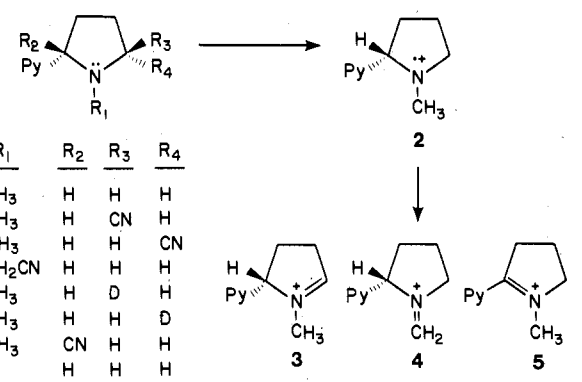
Regio- and Stereochemical Studies on the α-Carbon Oxidation of (S)-Nicotine by Cytochrome P-450 Model Systems

Lisa A. Peterson† and Neal Castagnoli, Jr.*

Division of Toxicology, School of Pharmacy, University of California, San Francisco, California 94143. *Received July 16, 1987*

Results from previous studies indicate that rabbit liver microsomal cytochrome P-450 catalyzes the C-5′ two-electron oxidation of (*S*)-nicotine stereoselectivity with preferential loss of the *pro*-(*E*)-hydrogen atom trans to the pyridine ring. We now have examined the regio- and stereochemical features of the oxidation of (*S*)-nicotine by peroxides in the presence of various hemoproteins and by electrochemical and photochemical methods. None of these systems gave rise to the stereochemical outcomes observed with the cytochrome P-450 mediated reaction. The results of these studies are interpreted as additional evidence for the formation of a highly ordered complex between (*S*)-nicotine and cytochrome P-450 that directs the regio- and diasterioselective α-carbon oxidation of this substrate.

The generally accepted mechanism for the cytochrome P-450 catalyzed α-carbon oxidation of tertiary amines involves initial transfer of an electron from the nitrogen lone pair to an electron-deficient heme-bound oxygen atom.[1-4] In the case of the tobacco alkaloid (*S*)-nicotine (1), this pathway leads to the aminium radical intermediate **2**, which then undergoes a second one-electron oxidation that results in the net loss of a hydrogen atom from either the 5′-carbon atom for *N*-methyl group with the eventual formation of the corresponding iminium species **3** and **4**. Metabolic formation of the third possible iminium species, **5**, has not been reported. The iminium ion metabolites can be trapped in liver microsomal preparations as the corresponding (*E*)-**6** and (*Z*)-**7** α-cyano amines and the *N*-cyanomethyl adduct **8**, respectively. We have employed this reaction and a capillary column GC–EIMS selected ion monitoring assay to examine the stereochemistry of proton/deuteron loss from the *E* and *Z* diastereoisomers of (*S*)-nicotine-5′-d_1 (**9** and **10**, respectively).[5] The results of our studies revealed that the microsomal cytochrome P-450 catalyzed C-5′ oxidation of **9** and **10** proceeds with the selective loss of the deuterium atom from **9** and the hydrogen atom from **10** [equivalent to loss of the *pro*-(*E*)-5′ proton trans to the pyridine moiety of (*S*)-nicotine]. No significant deuterium isotope effects could be detected.



	R_1	R_2	R_3	R_4
1	CH_3	H	H	H
6	CH_3	H	CN	H
7	CH_3	H	H	CN
8	CH_2CN	H	H	H
9	CH_3	H	D	H
10	CH_3	H	H	D
11	CH_3	CN	H	H
12	H	H	H	H

In the present paper, we report the results of our regio- (Table I) and stereochemical (Table II) studies on the α-carbon oxidation of (*S*)-nicotine by a variety of other oxidizing systems, which were chosen for their ability to mimic various aspects of the cytochrome P-450 mediated process. Estimations of the regioselectivity (C-5′ vs C-2′ vs *N*-methyl) were obtained by capillary column GC analysis of the corresponding α-cyano adducts **6** and **7**, **11** and **8**. Since cyanide ion is inhibitory to the peroxidases, sodium cyanide was added following the incubations in these cases. Under these conditions, the methyliminium species **4** underwent hydrolysis, and therefore, the extent of N-demethylation was determined by GC analysis of nornicotine (**12**). These GC tracings also provided rough estimates of the extent to which (*S*)-nicotine was consumed during the 30-min incubation periods—about 5% for the hemoprotein-dependent reactions and up to 30% for the electrochemical and photochemical oxidations. The stereoselectivity of the C-5′ oxidation was examined with substrates **9** and **10**. The deuterium compositions of the 5′-cyano adducts generated in these experiments were measured by monitoring the fragment ion $(M - C_5H_4N)^+$ at m/z 110. The extent to which deuterium was lost or retained was estimated by calculating the ratios of ion currents at m/z 109 and 110 to the sum of these ion currents. Minor corrections for ^{13}C satellite contributions, substrate enantiomeric composition, and the presence of small amounts of d_2 and/or d_0 contaminants in the substrates were required.[5]

Table I. Regioselectivity Observed in the Oxidation of (*S*)-Nicotine by Microsomal Preparations and Model Oxidizing Systems

oxidizing system	relative yield[a] 5′	NCH_3	2′
dutch rabbit liver microsomes: NADPH/O_2 (n = 8)	9	1	0
dutch rabbit liver microsomes: cumene hydroperoxide (n = 3)	9	1	0
horseradish peroxidase: HOOH (n = 3)	9	4.5	1
methemoglobin: HOOH (n = 3)	9	trace	1
chloroperoxidase: EtOOH (n = 2)	4	trace	1
electrochemical oxidation (n = 3)	6	1	3
photochemical oxidation (n = 2)	3	1	0

[a] As determined by relative GC peak areas of the respective α-amino nitriles or nornicotine.

Results

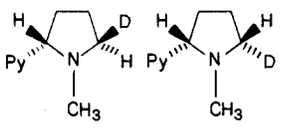
The influence of the protein environment on the course of the α-carbon oxidation of (*S*)-nicotine was explored with horseradish peroxidase,[2,6,7] methemoglobin,[2,8,9] and chlo-

†Department of Biochemistry, Vanderbilt University, Nashville, TN 37232.

(1) Shono, J.; Toda, J.; Oshino, N. *J. Am. Chem. Soc.* **1982**, *104*, 2639.
(2) Miwa, G. T.; Walsh, J. S.; Kedderis, G. L.; Hollenberg, P. F. *J. Biol. Chem.* **1983**, *258*, 14445.
(3) Hanzlik, R. P.; Tullman, R. H. *J. Am. Chem. Soc.* **1982**, *104*, 2048. Augusto, O.; Beilan, H. S.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1982**, *257*, 111288.
(4) Hollenberg, P. F.; Miwa, G. T.; Walsh, J. S.; Dwyer, L. A.; Rickert, D. E.; Kedderis, G. L. *Drug Metab. Dispos.* **1985**, *13*, 272. Abdel-Monem, M. M. *J. Med. Chem.* **1975**, *18*, 427. Groves, J. T.; McCluskey, G. A.; White, R. E.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 154.
(5) Peterson, L. A.; Trevor, A.; Castagnoli, N., Jr., *J. Med. Chem.* **1987**, *30*, 249.
(6) Kedderis, G. L.; Hollenberg, P. F. *J. Biol. Chem.* **1983**, *258*, 8129.

0022-2623/88/1831-0637$01.50/0

Table II. Stereoselectivity Observed in the Oxidation of the (*S*)-Nicotine-d_1 Analogues **9** and **10** by Liver Microsomal Preparations and Model Oxidizing Systems

	% deuterium retention in the 5′-cyano adducts **6** and **7**	
		
oxidizing system	**9**	**10**
dutch rabbit liver microsomes: NADPH/O_2 (n = 8)	26 ± 15	84 ± 2
dutch rabbit liver microsomes: cumene hydroperoxide (n = 8)	22 ± 3	88 ± 2
methemoglobin: HOOH (n = 3)	71 ± 1	62 ± 1
horseradish peroxidase: HOOH (n = 3)	78 ± 5	68 ± 7
chloroperoxiase: EtOOH (n = 2)	94 ± 0	72 ± 0
electrochemical oxidation (n = 2)	80 ± 1	37 ± 1
photochemical oxidation (n = 2)	98 ± 1	15 ± 2

roperoxidase,[2,10] hemoproteins that have been demonstrated to catalyze the α-carbon oxidation of tertiary amines. (*S*)-Nicotine underwent oxidation in the presence of all three proteins when the incubation mixtures were supplemented with the appropriate oxidant (hydrogen peroxide for horseradish peroxidase and methemoglobin and ethyl peroxide for chloroperoxidase). GC analysis of the organic extracts showed the 5′-carbon atom to be the dominant α-carbon oxidation site. All three hemoproteins also catalyzed the C-2′ oxidation of this substrate while only horseradish peroxidase catalyzed attack at the *N*-methyl group (Table I). Only low levels of products were detected when the peroxide or the protein was omitted from the incubation mixture. Analysis of the deuterium content of the 5′-cyano adducts generated from **9** and **10** established that C-5′ oxidation in all three reactions resulted in the selective loss of the proton from both monodeuteriated substrates (Table II). This result indicates that the stereochemical course of these oxidations is controlled by deuterium isotope effects, an outcome that differs from the stereochemical control observed with the cytochrome P-450 mediated reactions. The possibility that a peroxide-supported cytochrome P-450 catalyzed oxidation of (*S*)-nicotine might behave in a manner similar to that observed with these hemoproteins could be eliminated since the regio- (Table I) and stereochemical (Table II) results obtained with the cumene hydroperoxide supported cytochrome P-450 catalyzed oxidation of (*S*)-nicotine were essentially identical with those obtained with the NADPH/O_2 supported reaction.

The electrochemical α-carbon oxidation of tertiary amines presumably proceeds by initial loss of an electron from the nitrogen lone pair.[11] Therefore, this reaction was examined with (*S*)-nicotine as a chemical model for the cytochrome P-450 catalyzed reaction. The cyclic voltammogram of (*S*)-nicotine in the presence of sodium cyanide revealed irreversible oxidation waves at 1.1 V (first two-electron oxidation) and 1.5 V (subsequent oxidation of the initial oxidation products). Accordingly, the controlled potential electrolysis of (*S*)-nicotine was carried out at 1.2 V. In the presence of sodium cyanide, this reaction led to the formation of the 5′-cyano, 2′-cyano, and *N*-cyanomethyl adducts in the ratio of 6:3:1. The preferential oxidation of the 5′- and 2′-positions is consistent with reports that the electrochemical α-carbon oxidation of cyclic tertiary amines tends to occur at ring carbon atoms.[12] GC–EIMS analysis of the 5′-cyano adducts demonstrated the stereoselective loss of the proton from **9** and deuteron from **10** (Table II). Only a modest preference for proton (from **9**) vs deuteron (from **10**) loss was observed. Thus, in contrast to the cytochrome P-450 catalyzed reaction, the C-5′-*pro*-(*Z*) proton cis to the pyridine ring of (*S*)-nicotine is selectively lost in this chemical model reaction.

The methylene blue sensitized photochemical oxidation of tertiary amines also is thought to proceed via aminium radical species.[13] Irradiation of an aqueous solution of (*S*)-nicotine buffered at pH 8.8 in the presence of sodium cyanide and methylene blue gave a 3:1 mixture of the 5′-cyano and *N*-cyanomethyl adducts. No 5′-cyano adduct was detected. Brierre et al. reported the formation of only the 5′-cyano adducts when methanol was used as solvent.[14] The stereoselective loss of the proton/deuteron cis to the pyridine ring in this reaction was similar to that observed in the electrochemical studies (Table II).

Discussion

Although the substrate specificity for the cytochrome P-450 family of enzymes appears to be rather low,[15] recent studies have indicated that certain reaction pathways may lead to highly selective regio- and stereochemical outcomes.[16] Relatively little is known about the stereochemical features of the cytochrome P-450 catalyzed α-carbon oxidations of tertiary amines, perhaps, in part, because of the instability of the resulting iminium intermediates. A general consideration of reaction products,[17] the absence of large isotope effects.[4] and similarities between the enzyme-catalyzed reaction and the stepwise electrochemical oxidation[18] have led investigators to propose a radical pathway as illustrated by the C-5′ oxidation of (*S*)-nicotine via the aminium radical intermediate **2**. In the absence of intermolecular enzyme–substrate steric constraints, arguments based on literature reports[19] and stereoelectronic considerations[5] led us to predict the diastereoselective loss of the *pro*-(*Z*) proton cis to the pyridine ring of the putative aminium radical **2**. Our experimental observations, however, led to the conclusion that this cytochrome P-450 mediated oxidation proceeds with the diastereoselective loss of the *pro*-(*E*) proton trans to the pyridine ring.

(7) Sarislani, F. S.; Duffel, M. W.; Rosazza, J. P. *J. Med. Chem.* **1985**, *28*, 629.
(8) Griffin, B. W.; Ting, P. L. *Biochemistry* **1978**, *17*, 2206. Griffin, B. W.; Davis, D. V.; Bruno, G. V. *Bioorg. Chem.* **1981**, *10*, 342.
(9) Zaide, S.; Gelhar, D.; Selman-Greenspan, A. *Pharm. Res.* **1984**, 277. Starke, D. W.; Blisard, K. S.; Mieyal, J. *Mol. Pharmacol.* **1984**, *25*, 467.
(10) Kedderis, G. L.; Kopp, D. R.; Hollenberg, P. F. *J. Biol. Chem.* **1972**, *255*, 10 174.
(11) Chow, Y. L.; Danen, W. C.; Nelson, S. F.; Rosenblatt, D. H. *Chem. Rev.* **1978**, *78*, 243.
(12) Chiba, T.; Takata, Y. *J. Org. Chem.* **1975**, *42*, 2973.
(13) Herlem, D.; Hubert-Brierre, Y.; Khuong-Huu, F. *Tetrahedron Lett.* **1976**, 4173.
(14) Hubert-Brierre, Y. D.; Herlem, D.; Khuong-Huu, F. *Tetrahedron* **1975**, *31*, 3049.
(15) Low, L. K.; Castagnoli, N., Jr. In *Burger's Medicinal Chemistry*; Wolff, M. E., Ed.; Wiley: New York, 1980; pp 107–226.
(16) Gelb, M. H.; Heimbrook, D. C.; Malkonen, P.; Sligar, S. G. *Biochemistry* **1982**, *21*, 370. Wood, A. W.; Ryan, R. E.; Thomas, P. E.; Levin, W. *J. Biol. Chem.* **1983**, *258*, 8839. Porter, W. R.; Wheeler, C.; Trager, W. F. *Biochem. Pharmacol.* **1981**, *30*, 3099.
(17) Rose, J.; Castagnoli, H., Jr. *Med. Res. Rev.* **1983**, *3*, 73.
(18) Shono, T.; Toda, T.; Oshino, N. *Drug. Metab. Dispos.* **1981**, *9*, 481.
(19) Lewis, F. D.; Ho, T. L. *J. Am. Chem. Soc.* **1980**, *102*, 1751.

The results obtained in the present study emphasize the unique features of the cytochrome P-450 catalyzed α-carbon oxidation of (*S*)-nicotine. Although the corresponding peroxide-supported oxidation in the presence of the hemoproteins horseradish peroxidase, methemoglobin, and chloroperoxidase gave regiochemical profiles similar to those observed with cytochrome P-450, the dominant stereochemical feature associated with all three hemoproteins was the selective loss of the C-5′ proton from both monodeuteriated substrates **9** and **10**, suggesting that bond cleavage in these reactions is subject to a deuterium isotope effect. Since these substrates are diastereoisomers, estimation of an intrinsic isotope effect[20] is not possible. Kinetic isotope effect measurements, however, have been reported for the oxidative N-demethylation of *N*-methyl-*N*-(trideuteriomethyl)aniline.[2] The reported k_H/k_D value for the cytochrome P-450 catalyzed reaction, 1.78, was dramatically lower than the corresponding values obtained with horseradish peroxidase and methemoglobin, both of which were greater than 8. The differences in these kinetic isotope effect values together with the results obtained in our studies suggest that the pathway followed in the cytochrome P-450 catalyzed reaction may be different from that followed in the reactions catalyzed by these two hemoproteins. Large isotope effects also have been reported for cytochrome P-450 catalyzed carbon hydroxylations, which, unlike the α-carbon oxidations of tertiary amines, are thought to proceed via a hydrogen atom abstraction pathway.[21] Our results also suggest that the chloroperoxidase-catalyzed oxidation of (*S*)-nicotine follows a pathway similar to that of horseradish peroxidase and methemoglobin. This conclusion, however, is not supported by other observations, which indicate that the active site[22] and catalytic mechanism[2] of this peroxidase are similar to those of cytochrome P-450.

The electrochemical and photochemical oxidations of (*S*)-nicotine were examined as chemical models for the aminium radical pathway. The electrochemical oxidation was of particular interest since the fate of intermediate **2** was most likely to be unencumbered by intermolecular enzyme–substrate steric effects. Assuming a radical mechanism, the low α-carbon selectivity of this reaction may be interpreted as evidence that spontaneous proton loss from all three α-cation atoms of the aminium radical intermediate is energetically feasible and that the high C-5′ selectivity observed with cytochrome P-450 is a reflection of intermolecular enzyme–substrate steric effects. The stereoselectivity of the electrochemical oxidation (loss of the proton/deuteron cis to the pyridine ring) is consistent with the predicted reaction pathway that allows maximal overlap of the developing p orbital on the α-carbon atom with the half-empty p orbital of the planar aminium radical.[19] In the case of (*S*)-nicotine, molecular models show that considerably less steric interaction occurs between the *N*-methyl and pyridyl groups with loss of the 5′-*pro*-(*Z*) hydrogen atom than with loss of the 5′-*pro*-(*E*) hydrogen atom.[5]

The regioselectivity observed in the methylene blue sensitized photochemical oxidation may be rationalized further in terms of a π–π complex (Figure 1) similar to that postulated for the corresponding photochemical oxidation

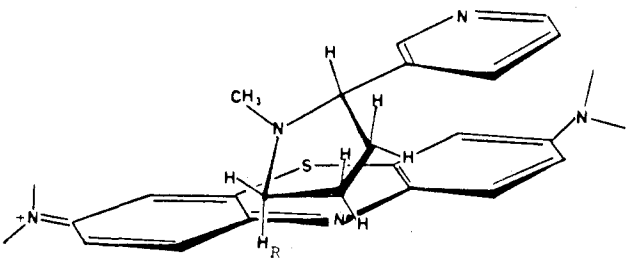


Figure 1. Proposed π–π interactions between (*S*)-nicotine and methylene blue.

of arylamines.[23] This complex allows proton transfer from the aminium radical **2** to the methylene blue acceptor molecule only from the *N*-methyl and C-5′ carbon atoms and not the C-2′ carbon atom since the proton attached to this position points away from the methylene blue. This model also predicts the selective loss of the C-5′ proton from **9** and the C-5′ deuteron from **10** [equivalent to the 5′-*pro*-(*Z*) proton of (*S*)-nicotine] as observed experimentally.

Since the cytochrome P-450 catalyzed reaction results in the preferential loss of the energetically unfavored 5′-*pro*-(*E*) proton of (*S*)-nicotine, one might postulate that the enzymatic process does not involve initial aminium radical formation. This postulate assumes that the reaction pathway is determined solely by intramolecular steric factors. The high degree of regioselectivity observed in the cytochrome P-450 catalyzed reaction, however, suggests an ordered enzyme–substrate complex that restricts the conformational possibilities of the postulated aminium radical intermediate. Furthermore, proton loss from the aminium radical intermediate may be facilitated by a basic site on the protein. The enhanced regio- and stereoselectivity of the methylene blue sensitized photochemical oxidation of (*S*)-nicotine relative to that observed in the electrochemical oxidation may serve as a model for such a process. Our results suggest that (*S*)-nicotine binds at the active site with the pyridine ring extended away from the porphyrin ring system, thus placing the 5′-*pro*-(*E*) hydrogen atom in close proximity to the iron oxo species. These binding constraints would require that the *N*-methyl group be positioned cis to the pyridine ring. Molecular models suggest that such a binding configuration would allow the closest approach of the aliphatic nitrogen atom's lone pair of electrons to the enzyme's oxidizing system. Although such a complex also might satisfy a direct hydrogen atom abstraction pathway, such a mechanism would be expected to result in a large isotope effect as has been observed for carbon hydroxylation reactions.[21] Although it might be masked in the presence case, the lack of evidence demonstrating such an isotope effect with this and other cytochrome P-450 tertiary amine substrates argues against this possibility.

The results of these model studies provide additional evidence that the interactions between cytochrome P-450 and (*S*)-nicotine result in the formation of an ordered complex that controls the regio- and stereochemical course of this oxidation. Further information on the mechanism of the enzyme-catalyzed reaction pathway should help to elucidate the details of the structure of this complex.

Experimental Section

(*S*)-Nicotine was obtained from Aldrich Chemical Co. and was purified by vacuum distillation prior to conversion to its bistartrate salt.[24] The cyano adducts **6–8** and **11** and the bistartrate salts

(20) Melander, L.; Saunders, W. H. In *Reaction Rates of Isotopic Molecules*; Wiley-Interscience: New York, pp 91–128.
(21) Hjelmeland, L. M.; Aronow, L.; Trudell, J. R. *Biochem. Biophys. Res. Commun.* **1977**, *76*, 541. Shapiro, S.; Piper, J. W.; Caspi, E. *J. Am. Chem. Soc.* **1982**, *104*, 2301.
(22) Cranier, S. P.; Dawson, J. H.; Hodgson, K. O.; Hager, L. P. *J. Am. Chem. Soc.* **1980**, *102*, 7282.
(23) Kayser, R. H.; Young, R. H. *Photochem. Photobiol.* **1976**, *24*, 395.

of the monodeuteriodiastereomers **9** and **10** were synthesized as described previously.[5] The dichloromethane used for the GC–EIMS analyses was HPLC grade. Horseradish peroxidase (Type IV), methemoglobin, and chloroperoxidase were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

GC and GC–EIMS Assays. GC analyses were performed on a 30 m long J & W 10 mm × 0.0325 mm DB5 fused silica gel capillary column and a Varian 3700 chromatograph employing a nitrogen/phosphorus detector under the following conditions: carrier gas (3.5 mL/min); injector temperature 220 °C; detector temperature 250 °C; column temperature 90 °C (1 min) increased by 30 °C/min to 180 °C. GC–EIMS studies used the same silica gel column and a Varian 3700 gas chromatograph linked to a Kratos MS25S mass spectrometer. The temperature of the injection port of the GC was maintained at 220 °C, and the temperatures of the ion source and inlet line of the mass spectrometer were maintained at 150 and 200 °C, respectively. The column temperature initially was ambient and following sample injection and solvent elution was increased rapidly (30 °C/min) to and maintained at 180 °C. Computer-assisted selected ion monitoring techniques were used to measure the ion current at m/z 109, 110, and 111, and the resulting values were used to calculate the percent deuterium retained in the cyano adducts.[5,25]

Dutch Rabbit Liver Microsomal Incubations. Dutch male rabbit liver microsomes were prepared according to previously reported methods.[26] The bistartrates of (*S*)-nicotine, **9** or **10** (0.5 mM) were incubated in a rotating water bath with the microsomes (3 mg of protein/mL) in 0.2 M potassium phosphate buffer, pH 7.4 (final volume 2.5 mL), containing magnesium chloride (1.5 mM) and sodium cyanide (1 mM) in the presence or absence of cumene hydroperoxide (1 mM). The incubations (37 °C) were terminated after 30 min by cooling the mixtures in a dry ice–methanol bath. Subsequent analyses were performed by extracting the thawed mixtures with 5 mL of ferrous sulfate washed ether. The extracts were frozen on dry ice, and the ether was decanted into prewashed vials. After the solvent was removed under a stream of nitrogen, the residues were dissolved in methylene chloride (50 mL) and submitted to GC or GC–EIMS analysis.

Horseradish Peroxidase Incubations.[7] Incubation mixtures (5 mL) were composed of horseradish peroxidase (0.1 mg of protein/mL), hydrogen peroxide (0.136 mM), and the bistartrates of (*S*)-nicotine, **9** or **10** (0.85 mM), dissolved in pH 7.4 phosphate buffer. Following 30 min in a rotating water bath (37 °C), the mixtures were cooled in an ice bath and treated with sodium cyanide (30 mg). After an additional 15 min at room temperature, the mixtures were extracted with methylene chloride (5 mL), and the extracts were concentrated to about 100 mL and submitted to GC or GC–EIMS analysis. Incubations were carried out in triplicate and control incubations were run in the absence of hydrogen peroxide or enzyme.

Methemoglobin Incubations.[27] Similarly, incubations (2.5 mL) were carried out with methemoglobin (1.8 mg) and the bistartrates of (*S*)-nicotine, **9** or **10** (10 mM), in the presence of hydrogen peroxide (0.6 mM) in pH 7.4 phosphate buffer at 37 °C for 30 min. Sodium cyanide (30 mg) was added, and the samples were treated as described above. The studies were performed in triplicate and controls were run in the absence of either hydrogen peroxide or hemoprotein.

Chloroperoxidase Incubations.[2] Analogous studies were carried out in duplicate with substrates (0.85 mM), chloroperoxidase (66 mg of protein/mL), and ethyl peroxide (1.7 mM) in pH 6.8 phosphate buffer (final volume 2.5 mL) at 25 °C for 30 min.

Electrochemical Oxidation.[12] A 25-mL H-type electrolysis cell (The Electrosynthesis Co., Inc.) with porous glass frits was employed for these studes. The bistartrates of (*S*)-nicotine, **9** or **10** (0.2 mM), were dissolved in 15 mL of methanol–water (1:1) containing 0.2 M sodium cyanide. The reaction was carried out between 0 and 5 °C at 1.2 V vs a saturated calomel electrode with platinum wire electrodes with a BAS voltammograph power source. After 15 min, the solutions were extracted with methylene chloride (5 mL), and after concentration under a nitrogen stream to 50 mL, the concentrates were analyzed by GC or GC–EIMS. The experiment was repeated three times with each substrate.

Methylene Blue Sensitized Photochemical Oxidation.[14] To a solution of methylene blue (0.8 mg, 2.1 mmol), potassium cyanide (3.8 mg, 78 mmol), and sodium pyruvate (6.5 mg, 59 mmol) in 1.5 mL of 0.2 M potassium phosphate phosphate buffer adjusted to pH 8.8 with 5 N sodium hydroxide was added the bistartrate of (*S*)-nicotine, **9** or **10** (27.1 mg, 58.5 mmol). Each sample was irradiated with a medium-pressure (450-W) Hannovia lamp filtered through pyrex for 30 min during which time dioxygen was bubbled through the solution. The reaction mixtures were worked-up as described above and analyzed by GC or GC–EIMS. The experiments were run in duplicate.

Acknowledgment. We acknowledge the Bio-organic, Biomedical Mass Spectrometry Resource at the University of California, San Francisco (A. L. Burlingame, Director) supported by NIH Division of Research Resources Grant RR-01614. This work was supported by NIH Training Grant GMO-7175 and NCI Grant CA-35678.

Registry No. 1, 54-11-5; H_2O_2, 7722-84-1; ethyl peroxide, 628-37-5; cytochrome P-450, 9035-51-2; peroxidase, 9003-99-0; chloroperoxidase, 9055-20-3.

(24) Aceto, A. D.; Martin, B. R.; Uwaydah, I. M.; May, E. L.; Harris, L. S.; Izazola-Conde, C.; Dewey, W. L.; Bradshaw, T. J.; Vincek, W. C. *J. Med. Chem.* **1979**, *22*, 174.

(25) Peterson, L. A. *Stereochemical Studies of the Cytochrome P-450 Catalyzed Oxidation of (S)-Nicotine to the Nicotine-D1′,5′-Iminium Ion*; Dissertation; University of California: San Francisco, 1986; pp 75–80.

(26) Hoag, M. K. P.; Trevor, A. J.; Asscher. Y.; Weissman, J.; Castagnoli, N., Jr., *Drug Metab. Dispos.* **1984**, *12*, 371.

(27) Ortiz de Montellano, P. R.; Catalano, C. E. *J. Biol. Chem.* **1985**, *260*, 9265.