		
	3.21 (m, 1 H)	C-5′		
	7.18 (m, 1 H)	C-5		
	7.56 (m, 1 H)	C-4		
	8.41 (m, 2 H)	C-2, C-6		
8	Same as 7 with resonance at 2.88 (s, 1 H) absent		191 (18)	26.2
			135 (46)	
			134 (100)	
			120 (4)	
			113 (8)	

[a] In CCl_4 with TMS as the internal standard. [b] At 70 eV. [c] Glpc was carried on a column of 10% KOH, 10% polybutylene glycol on 60–80 firebrick (column length: 15 ft × 0.25 in.; column temperature: 182°, flow rate: 100 cm^3 of He/min). [d] For the reported mass spectrum and partially assigned nmr of **1**, see W. L. Alworth, Ph.D. Thesis, University of California, 1964, and "Varian High Resolution NMR Spectra Catalog," Spectrum No. 269, respectively. The nmr spectrum of **1** reported above was obtained by us.

optical rotary dispersion measurements.[25] The CD curve of biosynthetic **3** (in 95% C_2H_5OH) gave a molecular ellipticity $[\theta]$ at 260 nm of +22,800 (peak); **1** showed $[\theta]_{270}$ −7090 (trough) in addition to $[\theta]_{261}$ +24,800 (peak). Although **1** showed a weaker negative Cotton effect at 273 nm in the ORD,[25] this absorption was absent in both the CD and ORD of biosynthetic **3**. On the basis of the CD curve of biosynthetic **3**, the *S* configuration is assigned at the 2′-carbon. In addition, as a result of the nmr spectral differences between *cis*- (**5**) and *trans*-3′-methylnicotine (**3**) as discussed previously, the absolute configuration at the 3′ carbon of biosynthetic **3** can also be assigned the *S* chirality. Clearly, only one of the four possible diastereomers was formed biosynthetically from precursor **4**.

The product (**6**) arising from the administration of 1,2-dimethyl-1-pyrrolinium-*2-methyl-*^{14}C chloride (**9b**) to *N. glutinosa* was characterized solely on the basis of mass spectroscopy due to the low incorporation (0.04%) of **9b**. The mass spectrum of **6** and **1** in a ratio of 1 to 130 showed m/e 176 and 98 in addition to the normal mass spectrum of nicotine. High-resolution mass spectroscopy established a molecular formula of $C_{11}H_{16}N_2$ for m/e 176 (calcd: 176.1313; found: 176.1313) and $C_6H_{12}N$ for m/e 98 (calcd: 98.0970; found: 98.0971) in agreement with formulation of

(23) P. Karrer and R. Widmer, *Helv. Chim. Acta*, **8**, 364 (1925).
(24) C. S. Hudson and A. Neuberger, *J. Org. Chem.*, **15**, 24 (1950).
(25) J. C. Craig and S. K. Roy, *Tetrahedron*, 21, 401 (1965).

the biosynthetic product as **6**. No additional characterization was possible due to the small amount of material available.

Administration of 1,3,3-trimethyl-1-pyrrolinium-*3,3-dimethyl*-^{14}C chloride (**10b**) to the plants, subsequent isolation, and preparative glpc gave 3′,3′-dimethylnicotine (**7**) in 0.77% yield. The characterization of biosynthetic **7** was by direct comparison with synthetic **7**. Synthetic and biosynthetic **7** were identical in glpc retention time (established by coinjection) and mass spectrally. The CD curve of **7** (in 95% C_2H_5OH) gave molecular ellipticities $[\theta]$ of +1950 (peak) and +2100 (peak) at 263 and 270 nm, respectively. The large decrease in the molecular ellipticity at 263 nm makes assignment of the *S* configuration of the 2′ carbon of **7** tenuous although, biogenetically, the *S* configuration might be expected. The possibility that the changes observed in the CD curve of **7** are due to the presence of an unequal mixture of enantiomers cannot be eliminated at this time.

Conclusions

The present work has shown that the enzyme system which catalyzes the condensation of 1-methyl-1-pyrrolinium salt with a 1,6-dihydronicotinic acid derivative is not completely specific. Furthermore, its specificity has been partially defined by the present experiments. The great differences observed in the efficiency of incorporation of the three substituted precursors examined can be rationalized in a consistent manner on the basis of differences in steric hindrance in the condensation reaction with a 1,6-dihydronicotinic acid derivative. The present experiments, therefore, furnish additional support for this hypothesized step in nicotine biosynthesis. Alternately, the differences in the efficiency of incorporation of **4**, **9b**, and **10b** may reflect differences in the metabolism of the precursors *in vivo;* however, we regard possible metabolic differences to be of secondary importance. Finally, it seems possible that a single enzyme system might produce the four common *Nicotiana* alkaloids, nicotine (**1**), nornicotine (**24**), anabasine (**25**), and

24 **25** **26**

anatabine (**26**), by similar condensations when provided with proper substrates.

The present approach also has broad potential applications for the preparation of analogs of biologically active natural products since, in general, it is easier to synthesize a substituted precursor than to carry out a total synthesis of an analog of a complex natural product. Additional experiments are planned with *Nicotiana* and other species in order to examine the generality of this latter concept.

Experimental Section[26]

***trans*-3′-Methylnicotine (3) and *cis*-3′-Methylnicotine (5).** The method of preparation of **5** and **3** is exactly as described for the synthesis of 7 below, with the following exceptions: (a) 5 mmol (565 mg) of 1,3-dimethyl-2-pyrrolidinone was used in place of **18** and (b) all of the crude iminium salt **22b** was reduced with $NaBH_4$. Isolation as described for **7** and preparative glpc gave 86 mg (10%) of **3** and 36 mg (4%) of **5**. *Molecular Formula:* Calcd for $C_{11}H_{16}N_2$: 176.1313. *Found:* 176.1313.

3′,3′-Dimethylnicotine (7). Under a nitrogen atmosphere was placed 50 ml of anhydrous diethyl ether. After adding 5 mmoles (790 mg) of 3-bromopyridine, the reaction mixture was cooled to −78°, and 3.1 ml (5 mmol) of *n*-butyllithium in hexane was added, followed by stirring for 20 min at −78° and addition of 5 mmoles (635 mg) of 1,3,3-trimethyl-2-pyrrolidinone (**18**). After stirring at −78° for 5 hr and room temperature for 13 hr, 20 ml of 6 *N* NaOH was added, the ether layer was separated, and the aqueous phase was extracted with ether (2 × 30 ml). The combined ethereal solutions were then extracted with 30 ml of 10% HCl and evaporation of the aqueous solution *in vacuo* gave the crude iminium salt **22a**. An aliquot (40%) of this iminium salt was dissolved in 10 ml of H_2O, sodium borohydride was added until the solution reached pH 8, and it was allowed to stand alkaline at room temperature for 30 min. Excess borohydride was destroyed by acidification with 10% HCl, and the resulting acidic solution was made alkaline with 6 *N* NaO*H* to pH 11. After extracting with methylene chloride (3 × 25 ml), drying the resulting methylene chloride solution over K_2CO_3, and filtering, concentration *in vacuo* gave the crude nicotine analog. Preparative glpc gave 64 mg (17%) of pure 3′,3′-dimethylnicotine (**7**). *Molecular Formula:* Calcd for $C_{12}H_{18}N_2$: 190.1469. Found: 190.1449.

3′,3′-Dimethylnicotine-*2′-d* (8). The iminium salt **22a**, not utilized in the preparation of 7, was used to prepare **8**. The reduction and isolation were carried out as described for **7** except that $NaBD_4$ in D_2O solution was used instead of $NaBH_4$ in H_2O. Preparative glpc of the crude nicotine analog gave 90 mg (16%) of pure **8**. The nmr indicated the presence of 93% of the 2′-dueterio species. *Molecular Formula:* Calcd for $C_{12}H_{17}DN_2$: 191.1533. Found: 191.1531.

1,2-Dimethyl-1-pyrrolinium-*2-methyl*-^{14}C Perchlorate (9a). In a flask equipped with two dropping funnels, a condenser, stir bar, and nitrogen sweep was placed 50 mg-atoms (1.22 g) of magnesium and 100 ml of anhydrous diethyl ether. Then 0.25 mCi of methyl-^{14}C iodide[8] (9.9 mg) and 50 mmol (7.1 g) of methyl iodide dissolved in 25 ml of ether were added slowly with stirring. After nearly all the magnesium had dissolved, 37.5 mmol (3.72 g) of 1-methyl-2-pyrrolidinone in 25 ml of diethyl ether was added over 30 min, the solution was allowed to stand 20 hr at room temperature, and 100 ml of 6 *N* NaOH was added. The ether layer was removed, the aqueous phase was extracted with diethyl ether (6 × 50 ml), the combined ethereal solutions were extracted with 10% hydrochloric acid (three times with 50 ml), and the aqueous solution was evaporated *in vacuo* at 40° to give the crude pyrrolinium salt. This residue was dissolved in 50 ml of 3 *N* NaOH, which was extracted with methylene chloride (four times with 25 ml). The methylene chloride extracts were added to 200 ml of absolute ethanol and 70% aqueous perchloric acid was added until the solution was slightly acidic (pH 3). Concentration *in vacuo* to 150 ml and cooling to 0° resulted in a precipitate which was dried at 10 μ for 18 hr giving 2.62 g (27%) of **9a**: mp 225–230° dec (lit.[10–12] mp 238°, 235–236°, 239–240°); nmr (D_2O) 4.17 (t, 2 H), 3.46 (s, 3 H), 3.23 (t, 2 H), 2.44 (s, 3 H), 2.20 (m, 2 H); tlc (EtOH–0.1 *N* HCl, 2:1; I_2 detection) one spot at R_f 0.27; tlc (*n*-BuOH–HOAc–H_2O, 4:1:5; I_2 detection) one spot at R_f 0.10. *Anal.*

(26) All melting points are uncorrected. Infrared (ir) spectra were measured on a Perkin-Elmer 137 spectrophotometer, ultraviolet (uv) spectra were recorded on a Cary Model 14 instrument, and nuclear magnetic resonance (nmr) spectra were obtained with either a Varian A-60 or T-60 spectrometer and are reported as δ (ppm) values downfield from internal tetramethylsilane or sodium trimethylsilylpropanesulfonate (δ 0). Mass spectra were obtained on a CEC 103C or 21-110B instrument. All radioactive counting was performed on a Nuclear Chicago Corporation Mark I liquid scintillation computer (Model 6880) and are in disintegrations per minute (dpm) relative to an external standard, corrected for background. Counting was carried out with 15-ml aliquots of either a solution of 18.0 g of 2,5-diphenyloxazole (PPO), 0.4 g of *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP), and 4 l. of toluene or a solution of 18.0 g of PPO, 0.4 g of POPOP, 200 g of naphthalene, 1 l. of ethanol, 1.4 l. of toluene, and 1.6 l. of dioxane. Whenever necessary, 1 ml of NCS Solubilizer (Nuclear Chicago Corp.) was added to the liquid scintillation sample vial in order to ensure complete solubility. All elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif. CD and ORD spectra were run on a Cary 60 instrument. Glpc's were carried out on a Varian Aerograph A-90-P instrument.

Calcd for $C_8H_{12}ClNO_4$: C, 36.5; H, 6.1; N, 7.1. Found: C, 36.2; H, 6.1; N, 7.2.

1,2-Dimethyl-1-pyrrolinium-*2-methyl-^{14}C* Chloride (9b). Approximately 8 mmoles of **9a** was dissolved in 50 ml of 3 *N* NaOH, and the alkaline solution was extracted with methylene chloride (three times with 25 ml). The combined extracts were shaken with 25 ml of 10% HCl, and the aqueous solution was evaporated *in vacuo*. The residue was dissolved in 100 ml of distilled water prior to administering aliquots to *N. glutinosa* plants.

1,3,3-Trimethyl-1-pyrrolinium-*3,3-dimethyl-^{14}C* Perchlorate (10a). To 25 ml of anhydrous diethyl ether was added 20 mmol (2.54 g) of 1,3,3-trimethyl-1-pyrrolidinone-*3,3-dimethyl-^{14}C* and 6.75 ml (20 mmol) of lithium aluminum hydride in ether (0.74 mmol/ml). After refluxing for 1 hr, the reaction mixture was cooled, 50 ml of ether and 50 ml of 3 *N* NaOH were added, the ether layer was removed, and the aqueous solution was extracted with ether (six times with 30 ml). The combined ether extracts were washed with 10% HCl (four times with 25 ml), and the aqueous solution was concentrated *in vacuo* at 40° to give a mixture of **10b** (66%) and 1,3,3-trimethylpyrrolidine-*3,3-dimethyl-^{14}C* hydrochloride (**19**) (34%) as determined by nmr: tlc (*n*-BuOH–H_2O–HOAc, 4:5:1), **19** at R_f 0.14 and **10b** at R_f 0.10; tlc (EtOH–0.1 *N* HCl, 2:1), **19** at R_f 0.51 and **10b** at R_f 0.37.

The mixture of **10b** and **19** was dissolved in 50 ml of 6 *N* NaOH and extracted with methylene chloride (four times with 25 ml). The methylene chloride extracts were added to 150 ml of absolute ethanol, 70% aqueous perchloric acid was added until the ethanolic solution became acidic (pH 3), the methylene chloride was removed *in vacuo*, and the ethanolic solution was cooled to −10°. The resulting precipitate was removed and dried to give 1.14 g (27%) of **10a**: mp 110–112°; nmr (D_2O) 8.41 (s, 1 H), 4.24 (t, 2 H), 3.58 (s, 3 H), 2.19 (t, 2 H), 1.36 (s, 6 H). *Anal.* Calcd for $C_7H_{14}ClNO_4$: C, 39.7; H, 6.7; N, 6.6. Found: C, 39.4; H, 6.7; N, 6.5.

1,3,3-Trimethyl-1-pyrrolinium-*3,3-dimethyl-^{14}C* chloride (10b) was obtained from the corresponding perchlorate **10a** by the procedure given above for obtaining the chloride **9b** from the perchlorate **9a**.

3-Ethoxycarbonyl-1-methyl-2-pyrrolidinone (12). A mixture of 500 g of diethyl carbonate, 99.1 g (1 mol) of 1-methyl-2-pyrrolidinone, and 2500 ml of anhydrous benzene was refluxed overnight under a water separator. The mixture was cooled to room temperature, 85.3 g of 56.3% NaH dispersion was slowly added, and reaction was allowed to proceed at room temperature for 15 min and then refluxed for 12 hr at which time hydrogen evolution had ceased. Cooling in an ice bath was followed by addition of 103 g of glacial acetic acid and 200 ml of benzene to decompose the excess sodium hydride and sodium enolate. The resulting slurry was filtered, the precipitate was washed with methylene chloride, and the filtrate and washings were concentrated *in vacuo* and then fractionally distilled at 119° and 1.8 mm to give the desired product contaminated with a small amount of mineral oil. Column chromatography on silica gel using benzene and benzene–ethanol (1:1) as eluents followed by redistillation gave 70.4 g (41.2%) of **12**: ir (thin film) 1680 (amide C=O) and 1740 cm^{-1} (ester C=O); nmr (neat) 4.17 (q, 2 H), 3.42 (m, 3 H), 2.83 (s, 3 H), 2.35 (m, 2 H), 1.27 (t, 3 H). *Anal.* Calcd for $C_8H_{13}NO_3$: C, 56.1; H, 7.7; N, 8.2. Found: C, 55.9; H, 7.7; N, 8.3.

3-Ethoxycarbonyl-1,3-dimethyl-2-pyrrolidinone-*3-methyl-^{14}C* (13). Petroleum ether was added to 1.71 g (40 mmol) of 56% sodium hydride dispersion and then drained, leaving sodium hydride free of mineral oil. A solution of 5.13 g (30 mmol) of 3-ethoxycarbonyl-1-methyl-2-pyrrolidinone (**12**) and 165 ml of tetrahydrofuran was added and stirred with the sodium hydride for 2 hr. Then 2.18 ml (35 mmol) of methyl-*^{14}C* iodide[8] was added, the mixture was stirred overnight, and the solvent was removed *in vacuo*. The product was extracted from the sodium salts with benzene (four times with 25 ml), and distillation at 105–109° and 1.4 mm gave 3.73 g (66%) of **13**: ir (thin film) 1680 (amide C=O) and 1740 cm^{-1} (ester C=O); nmr ($CDCl_3$) 4.17 (q, 2 H), 3.40 (m, 2 H), 2.84 (s, 3 H), 1.7–2.5 (m, 2 H), 1.27 (m, 6 H); glpc on 30% QF-1 on Chromosorb P (5 ft × 0.25 in.; 100 ml/min; 146°) gave one peak, retention time 6.4 min (starting material **12**, 8.2 min retention time). *Anal.* Calcd for $C_9H_{15}NO_3$: C, 58.4; H, 8.2; N, 7.6. Found: C, 58.3; H, 8.0; N, 7.5.

3-Carboxy-1,3-dimethyl-2-pyrrolidinone-*3-methyl-^{14}C* (14). 3-Carbethoxy-1,3-dimethyl-2-pyrrolidinone-*3-methyl-^{14}C*, 1.85 g (10 mmoles), and 25 ml of 10% NaOH were stirred at room temperature for 16 hr. The reaction mixture was adjusted to pH 1 with concentrated hydrochloric acid and continuously extracted with methylene chloride for 90 hr. Removal of the solvent *in vacuo* gave 1.57 g (100%) of acid **14**, melting at 142–144° dec after recrystallization from ethyl acetate: nmr ($CDCl_3$) 10.91 (s, 1 H), 3.42 (m, 2 H), 2.90 (s, 3 H), 1.8–2.6 (m, 2 H), 1.42 (s, 3 H). *Anal.* Calcd for $C_7H_{11}NO_3$: C, 53.5; H, 7.1; N, 8.9. Found: C, 53.5; H, 7.2; N, 8.8.

1,3-Dimethyl-2-pyrrolidinone-*3-methyl-^{14}C* (15). The acid **14**, 1.16 g (7.4 mmoles), was heated at 150–160° until decarboxylation was complete to give 836 mg (100%) of **15**: nmr ($CDCl_3$) 3.25 (m, 2 H), 2.80 (s, 3 H), 1.5–2.6 (m, 3 H), 1.18 (d, 3 H). *Anal.* Calcd for $C_6H_{11}NO$: C, 63.7; H, 9.8; N, 12.4. Found: C, 63.6; H, 10.0; N, 12.3.

Lithium Aluminum Hydride Reduction of 1,3-Dimethyl-2-pyrrolidinone-*3-methyl-^{14}C* (15). The acid **14**, 3.144 g (20 mmol), was heated at 140–160° until the evolution of carbon dioxide had ceased. After cooling, 25 ml of anhydrous ether was added followed by 5 ml of a 1.2 *M* ethereal lithium aluminum hydride solution, and the solution was refluxed for 1 hr. Water (5 ml) was added after cooling, then 100 ml of 6 *N* NaOH. The ether layer was removed, the aqueous solution was extracted with ether (seven times with 25 ml), the combined ethereal extracts were extracted with 10% HCl (six times with 25 ml), and aqueous acid solution was evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml of D_2O and its nmr showed a doublet at 1.33 (3 H), a singlet at 3.61 (3 H), a triplet at 4.17 (2 H), and a singlet at 8.6 (1 H) assignable to **4**; signals assignable to **16** occurred at 1.10 (overlapping doublets, 3 H) and 2.92 (s, 3 H). Thus, nmr analysis indicated that the product was a mixture of **4** (63%) and **16** (37%). Scintillation counting of an aliquot of the aqueous solution of the reduction products gave a yield of 92%: tlc [I_2 detection, EtOH–0.1 *N* HCl, 2:1] gave **16** and **4** at R_f's of 0.54 and 0.41, respectively; tlc [I_2 detection, *n*-BuOH–HOAc–H_2O, 4:1:5] gave, after two elutions, **16** and **4** at R_f's of 0.45 and 0.33, respectively.

1,3-Dimethylpyrrolidine-*3-methyl-^{14}C* Hydrochloride (16). In a Parr hydrogenation bottle was placed 3 mmol of a mixture of **4** (63%) and **16** (37%) in 50 ml of H_2O and 50 mg of 10% Pd/C was added. After hydrogenation at 40 psi of hydrogen for 18 hr, the solution was filtered through Celite, and the filter pad was washed with 100 ml of hot water. The solvent was removed *in vacuo* at 40° to give a 97% yield of **16** by radioactive assay; nmr (D_2O) 3.0–3.9 (m, 4 H), 2.92 (s, 3 H), 1.6–2.8 (m, 3 H), 1.10 (overlapping doublets, 3 H); tlc [I_2 detection, EtOH–0.1 *N* HCl, 2:1] one spot at R_f 0.53; tlc [I_2 detection, *n*-BuOH–HOAc–H_2O, 4:1:5] one spot at R_f 0.47 after two elutions.

A portion of **16** was converted to the free base to which was added picric acid. The picrate was crystallized from 2-propanol, mp 180–183° (lit.[12] mp 183–184°). *Anal.* Calcd for $C_{12}H_{16}N_4O_7$: C, 43.9; H, 4.9; N, 17.1. Found: C, 44.2; H, 4.9; N, 17.1.

Separation of 1,3-Dimethyl-1-pyrrolinium-*3-methyl-^{14}C* Chloride (4) and 1,3-Dimethylpyrrolidine-*3-methyl-^{14}C* Hydrochloride (16). A column (5 × 64 cm) was prepared using 550 g of silica gel slurried in EtOH–0.1 *N* HCl (2:1), the eluting solvent. A mixture (4 mmoles) of **4** (63%) and **16** (37%) was applied to the column in 25 ml of the eluting solvent, using two 25-ml portions of the eluting solvent for rinsing the compounds onto the column. Fractions of approximately 50 ml were collected at a flow rate of approximately 50 ml/hr and the chromatography was followed by scintillation counting; 200 μl ± 10 μl of each fraction was dissolved in 15 ml of dioxane scintillation counting solution. The pyrrolidine **16** was eluted in fractions 18–26 and the pyrrolinium salt **4** in fractions 26–50. Fractions 27–50 were combined, evaporated to dryness *in vacuo* at 40°, and reapplied to the silica gel column as a slurry in EtOH–0.1 *N* HCl (2:1), carrying out the chromatography in the same manner. The desired product **4** was eluted in fractions 39–71 which were combined, concentrated to approximately 200 ml *in vacuo* at 40°, and applied to a cation-exchange column (AG-50W-X8; H^+ form; 200–400 mesh; approximately 150 ml of resin). The column was washed until neutral with distilled water; elution of **4** from the column followed with 1.5 *N* HCl, the volume of each fraction being 75 ml and the flow rate 75 ml/hr, monitored by scintillation counting as described above. Pyrrolinium salt **4** was eluted in fractions 16–24; in addition, each of these fractions gave positive Dragendorf's test.[27] Fractions 16–24 were combined and evaporated at 40° *in vacuo* to give 85% (2.08 mmoles) of the **4** applied to the initial column: $\lambda_{max}^{C_2H_5OH}$ 262 nm (ε 21); $\lambda_{max}^{H_2O}$ 249

(27) K. Randerath, "Thin Layer Chromatography," Academic Press, New York, N. Y., 1963, p 74.

nm (ϵ 14); nmr (D_2O) 8.55 (s, 1 H), 4.18 (t, 2 H), 3.63 (singlet methyl superimposed on multiplet, 4 H), 1.7–2.8 (m, 2 H), 1.33 (d, 3 H); tlc [I_2 detection, EtOH–0.1 *N* HCl, 2:1] one spot, R_f 0.43; tlc [I_2 detection, *n*-BuOH–HOAc–H_2O, 4:1:5] one spot, R_f 0.31 after two elutions; mass spectrum (70 eV) *m/e* (rel intensity) 97 (54, M^+ − HCl), 96 (100), 82 (34). *Molecular Formula:* Calcd for $C_6H_{11}N$ (M^+ − HCl): 97.0891. Found: 97.0887.

1,3,3-Trimethyl-2-pyrrolidinone-*3,3-dimethyl-^{14}C* (18). To 500 ml of anhydrous diethyl ether and 220 mmole (31 ml) of diisopropylamine (freshly distilled from BaO), cooled to −70°, was added 131 ml (210 mmol) of *n*-butyllithium (1.6 *M* in hexane). Then 4.95 g (50 mmol; 4.85 ml) of 1-methyl-2-pyrrolidinone was added, the solution was stirred for 15 min at −70°, and 13.8 ml (220 mmol) of CH_3I-^{14}C[8] was added. Stirring was continued at room temperature for 16 hr, 150 ml of H_2O was added, the ether layer was removed and evaporated *in vacuo*, and the residue was dissolved in 100 ml of H_2O. The combined aqueous solutions were continuously extracted with methylene chloride for 24 hr. Removal of the solvent and distillation of the residue at 95–97° (27 mm) gave 4.79 g (78%) of **18**: nmr ($CDCl_3$) 3.29 (t, 2 H), 2.79 (s, 3 H), 1.81 (t, 2 H), 1.04 (s, 6 H); glpc on 30% QF-1 on Chromosorb P (168°; 100 ml/min; 10 ft × 0.25 in.) one peak, 9.0 min. *Anal.* Calcd for $C_7H_{13}NO$: C, 66.1; H, 10.3; N, 11.0. Found: C, 65.9; H, 10.2; N, 10.9.

2-Acetonyl-1,3-dimethylpyrrolidine (17). To 6 mmol of 1,3-dimethyl-1-pyrrolinium chloride and 6 ml of H_2O were added 35 ml of 1 *N* NaOH, 10 ml of H_2O, 20 ml of ethanol, and 15 ml of ethyl acetoacetate. After stirring in the dark under nitrogen for 17 days, 50 ml of concentrated HCl was added. The reaction mixture was warmed on a steam bath for 5 hr, and concentrated to 5 ml *in vacuo*. The residue was dissolved in 50 ml of water and made strongly alkaline with 6 *N* NaOH; the resulting aqueous solution was continuously extracted with methylene chloride for 4 days. Removal of the solvent gave 440 mg (47%) of **17**: ir (thin film) 1730 cm^{-1} (C=O); nmr ($CDCl_3$) 2.7–3.7 (m, 5 H), 2.69 (s, 3 H), 1.4–2.5 (m, 3 H), 2.30 (s, 3 H), 1.12 (d, 3 H); mass spectrum (70 eV) *m/e* (rel intensity) 155 (4, M^+), 140 (2), 124 (28), 109 (21), 98 (100).

Warming **17** with a saturated ethanolic solution of picric acid gave the picrate which was recrystallized from absolute ethanol: mp 147–151 dec; nmr (pyridine-d_5) 8.97 (s, 2 H), 3.1–4.0 (m, 5 H), 3.08 (s, 3 H), 1.6–2.6 (m, 3 H), 2.25 (s, 3 H), 1.01 (overlapping doublets, 3 H). *Anal.* Calcd for $C_{15}H_{20}N_4O_8$: C, 46.9; H, 5.3; N, 14.6; O, 33.3. Found: C, 47.1; H, 5.3; N, 14.7.

Free Radical Analogs of Histidine

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Abstract: Some stable free radical analogs of histidine have been prepared which contain a nitronyl nitroxide ring system in place of the imidazole function. The esr spectra of these amino acids show magnetically nonequivalent methylene protons as shown by different hyperfine coupling constants of the β-methylene hydrogens. The spectra also show coupling constant changes with pH which have been correlated with an ion–dipole interaction between the nitronyl nitroxyl ring and the carboxylate anion in a manner which is analogous to histidine. The analogs which show this interaction also show esr line broadening effects consistent with a model involving hindered interconversion of conformers.

Stable free radicals have recently been used in the investigation of a number of biologically important systems. This method utilizes spin labels[1,2] or paramagnetic ions[3,4] to obtain information about the environment surrounding the paramagnetic species. Electron spin resonance is the major spectroscopic tool applied to these systems but the nmr,[5] visible, ultraviolet,[6] and fluorescence[7] spectra of the host system may also be affected by the free radical.

Where the molecule or biological system is not normally paramagnetic the free radical function may be added as a spin label. Ideally the addition is carried out in such a way that the label, usually a nitroxide radical, does not significantly alter the molecular structure or biological activity. The nitroxide radical can reflect large changes in solvent polarity[8] and losses of rotational freedom.[9,10] Since the nitroxide group is not coupled to a proton, large spectral changes are, in general, observed only when the radical has a low rate of rotation relative to the magnetic field ($\tau > 5 \times 10^{-11}$ sec). This effectively limits the use of spin labels to larger rigid molecules or to systems within viscous oriented media such as crystals or membranes.

These limitations precluded the use of the spin label technique in our investigation of conformations and intramolecular interactions in smaller peptides. In order to retain the unique advantages that electron spin resonance spectroscopy offers in the study of complex molecules we have attempted to construct a system which uses the free radical as a functioning portion of the amino acid rather than as a reporter group. For such a system to have significant utility the free radical amino acid must have a size, shape, and polarity similar to its analog.

We would like to report such a free radical analog of histidine. This model, β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanine (**1**), contains the nitronyl nitroxide ring system in place of the im-

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