Inhibition of Purified and Membrane-Bound Flavin-Containing Monooxygenase 1 by (*N*,*N*-Dimethylamino)stilbene Carboxylates†

Bernd Clement,*,‡ Matthias Weide,‡ and Daniel M. Ziegler§

Pharmazeutisches Institut, Christian-Albrechts-Universität Kiel, Gutenbergstrasse 76, D-24118 Kiel, Germany, and Biochemical Institute, Department of Chemistry and Biochemistry, The University of Texas at Austin, Austin, Texas 78712

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(*E*)-[2-(4-(Dimethylamino)phenyl)vinyl]benzenes bearing a nitrile or carboxyl group in the 2′, 3′, or 4′ position were synthesized and tested for substrate activity with purified pig liver flavin-containing monooxygenase (FMO1). Although the nitrile derivatives were too insoluble to saturate the catalytic site at pH 7.4, they appeared to be substrates with K_m's somewhat above their maximum solubility (≅0.1 mM) in the assay medium. Of the three carboxylic acid analogs, (*E*)-4-[2-(4-(dimethylamino)phenyl)vinyl]benzoic acid had no detectable water solubility at pH 7.4, and measurements were restricted to (*E*)-3-[2-(4-(dimethylamino)phenyl)vinyl]benzoic acid (DS3CO) and (*E*)-2-[2-(4-(dimethylamino)phenyl)vinyl]benzoic acid (DS2CO). While DS3CO and DS2CO were substrates, they also inhibited FMO1 turnover. DS3CO was the more effective inhibitor, and at 2 mM it inhibited FMO1 and microsomal-catalyzed oxidation of methimazole (*N*-methyl-2-mercaptoimidazole) by 80−90%. Kinetic studies indicated that the aminostilbene carboxylates were noncompetitive with both the xenobiotic substrate, methimazole, and NADPH. However, inhibition constants calculated from double reciprocal plots of velocity vs NADPH were K_i(comp) 130 and 150 μM for DS3CO and DS2CO, respectively, whereas the uncompetitive K_i's were 10−15 times higher, which suggests that inhibition of NADPH binding may be primarily responsible for inhibition of FMO1 by the aminostilbene carboxylates. This model is also consistent with inhibition of cyclohexanone monooxygenase, a bacterial analog of FMO. DS3CO and DS2CO were again noncompetitive with methimazole but primarily competitive with NADPH. The aminostilbene carboxylates had no detectable effects on activity of pig or rat liver NADPH−cytochrome P450 reductase, which suggests that they are not nonspecific flavoprotein antagonists.

Introduction

Microsomal flavin-containing monooxygenases (FMO)[1] catalyze the oxidation of structurally diverse xenobiotics bearing electron rich centers, typically nitrogen, sulfur, phosphorus, or selenium (*1*−*3*). While substrates accepted by these enzymes lack common structural features, all are soft nucleophiles readily oxidized upon contact with the enzyme-bound 4a-hydroperoxyflavin. Kinetic and other studies defining the mechanism underlying such lack of specificity have been reviewed (*4*). The data also suggest that essential cellular nucleophiles are effectively excluded from the catalytic site, but structural elements that prevent their contact with the enzyme-bound oxidant are still poorly defined. While overall size (*5*) and physical dimensions (*6*) appear to limit access to the active site, charge is also an important factor (*7*). Most compounds bearing ionic groups are excluded, but sulindac sulfide (*8*) and a few other sulfur compounds (*7*) which exist as anions at physiological pH are substrates. Investigators of the latter two reports concluded that position relative to the nucleophilic heteroatom rather than mere presence of a negative charge—especially on a rigid molecule like sulindac sulfide—determines substrate activity.

This study was undertaken to test this interpretation by measuring substrate activity of positional isomers of (*N*,*N*-dimethylamino)stilbene carboxylic acids (Chart 1). However, these compounds proved to be better inhibitors than substrates that, unlike anionic detergent FMO inhibitors (*7*), also inhibited microsomal-bound FMO at pH 7.4. This report describes the synthesis and effects of aminostilbenes bearing a carboxylate group on activities of purified and membrane-bound FMO1.

Experimental Procedures

Materials. The following reagents were obtained from the sources indicated: $NADP^+$, glucose 6-phosphate, acetylthiocholine, 5,5′-dithiobis(2-nitrobenzoic acid), methimazole (*N*-methyl-2-mercaptoimidazole), *L-mesenteriodes* glucose-6-phosphate dehydrogenase, and catalase, Sigma Chemical Co. (St. Louis, MO). 4-(Dimethylamino)cinnamic acid and its nitrile were purchased from Aldrich (Steinheim, Germany) and recrystallized from ethanol. All reagents used for the organic syntheses were synthetic grade purchased from either Firma Merck (Darmstadt, Germany) or Aldrich (Steinheim, Germany).

Enzyme Preparations. FMO was isolated from pig liver microsomes by minor modifications of the original procedure as described in a recent report (*9*). Liver tissue from adult male

† This paper is dedicated to Professor Dr. G. Seitz on the occasion of his 60th birthday.

* Address correspondence to this author at the Pharmazeutisches Institut, Christian-Albrechts-Universität Kiel, Gutenbergstrasse 76, D-24118 Kiel, Germany. Tel +431/880-1126, FAX +431/880-1352.

‡ Pharmazeutisches Institut, Christian-Albrechts-Universität Kiel.

§ Biochemical Institute, The University of Texas at Austin.

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[1] Abbreviations: FMO, flavin-containing monooxygenase; CMO, cyclohexanone monooxygenase; DMPU, *N*,*N*′-dimethyl-*N*,*N*′-propyleneurea. Also see Chart 1 for abbreviations of the stilbene and cinnamic acid derivatives used in this report.

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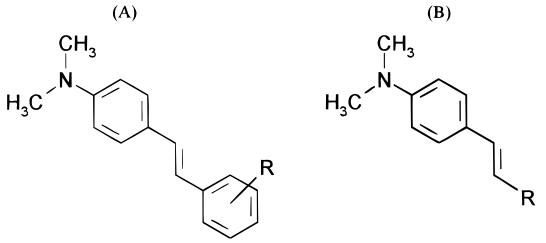
Chart 1. Structures of the Stilbenes (A) and Related Compounds (B) Used in This Study[a]



Structure	R	Abbreviation
A	2-CN	DS2CN
A	3-CN	DS3CN
A	4-CN	DS4CN
B	-CN	CCN
A	2-COOH	DS2CO
A	3-COOH	DS3CO
A	4-COOH	DS4CO
B	-COOH	CCO

[a] Abbreviations used: (A) The 2, 3, and 4 positional isomers of [2-(4-(dimethylamino)phenyl)vinyl]benzonitrile and [2-(4-(dimethylamino)phenyl)vinyl]benzoic acid, respectively. (B) 4-(Dimethylamino)cinnamic acid and its nitrile.

Sprague-Dawley rats was obtained locally (Austin). Microsomes isolated by differential centrifugation from rat liver homogenates prepared in 0.25 M sucrose containing 0.1 mM butylated hydroxytoluene were resuspended in 10 volumes of 0.25 M sucrose and resedimented. The pellet from each liver was resuspended in 0.25 M sucrose and stored in small aliquots at −70 °C. The preparations were thawed and stored on ice no more than 6 h before use. Any of the sample not used in this time was discarded. Cyclohexanone monooxygenase was isolated from *Acinetobacter* by the procedure described by Donoghue *et al.* (*10*).

Activity Measurements. Activities of purified FMO1 were measured polarographically by following substrate-dependent oxygen uptake at 37 °C in 0.1 M potassium phosphate (pH 7.4) containing 0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, 1 unit/mL glucose-6-phosphate dehydrogenase, and 1000 units/mL catalase. After 3−4 min temperature equilibration, 0.2−0.3 nmol of FMO1 in 10−30 μL was added through the capillary stem of the 2 mL sealed reaction vessel, and the endogenous rate of oxygen uptake was recorded for 1−1.5 min before adding the xenobiotic substrate. The change in oxygen concentration was recorded for 5−10 min. Methimazole was then routinely added as a preliminary test for possible effects on FMO1 activity of the xenobiotics added initially. Activities reported were calculated from initial rates of oxygen uptake.

The oxidation of methimazole catalyzed by microsomes and the purified flavoenzymes was measured by following methimazole-dependent thiocholine oxidation as described by Guo *et al.* (*6*) in reaction medium identical to that used for the oxygen uptake measurements except that it also contained 0.3 mM thiocholine. The complete reaction medium minus the substrate and enzyme preparation was preincubated in a metabolic shaker at 37 °C for 3−4 min. Microsomes (or FMO1) were added, and 1 min later, the reaction was started by adding 1.0 mM methimazole. Aliquots removed at 0, 3, 6, 9, and 12 min were deproteinized with trichloracetic acid, and the concentration of thiocholine was measured as described (*6*). The rate of H_2O_2 formation was measured by following the peroxidation of methanol by the procedure described earlier (*11*).

Synthesis. The melting points were determined with a Büchi 510 apparatus and are uncorrected. The IR spectra were recorded with a Perkin-Elmer Fourier FTIR 16 PC spectrometer. The ^{1}H-NMR spectra were recorded with a Bruker AM 360 (360.15 MHz) and Bruker AM 400 (400.13 MHz) spectrometer. The elemental analysis of compounds described in this section were performed by the Ilse Betz Laboratory, Kronach, Germany. The (*E*)-[2-(4-(dimethylamino)phenyl)vinyl]benzonitriles were synthesized by minor modifications of the procedure described by Takahaschi *et al.* (*12*) for the base-catalyzed condensation of 4-(dimethylamino)benzaldehyde with methylbenzonitriles as follows:

DMPU (5 mL) containing 1.49 g (10 mmol) of 4-(dimethylamino)benzaldehyde and 1.17 g (10 mmol) of the 2-, 3-, or 4-methylbenzonitrile was added dropwise to a stirred solution of 2.47 g of *t*-BuOK (11 mmol) in 15 mL of DMPU at 10 °C (or 20 °C for DS3CN) under nitrogen. After stirring for 5 h, the reaction mixture was poured into 100 mL of ice water containing 1.08 g (20 mmol) of ammonium chloride. The products were extracted into ether or dichloromethane and crystallized. The structure of each compound was determined by IR, ^{1}H-NMR, and ^{13}C-NMR. The analytical data including mp and elemental composition along with precise details for the extraction and crystallization of each compound will be supplied upon request. Only the *E* configurations were detected in the final crystalline products of all positional isomers of the [2-(4-(dimethylamino)-phenyl)vinyl]benzonitriles and the corresponding benzoic acids. The vinyl protons of the ^{1}H-NMR spectra are in the range of (*E*)-stilbene ($\delta = 7.1$ ppm) and not of (*Z*)-stilbene ($\delta = 6.55$ppm) (*13*), and their coupling constants $^3J(H_\alpha\text{-}H_\beta)$ of about 16 Hz are also only in agreement with the (*E*)-configuration (*14*).

(*E*)-4-[2-(4-(Dimethylamino)phenyl)vinyl]benzoic Acid. One millimole (0.248 g) of the nitrile, DS4CN, was heated to 90 °C in 6 mL of 50% sulfuric acid for 27 h. After cooling, 6 mL of water was added and the pH adjusted to between 3 and 4 with concentrated ammonia. The precipitate was collected, washed with water, and then sublimed at 180 °C under vacuum ($<10^{-2}$ mbar). The yield of yellow powdery product was 56% of theoretical; mp 330 °C dec; IR (KBr): 3440, 2856, 1678, 1592, 1522, 962 cm^{-1}; ^{1}H-NMR (360 MHz, [D$_6$]DMSO) δ (ppm) = 2.95 (s, 6H, −N(CH$_3$)$_2$), 7.1 (mc, AA′BB′, 4H, ArH), 7.76 (mc, AA′BB′, 4H, ArH), 7.17 (mc, AB, ^3J = 16.4 Hz, 2H, olef H), 12.81 (br, 1H, −COOH). Calcd for $C_{17}H_{17}NO_2$: C, 76.38; H, 6.41; N, 5.24. Found: C, 76.40; H, 6.32; N, 5.24.

(*E*)-3-[2-(4-(Dimethylamino)phenyl)vinyl]benzoic Acid. This compound was also synthesized by hydrolysis of the corresponding nitrile and purified by sublimation. Yield = 44% of theoretical; mp 220−222 °C dec; IR (KBr): 3440, 2850, 2590, 1682, 1630, 1606, 1578, 1522, 960 cm^{-1}; ^{1}H-NMR (400 MHz, [D$_6$]DMSO) δ (ppm) = 2.95 (s, 6H, −N(CH$_3$)$_2$), 6.72 (d, 2H, ArH), 7.13 (mc, AB, ^3J = 16.4 Hz, 2H, olef H), 7.45 (mc, 3H, ArH), 7.77 (mc, 1H, ArH), 8.07 (s, 2H, ArH), 13 (br, ^{1}H, −COOH). Calcd for $C_{17}H_{17}NO_2$: C, 76.38; H, 6.41; N, 5.24. Found: C, 76.37; H, 6.33; N 5.22.

(*E*)-2-[2-(4-(Dimethylamino)phenyl)vinyl]benzoic Acid. This compound could not be prepared by hydrolysis of the nitrile (DS2CN), and it was synthesized as follows: Five milliliters of DMPU containing 1.49 g (10 mmol) of 4-(dimethylamino)-benzaldehyde and 1.64 g (10 mmol) of ethyl 2-methylbenzoate was added dropwise at 10 °C to a stirred solution of 22 mmol (2.47 g) of *t*-BuOK in 15 mL of DMPU under nitrogen. After stirring for 8 h under nitrogen, the reaction mixture was poured into 100 mL of 0.2 mM ice-cold ammonium chloride. After extracting three times with 100 mL of diethyl ether, the aqueous phase was adjusted to pH 5−6 on ice. The product precipitated as a yellow solid, which upon analysis proved to be the free acid. The ethyl ester apparently hydrolyzed during extraction. The precipitate was collected, washed once with cold water, pH 5−6, dried, and recrystallized from acetone−water. Yield = 36% theoretical; mp 136 °C dec; IR (KBr): 3426, 2892, 2804, 1678, 1606, 1592, 1524, 968 cm^{-1}; ^{1}H-NMR (360 MHz, [D$_6$]DMSO) δ (ppm) = 2.94 (s, 6H, −N(CH$_3$)$_2$), 7.06 (mc, AA′BB′, 4H, ArH), 7.3 (t, 1H, ArH), 7.38 (mc, AB, ^3J = 16.3 Hz, 2H, olef H), 7.51 (mc, 1H, ArH), 7.8 (mc, 2H, ArH), 12.96 (br, 1H, −COOH). Calcd for $C_{17}H_{17}NO_2$: C, 76.38; H, 6.41; N, 5.24. Found: C, 76.32; H, 6.36; N, 5.20.

Results

(*E*)-[2-(4-(Dimethylamino)phenyl)vinyl]benzonitriles. All of the nitriles (Chart 1) stimulated FMO1 and NADPH-dependent oxygen uptake, which is good

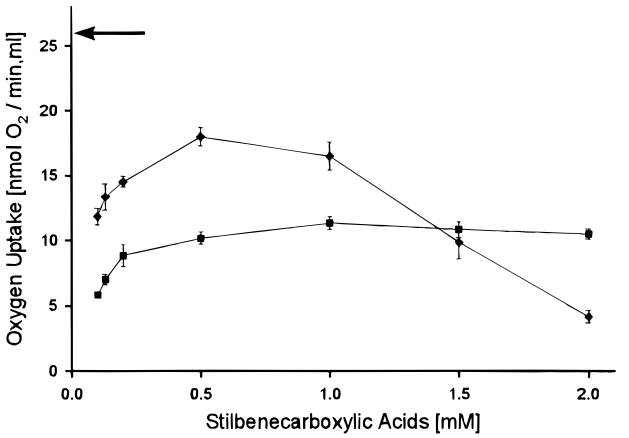


Figure 1. FMO1- and NADPH-dependent O_2 uptake as a function of DS2CO (■) and DS3CO (♦) concentration. Oxygen uptake was measured polarographically in a 2 mL cell at 37 °C containing 0.1 M phosphate, pH 7.4, 0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, 1.0 unit/mL glucose-6-phosphate dehydrogenase, 1000 units/mL catalase, and 5 mg/mL bovine serum albumin. After 4−5 min temperature equilibration, FMO (0.9 nmol in 20 μL) was added through the capillary stem, and 1.5−2.0 min later the stilbenes were added in no more than 10 μL of DMPU or 20 μL of acetonitrile. The rates listed are averages ± SD calculated from initial rates of substrate-dependent oxygen uptake for 3 or more preparations of FMO1. The arrow indicates activity with 0.15 mM methimazole under the same conditions.

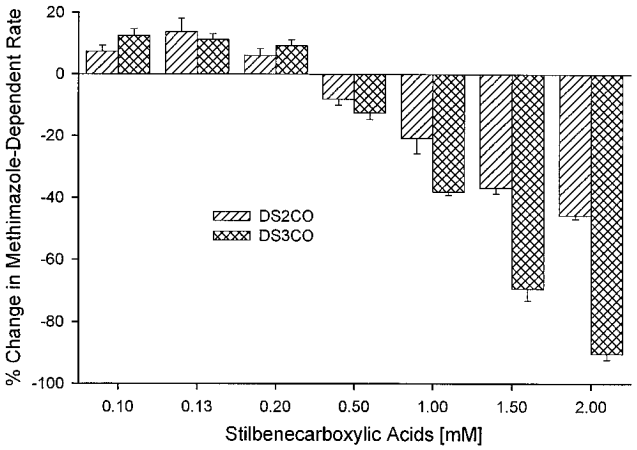


Figure 2. Effect of increasing concentrations of DS2CO and DS3CO on methimazole-dependent O_2 uptake catalyzed by FMO1. The reactions were carried out as described in the legend to Figure 1 in 0.1 M phosphate, pH 7.4, containing 0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, 1.0 unit/mL glucose-6-phosphate dehydrogenase, and 1000 units/mL catalase. After 3−4 min temperature equilibration, the stilbenes were added at the concentrations indicated, followed 2 min later by 1.5 mM methimazole. Oxygen uptake was recorded for another 2 min. The percent change in rates from methimazole-dependent rate in the absence of the stilbenes is the average ± SD for 3−4 determinations.

presumptive evidence for substrate activity. However, the concentrations of the (*N*,*N*-dimethylamino)stilbenenitriles required to half-saturate the enzyme were greater than their solubility in the assay medium, which essentially precluded collecting accurate kinetic constants. From the limited values at low concentrations, the K_m's for DS2CN, DS3CN, and DS4CN were near or somewhat above their upper limits of solubility (≅0.1 mM). However, CCN (Chart 1) was an excellent substrate with a V_{max} equal to that of *N*,*N*-dimethylaniline (data not shown).

(*E*)-[2-(4-(Dimethylamino)phenyl)vinyl]benzoic Acid. The solubility of DS4CO in the assay medium was

Table 1. DS2CO and DS3CO Inhibition of FMO1-Catalyzed Oxidation of Amine Drugs[a]

substrate	concn (mM)	% inhibition by 1 mM DS2CO	% inhibition by 1 mM DS3CO
tamoxifen	0.20	51 ± 10	71 ± 6
trifluoperazine	0.50	47 ± 6	70 ± 8
imipramine	0.50	42 ± 2	58 ± 6
methimazole[b]	0.50	50 ± 5	54 ± 12

[a] Activities were measured by following substrate-dependent O_2 uptake at 37 °C in 0.1 M phosphate, pH 7.4, containing the NADPH generating system and catalase at the concentrations listed under Experimental Procedures, except that $NADP^+$ was reduced to 0.1 mM. After 4 min temperature equilibration, FMO1 was added, followed 1 min later with substrate. O_2 uptake was recorded for 3−5 min. The assay was then repeated in the presence of the aminostilbene carboxylates. The values are average ± SD % decrease in O_2 reduction in the presence of the inhibitors for no less than 3 determinations. [b] The S-oxygenation of methimazole, the most widely used marker activity of FMO1, is included for comparison.

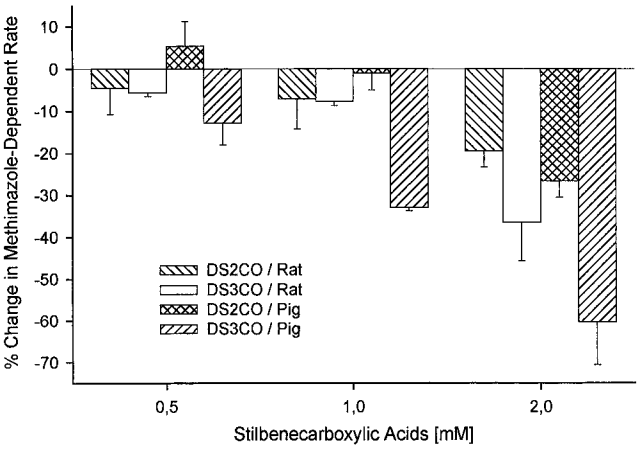


Figure 3. Effects of DS2CO and DS3CO on the methimazole *S*-oxidase activity of rat and pig liver microsomes. Activities were calculated from methimazole-dependent thiocholine oxidation at pH 7.4 in media identical to that listed in the legend to Figure 1 except that it also contained 0.3 mM thiocholine. The reactions were carried out in 10 mL Erlenmeyer flasks mounted in a shaker bath at 37 °C. After 4−5 min temperature equilibration, microsomes (2−3 mg/mL) were added, and 1 min later the reaction was started by adding methimazole (1.5 mM). The methimazole-dependent thiocholine oxidation was calculated from loss of thiocholine in aliquots removed at 0, 3, 6, and 9 min by the procedure described in ref 6.

near the limits of detection spectrophotometrically, which prevented substrate activity measurements with this compound. Although considerably more soluble above 1 mM, DS2CN and DS3CN would often slowly come out of solution. This could be largely prevented by adding bovine serum albumin (5 mg/mL) to the assay medium. Serum albumin had little, if any, effects on activity at low concentrations of the aminostilbene carboxylates, and where specified, the kinetic studies were carried out in the presence of this protein.

Both DS2CO and DS3CO stimulated initial rates of FMO1 and NADPH-dependent oxygen uptake, but neither affected the endogenous rate of H_2O_2 generation (data not shown), which suggests that the increase in oxygen uptake was not due to uncoupling of oxygen reduction from substrate oxygenation. However, reaction rates as a function of DS2CO or DS3CO concentration did not follow typical saturation kinetics, and the rates decreased with increasing concentrations above 1.0 mM (Figure 1). Both DS2CO and DS3CO also inhibited the oxidation of methimazole (Figure 2) at concentrations

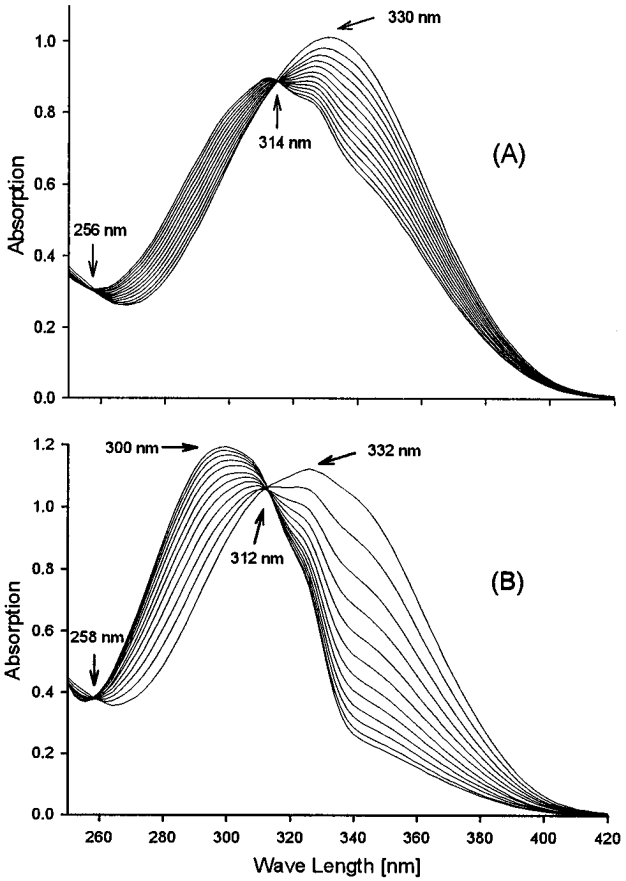


Figure 4. Changes in the spectra of DS2CO (A) and DS3CO (B) catalyzed by FMO1. The reactions were carried out in a thermostated, stirred cell mounted in a Hewlett-Packard diode array spectrophotometer, Model 8452A. The composition of the assay medium was identical to that listed in Figure 1 except FMO1 was decreased to about 0.1 μM, glucose 6-phosphate to 1.25 mM, and $NADP^+$ to 50 μM. After setting the base line to zero absorbance, 49 μM DS2CO (panel A) or DS3CO (panel B) was added and spectra were recorded every 10 s for 10 min. Only spectra taken at 1 min intervals are shown. In the absence of either NADPH or FMO, there was no detectable change in the spectra, but with both present the longer wavelength absorbance decreased with a concomitant increase in absorbance at shorter wavelengths with isosbestic points at the wavelengths indicated.

above 0.2−0.3 mM. DS3CO was a more effective inhibitor, and at 2 mM it decreased FMO-dependent O_2 uptake almost 80% with methimazole and with all other substrates tested (Table 1). However, below 0.2 mM, both DS2CO and DS3CO stimulated the oxidation of methimazole 5−10%. Although small, the stimulation was reproducible and observed consistently with all preparations of FMO1.

DS2CO and DS3CO also inhibited the oxidation of methimazole catalyzed by the microsomal-bound FMO (Figure 3). The pattern of inhibition was similar to that observed with purified FMO1. DS3CO was again the more effective inhibitor with both rat and pig liver microsomes, but methimazole oxidase activity of rat liver was less sensitive to DS3CO than that of pig liver microsomes. While inhibition of FMO turnover by a metabolite cannot be completely excluded, preincubation for several minutes before adding methimazole did not increase inhibition. Furthermore, the rates shown were calculated from initial rates when the concentration of product (or products) should be negligible.

The (dimethylamino)stilbene carboxylates were presumably oxidized to *N*-oxides, but a procedure for the chemical synthesis of *N*-oxides to serve as chromatographic standards could not be developed. Because of the pronounced inhibition, the amount of product that could be generated with FMO1 was too limited for isolation and identification without an authentic standard. Although the products were not identified, spectra (Figure 4A,B) indicate that FMO1 catalyzed the oxidation of both DS2CO and DS3CO to compounds that are spectroscopically different from the parent compounds.

Table 2. DS2CO and DS3CO Inhibition Constants for the Oxidation of Methimazole Catalyzed by FMO1 and CMO[a]

		K_i's (mM)			
		FMO1		CMO	
inhibitor	varied substrate	comp[b]	uncomp	comp	uncomp
DS2CO	methimazole	1.1	0.65	0.63	0.54
	NADPH	0.15	2.2	0.070	1.91
DS3CO	methimazole	0.30	1.9	0.20	0.17
	NADPH	0.13	1.2	0.062	0.69

[a] The inhibition constants were calculated from the slope and intercept changes (Figures 5 and 6) with the following equations: $K(\text{comp}) = [I]V^*_{max}K_m/V_{max}K^*_m - V^*_mK_m$; and $K(\text{comp}) = [I]V^*_{max}/V_{max} - V^*_{max}$; where [I] = concentration of inhibitor; V_{max}, K_m and V^*_{max}, K^*_m are the *x* and *y* intercepts in the absence and (*) presence of [I], respectively. The values are averages of 2−3 determinations with different preparations of FMO1 or CMO and concentrations of [I] that varied from 0.05 to 0.5 mM. None of the values included in the averages varied more than ±2.5 times the K_i's listed. [b] Abbreviations: comp = competitive; uncomp = uncompetitive.

Kinetic studies on the mechanism of DS2CO inhibition of methimazole oxidation gave a noncompetitive (mixed) pattern (Figure 5) with variable methimazole. However, at saturating methimazole (1.5 mM) and variable NADPH, the pattern was largely competitive (Figure 5B), as indicated by the magnitude of inhibition constants (Table 2) calculated from slope and intercept changes of double reciprocal plots of rates as a function of NADPH concentration (Figure 5B).

DS2CO and DS3CO also inhibit CMO, the bacterial analog of FMO (*15*), and as with the mammalian enzyme, DS3CO was the more effective inhibitor. The inhibition of CMO by the aminostilbene carboxylic acids gave a classic noncompetitive pattern with methimazole as the variable substrate, with K_i's of 0.20 mM for DS3CO and 0.63 mM for DS2CO (Figure 6A, Table 2). However, with NADPH as the variable substrate, the pattern was again largely competitive (Figure 6B, Table 2), which suggests that, as with FMO1, the aminostilbene carboxylates compete with NADPH for the same catalytic intermediate of CMO. They are not, however, nonspecific flavoprotein antagonists. At 2 mM, neither DS2CO nor DS3CO had any detectable effects on activity of rat or pig liver NADPH−cytochrome P450 reductase measured by following reduction of cytochrome *c* (*16*).

Discussion

The pronounced inhibition of FMO1 by the (dimethylamino)stilbene carboxylic acids was unexpected. Earlier studies with sulindac sulfide (*8*) and analogs of lipoic acid (*7*) suggested that an anionic group on a rigid side chain far enough removed from the nucleophilic heteroatom would not influence interactions of the substrate with the enzyme. While it appears that increasing distance between the amine and carboxylate groups does facilitate interactions with the catalytic site (DS3CO vs DS2CO, Figure 1), this also increases inhibitor activity

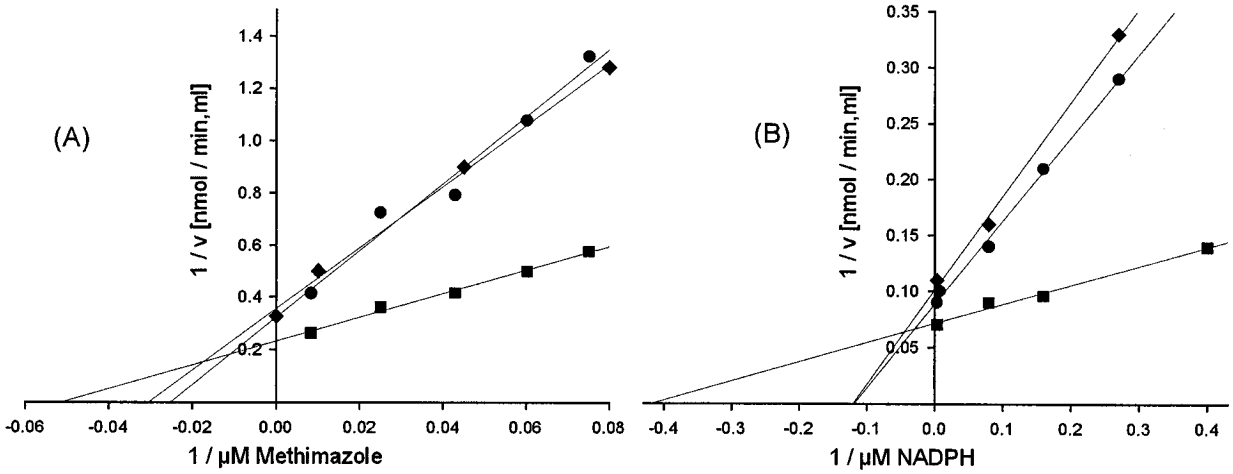


Figure 5. Lineweaver−Burk plots for FMO1-catalyzed oxidation of methimazole ± DS2CO or DS3CO with (A) methimazole and (B) NADPH as the varied substrate. The reaction rates for the plots shown were calculated from methimazole-dependent thiocholine oxidation at pH 7.4 minus (■) and plus 0.33 mM DS2CO (●) or 0.25 mM DS3CO (♦) with 0.25 mM NADPH and variable methimazole (A) or 1.5 mM methimazole and variable NADPH with 0.5 mM DS2CO (●) or DS3CO (♦) (B) measured as described in the legend to Figure 3 by following methimazole-dependent thiocholine oxidation in aliquots withdrawn at 0, 3, 6, and 9 min. The concentration of FMO1 was 0.6 μM.

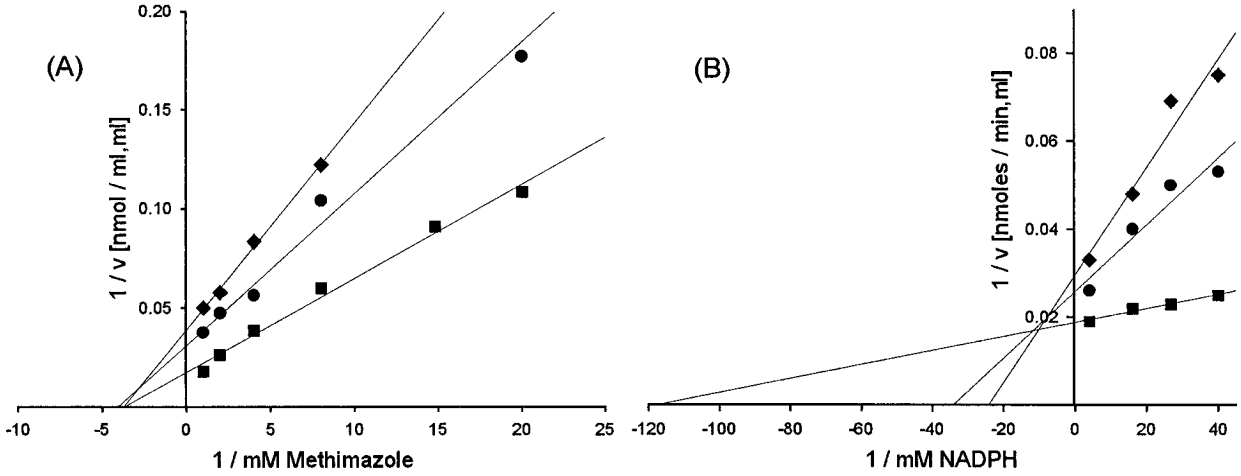


Figure 6. Lineweaver−Burk plots for CMO-catalyzed oxidation of methimazole ± DS2CO or DS3CO with (A) methimazole and (B) NADPH as the varied substrate. Reaction rates were calculated from initial rate of methimazole-dependent oxygen uptake as described in media described in the legend to Figure 2 minus (■) and plus (●) 0.5 mM DS2CO or (♦) 0.5 mM DS3CO. Because of the thermal instability of CMO, the reactions were carried out at 15 °C with variable methimazole and at 20 °C with variable NADPH. Neither DS2CO nor DS3CO had detectable substrate activity with CMO.

at concentrations above 1 mM. The distance between the nucleophilic center and the anionic group is not, however, the only factor. *N*,*N*-Dimethylcinnamic acid (Chart 1, CCO) had no detectable substrate or inhibitor activities even though molecular models suggest that the distance between the amino and carboxylate groups is not that different from DS2CO. This suggests that the second planar ring may be essential for both substrate and inhibitor activity of the aminostilbene carboxylic acids.

Kinetic studies with methimazole as the variable substrate (Figure 5A) indicate that DS3CO and DS2CO interact with two different catalytic intermediates of the enzyme. The competitive K_i is probably due to the interaction of these stilbene derivatives with the enzyme-bound 4a-hydroperoxyflavin. While this form of the enzyme has a bound $NADP^+$, the pronounced competitive inhibition with NADPH strongly suggests that DS3CO and DS2CO also bind (or interact) with the oxidized flavoprotein which is the only enzyme intermediate that binds NADPH (*4*). While NADPH and the inhibitors both interact with the oxidized form of the flavoprotein, they may bind at different sites and conformational changes induced by these stilbene derivatives prevent NADPH binding.

This interpretation is also supported by the kinetic studies carried out with CMO—a bacterial analog of FMO. While CMO catalyzes NADPH- and O_2-dependent oxidation of xenobiotic sulfur compounds (*15*), it does not accept amine or other FMO substrates bearing nitrogen. Because DS3CO and DS2CO are not substrates for CMO, they do not compete with methimazole for the enzyme-bound hydroperoxyflavin. However, the inhibition is at least partially competitive (Table 2), which suggests that DS2CO and DS3CO bind with a form of the enzyme that also interacts with methimazole. The classic noncompetitive pattern (Figure 6A) of double reciprocal plots of velocity as a function of methimazole concentrations ± DS2CO or DS3CO suggests that the inhibitors effectively remove a fraction of the enzyme from the catalytic cycle, apparently by blocking NADPH binding to the oxidized enzyme (Figure 6B), but have less effect on the other intermediate forms of the enzyme. The kinetic data strongly indicate that inhibition of NADPH

binding is primarily responsible for the inhibition of CMO by the aminostilbene carboxylic acids. Whether only flavoenzymes similar in mechanism to FMO will be inhibited by the aminostilbene carboxylates remains to be determined. However, the aminostilbene carboxylates have no effect on activity of NADPH−cytochrome P450 reductase, which indicates that they are not nonspecific flavoenzyme antagonists.

DS2CO and DS3CO are the first lipophilic anionic compounds tested that inhibit the membrane-bound flavoenzyme as effectively as purified FMO1 (Figures 2 and 3). Perhaps interference with NADPH binding (which must bind to an exposed site on the purified and microsomal-bound enzyme) accounts for this phenomenon.

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