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First evidence that cytochrome P450 may catalyze both S-oxidation and epoxidation of thiophene derivatives

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Abstract

Oxidation of 2-phenylthiophene (2PT) by rat liver microsomes, in the presence of NADPH and glutathione (GSH), led to three kinds of metabolites whose structures were established by ^{1}H NMR and mass spectrometry. The first ones were 2PT-*S*-oxide dimers formed by Diels–Alder type dimerization of 2PT-*S*-oxide, while the second ones were GSH adducts derived from the 1,4-Michaël-type addition of GSH to 2PT-*S*-oxide. The third metabolites were GSH adducts resulting from a nucleophilic attack of GSH to the 4,5-epoxide of 2PT. Oxidation of 2PT by recombinant, human cytochrome P4501A1, in the presence of NADPH and GSH, also led to these three kinds of metabolites. These results provide the first evidence that cytochrome P450 may catalyze the oxidation of thiophene compounds with the simultaneous formation of two reactive intermediates, a thiophene-*S*-oxide and a thiophene epoxide.
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Keywords: 2-Phenylthiophene; Thiophene-*S*-oxide dimers; Thiophene-*S*-oxide–glutathione adduct; Thiophene epoxide–glutathione adduct; CYP1A1; Rat liver microsomes; Arene oxide

Arene oxides derived from cytochrome P450-dependent oxidation of aromatic compounds play a central role in the oxidative metabolism of these compounds [1,2]. The metabolism of furan derivatives also involves the formation of arene oxides as the main primary reactive intermediates [2]. The situation is more complex in the case of thiophene compounds, as two electrophilic, unstable intermediates are a priori possible in the oxidative metabolism of these compounds in mammals, the thiophene-*S*-oxides and thiophene epoxides [3,4]. Thiophene derivatives are ubiquitous in nature; some of them are liberated in the environment during cooking processes and the combustion of fossil fuels [5,6]. Polythiophenes are commonly used in materials chemistry, and thiophene rings are present in many drugs [4]. There is evidence that several thiophene derivatives cause toxic effects [7–11], and, at least in some cases, these toxic effects have been related to the formation of reactive, electrophilic intermediates during the oxidative metabolism of the thiophene ring [7,9,11]. Thus, tienilic acid, a diuretic drug that was involved in the appearance of immunoallergic hepatitis, is mainly oxidized by cytochrome P450 2C9 in human liver [9]. This oxidation leads to a suicide inactivation of CYP2C9 [12], as well as the covalent binding of tienilic acid to liver proteins, which could play a key role in the appearance of the toxic effects of this drug [9,13].

So far, several publications have provided evidence for the formation of thiophene-*S*-oxides as major intermediates in the oxidative metabolism of thiophene compounds in mammals [3,14–17], whereas no direct evidence for the formation of thiophene epoxide intermediates has been reported. Thus, in rats, the metabolites of thiophene itself found in vitro and in vivo are sulfoxide dimers resulting from the Diels–Alder type dimerization of thiophene-*S*-oxide [16] and glutathione adducts derived from the Michaël-type addition of glutathione to thiophene-*S*-oxide [3,16] (Fig. 1). The oxidative metabolism of a 3-aroylthiophene analog of tienilic acid also leads to metabolites derived from the reaction of glutathione with its *S*-oxide intermediate [15]. In vivo and in vitro oxidation of several

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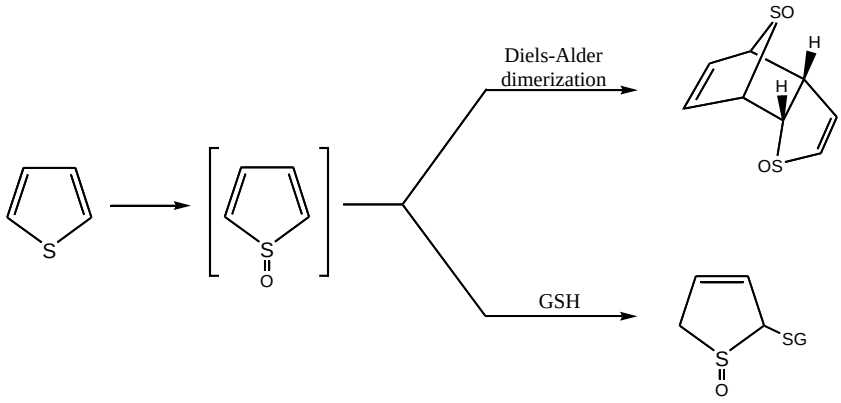

Fig. 1. In vivo and in vitro metabolism of thiophene in rats (from [3,16]). The glutathione (GSH) adduct shown in this figure is only an intermediate; the final metabolite found in urine is the corresponding mercapturate.

2-aroylthiophenes mainly leads to metabolites resulting from their hydroxylation at position 5 [18–24]. These 5-hydroxy metabolites could result from an isomerization of either a thiophene-*S*-oxide intermediate or a 4,5-epoxide intermediate [25].

We have recently studied the oxidation of 2-phenylthiophene (2PT) either by rat liver microsomes or by recombinant cytochrome P4501A1 (CYP1A1), and observed the formation of metabolites derived from both 2-phenylthiophene-*S*-oxide and from 2-phenylthiophene epoxide. These results provide the first evidence that cytochrome P450-dependent oxidation of thiophenes may occur with the simultaneous formation of *S*-oxide and epoxide intermediates.

Materials and methods

All reagents were of the highest quality commercially available. 2PT was purchased from Aldrich (L'Isle d'Abeau Chesnes, France).

Tritiation of 2PT. Tritiation at position 5 of 2PT was performed by taking advantage of the previously reported easy exchange of thiophene hydrogens at C_2 and C_5 under acidic conditions [3,16]. A mixture of 20 mg 2PT, 200 μl trifluoroacetic anhydride, and 30 μl tritiated water (16 μCi/μmol) in 2 ml CH_2Cl_2 was stirred for 24 h at 40 °C. After dilution with 10 ml CH_2Cl_2, the solution was washed eight times with H_2O, dried on $MgSO_4$, and evaporated to dryness, leading to 20 mg of [5-^{3}H]-2PT (0.7 μCi/μmol).

Microsomal incubations and HPLC analysis. Liver microsomes from rats pretreated with β-naphthoflavone (50 mg/kg/day i.p. for 3 days) were prepared as described previously [26]; they contained 1.3 nmol P450/mg protein. Microsomes from yeast expressing human CYP1A1 and yeast NADPH-cytochrome P450 reductase were obtained according to previously described techniques [27]; they contained 0.18 nmol CYP1A1/mg protein.

For analytical measurements (HPLC and HPLC–MS analyses), incubations were done in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, 2PT (0.1–1.5 mM), microsomes (protein amounts corresponding to 0.3 μM P450), and a NADPH-generating system (1 mM NADP, 10 mM glucose-6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase), at 28 °C (yeast microsomes) or 37 °C (rat microsomes), for times between 1 and 30 min. Reactions were stopped with the addition of 0.5 volume of CH_3CN containing 4% acetic acid. After centrifugation at 10,000*g* for 10 min, the supernatant was analyzed by HPLC using a Hypersil MOS column (5 μm, 250 × 4.6 mm) and a 20 min linear gradient from A = 0.1 M ammonium acetate, pH 4.6, to B = CH_3CN, CH_3OH, H_2O (7:2:1) for 20 min at a flow rate of 1 ml/min. For radioactivity analyses, 0.5 min fractions were collected in 3 ml polyethylene tubes and counted after addition of 2 ml picofluor 40 in a scintillation counter (Perkin-Elmer, Saint Quentin en Yvelines, France).

HPLC–MS studies. Incubations were performed as described above in 1 ml total volume; the supernatant was loaded onto OASIS columns (Waters, Saint Quentin en Yvelines, France), washed with 1 ml H_2O, and eluted with 1 ml CH_3OH. After concentration to 0.2 ml, 20 μl of the final solution was analyzed with a HPLC Surveyor coupled to an ion-trap mass spectrometer (Thermofinnigan LCQ Advantage, Orsay, France), using a Kromasil C18 column (3.5 μm, 100 mm × 2.1 mm) and a linear gradient of A/B mixture from 0% B to 80% B in 20 min at 200 μl/min (A = 0.1% formic acid in H_2O, B = CH_3CN containing 0.1% formic acid). MS parameters were 275 °C for capillary temperature and 5 keV for capillary voltage.

^{1}H NMR studies. ^{1}H NMR spectra were recorded on Bruker WM250 and AM 500 spectrometers. Chemical shifts (δ) are given in ppm relative to $(CH_3)_4Si$ and *J* in Hz. Abbreviations used for singlet, doublet, doublet of doublets, broad singlet, and massif are s, d, dd, bs, and m, respectively. Cys, Gly, and Glu are used for cystein, glycine, and glutamate, respectively. For the preparation of larger amounts of metabolites necessary for ^{1}H NMR analysis, incubations of 80 ml rat liver microsomal suspensions containing 98 nmol P450 and 500 μM 2PT, in the presence of the NAPDH-generating system, were performed for 1 h at 37 °C. After addition of 1.6 ml acetic acid and centrifugation, the supernatant was loaded on SepPak C18 cartridges (20 ml/cartridge). Each cartridge was washed with 3 ml H_2O and eluted with 2 ml CH_3OH. The metabolites were separated by HPLC (see above), lyophilized, dissolved in 0.5 ml D_2O or $CDCl_3$, and studied by ^{1}H NMR spectroscopy.

Metabolites 2PTSOD. Their ^{1}H NMR spectra (in $CDCl_3$) were found to be identical to those of previously described 2PT-*S*-oxide dimers [28]. The major isomer was 2,4-diphenyl-*cis*-3a,4,7,7a-tetrahydro-*cis*-4,7-epithio-1-benzothiophene, *trans*-1, *syn*-8-dioxide (Fig. 2), whereas the minor one was the corresponding *cis*-1, *syn*-8 dioxide isomer.

Metabolite 2PTGA. Electrospray ionization mass spectrometry (ESIMS), *m/z* = 484 (M + H); MS–MS on ion 484, *m/z* = 466(–H_2O, 15%), 436(–SO, 50%), 409(10%), 355(100%), 337(20%), 191(20%), 177(15%); UV spectrum: λ_{max} = 246 nm. ^{1}H NMR (250 MHz, D_2O) δ = 7.3–7.5(m, 5H, phenyl), 6.34(m, 1H, H_3), 6.30(d, 5.7 Hz, 1H, H_4), 5.91(bs, 1H, H_5), 5.23(bd, 2.5 Hz, 1H, H_2), 4.66(m, 1H, αCys), 3.85(m, 3H, αGlu + αGly), 3.2–3.5(m, 2H, βCys), 2.62(m, 2H, γGlu), 2.2(m, 2H, βGlu). From the shape of the signals observed for H_3 and the β protons of the Glu residue, 2PTGA appeared to be a mixture of at least two diastereoisomers.

Metabolite 2PTGB. ESIMS, *m/z* = 484 (M + H); MS–MS on ion 484, *m/z* = 466(–H_2O, 100%), 337(25%), 308(60%). UV spectrum: λ_{max} = 286 nm. The ^{1}H NMR spectrum corresponds to a mixture of two diastereoisomers A and B in a 3:2 ratio. ^{1}H NMR(500 MHz, D_2O): δ = 7.65(d, 7.7 Hz, 2H, *ortho*-phenyl), 7.50(m, 3H, phenyl), 6.19 (A) and 6.16 (B) (d, 3.5 Hz, 1H, H_3), 5.71 (B) and 5.70 (A) (s, 1H, H_5), 4.70(m, 1H, αCys), 4.41

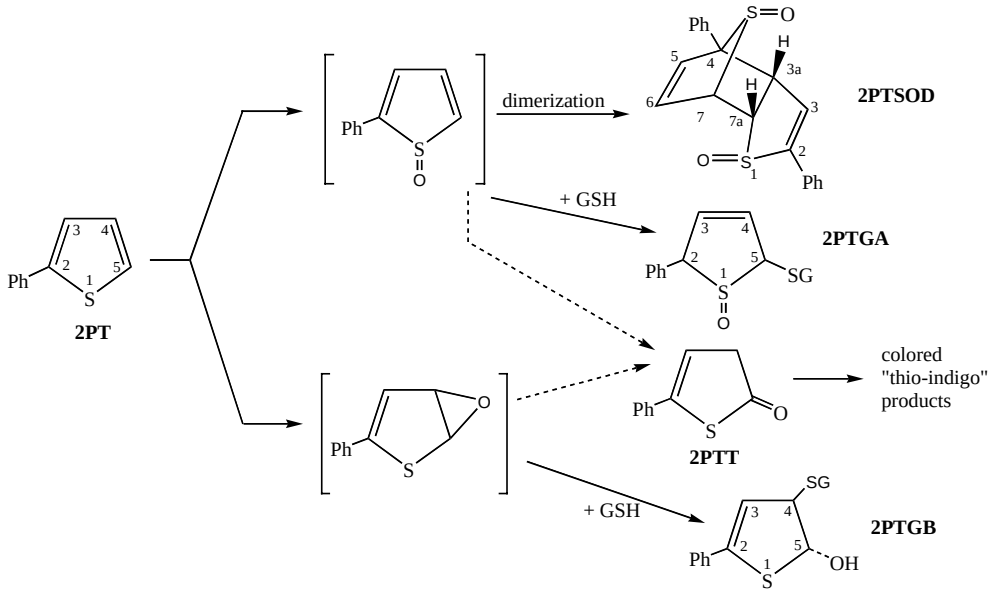

Fig. 2. Oxidative metabolism of 2PT by rat liver microsomes or recombinant human CYP1A1 in the presence of GSH. The stereochemistry shown for 2PTSOD is that of the major stereoisomer. The relative configuration of the C_2, C_5, and S_1 centers of 2PTGA is not known. In the case of 2PTGB, only the structure of one of the two main diastereoisomers formed is shown. The second diastereoisomer should derive from an inversion of configuration of C_4 and C_5. In order to facilitate the understanding of the involved reactions, the numbering of the thiophene atoms of 2PT was also used for the 2PTGA and 2PTGB metabolites. The official nomenclature for 2PTGA and 2PTGB would have required a different numbering, with the phenyl substituent being at position 5.

(B) and 4.39 (A) (d, 3.5 Hz, 1H, H_4), 3.76(m, 3H, αGlu and αGly), 3.2 (A) and 3.15 (B) (dd, 4.5 and 14 Hz, 1H, βCys), 3.02 (A) and 2.9 (B) (dd, $J = 9$ and 14 Hz, 1H, βCys), 2.5(m, 2H, γGlu), 2.1(m, 2H, βGlu). The nuclear Overhauser effect spectroscopy (NOESY) spectrum of 2PTGB clearly showed an interaction between the vinyl proton and the *ortho*-phenyl protons, which is only compatible with the presence of this proton on C_3.

The use of the heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple band correlation (HMBC) and heteronuclear single quantum correlation (HSQC) two-dimensional NMR techniques [29] allowed us to determine the ^{13}C chemical shifts of some carbons of 2PTGB: $\delta = 173$(COGlu), 169(COGly), 168(COCys), 128, 126.7, 123.9, and 123.8 (phenyl), 112.4 (C_3), 84.5 (C_5), 58.3 (C_4), 52 (Cα Cys), 51 (Cα Glu), 41 (Cα Gly), 28.6 (Cγ Glu), and 23.8 (CβGlu). The signals corresponding to C_3, C_4, and C_5 also showed the presence of two diastereoisomers.

Results

2-Phenylthiophene (2PT), partially tritiated at position 5, [5-^{3}H]-2-phenylthiophene, was incubated with liver microsomes from β-naphthoflavone-pretreated rats in the presence of NADPH. Analysis of the incubation mixture by HPLC–MS showed the major formation of two 2-phenylthiophene-*S*-oxide dimers, 2PTSOD, that should result from the Diels–Alder type dimerization of 2-phenylthiophene-*S*-oxide. These 2PTSOD metabolites were fully characterized by their mass and ^{1}H NMR spectra, by comparison with those of authentic samples prepared by oxidation of 2PT with *meta*-chloroperbenzoic acid in the presence of BF_3 etherate, a method previously described for the synthesis of other thiophene-*S*-oxide dimers by dimerization of thiophene-*S*-oxide intermediates generated in situ [30]. The spectral data of the two 2PTSOD metabolites, that were formed in a 5:1 molar ratio, were found to be in complete agreement with those of two previously described stereoisomers of 2PT-*S*-oxide dimers [28]. The stereochemistry of the major isomer is shown in Fig. 2; it only differs from that of the minor isomer by the configuration of the S_1 atom (*trans*-1-oxide instead of *cis*-1-oxide). It is noteworthy that the stereochemistry observed for the 2PTSOD metabolites completely corresponded to those described previously for other thiophene-*S*-oxide dimers; it should derive from the stereoelectronic control of the Diels–Alder type dimerization [16,28,30].

The HPLC–MS analysis of the reaction mixture also showed the formation of trace amounts of 2PTT (2-phenylthiolene thiolactone) (Fig. 2) that could derive from a 5-hydroxylation of 2PT. This metabolite was found to be identical (HPLC retention time and mass spectrum) to an authentic sample of 2PTT that was synthesized according to a previously described procedure [31]. It is an unstable compound that rapidly leads to ''thio-indigo-like'' condensed, colored products [32,33]. Accordingly, after incubation with 2PT and NADPH, liver microsomes became highly colored (from blue to brown depending upon the experimental conditions). Because of these further complex reactions of 2PTT, the only way to evaluate the extent of 2PTT formation was to measure the amount of tritiated water released upon microsomal oxidation of [5-^{3}H]-2-phenylthiophene. Incubation (30 min) of [5-^{3}H]-2PT(300μM) with rat liver microsomes in the presence of a NADPH generating system led to a 35% conversion of [5-^{3}H]-2PT into 2PTSOD and to the release of ^3H_2O(30%) under the used conditions (Table 1). Identical incubations but in the absence of NADPH failed to lead to any ^3H_2O release and to any formation of 2PTSOD.

Table 1
Relative amounts of metabolites formed upon oxidation of 2PT by β-naphthoflavone-pretreated rat liver microsomes or yeast-expressed human CYP1A1, in the presence (or absence) of GSH[a]

Metabolites	Yields (%)[b]		
	Rat liver microsomes		CYP1A1 + GSH
	−GSH	+GSH	
2PTSOD	35	12	31
2PTT (from ^3H_2O release)	30	3	4
2PTGA	0	20	6
2PTGB	0	30	3

[a] Conditions: 30 min incubation of 300 μM [5-^{3}H]-2PT with either β-naphthoflavone-pretreated rat liver microsomes (0.3 μM P450) or microsomes of yeast expressing human CYP1A1 (0.28 μM) and yeast NADPH cytochrome P450 reductase, in the presence of a NADPH-generating system (1 mM NADPH). +GSH: presence of 5 mM GSH in the incubation mixture.

[b] Yields relative to starting [5-^{3}H]-2PT were calculated from the radioactivity of each metabolite separated by HPLC (see Materials and methods).

Microsomal incubations under identical conditions but in the presence of 5 mM glutathione (GSH) led to a dramatic decrease in the formation of 2PTSOD and tritiated water (Table 1), and in blue coloration of the microsomes. HPLC–MS analysis of the reaction mixture showed the formation of two new metabolites with respective yields of 20% and 30% based on starting [5-^{3}H]-2PT (Table 1). Analysis of these metabolites by mass spectrometry and ^{1}H NMR spectroscopy using correlated spectroscopy (COSY), NOESY, HMQC, HBQC, and HSQC techniques [29] showed that the new metabolites were glutathione adducts. The mass spectra of these two metabolites all exhibited a molecular ion at $M + H = 484$, corresponding to $2PT + O + GSH + H$. The ^{1}H NMR spectrum of the most polar metabolite, 2PTGA, showed the presence of four protons that are characteristic of a 2,5-dihydrothiophene ring (see Materials and methods). This spectrum was highly similar to those previously described for compounds derived from the Michaël-type addition of thiols to thiophene-*S*-oxides [3,15,16,34]. It was in complete agreement with the structure of 2PTGA shown in Fig. 2, that results from the 1,4-addition of glutathione to 2PT-*S*-oxide. From its ^{1}H NMR spectrum, 2PTGA appeared to be a mixture of at least two major stereoisomers; however, this spectrum did not allow one to determine the relative configurations of the C_2, S_1, and C_5 centers. The less polar glutathione adducts, 2PTGB, were a 3:2 mixture of two diastereoisomers whose ^{1}H NMR spectrum showed the presence of only three protons for the dihydrothiophene ring. Two dimensional ^{1}H NMR analysis was in agreement with a structure resulting from the addition of glutathione to the 4,5-epoxide of 2PT, with a hydroxy group on carbon 5 and the GS group on carbon 4 (Fig. 2). This regiochemistry is in agreement with the ^{13}C chemical shifts found for C_5 and C_4 (84.5 and 58.3 ppm, respectively), as a function of literature data for carbons bearing a SR and an OH substituent (for C_5), and a SR and a vinyl substituent (for C_4) respectively [35]. The *trans* relative position of the OH and SG substituents is in agreement with the low coupling constant observed between H_4 and H_5 (<1 Hz). A *cis* relative position should have led to a larger coupling constant, as *cis*-4,5-diols of 4,5-dihydrothiophenes exhibit a coupling constant between the H_4 and H_5 protons larger than 5 Hz [28]. These data suggest that 2PTGB is a 3:2 mixture of diastereoisomers of 2-phenyl-*trans*-5-hydroxy-4-*S*-glutathionyl-4,5-dihydrothiophene.

The structures indicated (Fig. 2) for these two different kinds of glutathione adducts, 2PTGA and 2PTGB, were in agreement with their different behaviors in MS–MS spectrometry (2PTGA loses 48 corresponding to SO, whereas 2PTGB mainly loses 18 corresponding to H_2O), and in an acidic medium (2PTGA is stable for more than 1 h at pH 1, whereas 2PTGB undergoes a very fast dehydration to 2-phenyl-*S*-glutathionyl thiophene ($M + H = 466$) under identical conditions).

These data show that microsomal oxidation of 2PT occurred with simultaneous formation of a thiophene-*S*-oxide and an arene oxide on the thiophene ring. Microsomal incubations under identical conditions, except for the presence of usual cytochromes P450 inhibitors, such as α-naphthoflavone and miconazole [36], led to an almost complete inhibition of the formation of 2PTSOD, 2PTGA, and 2PTGB (92% inhibition with 100 μM α-naphthoflavone + 100 μM miconazole; data not shown). This indicates that the intermediate formation of 2PT *S*-oxide and arene oxide is catalyzed by cytochromes P450. Since liver microsomes contained several cytochrome P450 isozymes, it was not possible to conclude from these results whether these two intermediates were formed by the same cytochrome P450 or by two different isozymes. In order to solve this problem, we then studied the oxidation of 2PT by recombinant human CYP1A1. A similar study of incubations of 2PT with microsomes of yeast co-expressing human CYP1A1 and yeast NADPH cytochrome P450 reductase, in the presence of NADPH and glutathione, led to results similar to those obtained with rat liver microsomes, with the formation of the same metabolites (Table 1). The less efficient trapping of the *S*-oxide and epoxide intermediates by GSH in the case of recombinant CYP1A1, relative to what it is in the case of liver microsomes, could be due to a more efficient catalysis of these GSH reactions by glutathione transferase(s) in rat liver microsomes. The fact that the oxidation of 2PT to 2PTSOD, 2PTGA, and 2PTGB by yeast microsomes is catalyzed by CYP1A1 was established by control experiments using either identical incubations, except for the presence of 100 μM α-naphthoflavone, an usual inhibitor of cytochromes P450 of the 1A subfamily [36] (97% inhibition of 2PTSOD, 2PTGA, and 2PTGB formation), or microsomes of the same yeast not expressing CYP1A1 (no appearance of these metabolites). Kinetic studies on the CYP1A1-catalyzed oxidation of 2PT to 2PTSOD led to apparent k_{cat} and K_m values of

$30 \pm 5\ min^{-1}$ and $540 \pm 50\ \mu M$, respectively (data not shown).

Discussion

The aforementioned results provide a supplementary evidence for the intermediate formation of thiophene-*S*-oxides in the microsomal oxidation of thiophene derivatives. As previously described in the case of thiophene itself [16] and of the thiophene-containing drug, ticlopidine [17], 2PT is oxidized by liver microsomes, with the formation of thiophene-*S*-oxide dimers resulting from Diels–Alder dimerization of the corresponding thiophene-*S*-oxide intermediate. As in the case of thiophene [3,16] and a 3-aroylthiophene [14,15], oxidation of 2PT by rat liver microsomes in the presence of a thiol nucleophile (GSH here) led to the formation of metabolites derived from the addition of the thiol to the intermediate thiophene-*S*-oxide. The ^1H NMR study of these metabolites showed that the major isomer formed results from a 1,4-Michaël-type addition of the thiol to the thiophene-*S*-oxide (as depicted in Fig. 2). This regiochemistry of the thiol addition was previously reported for other chemically prepared thiophene-*S*-oxides [34].

More interestingly, the aforementioned results show for the first time that microsomal oxidation of a thiophene derivative not only leads to the intermediate formation of an *S*-oxide but also to that of an arene-oxide of the thiophene ring. The precise stereochemistry of the two diastereoisomers of the 2PTGB metabolite is not definitely established; however, a detailed analysis of their ^1H NMR spectrum strongly suggests that they derive from a nucleophilic attack of GSH to the 4,5-oxide of 2PT, at position 4 (Fig. 2). Thus, the formation of 2PTSOD and 2PTGA, as well as of 2PTGB, in recombinant CYP1A1-dependent reactions clearly shows that a cytochrome P450 may oxidize a thiophene compound with the simultaneous formation of two intermediates, a thiophene-*S*-oxide and a thiophene epoxide. Further studies are necessary to know whether this phenomenon is general; recent results obtained in this laboratory indicate that microsomal oxidation of 3-phenylthiophene also leads to metabolites derived from the corresponding *S*-oxide and epoxide intermediates (P. Dansette and D. Mansuy, in preparation). Another question that requires further studies is concerned with the mechanism of formation of 2PTT, the 5-hydroxylated metabolite of 2PT. As previously discussed for the 5-hydroxylated products of several 2-aroylthiophenes [25], these metabolites could derive from an isomerization of either a thiophene epoxide or a thiophene-*S*-oxide.

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