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Research Article

Tertiary Amines Related to Brompheniramine: Preferred Conformations for N-Oxygenation by the Hog Liver Flavin-Containing Monooxygenase

John R. Cashman,[1,3] Joy R. Celestial,[1] Andrew Leach,[2] Julie Newdoll,[1] and Sang Bum Park[1]

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The metabolism of racemic, (*D*)- and (*L*)-brompheniramine, a widely used antihistamine, was studied with microsomes and with highly purified flavin-containing monooxygenase (FMO) from hog liver. In addition, a number of other similar tertiary amines were evaluated as substrates for FMO activity from hog liver and the kinetic constants obtained were compared with brompheniramine. Although some N-demethylation was observed, the major metabolite of brompheniramine and the other tertiary amines examined in hog liver microsomes was the metabolite containing an aliphatic nitrogen *N*-oxide. Brompheniramine was extensively N-oxygenated by the highly purified FMO from hog liver. N-Oxygenation of brompheniramine in both microsomes and with highly purified FMO from hog liver was enantioselective. The K_m for N-oxygenation of (*D*)-brompheniramine was markedly lower than the K_m for (*L*)-brompheniramine. (*E*)- and (*Z*)-zimeldine are less conformationally flexible model compounds of brompheniramine, and these compounds were also examined and were found to be stereoselectively N-oxygenated by the highly purified FMO from hog liver. The similarities and differences in K_m and V_{max} values were evaluated in terms of possible conformations of the substrates determined by SYBYL molecular mechanics calculations. Distance map data indicated that FMO preferentially accommodated selected conformations of tertiary amines. Thus, (*D*)-bromopheniramine and (*Z*)-zimeldine presumably have the aliphatic tertiary amine nitrogen atom and aromatic ring center at a defined distance and geometry and were more efficiently N-oxygenated than their respective isomers.

KEY WORDS: bromopheniramine enantiomers; hog liver flavin-containing monooxygenase; zimeldine; molecular mechanics.

INTRODUCTION

Brompheniramine [2-*p*-bromo-α-(2-dimethylaminoethyl)-benzylpyridine], 1 (Fig. 1), is an antihistamine drug that is widely used in pharmaceutical preparations to relieve nasal itching, sneezing, and rhinorrhea in patients with allergic rhinitis (1). In humans and animals, brompheniramine is rapidly absorbed, with symptomatic relief beginning at 15 min and becoming maximal at 1 to 3 hr with the short-acting forms. The duration of action of brompheniramine is variable, with the serum elimination half-life 20–25 hr (2). Brompheniramine undergoes extensive metabolism (3), and in man, the mono (i.e., compound 3)- and didesmethylated urinary metabolites have been identified (4). Although the aliphatic nitrogen *N*-oxide metabolite of other similar tertiary amines (i.e., chlorpheniramine and diphenylhydramine) has been reported to be excreted in considerable amounts in the urine of dogs and humans (5,6), the aliphatic nitrogen *N*-oxide metabolite of brompheniramine, 2, has not been reported. It is not clear whether the absence of *N*-oxide metabolite formation *in vivo* reflects a low affinity of brompheniramine for the N-oxygenation monooxygenase system, whether reduction is efficient, or whether analytical difficulties precluded detection of the tertiary amine *N*-oxide. In addition to cytochrome P-450-catalyzed N-demethylation (7) of brompheniramine, the flavin-containing monooxygenase (FMO)[4] may also contribute to the oxidative metabolism of this tertiary amine (for a general review of tertiary amine metabolism by FMO, see Ref. 8). However, in adult humans, the major form of FMO (form II) (9) is quite distinct from hog liver FMO (form I),[5] and form II FMO quite possibly N-oxygenates brompheniramine and other related aliphatic tertiary amines with a substrate specificity different from that

[1] Department of Pharmaceutical Chemistry and the Liver Center, University of California, San Francisco, California 94143.

[2] Department of Chemistry, University of Southampton, Hampshire, UK S095HN.

[3] To whom correspondence should be addressed at IGEN Research Institute, 130 5th Avenue N., Seattle, Washington 98109.

[4] *Abbreviations used:* FMO, flavin-containing monooxygenase; MS, mass spectrometer; mCPBA, metachloroperbenzoic acid; NMR, nuclear magnetic resonance; CI, chemical ionization; LSIMS, liquid secondary ion mass spectrometry

[5] An alternative nomenclature for FMO form I (FMO 1A1) and FMO form II (FMO 1D1) has been proposed based on the rabbit liver enzymes.

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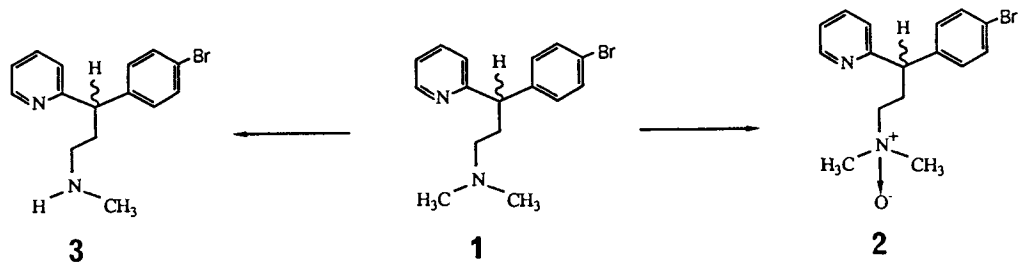

Fig. 1. N-Oxygenation and N-demethylation of brompheniramine.

of human liver form I FMO (10). In addition, it is possible that humans possess a hepatic cytochrome P-450 which also efficiently forms brompheniramine *N*-oxide.

Zimeldine [i.e., (*Z*)-3-(4-bromophenyl)-*N*,*N*-dimethyl-3-(3-pyridyl)-allylamine], **4** (Fig. 2), is a clinically effective antidepressant drug developed as a selective neuronal serotonin uptake inhibitor (11), although it was withdrawn from the market due to incidences of Guillian Barré syndrome. In humans and animals, zimeldine is extensively metabolized (12). In hog liver microsomes, stereoselective zimeldine aliphatic nitrogen N-oxygenation (i.e., to produce **5**) was shown to be dependent on FMO (13). Even though zimeldine shows a striking structural similarity to brompheniramine, it is without activity on histaminergic, muscarinic, adrenergic, and serotonergic neurotransmitter receptors (14). Because zimeldine can be considered a less flexible analogue of brompheniramine and because brompheniramine possesses a center of chirality, comparison of the relative affinity of isomers of brompheniramine and zimeldine for *N*-oxygenation by the hepatic FMO may reveal details about the substrate binding channel and enzyme active site topology.

10-(*N*,*N*-Dimethylaminoalkyl)phenothiazines are among the most widely used neuroleptic agents and possess a wide variety of pharmacological properties depending upon their chemical structure. 10-(*N*,*N*-Dimethylaminoalkyl)-phenothiazines undergo extensive metabolism and are excellent substrates for the hepatic flavin-containing monooxygenase (15). In contrast to hepatic FMO, the pulmonary form of FMO from rabbit does not N-oxygenate 10-(*N*,*N*-dimethylaminoalkyl)phenothiazines with short aliphatic tertiary amine side chains but does N-oxygenate 10-(*N*,*N*-dimethylaminoalkyl)phenothiazines containing C_6 and C_7 alkyl side chains (16). Unlike hepatic FMO, the pulmonary enzyme active site excludes 10-(*N*,*N*-dimethylaminoalkyl)-phenothiazines with a short tertiary amine side chain but not phenothiazine side chains containing C_5–C_7 dimethyl amino alkyl groups. In agreement with other studies (13,21), the data for 10-(*N*,*N*-dimethylaminoalkyl)phenothiazine N-oxygenation was interpreted to suggest specific dimensions and size constraints to the binding and stereoselective N-oxygenation of aliphatic tertiary amines by FMO (14,15).

Herein we report the characterization of the *in vitro* metabolism of racemic and (*D*)- and (*L*)-brompheniramine by microsomes and highly purified FMO from hog liver. The results of these studies provide evidence that microsomes and highly purified FMO from hog liver enantioselectively N-oxygenate brompheniramine. Because zimeldine can be viewed as a less flexible analogue of brompheniramine, the stereoselective N-oxygenation of zimeldine to zimeldine *N*-oxide by highly purified FMO was studied, employing (*E*)- and (*Z*)-zimeldine (Fig. 2). Comparison of zimeldine and brompheniramine N-oxygenation provided insight into the stereoselectivity of FMO-catalyzed N-oxygenations. In addition, conformational analysis of selected compounds by molecular modeling calculations was performed to determine whether FMO-mediated N-oxygenation correlated with substrate conformational preference.

METHODS

General Procedures

^{1}H NMR spectra were recorded on a General Electric 300-MHz spectrometer. Ultraviolet spectra were recorded on a Perkin Elmer 559A spectrometer. Chemical ionization (CI) mass spectra of *N*-oxides were taken on a modified Kratos AEI MS 9025 at 8 kV using isobutane (approximately 1 Torr) at a source temperature of 120°C. Liquid secondary ion mass spectral analyses were performed with a Kratos MS 50 at 6 kV and a source temperature of 50°C. Gas chromatography–mass spectra were obtained with a VG70S spectrometer fitted with a Varian Model 3600 gas chromatograph equipped with a DB-1 capillary column.

Reagents

Dextro-, levo- (or *R*- and *S*-brompheniramine, respec-

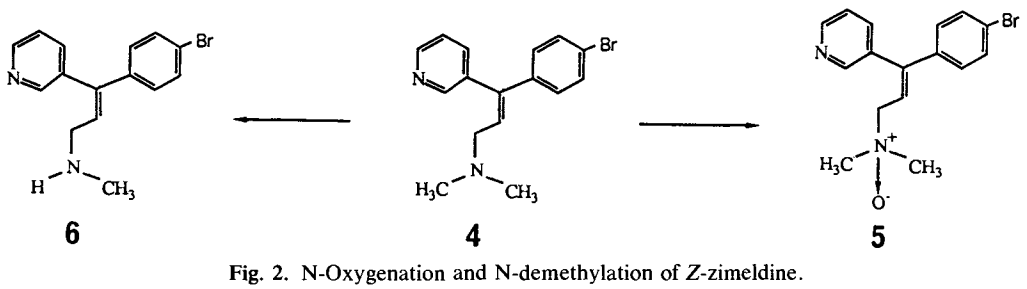

Fig. 2. N-Oxygenation and N-demethylation of *Z*-zimeldine.

tively) and racemic brompheniramine maleates were used as supplied by S. Symchowicz of Schering Corp. (Bloomfield, NJ). Dextro-3-(2-pyridyl)-3-*p*-bromophenyl-*N*,*N*-dimethylpropylamine-*d*-phenyl succinate, m.p. 152–154°C, had a specific rotation $[\alpha]_D^{25}$ (+)91° (at a concentration of 1% in dimethylformamide). Levo-3-(2-pyridyl)-3-*p*-bromophenyl-*N*,*N*-dimethylpropylamine-l-phenylsuccinate, m.p. 153–154°C, had a specific rotation $[\alpha]_D^{25}$ (−)91° (at a concentration of 1% in dimethylformamide. The free bases of dextro- and levobrompheniramine were obtained from the salts by mixing with diethyl ether and aqueous potassium carbonate to give $[\alpha h_D^{25}$ (+)42.7° and (−)42.7°, respectively. (*E*)- and (*Z*)-zimeldine hydrochlorides were supplied by T. Högberg of Astra Alab (Södertälje, Sweden); the *N*-oxides (*E*- and *Z*-zimeldine *N*-oxides) were synthesized and characterized by the method previously described (13). All compounds of the NADPH generating system were obtained from Sigma Chemical Co. All other chemicals were of the highest purity and were purchased from commercial sources.

Synthesis of *N*-Oxides

Levo- and Dextro-Brompheniramine N-*Oxide*. To a stirred solution of brompheniramine base (50 mg, 158 μmol) in CH_2Cl_2 (1 mL) at 0°C was added mCPBA (80% technical grade, 39 mg, 178 μmol). The reaction mixture was stirred for 4 hr. Analysis by thin-layer chromatography showed that no starting material remained. The crude mixture was chromatographed directly on basic alumina (eluent CH_2Cl_2/MeOH gradient) to give the levo or dextro *N*-oxide (45 mg, 85%). ^{1}H NMR ($CDCl_3$): δ2.65 [m, 1H,(O)N CH_2CH'], δ2.86 [m, 1H, (O)N $CHCH^2$], δ3.16 (s, 3H, N–CH_3), δ3.18 (s, 3H, N–$^2CH_3$), δ3.22 (m, 1H, CH–aryl), δ4.07 [t, J = 7.8 Hz, 2H, (O)N–CH_2], δ7.10–7.43 (m,6H, arom-H), δ7.57 (t, J = 7.8 Hz, 1H, pyr–^{5}H), δ8.56 (d, J = 7.2 Hz, 1H, pyr–^{6}H). Mass spectrum (LSIMS) *m*/*z* (relative intensity) 337/335 (MH^+, 50/52), 321/319 (21/23), 305/303 (27/25), 276/274 (49/44) (CI): *m*/*z* (relative intensity) 335/333 ($M^+ - 1$, 9.3/8.1), 319/317 (100/14.3), 307/305 (25/31), 292/290 (25/27), 276/274 (72/75), 241 (31), 227 (38). High-resolution mass spectrometry or CHN analysis was not possible because the *N*-oxide was too labile. UV (MeOH): 226 nm, 262 nm (dextro-brompheniramine) and 218 nm, 262 nm (levo-brompheniramine).

Liver Preparations

Untreated hog liver microsomes were a generous gift of Professor D. M. Ziegler, University of Texas, Austin. Highly purified flavin-containing monooxygenase (FMO) was isolated and purified from hog liver microsomes by a modified procedure previously described (17). To minimize inactivation of the FMO, all steps were carried out as quickly as possible at 4°C. The concentration of microsomal and highly purified FMO protein was determined by the Bio-Rad (Richmond, CA) and the Pierce BCA (Rockford, IL) methods, respectively, using serum albumin as a standard.

Heat inactivation of hog liver microsomes was carried out as described previously (13).

Metabolic Incubation Systems

A typical incubation mixture contained 50 m*M* potassium phosphate, pH 8.4, 0.5 m*M* $NADP^+$, 2.0 m*M* glucose-6-phosphate, 1 IU of glucose-6-phosphate dehydrogenase (EC 1.1.1.49 from baker's yeast), and 600 μg of hog liver microsomes or 8–20 μg of highly purified hog liver FMO. The reaction was initiated by the addition of substrate and incubation was continued with constant shaking to maintain adequate oxygen. At timed intervals the reaction was stopped by the addition of 2 vol of cold dichloromethane, mixed thoroughly, and centrifuged to separate the aqueous and organic fractions. After filtration of the dichloromethane fraction through a 4-μm Nylon filter and evaporation, the extract was taken up in methanol for separation and quantification by HPLC as described previously (18,19).

Chromatography was done on an IBM Model 9533 system with UV detection and a Hewlett Packard Model 3396 integrator. A 5-μm, 25 cm × 4.5-mm silica column (AXXIOM chromatography, Richard Scientific, Novato, CA) was used for all separations. The HPLC mobile phase consisted of 60% perchloric acid–methanol (0.2–0.8%/1000, v:v) and was pumped through the column at a flow rate of 1.0 mL/min.

Liver microsomes from untreated hogs or highly purified hog liver FMO had characteristically high *p*-methoxyphenyl-1,3-dithiolane *S*-oxygenase activity (3.9 nmol/min/mg of microsomal protein at pH 7.4 or 548 nmol/min/mg of purified protein at pH 9.0, respectively), which was assayed as described before (20). Testosterone hydroxylase activity was also evaluated as a marker for cytochrome P-450 activity as described previously (21). Hog liver microsomes possessed testosterone hydroxylase activity similar to that of untreated rat liver microsomes (i.e., 15β-, 6β-, 16β-, and 2β-testosterone hydroxylase activities of 0.09, 1.6, 0.24, and 0.45 nmol/min/mg of protein, respectively, at pH 7.4).

RESULTS

(*L*)- and (*D*)-Brompheniramine Metabolism in Hog Liver Microsomes

Preliminary studies showed that hog liver microsomes supplemented with NADPH catalyzed the oxygenation of brompheniramine to brompheniramine *N*-oxide. Although desmethylbrompheniramine could be detected, its rate of formation at pH 8.4 was approximately 10-fold less than the rate of brompheniramine *N*-oxide formation. The formation of (*L*)- and (*D*)-brompheniramine *N*-oxide was a linear function of protein concentration (0.3–1.0 mg of microsomal protein) and incubation time for at least 10 min.

The molecular basis for formation of brompheniramine *N*-oxide was further investigated by examining the effects of various treatments on the N-oxygenation of racemic brompheniramine. Formation of brompheniramine *N*-oxide was dependent on NADPH. *n*-Octylamine-stimulated hog liver microsomes catalyzed the formation of brompheniramine *N*-oxide approximately 1.4-fold (i.e., 17.5 ± 2.1 vs. 12.0 ± 1.6 nmol/min/mg of microsomal protein). Formation of brompheniramine *N*-oxide was strongly dependent on NADPH and temperature. In the presence of microsomes pretreated with heat to almost completely inactivate FMO but leave cytochrome P-450 activity approximately 80% intact (13), no detectable amount of brompheniramine *N*-oxide

was observed. Thiourea (0.5 m*M*), a well-documented specific alternate substrate competitive inhibitor of FMO (16), completely abolished brompheniramine *N*-oxide formation. The major product detected in dichloromethane extracts of microsome reactions eluted with a retention volume identical to that of authentic brompheniramine *N*-oxide upon separation by HPLC. The electron impact mass spectrum of this metabolite gave prominent ions at *m/z* (relative abundance) 247/249 (21/6), 89(81), and 72(62) which apparently arose from fragmentation alpha to the benzylic carbon in the mass spectrometer. A minor metabolite from dichloromethane extracts of reactions catalyzed by microsomes eluted with a retention volume identical to that of authentic desmethylbrompheniramine, **6**. The gas chromatography mass spectrum of this metabolite gave prominent ions of *m/z* (relative abundance) 247/249 (100/97), 167 (25), 72 (24), and 58 (73), which was virtually identical to the gas chromatography/electron-impact mass spectrum of the authentic material. Under the conditions of the analysis by electron-impact mass spectrometry, neither metabolite gave prominent molecular ions because efficient alpha cleavage dominated the mass spectrum.

Enantioselective Brompheniramine N-Oxygenation

Kinetic constants for the N-oxygenation of (*L*)- (*D*)-, and racemic brompheniramine catalyzed by hog liver microsomes and highly purified hog liver FMO were calculated from the rate of *N*-oxide formation at variable substrate concentrations by the HPLC procedure described under Methods. The K_m and V_{max} values were obtained from double-reciprocal plots of velocity versus substrate using three different preparations of microsomes and highly purified FMO from hog liver. As shown by the kinetic constants listed in Table I; brompheniramine was enantioselectively N-oxygenated by both microsomes and highly purified FMO from hog liver. Thus, in the presence of hog liver microsomes, the K_m value for (*D*)-brompheniramine was markedly lower than the K_m value for (*L*)-brompheniramine. As shown by the kinetic constants listed in Table I, brompheniramine was also a good substrate for the highly purified hog liver FMO. The concentration of brompheniramine required for half-maximal activity was similar to that required for hog liver microsomes and was comparable with that of other similar aliphatic tertiary amine substrates for this enzyme (8,15,16,22–25). Brompheniramine was also N-demethylated in the presence of hog liver microsomes. As shown in Table I, some enantioselectivity was observed but the rate of *N*-desmethyl brompheniramine formation at a concentration required for half-maximal activity was considerably less than that observed for N-oxygenation.

Regioselective N-Oxygenation of Zimeldine by the Highly Purified Flavin-Containing Monooxygenase

To investigate the N-oxygenation of a compound closely related to brompheniramine, the regioselective N-oxygenation of (*E*)- and (*Z*)-zimeldine was studied *in vitro*. Because the structures of brompheniramine and zimeldine are similar, we anticipated that zimeldine would be a good substrate for highly purified hog liver FMO (13). The formation of (*E*)- and (*Z*)-zimeldine *N*-oxide was a linear function of protein concentration (4–20 μg of highly purified FMO) and of incubation time (1–4 min). In agreement with previous studies employing hog liver microsomes (13), the concentration of (*Z*)-zimeldine required for half-maximal activity was lower than that of (*E*)-zimeldine. The K_m and V_{max} values obtained from double-reciprocal plots of velocity versus substrate for (*Z*)- and (*E*)-zimeldine were 29 μ*M* and 758 nmol/min/mg of protein and 80 μ*M* and 701 nmol/min/mg of protein, respectively (Table I).

Molecular Modeling Studies

Molecular modeling studies were carried out on selected aliphatic tertiary amines to determine if N-oxygenation

Table I. Kinetic Constants for Oxidation of Brompheniramine by Microsomes and Highly Purified Flavin-Containing Monooxygenase from Hog Liver

		N-Oxygenation[a]		N-Demethylation	
Condition	Substrate	K_m (μ*M*)	V_{max} (nmol/min/mg protein)	K_m (μ*M*)	V_{max} (nmol/min/mg protein)
Hog liver microsomes	(*D*)-Brompheniramine	87 ± 3[b]	3.8 ± 0.5	200 ± 10	0.9 ± 0.3
Hog liver microsomes	(*L*)-Brompheniramine	202 ± 31*	5.5 ± 0.3	800 ± 53	0.6 ± 0.2
Hog liver microsomes	Brompheniramine	159 ± 1	5.2 ± 0.4	334 ± 17	0.8 ± 0.4
FMO[c]	(*D*)-Brompheniramine	112 ± 6	634 ± 21		
FMO	(*L*)-Brompheniramine	366 ± 19*	670 ± 17		
FMO	Brompheniramine	212 ± 4	608 ± 28		
FMO	(*Z*)-Zimeldine	29 ± 2	758 ± 31		
FMO	(*E*)-Zimeldine	80 ± 5	701 ± 19		

[a] Kinetic constants were calculated from double-reciprocal plots of velocity versus variable substrate concentration. The values are the mean of two or three determinations using microsomes and FMO from two or three different preparations. Metabolites were quantitated by the HPLC procedure described under Methods using six substrate concentrations from 10 to 1000 μ*M*.

[b] Data are expressed as the mean ± SE. Statistical analysis was performed by the Student *t* test for evaluation of the difference between two means.

[c] Highly purified flavin-containing monooxygenase from hog liver.

* Statistically significant difference from (*D*)-brompheniramine, $P < 0.1$ (38).

could be correlated with conformational preference and to try and rationalize the modest difference in FMO substrate binding of the various tertiary amine substrates examined. The objective of these studies was to derive a qualitative structural model of the binding region of the enzyme from a knowledge of the conformational space of the various substrates. Zimeldine and brompheniramine are very similar in structure and we therefore assumed that there was some optimal arrangement of the two aromatic rings, the acyclic side chain and the aliphatic tertiary amine nitrogen atom proximal to or within the active site. We assumed that these structural elements were adopted by both *Z*-zimeldine and *D*-brompheniramine in a low-energy conformation that *E*-zimeldine and *L*-brompheniramine could not adopt. It is known that the binding channel leading to the active site of FMO is relatively rigid, and we assumed not only that one isomer was preferred, but that the preferred conformer was in a low-energy conformation.

Results of Conformational Analyses

The accessible conformational space of *Z*-zimeldine was investigated using the systematic search algorithm implemented in the SYBYL software system (26) (version 5.4, Tripos Associates Inc., 1699 Hanley Road, Suite 303, St. Louis, MO 63144) (27). A systematic conformational search was employed to elucidate the differences between the four molecules. The four rotatable bonds ($\tau 1$–$\tau 4$; Fig. 3) in *Z*- and *E*-zimeldine and the five rotatable bonds ($\tau 1$–$\tau 5$; Fig. 3) in *D*- and *L*-brompheniramine were each varied between 0 and 360° in increments of 15° using the SYBYL systematic search algorithm. Conformations with high-energy nonbonded interactions were rejected. The energy of each conformation was computed using the COSMIC force field (28). The distances between the aliphatic tertiary amine nitrogen atom and the centers of the two rings were monitored during the search. These sets of distances were then compared, after eliminating conformations which were more than 10 kcal/mol higher in energy than the lowest-energy structure for each molecule. No conformation of *E*-zimeldine was able to position the aliphatic tertiary amine nitrogen so that the distance from it to the centers of the aromatic rings was equivalent to the equivalent distances in any of the conformations of *Z*-zimeldine. However, for both enantiomers of brompheniramine there was a large degree of overlap with the distance map of *Z*-zimeldine. The data were thus analyzed further to provide an explanation for the different binding of *D*- and *L*-brompheniramine, which cannot be distinguished using only two distances (i.e., a minimum of four distances are required to differentiate between enantiomers). Because *D*-brompheniramine has a lower K_m than the isomer, we sought to identify those structures common to both *Z*-zimeldine and the D isomer of brompheniramine (but not common to the L isomer), in order to define possible binding site models which would qualitatively explain the data. The assumption underlying such an analysis was that the active site of FMO was capable of N-oxygenating both *Z*-zimeldine and *D*-brompheniramine preferentially, and the preferred isomer could adopt a low-energy conformation which was complementary but that L-brompheniramine could not adopt the same conformational geometry as readily.

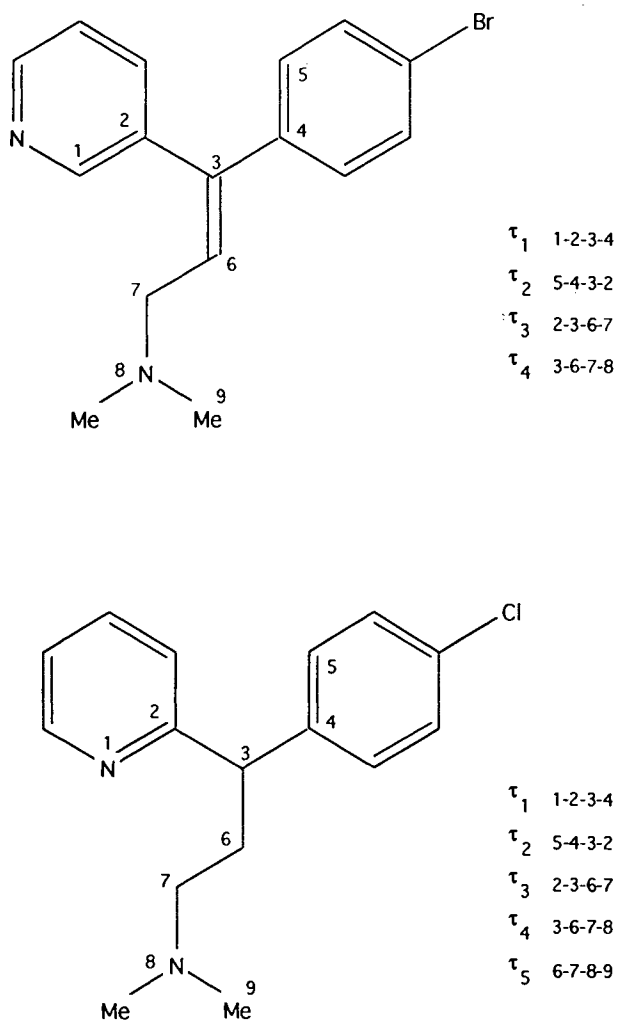

Fig. 3. Schematic illustration of *Z*-zimeldine and brompheniramine indicating the torsion angles (i.e., τ) permitted to vary in the studies described in the text.

Three criteria were used to find matching pairs of conformations of *Z*-zimeldine and *D*-brompheniramine. First, the aromatic ring systems had to be in the same relative orientation (i.e., two matching conformations had to have the same values of $\tau 1$ and $\tau 2$). Second, when the ring systems were overlaid the two aliphatic tertiary amine nitrogens were required to be within 0.5 Å of each other. Finally, the two methyl groups had to occupy approximately the same region of space. The latter conditions were monitored by calculating the vector from the average tertiary amine methyl carbon coordinates to the aliphatic tertiary amine. The angle between the two vectors in the two molecules was then required to be less than 45° (Fig. 4). Having identified a matching pair of *Z*-zimeldine and *D*-brompheniramine conformations, the entire set of *L*-brompheniramine structures was examined to ensure that no conformation of *L*-brompheniramine could also satisfy the same three criteria. If no conformation of *L*-brompheniramine could indeed be found, the combined *Z*-zimeldine/*D*-brompheniramine structures were stored. By this means we determined conformations of *Z*-zimeldine and *D*-brompheniramine in which the two aro-

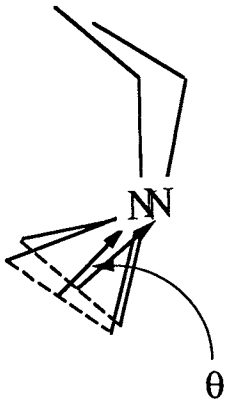

Fig. 4. Illustration of the approach used to determine whether the methyl substituents on the aliphatic amine nitrogen atoms of the two molecules occupy approximately the same region of space. For each molecule, the vector from the average positions of the two carbon atoms to the nitrogen was calculated. The angle between the vectors for the two molecules was then calculated. If the angle was ≤45°, the conformations were accepted.

matic rings adopted an equivalent conformation and the aliphatic tertiary amine nitrogen atom was in the same position relative to the rings (Fig. 5).

A total of 244 pairs of conformations of *Z*-zimeldine and *D*-brompheniramine was obtained in this way. In all of these structures the aliphatic tertiary amine group was positioned on one side of the plane defined by the two centers of the two aromatic rings and the asymmetric carbon atom (Fig. 5). Two representative pairs of structures are shown in Fig. 6. *L*-Brompheniramine was unable to adopt a conformation satisfying these requirements. The family of 244 structures thus defined a set of conformations, one of which we anticipated to be complementary in shape to the binding site of the enzyme. The values adopted by the torsion angles $\tau 1$ and $\tau 2$ in these conformations fell into approximately four groups, corresponding to ($\tau 1$, $\tau 2$) values centered around (10°, 20°), (150°, 160°), (−160°, 30°), and (−140°, 170°). In a previous study, Höberg *et al.* found similar conformational minima for the two aromatic ring portions of *Z*-zimeldine using a different force field (29). We also repeated the calculations of *Z*-zimeldine and *D*-brompheniramine with the pyridine nitrogen atom protonated; essentially the same results were obtained as for the unprotonated forms. We conclude that the protonation state of the pyridine ring does not affect the model we present here.

It was instructive to compare the results of these investigations with the kinetic data for the selectivity of N-oxygenation of 10-(*N*,*N*-dimethylaminoalkyl)-2-(trifluoromethyl)phenothiazines against pulmonary and hepatic FMOs as determined by Nagata *et al.* (16). In the study reported by Nagata *et al.*, it was shown that the alkyl side-chain length was a significant factor in substrate N-oxygenation activity, with a 2-carbon aliphatic side chain being less active against hog liver FMO than analogues with longer aliphatic chains. A much more significant effect was noted for rabbit lung FMO. Aliphatic side chains of methylene carbon length less than six showed little measurable *N*-oxyge-

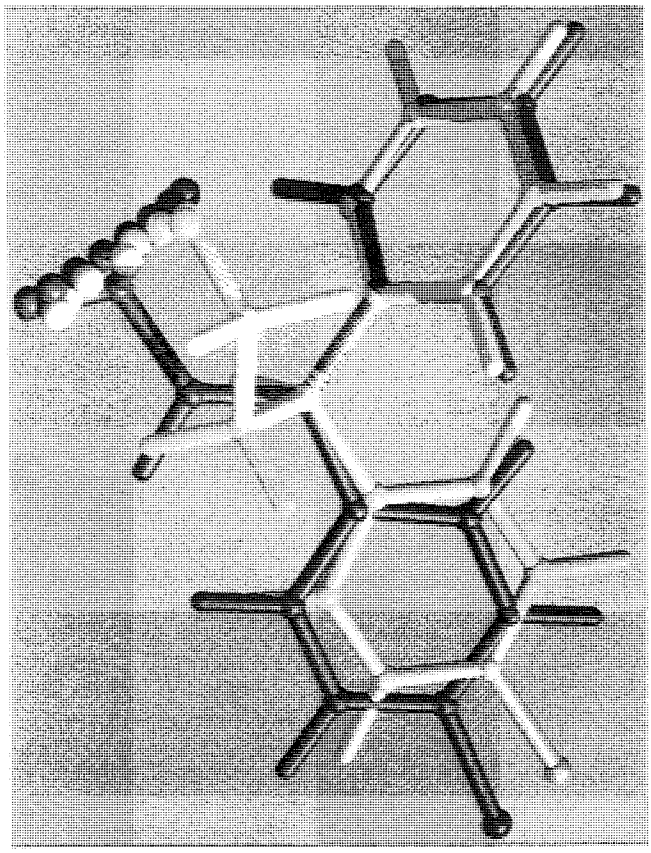

Fig. 5. Locus of points occupied by the amine nitrogen atom (balls) in the complementary pairs of conformations of *Z*-zimeldine (gray) and *D*-brompheniramine (white) which are inaccessible to *L*-brompheniramine.

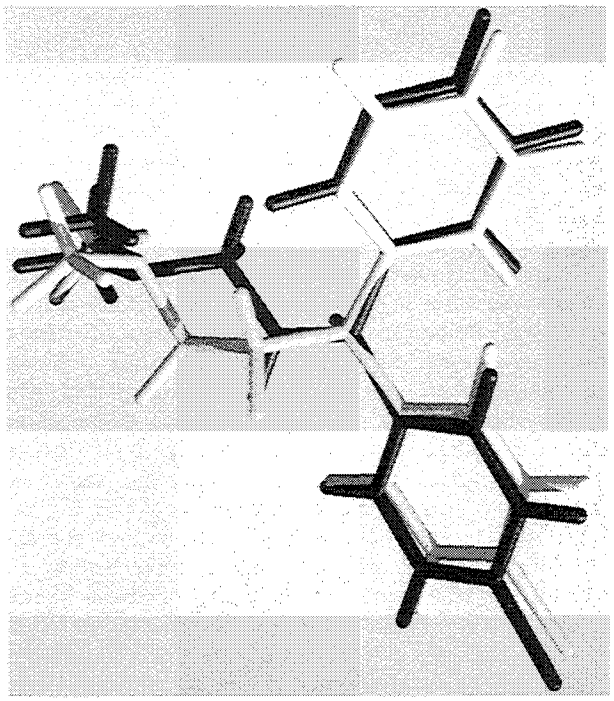

Fig. 6. Two representative conformations of *Z*-zimeldine (gray) and *D*-brompheniramine (white) which contribute to the locus of points in Fig. 5.

nase substrate activity. As a result of these observations it was proposed that the substrate channel for the pulmonary enzyme was significantly less open than the substrate channel for the hepatic enzyme. The 10-(*N,N*-dimethylaminoethyl)-2-(trifluoromethyl)phenothiazine (i.e., 2-carbon phenothiazine analogue) has an *N*-oxygenase activity against hog liver FMO intermediate between that of *Z*-zimeldine and that of *D*-brompheniramine. However, the puckered conformation of the phenothiazine ring system is not among those found to be stable for the diaryl ring system of zimeldine and brompheniramine. High-energy steric interactions between the two aromatic rings could be present. Consequently, substituted phenothiazines cannot be directly compared to the conformations of *Z*-zimeldine and *D*-brompheniramine. Nevertheless, a systematic search over the three rotatable bonds in 10-(*N,N*-dimethylaminoethyl)-2-(trifluoromethyl)-phenothiazine did generate a large number of conformations in which the distances from the amine nitrogen to the centers of aromatic rings were the same as in the *Z*-zimeldine/*D*-brompheniramine structures, and the methyl groups occupied approximately the same region of space. An example is shown in Fig. 7. For the model to be applicable to 10-(*N,N*-dimethylaminoalkyl)-2-(trifluoromethyl)phenothiazines, it is clear that the region of the substrate binding channel where the aromatic ring systems are positioned must be large enough to be able to accommodate both types of ring systems. This is in agreement with earlier work which reported that "the channel leading to the oxidant in [hog liver] FMO appears more open and readily admits compounds bearing a tricyclic ring" (16).

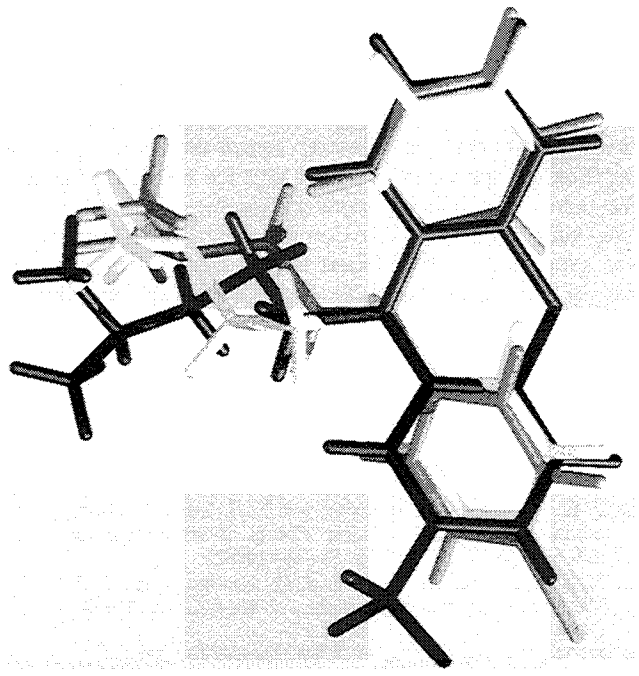

Fig. 7. Superimposition of conformations of *Z*-zimeldine (gray), *D*-brompheniramine (white), and 10-(*N,N*-dimethylaminoethyl)-2-(trifluoromethyl)phenothiazine (black), in which the centers of the aromatic rings and the aliphatic amine nitrogen atoms are overlaid and the methyl substituents on the amine nitrogen occupy the same region of space.

DISCUSSION

Metabolic Considerations

The kinetic constants listed in Table I for brompheniramine and presented above for zimeldine suggested that brompheniramine and zimeldine were good substrates for the highly purified hog liver FMO. The concentration of brompheniramine and zimeldine required to half-saturate the enzyme was in the range for other similar tertiary amine substrates for hog liver FMO, and turnover at infinite substrate concentration was large and equal to that of other similar aliphatic tertiary amine substrates for this enzyme (8). In addition to formation of brompheniramine *N*-oxide, a minor amount of desmethyl brompheniramine was formed, albeit in 10-fold lesser amounts in the presence of microsomes from hog liver. The administration of brompheniramine (4) and zimeldine (12) to animals and humans showed that considerable quantities of metabolites arising from N-demethylation and deamination reactions occurred *in vivo*. The large difference in the *in vitro* and *in vivo* ratio of metabolites arising from N-demethylation of brompheniramine or zimeldine to brompheniramine or zimeldine *N*-oxide, respectively, that are reported here and elsewhere (4,12), cannot be due solely to changes in the concentration of monooxygenases in the intact liver. In fact, the level and type of cytochrome P-450 isozymes present in hog liver microsomes are similar to those of untreated rat liver microsomes (e.g., see Methods). It is possible that, *in vivo*, brompheniramine and zimeldine *N*-oxide are reduced to parent tertiary amine and the parent compounds are ultimately biotransformed to demethylated metabolic products. It is also possible that difficulties associated with the isolation and characterization of brompheniramine *N*-oxide metabolites also contributed to the low ratio of *N*-oxide to N-demethylated metabolites observed *in vivo*. Most likely, however, is the possibility that the type and amount of FMO in adult human liver (mainly form II) do not efficiently catalyze N-oxygenation of brompheniramine (9).

The *N*-oxide metabolites of brompheniramine produced by microsomal enzymes may represent routes of drug deactivation presumably leading to detoxication and excretion. Tertiary amine *N*-oxides tend to be excreted from the body and generally represent a detoxication process for tertiary amine drugs (15). *n*-Octylamine-stimulated hog liver microsomes catalyzed the formation of *N*-oxide about 1.4-fold, and in the presence of this primary amine, the *N*-oxide was the only metabolite detected. This result suggests that *N*-oxygenation of brompheniramine was catalyzed largely by the FMO since *n*-octylamine is a good inhibitor of cytochromes P-450 (30) and a known positive effector for FMO (8). Thiourea, a well-documented specific alternate substrate competitive inhibitor for FMO (14), completely inhibited brompheniramine N-oxygenation. Heat inactivation of the microsomal suspension, under conditions that destroyed FMO (8,13) but did not inactivate cytochrome P-450, completely abolished brompheniramine *N*-oxide formation. Taken together, these results suggest a major role of FMO for brompheniramine N-oxygenation in hog liver microsomes.

Histamine H_1 antagonists such as brompheniramine and chlorpheniramine are known to bind enantioselectively to

histamine H_1 receptors (31) and to antagonize enantioselectively the pharmacological effects of histamine (32). Thus, dextrorotatory pheniramines appear to be more effective antihistamines than their levorotatory enantiomers. Because (*D*)-brompheniramine has a higher affinity for the hog liver microsomal FMO, it is possible that in man, (*D*)-brompheniramine is extensively N-oxygenated. However, this suggestion must be verified experimentally because it is possible that adult human liver FMO and hog liver FMO possess distinct stereoselectivities. The extent of stereoselectivity observed for brompheniramine N-oxygenation catalyzed by FMO was similar to that reported for other similar aliphatic tertiary amines (23,25,33). Presently, it is not clear what role human liver FMO plays in tertiary amine drug metabolism, but presumably, enantioselective N-oxygenation may contribute to the pharmacodynamics and pharmacological properties of brompheniramine *in vivo* (34). If the adult human liver FMO behaves in a fashion similar to that observed for the hog liver FMO, it is anticipated that N-oxygenation of the *D* enantiomer of racemic brompheniramine would be expected to predominate at low drug concentrations.

In agreement with previous studies concerning heteroatom oxygenations (20,35,36) microsomes and highly purified FMO from hog liver stereoselectively N-oxygenated brompheniramine. Structure–N-oxygenation activity relations established previously for related phenothiazines (16) showed that the hepatic FMO substrate binding site "appears more open and readily admits compounds bearing a tricyclic ring," in contrast to pulmonary FMO, which is viewed as possessing a narrow channel with considerable steric constraints at the mouth of the channel. Because brompheniramine bears considerable structural similarity to phenothiazines, the stereoselectivity studies described above help to define the binding site more clearly. Unlike phenothiazines, which possess *N,N*-dimethylaminoalkyl groups which are geometrically constrained in space, brompheniramine enantiomers have *N,N*-dimethylaminoalkyl groups which may occupy quite different regions of the FMO enzyme active site. Zimeldine is similar in structure to brompheniramine, with the notable difference that a carbon–carbon double bond replaces the center of chirality. Although considerable stereoselectivity was observed for zimeldine N-oxygenation by the highly purified hog liver flavin-containing monooxygenase, the K_m values were markedly greater than those observed for brompheniramine. This result may be a consequence of the greater rigidity of zimeldine or it may be due to the lower basicity of the allylamine zimeldine, which could make it less readily oxidized. As shown in Fig. 2, attack of oxygen by FMO must proceed as depicted to give the *N*-oxide products determined. Although only approximate, substrate stereoselectivity studies can provide a crude picture of the enzymatic steric bulk at the binding site of FMO.

Molecular modeling studies were done to gain insight into the nature of tertiary amine substrate–FMO binding. Molecular modeling techniques can provide means of estimating geometrical restrictions of enzyme–substrate binding site regions because it is believed that the substrate binding channel is relatively rigid. Because pheniramines and closely related phenothiazines have readily determined structures, the minimal size and shape requirements for the aromatic rings and distance between the rings and the aliphatic tertiary amine can be calculated. Further, the K_m values of the two enantiomers of brompheniramine and the two isomers of zimeldine suggest that additional restrictions on the conformation of the aliphatic tertiary amine side chain with respect to the two aromatic rings are in place and contribute to the stereoselectivity of FMO. Our studies suggest that the aromatic ring systems of *Z*-zimeldine and brompheniramine are small enough to fit into the substrate binding channel and active site of hog liver FMO. However, in addition to steric constraints about the mouth of the active site, the enzyme active site requires further precise alignment of the aliphatic tertiary amine atom and enzyme peroxyflavin as revealed by stereoselectivity studies of stereochemically related substrates. Thus, related studies of chlorpheniramine N-oxygenation (37) by hog liver flavin-containing monooxygenase also illustrate the conformational space required by the substrate for optimal active site binding. Future studies will be directed toward further characterization of the active site of the flavin-containing monooxygenase.

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REFERENCES

1. M. A. Drouin. H_1 antihistamines: Perspective on the use of the conventional and new agents. *Ann. Allergy* 55:747–752 (1985).
2. F. E. R. Simons, E. M. Frith, and K. J. Simons. The pharmacokinetics and antihistaminic effects of brompheniramine. *J. Allergy Clin. Immunol.* 70:458–464 (1982).
3. R. B. Bruce, L. B. Turnbull, J. H. Newman, and J. E. Pitts. Metabolism of brompheniramine. *J. Med. Chem.* 11:1031–1034 (1968).
4. H. Maurer and K. Pfleger. Identification and differentiation of alkylamine antihistamines and their metabolites in urine by computerized gas chromatography-mass spectrometry. *J. Chromatogr.* 430:31–41 (1988).
5. S. G. Carruthers, D. W. Shoeman, C. E. Hignite, and D. L. Azarnoff. Correlation between plasma diphenylhydramine level and sedative and antihistamine effects. *Clin. Pharmacol. Ther.* 23:375–381 (1978).

6. J. D. Osterloh, A. Karakaya, and D. E. Carter. Isolation and identification of the polar metabolites of chlorpheniramine in the dog. *Drug Metab. Disp.* 8:12–15 (1980).
7. B. Lindeke and A. K. Cho. N-Dealkylation and deamination. In W. B. Jakoby, J. R. Bend, and J. Caldwell (eds.), *Metabolic Basis of Detoxication*, Academic Press, New York, 1982, Vol. 1, pp. 105–126.
8. D. M. Ziegler. Microsomal flavin-containing monooxygenase: Oxygenation of nucleophilic nitrogen and sulfur compounds. In W. Jakoby (ed.), *Metabolic Basis of Detoxication*, Academic Press, New York, 1980, Vol. 1, pp. 201–227.
9. N. Lomri, Q. Gu, and J. R. Cashman. Molecular cloning of the flavin-containing monooxygenase (form II) cDNA from adult human liver. *Proc. Natil. Acad. Sci. USA* 89:1685–1689 (1992).
10. C. Dolphin, E. A. Shephard, S. Povey, C. N. A. Palmer, D. M. Ziegler, R. Ayesh, R. L. Smith, and I. R. Phillips. Cloning, primary sequence, and chromosomal mapping of a human flavin-containing monnoxygenase (FMO1). *J. Biol Chem.* 266:12379–12385 (1991).
11. S. B. Ross and A. L. Renyi. Inhibition of the neuronal uptake of 5-hydroxytryptamine and noradrenaline by (Z) and (E)-3-(4-bromophenyl)-N,N-dimethyl-3-(3-pyridyl)allylamines and their secondary analogs. *Neuropharmacology* 16:57–63 (1977).
12. J. Lundström, T. Högberg, T. Gosztonyi, and T. de Paulis. Metabolism of zimelidine in rat, dog and man. *Arzneim.-Forsch./Drug Res.* 31:486–494 (1981).
13. J. R. Cashman, J. Proudfoot, D. W. Pate, and T. Högberg. Stereoselective N-oxygenation of zimeldine and homozimeldine by the flavin-containing monooxygenase. *Drug Metab. Dispos.* 16:616–622 (1988).
14. S. O. Ögren, S. B. Ross, H. Hall, A. C. Holm, and A. L. Renyi. The pharmacology of zimelidine: A 5-HT selective reuptake inhibitor. *Acta Psychiat. Scand. Suppl. 290* 63:127–151 (1981).
15. D. M. Ziegler. Flavin-containing monooxygenases: Catalytic mechanism and substrate specificities. *Drug Metab. Rev.* 19:1–32 (1988).
16. T. Nagata, D. E. Williams, and D. M. Ziegler. Substrate specificities of rabbit lung and porcine liver flavin-containing monooxygenases: Differences due to substrate size. *Chem. Res. Toxicol.* 3:372–376 (1990).
17. P. J. Sabourin, B. P. Smyser, and E. Hodgson. Purification of the flavin-containing monooxygenase from mouse and pig liver microsomes. *Int. J. Biochem.* 16:713–720 (1984).
18. J. R. Cashman and Z.-C. Yang. Analysis of amine metabolites by high-performance liquid chromatography on silica gel with a non-aqueous ionic eluent. *J. Chromatogr.* 532:405–410 (1990).
19. R. J. Y. Shi, W. L. Gee, R. L. Williams, and E. T. Lin. High performance liquid chromatography assay for basic amine drugs in plasma and urine using a silica gel column and an aqueous mobile phase. II. Chlorpheniramine. *J. Liquid Chromatogr.* 10:3101–3112 (1987).
20. J. R. Cashman and L. D. Olsen. Stereoselective S-oxygenation of 2-aryl-1,3-dithiolanes by the flavin-containing and cytochrome P-450 monooxygenases. *Mol. Pharmacol.* 38:573–585 (1990).
21. J. R. Cashman, L. D. Olsen, G. Young, and H. Bern. S-Oxygenation of eptam in hepatic microsomes from fresh- and salt-water striped bass (*Morone saxatilis*). *Chem. Res. Toxicol.* 2:392–399 (1989).
22. J. R. Cashman. A radiometric assay for flavin-containing monooxygenase activity. *Anal. Biochem.* 160:294–300 (1987).
23. D. M. Ziegler, D. Jollow, and D. E. Cook. Properties of a purified liver microsomal mixed function amine oxidase. In H. Kamin (eds.), *Flavins and Flavoproteins*, University Park Press, Baltimore, MD, 1971, pp. 507–522.
24. J. R. Cashman and D. M. Ziegler. Contribution of *N*-oxygenation to the metabolism of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) by various liver preparations. *Mol. Pharmacol.* 29:163–167 (1986).
25. J. R. Cashman. Enantioselective *N*-oxygenation of verapamil by the hepatic flavin-containing monooxygenase. *Mol. Pharmacol.* 36:497–503 (1989).
26. T. E. Ferrin, C. C. Huang, L. E. Jarvis, and R. Langridge. The MIDAS display system. *J. Mol. Graph.* 6:13–27 (1988).
27. M. Clark, R. D. Cramer, and N. van Opdenbosch. Validation of the general purpose Tripos 5.2 force field. *J. Comp. Chem.* 10:982–1012 (1989).
28. J. G. Vinter, A. Davis, and M. R. Saunders. Strategic approaches to drug design. I. An integrated software framework for molecular modeling. *J. Comp-Aided Mol. Design.* 1:31–51 (1987).
29. T. Högberg, S. B. Ross, P. Ström, G. L. Grunewald, M. W. Creese, and J. D. Bunce. Homoallylic amines related to zimeldine. A comparative study on neuronal serotonin and norepinephrine reuptake based on conformational analysis. *J. Med. Chem.* 31:913–919 (1988).
30. C. R. E. Jefcoate, J. L. Gaylor, and R. L. Calabrese. Ligand interactions with cytochrome P-450. I. Binding of primary amines. *Biochemistry* 8:3455–3463 (1969).
31. S. J. Peroutka and S. H. Snyder. [^{3}H]-Mianserin: Differential labeling of serotonin$_2$ and histamine$_1$ receptors in rat brain. *J. Pharmacol. Exp. Ther.* 216:142–148 (1981).
32. F. E. Roth and W. M. Govier. Comparative pharmacology of chlorpheniramine (chlortrimeton) and its optical isomers. *J. Pharmacol. Exp. Ther.* 124:347–349 (1958).
33. L. A. Damani, W. F. Pool, P. A. Crooks, R. K. Kaderlik, and D. M. Ziegler. Stereoselectivity in the *N'*-oxidation of nicotine isomers by flavin-containing monooxygenase. *Mol. Pharmacol.* 33:702–705 (1988).
34. D. D. Shen, R. Milsap, and J. W. Georgitis. Pharmacodynamics of brompheniramine and pseudoephedrine in the treatment of perennial rhinitis. *J. Allergy Clin. Immunol.* 75:186 (1985).
35. J. R. Cashman, L. D. Olsen, and L. M. Bornheim. Oxygenation of dialkyl sulfides by a modified Sharpless reagent: A model system for the flavin-containing monooxygenase. *J. Am. Chem. Soc.* 112:3191–3195 (1990).
36. J. R. Cashman and D. E. Williams. Enantioselective *S*-oxygenation of 2-aryl-1,3-dithiolanes by rabbit lung enzyme preparations. *Mol. Pharmacol.* 37:333–339 (1990).
37. J. R. Cashman, J. R. Celestial, and A. R. Leach. Enantioselective *N*-oxygenation of chlorpheniramine by the flavin-containing monooxygenase from hog liver. *Xenobiotica* 22:459–469 (1992).
38. R. R. Sokal and F. J. Rohlf. Single classification analysis of variance. In *Biometry, the Principles and Practice of Statistics in Biological Research*, Freeman, San Francisco, 1981, pp. 239–246.