3), 105538-39-4; **IVb** (R_1 = CH_3, R_2 = H, R_3 = 4-ClC_6H_4, n = 3), 105538-40-7; **IVb** (R_1 = R_2 = H, R_3 = 4-ClC_6H_4, n = 3), 105538-41-8; $H_2NCONH(CH_2)_3CO_2H$, 2609-10-1; $BOCNH(CH_2)_3CO_2H$, 57294-38-9; 4-$O_2NC_6H_4COCH_2CH(OH)CO_2H$, 81008-13-1; $Br(CH_2)_3CO_2Et$, 2969-81-5; $Br(CH_2)_3CN$, 5332-06-9; $BrCH_2CO_2H$, 79-08-3; $H_3CCHBr(CH_2)_2CO_2Et$, 27126-42-7; $BrCH_2CH(OEt)_2$, 2032-35-1; $EtO_2CCH_2CO_2Et$, 105-53-3; CH_3I, 74-88-4; H_2NNH_2, 302-01-2; $OHCCO_2Et$, 924-44-7; 4-$O_2NC_6H_4Ac$, 100-19-6; $OHCCo_2H$, 298-12-4; $(CH_3)_2CHCH_2COCH_3$, 108-10-1; $H_3CCOCH_2C_6H_5$, 103-79-7; $Br(CH_2)_2CO_2Et$, 539-74-2; $Br(CH_2)_4CO_2Et$, 14660-52-7; $Br(CH_2)_5CO_2Et$, 25542-62-5; $Br(CH_2)_2CH(CH_3)CO_2Et$, 2213-09-4; Pr_2NH, 142-84-7; potassium pyruvate, 4151-33-1; morpholine, 110-91-8; methylcyclohexylketone, 823-76-7; α-methyl-γ-hydroxybutyrolactone, 53561-62-9; 4-methyl-6-(*p*-nitrophenyl)-3(2*H*)-pyridazinone, 105537-88-0; 4-methyl-6-phenyl-3(2*H*)-pyridazinone, 13300-09-9; 4-pyrrolidinobutyric acid, 85614-44-4; 4-pyrrolidinobutyric acid hydrochloride, 49637-21-0; (+)-bicuculline methiodide, 40709-69-1.

Stereochemical Studies on the Cytochrome P-450 Catalyzed Oxidation of (*S*)-Nicotine to the (*S*)-Nicotine $\Delta^{1'(5')}$-Iminium Species

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Mammals metabolize the tobacco alkaloid (*S*)-nicotine primarily to the lactam (*S*)-cotinine by a pathway involving an initial cytochrome P-450 catalyzed two-electron oxidation at the prochiral 5′-carbon atom. The stereochemical course of this oxidation was examined with human microsomal preparations and the *E* and *Z* diastereomers of (*S*)-nicotine-5′-d_1. The metabolically generated $\Delta^{1'(5')}$-iminium ion intermediate was trapped and analyzed as the corresponding diastereomeric 5′-cyano derivatives by a capillary column GC–EIMS selected ion monitoring assay. The results of these studies established that this biotransformation proceeds with the stereoselective abstraction of the 5′-*pro-E* proton, that is, the C-5′ proton trans to the bulky pyridyl group. The observed stereoselectivity was independent of proton vs. deuteron abstraction. Additionally, the extent of (*S*)-cotinine formation was minor and did not influence the stereochemical composition of the metabolically derived α-cyano amines. Studies with male Dutch rabbit liver microsomal preparations gave similar results. These findings suggest that the structure of the complex formed between (*S*)-nicotine and the active site of cytochrome P-450 is highly ordered and dictates the stereochemical course of the reaction pathway.

The cytochrome P-450 catalyzed[1] overall two-electron oxidation of (*S*)-nicotine (1) at the prochiral C-5′ position to form the iminium intermediate **2** is the initial step in the biotransformation of this alkaloid to (*S*)-cotinine (**3**), the principal metabolite of (*S*)-nicotine in humans.[2] Since the two methylene protons at the C-5′ position are diastereotopic, this α-carbon oxidation may proceed by a process that could result in the selective abstraction of the 5′-*pro-E* (Ha) or 5′-*pro-Z* (Hb) proton. In order to understand better the detailed molecular mechanisms associated with the cytochrome P-450 catalyzed α-carbon oxidation of this and other aliphatic tertiary amines, we have undertaken studies to characterize the stereochemical course followed in this biotransformation.

The available experimental evidence relating to this type of transformation supports a stepwise reaction pathway involving an initial transfer of one electron from the lone pair of the pyrrolidine nitrogen atom to a heme-bound, electron-deficient oxygen atom.[3] The subsequent one-electron oxidation of the resulting radical cation **4** is the step that is accompanied by the net loss of hydrogen from the C-5′ position. This step may proceed directly to the iminium species **2** (loss of a hydrogen atom) or via the carbon-centered radical **5** (loss of a proton) and the carbinolamine **6**, which will be in equilibrium with **2**. The iminium intermediate, which in vivo is oxidized further to (*S*)-cotinine (**3**) in a reaction catalyzed by cytosolic enzymes,[4] may be trapped as the corresponding diastereomeric α-cyano adducts **7** and **8**.[1,5]

Several studies concerned with substrate diastereoselective biooxidations at prochiral methylene carbon atoms have been reported. For example, using cholesterol-7α-t_1 and -7β-t_1, Bergstrom et al. established that the 7α-hydroxylation of cholesterol in the rat proceeds with greater than 90% loss of the 7α-hydrogen atom.[6] A somewhat similar experiment showed that the 11β-hydroxylation of pregnanedione-11α, 12α-t_2 by perfused bovine adrenal glands proceeds with complete retention of label.[7] On the other hand, the cytochrome $P\text{-}450_{cam}$ catalyzed oxidations of both camphor-5-*exo*- and 5-*endo*-d_1 yield principally 5-*exo*-hydroxycamphor-5-*endo*-d_1.[8] According to our knowledge, analogous studies on aliphatic tertiary amines have not been reported.

This paper summarizes the results of our stereochemical investigations on the α-carbon oxidation of (*S*)-nicotine by human and male Dutch rabbit liver microsomal preparations with use of the specifically labeled (*E*)- and (*Z*)-5′-monodeuterio diastereomers **9** and **10**, respectively, of (*S*)-nicotine. The synthesis of **9** was achieved by the stereoselective[9] catalytic deuteriation of the corresponding $\Delta^{1'(5')}$-iminium species **2**. Similarly, catalytic hydrogenation of **2**-d_1 provided the corresponding *Z* diastereomer **10**.

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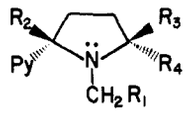
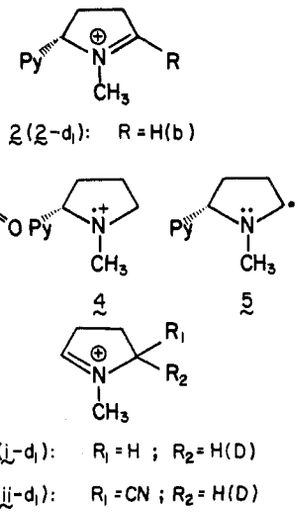
Chart I



	R_1	R_2	R_3	R_4
1 (1-d_2)	H	H	Ha(D)	Hb(D)
6	H	H	H,OH	OH,H
7 (7-d_1)	H	H	CN	H(D)
8 (8-d_1)	H	H	H(D)	CN
9	H	H	D	Hb
10	H	H	Ha	D
11	CN	H	H	H
12	H	CN	H	H



Analysis of the deuterium content of the metabolically generated iminium species was achieved by a capillary column GC–EIMS selected ion monitoring assay of the corresponding α-cyano amines **7** (**7**-d_1) and **8** (**8**-d_1) (Chart I).

Results

Chemistry. The synthesis of the $\Delta^{1'(5')}$-iminium species **2** was achieved by the two-electron reduction of (*S*)-cotinine with sodium dihydrobis(2-methoxyethoxy)aluminate (Red-Al).[10] The product was isolated as the corresponding (*E*)- and (*Z*)-α-cyano amine isomers **7** and **8**, respectively, by addition of potassium cyanide to the crude reaction mixture. Following column chromatographic purification, this diastereomeric mixture was treated with perchloric acid, which led to the formation of the crystalline bis-perchlorate salt of **2**. Deuteriation of this product over a platinum catalyst proceeded smoothly in a solution of deuterium oxide containing deuterium chloride. The resulting product was purified as its bis-*l*-tartrate salt.[11]

The deuterium content of the product was estimated by capillary column GC–EIMS analysis. Due to the presence of a strong $(M - 1)^+$ fragment ion in the EI mass spectrum of (*S*)-nicotine,[12] the base peak (fragment ion i) was monitored. This base peak in the spectrum of the reaction product appeared at m/z 85, corresponding to fragment ion i-d_1, consistent with the formation of (*S*)-nicotine-d_1. The ratios of the ^{13}C-satellite corrected ion intensities at masses 84:85:86 were found to be 2:88:10. Comparison of these ratios with the corresponding values obtained with standard (*S*)-nicotine led us to conclude that the product contained about 10% of a dideuteriated species, which presumably is formed via partial proton–deuteron exchange at C-4′ of the iminium intermediate.[13]

Analysis of the 240-MHz ^{1}H NMR spectrum allowed us to assign the structure of this product as the desired *E* diastereomer **9** of (*S*)-nicotine-5′-d_1. Previous ^{1}H NMR studies have established that the signal for the 5′-*pro-Z* proton (Hb) of (*S*)-nicotine appears at 3.3 ppm, 1.0 ppm downfield from the corresponding signal for the 5′-*pro-E* proton (Ha).[14] The ^{1}H NMR spectrum of the product obtained from the catalytic deuteriation of **2** (Figure 1, middle panel) differs from that of (*S*)-nicotine-d_0 (Figure 1, upper panel) in that the quartet corresponding to the signal for the 5′-*pro-E* proton (Ha) at 2.3 ppm is absent and the multiplet at 3.3 ppm for the Hb proton has simplified to a broad doublet, reflecting the loss of the geminal coupling with Ha. Careful and repeated integration of the spectrum indicated that the reduction had proceeded with ca. 87% stereoselectivity. Delay times greater than 5 times t_1 were used to allow the protons to relax completely between radiofrequency pulses to insure accurate integration of the signals.

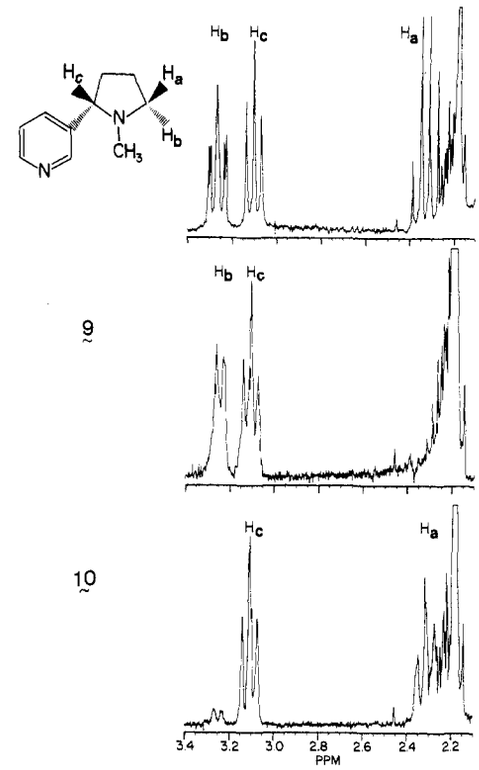


Figure 1. Relevant regions of the 240-MHz ^{1}H NMR spectra of (*S*)-nicotine-d_0 (upper panel) and the *E* (middle panel) and *Z* (lower panel) diastereomers of (*S*)-nicotine-5′-d_1 (**9** and **10**, respectively).

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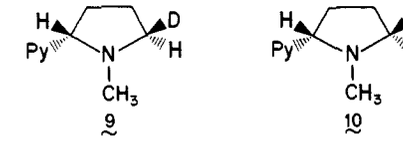
The synthesis of the *Z* diastereomer **10** of (*S*)-nicotine-5′-d_1 was achieved in a similar manner. The controlled reduction of (*S*)-cotinine with freshly prepared sodium aluminum deuteride[15] provided the required $\Delta^{1'(5')}$-iminium 5′-d_1 intermediate **2**-d_1, which was purified via the corresponding mixture of deuteriated α-cyano amines **7**-d_1 and **8**-d_1. Catalytic hydrogenation of **2**-d_1 gave **10**, which was obtained as its bis-*l*-tartrate salt. Unlike the *E* isomer, **10** was not contaminated with any multideuteriated species (m/z 84:85:86 = 5:94:1). The replacement of Hb with deuterium led to loss of the ^{1}H NMR signal at 3.3 ppm and to the simplification of the signal for Ha to a triplet due to loss of geminal coupling with Hb (Figure 1, lower panel). Integration of the ^{1}H NMR spectrum indicated that the reduction was 92% stereoselective.

Metabolic Studies. Previous studies have shown that rabbit liver microsomal enzymes catalyze the oxidation of (*S*)-nicotine to the chemically unstable iminium metabolite **2**, which, in the presence of 1 mM sodium cyanide, is converted to the corresponding α-cyano amines **7** and **8**.[1,5] In a preliminary study, capillary column GC analysis of human microsomal incubation mixture extracts of (*S*)-nicotine and sodium cyanide showed the presence of the diastereomeric 5′-cyanonicotine adducts and small amounts of (*S*)-cotinine. Neither the *N*-cyanomethyl isomer **11**, previously observed in rabbit liver microsomal incubation mixtures,[5] nor the 2′-cyano isomer **12** was observed in the GC tracings. These results suggested that the human cytochrome P-450 catalyzed carbon oxidation of (*S*)-nicotine is highly regioselective.

A capillary column GC–EIMS selected ion monitoring assay was developed to estimate the deuterium composition of the α-cyano amines **7** and **8** generated from **9** and **10** in liver microsomal preparations containing substrate and sodium cyanide. The capillary GC–EI mass spectra of a mixture of the synthetic (*Z*)- and (*E*)-α-cyano amines were essentially identical. As with (*S*)-nicotine, the molecular ion of this system was not useful for deuterium composition estimations due to the fragmentation that results in loss of a hydrogen atom. The base peak in both spectra, which appears at m/z 109, corresponds to the (M $- C_5H_4N)^+$ fragment ion ii. This ion and the corresponding deuterium-containing ion ii-d_1 at m/z 110 were chosen for selected ion monitoring. The ratios of the ion currents at m/z 109 and 110 to the sum of these ion currents provided an estimate of the extent to which deuterium was lost or retained during the metabolic oxidation of **9** and **10**. In all cases, small corrections had to be made to account for the small d_0 contaminants present in **9** and **10**, for the ^{13}C-satellite contribution of the m/z 109 ion to the m/z 110 ion, and for the stereochemical composition of the substrates. Additionally, when **9** was employed as the substrate, it was necessary to correct for the dideuteriated contaminant. Although standard curves were not run, the intensities of the principal ions observed in the spectrum of a 1:1 mixture of **7** (**7**-d_1) and **8** (**8**-d_1) were equal, suggesting that any unexpected differences in the GC–EIMS behavior of these two species, such as fragmentation differences due to isotope effects, must be minor.

The results of the human microsomal incubation experiments are summarized in Chart II. The three liver microsomal preparations were obtained from three different sources (see Acknowledgment), and therefore differences in isozyme composition due to differences in

Chart II



9 **10**

Source of tissue	% Deuterium retention in **7** and **8**	
Human		
I	3(4)	92(4)
II	24(4)	84(4)
III	3(4)	91(4)
Mean	10	89
Dutch rabbit		
I	34(6)	87(6)
II	22(6)	81(6)
III	30(4)	89(4)
IV	28(2)	79(2)
V	27(2)	77(2)
VI	16(3)	88(3)
Mean	26	84

handling cannot be ruled out. Analysis of the deuterium content of the 5′-cyanonicotine isomers established that 90% of the product generated from **9** was proton enriched while 89% of the product isolated from **10** was deuteron enriched. Additionally, the deuterium/hydrogen ratio of the α-cyano amines generated from a 1:1 mixture of **9** and **10** was found to be 1:1, consistent with the absence of an intermolecular deuterium isotope effect. On the basis of these data, we have concluded that the oxidation of (*S*)-nicotine by human cytochrome P-450 enzymes proceeds in a highly stereoselective manner involving loss of the 5′-*pro-E* proton.

The stereochemical course of this oxidation also was examined with male Dutch rabbit liver microsomal preparations. Attack occurred principally at the 5′-position although the *N*-methyl group also was oxidized to a limited extent since small amounts of *N*-(cyanomethyl)nornicotine (**11**) were detected in the capillary column GC tracings. No 2′-cyano product was observed although trace amounts of (*S*)-cotinine again were detected. The results of the stereochemical studies (Chart II) demonstrate that male Dutch rabbit liver microsomal enzymes also catalyze the oxidation of the 5′-position of (*S*)-nicotine with the selective loss of the 5′-*pro-E* proton. These preparations, however, tended to be less selective, with an average loss of 74% of the label with **9** as substrate and 83% retention of the label with **10** as substrate. The results did not vary significantly with the length of the incubation time (data not shown).

The presence of (*S*)-cotinine in the incubation mixture isolates led to the possibility that the deuterium content of the α-cyano amines was not an accurate measure of the diastereoselectivity operating in the oxidation of (*S*)-nicotine since further oxidation of the iminium intermediate or of the α-cyano amines could be accompanied by a deuterium isotope effect. This possibility was eliminated by results obtained from the following experiment. A 1:1 (d_0:d_1) mixture of the diastereomeric 5′-cyanonicotines (50 μM) was incubated with rabbit liver microsomal preparations in the presence of 1 mM sodium cyanide. The concentrations of (*S*)-cotinine were determined by a capillary column GC assay using an N/P detector.[16] In the absence of NADPH, up to 4% of (*S*)-cotinine was formed after a 30-min incubation period. In the presence of NADPH, up to 20% of the mixed substrate was converted

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to (*S*)-cotinine. The deuterium content (40%) of the recovered α-cyano amines, however, was found be essentially the same as that of the starting substrate (41%).

Discussion

The results of these studies demonstrate that the cytochrome P-450 catalyzed oxidation of (*S*)-nicotine to the iminium ion **2** proceeds with the selective loss of the 5′-*pro-E* proton in human and male Dutch rabbit liver microsomal preparations. Differences in the degree of stereoselectivity (90% for the human and about 78% for the rabbit) may reflect species differences in isozyme composition. Both regio- and enantioselective variations in steroid hydroxylations have been reported for a number of cytochrome P-450 isozymes isolated from the rat.[17] More detailed studies with purified enzyme preparations will be required to address this question.

The possibility that cleavage of the carbon–hydrogen vs. carbon–deuterium bond may be accompanied by an intrinsic deuterium isotope effect in this system is difficult to assess because this transformation does not lend itself to intramolecular isotope effect measurements. No intermolecular isotope effects were detected when a 1:1 mixture of (*S*)-nicotine-d_0 and (*S*)-nicotine-d_1 served as substrate. Also, if present, deuterium isotope effects did not alter the stereoselectivity of the reaction. Finally, the limited extent to which the initial (*S*)-nicotine oxidation products were oxidized to (*S*)-cotinine did not alter the deuterium content of the α-cyano amines and, therefore, had no detectable influence on the stereochemical outcome of the initial two-electron oxidation. Consistent with these observations, literature values for both inter- and intramolecular isotope effects in the enzyme-catalyzed α-carbon oxidations of tertiary amines are small.[18]

The regio- and stereochemical profiles observed in this biooxidation suggest that the reaction is under steric constraints imposed by the enzyme. This result contrasts with those observed in related cytochrome P-450 catalyzed carbon oxidations. For example, replacement of the exo protons of norbornane with deuterons leads to a change in the exo to endo product ratio from 3.4:1 to 0.76:1.[19] Similarly, the extent of abstraction of the exo vs. endo hydrogen/deuterium atom during the 5-exo-hydroxylation of camphor by cytochrome P-450$_{cam}$ was dictated principally by deuterium isotope and not by stereochemical effects.[8] These results suggest that the species responsible for the proton abstraction step in these non-amine-containing systems has access to both the *pro-E* and *pro-Z* protons.

The regiochemistry followed in the carbon–hydrogen bond cleavage of aminium radical cations generated by singlet *trans*-stilbene has been examined with tertiary amines such as *N*-methyl-*N*,*N*-diisopropylamine.[20] The aminium radical cation formed from this amine loses hydrogen exclusively from the *N*-methyl group. The regioselectivity of this reaction appears to be governed by steric factors, which restrict overlap of the p orbital on the

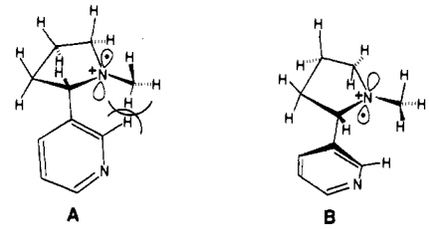

Figure 2. Drawings of the aminium radical 4 showing the steric interactions that accompany overlap of the half-filled p orbital on nitrogen with the C-5′*Z* bond (A) and the absence of such steric interactions with the corresponding overlap involving the C-5′*E* bond (B).

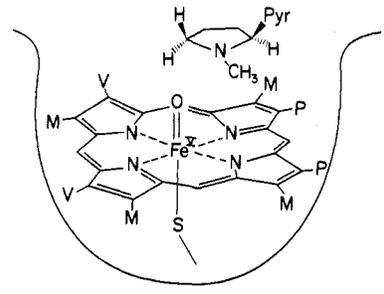

Figure 3. A depiction of (*S*)-nicotine in the active site of cytochrome P-450 oriented to explain selective loss of the 5′-*pro-E* proton.

developing methine carbon radical of the *N*-isopropyl groups with the half-empty p orbital of the planar aminium radical. A similar analysis of the radical cation **2** formed from the one-electron oxidation of (*S*)-nicotine is illustrated in Figure 2. Considerably more steric interaction between the methyl and pyridyl groups accompanies the overlap of the carbon and nitrogen p orbitals with loss of the 5′-*pro-E* proton compared with loss of the 5′-*pro-Z* proton. This model, therefore, predicts the selective loss of the 5′-*pro-Z* proton, contrary to the observed experimental results.

Studies reported by Whidby and Seeman have shown that the pyridyl and methyl substituents of (*S*)-nicotine prefer a trans orientation.[21] The energy barrier to the inversion of the *N*-methyl group, however, is likely to be relatively small, and therefore the orientation of the molecule in the active site of the enzyme may be determined by binding of the lipophilic pyridyl group to a hydrophobic pocket. In any event, we conclude that the mode of binding of (*S*)-nicotine at the active site of the enzyme is an important determinant in the stereochemical course of this oxidation. If (*S*)-nicotine binds with the bulky pyridyl group pointing away from the planar porphyrin ring of this heme protein as shown in Figure 3, the 5′-*pro-E* proton will be situated more closely to the proposed iron–oxene species than the 5′-*pro-Z* proton. According to this hypothesis, transfer of the 5′-*pro-E* proton directly to the active iron–oxene species would be facilitated by the steric constraints imposed on the substrate by the enzyme. This proposal currently is being tested with various model systems.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^1H and ^2H NMR spectra (obtained in $CDCl_3$ or H_2O) were recorded on a Varian FT-80 or a homebuilt 240-MHz instrument linked to a Nicolet 1180 computer. Chemical shifts are reported in ppm relative to Me_4Si or DSS as internal standard. Analytical GC was performed on a Hewlett-Packard 5880A series instrument with a nitro-

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gen/phosphorus detector utilizing a 25 m × 0.2 mm i.d. silica SE 54 capillary column with helium as the carrier gas. Preparative HPLC was carried out with use of a Beckman Model 100A pump with a Whatman M9 Partisil column linked to an Altex Model 153 UV detector. Analytical HPLC was performed with an Altex 110A pump connected to a Lichrosorb SI-60 silica gel column from Altex Scientific Inc. and a Hitachi 100-10 variable UV detector. GC–EIMS was performed on a J&W 30 m 0.325 mm i.d. DB5 fused silica capillary column with use of a Varian 3700 GC linked to a Kratos MS25S instrument. Elemental analyses were determined by the Microanalytical Laboratory of the University of California, Berkeley, CA. All values were within 0.4% of theory.

(*S*)-Nicotine-5′(*E*)-d_1 (9). A 70% solution of sodium dihydrobis(2-methoxyethoxy)aluminate (4.3 g, 28.8 mequiv) in 100 mL of dry ether was added dropwise to an ice-cold, stirred solution of (*S*)-cotinine[22] (6 g, 34 mmol) in 120 mL of dry ether in a flame-dried flask under N_2 over a 30-min period. After the mixture was stirred for 3 h, 50 mL of an aqueous solution of KCN (12 g, 182 mmol) was added slowly, following which the pH was adjusted to 5 with acetic acid. The reaction mixture was allowed to warm to room temperature, was made basic with a saturated solution of K_2CO_3, and was extracted with 3 × 50 mL of ether. The extracts were combined, dried over K_2CO_3, and filtered, and the solvent was removed under reduced pressure to yield a yellow oil, which was purified by column chromatography on silica gel with ether/ammonia (2000:1). Distillation [125 °C (0.8 mm)] gave 3.8 g (62%) of the diastereomeric mixture of **7** and **8** as a pale yellow oil. An ice-cold solution of this mixture (2.6 g, 13.8 mmol) in 20 mL of ethanol was treated with ethanolic perchloric acid [2.6 mL (230.2 mmol) of 70% perchloric acid dissolved in 10 mL of ethanol]. After the mixture stood for 2 days at 5 °C, the white crystalline product was collected and recrystallized from methanol containing a trace of perchloric acid to yield 2.3 g (5.9 mmol, 42%) of the bisperchlorate salt of the (*S*)-nicotine $\Delta^{1',5'}$-iminium species **2**: mp 237–241 °C dec (lit.[10] mp 230–232 °C); ^{1}H NMR (10% DCl in D_2O) δ 2.0–2.35 (m, 4 H, C3′ and C4′), 3.28 (br s, 3 H, NCH_3), 7.5–9.0 (m, 5 H, 4 aromatic pyridine H and C5′). Anal. ($C_{10}H_{15}N_2Cl_2O_6$) C, H, N.

A suspension of platinum oxide (700 mg) in 70 mL of a 2.5% DCl/D_2O solution was prereduced under 1 atm of D_2 for 30 min with vigorous stirring in a 250-mL round-bottomed flask. The iminium species **2** (7.1 g, 17.3 mmol) was added, and the flask was recharged with D_2. The reaction mixture was stirred at room temperature for 3.5 h and then was filtered through a pad of Celite. The filtrate was made basic with a saturated K_2CO_3 solution, extracted with CH_2Cl_2 (5 × 70 mL), dried (K_2CO_3), and filtered. The solvent was removed by distillation at atmospheric pressure to yield 4.34 g of (*S*)-nicotine-5′(*E*)-d_1 (**9**) as a yellow oil: 240-MHz ^{1}H NMR ($CDCl_3$) δ 1.5–2.1 (m, 4 H, C3′ and C4′), 2.2 (s, 3 H, NCH_3), 3.1 (t, 1 H, C2′), 3.3 (br d, 0.86 H, C5′-*pro*-*Z*), 7.3 (m, 1 H, C5), 7.6 (d of t, 1 H, C4), 8.3–8.5 (m, 2 H, C2 and C6); MS, *m*/*z* 163 (M^+, 40%), 134 (46%), 85 (100%). The bis-*l*-tartrate salt of **9** (5.16 g, 56.8% overall yield) was prepared in ethanol:[11] mp 138.5–139.5 °C; ^{2}H NMR (H_2O) δ 3.4. Anal. ($C_{18}H_{25}DN_2O_{12}$) C, H, N.

(*S*)-Nicotine-5′(*Z*)-d_1 (10). A solution of freshly prepared $NaAlD_4$[15] (3 mL, 20 mmol of deuteride) in 50 mL of dry THF was added dropwise to an ice-cooled, stirred solution of (*S*)-cotinine (3 g, 17 mmol) in 50 mL of dry THF under N_2. The reaction mixture was allowed to warm slowly to room temperature. After 3 h, the mixture was cooled and another aliquot of $NaAlD_4$ (1 mL) was added slowly. The system was allowed to warm to room temperature and was stirred for an additional 45 min. A solution of KCN (6 g, 17 mmol) in 50 mL of water was added, and the pH of the resulting mixture was adjusted to 5 with acetic acid. After the mixture was stirred at room temperature for 3 h, a saturated K_2CO_3 solution (20 mL) was added, the THF layer was separated, and the aqueous phase was extracted with ether (4 × 50 mL). The organic layers were combined and dried (K_2CO_3), and the solvent was removed under reduced pressure to yield 2.04 g of crude product, which was purified on preparative HPLC (silica column; mobile phase, ether/ammonia, 2000:1; flow rate, 5 mL/min) to provide 1.6 g (8.5 mmol, 50%) of a mixture of **7**-d_1 and **8**-d_1 as a pale yellow oil: 80-MHz ^{1}H NMR ($CDCl_3$) δ 1.9–2.6 (m, 4 H, C3′ and C4′), 2.30 and 2.32 (2 s, 3 H, NCH_3), 3.5 (m, 1 H, C2′), 7.3 (m, 1 H, C5), 7.6 (m, 1 H, C4), 8.55 (m, 2 H, C2 and C6); GC–EIMS, *m*/*z* 188 (M^+) and 110 (100%). Treatment of this mixture with ethanolic perchloric acid as described above gave, after recrystallization from methanolic perchloric acid, a 44% yield of the desired monodeuterio iminium species **2**-d_1: mp 240–243 °C dec; 80-MHz ^{1}H NMR (10% DCl in D_2O) δ 2.0–3.0 (m, 4 H, C3′ and C4′), 3.37 (br s, 3 H, NCH_3), 5.6 (t, 1 H, C2′), 8.0 (m, 1 H, C5), 8.25–8.9 (m, 3 H, C2, C4, and C6). Hydrogenation of **2**-d_1 over a platinum catalyst as described above provided an 83% yield of the crude (*S*)-nicotine-5′(*Z*)-d_1 (**10**), which was purified by silica gel chromatography using ether saturated with concentrated aqueous NH_4OH as the eluent: 240-MHz ^{1}H NMR ($CDCl_3$) δ 1.5–2.1 (m, 4 H, C3′ and C4′), 2.17 (s, 3 H, NCH_3), 2.3 (m, 1 H, $H_{5'E}$), 3.1 (t, 1 H, C2′), 7.3 (m, 1 H, C5), 7.6 (d of t, 1 H, C4), 8.3–8.5 (m, C2 and C4); GC–EIMS, *m*/*z* 163 (M^+), 134, 85 (100%). The bis-*l*-tartrate salt of **10** was obtained from ethanol:[11] mp 138.5–139 °C; ^{2}H NMR (D_2O) δ 3.8. Anal. ($C_{18}H_{25}DN_2O_{12}$) C, H, N.

2′-Cyanonicotine (12). A stirred solution of (*S*)-nicotine (2.0 g, 12.4 mmol) and mercuric acetate (15 g, 47 mmol) in 50 mL of acetic acid was heated at 80 °C for 2 h. The cooled reaction mixture was filtered, and H_2S gas was bubbled through the filtrate. The resulting black precipitate was filtered, and the process was repeated two times. The solution then was made basic with saturated K_2CO_3, and KCN (1.7 g, 26 mmol) was added. The reaction mixture was stirred for 1 h at room temperature and then was extracted with 3 × 50 mL of ether. The combined extracts were dried, filtered, and concentrated to yield 1.04 g of crude product. Chromatography on a silica gel column (30 g) gave 22.8 mg (1%) of the desired 2′-cyanonicotine and 421 mg of the 5′-cyanonicotine diastereomers. The 2′-isomer was characterized by 80-MHz ^{1}H NMR [($CDCl_3$) δ 1.9–2.4 (m, 3 H), 2.24 (s, 3 H, NCH_3), 2.4–2.9 (m, 2 H), 3.1–3.5 (m, 1 H), 7.25 (q, 1 H, C5), 7.9 (d of t, 1 H, C4), 8.6 (d, 1 H, C6), and 8.85 (m, 1 H, C2)] and by GC–EIMS [*m*/*z* 187 (M^+)]. Limited quantities precluded full characterization of this product.

Metabolic Studies. Human livers were obtained from Drs. J. Trudell (Stanford University, Stanford, CA) and L. Waskell (Veteran's Administration Hospital, San Francisco, CA). A human liver microsomal preparation also was obtained from Dr. F. Kadlubar (National Center for Toxicological Research, Jefferson, AR). Both male Dutch rabbit and human liver microsomes were prepared according to previously described methods[23] except that in the case of human tissue the initial homogenization was performed with 4 mL of KCl solution/g of liver instead of the 2 mL/g of liver used for the rabbit tissue.

The deuteriated nicotine derivatives (0.5 mM) were incubated with microsomal preparations (3 mg of protein/mL) in 0.2 M potassium phosphate buffer, pH 7.4, containing magnesium chloride (1.5 mM), sodium cyanide (1.0 mM), and NADPH (1 mg/mL) in a final volume of 5 mL. Incubations were carried out for 30 min on a metabolic shaker at 37 °C, following which the samples were cooled on ice and then extracted with an equal volume of peroxide-free ether. The aqueous phase was frozen in a dry ice bath, and the ether phase was decanted. After removal of the ether under a stream of N_2, the residue was dissolved in 50 μL of CH_2Cl_2 in preparation for GC–EIMS analysis. The sample was injected onto the column at room temperature. After the solvent had eluted (about 2 min), the column was heated rapidly to and maintained at 180 °C. The injection-port, ion-source, and inlet-line temperatures were maintained at 220, 150, and 200 °C, respectively. The ion currents at *m*/*z* 109, 110, and 111 were determined by computer-assisted selected ion monitoring techniques. A series of equations were developed to correct for the ^{13}C-satellite contributions and the enantiomeric and d_0 and d_2 contaminants present in the substrates.[24]

Incubations performed with the 1:1 mixture of the d_0 and d_1 α-cyanonicotines followed the procedures described above except

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that the total concentration of the α-cyanonicotines was 50 μM. The deuterium composition of the unmetabolized substrate was determined by the same GC-EIMS procedure. (S)-Cotinine analyses were performed on 1-mL aliquots of the incubation mixtures according to the procedure described by Jacob et al.[16]

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Registry No. 1, 54-11-5; 2, 105090-00-4; 2 (bisperchlorate), 71014-67-0; 3, 486-56-6; 7-d_1, 105089-99-4; 8-d_1, 105120-31-8; 9, 105120-32-9; 10, 105089-96-1; 10 (bis-*l*-tartrate), 105089-97-2; 12, 105089-98-3; cytochrome P-450, 9035-51-2.

Structural Requirements for the Inhibition of 5-Lipoxygenase by 15-Hydroxyeicosa-5,8,11,13-tetraenoic Acid Analogues

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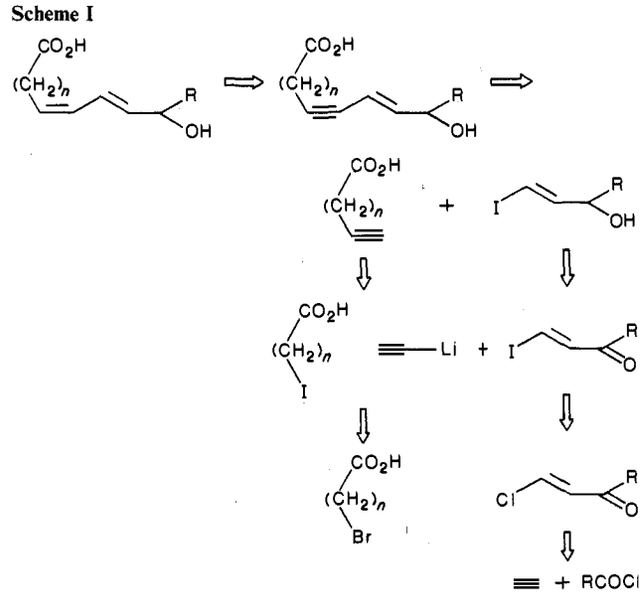
The structural requirements for inhibition of RBL-1 (rat basophilic leukemia) 5-lipoxygenase by 15-hydroxyeicosa-5,8,11,13-tetraenoic acid (15-HETE, 1) were studied by systematic chemical modifications of the molecule at the hydroxyl and carboxyl groups, the double bonds, and the carboxylate and ω side chains. The most potent inhibitors were analogues that contained a 5,8-*cis*,*cis*-diene system and acted as alternate substrates for the enzyme. However, several analogues in which the 5,8-diene had been reduced were also found to inhibit the enzyme. Inhibition of 5-lipoxygenase by 15-hydroxyeicosa-11,13-dienoic acid (15-HEDE) analogues was optimal in compounds that generally contained a free carboxyl group, a carboxylate side chain of nine carbons, an ω side chain of five or six carbons, a *cis*,*trans*- or *trans*,*cis*-11,13-diene or 11,13-diyne system, and a 15-hydroxyl group. Conversion of 15-HEDE to its 16-membered lactone reduced but did not eliminate 5-lipoxygenase inhibitory activity. In contrast, a 3- to 10-fold enhancement of activity occurred when 5,15-diHETE (**58**) or 5-HETE (**56**) were cyclized to their respective δ-lactones. Molecular modeling of 15-HEDE analogues, modified in the C_{11}–C_{15} region, showed that inactive analogues protrude into regions in space not occupied by active analogues. These structural studies indicate that multiple regions are important for 5-lipoxygenase inhibition by both 15-HETE and 15-HEDE analogues and that no single region plays a predominant role in inhibition.

Several laboratories have reported that 15-hydroxyeicosa-5,8,11,13-tetraenoic acid (15-HETE) is a potent inhibitor of platelet 12-lipoxygenase[1] and neutrophil 5-lipoxygenase.[2-5] Since neutrophils generate both 15-HETE and 5-HETE,[6] a possible regulatory role for 15-HETE in the control of cellular 5-lipoxygenase activity has been suggested.[1,5] Additionally, the formation of 5,15-diHETE, when 15-HPETE was incubated with human neutrophils,[4] led to the suggestion that 15-HPETE and possibly 15-HETE could serve as alternate substrates for 5-lipoxygenase. These interesting findings prompted us to investigate the structural features of 15-HETE required for 5-lipoxygenase inhibitory activity as departure point for the design of therapeutically useful 5-lipoxygenase inhibitors.

Scheme I



Synthesis

Gram quantities of 15-HETE (1) and analogues **7**, **8**, **10**, **12**, and **14** were prepared from the corresponding ω6 fatty

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(6) Abbreviations: 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; 5,15-diHETE, 5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid; 15-HEDE, 15-hydroxy-11,13-eicosadienoic acid.

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