Chiral Multisubstrate Inhibitors of Dopamine β-Monooxygenase: Evidence for Dual Modes of Interaction†

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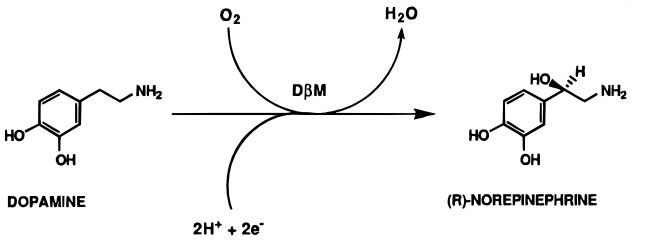
ABSTRACT: The electronic and steric constraints of the dopamine β-monooxygenase (DβM; E.C.1.14.17.1) active site were studied using a series of chiral bisubstrate inhibitors. The (R) and (S) enantiomers of 5-phenyl-2-thiooxazolidone were apparent bisubstrate inhibitors for DβM with respect to tyramine and dioxygen, but with small enantiomeric selectivity. In contrast to the substrate specificity of the enzyme, N-methylation of both inhibitors increased the potency without altering the enantiomeric selectivity. The (S) C-4-methyl substitution was more detrimental toward the inhibition potency compared to (R) C-4-methyl substitution for both the (R) and (S) series, which was also opposite of the substrate specificity of the enzyme. The high inhibition potency and apparent bisubstrate behavior of 3-phenyl-1,5-bisthioglutarimide (**XVI**), a probe designed to mimic two distinct binding modes for the (R) and (S) inhibitors, suggested that they may interact with the enzyme by two different modes involving both coppers in the active site. Direct support for the interaction of the thione group(s) of **XVI** with the reduced DβM copper(s) is provided by the UV−vis spectroscopic studies. The complete disappearance of the characteristic UV absorption of **XVI** at 336 nm in the presence of stoichiometric amounts of reduced DβM demonstrate that it could be an active site titrant for reduced DβM. The ability of the enzyme to interact with these inhibitors by more than one mode suggests that the DβM active site possesses high steric and electronic tolerance.

Dopamine β-monooxygenase [DβM;[1] E.C.1.14.17.1], a copper containing mammalian enzyme, catalyzes the biosynthetic conversion of dopamine to (*R*)-norepinephrine in the sympathetic nervous system (Kaufman et al., 1962; Goldstein et al., 1965; Kaufman & Friedman, 1965; Taylor, 1974; Rosenberg & Lovenberg, 1980; Ljones & Skotland, 1986; Fitzpatrick & Villafranca, 1987; Stewart & Klinman, 1988). In addition to the physiological benzilic hydroxylation reaction, DβM has also been shown to catalyze a wide variety of monooxygenations, including ketonization of phenylethanolamines (May et al., 1981,1982) and β-halo phenylethylamines (Klinman & Krueger, 1982; Mangold & Klinman, 1984; Bossard & Klinman, 1986), oxygenation of sulfides (May & Phillips, 1980) and selenides (May et al., 1987), epoxidation of olefins (May et al., 1983; Padgette et al., 1985), N-dealkylation of benzylic N-substituted analogs (May et al., 1983; Padgette et al., 1985; Wimalasena & May, 1987), allylic hydroxylation (Sirimanne & May, 1988), and oxidative aromatization of cyclohexadienes (Wimalasena & May, 1989). While ascorbic acid is presumed to be the physiological electron donor for the enzyme, other well-known electron donors such as $K_4Fe(CN)_6$, dopamine, hydroquinone, and dichlorophenolindophenol (Skotland et al., 1980; Skotland & Ljones, 1980; Rosenberg et al., 1980; Diliberto & Allen, 1981; Stewart & Klinman, 1987) are also weak electron donors for the enzyme. Work in our laboratory has shown that N-substituted phenylenediamines (Wimalasena & Wimalasena, 1991a,b) and the ascorbate derivative 2-aminoascorbic acid (Wimalasena & Wimalasena, 1991b) are well behaved chromophoric single electron donors for the enzyme with the efficiency similar to that of ascorbic acid. In addition, we have recently demonstrated (Wimalasena et al., 1994, 1996) that 6-O-phenyl (or alkylphenyl) and 6-S-phenyl (or alkylphenyl) derivatives of ascorbic acid are much more efficient reductants for the enzyme compared to ascorbic acid.



The copper atoms in the DβM active site are believed to be responsible for the binding, activation, and the insertion of oxygen into the organic substrate. An optimum stoichiometry of two catalytically essential coppers per DβM active site has been proposed on the basis of the copper dependency of the catalytic activity with the phenylethylamine substrate (Klinman et al., 1984) and the rate of enzyme inactivation by suicide inactivators (Ash et al., 1984). However, unlike

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[1] Abbreviations: EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; DβM, soluble chromaffin granule dopamine β-monooxygenase; DMPD, dimethylphenylenediamine; Abs EtOH, absolute ethanol; MOPAC, molecular orbital package; MS (EI), mass spectrometry with electron impact detection; ^{1}H-NMR, proton nuclear magnetic resonance; THF, tetrahydrofuran.

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other dioxygen binding copper proteins such as hemocyanin or tyrosinase (Solomon, 1981; Preaux & Gielens, 1984), spectroscopic evidence suggests that the copper centers in the DβM active site are mononuclear and magnetically noninteractive. EXAFS (Hasnain et al., 1984) and EPR (Blackburn et al., 1984, 1988; Scott et al., 1988; Blumberg et al., 1989) studies have suggested that, in the oxidized state of the enzyme, each Cu(II) is coordinated to a maximum of three histidines per copper, one water, and an additional nonaromatic ligand, and in the reduced form, Cu(I) is coordinated with two to three histidines per copper atom and a sulfur ligand (Pettingill et al., 1991). Recent stoichiometric characterization of the carbon monoxide (Blackburn et al., 1990; Pettingill et al., 1991) and 2,6-dimethylphenyl isocyanide (Reedy et al., 1995) bound reduced DβM has suggested that the two copper centers of DβM are chemically inequivalent and only one copper center of the reduced enzyme is capable of interacting with dioxygen during the catalytic turnover.

In the present study, we have used a series of novel, potent, rigid, chiral bisubstrate inhibitors of DβM which were designed to mimic phenylethylamine and dioxygen substrates of the enzyme in order to examine the electronic, steric, and proximity constraints of the DβM active site. Our results strongly suggest that the DβM active site possesses a remarkably unexpected steric and electronic tolerance toward these inhibitors, allowing enantiomeric pairs of inhibitors to interact with the enzyme by two distinct modes which appear to be different from the regular substrate binding mode. A structure−activity analysis of a large number of chiral inhibitors has led us to design and characterize a dual acting, extremely potent inhibitor which appears to interact with both copper centers in the reduced enzyme active site mimicking both inhibitor binding modes. The significance of these findings to the structure and functions of the active site coppers of the enzyme are discussed.

EXPERIMENTAL PROCEDURES

Materials and Methods. Tyramine hydrochloride was from Sigma; disodium fumarate, *N*,*N*-dimethyl-1,4-phenylenediamine dihydrochloride (DMPD), and ascorbic acid were from Aldrich. Beef liver catalase (65 000 units/mg of protein) was from Boehringer-Mannheim. Soluble DβM was purified (sp act = 16−20 units/mg), according to the procedure of Ljones et al. (Ljones et al., 1976) with minor modifications using freshly prepared bovine adrenal chromaffin granules (Kirshner, 1962; Njus & Radda, 1979). All spectrophotometric measurements were carried out on an HP 8452 diode array spectrophotometer equipped with a temperature-regulated multicell compartment. Oxygen monitor assays were performed on a Yellow Springs Instrument model 5300 biological oxygen monitor system. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL-300 (300 MHz) NMR spectrometer using tetramethylsilane (TMS) as the internal standard. The optical rotations were measured on a JASCO model DIP-360 digital polarimeter.

Spectrophotometric Assays of Dopamine β-Monooxygenase. This assay was used exclusively when tyramine or the reductant DMPD was the variable substrate. The standard assay solution contained 10 mM fumarate, 100 μg/mL catalase, and 0.5 μM $CuSO_4$ in 125 mM acetate buffer at pH 5.5 in a total volume of 1.0 mL at 37 °C unless otherwise stated. The concentrations of DβM were kept at a constant of 1.2 μg of protein per assay. The enzymatic reactions were usually initiated with the reductant, DMPD. The rate of increase in absorbance at 515 nm due to the enzyme-mediated formation of the DMPD cation radical was measured against a reference identical to the enzymatic reaction mixture but without the enzyme as we have previously described (Wimalasena & Wimalasena, 1991b). In assays where tyramine was the variable substrate, the DMPD concentration was kept at 12.5 mM. When DMPD was the varied substrate, tyramine was kept at a constant concentration of 10 mM.

Standard Oxygen Monitor Assay of Dopamine β-Monooxygenase. This assay was used when either ascorbic acid was used as the reductant or oxygen was the variable substrate. All enzymatic reactions were carried out under standard assay conditions in a total volume of 2.6 mL at 37 °C unless otherwise indicated. DβM concentrations were kept at 3.0 μg of protein per assay. Enzymatic reactions were initiated with an appropriate concentration of the variable substrate, and initial rates were measured as the rate of oxygen consumption minus the small background rates due to autoxidation of ascorbic acid. When ascorbate was the variable substrate, tyramine was kept at a constant concentration of 10 mM, and when tyramine was the variable substrate, the ascorbate concentration was kept at 10 mM. In experiments where oxygen was the variable substrate, the inhibition constants were determined under both random and ordered binding conditions (see Results and Discussion). When the random binding conditions were necessary, the assay solution was made to contain 50 mM phosphate buffer, pH 6.6 (with an ionic strength of 0.2 adjusted with 50 mM NaCl), 1.0 μM $CuSO_4$, 2 mM tyramine, and no fumarate. The standard assay conditions (125 mM acetate buffer, pH 5.5 in the presence of 10 mM fumarate) were employed when ordered conditions were necessary. The assay solution (2.5 mL) was incubated at 37 °C for about 3 min, and the oxygen or oxygen−nitrogen mixtures were passed through the solution for about 3 additional min, to achieve the desired concentrations of oxygen. Then catalase (100 μg/mL) followed by ascorbate (10 mM) were added, and the background rate due to the autoxidation of ascorbic acid was recorded before initiating the reaction with the enzyme.

Kinetic Data Analysis. Kinetic constants, K_m and V_{max} for the various substrates were determined by the computer fit of data directly to the hyperbolic form of the Michaelis−Menten equation. All inhibitor constants, K_i, were determined by fitting the initial velocity data to standard competitive, noncompetitive, and uncompetitive forms of the Michaelis−Menten equation, employing the standard regression analysis of Cleland (Cleland, 1979), and suggested standard statistical criteria were applied to decide between inhibition patterns. The desired concentration ranges of each inhibitor for accurate determination of K_i were estimated by screening experiments where approximately 50% of the enzyme activity was inhibited at the highest concentration of the inhibitor. In all K_i determinations, five different inhibitor concentrations and six substrate concentrations were employed.

Reconstitution of Depleted Copper in Purified DβM. Purified DβM (500 μL, 1.2 μg/mL) was incubated with 1000-fold excess of $CuSO_4$ for 1 h at 0 °C. The excess copper was separated by gel filteration using 100 mM BES and 100

mM KCl, pH 7.0, on a Superdex 75 HR 10/30 gel filtration column (Pharmacia Biotech) connected to an FPLC system. The protein containing fractions were pooled and concentrated to 500 μL using an Amicon cell with a YM-30 membrane.

UV−Vis Spectroscopic Studies of the Enzyme−3-Phenyl-1,5-bisthioglutarimide Complex. All the solvents and enzyme samples were thoroughly deoxygenated by flushing with N_2 and evacuating while freezing and rethawing the solution (this was repeated four times). All mixing and preparations were carried out within an Atmosbag (Aldrich) under a positive pressure stream of N_2. The UV−vis spectrum of the control sample containing 3-phenyl-1,5-bisthioglutarimide and Cu(II) in a total of 305 μL of anaerobic solution (300 μL of sodium acetate buffer, pH 5.2, and 5 μL of ethanol) in an air-tight anaerobic UV−vis cell was recorded initially. Then, 100 μL of deoxygenated enzyme-free buffer containing the desired amount of ascorbic acid was added, and the UV−vis spectrum was recorded. The final concentrations of the control sample were 3-phenyl-1,5-bisthioglutarimide, 10 μM; Cu(II), 18 μM; and ascorbic acid, 40 μM. The identical process was repeated for the test experiment except that a deoxygenated 100 μL solution of Cu(II) reconstituted purified DβM was used in place of the Amicon filtrate. The final concentrations for the test sample were 3-phenyl-1,5-bisthioglutarimide, 10 μM; ascorbic acid, 40 μM; and DβM monomers, 9 μM.

Chemical Synthesis

*3-Phenyl-3,4,5,6-tetrahydro-2-pyrimidinethiol (**XIV**).* A solution of 1.0 g (6.8 mmol) of 2-phenyl-1,3-diaminopropane (Weinhard et al., 1985) and 0.38 g (6.8 mmol) of 85% KOH in 5 mL of water was cooled in an ice bath, and 0.53 g (6.8 mmol) of CS_2 in 5 mL of dioxane was added with stirring. The reaction mixture was warmed to room temperature and an additional 0.38 g (6.8 mmol) of 85% KOH in 5 mL of water followed by 2.32 g (7.0 mmol) of $Pb(NO_3)_2$ in 7 mL of water were added and stirring was continued for an additional 10 min. The reaction mixture was heated in a boiling water bath for 45 min and filtered to remove the black PbS precipitate. The supernatant was evaporated to dryness and partitioned between NaCl-saturated water and ethyl acetate. The ethyl acetate layer was dried with anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The resultant white product was recrystallized twice with ethyl acetate and hexane. Yield: 0.35 g (26.6%); mp 228−230 °C. ^{1}H-NMR ($CDCl_3$): δ 1.6 (s, 2H), 3.2 (m, 1H), 3.4−3.6 (m, 4H), 7.2−7.4 (m, 5H). MS (EI): *m*/*e* 192 (M^+); 104 (100%). Exact mass calculated for $C_{10}H_{12}N_2S$: 192.0721; found, 192.0717.

*5-Phenyl-2-thio-3,4,5,6-tetrahydroxazine (**XV**).* This compound was also synthesized by the $Pb(NO_3)_2$ procedure using 2-phenyl-3-aminopropanol [prepared from α-phenyl-β-aminopropionic acid ethyl ester (Testa et al., 1958) using the procedure of Testa et al. (1961)] as the starting material. A solution of 0.4 g (2.7 mmol) of 2-phenyl-3-aminopropanol in 3.0 mL of water was treated with 2.7 mL (2.7 mmol) of 1 N KOH and cooled in ice. Then, 0.2 g (2.7 mmol) of CS_2 in 5.0 mL of dioxane was added and stirred for 10 min while allowing the mixture to gradually warm up to room temperature. The reaction mixture was treated with a second aliquot of 2.7 mL (2.7 mmol) of 1 N KOH and 2.7 mL (2.7 mmol) of 1 M $Pb(NO_3)_2$, and stirring was continued for an additional 5 min. After that period, the reaction mixture was heated in a water bath at 80 °C for 1 h, filtered, and the black precipitate washed several times with ethyl acetate. The combined filtrate and the washings were evaporated to dryness under reduced pressure. The residue was taken up with a mixture of water and ethyl acetate, the organic layer was separated, dried over anhydrous Na_2SO_4, and evaporated to dryness. The resultant crystalline product was recrystallized twice from a mixture of ethyl acetate and hexane to give 0.23 g (40%) of the pure compound. Mp 140−143 °C. ^{1}H-NMR ($CDCl_3$): δ 1.57 (s, broad, 1H), 3.39−3.29 (m, 1H), 3.55−3.48 (t, J = 11 Hz, 1H), 3.70−3.57 (m, 1H), 4.38−4.31 (t, 11 Hz, 1H), 4.57−4.51 (ddd, 11 Hz, 4.2 Hz, 2.2 Hz, 1H), 7.41−7.19 (m, 5H).

*3-Phenylglutarimide (**XVIII**).* A solution of 6.0 g (28.8 mmol) of 3-phenylglutaric acid in 75 mL of acetic anhydride was heated at 80−100 °C in an oil bath for 1 h under N_2. The reaction mixture was cooled to room temperature and evaporated to dryness under reduced pressure. The crude anhydride was dissolved in 250 mL of methanolic ammonia and stirred overnight. The solvent was removed under reduced pressure to give white crystalline 3-phenylglutaric acid monoamide, which was used in the subsequent step without further purification [pure by ^{1}H-NMR (acetone-d_6/D_2O): δ 2.5 −2.7 (m, 4H); 3.60 (m, 1H); 7.25 (m, 5H)]. Five grams of monoamide was heated in an oil bath at 220 °C for 4 h (until no more water evolved). The resultant material was cooled, dissolved in hot 50% (v/v) ethanol, and decolorized with 1.0 g of activated charcoal. The solvent was removed under reduced pressure to yield a white crystalline material, which was recrystallized twice with a mixture of ethanol and water. Yield: 2.5 g (55%); mp 174−175 °C. ^{1}H-NMR (acetone-d_6): δ 2.8 (m, 4H), 3.50 (m, 1H), 7.25−7.50 (m, 5H), 9.65 (s, broad, 1H).

*3-Phenyl-1,5-bisthioglutarimide (**XVI**).* A solution of 0.48 g (2.5 mmol) of 3-phenylglutarimide and 1.2 g of Lawesson's reagent (Cava & Levinson, 1985) in 20 mL of dry toluene was refluxed under N_2 for 2 h, cooled to room temperature, and evaporated to dryness under reduced pressure. The yellow solid residue was chromatographed on silica gel using a mixture of 80% CH_2Cl_2 and 20% hexane. The desired fractions were pooled and evaporated to dryness. The residue was dissolved in warm ether, and the insoluble white residue was removed by filtration. The product was recrystallized twice from a mixture of hexane and ether to yield golden yellow needles. Yield: 0.35 g (63%); mp 155−156 °C. ^{1}H-NMR ($CDCl_3$): δ 3.0 (dd, J = 9 Hz, 8 Hz, 2H), 3.35 (m, 1H), 3.5 (dd, J = 9 Hz, 2 Hz, 2H), 7.2−7.4 (m, 5H), 10.75 (s, broad, 1H); MS (EI): *m*/*e*, 221 (M^+). Exact mass calculated for $C_{11}H_{11}NS_2$: 221.0333; found, 221.0333.

*3-Phenyl-5 thioglutarimide (**XVII**).* A solution of 538 mg of 3-phenyl-1,5-bis thioglutarimide (2.43 mmol) in anhydrous THF (2 mL) was slowly introduced to a suspension of NaH (38 mg, 2.43 mmol) in anhydrous THF (5 mL) under N_2, and the resulting solution was stirred at 20 °C for 1 h. The reaction mixture was treated with water (10 mL), and stirring was continued at 50 °C for an additional 1 h. Then, 6 N H_2SO_4 (10 mL) was added, the solution was stirred for 3 h at room temperature, and products were extracted with $CHCl_3$. The combined $CHCl_3$ extracts were washed with water, dried with Na_2SO_4, and evaporated under reduced pressure to yield a yellow solid. Fractionation of the product

by silica gel column chromatography using CH_2Cl_2 as a the eluent gave the desired compound. Recrystallization with CH_2Cl_2 and hexane yielded a pale yellow solid. Yield: 11% (54 mg), mp 147−148 °C. ^{1}H-NMR ($CDCl_3$): δ 2.7−2.8 (m, 1H), 2.9−3.1 (m, 2H), 3.3 (m, 2H), 7.1−7.4 (m, 5H), 9.7 (s, broad, 1H). ^{13}C-NMR ($CDCl_3$): δ 36.9, 38.3, 47.7, 126.3, 127.6, 129.1, 140.2, 169.0, 206.9.

2-Phenylsuccinimide. A solution of 15.0 g (85.1 mmol) of phenylsuccinic anhydride in 250 mL methanolic ammonia (300 mmol) was stirred overnight at room temperature and then evaporated to dryness under reduced pressure. The crude product, 9.0 g of ammonium salt of 2-phenylsuccinamic acid (42.7 mmol), was heated in an oil bath to 190 °C for 2 h until no more water evolved. The resulting brown/white solid was chromatographed on silica gel using 20% ethyl acetate and 80% hexane as the eluent to yield 7.22 g (yield 95%) of the pure product; mp 74 °C. ^{1}H-NMR ($CDCl_3$): δ 2.84 (dd, J = 5.1, 18.6, 1H), 3.21 (dd, J = 9.6, 18.6, 1H), 4.05 (dd, J = 5.1, 9.5, 1H), 7.22−7.42 (m, 5H), 9.32 (s, broad, 1H). ^{13}C-NMR ($CDCl_3$): δ 38.2, 47.3, 127.4, 128.0, 129.1, 126.7, 176.7, 176.8, 178.6.

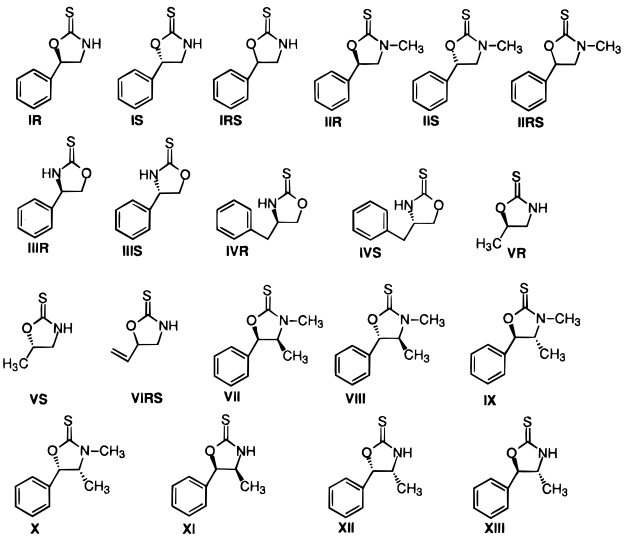
*2-Phenyl-4-thiosuccinimide (**XIX**).* A solution of 1.03 g (5.9 mmol) of 2-phenylsuccinimide and 3.06 g (7.6 mmol) Lawesson's reagent in 40 mL dry toluene was refluxed under N_2 for 1.5 h and then cooled to room temperature and evaporated to dryness under reduced pressure. The yellow crude solid was chromatographed on silica gel using 15% ethyl acetate and 85% hexane as the eluent to obtain a yellow solid, which was recrystallized from hot ethyl acetate. Yield: 0.250 g (22%); mp 147 °C. ^{1}H-NMR ($CDCl_3$): δ 3.27 (dd, J = 4.8, 19.7, 1H), 3.64 (dd, J = 9.2, 19.7, 1H), 4.11 (dd, J = 4.9, 9.3, 1H), 7.23−7.42 (m, 5H), 9.23 (s, broad, 1H). Calcd: C, 62.80%; H, 4.74%; N, 7.32%. Found: C, 62.61%; H, 4.72%; N, 7.37%.

*2-Methyl-2-phenyl-1,4-bisthiosuccinimide (**XX**) and 2-Methyl-2-phenyl-4-thiosuccinimide (**XXI**).* A solution of 0.20 g (1.1 mmol) of 2-methyl-2-phenylsuccinimide and 1.02 g (5.4 mmol) of Lawesson's reagent in 30 mL dry toluene was refluxed under N_2 for 2 h and then cooled to room temperature and evaporated to dryness under reduced pressure. The yellow crude solid was chromatographed on silica gel using 10% ethyl acetate and 90% hexane as the eluent to obtain two different yellow products, which were recrystallized from hot ethyl acetate. The more polar product was identified as 2-methyl-2-phenyl-4-thiosuccinimide. Yield: 0.160 g (73%); mp 104 °C. ^{1}H-NMR ($CDCl_3$): δ 1.73 (s, 3H), 3.28 (d, J = 19.4, 1H), 3.57 (d, J = 19.4, 1H), 7.26−7.44 (m, 5H), 9.11 (s, broad, 1H). ^{13}C-NMR ($CDCl_3$): δ 25.0, 56.6, 125.5, 127.6, 128.9, 140.9, 182.8, 209.4. Calcd: C, 64.36%; H, 5.40%; N, 6.82%. Found: C, 64.12%; H, 5.31%; N, 6.68%. The less polar product was identified as 2-methyl-2-phenyl-1,4-bisthiosuccinimide. Yield: 0.050 g (21%); mp 101 °C. ^{1}H-NMR ($CDCl_3$): δ 1.84 (s, 3H), 3.32 (d, J = 19.7, 1H), 3.62 (d, J = 19.7, 1H), 7.25−7.37 (m, 5H), 10.02 (s, broad, 1H). ^{13}C-NMR ($CDCl_3$): δ 26.9, 59.2, 125.6, 127.6, 128.8, 143.1, 210.0, 219.1. MS (EI): *m*/*e* 221 (M^+). Calcd: C, 59.69%; H, 5.01%; N, 6.33%. Found: C, 59.45%; H, 5.01%; N, 6.13%.

RESULTS AND DISCUSSION

The absolute stereospecificity of the Dβ M reaction toward (R) hydroxylation has been well established. This high stereospecificity of the enzyme has been shown to be achieved through stereocontrolled abstraction of the pro (R) benzilic hydrogen followed by rebinding of oxygen with the retention of configuration (Taylor, 1974). Therefore, the pro (R) benzilic hydrogen of the enzyme bound amine substrate must be accessible to the activated copper oxygen species in the active site, while the pro (S) hydrogen is not accessible. Thus, (*R*)-phenyl-2-thiooxazolidone (**IR**), the basic skeleton of which was derived from (*R*)-phenylethanolamine, was expected to mimic the phenylethylamine substrate and/or phenylethanolamine product binding modes of the enzyme effectively. The 2-thione functionality of the molecule, which is known to be an excellent ligand for Cu(I) (Rorabacher et al., 1983), was expected to be positioned in the same direction as the pro (R) hydrogen of the enzyme-bound substrate [or (R) hydroxyl of the ethanolamine product] and therefore expected to effectively interact with the copper center, mimicking the enzyme-bound dioxygen substrate. The (S) enantiomer, 5-(*S*)-phenyl-2-thiooxazolidone (**IS**), on the other hand, was expected to interact with the enzyme as a weak monosubstrate inhibitor mimicking only the phenylethylamine substrate (or phenylethanolamine product) and not the enzyme-bound dioxygen substrate yielding a significant enantiomeric discrimination between the two isomers.

Chart 1: Structures of Chiral Thiooxazolidone Inhibitors



In sharp contrast to the above predictions, both **IR** and **IS** were found to be well-behaved potent competitive inhibitors for Dβ M with respect to tyramine under standard assay conditions (for structures see Chart 1) with the enantiomeric selectivity only about three times in favor of the (R) enantiomer (Table 1). The transposition of the oxygen and nitrogen in the thiooxazolidone ring of **IR** and **IS** by changing 5-phenyl to 4-phenyl (**IIIR** and **IIIS**), reduces the inhibition potency considerably, without affecting the chiral selectivity significantly. Increasing the flexibility of the thiooxazolidone ring relative to the phenyl ring in **IIIR** and **IIIS** by insertion of a methylene group in between the two rings (**IVR** and **IVS**) does not alter the inhibition potency significantly, but reduces the chiral selectivity slightly (Table 1). Furthermore, the replacement of the phenyl substituent of **IR** or **IS** with a much less bulky methyl group (**VR** and **VS**) as expected reduces the inhibition potency considerably, but reduces the chiral selectivity only very slightly (Table 1). The racemic goitrogenic natural product 5-vinyl-2-

Table 1: Apparent Inhibition Constants for Thiooxazolidone Inhibitors with Respect to Tyramine[a]

inhibitor	K_i (μM)	inhibitor	K_i (μM)	inhibitor	K_i (μM)
IR	4.1 ± 0.3	IS	13.7 ± 0.1	IRS	6.9 ± 0.2
IIR	0.95 ± 0.06	IIS	2.4 ± 0.2	IIRS	1.4 ± 0.1
IIIR	47 ± 4	IIIS	142 ± 7	IVR	39 ± 4
IVS	78 ± 6	VR	316 ± 17	VS	433 ± 34
VIRS	68 ± 6	VII	216 ± 25	VIII	35.4 ± 4.9
IX	5.4 ± 0.2	X	1.6 ± 0.1	XI	134 ± 8
XII	7.9 ± 0.8	XIII	7.3 ± 0.7		

[a] Inhibition constants (K_i) were determined at pH 5.5 in the presence of 10 mM fumarate under standard assay conditions (ordered binding) using the DMPD-based spectrophotometric assay as detailed in Experimental Procedures. All the inhibitors displayed well-behaved competitive kinetic patterns with tyramine under the experimental conditions.

thiooxazolidone [goitrin; (**VIRS**)] is a stronger inhibitor than both **VR** and **VS,** but it is a much weaker inhibitor than either **IR** or **IS**, suggesting that the increase in the hydrophobic bulkiness at the 5 position of the thiooxazolidone ring enhances the inhibition potency considerably without significantly altering the chiral selectivity.

Although N-methylated derivatives of the parent compounds, **IIR** and **IIS**, were expected to yield a better chiral selectivity due to the presence of a bulky N-methyl group, the data presented in Table 1 clearly shows that the chiral selectivity of **IIR** and **IIS** are even less pronounced than that of **IR** and **IS**. However, unexpectedly, the inhibition potencies of both enantiomers were significantly increased by N-methylation. In spite of the broad substrate specificity of DβM, N-methylated derivatives of phenylethylamines or similar analogs are known to be very weak substrates (or inhibitors) or not substrates at all for the enzyme (Creveling et al., 1962), suggesting the highly unfavorable effect of the N-methyl group toward the efficient enzyme−substrate interaction. However, the above results show that the N-methyl groups of *both* **IIR** and **IIS** positively contribute to the enzyme−inhibitor interaction, suggesting that the mode(s) of interaction of these inhibitors may not exactly mimic the substrate binding mode of the enzyme. In addition, the high inhibition potencies of these N-methylated analogs in comparison to nonmethylated analogs also suggest that the acidic amide hydrogens of **IR** and **IS** do not significantly contribute toward the interactions with the enzyme.

In order to further examine the nature of the interaction of the above compounds with the enzyme, a series of chiral C-4-methyl substituted 5-phenyl-*N*-methyl-2-thiooxazolidones (**VII**−**X**) have been synthesized, characterized, and examined with the enzyme. The results presented in Table 1 demonstrate that the effect of substitution of a (S) C-4-methyl group in both **IIR** and **IIS** (**VII**, **VIII**) was much more detrimental toward their inhibition potencies in comparison to the substitution of a (R) C-4-methyl group (**IX**, **X**). In fact, this effect is so pronounced and contrasting for the two series of compounds that a reversal of the order of inhibition potencies of the parent compounds from the (R) series to the (S) series is observed. This similar trend is also observed for the chiral C-4-methyl substitution of parent compounds **IR** and **IS** which lack the N-methyl groups (**XI**−**XIII**), suggesting that this effect is not related to the presence of an N-methyl substituent (Table 1). Again, this observation contrasts the normal substrate specificity of the enzyme. Structure−activity studies of DβM have clearly demonstrated that while (R) α-methyl substitution on the ethylamine side chain of DβM substrates decreases both the binding and the turnover activity dramatically, the substitution of an (S) α-methyl group does not (note that the α-carbon of the substrate is analogous to the C-4 of the inhibitor). For example, (*S*)-amphetamine is a much better substrate for DβM than (R) amphetamine (Taylor, 1974). Similarly, Herman et al. (1991) have demonstrated that the (S) enantiomer of (phenylthio)-2-aminopropane is a seven times (k_{cat}/K_m ratio) better sulfoxidizing alternate substrate for DβM, than the corresponding (R) enantiomer. Therefore, the contrasting effects of the chiral C-4-methyl substitution on the inhibition potencies of the above compounds together with the α-methyl substitution on the relative activity of DβM substrates further confirm the above proposal that the mode of interaction of the above inhibitors with the enzyme may *not* be identical to that of enzyme−substrate interaction.

Detailed kinetic examination of the most potent thiooxazolidone inhibitors, **IIR** and **IIS**, revealed that they are well-behaved highly potent competitive inhibitors with respect to tyramine and uncompetitive inhibitors with respect to the reductant, ascorbate or DMPD, under both random and ordered conditions [for the kinetic mechanism of DβM, see Klinman et al. (1980), Ahn and Klinman (1983), and Miller and Klinman (1983, 1985)]. In addition, both these compounds are found to be competitive with respect to dioxygen under random conditions and noncompetitive under ordered conditions (Table 2). These kinetic patterns are identical to the kinetic patterns predicted and observed by Kruse et al. (1986) for their 1-benzylimidazole-2-thiol bisubstrate inhibitors of DβM, which were also designed to mimic both oxygen and phenylethylamine substrates. Therefore, again in contrast to the original predictions, both **IIR** and **IIS** appear to behave as potent apparent bisubstrate inhibitors with respect to the amine and dioxygen substrates without significant enantiomeric selectivity. Although the other thiooxazolidone derivatives have not been fully kinetically characterized with respect to dioxygen and the reductant, we believe they may also behave as apparent bisubstrate inhibitors for the enzyme similar to **IIR** and **IIS** due to their structural similarities and similar inhibition potencies to the parent compounds. Since the high inhibition potency of N-substituted imidazole-2-thiones was shown to be due to the interaction of the thione group with one of the copper centers of the reduced enzyme directly (Scott et al., 1988), the identical kinetic behavior and even better inhibition potency of both the (R) and (S) series of the above thiooxazolidone inhibitors could also be due to the direct efficient interaction of their thione groups with the Cu(I) in the reduced enzyme.

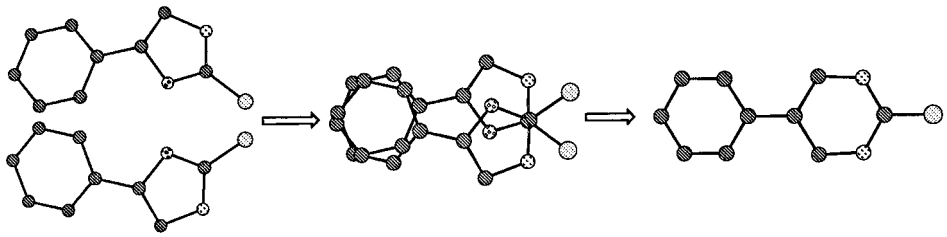
As mentioned above, the phenyl groups of both (R) and (S) series of inhibitors appear to interact with the phenyl binding site of the enzyme with similar high affinities (compare **IR** and **IS** with **VR** and **VS**). Therefore, the thione group (as argued above) and the phenyl ring of both the (R) and (S) series of inhibitors could occupy similar positions with similar orientations in the active site when bound to the enzyme. Such a mode of interaction could roughly be envisioned by projecting the heterocyclic rings of the (R) and (S) pairs of enantiomers in opposite directions and keeping the phenyl group in the same position to bring the thione groups together (Scheme 1). Two synthetically

Table 2: Dependence of the Apparent Kinetic Constants and Kinetic Patterns on pH and Fumarate for Some Selected Inhibitors[a]

	variable substrate						
	tyramine		DMPD	ascorbate		oxygen	
inhibitor	pH 5.5[b]	pH 5.5[c]	pH 5.5[b]	pH 5.5[c]	pH 6.6[c]	pH 5.5[c,d]	pH 6.6[c,e]
IIR	0.95 ± 0.06 (C)	0.59 ± 0.10 (C)	5.8 ± 0.2 (U)	5.0 ± 0.3 (U)	2.2 ± 0.8 (U)	K_{is} = 0.78 ± 0.23 K_{ii} = 2.54 ± 0.22 (N)	2.9 ± 0.5 (C)
IIS	2.4 ± 0.2 (C)	2.9 ± 0.4 (C)	10.8 ± 0.5 (U)	8.2 ± 0.5 (U)	10.1 ± 1.6 (U)	K_{is} = 5.52 ± 2.55 K_{ii} = 4.94 ± 0.48 (N)	5.3 ± 0.5 (C)
XIV	270 ± 20 (U)		66 ± 7 (C)				
XV	72 ± 4 (U)		60 ± 8 (C)				
XVI	0.048 ± 0.003 (C)		0.166 ± 0.007 (U)			K_{is} = 1.16 ± 0.56 K_{ii} = 4.10 ± 1.36 (N)	1.44 ± 0.28 (C)
XVII	0.75 ± 0.07 (C)		0.37 ± 0.03 (U)				
XVIII	6900 ± 400 (C)						
XIX	K_{is} = 10 ± 2 K_{ii} = 25 ± 5 (N)						
XX	K_{is} = 18 ± 8 K_{ii} = 25 ± 4 (N)						
XXI	K_{is} = 63 ± 15 K_{ii} = 162 ± 23 (N)						

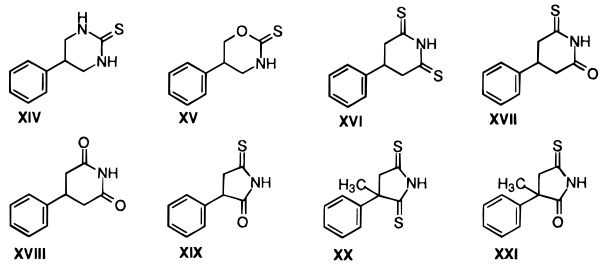
[a] Experimental conditions and data analysis are detailed in Materials and Methods. All inhibition constants are reported in micromolar units with standard errors. Experimentally observed kinetic patterns are given in parentheses; (C), competitive; (N), noncompetitive; (U), uncompetitive. [b] Inhibition constants were determined by the DMPD-based spectrophotometric assay. [c] Inhibition constants were determined by the polarographic oxygen monitor assay. [d] Inhibition constants were determined by the polarographic oxygen monitor assay in the presence of 10 mM fumarate [ordered binding conditions for oxygen and tyramine (tyramine first)]. [e] Inhibition constants were determined by the polarographic oxygen monitor assay in the absence of fumarate (random binding conditions for oxygen and tyramine).

Scheme 1: Possible Single Binding Modes for (R) and (S) Thiooxazolidone Inhibitors[a]



[a] All the structures were calculated using the MM2 parameter set.

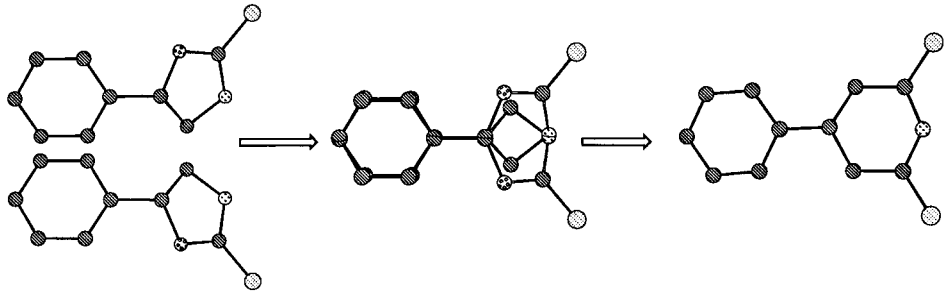
Chart 2: Structures of Binding Mode Probes



accessible molecules, 5-phenyl-3,4,5,6-tetrahydro-2-pyridinethiol (**XIV**) and 5-phenyl-3,4,5,6-tetrahydro-2-oxazinethiol (**XV**), appear to satisfy the major steric and electronic requirements of such a binding model and therefore should behave as mimics of both binding modes (for structures see Chart 2). However, the results presented in Table 2 indicate that both **XIV** and **XV** are very weak inhibitors with inhibition potencies much less than **IR**, **IS**, or racemic **IRS**, with respect to tyramine under standard experimental conditions. In addition, these compounds behave as uncompetitive inhibitors with respect to tyramine and competitive with respect to the reductant, DMPD, demonstrating their preferential interaction with the oxidized form of the enzyme, unlike the above thiooxazolidone inhibitors. This behavior also suggests that these molecules could not behave as competitive inhibitors with respect to dioxygen, since dioxygen is known to interact only with the reduced form of the enzyme. Therefore, clearly the nature of the interaction of these two molecules with the enzyme must be significantly different from the above thiooxazolidone inhibitors (**IIR** and **IIS**).

In addition to the inconsistent kinetic behavior of the representative molecules, **XIV** and **XV**, there are several fundamental drawbacks in the above model. First of all, the model suggests that the oxygen atoms of the phenylethanolamine backbone of both (R) and (S) enantiomers are positioned in a close spatial arrangement, which may not explain the experimentally observed absolute stereospecificity of the DβM reaction satisfactorily. Second, this model requires the nitrogen atoms of the (R) and (S) enantiomeric pairs of the inhibitor to occupy two opposite symmetrical locations in the enzyme active site (Scheme 1). Since, N-methylation causes a similar increase in the inhibition potency of both (R) and (S) series of inhibitors, both these locations must possess similar favorable steric and/or electronic tolerances to accommodate the bulky N-methyl groups, which is highly unlikely for a specific enzyme like DβM. On the other hand, since DβM is known to contain two mononuclear copper centers per active site, the interaction of the above chiral inhibitors with the enzyme may be explained by a model in which the (R) series may interact with one of the copper centers (most likely the center that involves the dioxygen binding), while the (S) series may interact with the second copper center in the active site. These

Scheme 2: Possible Dual Binding Modes for (R) and (S) Thiooxazolidone Inhibitors[a]



[a] All the structures were calculated using the MM2 parameter set.

two distinct binding modes of (R) and (S) enantiomeric pairs of inhibitors could also be constructed into a dual acting single molecule. As shown in Scheme 2, if such two distinct binding modes are operative, then the synthetically accessible bisthio derivative, 3-phenyl-1,5-bisthioglutarimide (**XVI**), should effectively interact with both the copper centers of the enzyme and be a much more potent inhibitor than the racemic **IRS** (Byers, 1978).

The bisthione **XVI** was found to be a kinetically well-behaved extremely potent competitive inhibitor with respect to tyramine (one of the most potent inhibitors known for the enzyme) under standard experimental conditions. In addition, **XVI** is also a well-behaved uncompetitive inhibitor with respect to the reductant, DMPD, demonstrating its predominant interaction with the reduced form of the enzyme similar to the thiooxazolidone inhibitors. Furthermore, **XVI** displayed competitive inhibition with respect to dioxygen under random conditions and noncompetitive inhibition under ordered conditions, as expected. These results clearly demonstrate that **XVI** is a well-behaved[2] extremely potent bisubstrate inhibitor for the enzyme with respect to phenylethylamine and dioxygen, which is kinetically indistinguishable from but 1.4×10^2 times stronger than the racemic **IRS** (Tables 1 and 2). On the other hand, the bisoxygen analog of **XVI** (**XVIII**) is a 1.4×10^5 times weaker inhibitor for the enzyme than **XVI**, with an inhibition constant in the millimolar range, suggesting that the sulfur atoms of **XVI** are crucial for the effective interaction with the enzyme. Furthermore, the corresponding monothio analog, 3-phenylthioglutarimide (**XVII**), is a 16 times weaker inhibitor for the enzyme than **XVI**, suggesting that while the first thione group of **XVI** is extremely important for the efficient interaction with the enzyme, the contribution from the second thione moiety is apparently less, but significant.

In order to further examine the effect of the geometric and electronic alterations of the thione functionalities of **XVI** on the inhibition potency, we attempted to synthesize 2-phenyl-1,4-bisthiosuccinimide (for structures see Chart 2). However, while bisthione could not be synthesized, we were able to synthesize the monothione, 2-phenyl-4-thiosuccinimide (**XIX**) and both bis and monothiones of the 2-methyl analog, 2-methyl-2-phenylsuccinimide (**XX** and **XXI**), using the same protocols that we have used for the synthesis of **XVI**. The kinetic data presented in Table 2 indicate that the interaction of these compounds with the enzyme may not be as straightforward as **XVI**. For example, in contrast to **XVI**, all of these derivatives appear to display mixed-type inhibition patterns, suggesting more than one mode of interaction with the enzyme, probably due to their asymmetric nature and/or other geometric and electronic properties. However, regardless of this complexity, comparison of the K_{is} component of the inhibition constants clearly indicate that both monothiones and the dithione are much more potent inhibitors for the enzyme than their oxygen analogs [corresponding dioxo compounds were found to be very weak inhibitors with inhibition constants in the millimolar range (data not shown)]. In addition, although not as pronounced as in the case of **XVI**, the inhibition potency of dithione **XX** is noticeably higher than the corresponding monothione (**XXI**) again confirming the positive role of the second thione functionality in interacting with the enzyme. Therefore, these results further support the proposal that the (R) and (S) series of the above thiooxazolidone inhibitors may interact with the enzyme by the proposed two distinctly different modes in contrast to our original predictions.

As shown in Figure 1A, an anaerobic solution of **XVI** in sodium acetate buffer, pH 5.2, displays a strong absorption band at 336 nm. While the addition of excess Cu(II) (10 M excess) does not significantly change the spectrum, addition of 1 equiv[3] of Cu(I) [Cu(II) with a 2-fold excess of ascorbic acid] under anaerobic conditions completely abolishes the strong absorption band of **XVI** at 336 nm (Figure 1A). Although no new well-defined absorption bands are observable above 300 nm at low concentrations of **XVI** and Cu(I), studies at higher concentrations have indicated that there is a very weak broad absorption band at about 466 nm (Figure 1A). Since the highly intense UV absorption band of **XVI** at 336 nm is known to be due to the characteristic π to π^* transition of the cyclic thioimide functionality [$\epsilon = 32\,200$ M^{-1} cm^{-1} for bisthioglutarimide (Berg, & Sandstrom, 1966)], the observed complete abolition (reversible) of this absorption band in the presence of free solution Cu(I), under anaerobic conditions, must be due to the strong complexation of **XVI** with Cu(I). The lack of significant changes in the intensity or the position of this band in the presence of free

[2] The lack of a significant effect on the inhibition potency of **XVI** in the presence of exogenous Cu(II) in the assay medium (up to 2−3-fold in excess of the inhibitor concentration) clearly demonstrates that the inhibition of the enzyme by **XVI** could not simply be due to the extraction of copper from the active site of the enzyme (data not shown).

[3] The observed stoichiometry of 1:1 with respect to Cu(I) and **XVI** appears to suggest that if four ligands are required to fill the coordination sphere of Cu(I), the remaining positions must be occupied by solvent or acetate ions present in the solution. Since the thione functionalities of **XVI** are very strong ligands for Cu(I), this must be due to geometric and/or steric constraints of the coordinated ligand. Regardless of these complexities, the apparent 1:1 stoichiometry assures that both the thione functionalities of **XVI** are in coordination with Cu(I) and therefore the observed UV−vis characteristics of the Cu(I)−**XVI** complex must be due to a species in which both thiones are coordinated.

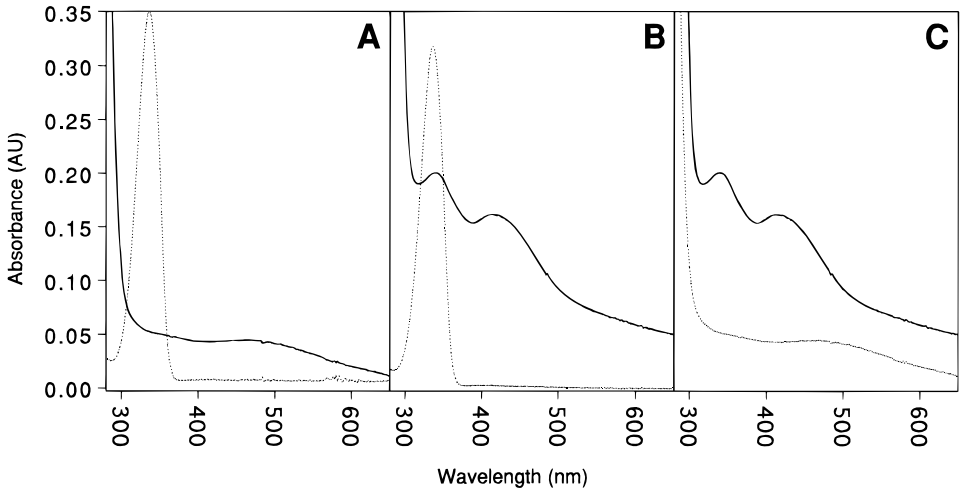


FIGURE 1: UV−vis spectroscopic studies of the enzyme−3-phenyl-1,5-bisthioglutarimide complex. (A) (-----) The UV−vis spectrum of the control sample containing **XVI** and solution Cu(II) under anaerobic conditions; (—) the UV−vis spectrum after addition of deoxygenated enzyme free buffer containing desired amount of ascorbic acid. The final concentrations of the control sample were **XVI**, 10 μM; Cu(II), 18 μM; and ascorbic acid, 40 μM. (B) Same as the control except that enzyme free buffer was replaced with Cu(II) reconstituted purified DβM containing desired amount of ascorbic acid. The final concentrations for the test sample were **XVI**, 10 μM; ascorbic acid, 40 μM; and DβM monomers, 9 μM. (C) Comparison of the spectra of **XVI**−Cu(I) complex (-----) and reduced DβM-**XVI** complex (—) (data taken from Figure 1, panels A and B).

solution Cu(II) demonstrates that **XVI** interacts much more tightly with Cu(I) than Cu(II). The ^{1}H-NMR characterization of **XVI** reisolated from the Cu(I) complex confirmed that the complexation is reversible and no irreversible chemical alterations to the ligand occurs (data not shown). These results strongly indicate that the interaction of the thione functionalities of **XVI** with Cu(I) completely abolishes its strong UV absorption band at 336 nm.

Results of a similar experiment carried out with copper reconstituted purified DβM is shown in Figure 1, panels B and C. These results clearly demonstrate that the addition of a stoichiometric amount of ascorbate reduced DβM to a solution of **XVI** under anaerobic conditions completely abolishes the strong absorption band at 336 nm as observed in the free solution copper experiment. Control experiments also revealed that the addition of copper reconstituted oxidized enzyme to a solution of **XVI** under identical conditions does not alter the intensity or the position of the 336 nm absorption band. Furthermore, a control experiment similar to that above with the Amicon filtrate (saved during the concentration of the reconstituted enzyme) which lacks only the enzyme, does not significantly alter the 336 nm absorbance band, confirming that the free solution copper (if any) in the DβM sample is not responsible for the above spectral changes (data not shown). The careful comparative examination of the results of free copper and reduced DβM experiments revealed that at least two new moderately intense absorption bands around 350 and 425 nm are present in the enzyme experiment which were absent in the free solution copper experiment (Figure 1C). These results together with the results of the above free solution Cu(I) experiments with **XVI** unequivocally demonstrate that the thione group(s) of **XVI** directly interacts with the active site Cu(I) center(s) of the reduced DβM. In addition, the lack of significant changes in the position or the intensity of the 336 nm band of **XVI** in the presence of an identical concentration of oxidized DβM further demonstrates that **XVI** exclusively interacts with the reduced enzyme as predicted by the inhibition kinetic patterns. Although not completely analyzed, the observed moderately intense two new enzyme-dependent absorption bands at 350 and 425 nm, which were not observable in the free solution Cu(I) controls, could be due to the well-known metal to ligand charge transfer bands [for example, see Munakata and Kitagawa (1982); detailed examination of the origin of these UV absorption bands are currently in progress].

Although our present results may not unequivocally demonstrate that both thione functionalities of **XVI** interact with both the copper centers in the enzyme, there is substantial indirect evidence that this may be the case. First, the significant and consistent positive contribution of the second thione functionality of **XVI** and **XX** toward the enzyme inhibition relative to the carbonyl functionality of corresponding monothione derivatives could not be explained unless we assume that the second thione group interacts with the second copper center in the enzyme, especially since other possible interactions such as ionic or hydrogen bonding must be stronger with oxygen than with sulfur. Second, very similar UV spectroscopic characteristics between the free solution Cu(I)−**XVI** complex in which both thione groups must be interacting with Cu(I)[3] and the reduced enzyme−**XVI** complex strongly suggest that the second thione of **XVI** may also interact with the second copper center in the enzyme at least weakly. On the other hand, the apparently smaller contribution of the second thione group in **XVI** and **XX** toward the enzyme inhibition, in contrast to the similar potencies of the (R) and (S) series of thiooxazolidone inhibitors, must be a consequence of the weakening of both thione Cu(I) interactions due to the relatively high demand for electrons of the bisthioamide functionality in these dual acting molecules. Alternatively, this could also be due to the nonoptimum arrangement of both thione functionalities to obtain the maximum interaction with both Cu(I) centers due to the structural rigidity of **XVI** and **XX** and/or the nonchemical equivalence of the two copper centers in DβM [for example, see Pettingill et al. (1991), Reedy and Blackburn (1994), and Reedy et al. (1995)].

Overall, the proposed dual binding model for the interaction of the above chiral thiooxazolidone inhibitors is in good agreement with all the experimental observations (Figure 2).

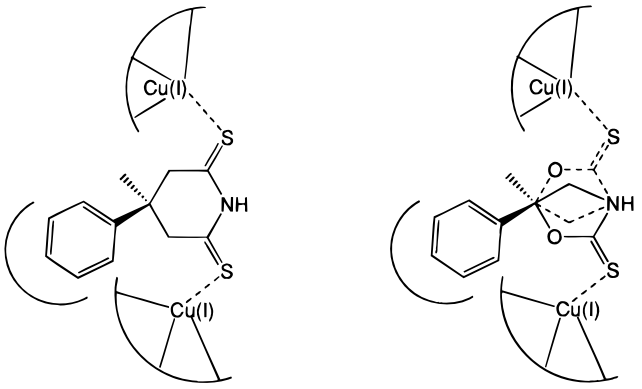


FIGURE 2: Proposed modes of interactions of **XVI** and (R) and (S) thiooxazolidone inhibitors with DβM.

The lack of significant enantiomeric selectivity as well as the lack of correlation between inhibition potencies and enantiomeric selectivities among the (R) and (S) series of inhibitors could be explained, assuming that they interact with the enzyme by the two different modes described above. As depicted in Figure 2 in the dual binding model, the nitrogen atoms of the thiooxazolidone rings of both (R) and (S) series of inhibitors occupy relatively similar positions in the DβM active site suggesting that the methyl groups of N-methylated derivatives should also occupy similar positions in the active site for both series. Therefore, in contrast to our initial predictions, N-methylation of **IR** and **IS** may not introduce significant differential steric and/or electronic interactions that would result in better enantiomeric selectivity. The increased inhibition potency by N-methylation, which is the reverse of the substrate specificity of the enzyme, strongly suggests that the nitrogen atom of the thiooxazolidone rings of both (R) and (S) series of inhibitors may not occupy the same position in the active site as the nitrogen atom of the enzyme-bound phenylethylamine substrate. This conclusion is further supported by the observation that the (S) C-4-methyl substitution is highly detrimental toward the inhibition potency in comparison to (R) C-4-methyl substitution for both (R) and (S) series of thiooxazolidone inhibitors which is also opposite of the effect of corresponding chiral C-α-methyl substitution in regular DβM amine substrates. The proposed dual interacting model is also consistent with the recent studies of Reedy et al. (Reedy et al., 1995) where they have demonstrated that the reaction of 2,6-dimethylphenyl isocyanide with reduced DβM produces monoisocyanide complexes at each of the two copper centers. The overall small but consistently better inhibition potency of the (R) series of inhibitors in comparison to the (S) series (except C-4-methylated compounds) may be due to the favorable electronic, steric, and/or proximity complementarity of the active site of the enzyme toward the (R) series. On the other hand, if all the other steric and electronic constraints in the DβM active site are same for both (R) and (S) series of inhibitors, the small but consistently higher inhibition potency of the (R) series in comparison to the (S) series of inhibitors could be due to the proposed chemical inequivalency of the copper centers in the reduced enzyme (Pettingill et al., 1991; Reedy & Blackburn, 1994; Reedy et al., 1995). In addition, the unexpected apparent bisubstrate behavior of (S) series of inhibitors may simply be due to the steric interference of the enzyme-bound inhibitor with the oxygen binding site of the enzyme.

In summary, the present results clearly demonstrate that the active site of DβM unexpectedly possesses a remarkably high, steric and electronic tolerance toward these inhibitors especially in the region of the two copper centers. The complete disappearance of the characteristic UV absorption band of **XVI** at 336 nm in the presence of stoichiometric amounts of reduced DβM demonstrates that it could be used as a spectroscopic active site titrant for reduced DβM (first of its kind). The detailed UV−vis, ^{13}C-NMR, and FT-IR characterization of the reduced DβM−**XVI** complex, which is in progress, should provide strong additional support for the above dual binding model. Structure−activity analysis of properly designed dual interacting inhibitors may provide additional information regarding the steric and electronic nature of the copper centers in DβM. In addition, it is now possible to develop more refined extremely potent dual acting inhibitors for DβM that may be valuable for the *in vivo* modulation of adrenergic activity for therapeutic purposes.

SUPPORTING INFORMATION AVAILABLE

Analysis of **IR**, **IS**, **IIR**, **IIS**, **IIIR**, **IIIS**, **IVR**, **IVS**, **VR**, **VS**, **VIRS**, **VII**, **VIII**, **IX**, **X**, **XI**, **XII**, and **XIII** (5 pages). Ordering information is given on any current masthead page.

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