1,2,3-Thiadiazole: A Novel Heterocyclic Heme Ligand for the Design of Cytochrome P450 Inhibitors†

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Received September 16, 1996; Revised Manuscript Received April 8, 1997⊗

ABSTRACT: The 1,2,3-thiadiazole heterocycle has been explored as a heme ligand and mechanism-based inactivator for the design of cytochrome P450 inhibitors. One 4,5-fused bicyclic and three 4,5-disubstituted monocyclic 1,2,3-thiadiazoles have been examined for their spectral interactions, inhibition, mechanism-based inactivation, and oxidation products by the versatile microsomal P450s 2B4, 2E1, and 1A2. The compounds generally show heteroatom coordination to the heme iron; however, the binding mode is influenced by the architecture of the active site. For example, 4,5-diphenyl-1,2,3-thiadiazole shows type I and type II difference spectra with P450s 2B4 and 2E1, respectively, and no spectral perturbation with P450 1A2. Except for the fused bicyclic compound, the spectral dissociation constants are in the 2−50 μM range. The effectiveness as an inhibitor depends on the substituents at the 4- and 5- positions and on the P450 examined. Inhibition of the P450-catalyzed 1-phenylethanol oxidation to acetophenone by the thiadiazoles does not correlate with either the type of binding spectra or the spectral dissociation constants of the compounds. P450s 2E1 and 2B4 are inactivated by the 4,5-fused bicyclic 1,2,3-thiadiazole in a mechanism-based manner. Inactivation of the P450 correlates with loss in absorbance at 450 nm for the ferrous−CO complex. The monocyclic 1,2,3-thiadiazoles do not inactivate any of the P450s examined. The 1,2,3-thiadiazole ring is oxidized by the P450 system. Oxidation of the monocyclic compounds results in extrusion of the three heteroatoms and formation of the corresponding acetylenes, whereas oxidation of the fused bicyclic compound does not yield an acetylenic product.

The cytochrome P450 (P450)[1] enzymes are a superfamily of oxidative catalysts important in the biosynthesis and metabolism of various endobiotics such as steroids and steroid hormones including cholesterol, bile acids, androgens, estrogens, progestins, and corticosteroids, the neurotransmitter nitric oxide, and vitamins A and D and in the metabolism of xenobiotics including natural products, drugs, and other organic chemicals (Guengerich, 1994; Coon et al., 1992). The remarkable diversity of reactions catalyzed by these heme proteins has prompted the term "diversozymes" (Coon et al., 1996). The central role of several P450s in biochemical pathways and the roles played by their metabolic products in physiological processes has made some of these enzymes attractive targets for the development of therapeutic agents (Vanden Bossche et al., 1990, 1995). Success in the treatment of estrogen-dependent endometrial, breast, and colon cancers with inhibitors of the aromatase P450 enzyme (Kimmick & Muss, 1995; Davies et al., 1992; James et al., 1992; Perez & Borja, 1992; Evans et al., 1992; Hortobagyi, 1992) has led to an explosion in the search for novel therapeutic agents that selectively inhibit P450 enzymes involved in steroid hormone biosynthesis (Defaye et al., 1996; Lesuisse et al., 1996; Kato et al., 1995; Numazawa & Oshibe, 1995; Numazawa et al., 1995; Rowlands et al., 1995; Kudoh et al., 1995; Abul-Hajj et al., 1995a,b; O'Reilly et al., 1995; Shimizu et al., 1995; Ibrahim & Buzdar, 1995; Vanden Bossche et al., 1994c; Lang et al., 1993; Rahier & Taton, 1992).

P450 inhibitors can be broadly classified into two structural groups. One group is based on structural similarity of the inhibitor to that of the physiological substrate, such as steroid-based inhibitors of aromatase, lanosterol-14α-demethylase, cholesterol-7α-hydroxylase, and aldosterone synthase (Defaye et al., 1996; Lesuisse et al., 1996; Shimizu et al., 1995; Trzaskos et al., 1995a,b; Warren et al., 1995; Barrie et al., 1994; Dowsett, 1994; Peet et al., 1993; Rahier & Taton, 1992; Ator et al., 1992). The other group consists of compounds that can coordinate to the sixth position of the heme iron (Baroudi et al., 1996; Halpert, 1995; Ibrahim & Buzdar, 1995; Ito et al., 1994; Koymans et al., 1995; Lang et al., 1993; Vanden Bossche et al., 1990). Compounds in this class, such as ketoconazole, itraconazole, and miconazole, are used as antimycotic agents (Sawyer, 1975; Thiery et al., 1972; Brugmans et al., 1970). They are generally nonselective and inhibit various P450 enzymes; however, recent developments in specificity and affinity have been achieved by appending appropriate substituents to the heme ligands (Koymans et al., 1995; Ito et al., 1994; Swinney et al., 1994; Lang et al., 1993; Aoyama et al., 1992). The imidazole ring is the most commonly used heme ligand in this class of inhibitors. Other heterocyclic rings such as pyrazole, triazole, thiophene, pyridine, and pyrimidine have received limited attention (Mancy et al., 1995; Rowlands et

† This investigation was supported by Grant GM-46807 from the National Institutes of Health.

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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1997.

[1] Abbrevations: P450, cytochrome P450; reductase, NADPH−cytochrome P450 reductase; DLPC, dilauroylglycero-3-phosphocholine; MTTC, methyl thieno[3,2-d][1,2,3]-thiadiazole-6-carboxylate; DPT, 4,5-diphenyl-1,2,3-thiadiazole; PMT, 4-phenyl-5-methyl-1,2,3-thiadiazole; PT, 4-phenyl-1,2,3-thiadiazole; DMF, dimethylformamide.

S0006-2960(96)02318-5 CCC: $14.00

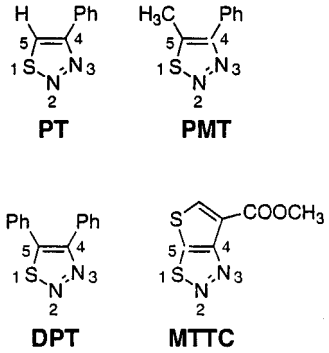

FIGURE 1: Structures of the 1,2,3-thiadiazoles.

al., 1995; Hartmann et al., 1994; Vaz et al., 1992; Dansette, 1991; Jones et al., 1990; Moawad et al., 1989). Besides high specificity and affinity with the biological target, a desirable attribute of a potential therapeutic agent is its ability to function as a mechanism-based inhibitor with a low partition number (Walsh, 1982). In this connection several mechanism-based inhibitors have been developed for specific P450 enzymes (Johnston et al., 1995, 1990; Warren et al., 1995; Brueggemeier, 1994; Numazawa et al., 1993; Peet et al., 1993; Lesuisse et al., 1992). P450 enzymes oxidize the acetylenic group to a reactive intermediate that can inactivate the P450 by alkylation of the protein or the heme prosthetic group (Ortiz de Montellano & Reich, 1986). This group has been incorporated into various structures with the intent of introducing selective mechanism-based inactivation of P450 isoforms (Johnston et al., 1995, 1990; Roberts et al., 1994; Burger et al., 1993; Hopkins et al., 1992).

Except for reports by Ortiz de Montellano and Mathews (1981) on the inactivation of hepatic microsomal P450 by 1,2,3-benzothiadiazoles, the 1,2,3-thiadiazole heterocyclic ring has received little attention as a potential heme-binding ligand for the design of P450 inhibitors. Besides the nitrogen and sulfur heteroatoms that may coordinate to heme iron, the 1,2,3-thiadiazoles are sensitive to oxidation and may be readily oxidized by the P450 system to reactive intermediates. In this study we have examined the 1,2,3-thiadiazole ring system as a heme ligand-based inhibitor of P450s with the potential for mechanism-based inactivation. P450s 2B4, 2E1, and 1A2 and the 1,2,3-thiadiazoles shown in Figure 1 have been examined for (1) spectral interactions, (2) inhibition of 1-phenylethanol oxidation, (3) mechanism-based inactivation, and (4) oxidation products. Our results indicate that the type of spectral interaction and the effectiveness as an inhibitor depends on the structure of the compound as well as the isoform of P450 examined. Oxidation of the monocyclic 1,2,3-thiadiazoles results in loss of the heteroatoms and the unmasking of an acetylenic function; however, a transient reactive intermediate capable of inactivating the cytochrome does not appear to be generated by these structures. The fused bicyclic thiadiazole inactivates the P450 consistent with a mechanism-based process and is correlated with loss of the ferrous−CO complex at 450 nm.

MATERIALS AND METHODS

Enzymes. P450 2B4 and reductase were purified to electrophoretic homogeneity from the microsomal fraction of the livers of white male New Zealand rabbits that had been pretreated with the inducing agent phenobarbital by described methods (Coon et al., 1978; French & Coon, 1979). P450s 2E1 and 1A2 were gifts of Dr. M. J. Coon, University of Michigan.

Chemicals. NADPH and DLPC were obtained from Sigma Chemical Co., St. Louis, MO. The 1,2,3-thiadiazoles 4,5-diphenyl-1,2,3-thiadiazole (DPT), 4-phenyl-5-methyl-1,2,3-thiadiazole (PMT), 4-phenyl-1,2,3-thiadiazole (PT), and methyl thieno[3,2-d][1,2,3]-thiadiazole-6-carboxylate (MTTC) were synthesized by reported methods (Britton et al., 1984; Babu et al., 1990) and were characterized by IR, NMR, and mass spectroscopy, and their purity was judged by thin-layer chromatography and NMR.

Spectral Interactions with P450 Isoforms. Difference absorbance spectra were recorded on a Cary Model 3E UV/VIS double-beam spectrophotometer. Typically 1.5 nmol of P450 (2B4, 2E1, or 1A2) in 1.0 mL of 100 mM potassium phosphate buffer, pH 7.4, was placed in the reference and sample cuvettes, and after thermal equilibration at 18 °C the baseline was zeroed between 350 and 550 nm. Successive 1.0 μL aliquots of a DMF or methanol solution of the compounds at the appropriate concentration was added to the sample cuvette to give final ligand concentrations in the range of 1−300 μM. An equal volume of DMF or methanol was added each time to the reference cuvette, and the difference spectrum was recorded. Spectral dissociation constants for type I spectra were obtained from double-reciprocal plots of the difference in absorbance between 385−393 nm (absorbance maximum) and 406−410 nm (isosbestic point), and for type II spectra, between 422−429 nm (absorbance maximum) and 408-419 nm (isosbestic point). The spectral dissociation constants were determined independently twice, and the results are presented as the mean.

Inhibition of 1-Phenylethanol Oxidation. The P450-catalyzed oxidation of 1-phenylethanol to acetophenone was used to examine the ability of thiadiazoles to function as inhibitors. DPT, PMT, PT, and MTTC were examined at 100, 250, and 500 μM with P450s 2B4, 2E1, and 1A2. Typically, a reaction mixture contained 0.1 nmol of P450 (2B4, 2E1, or 1A2), 0.2 nmol of reductase, 30 μg of DLPC, 50 μmol of potassium phosphate buffer, pH 7.4, 1.0 μmol of NADPH, the appropriate volume of a 50 mM stock solution of the inhibitor in methanol to give final concentrations of 100, 250, and 500 μM, and 1-phenylethanol (2.0 μmol for P450s 2B4 and 1A2; 0.5 μmol for P450 2E1) in a final volume of 1.0 mL. NADPH was added to initiate the reactions. Following incubation of the reaction mixtures at 37 °C for 30 min, 0.2 mL of 1 N HCl was added to quench the reaction. A 50 μL aliquot was analyzed for acetophenone by HPLC as previously described (Vaz & Coon, 1994). Each reaction was done in triplicate, and each experiment was repeated at least twice.

Mechanism-Based Inactivation of P450s 2B4 and 2E1 by Thiadiazoles. The inactivation experiments were setup as follows. P450s 2B4 and 2E1 were reconstituted with the reductase in a molar ratio of 1:2. Typically, a reaction mixture (1.0 mL) contained 1.0 nmol of P450 2B4 or 2E1, 2.0 nmol of reductase, 30 μg of freshly sonicated DLPC, 50 μmol of potassium phosphate buffer, pH 7.4, and the desired concentration of the inhibitor. The reaction mixture was incubated at 37 °C and the reaction was initiated by the addition of 2.0 μmol of NADPH. At the indicated time intervals an aliquot (50 μL, 0.05 nmol of P450) was removed and added to 200 μL of a solution containing 1.0 μmol of

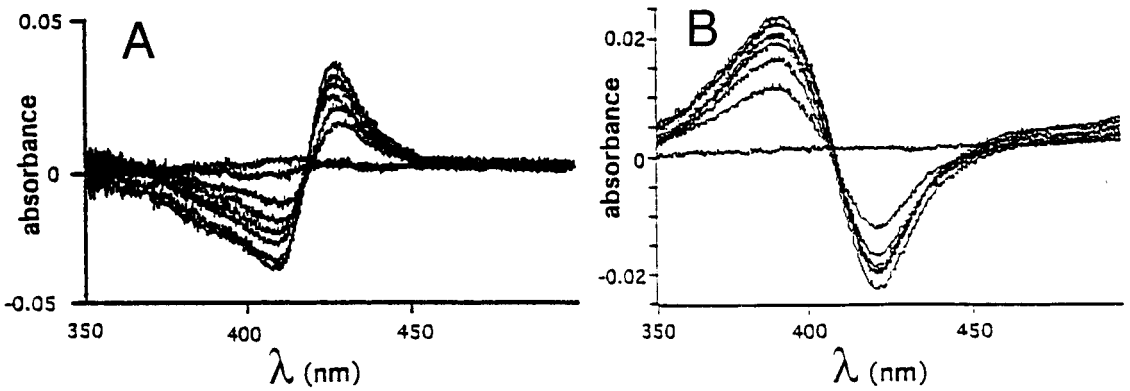

FIGURE 2: Typical difference binding spectra obtained with P450s and 1,2,3-thiadiazole compounds. Panel A, type II difference spectra of PT with P450 2B4; panel B, type I difference spectrum of PT with P450 1A2.

Table 1: Summary of Binding Spectra of the 1,2,3-Thiadiazoles with P450s 2B4, 2E1, and 1A2[a]

	2B4			1A2			2E1		
compound	spectrum type	λ_{max} (nm)	K_s (μM)	spectrum type	λ_{max} (nm)	K_s (μM)	spectrum type	λ_{max} (nm)	K_s (μM)
MTTC	II	429	3100	II	418	1400	II	420	1100
DPT	I	390	20	ND[b]			II	422	2
PMT	II	429	13	II[c]	424		II	423	3
PT	II	428	24	I	391	50	II	422	27

[a] The spectral dissociation constants are the mean of two independent determinations with a SD less than 5% from the mean. [b] Not detectable. [c] The difference spectrum was characteristically type II; however the signal intensity at 424 nm was extremely weak and the dissociation constant could not be determined.

1-phenylethanol, 10 μmol of potassium phosphate buffer, pH 7.4, and 0.5 μmol of NADPH. The second reaction mixture was incubated for 20 min at 37 °C, and then 200 μL of 1 N HCl was added to quench the reaction. An aliquot (50 μL) was analyzed for acetophenone by reversed-phase HPLC as previously described (Vaz & Coon, 1994). Each reaction was done in duplicate, and the experiment was repeated three times. To determine the residual spectrally detectable P450 as the reduced CO complex, the reaction mixtures were comparable to that described above except that 3.0 nmol of P450 2B4 was used in a reaction volume of 5.0 mL. At the indicated time intervals a 1.0 mL aliquot was removed and the P450 concentration was determined by the reduced CO difference spectrum (Omura & Sato, 1964) measured on a Hewlett-Packard 4752 diode array spectrophotometer. The experiments were done in duplicate and repeated three times.

Identification of Major Metabolites of the Thiadiazoles. Typically, a reaction mixture (0.5 mL) containing 0.5 nmol of P450, 0.75 nmol of reductase, 75 μg of DLPC, 25 μmol of potassium phosphate buffer, pH 7.4, 1.0 μmol of NADPH, and 0.5 μmol of 1,2,3-thiadiazole substrate was incubated at 37 °C for 30 min and then extracted twice with 1.0 mL aliquots of methylene chloride. The extract was concentrated to approximately 10 μL under reduced pressure in a vacuum centrifuge evaporator. A 2−5 μL aliquot was analyzed by GC/MS on a 30-m DB-5 fused silica capillary column fitted to a Finnigan 4021 GC-MSDS mass spectrophotometer under electron impact conditions at 70 eV. The injector temperature was 200 °C. The initial column temperature was maintained at 50 °C for 2 min and then ramped to 275 °C at 10 °C/min. The carrier gas was helium at a head pressure of 10 psi. Mass spectra were obtained by summing the scans within the peak and subtracting an equal number of scans just before the peak. The mass spectra were compared to authentic standards run under identical GC/MS conditions.

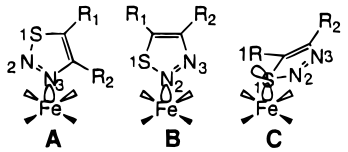

FIGURE 3: Hypothetical binding orientations for 1,2,3-thiadiazoles showing ligation to heme iron by the N_3, N_2, and S_1 heteroatoms. The heterocycle plane is shown perpendicular to the heme plane for coordination by the N_3 and N_2 heteroatoms (A and B). The steric effect of a substituent (R_1) with respect to the heme plane for coordination by N_3 is shown in A. The plane of the thiadiazole ring is shown at a 60° angle to the heme plane for binding by S_1 (C).

RESULTS AND DISCUSSION

Spectral Interactions. The binding of thiadiazoles to the heme proteins depends on the isoform of P450 examined as well as the structure of the thiadiazole. Panels A and B of Figure 2 show typical type II and type I difference spectra that were obtained with PT and P450s 2B4 and 1A2, respectively. A summary of the spectral interactions of the four thiadiazole compounds examined in this study is presented in Table 1. Generally, the compounds showed a type II spectrum, indicating coordination of a heteroatom to the heme iron. However, DPT showed type I, type II, and no spectral interactions with P450s 2B4, 2E1, and 1A2, respectively. Thus, the protein architecture at the active site influences the mode of binding of the thiadiazole.

The 1,2,3-thiadiazole ring system has three heteroatoms as potential electron-pair donors for coordination to the heme iron of P450 cytochromes. These heteroatoms can result in distinct binding orientations as shown in Figure 3. The nonbonded electron pair on either nitrogen is in the plane of the thiadiazole ring. Consequently, heme−iron complexes with either nitrogen have the thiadiazole and heme ring planes perpendicular to each other. This is similar to imidazole complexes with $P450_{cam}$ (Poulos & Howard, 1987).

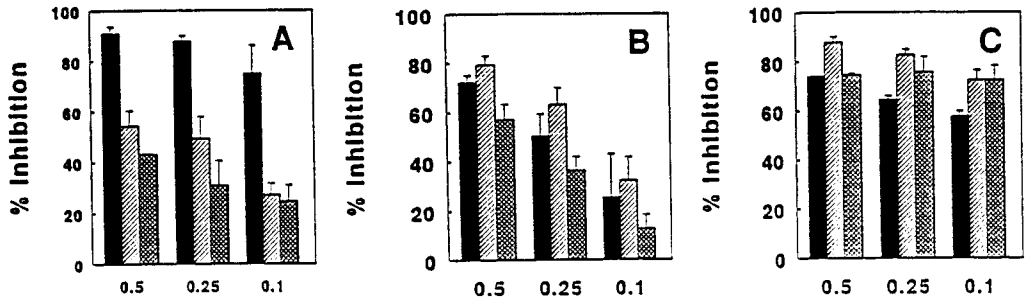

FIGURE 4: Bar graphs showing the extent of inhibition observed for the oxidation of 1-phenylethanol to acetophenone by P450 2B4 (panel A); P450 2E1 (panel B); and P450 1A2 (panel C) due to the inhibitors DPT (solid bars), PMT (hatched bars), and PT (crosshatched bars), at inhibitor concentrations of 0.5, 0.25, and 0.1 mM and a substrate concentration equal to the K_m for the respective isoform.

Figure 3A shows that N_3 as an electron-pair donor to heme iron is influenced significantly by steric considerations at the 4-position. Bulky groups would push against the heme plane, resulting in a decreased ability of N_3 to coordinate to heme iron. This is similar to the steric effect of the phenyl group for coordination of 2-phenylimidazole to heme (Poulos & Howard, 1987). The R_1 and R_2 substituents at the 4- and/or 5-positions do not have steric interactions with the heme when N_2 is coordinated to the heme iron (Figure 3B). In contrast to the nitrogen heteroatoms, the lone pairs of electrons on sulfur are oriented at a 60°/120° angle to the thiadiazole ring plane. This implies that binding to heme iron through the sulfur atom causes the thiadiazole and heme planes to be at a 60° angle (Figure 3C). The angular orientation causes substituents at the 5-position (next to sulfur) to have significantly less steric interaction with the heme plane when compared to substituents at the 4-position when N_3 is coordinated to the heme iron. These three distinct binding orientations may provide a significant advantage in designing inhibitors for specific P450 isoforms. Substituents at the 4- and 5-positions can have positive (hydrophobic, ionic, or hydrogen bonding) or negative (steric) effects with respect to the protein architecture at the active site. The phenyl substituent at the 4-position in DPT, PMT, and PT is expected to prevent heme coordination by the N_3 nitrogen. These compounds generally show type II spectral interactions with the P450s examined (Table 1). This indicates that coordination to the heme iron is either by N_2 or S_1. The spectra (400−600 nm) of sulfur ligands to the P450 heme iron are similar to those of nitrogen ligands (White & Coon, 1982). Thus, the spectral results obtained in this study do not permit a distinction between coordination by the N_2 nitrogen or sulfur for these compounds.

Inhibition of 1-Phenylethanol Oxidation. Figure 4 shows the extent of inhibition by DPT, PMT, and PT of the P450s 2B4-, 2E1-, and 1A2-catalyzed oxidation of 1-phenylethanol to acetophenone. The concentration of 1-phenylethanol at which the inhibition was examined is at the K_m for this substrate with isoforms 2B4 and 2E1 (Vaz & Coon, 1994) and 1A2 (data not shown). Accordingly, the extent of inhibition observed with the various thiadiazoles reflects their potency as inhibitors of the isoforms examined. For example, P450 2E1 is inhibited approximately 50% by DPT at 250 μM, whereas both P450 2B4 and 1A2 are inhibited to 60% or higher at 100 μM. Thus indicating that DPT is a more potent inhibitor of P450s 2B4 and 1A2 than of 2E1. PMT is slightly more inhibitory to P450s 2E1 and 1A2 than DPT. PT, which is a weaker inhibitor than DPT and PMT with P450s 2E1 and 2B4, is comparable to PMT and more effective than DPT with P450 1A2. Particularly noteworthy

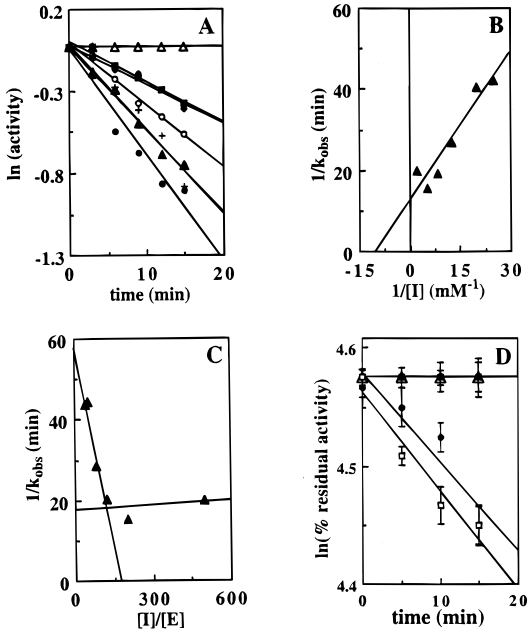

FIGURE 5: Mechanism-based inactivation of P450 2E1 by MTTC. Panel A: Semilog plots of the time course for inactivation of P450 2E1. Reactions were carried out as described in Table 2 except that the concentrations of MTTC were 0 (△), 0.04 (◆), 0.05 (■), 0.08 (○), 0.12 (+), 0.2 (▲), and 0.5 (●) mM. Each data point represents the mean of two reactions. The standard deviation from the mean was generally between 5% and 10%. The experiment was repeated three times. Each line represents a linear regression fit to the data points for each concentration, the correlation coefficients for the lines were greater than 0.93. The observed inactivation rate constant is the slope of the line. Panel B: Plot of $1/k_{obs}$ vs 1/[I], from which K_i and k_{inact} were determined. Panel C: Plot of $1/k_{obs}$ vs [I]/[E], from which the partition number was determined. The lines represent an iterative fit of the data points to linear regression analysis. The break point was chosen on the basis of a consistent drop in the correlation coefficient of the regression analysis when points were included. The lines for the two sections are linear regression analysis fits to the data points within each segment. Panel D: MTTC-dependent loss in 1-phenylethanol oxidation activity (□) of P450 2B4 correlated to the loss in the dithionite-reduced CO difference spectrum (●). Control experiments, where MTTC was omitted in the first incubation, showed no loss in P450 as determined either by the activity of 1-phenylethanol oxidation (△) or by the dithionite-reduced CO difference spectrum (■). Reactions were done as in Table 2 except that P450 2B4 was used and the concentration of MTTC was 1.0 mM. Reactions to determine the loss of P450 by the CO difference spectrum were comparable except that 3 nmol of P450 2B4 was used in a reaction volume of 5.0 mL. Each data point represents the mean of duplicate determinations.

is an apparent lack of correlation between the spectral dissociation constants and the extent of inhibition of the isoforms examined. P450 1A2 that shows the weakest

Table 2: Component Requirements for the Inactivation of P450 2E1 by MTTC

system	% inactivation
complete[a]	65 ± 5
NADPH omitted[b]	5 ± 2
reductase omitted	5 ± 3
O_2 replaced by N_2[c]	10 ± 5
MTTC omitted	5 ± 3
DLPC omitted	40 ± 5

[a] The complete system (first reaction mixture) contained 1.0 nmol of P450 2E1, 2.0 nmol of reductase, 30 μg of freshly sonicated DLPC, 0.5 μmol of MTTC, 2.0 μmol of NADPH, and 50 μmol of potassium phosphate buffer, pH 7.4, in a final volume of 1.0 mL. The first reaction mixture was incubated at 23 °C, and the reaction was initiated by the addition of the NADPH. After 10 min, 50 μL aliquots (0.05 nmol of P450) were removed and added to 200 μL solutions, each containing 1.0 μmol of 1-phenylethanol, 10 μmol of potassium phosphate buffer, pH 7.4, and 0.5 μmol of NADPH (second reaction mixture). The second reaction mixture was incubated for 20 min at 37 °C and then quenched by adding 200 μL of 1 N HCl. A 50 μL aliquot of each reaction mixture was analyzed for acetophenone by reversed-phase HPLC as previously described (Vaz & Coon, 1994). Each reaction was done in duplicate, and the experiment was repeated three times. [b] Components were omitted from the first reaction mixture only. The appropriate amount of the component was then added to the second reaction mixture prior to incubation at 37 °C. [c] The first reaction mixture was purged with N_2 prior to addition of the NADPH.

spectral interactions with the thiadiazoles, is the isoform most significantly inhibited. These results, in conjunction with the spectral binding studies, indicate that the 1,2,3-thiadiazole ring can serve as a heme ligand and the inhibitory potency is determined by the type of substituents at positions 4 and 5 on the 1,2,3-thadiazole ring. In this connection the results are very comparable to the imidazole ring, where substituents at the 4- and 5-positions profoundly influence enzyme selectivity as well as inhibitory potency.

Mechanism-Based Inactivation. Figure 5 shows the time-dependent loss in 1-phenylethanol oxidation activity of P450 2E1 as a result of mechanism-based inactivation by MTTC. Similar results were obtained for P450 2B4 (data not shown). The loss in activity was dependent on molecular oxygen, NADPH, and functional cytochrome P450 2E1 and reductase (Table 2). At 37 °C the rate of inactivation of P450 2E1 was extremely rapid as compared to P450 2B4; accordingly, the inactivation of 2E1 was determined at 23 °C. Inactivation rate constants (k_{inact}) determined from plots of $1/k_{obs}$ versus 1/[I] (Figure 5, panel B) were 0.08 ± 0.005 min^{-1} (at 23 °C) for 2E1 and 0.04 ± 0.003 min^{-1} for P450 2B4 (at 37 °C, data not shown), with K_is of 0.1 ± 0.007 mM and 2.0 ± 0.06 mM, respectively. Partition numbers of 110 ± 15 and 2100 ± 150 were determined from plots of $1/k_{obs}$ versus [I]/E for 2E1 (Figure 5, panel C) and 2B4 (data not shown), respectively.

P450 2E1 is significantly more sensitive than P450 2B4 to mechanism-based inactivation by MTTC when compared by the difference in partition numbers and inactivation rate constants for the two enzymes. With P450 2B4, the loss in 1-phenylethanol hydroxylase activity was established to be concurrent with loss in the 450 nm absorbance of the CO complex (Figure 5, panel D) without a corresponding increase at 420 nm, suggesting that heme destruction may occur during inactivation. DPT, PMT, and PT showed no inactivation of either P450 2E1 or 2B4, although these compounds are fairly effective inhibitors as shown above, and the thiadiazole ring is oxidized by the P450 system (discussed below).

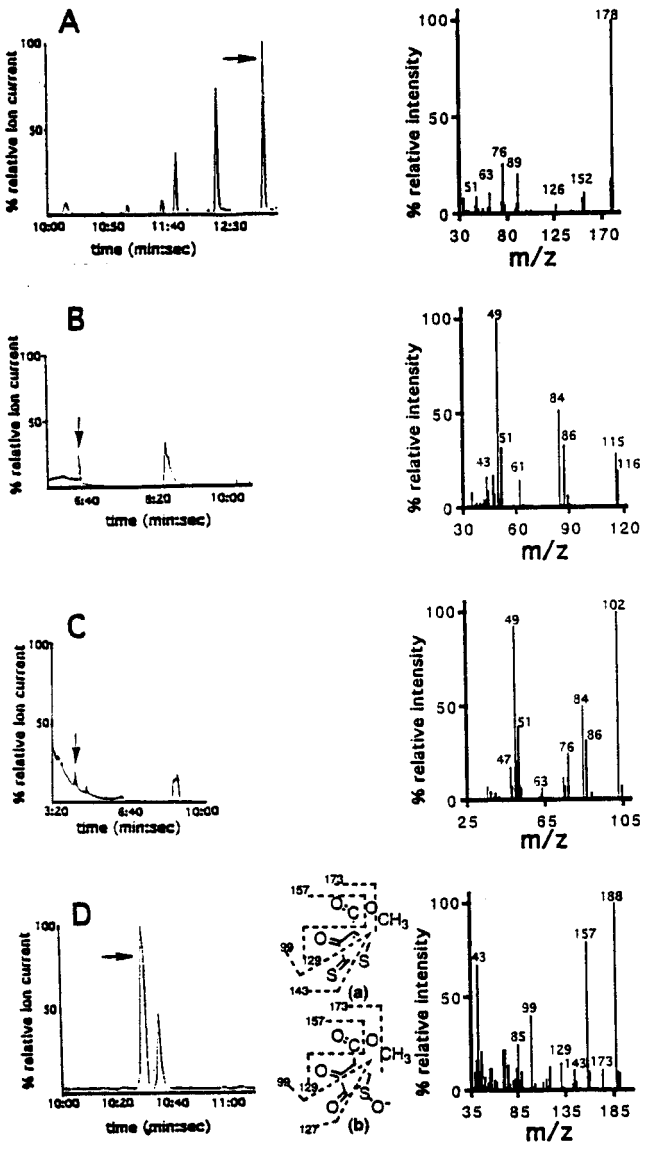

FIGURE 6: GC/MS analysis of the methylene chloride-extractable major metabolites of DPT (panel A), PMT (panel B), PT (panel C), and MTTC (panel D). The mass spectra shown are of the peaks marked with an arrow in the corresponding ion chromatograms. Mass spectra were obtained by summing the scans under the peak and subtracting an equal number of scans just prior to the peak. The GC/MS of authentic acetylenic standards were also determined and were found to have similar retention times and fragmentation patterns (data not shown). The structures shown in panel D are hypothetical and are based on assignment of ion masses observed in the mass chromatogram for the metabolite peak.

Products from Enzymatic Oxidation of 1,2,3-Thiadiazoles. The ability of MTTC and not of DPT, PMT, and PT to function as a mechanism-based inhibitor prompted an examination of the major oxidative products derived from these compounds. Figure 6 shows the results of GC/MS analysis of the methylene chloride-extractable components from the oxidative metabolism of DPT, PMT, PT, and MTTC. The mass spectra of peaks identified in the ion chromatograms indicate a loss of the heteroatoms from DPT, PMT, and PT with formation of diphenylacetylene (panel A), phenylmethylacetylene (panel B), and phenylacetylene (panel C), respectively. The identity of these products was confirmed by comparison to the mass spectra of authentic

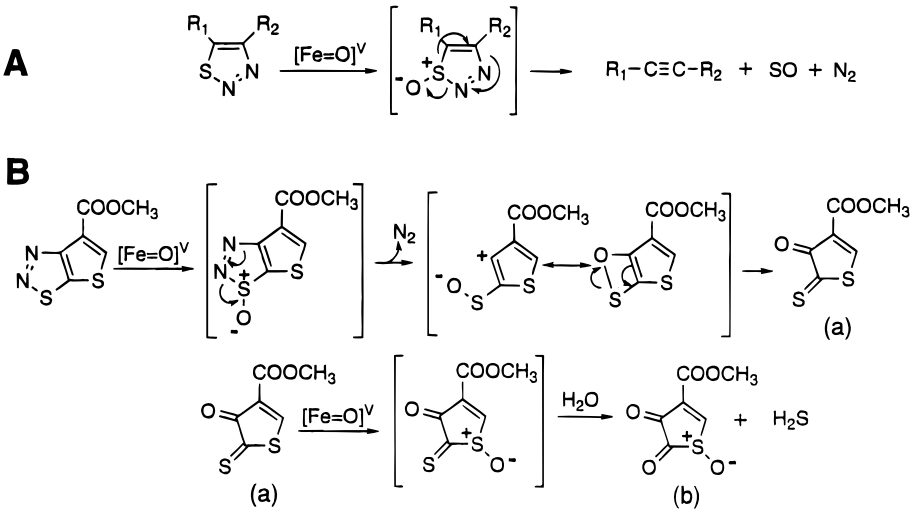

FIGURE 7: Mechanistic schemes showing (A) the formation of acetylenes from the monocyclic 1,2,3-thiadiazoles and (B) the putative metabolite from MTTC by (a) oxidation only of the 1,2,3-thiadiazole ring or (b) oxidation at both rings and hydrolytic desulfuration.

Table 3: Component Requirements for the Formation of 1,2-Diphenylacetylene from the Oxidation of DPT by P450 2B4

system	1,2-diphenylacetylene formed [pmol min^{-1} (nmol of P450)$^{-1}$]
complete[a]	2.8 ± 0.3
NADPH omitted	nd[b]
P450 2B4 omitted	nd
reductase omitted	nd
O_2 replaced by N_2	nd
DLPC omitted	2.0 ± 0.2

[a] The complete system consisted of 0.2 nmol of P450 2B4, 0.6 of nmol reductase, 30 μg of DLPC, 50 μmol of potassium phosphate buffer, pH 7.4, and 2 μmol of NADPH in a final volume of 1 mL. The reaction was incubated at 37 °C for 20 min and then quenched with 200 μL of 1 N HCl and the 1,2-diphenylacetylene was quantitated by HPLC on a Waters μBondapack C-18 column with acetonitrile/water (55/45) containing 0.1% trifluoroacetic acid as the mobile phase. The detector wavelength was set at 290 nm. Diphenylacetylene was quantitated on the basis of a standard curve that was linear from 0.5 to 100 pmol. [b] nd, not detected.

standards (data not shown). Table 3 shows that the formation of diphenylacetylene from DPT depends on molecular oxygen, NADPH, and a catalytically functional reconstituted system. Similar results were obtained with the other monocyclic thiadiazoles (data not shown).

In contrast to the acetylenic products derived from the monocyclic thiadiazoles, the oxidation of the fused bicyclic thiadiazole, MTTC, resulted in a product whose mass spectrum is shown in Figure 6, panel D. The molecular ion of this product may be accounted for in two ways: (1) by extrusion of nitrogen and the incorporation of an atom of oxygen in a single oxidative reaction to give a molecular mass of 188 or (2) by the loss of all heteroatoms from the thiadiazole half of the bicyclic compound and incorporation of three oxygen atoms for a molecular mass of 188; the insets (a) and (b) of Figure 6D show putative structures for the two possibilities. These alternative structures assigned to the metabolite have not been confirmed by other analytical methods. It may be noted that structure (b) could result in an ion mass of 127 m/z but not of 143 m/z; whereas, structure (a) can result in an ion mass of 143 m/z but not 127 m/z. The mass spectrum shows an ion mass fragment at 143 m/z but not at 127 m/z. However, distinction between these two putative structures is not possible with the limited information currently available.

Oxidation of the monocyclic 1,2,3-thiadiazoles examined in this study can be rationalized by initial oxidation of the sulfur followed by rearrangements. As shown in Figure 7A for DPT, PMT, and PT, the unstable S-oxide may rearrange by extrusion of nitrogen and sulfur oxide to form the acetylenic product. In contrast to the monocyclic compounds, the bicyclic structure does not permit rearrangement of the oxidized thiadiazole ring to an acetylene. The unstable thiadiazole S-oxide may rearrange with extrusion of nitrogen to yield the putative structure (a) shown in Figure 7B. Alternatively, the bicyclic thiadiazole may undergo a double oxidation on the thiadiazole and thiophene rings followed by hydrolytic desulfuration to yield the putative structure (b) shown in Figure 7B. The mass spectral fragmentation pattern shown in Figure 6D for a putative metabolite of MTTC does not permit a clear distinction between structures (a) and (b).

The difference in the oxidation of the monocyclic and bicyclic thaidiazoles may account for the ability of MTTC to function as a mechanism-based inactivator. Precedence for oxidation of a thiophene ring to reactive intermediates capable of inactivating P450 enzymes has been presented by Lopez-Garcia et al. (1994). The lack of inactivation of the P450 isoforms by DPT, PMT, and PT suggests that a transient reactive intermediate capable of either heme or protein modification is not formed on oxidation of the thiadiazole ring. Furthermore, the inability of the acetylenes to inactivate the P450s under the conditions of their generation from the monocyclic 1,2,3-thiadiazoles suggests that the acetylenes are formed nonenzymatically following dissociation of the oxidized thiadiazoles from the active site. The oxidative formation of acetylenes from the thiadiazoles is particularly interesting as the acetylenic group is used in the design of mechanism-based inhibitors of cytochrome P450 enzymes. Although inactivation of the P450s examined in this study does not occur by the acetylenes generated from the 1,2,3-thiadiazoles, it is conceivable that 1,2,3-thiadiazole can be designed where the oxidation results in a transient bound acetylenic product susceptible to further oxidation and subsequent inactivation of the P450. A unique advantage that may be gained from the unmasking of an acetylenic group is the potential ability to target an acetylenic function-based inhibitor as a thiadiazole prodrug.

Recent reports on the development of resistance to imidazole antifungal agents (Johnson et al., 1995; McCullough & Hume, 1995; Vanden Bossche et al., 1994a,b) portends the need for exploration of novel structures capable of specific inhibition of P450 enzymes. These structures must provide antifungal activity comparable to the imidazole-based drugs and circumvent the resistance pathways developed by fungi to these agents. The 1,2,3-thiadiazole ring clearly provides a heme ligand comparable to the imidazole ring. Furthermore, its oxidative metabolism has been shown to be distinct from that of imidazole. For these reasons the 1,2,3-thiadiazole may provide a novel heme ligand for the design of a new generation of antimycotic agents and other P450-specific inhibitors with the potential for targeted generation of an acetylenic group where P450 enzymes have been demonstrated to play a critical role in the etiology of the diseased state.

ACKNOWLEDGMENT

We thank Dr. M. J. Coon, Department of Biological Chemistry, The University of Michigan, for his constant support, encouragement, and discussions about this work.

REFERENCES

Abul-Hajj, Y. J., Liu, X. P., & Hedge, M. (1995a) *Steroids 60*, 423−427.

Abul-Hajj, Y. J., Liu, X. P., & Hedge, M. (1995b) *J. Steroid Biochem. Mol. Biol. 54*, 111−119.

Aoyama, Y., Ishida, K., Hori, K., Sakaguchi, A., Kudoh, M., & Yoshida, Y. (1992) *Biochem. Pharmacol. 44*, 1701−1705.

Ator, M. A., Schmidt, S. J., Adams, J. L., Dolle, R. E., Kruse, L. I., Frey, C. L., & Barone, J. M. (1992) *J. Med. Chem. 35*, 100−106.

Babu, B. R., Goto, M., Higaki, M., Sugiyama, T., Nakamura, K., & Ohno, A. (1990) *Bull. Chem. Soc. Jpn. 63*, 2742−2744.

Barrie, S. E., Potter, G. A., Goddard, P. M., Haynes, B. P., Dowsett, M., & Jarman, M. (1994) *J. Steroid Biochem. Mol. Biol. 50*, 267−273.

Baroudi, M., Robert, J., & Luu-Duc, C. (1996) *J. Steroid Biochem. Mol. Biol. 57*, 73−77.

Britton, T. C., Lobl, T. J., & Chidester, C. G. (1984) *J. Org. Chem. 49*, 4773−4780.

Brueggemeier, R. W. (1994) *Breast Cancer Res. Treat. 30*, 31−42.

Brugmans, J. P., Van Cutsem, J. M., & Thienpont, D. C. (1970) *Arch. Dermatol. 102*, 428−432.

Burger, A., Clark, J. E., Nishimoto, M., Muerhoff, A. S., Masters, B. S., & Ortiz de Montellano, P. R. (1993) *J. Med. Chem. 36*, 1418−1424.

Coon, M. J., Van der Hoven, T. A., Dahl, S. B., & Haugen, D. A. (1978) *Methods Enzymol. 52*, 109−117.

Coon, M. J., Ding, X., Pernecky, S. J., & Vaz, A. D. N. (1992) *FASEB J. 6*, 669−673.

Coon, M. J., Vaz, A. D. N., & Bestervelt, L. L. (1996) *FASEB J. 10*, 428−434.

Dansette, P. M., Amar, C., Valadon, P., Pons, C., Beaune, P. H., & Mansuy, D. (1991) *Biochem. Pharmacol. 41*, 553−560.

Davies, J. H., Dowsett, M., Jacobs, S., Coombes, R. C., Hedley, A., & Shearer, R. J. (1992) *Br. J. Cancer 66*, 139−142.

Defaye, G., Piffeteau, A., Delorme, C., & Marquet, A. (1996) *J. Steroid Biochem. Mol. Biol. 57*, 141−147.

Dowsett, M. (1994) *Ann. Oncol.* (Suppl. 7S), 3−5.

Evans, T. R., Di Salle, E., Ornati, G., Lassus, M., Benedetti, M. S., Pianezzola, E., & Coombes, R. C. (1992) *Cancer Res. 52*, 5933−5939.

French, J. S., & Coon, M. J. (1979) *Arch. Biochem. Biophys. 195*, 565−577.

Guengerich, F. P. (1994) *Toxicol. Lett. 70*, 133−138.

Halpert, J. R. (1995) *Annu. Rev. Pharmacol. Toxicol. 35*, 29−53.

Hartmann, R. W., Bayer, H., & Grun, G. (1994) *J. Med. Chem. 37*, 1275−1281.

Hopkins, N. E., Foroozesh, M. K., & Alworth, W. L. (1992) *Biochem. Pharmacol. 44*, 787−796.

Hortobagyi, G. N. (1992) *Breast Cancer Res. Treat. 21*, 3−13.

Ibrahim, N. K., & Buzdar, A. U. (1995) *Am. J. Clin. Oncol. 18*, 407−417.

Ito, T., Aoyama, Y., Ishida, K., Kudoh, M., Hori, K., Tsuchiya, S., & Yoshida, Y. (1994) *Biochem. Pharmacol. 48*, 1577−1582.

James, V. H., Reed, M. J., & Purohit, A. (1992) *J. Steroid Biochem. Mol. Biol. 43*, 149−153.

Johnson, E. M., Warnock, D. W., Luker, J., Porter, S. R., & Scully, C. (1995) *J. Antimicrob. Chemother. 35*, 103−114.

Johnston, J. O., Wright, C. L., Holbert, G. W., & Benson, H. D. (1990) *J. Enzyme Inhib. 4*, 137−142.

Johnston, J. O., Wright, C. L., & Holbert, G. W., (1995) *J. Steroid Biochem. Mol. Biol. 52*, 17−34.

Jones, C. D., Winter, M. A., Hirsch, K. S., Stamm, N., Taylor, H. M., Holden, H. E., Davenport, J. D., Krumkalns, E. V., & Suhr, R. G. (1990) *J. Med. Chem. 33*, 416−429.

Kato, A., Ikeda, Y., Sugita, N., Nitta, T., Enari, H., Kashima, A., Konno, M., & Niimura, K. (1995) *Chem. Pharm. Bull. 43*, 2152−2158.

Kimmick, G., & Muss, H. B. (1995) *Oncology 9*, 877−886, 889−894.

Koymans, L. M., Moereels, H., & Vanden Bossche, H. (1995) *J. Steroid Biochem. Mol. Biol. 53*, 191−197.

Kudoh, M., Susaki, Y., Ideyama, Y., Nanya, T., Okada, M., Shikama, H., & Fujikura, T. (1995) *J. Steroid Biochem. Mol. Biol. 54*, 265−71.

Lang, M., Batzl, C., Furet, P., Bowman, R., Hausler, A., & Bhatnagar, A. S. (1993) *J. Steroid Biochem. Mol. Biol. 44*, 421−428.

Lesuisse, D., Gourvest, J. F., Hartmann, C., Tric, B., Benslimane, O., Philibert, D., & Vevert, J. P. (1992) *J. Med. Chem. 35*, 1588−1597.

Lesuisse, D., Gourvest, J. F., Benslimane, O., Canu, F., Delaisi, C., Doucet, B., Hartmann, C., Lefrancois, J. M., Tric, B., Mansuy, D., Philibert, D., & Teutsch, G. (1996) *J. Med. Chem. 39*, 757−772.

Lopez-Garcia, M. P., Dansette, P. M., & Mansuy, D. (1994) *Biochemistry 33*, 166−175.

Mancy, A., Broto, P., Dijols, S., Dansette, P. M., & Mansuy, D. (1995) *Biochemistry 34*, 10365−10375.

McCullough, M., & Hume, S. (1995) *J. Med. Vet. Mycol. 33*, 33−37.

Moawad, E. B., Yousif, M. Y., & Metwally, M. A. (1989) *Pharmazie 44*, 820−822.

Numazawa, M., & Oshibe, M. (1995) *Steroids 60*, 506−511.

Numazawa, M., Mutsumi, A., Asano, N., & Ito, Y. (1993) *Steroids 58*, 40−46.

Numazawa, M., Mutsumi, A., Tachibana, M., & Nagaoka, M. (1995) *Biol. Pharm. Bull. 18*, 555−558.

Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370−2378.

Ortiz de Montellano, P. R., & Mathews, J. M. (1981) *Biochem. Pharmcol. 30*, 1138−1141.

Ortiz de Montellano, P. R., & Reich, N. O. (1986) in *Cytochrome P450: Structure, Mechanism and Biochemistry*, ed., (Ortiz de Montellano, P. R., Ed.) pp 273−314, Plenum Press, New York.

O'Reilly, J. M., Li, N., Duax, W. L., & Brueggemeier, R. W. (1995) *J. Med. Chem. 38*, 2842−2850.

Peet, N. P., Johnston, J. O., Burkhart, J. P., & Wright, C. L. (1993) *J. Steroid Biochem. Mol. Biol. 44*, 409−420.

Perez, N., & Borja, J. (1992) *J. Int. Med. Res. 20*, 303−312.

Poulos, T. L., & Howard, A. J. (1987) *Biochemistry 26*, 8165−8174.

Rahier, A., & Taton, M. (1992) *Biochim. Biophys. Acta 1125*, 215−222.

Roberts, E. S., Hopkins, N. E., Zaluzec, E. J. Gage, D. A., Alworth, W. L., & Hollenberg, P. F. (1994) *Biochemistry 33*, 3766−3771.

Rowlands, M. G., Barrie, S. E., Chan, F., Houghton, J., Jarman, M., McCague, R., & Potter, G. A. (1995) *J. Med. Chem. 38*, 4191−4197.

Sawyer, P. R., Brogden, R. N., Pinder, R. M., Speight, T. M., & Avery, G. S. (1975) *Drugs 9*, 406−423.
Shimizu, Y., Yarborough, C. P., Elger, W., Chwalisz, K., & Osawa, Y. (1995) *Steroids 60*, 234−238.
Swinney, D. C., So, O. Y., Watson, D. M., Berry, P. W., Webb, A. S., Kertesz, D. J., Shelton, E. J., Burton, P. M., & Walker, K. A. (1994) *Biochemistry 33*, 4702−4713.
Thiery, M., Mrozowski, B. J., & Van Kets, H. (1972) *Mykosen 15*, 35−37.
Trzaskos, J. M., Ko, S. S., Magolda, R. L., Favata, M. F., Fischer, R. T., Stam, S. H., Johnson, P. R., & Gaylor, J. L. (1995a) *Biochemistry 34*, 9670−9676.
Trzaskos, J. M., Fischer, R. T., Ko, S. S., Magolda, R. L., Stam, S., Johnson, P., & Gaylor, J. L. (1995b) *Biochemistry 34*, 9677−9681.
Vanden Bossche, H., Marichal, P., Gorrens, J., & Coene, M. C. (1990) *Br. J. Clin. Pract.—Symp. Suppl. 71*, 41−46.
Vanden Bossche, H., Marichal, P., & Odds, F. C. (1994a) *Trends Microbiol. 2*, 393−400.
Vanden Bossche, H., Warnock, D. W., Dupont, B., Kerridge, D., Sen Gupta, S., Improvisi, L., Marichal, P., Odds, F. C., Provost, F., & Ronin, O. (1994b) *J. Med. Vet. Mycol. 32* (Suppl. 1), 189−202.
Vanden Bossche, H. V., Moereels, H., & Koymans, L. M. (1994c) *Breast Cancer Res. Treat. 30*, 43−55.
Vanden Bossche, H., Koymans, L., & Moereels, H. (1995) *Pharmacol. Ther. 67*, 79−100.
Vaz, A. D. N., & Coon, M. J. (1994) *Biochemistry 33*, 6442−6449.
Vaz, A. D. N., Coon, M. J., Peegel, H., & Menon, K. M. J. (1992) *Drug Metab. Dispos. 20*, 108−112.
Walsh, C. (1982) *Tetrahedron 38*, 871−909.
Warren, J. T., Rybczynski, R., & Gilbert, L. I. (1995) *Insect Biochem. Mol. Biol. 25*, 679−695.
White, R. E., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 3073−3083.

BI9623183