Thiophene Sulfoxides as Reactive Metabolites: Formation upon Microsomal Oxidation of a 3-Aroylthiophene and Fate in the Presence of Nucleophiles *in Vitro* and *in Vivo*

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Oxidative metabolism of a 3-aroylthiophene, **1**, by rat liver microsomes in the presence of mercaptoethanol as a trapping agent led to the isolation of four main compounds, **2**−**5**, which have been isolated and characterized by UV, ^{1}H NMR, and mass spectroscopy. They all derive from two primary metabolites, **2** and **3**, which result from the nucleophilic addition of mercaptoethanol to a reactive, very electrophilic intermediate formed by sulfoxidation of the thiophene ring of **1**. Further reactions of diastereoisomers **2** and **3** with mercaptoethanol led to compound **4** that is opened at the level of its thiophene ring and, eventually, to a final metabolite **5** resulting formally from the addition of mercaptoethanol on the 4,5-double bond of the thiophene ring of **1**. Compound **5** is very stable even in the presence of a large excess of mercaptoethanol. Similar reactions were observed upon microsomal oxidation of **1** in the presence of another thiol, *N*-acetylcysteine. Final metabolites **8a** and **8b** equivalent to **5** except for the replacement of its mercaptoethanol substituent with an *N*-acetylcysteinyl group were isolated and characterized by UV, ^{1}H NMR, and mass spectroscopy. Interestingly, after treatment of rats with **1**, metabolites **8a** and **8b** could be detected in urine, indicating that the successive reactions, that were observed *in vitro* after microsomal oxidation of **1** in the presence of a thiol-containing trapping agent, also occur *in vivo*, glutathione acting as a nucleophile in that case. These data provide clear evidence for the intermediate formation of a reactive, electrophilic thiophene sulfoxide in metabolic oxidation of **1** *in vitro* and *in vivo*. They also provide the first data on the complex reactivity of such thiophene sulfoxides, whose chemistry is poorly known, and on their fates in living organisms.

Introduction

Although there is evidence that several thiophene derivatives cause toxic effects (*1*−*4*), very little is known not only on the molecular mechanisms of these effects but also, in a more general manner, on the oxidative metabolism of the thiophene ring in mammals (*5*, *6*). Oxidation of tienilic acid, a diuretic drug that was involved in the appearance of rare cases of immunoallergic hepatitis (*7*, *8*), leads to 5-hydroxytienilic acid as a major metabolite *in vivo* and *in vitro* (*9*). This 5-hydroxylation of the thiophene ring of tienilic acid is mainly catalyzed by cytochrome P450 2C9 in human liver (*10*, *11*) and leads to a suicide inactivation of this cytochrome (*12*). It has been recently proposed (*12*) that the thiophene sulfoxide of tienilic acid could be the reactive, electrophilic metabolite responsible both for the formation of 5-hydroxytienilic acid and for the covalent binding of tienilic acid to liver proteins including P450 2C9 itself. According to this mechanism (Figure 1), tienilic acid sulfoxide would rapidly react with various nucleophiles by a Michael-type addition at position 5 of the thiophene ring; reaction with H_2O would lead eventually to 5-hydroxytienilic acid whereas reaction with nucleophilic residues of proteins would result in covalent binding of tienilic acid to proteins. Intermediate formation of a thiophene sulfoxide metabolite has been recently established in the oxidative metabolism of two thiophene derivatives (*13*, *14*). Oxidation of the isomer **1** of tienilic acid with liver microsomes in the presence of a good nucleophile such as mercaptoethanol has led to the isolation of a 2,5-dihydrothiophene sulfoxide as a major metabolite which clearly derived from the addition of mercaptoethanol to the thiophene sulfoxide of **1** (*13*). The major urinary metabolite of thiophene itself in rats has been shown only recently to be a mercapturate deriving formally from the addition of *N*-acetylcysteine to thiophene sulfoxide (*14*) (Figure 1). These results suggest that thiophene sulfoxides, a new class of reactive metabolites, could play a central role in the oxidative metabolism of thiophene compounds.

However, it is noteworthy that the chemistry of thiophene sulfoxides is still poorly known (*15*). In fact, only very few thiophene sulfoxides have been prepared and identified so far. Most of them bear two bulky substituents at positions 2 and 5 (*16*), and it is only very recently that a complete X-ray structure has been reported for one of them (*17*). Very few data are available on their chemical reactivity if one excepts the Diels−Alder reactivity of thiophene sulfoxide itself and of a few substituted derivatives (*15*, *18*−*22*). As far as sulfoxides of thiophenes bearing a keto substituent, such as tienilic acid and its isomer **1**, are concerned, almost nothing was

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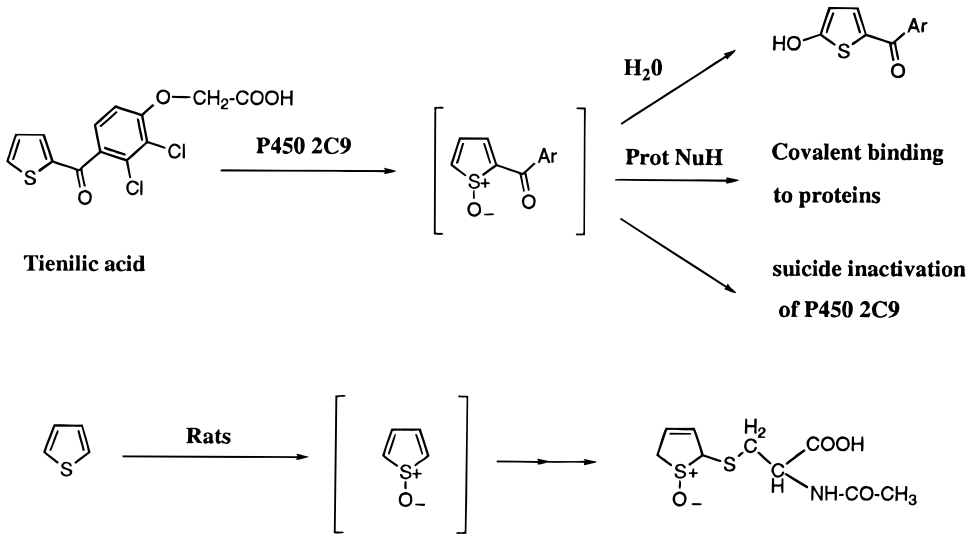


Figure 1. Involvement of thiophene sulfoxides as key intermediates in the *in vitro* microsomal oxidation of tienilic acid, and in the *in vivo* metabolism of thiophene in rats.

known about their chemical reactivity and possible behavior in a biological medium. This article describes in a complete manner the formation of a thiophene sulfoxide during metabolism of **1** *in vitro*, its trapping by thiol-containing nucleophiles, and the complex reactivity of the derived thiophene sulfoxide−nucleophile adducts as a function of the incubation conditions. This work provides insight into the reactions that occur *in vivo* after formation of this thiophene sulfoxide and its reaction with glutathione whose concentration is very high in many cells, particularly in the liver. It also provides a first view on the chemical reactivity of aroylthiophene sulfoxides and of their adducts with thiol-containing nucleophiles.

Experimental Section

All reagents were of the highest quality commercially available. Tienilic acid isomer **1** was a gift of Anphar-Rolland Laboratories (Chilly-Mazarin, France). Its labeled derivative (^{14}COAr, 25 Ci/mol, 98% radiochemical purity determined from HPLC as described previously (*10*)) was prepared by Amersham (Bucks, U.K.).

Preparation of Rat Liver Microsomes. Sprague-Dawley male rats (200 g) were treated with clofibrate (10% v/v in maize oil) according to the following protocol: day 0: 250 mg/kg ip, day 1: 500 mg/kg ip, and day 2: 1000 mg/kg ip. On day 4, rats were sacrified and microsomes of pooled livers were prepared as previously described (*23*). Protein concentrations were estimated by the method of Lowry (*24*); cytochrome P450 concentrations were measured according to the method described by Omura and Sato (*25*).

Microsomal Incubations and HPLC Analysis. In analytical conditions, rat liver microsomal suspensions containing 0.15 nmol of cytochrome P450 (1.6 nmol of P450·(mg of protein)$^{-1}$), 15 nmol of [^{14}C]-**1** (25 mCi/mmol), and mercaptoethanol were mixed at 4 °C in a hemolysis tube in 150 μL of 0.1 M phosphate buffer (pH 7.4), containing 1 mM diethylenetriaminepentaacetic acid (DETAPAC).[1] After a 3 min preincubation step at 37 °C with stirring under air, the reaction was started by the addition of an NADPH-generating system (2 U/mL glucose-6-phosphate dehydrogenase, 10 mM glucose 6-phosphate, and 1 mM NADP). Controls were done in the absence of the NADPH generating system. After 20 min, the reactions were stopped either in neutral conditions by the addition of 75 μL of acetonitrile or in acidic conditions by addition of 80 μL of CH_3CN/CH_3COOH, 72.5/7.5 v/v. After 10 min at 4 °C, proteins were separated by microcentrifugation and CH_3CN was evaporated under a nitrogen flux. Acetate buffer (0.1 M, pH 4.6) was then added to the samples prior to storage at −80 °C and analytical chromatography. Large-scale incubations of 10−50 mL were performed for preparative chromatography according to a similar protocol with gentle stirring to overcome microsome aggregation. After a 10 min, 2000*g* centrifugation, incubation supernatants were further purified on a SepPack C18 column (Waters-Millipore) and metabolites, as well as remaining **1**, eluted with 2 mL of methanol. High-pressure liquid chromatography (HPLC) was done on an Altex 320 gradient system using a C8 5 μm MOS Hypersil column (1 mL/min flow rate) with two main elution gradients: gradient A (solution C: 0.1 M ammonium acetate, pH 4.6; solution D: H_2O/CH_3CN, 50/50 v/v; 0−100% D in C in 20 min) and gradient B (same solutions, starting with 33% D in C isocratic for 6 min, 45% D in C isocratic for 7 min, followed by a gradient 45−100% in 7 min). UV and visible spectra were recorded on-line by a Spectra-Focus system (Spectra-Physics); 0.5 mL fractions were collected in 3 mL propylene tubes (LKB collector), and radioactivity was counted after addition of 2 mL of Picofluor 30 (Packard) on a Packard Tri-carb 4500 scintillation counter.

Covalent Binding to Microsomal Proteins. Covalent binding of metabolites of **1** to microsomal proteins was detected according to a previously described protocol (*26*−*28*) with the following modifications (*10*): 50 μL incubation medium aliquots were dropped on glass fiber filters (GF/B from Whatman) and immediately dipped in a large excess of acid (10% trifluoroacetic acid in water) in order to precipitate proteins. After two 10 min washes with methanol and one with CH_3COOEt, filters were air-dried and radioactivity was counted after addition of 3 mL of toluene scintillator (Packard).

Spectroscopic Studies. ^{1}H and ^{13}C NMR spectra were recorded on Bruker WM 250 and AM 400 spectrometers. Chemical shifts are given in ppm relative to $(CH_3)_4Si$ and J in Hz. The peak of monodeuteriated water (HOD) at 4.80 ppm was eliminated when necessary either by presaturation or by differential relaxation water elimination Fourier transform (WEFT) experiment, or displaced by heating at 70 °C. UV and visible spectra were recorded on a Kontron Uvikon 820 spectrophotometer; IR spectra were recorded on a Perkin FT IR spectrometer in Nujol. Mass spectra were recorded on a Riber-Mag R10-10C spectrometer with chemical ionization (NH_3); in the text, data are given after CIMS for chemical ionization mass spectrum.

Preparation of Compounds 2, 3, 4, and 5. These compounds were prepared from 35 min incubations at 37 °C of large volumes (20−40 mL) of 0.1 M phosphate buffer (pH 7.4) containing microsomal suspensions (1.2 μM P450), 70 μM **1**, 1

[1] Abbreviations: COSY: Correlation spectroscopy; DETAPAC: diethylenetriaminepentaacetic acid; HOD: monodeuterated water; NOE: nuclear Overhauser effect; WEFT: water elimination Fourier transform.

mM DETAPAC, an NADPH-generating system, and proper concentrations of mercaptoethanol (from Figure 3). After centrifugation at 2000*g* for 10 min, incubation supernatants were purified on a SepPack C_{18} column and metabolites eluted with methanol. After evaporation of methanol under nitrogen, the pH of the solution was adjusted to 3 by addition of HCl, and metabolites were extracted with CH_3COOEt. Their separation by HPLC gave pure compounds **2**, **3**, **4**, or **5** as ammonium salts after lyophilization, in sufficient amounts (ca. 1 mg for **2**, **3**, and **4**, and 2−7 mg for **5**) for structure determination by ^1H NMR, UV, and mass spectroscopy, but not for elemental analysis. The ^1H NMR characteristics of their ammonium salts are described in the tables. Therefore, only data corresponding to the ^1H NMR spectra of their methyl esters (prepared upon reaction with CH_2N_2 in Et_2O) and to the mass spectra of their ammonium salts are reported in the experimental part.

Compounds 2 and 3. Metabolites **2** and **3** were obtained in 60% yield from microsomal incubations in the presence of 100 μM mercaptoethanol. Preparative HPLC of metabolites **2** or **3** involved the use of ammonium acetate (50 mM) as solution A in order to prevent degradation upon lyophilization. Successive slow gradients such as 25−33% solution B (50% CH_3CN in H_2O) in A in 20 min allowed progressive enrichment and isolation of each metabolite as hygroscopic orange powders: IR (Nujol) ν = 1610, 1480, 1425, 1405, 1340, 1290, 1270, 1055, and 1030 cm^{-1}; CIMS (NH_3) *m/z* (%): 331 (25), 407 (100, $MH^+ - H_2O$), and 424 (10, $M^+ + NH_4 - H_2O$).

Compound 4. Fractions containing **4** and **5** were first isolated by HPLC (gradient A); **4** was further purified to homogeneity in isocratic conditions (40% solution B). It was obtained as a white powder. CIMS (NH_3) *m/z* (%): 172 (100), 331 (80), 348 (40), 377 (5), 391 (12), 408 (10), 424 (1), 451 (2), 467 (2), 485 (5), 502 (1), 545 (0.5), and 563 (1, MH^+). Methyl ester of **4**: ^1H NMR (250 MHz, CD_2Cl_2) δ = 7.29 (s, 1H, H_2), 7.17 (d, 9 Hz, 1H, $H_{2'}$), 6.80 (d, 9 Hz, 1H, $H_{3'}$), 4.77 (s, 2H), 4.39 (dd, 10 and 6 Hz, 1H, H_4), 3.85 (6H), 3.80 (s, 3H, CH_3), 3.55 (dd, 10 Hz, 1H, H_5), 3.27 (dd, 6 Hz, 1H, H_5), 2.9 (4H), 2.84 (t, 6 Hz, 2H), 2.32 (1H, OH), and 2.16 (2H, OH); CIMS (NH_3) *m/z* (%): 345 (100), 362 (50), 391 (90), 423 (10), 499 (30), and 577 (5, MH^+).

Compound 5. Compound **5** was purified by HPLC (gradient A) and obtained as a faint yellow powder (ammonium salt): CIMS (NH_3) *m/z* (%): 172 (100), 331 (50), 348 (60), 391 (2), 409 (10, MH^+), and 426 (10, $M + NH_4^+$). Methyl ester of **5**: ^1H NMR (250 MHz, CD_2Cl_2) δ = 7.20 (d, 9 Hz, 1H, $H_{2'}$), 7.14 (s, 1H, H_2), 6.80 (d, 9 Hz, 1H, $H_{3'}$), 4.81 (dd, 8 and 1.5 Hz, 1H, H_4), 4.78 (s, 2H), 3.98 (dd, 12 and 8 Hz, 1H, H_5), 3.83 (m, 6 Hz, 2H), 3.80 (s, 3H, CH_3), 3.50 (dd, 12 and 1.5 Hz, 1H, H_5), and 2.85 (m, 6 Hz, 2H); CIMS (NH_3) *m/z* (%): 345 (100), 362 (60), 423 (30, MH^+), and 440 (3, $M + NH_4^+$).

Compound 6. A mixture of **2** and **3** (0.5 mg in 0.5 mL of H_2O) was treated with 0.5 mL of 2 M HCl for 2 h at 37 °C. Metabolite **6** was then purified by HPLC in gradient A conditions and obtained as a white powder (ammonium salt). CIMS (NH_3) *m/z* (%): 331 (40), 348 (5), 353 (45), 371 (3), 388 (3), 407 (80, MH^+), and 424 (3, $M + NH_4^+$). Methyl ester of **6**: ^1H NMR (250 MHz, CD_2Cl_2) δ = 7.27 (d, 9 Hz, 1H, $H_{2'}$), 7.13 (d, 5.5 Hz, 1H, H_5), 6.97 (d, 5.5 Hz, 1H, H_4), 6.83 (d, 9.0 Hz, 1H, $H_{3'}$), 4.79 (s, 2H), 3.93 (q, 6 Hz, 2H), 3.81 (t, 6 Hz, 2H), and 3.25 (s, 3H, CH_3); CIMS (NH_3) *m/z* (%): 367 (60), 385 (15), 421 (100, MH^+), and 438 (1, $M + NH_4^+$).

Compound 7. HPLC fractions coming from partial purification of **2** and **3** were treated with 50 mM HCl for 45 min at 37 °C in the presence of 10 mM mercaptoethanol. HPLC analysis (gradient A or B) showed formation of **7** (70% yield) as well as little amounts of **6** (10% yield). Purification of **7** by HPLC (gradient A) gave 0.3 mg of **7**. CIMS (NH_3) *m/z* (%): 172 (100), 331 (85), 348 (40), 391 (10), 407 (35), 424 (5), and 485 (5, MH^+). Methyl ester of **7**: ^1H NMR (250 MHz, CD_2Cl_2) δ = 7.31 (d, 1.5 Hz, 1H, H_2), 7.22 (d, 9 Hz, 1H, $H_{2'}$), 6.80 (d, 9 Hz, 1H, $H_{3'}$), 6.49 (d, 10 Hz, 1H, H_5), 6.24 (dd, 10 and 1.5 Hz, 1H, H_4), 4.79 (s, 2H), 3.80 (7H, $H_{2''} + CH_3$), 2.96 (t, 6 Hz, 2H), and 2.86 (t, 6 Hz, 2H); CIMS (NH_3) *m/z* (%): 345 (100), 362(5), 389 (25), 421 (10), and 499 (20, MH^+).

Preparation of Compounds 8a and 8b. Incubation of **1** (100 μM) with microsomes containing 1.2 μM cytochrome P450 in the presence of *N*-acetylcysteine (5 mM) and an NADPH-generating system in a total volume of 40 mL was done for 2 h at 37 °C. After acidification to pH 4.5, centrifugation for 10 min at 2000*g*, SepPak extraction, and elution with CH_3OH, the solvent was evaporated and the residue taken in 0.5 mL of H_2O, acidified to pH 2 with HCl, extracted in CH_3COOEt, and immediately methylated with CH_2N_2 in Et_2O. Dimethyl esters of compounds **8a** and **8b** were purified by HPLC on a MOS hypersil column with isocratic elution (CH_3CN/H_2O, 45:55).

8a dimethyl ester: ^1H NMR (250 MHz, CD_2Cl_2) δ = 7.18 (d, 9 Hz, 1H, $H_{2'}$), 7.12 (s, 1H, H_2), 6.80 (d, 9 Hz, 1H, $H_{3'}$), 6.48 (dd, 5.2 Hz, 1H, NH), 4.83 (dd, 5−2 Hz, 1H, $H_{1''}$), 4.78 (s, 2H, OCH_2), 4.69 (dd, 8−1.5 Hz, 1H, H_4), 3.94 (dd, 13−8 Hz, 1H, H_{5a}), 3.80 (s, 3H, $OCH_{3'}$), 3.78 (s, 3H, $OCH_{3''}$), 3.45 (dd, 13−1.5 Hz, 1H, H_{5b}), 3.23 (dd, 14−5 Hz, 1H, $H_{2''a}$), 3.14 (dd, 14−5 Hz, 1H, $H_{2''b}$), and 2.00 (s, 3H, $COCH_3$); CIMS (NH_3) *m/z* (%): 345 (100), 362 (10), 522 (4, MH^+), and 539 (0.25, $M + NH_4^+$). $H_{1''}$ and $H_{2''}$ are the protons of C_α and C_β of the cysteine residue.

8b dimethyl ester: ^1H NMR (250 MHz, CD_2Cl_2) δ = 7.43 (d, 10 Hz, NH), 7.21 (d, 9 Hz, 1H, $H_{2'}$), 7.17 (s, 1H, H_2), 6.81 (d, 9 Hz, 1H, $H_{3'}$), 5.02 (dt, 10−5 Hz, 1H, H_{5a}), 4.79 (s, 2H, OCH_2), 4.70 (dd, 8−1 Hz, 1H, H_4), 3.95 (dd, 13−8 Hz, 1H, H_{5a}), 3.80 (s, 3H, $OCH_{3'}$), 3.72 (s, 3H, $OCH_{3''}$), 3.48 (dd, 13−1 Hz, 1H, H_{5b}), 3.27 (dd, 14−5 Hz, 1H, $H_{2''a}$), 3.01 (dd, 14−5 Hz, 1H, $H_{2''b}$), and 2.11 (s, 3H, $COCH_3$); CIMS (NH_3), *m/z* (%): 178 (100), 345 (80), 365 (5), 522 (35, MH^+), and 539 (4, $M + NH_4^+$).

Metabolism of 1 *in Vivo* in Rats. Two male Sprague-Dawley rats (300 g) were injected ip with **1** (30 mg/kg, 0.5 mCi/mmol, Tris salt in H_2O), and urine was collected in metabolic cages for 24 h. Each urine fraction (1−2, 2−4, 4−6, and 6−24 h) was extracted on a SepPak C_{18} with CH_3OH and analyzed by HPLC. The extracts were combined, evaporated under N_2 and taken up in water, acidified to pH 2 with HCl, back-extracted in CH_3COOEt, and methylated with CH_2N_2 in Et_2O. Three major metabolites were isolated. The first two ones exhibited ^1H NMR, UV, and CIMS spectra identical to those of **8a** and **8b** dimethyl esters described above. The third one was found to be relatively unstable, giving back to **1** methyl ester. It had a UV spectrum identical to those of **8a** and **8b** dimethyl esters. ^1HNMR (250 MHz, CD_2Cl_2 after exchange with D_2O) δ = 7.28 (d, 8 Hz, 1H, $H_{2'}$), 7.20 (s, 1H, H_2), 6.81 (d, 8 Hz, 1H, $H_{3'}$), 4.89 (dd, 8−1.5 Hz, 1H, H_4), 4.79 (s, 2H, OCH_2), 4.54 (t, 5 Hz, $H_{1''}$), 3.96 (dd, 13−8 Hz, 1H, H_{5a}), 3.80 (s, 3H, $COOCH_{3'}$), 3.76 (s, 3H, $COOCH_{3''}$), 3.51 (dd, 13−1.5 Hz, 1H, H_{5b}), 3.13 (dd, 14−5 Hz, 1H, $H_{2''a}$), 3.04 (dd, 14−5 Hz, 1H, $H_{2''b}$).

Results and Discussion

Oxidation of 1 by Liver Microsomes in the Presence or Absence of Mercaptoethanol. Oxidation of compound **1** (^{14}C-labeled on the keto group) with liver microsomes from rats pretreated with clofibrate (as an inducer (*29*)) in the presence of NADPH and O_2, the usual cofactors of cytochrome P450-dependent monooxygenases, led to soluble metabolites (57% of starting **1** under the used conditions) and to metabolites covalently bound to microsomal proteins (21% of starting **1**). The HPLC chromatogram of the soluble fraction (Figure 2A) revealed the formation of many metabolites less hydrophobic than **1**, none of which represented more than 5% of the total metabolism. Under identical conditions but in the presence of 100 μM mercaptoethanol in the incubation, the total transformation of **1** increased to 93%, with a marked decrease in the covalent binding of metabolites of **1** to protein (down to 6%) and a dramatic change in the HPLC chromatogram profile (Figure 2B). Three adducts, called metabolites **2**, **3**, and **4**, clearly appeared. The ratio of these metabolites was greatly dependent on the starting concentration of mercaptoethanol in the incubation medium. Under identical incubation condi-

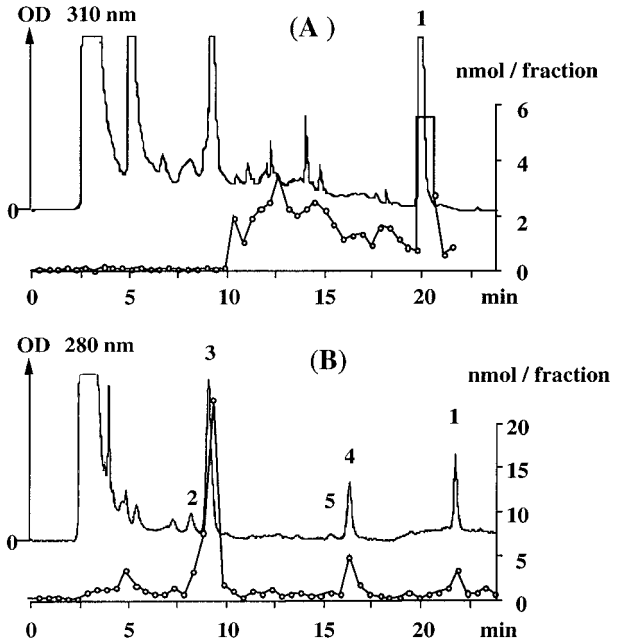


Figure 2. HPLC chromatograms from incubations of rat liver microsomes with 70 μM **1** and 1 mM NADPH in the presence (B) or absence (A) of mercaptoethanol (100 μM) for 20 min. Liver microsomes from clofibrate-preteated rats contained 1 μM cytochrome P450 (1.6 nmol of P450/mg of protein). (—) UV detection at 310 or 280 nm; (—∘—): radiochromatogram.

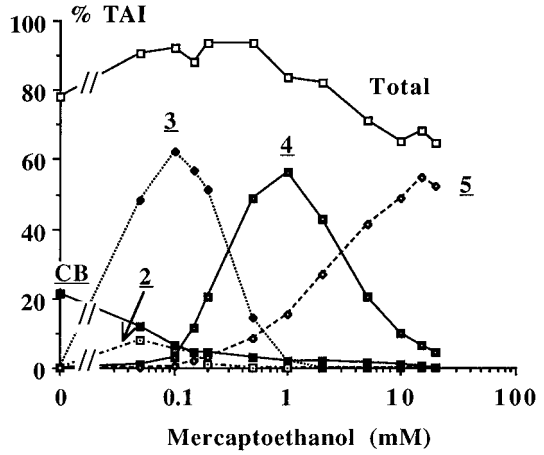


Figure 3. Proportions of microsomal metabolites of **1** as a function of mercaptoethanol concentration used in the incubate. Conditions as in Figure 2 except for mercaptoethanol concentration used in the incubate. CB: level of covalent binding to microsomal proteins. Yields are given in % relative to starting **1**.

tions (NADPH and protein concentrations, and incubation time), conversion of **1** slightly decreased when the starting concentration of mercaptoethanol increased from 1 to 20 mM (Figure 3). The covalent binding of metabolites of **1** to microsomal proteins greatly decreased in the presence of mercaptoethanol as a trapping agent in the incubation medium. It was almost completely suppressed in the presence of mercaptoethanol concentrations higher than 1 mM. Metabolites **2** and **3** appeared when small concentrations of mercaptoethanol were used (Figure 3). They reached a maximum level for a starting mercaptoethanol concentration around 100 μM. At 1 mM mercaptoethanol, they were almost absent while metabolite **4** now represented 57% of the total metabolism of **1**. At higher concentrations of mercaptoethanol, the amounts of **4** decreased while a new metabolite **5** began to appear. It was almost the only metabolite of **1** detected in the presence of 20 mM mercaptoethanol.

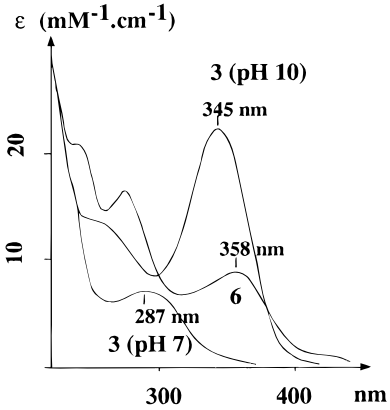
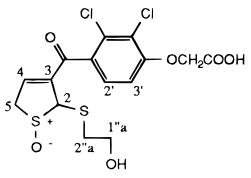
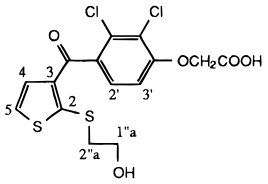


Figure 4. UV spectra of metabolite **3** at pH 7 and 10, and of metabolite **6** (in H_2O).

The aforementioned results suggested that microsomal oxidation of **1** produced a very electrophilic intermediate able to react with various nucleophiles present in the medium leading to an intense covalent binding to proteins and the formation of many reaction products in small amounts. Trapping of this intermediate with mercaptoethanol leads to initial metabolites **2** and **3** which are further transformed into metabolites **4** and **5** in the presence of mercaptoethanol in excess. The following section will focus on the characterization and chemical properties of these metabolites.

Isolation and Characterization of Compounds 2 and 3. In agreement with the data of Figure 3, preparative incubations of **1** with rat liver microsomes (containing 2 μM cytochrome P450) in the presence of 1 mM NADPH and 100 μM mercaptoethanol mainly gave compounds **2** and **3**. Both compounds were isolated by preparative HPLC. The major metabolite **3** exhibited a UV band at 287 nm (ϵ = 7200 $M^{-1}\cdot cm^{-1}$) in H_2O at neutral pH. At basic pHs (8.5−13), its UV spectrum was characterized by an intense red-shifted band at 345 nm (ϵ = 21 200 $M^{-1}\cdot cm^{-1}$) (Figure 4). The species absorbing at 287 and 345 nm were in equilibrium as a function of pH. ^{1}H NMR of **3** in D_2O at pH 4 (CD_3COOD) exhibited a set of signals expected for the protons of the aroyl moiety of **1** and the protons corresponding to a $S(CH_2)_2$-OH residue, and four additional signals: a singlet at 5.44 ppm and three signals at 7.02, 4.57, and 3.83 ppm corresponding to a $CHCH_aH_b$ system (Table 1). The two geminal protons absorbing at 3.83 and 4.57 ppm could be exchanged with D_2O at different rates. The latter was rapidly exchanged already at pH above 3 whereas the former was only exchanged at pH above 7. The irreversible transformation of **3** into **6** under acidic conditions (0.5−1 M HCl) was of great help for the determination of its structure. Actually, the structure of **6**, a very stable compound, could be easily deduced from its ^{1}H NMR spectrum, which exhibited the signals characteristic for the protons of the aroyl moiety of **1** and an $S(CH_2)_2OH$ group, and two doublets at 7.12 and 7.30 ppm with a coupling constant of 5.8 Hz as expected for two vicinal thiophene protons (*30*) (Table 1), and its mass spectrum displaying a molecular peak at 407 $(M + H)^+$. These data clearly showed that **6** was formally derived from **1** by introduction of a mercaptoethanol residue at position 2 of its thiophene ring (Figure 5). The UV spectrum of **6** (Figure 4) with an intense absorption at 358 nm (ϵ = 8200 $M^{-1}\cdot cm^{-1}$), which was found in thiophene derivatives such as **1**, was in good agreement with the presence of a thiophene ring in **6**.

Table 1. ^{1}H NMR[a] of Metabolites 2, 3, and 6

	 **2**	**3**	 **6**
H_2	5.67 (s, 1H)	5.44 (s, 1H)	
H_4	6.74 (broadened, 1H)	7.02 (d, 3.4, 1H)[b]	7.12 (d, 5.8, 1H)
H_5	under multiplet of $H_{1''}$[c]	3.83 (dd, 20−3.4, 1H)[d]	7.3 (d, 5.8, 1H)
	4.28 (dd, 3.4−18, 1H)	4.57 (dd, 20−2, 1H)[d]	
$H_{2'}$	7.42 (d, 8.8, 1H)	7.45 (d, 8.8, 1H)	7.37 (d, 8.8, 1H)
$H_{3'}$	6.95 (d, 8.8, 1H)	6.96 (d, 8.8, 1H)	6.97 (d, 8.8, 1H)
OCH_2	4.66 (s, 2H)	4.66 (s, 2H)	4.66 (s, 2H)
$H_{1''}$	3.78 (t, 6, 2H)	3.89 (t, 6, 2H)	3.93 (t, 6, 2H)
$H_{2''}$	2.89 (m, 2H)	3.02 (m, 2H)	3.33 (t, 6, 2H)

[a] Metabolites **2** and **3** as NH_4^+ salt in D_2O/CD_3COOD, pH 4.0 (400 MHz), or D_2O at pH 6.5 (250 MHz); **6** as NH_4^+ salt in D_2O; δ in ppm/Si$(CH_3)_4$; J in Hz. [b] Doublet because of partial exchange of the H absorbing at 4.57 ppm at pH >3, but quadruplet (dd, J = 3.7 and 1.5) in CD_3CN-D_2O-DCl mixtures in which no exchange of H_5 occurred. [c] Irradiation at 3.8 ppm led to the collapse of the signal at 4.28 ppm to a doublet (J = 3.4). [d] Slow exchange with D_2O of H absorbing at 3.83 and fast exchange of H at 4.57 at pH 6.5.

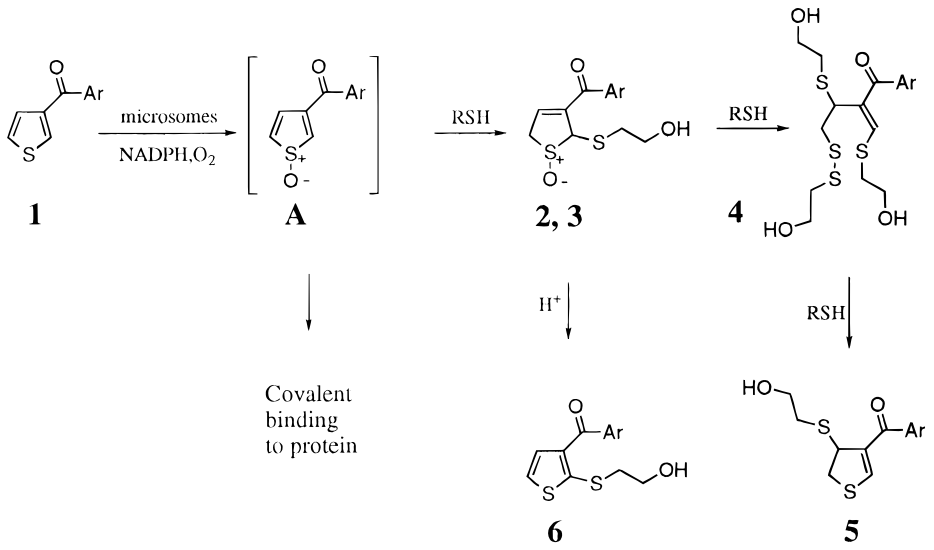


Figure 5. Main products formed upon metabolic oxidation of **1** in the presence of rat liver microsomes, NADPH, and mercaptoethanol. **2** and **3** are diastereoisomers. Compound **6** is not formed under these conditions; it was obtained afterward upon acidic treatment of **2** and **3**.

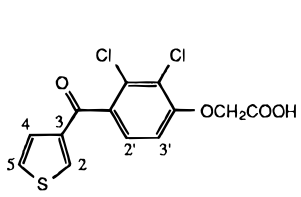
Metabolite **2** showed characteristics very similar to those previously described for **3**: (i) both compounds underwent an irreversible transformation to **6** in almost quantitative yields under acidic treatment; (ii) both compounds exhibited almost identical UV spectra at neutral (λ ~290 nm) and basic (λ ~345 nm) pH, with a reversible passage from the neutral to the basic form as a function of pH; (iii) the ^{1}H NMR spectrum of **2** was very similar to that of **3**, with the same kind of signals (identical multiplicity and integration, slightly different chemical shifts for some of them, as shown in Table 1).

All these data are in agreement with a 2,5-dihydrothiophene sulfoxide structure substituted at position 2 by a $S(CH_2)_2OH$ group, for compounds **2** and **3** which are diastereoisomers with a different relative configuration of their O and $S(CH_2)_2OH$ substituents (Figure 5). The presence of a band at 1030 cm^{-1} in the IR spectra of **2** and **3** could be assigned to the presence of a sulfoxide function in these compounds. Dehydratation of **2** and **3** with formation of **6** under acidic conditions (pH <1) is well-known for dihydrothiophene sulfoxides (*31*, *32*); it could explain the mass spectra (CI, NH_3) of **2** and **3** which both exhibit a peak at 407 corresponding to $[MH - H_2O]^+$ with the expected isotopic cluster (for the presence of two Cl) and fragments identical with those of the mass spectrum of **6**. The structures indicated for **2** and **3** also explain why the two geminal protons detected by ^{1}H NMR spectroscopy are easily exchanged in D_2O; their acidic character is due to their position α to two strong electron-withdrawing groups (SO and CH═CHCOAr). Finally, the presence of these acidic protons allows one to understand the pH-dependent UV spectra of **2** and **3** which are those of a conjugated ketone at neutral pH and of a highly conjugated anion at basic pH.

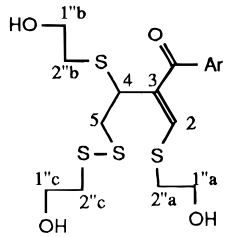
Reactivity of Compounds 2 and 3 with Mercaptoethanol. The data described in the preceding section showed that compounds **2** and **3** were reactive toward an excess of mercaptoethanol (Figure 3) as well as toward strong acids (formation of **6**). They could be isolated only after incubation of **1** with microsomes in the presence of low concentrations of mercaptoethanol and careful separation using HPLC at neutral pH. Upon reaction of freshly purified **2** and **3** with mercaptoethanol at 37 °C, it was found that the nature of the products was greatly dependent on the buffer pH.

(A) Reaction of 2 and 3 at pH between 4.5 and 6.5 and Isolation of Compound 4. In this pH range, **2** and **3** were quantitatively transformed into compound

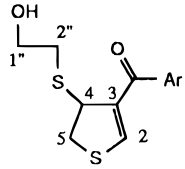
Table 2. ^{1}H NMR[a] of 1 and Metabolites 4, 5, and 7 (and Their Deuteriated Equivalents Obtained from 2 Bisdeuteriated at Position 5)



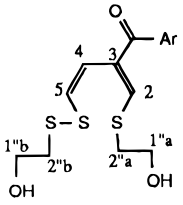
1[b]



4



5



7

	1[b]	**4**	**5**	**7**
H_2	8.12 (t, 1H)[b]	7.73 (s, 1H)	7.71 (s, 1H)	7.70 (s, 1H)
H_4	7.56[b]	4.42 (dd, 10−6, 1H) [s][c]	4.80 (dd, 8.5−1.5, 1H)	6.29 (d, 10, 1H) [s][c]
H_5	7.56[b]	3.31 (dd, 14−10, 1H) [D][c]	3.58 (dd, 13−1.5, 1H) [D][c]	6.65 (d, 10, 1H) [D][c]
		3.53 (dd, 14−6, 1H) [D][c]	4.03 (dd, 13−8.5, 1H) [D][c]	
$H_{2'}$	7.44 (d, 9, 1H)	7.30 (d, 8.8, 1H)	7.29 (d, 8.5, 1H)	7.34 (d, 8.8, 1H)
$H_{3'}$	6.97 (d, 9, 1H)	6.96 (d, 8.8, 1H)	6.96 (d, 8.5, 1H)	6.94 (d, 8.8, 1H)
OCH_2	4.67 (s, 2H)	4.66 (s, 2H)	4.66 (s, 2H)	4.65 (s, 2H)
$H_{1''a}$		3.73 (t, 6, 2H)	3.82 (t, 6, 2H)	3.73 (t, 6, 2H)
$H_{2''a}$		3.00 (t, 6, 2H)	2.91 (m, 6, 2H)	3.01 (t, 6, 2H)
$H_{1''b}$		3.82 (m, 2H)		3.79 (t, 6, 2H)
$H_{2''b}$		2.87 (m, 2H)		2.89 (t, 6, 2H)
$H_{1''c}$		3.87 (t, 6, 2H)		
$H_{2''c}$		2.94 (t, 6, 2H)		

[a] NH_4^+ salt in D_2O (250 MHz, and 400 MHz for **4**); δ in ppm/Si$(CH_3)_4$; J in Hz. [b] In D_2O, as NH_4 salt H_2 appears as a collapsed triplet ($J \approx 3$ Hz), H_4 and H_5 are not separated; in DMSO-d_6 all signals are separated: 8.09 (dd, 3−1.5, 1H, H_2) [d, 1.5 Hz],[c] 7.68 (dd, 5−3, 1H, H_5) [D],[c] 7.46 (dd, 5−1.5, 1H, H_4) [d, 1.5 Hz],[c] 7.38 (d, 8.5, 1H, $H_{2'}$), 6.92 (d, 8.5, 1H, $H_{3'}$), and 4.39 (s, 2H, OCH_2). [c] Data in brackets correspond to deuteriated compounds obtained from **2** (or **3**) bisdeuteriated at position 5 (see text); [D] indicates the presence of a deuterium at the corresponding position.

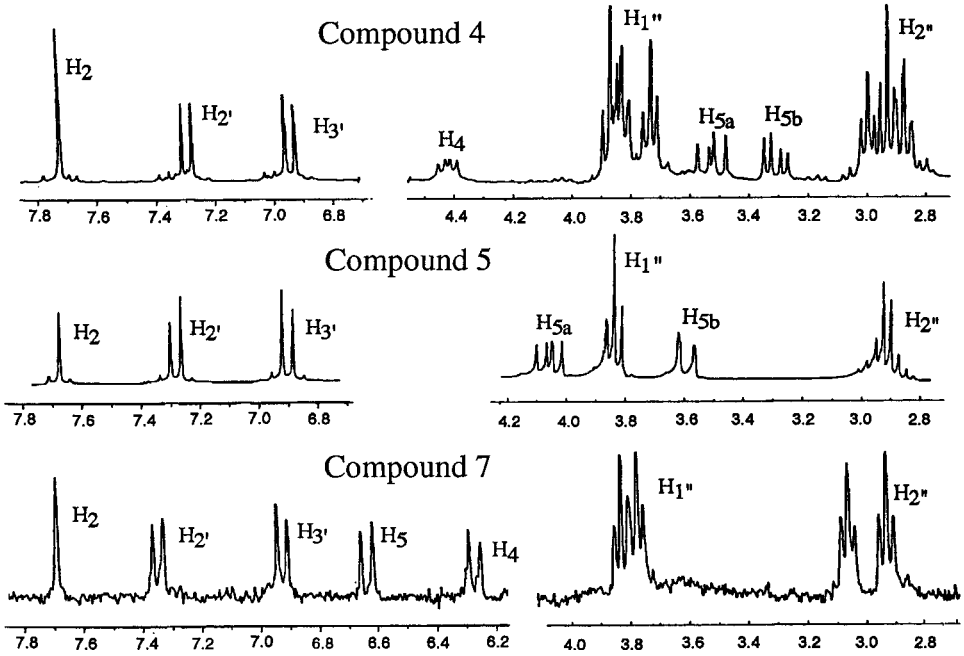
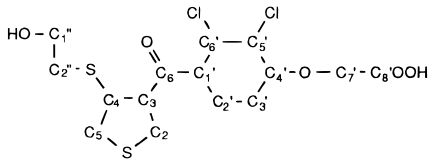


Figure 6. ^{1}H NMR spectra of compounds **4**, **5**, and **7** (D_2O, 250 MHz). The 4−6 ppm region involving the signals of OCH_2 and (HOD)[1] has been omitted for the sake of clarity. Labeling of protons are those shown in Table 2.

4. The reaction rate increased upon increasing the pH and/or mercaptoethanol concentration. For instance, at pH 4.6 and in the presence of 5 mM mercaptoethanol, formation of **4** was almost complete after 20 min whereas only 10% **4** was obtained under identical conditions in the presence of 0.5 mM mercaptoethanol. With this mercaptoethanol concentration but at pH 6.5, the formation of **4** was again quantitative in less than 20 min. Compound **4** was isolated by preparative HPLC. Its ^{1}H NMR spectrum in D_2O (or in CD_2Cl_2 for its methyl ester, see Experimental Section) revealed a set of signals corresponding to the intact aroyl moiety of **1**, the signals of three different $S(CH_2)_2OH$ groups that were clearly separated at 400 MHz, a singlet at low field (1H, 7.73 ppm), and a system of three coupled signals each integrating for one proton at 3.31 (J = 10 and 14 Hz), 3.53 (J = 6 and 14 Hz), and 4.42 ppm (J = 6 and 10 Hz) (Table 2 and Figure 6). The intense UV absorption of **4** (λ = 317 nm, ϵ = 20 800 $M^{-1}\cdot cm^{-1}$) and the presence of a low-field singlet at ca. 7.7 ppm that could be assigned to a vinylic proton indicated the presence of a double bond conjugated with the COAr group. The opened structure shown for compound **4** in Figure 5 and Table 2 is in agreement with its mass spectrum (m/z = 563 for MH^+) and a detailed analysis of its ^{1}H NMR spectrum. The presence of the $ArCOC{=}CHS(CH_2)_2OH$ moiety was indicated by an intense nuclear Overhauser effect (NOE) between the vinylic proton H_2 and the aromatic proton $H_{2'}$ in ortho position to the CO group and a ^4J coupling between H_2 and the proximal protons $H_{2''}$ of a mercap-

Table 3. ^{13}C NMR[a] of 1 and Compound 5



	1 (DMSO-d_6)	**1** (D_2O)	**5** (D_2O)
C_2	137.35 (CH)[b]	141.92 (CH)	161.33 (CH)
C_3	141.3	143.52	139.85
C_4	128.42 (CH)	131.16 (CH)[d]	51.37 (CH)
C_5	127.18 (CH)	129.92 (CH)[d]	45.32 (CH_2)
C_6	187.02	186.37	191.53
$C_{1'}$[c]	131.62	134.5	134.35
$C_{2'}$	127.71 (CH)	130.86 (CH)	129.80 (CH)
$C_{3'}$	111.95 (CH)	113.83 (CH)	113.65 (CH)
$C_{4'}$	157.06	159.29	158.44
$C_{5'}$	121.09	123	124.50
$C_{6'}$	129.41	133.36	132.42
$C_{7'}$	68.31 (CH_2)	70.53 (CH_2)	70.15 (CH_2)
$C_{8'}$	169.26	178.03	177.70
$C_{1''}$			63.32
$C_{2''}$			35.10

[a] **1** (10 mg) and **5** (7 mg) as NH_4^+ salts in D_2O or DMSO-d_6 (250 MHz); δ in ppm/Si$(CH_3)_4$, J in Hz. [b] Multiplicity determined by DEPT experiments (41). [c] Signals of the phenyl ring were assigned by selective INEPT experiments (42). [d] Assignment of C_4 and C_5 signals was only done in DMSO-d_6 (see text).

toethanol residue (see Table 2). The two geminal protons were assigned by their negative coupling constant ($J = -14$ Hz) determined from a correlation spectroscopy (COSY) 45 experiment (H_5, see Table 2). The presence of three $S(CH_2)_2OH$ groups in **4** was shown by ^1H NMR and mass spectroscopy. Moreover, the presence of a dithioether function was suggested by the easy transformation of **4** into **5** in the presence of mercaptoethanol (1 mM, 30 min at 37 °C and pH 7.4, vide infra), which could be replaced with either dithiothreitol or $SnCl_2$.

At that point, the opened structure shown in Figure 5 for **4** appeared likely and in agreement with the spectroscopic data; however, it was not possible to exclude another structure obtained by permutation of the $S(CH_2)_2OH$ and $SS(CH_2)_2OH$ substituents on positions 4 and 5.

(B) Reaction of 2 and 3 at Basic pH and Isolation of Compound 5. Reaction of pure compounds **2** and **3** with 0.5 mM mercaptoethanol in phosphate buffer (pH 7.4) led to a 90:10 mixture of **4** and **5**. However, at pH 12 and in the presence of 5 mM mercaptoethanol, **2** and **3** were quantitatively transformed into compound **5** in less than 3 min. This reaction allowed us to prepare **5** which was purified by HPLC and characterized by ^1H and ^{13}C NMR, UV, and mass spectroscopy. Its ^1H NMR spectrum (in D_2O) exhibited the set of signals corresponding to the aroyl moiety of **1**, a vinyl singlet at 7.71 ppm (showing an intense (NOE)[1] with the $H_{2'}$ of the aroyl group) integrating for 1H, a system of three coupled signals at 3.58 (1H, dd, 13 and 1.5 Hz), 4.03 (1H, dd, 13 and 8.5 Hz), and 4.80 ppm (1H, dd, 8.5 and 1.5 Hz), and the signals expected for one $S(CH_2)_2OH$ group (Table 2 and Figure 6). Comparison of the ^{13}C NMR spectrum of **5** with that of **1** showed almost identical signals for the carbons of the aroyl group. Supplementary signals at 35.1 and 63.32 ppm corresponding to the two carbons of the $S(CH_2)_2OH$ group were found in the spectrum of **5**. This spectrum also exhibited signals for the carbons derived from the thiophene ring of **1**, as a quaternary carbon, 2 CH carbons, and a CH_2 carbon (Table 3). All these data were in agreement with a 4,5-dihydrothiophene structure substituted at position 4 (or 5) by an $S(CH_2)_2$-OH residue for compound **5** (Figure 5). This structure, which formally derives from the addition of mercaptoethanol to one double bond of the thiophene ring of **1**, was further confirmed by the mass spectrum of **5** exhibiting a MH^+ ion at $m/z = 409$, and by the transformation of **5** into **1** in the presence of NaOH (pH = 14). This reaction corresponds to a base-catalyzed loss of mercaptoethanol from **5**.

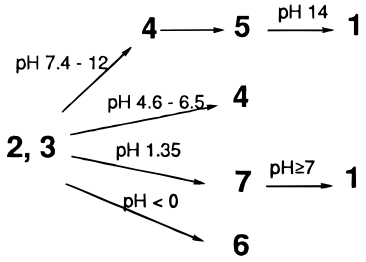


Figure 7. Summary of the various reactions observed upon treatment of **2** and **3** with mercaptoethanol as a function of pH. Formations of **1** from **5** and **6** from **2** (or **3**) occur without mercaptoethanol.

(C) Reaction of 2 and 3 with Mercaptoethanol at pH ca. 1. Reaction of compounds **2** and **3** with 10 mM mercaptoethanol in the presence of 0.05 M HCl at 37 °C led after 45 min to minor amounts of **5** and **6** (~10% and 2%) and to a major new product **7** (70% yield). Compound **7** was isolated by HPLC. Its ^1H NMR spectrum (D_2O) exhibited, in addition to the signals corresponding to the protons of the aroyl moiety, a singlet at 7.7 ppm (1H), 2 coupled doublets at 6.29 and 6.65 ppm (2 × 1H), and the signals corresponding to two nonequivalent $S(CH_2)_2OH$ groups (Table 2 and Figure 6). These NMR data and the mass spectrum of **7** exhibiting a peak MH^+ at $m/z = 458$ are in agreement with the opened structure shown in Table 2. The coupling constant of 10 Hz between the protons absorbing at 6.29 and 6.65 ppm indicated their *cis* relative position (*33*). The signal of H_4 was assigned by the presence of a ^3J coupling (1.5 Hz) with H_2 (NMR study on the methyl ester of **7**), which also confirmed that position 4 of **7** was substituted by a proton. The presence of a dithioether function was indicated by the easy transformation of **7** into **1** (with loss of two $S(CH_2)_2OH$ groups) in the presence of low concentrations of mercaptoethanol or dithiothreitol. The dithioether function could have been located either on carbon 2 or on carbon 5. However, the almost identical chemical shifts of H_2, of the aroyl group protons, and of the protons of one $S(CH_2)_2OH$ group in **7** and **4** argued in favor of the dithioether function at position 5 rather than at position 2.

Complementary Structure Determination by Deuteriation Experiments. As shown in Figure 5, metabolic oxidation of **1** with liver microsomes in the presence of mercaptoethanol leads to compounds **2** and **3** as primary metabolites. Further reaction of **2** and **3** with an excess of mercaptoethanol leads successively to the opened compound **4** then to the dihydrothiophene **5**. Interestingly, treatment of **5** with a strong base gives back to **1**. Moreover, treatment of **2** and **3** at pH ca. 1, in the presence or in the absence of mercaptoethanol, respectively, leads to two other products **7** and **6** (Figure 7).

The spectroscopic data described above allowed us to establish the structures indicated in Figure 5 for compounds **2**, **3**, and **6**, without any ambiguity. In the case of compounds **4** and **5**, the available data did not allow us to exclude structures with an $S(CH_2)_2OH$ group at

position 5 instead of position 4, as indicated in Figure 5. Therefore, supplementary experiments have been performed on compound **3** deuteriated at position 5 of its dihydrothiophene ring. Exchange of the two hydrogens H_5 of **3** was followed by ^1H NMR in D_2O containing NaOD. After complete disappearance of the H_5 signals, deuteriated **3** was isolated by HPLC and treated with 50 mM mercaptoethanol at pH 4.5 (in D_2O containing DCl) to give **4**. The ^1H NMR spectrum of deuteriated **4** obtained by this method showed an almost complete loss of the signals assigned to H_5, whereas the signal assigned to H_4 remained and now appeared as a singlet. Deuteriated **4** was then transformed into **5** in H_2O in the presence of excess mercaptoethanol. The ^1H NMR spectrum of deuteriated **5** also showed an almost complete disappearance of the signals at 3.58 and 4.03 ppm assigned to H_5. Finally, aromatization of deuteriated **5** into **1** in H_2O containing NaOH required heating at 70 °C to get to completion. This more difficult reaction with deuteriated **5** than with **5** itself would be in agreement with the removal of a deuteron (instead of a proton) at position 5 as a first, rate limiting step of elimination of mercaptoethanol from **5**. The ^1H NMR spectrum of deuteriated **1** in DMSO-d_6 (a solvent in which all the thiophene proton signals are separated) showed the almost complete disappearance of one thiophene signal (at 7.68 ppm).

Assignment of the ^1H NMR signals of the thiophene protons of nondeuteriated **1** was done as follows. The H_2 signal was identified because of its downfield shift at 8.09 ppm (in DMSO) and the existence of W couplings with the two other thiophene signals. After irradiation of the $H_{3'}$ signal, an NOE transferred to the $H_{2'}$ signal and the signal at 7.48 ppm at 70 °C. This signal was thus assigned to H_4 as H_5 would not have been sufficiently close to $H_{2'}$ in space for such an NOE effect. In fact, assignment of the signals of H_5 and H_2 was further confirmed by experiments in which **1** was treated for 24 h at 105 °C with $CF_3CF_2CF_2COOD$. Such conditions were known to lead to a partial H/D exchange at positions 2 and 5 of 3-aroylthiophenes (*34*). Accordingly, this treatment led to a partial disappearance of the signals at 8.1 (90%) and 7.68 (50%) ppm (spectrum in DMSO). These results allowed the assignment of the thiophene signals of **1** indicated in Table 2. Thus, the signal (at 7.68 ppm) that has almost completely disappeared in deuteriated **1** prepared from deuteriated **3** was that of H_5. These data clearly showed that the $S(CH_2)_2OH$ moiety present in compound **5** is located on carbon 4 and confirmed the structures shown in Figure 5 for compounds **4** and **5**.

Mechanisms for the Formation of the Various Metabolites of 1. The structure found for compounds **2** and **3** strongly suggests that they derive from a Michael-type addition of mercaptoethanol to the most reactive position (C_2) of the thiophene sulfoxide metabolite **A** formed by microsomal monooxygenation of **1** (Figure 5). Thiophene sulfoxide derived from **1** should be unstable as most thiophene sulfoxides (*15*), the presence of a COAr substituent making it especially electrophilic in nature. Its adducts with mercaptoethanol, **2** and **3**, could be isolated under selected conditions since they appeared to be unstable in the presence of acids (formation of **6**) or thiols such as mercaptoethanol in excess. Several compounds are formed upon reaction of **2** and **3** with mercaptoethanol as a function of pH (Figure 7). Moreover, it was also observed that **2** and **3** are interconverted into each other after treatment by NaOH in H_2O and neutralization of the medium. This rich chemistry of **2** and **3** may be interpreted if one takes into account two starting characteristics of these compounds: (i) their ability to act as Michael acceptors for nucleophiles because of the presence of their conjugated double bond, and (ii) the acidity of their H_5 protons whose abstraction leads to a stabilized anion. Interestingly, both situations should lead to the appearance of an intermediate conjugated anion located in part on carbon 3. These considerations have helped us to propose mechanisms for the formation of **4**, **5**, and **7**.

Possible Mechanism for the Formation of 4 and 5 from 2 and 3 (Figure 8). Nucleophilic addition of the thiol group of mercaptoethanol on the conjugated double bond of **2** (or **3**) should lead to an intermediate anion at C_3, which may evolve by cleavage of the C_2-S bond in the β-position. After protonation, the sulfenic acid **B** derived from that reaction may either recyclize to give back to **2** or react with mercaptoethanol to give **4**. The latter reaction is likely since sulfenates are known to rapidly react with thiols in excess to give dithioethers (*35*). In fact, the formation of a dithioether function should drive the reaction toward **4**. At neutral and basic pH, the SH group of mercaptoethanol should reduce the dithioether function of **4**, leading, as shown in Figure 8, to an S^- (or SH) intermediate able to add to the conjugated double bond in an intramolecular manner. The resulting cyclic anion should then undergo a β-elimination of a $^-S(CH_2)_2OH$ residue eventually leading to **5**.

Transformation of **5** into **1** in the presence of a base simply results from a base-catalyzed 1,2-elimination of $HSCH_2CH_2OH$.

Possible Mechanisms for the Interconversion of 2 and 3 and Formation of 7. Interconversion between **2** and **3**, which was observed after treatment of these compounds with NaOH followed by a quick neutralization of the medium, should not be due to a change of the sulfoxide stereochemistry, as it is known that H/D exchanges in position α to dialkyl sulfoxides occur with complete retention of the stereochemistry of the sulfoxide function (*36*−*38*). Thus, interconversion between **2** and **3** requires a bond to be broken. A likely mechanism for this reaction is depicted in Figure 9. It also involves the intermediate formation of an anion, similar to that proposed in Figure 8. Here this anion is not formed by addition of a nucleophile to the conjugated double bond of **2** (or **3**) (as in Figure 8), but by simple abstraction of a proton at C_5. Anion **C** (Figure 9) also could undergo a cleavage of the C−S bond in β-position leading to the opened sulfenate anion **D**. Recyclization of **D** by intramolecular Michael-type addition of $-SO^-$ to the conjugated double bond should lead back to the starting anion **C** or to its isomer **E**. Upon neutralization of the medium, protonation of anions **C**, **D**, and **E**, that are in equilibrium, should lead to a mixture of **2** and **3** as major products and to a very minor formation of the opened isomer **DH** which has never been detected. The final **2**:**3** ratio was found to mainly depend on the pH of the "neutralization" medium, but was independent on the nature of the starting product (**2** or **3**). It was maximum (0.2) at pH 7 and lower at higher pH (0.01 at pH 13, 0.05 at pH 10, and 0.16 at pH 8). Such openings of thiophene rings have been proposed as intermediate steps before sulfenate methylation (*39*).

Formation of **7**, which was observed upon treatment of **2** (or **3**) with mercaptoethanol at acidic pH, could be due to a trapping of the opened isomer **DH** by mercaptoethanol after protonation of its OH group, loss of H_2O,

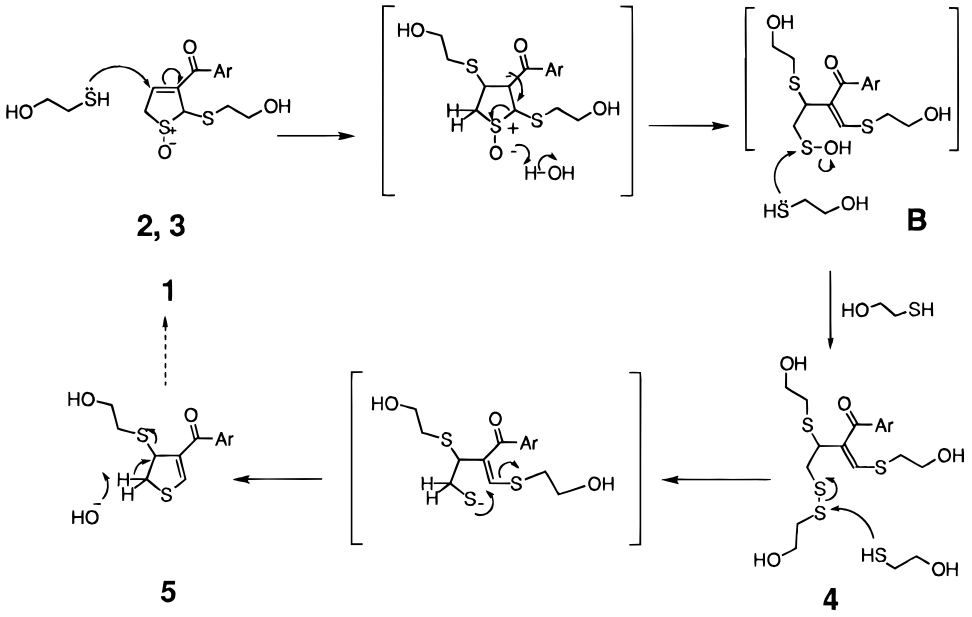


Figure 8. Possible mechanisms for the formation of **4** and **5** from reaction of **2** and **3** with mercaptoethanol. Anionic intermediates are indicated in order to illustrate similarities of mechanisms in Figures 8 and 9 (similar openings of the thiophene ring in the case of an anionic intermediate at C-3, for instance). However, there is no direct evidence for such intermediates; concerted mechanisms are as likely.

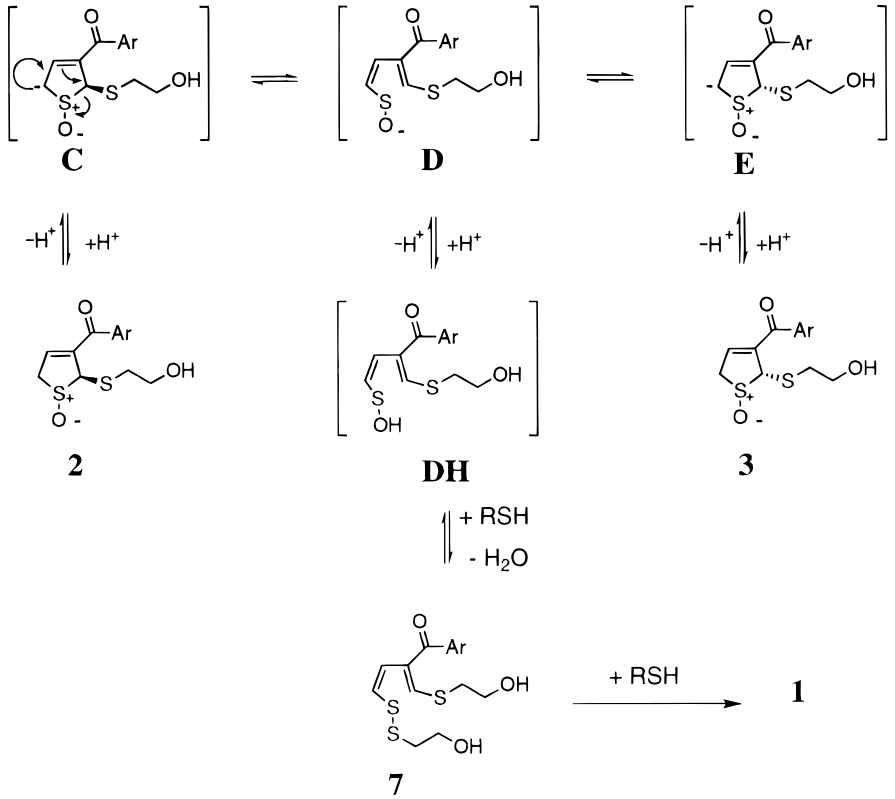


Figure 9. Possible mechanism for the interconversion between **2** and **3** after basic treatment, and for the formation of **7** upon reaction of **2** and **3** with mercaptoethanol at pH 1. Absolute and relative stereochemistry of the sulfoxide and C-2 is presently unknown for compounds **2** and **3**, and for the derived intermediates **C** and **E**. Formulas used for **2**, **3**, **C**, and **E** only indicate that **2** and **3** (as well as **C** and **E**) have different relative stereochemistry.

and formation of a dithioether function. Formation of **1** upon treatment of **7** with mercaptoethanol should involve a mechanism very similar to that proposed for reaction of **4** with mercaptoethanol that leads to **5** (Figure 8): (i) nucleophilic attack of the S−S bond of **7** by the S atom of mercaptoethanol, (ii) intramolecular addition of the S center to the conjugated double bond, and (iii) elimination of $HSCH_2CH_2OH$.

Microsomal Metabolism of 1 in the Presence of a Thiol in Excess: Relationship with Its Metabolism *in Vivo*. A complete view of the microsomal metabolism of **1** in the presence of mercaptoethanol is shown in Figure 5. In microsomal incubations performed under usual conditions (pH 7.4) and in the presence of mercaptoethanol in large excess (10−20 mM), almost only one product is formed (with an overall yield based on **1** up to 70%). This product, **5**, is the final stable metabolite resulting from the cascade of reactions occurring on **2** and **3** (Figure 5). Under *in vivo* conditions, the thiol-containing nucleophile that is present in large amounts in many

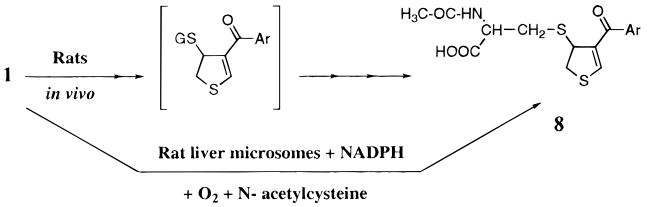


Figure 10. Formation of **8** as a metabolite of **1** *in vivo* in rats, and *in vitro* after incubation with rat liver microsomes, NADPH, and *N*-acetylcysteine. GSH is used for glutathione and Ar for 4-(2,3-dichlorophenoxy)-2-acetic acid. Compound **8** is a mixture of two diastereoisomers, **8a** and **8b**.

cells is glutathione. In the hepatocytes, which are greatly involved in the metabolism of xenobiotics, the glutathione concentration is high (1−5 mM). It was thus tempting to propose that the *in vivo* metabolism of **1** in rats led to an equivalent of **5** in which the mercaptoethanol residue was replaced with a glutathione residue. It is well-known that such glutathione adducts are very often further transformed into the corresponding mercapturates (*40*). Thus, formation of a mercapturate corresponding to **5** in which the mercaptoethanol group is replaced with an *N*-acetylcysteine group (**8** in Figure 10) appeared very likely, as a potential *in vivo* metabolite of **1**. In order to verify this proposition based on the scheme established for the microsomal metabolism of **1** in the presence of a thiol-containing nucleophile, two series of experiments were performed. In the first one, rats were treated with **1** and their urinary metabolites were studied after separation by HPLC. In the second one, the expected mercapturate **8** was prepared from reaction of **1** with rat liver microsomes in the presence of NADPH, O_2, and an excess of *N*-acetylcysteine under conditions similar to those used to prepare **5**.

(A) Preparation of 8 upon Microsomal Transformation of 1 in the Presence of *N*-Acetylcysteine. As expected, this reaction led to two major products which were isolated by preparative HPLC and whose UV spectra were almost identical to those of **4** and **5**, respectively. Compound **8** which exhibited a UV spectrum identical to that of **5** (λ = 334 nm) was in fact a mixture of two diastereoisomers, **8a** and **8b**, that were separated by HPLC after methylation of their carboxylic acid functions with CH_2N_2. Their ^{1}H NMR and mass spectra were in complete agreement with a structure derived from that of **5** upon replacement of its mercaptoethanol substituent with an *N*-acetylcysteinyl group (under the form of its methyl ester). Their mass spectrum (CI, NH_3) exhibited peaks at m/z = 522 $(M + H)^+$ and 539 $(M + NH_4)^+$, and their ^{1}H NMR spectra showed signals expected for a 3-aroyl-4,5-dihydrothiophene structure, as in **5**, in addition to those corresponding to an *N*-acetylcysteinyl substituent at position 4. This was particularly clear when comparing the ^{1}H NMR spectra of **8a** (and **8b**) and **5** (Table 4).

(B) *In Vivo* Metabolism of 1 in Rats. For the *in vivo* experiments, rats were treated with radiolabeled **1** (30 mg/kg, ^{14}C at the keto group, 0.5 mCi/mmol), and their urine was collected during 24 h. 20% of the dose was recovered in the urine with a maximum extracted between 2 and 4 h. The 2−4 h fraction was studied by HPLC; four radioactive peaks were observed. After extraction with ethyl acetate and methylation with CH_2N_2, the four compounds were studied by UV, ^{1}H NMR, and mass spectroscopy. One of them was unchanged **1** (as methyl ester), whereas two of those metabolites could be identified as **8a** and **8b** (dimethyl esters). They showed HPLC retention times, UV, ^{1}H NMR, and mass spectra identical to those previously found for dimethyl esters of **8a** and **8b**. They represented 1.3% and 0.8% of administered **1**, and 10% and 5% of the total radioactivity found in the urine. The fourth isolated compound (1% of starting **1**) showed a UV spectrum identical to those of **8a** and **8b**. Its ^{1}H NMR spectrum was very similar to that of **8a** and **8b** except for the absence of the $COCH_3$ singlet. We did not try to completely identify this compound; however, its UV and ^{1}H NMR characteristics suggested that it could be **8a** (or **8b**) with a free NH_2 function (instead of $NHCOCH_3$).

Table 4. Comparison of the ^{1}H NMR Characteristics of the Methyl Esters of Compounds 8a, 8b, and 5[a]

	8a	8b	5
H_2	7.12 (s, 1H)	7.17 (s, 1H)	7.14 (s, 1H)
H_4	4.69 (dd, 8−1.5, 1H)	4.70 (dd, 8−1, 1H)	4.81 (dd, 8−1.5, 1H)
H_5	3.94 (dd, 13−8, 1H)	3.95 (dd, 13−8, 1H)	3.98 (dd, 12−8, 1H)
	3.45 (dd, 13−1.5, 1H)	3.48 (dd, 13−1, 1H)	3.50 (dd, 12−1.5, 1H)
$H_{2'}$	7.18 (d, 9, 1H)	7.21 (d, 9, 1H)	7.20 (d, 9, 1H)
$H_{3'}$	6.80 (d, 9, 1H)	6.81 (d, 9, 1H)	6.80 (d, 9, 1H)
OCH_2	4.78 (s, 2H)	4.79 (s, 2H)	4.78 (s, 2H)

[a] Methyl ester of **5** and dimethyl esters of **8a** and **8b** in CD_2Cl_2 (250 MHz); δ in ppm/Si$(CH_3)_4$; J in Hz. The signals of the thioether substituent at position 4 and of $COOCH_3$ groups are not indicated; they are given in the Experimental Section. Labeling of protons as for **5** in Table 2.

These results show that the relatively complex series of reactions occurring after S-oxidation of **1** and trapping of **1** sulfoxide with a thiol, that was established *in vitro* using liver microsomes and mercaptoethanol, also occurs *in vivo* in rats. The final stable metabolite is a 3-aroyl, 4-*S*-alkyl 4,5-dihydrothiophene both *in vitro* and *in vivo* (**5** and **8**, respectively). Thus, it is very likely that compounds **8** detected in the urine of rats treated with **1** are derived from (i) a monooxygenase-dependent S-oxidation of **1**, (ii) a nucleophilic addition of glutathione at position 2 of **1** sulfoxide, (iii) a transformation of the resulting equivalent of **2** into **8** bearing a glutathione residue instead of an *N*-acetylcysteinyl residue, and (iv) the usual processing of glutathione adducts (*40*) into corresponding mercapturates **8**.

Conclusion

The aforementioned results provide a clear indirect evidence for the intermediate formation of a reactive, electrophilic thiophene sulfoxide in metabolic oxidation of **1** in *vitro* and *in vivo*. They also indicate how to trap such intermediates using low concentrations of a thiol trapping agent and a careful control of pH during isolation of substituted 2,5-dihydrothiophene sulfoxides such as **2**. Moreover, they give a first idea of the various reactions that may occur when such 3-aroylthiophene sulfoxides react with a thiol in excess (Figure 5). This allowed us to explain the global transformation of **1** into the final stable metabolite **5** *in vitro* in the presence of excess mercaptoethanol, and of **1** into **8** *in vivo*. Transformation of **1** into **5** with liver microsomes and mercaptoethanol in excess, that we had observed in the beginning of this study, was very difficult to explain without isolation of intermediates such as **2** and **4** and study of their reactivity toward thiols.

Recent data on the oxidative metabolism of thiophene itself and of 2-aroylthiophenes have indicated the key role of thiophene *S*-oxides as reactive intermediates (*12*, *14*).

The results described above provide the first data on the reactivity of such thiophene *S*-oxides, whose chemistry is poorly known (*15*), and on their possible fates in living organisms.

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