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Further Insights into the Oxidative Pathway of Thiocarbonyl-Type Antitubercular Prodrugs: Ethionamide, Thioacetazone, and Isoxyl

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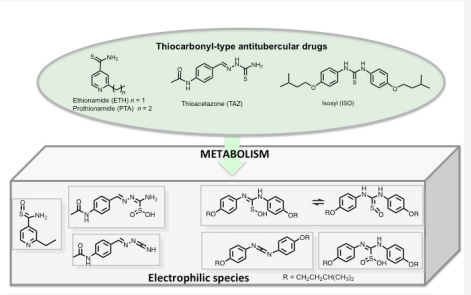
ABSTRACT: A chemical activation study of the thiocarbonyl-type antitubercular prodrugs, ethionamide (ETH), thioacetazone (TAZ), and isoxyl (ISO), was performed. Biomimetic oxidation of ethionamide using H_2O_2 (1 equiv) led to ETH−SO as the only stable *S*-oxide compound, which was found to occur in solution in the preferential form of a sulfine (ETH═S═O vs the sulfenic acid tautomer ETH−S−OH), as previously observed in the crystal state. It was also demonstrated that ETH−SO is capable of reacting with amines, as the putative sulfinic derivative (ETH−SO_2H) was supposed to do. Unlike ETH, oxidation of TAZ did not allow observation of the mono-oxygenated species (TAZ−SO), leading directly to the more stable sulfinic acid derivative (TAZ−SO_2H), which can then lose a SO_xH group after further oxidation or when placed in a basic medium. It was also noticed that the unstable TAZ−SO intermediate can lead to the carbodiimide derivative as another electrophilic species. It is suggested that TAZ−SOH, TAZ−SO_2H, and the carbodiimide compound can also react with NH_2-containing nucleophilic species, and therefore be involved in toxic effects. Finally, ISO showed a very complex reactivity, here assigned to the coexistence of two mono-oxygenated structures, the sulfine and sulfenic acid tautomers. The mono- and dioxygenated derivatives of ISO are also highly unstable, leading to a panel of multiple metabolites, which are still reactive and likely contribute to the toxicity of this prodrug.



1. INTRODUCTION

The resistance of *Mycobacterium tuberculosis* (*Mtb*) strains to first-line antitubercular drugs, especially to rifampicin and isoniazid, has been known for some time and is currently spreading around the world, provoking an actual health issue.[1] Awaiting the discovery of new anti-TB drugs, traditional second-line drugs have regained deep interest as a therapeutic alternative. Among them, thiocarbonyl-type compounds constitute a chemical family of antitubercular agents often associated with toxic side effects, which limit their clinical use.[2] Although representatives such as ethionamide (ETH), thioacetazone (TAZ), and isoxyl (ISO, also named thiocarlide) are old drugs (Figure 1), discovered more than 60 years ago, their mechanisms of action, toxicity, and chemical nature of their active/toxic metabolites are not completely understood to date. It is, however, known that these compounds act as prodrugs, i.e., require a biotransformation step to become active.[3] Although the three molecules are activated within *Mtb* by the same enzyme, called EthA,[3,4] their mechanisms of action and the chemical reactivity of their oxidative metabolites are reported to be quite different.[3] Moreover, the structural identity of those metabolites able to act against *Mtb* growth is not yet fully established and remains a matter of investigation. The scientific community is in agreement that this oxidative mechanism can also take place in the patient's body by action of human flavin-containing monooxygenase enzymes (FMO). Thus, this process can be involved in the well-known toxic pathway of several thiocarbonyl compounds such as thioacetamide.[3c,5]

With the view to gathering more information about the oxidative metabolites formed during the activation step of thiocarbonyl-type drugs (via EthA) and their possible toxicity (via FMO), a biomimetic chemical oxidation of ETH, TAZ, and ISO using H_2O_2 was carried out. The structure of the products was then elucidated using Raman, NMR, and mass spectrometry (MS), and their reactivity with amino nucleophiles was investigated.

2. EXPERIMENTAL PROCEDURES

Reagents and Material. Ethionamide (Sigma-Aldrich), thioacetazone (Acros), isoxyl (Cayman Chem), absolute ethanol (Aldrich), acetic acid (Aldrich), H_2O_2 (Aldrich), methylamine (Fluka), and 2-methoxyethylamine (Fluka) were used without further purification. The water used in the experiments was purified using a Milli-Q (Millipore) water system.

^{1}H NMR spectra were recorded on a Bruker spectrometer at 300, 400, and 600 MHz using DMSO-d_6 as the solvent and trimethylsilane as

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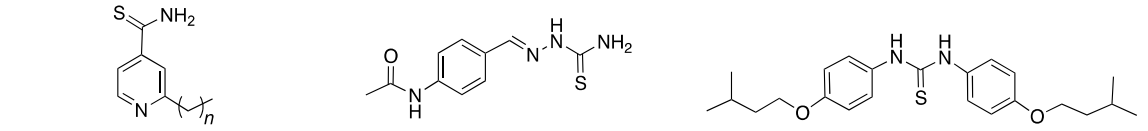

Figure 1. Chemical structures of thiocarbonyl-type compounds with antitubercular activity.

the reference. ^{13}C NMR spectra were recorded on a Bruker spectrometer at 101 and 151 MHz using DMSO-d_6 as the solvent. ^{15}N NMR spectra were recorded on a Bruker spectrometer at 50.7 MHz, and an external sample of CH_3NO_2 was used as the reference. Electrospray mass spectra (ESI) were acquired on a PerkinElmer SCIEX API 365 instrument, while high-resolution and desorption chemical ionization (DCI) mass spectra were recorded on a Finnigan TQS 7000 spectrometer. Normal Raman (NR) spectra of the samples were acquired by means of an Xplora (Horiba) microspectrometer: the excitation radiation was the 785 nm line, and the laser beam was focused on the sample by a 50× long distance objective.

Computational Details. All calculations were performed at the density functional theory (DFT) level using the hybrid functional B3LYP as implemented in the Gaussian 09 software package.[6] The 6-311++G(d,p) basis set was used for geometry optimization and vibrational frequencies calculation. A scaling factor of 0.9679 was applied for calculated harmonic vibrational wavenumbers considering the DFT/B3LYP/6-311++G(d,p) level of theory.[7] The Raman intensity was calculated from the Raman activity using Gausssum 3.0.[8]

Reactivity of ETH═S═O Toward Methylamine. To a solution of ETH═S═O (14 mg, 0.077 mmol) in EtOH was added 10 μL (0.116 mmol) of a 40% methylamine aqueous solution. The mixture was stirred for 15 min. The solvent was then evaporated, and the residue was analyzed by mass spectrometry. MS (ESI^-): m/z = 164.1 ($[M + H]^+$, 100%) and 180.1 ($[M + CH_4]^+$, 50%). M = $C_9H_{12}N_2O$.

Synthesis of TAZ−Sulfinic Acid (TAZ−SO_2H). The synthetic procedure for TAZ−sulfinic acid is based on the literature method[3c] with some modifications: 100 mg of TAZ was slowly added to a mixture of H_2O_2 (0.25 mL) and acetic acid (4 mL) in an ice bath (at 0 °C). This mixture was stirred for 10 min. This solution was then poured into cold water (20 mL), and the mixture was allowed to stand at 4 °C for 30 min until a white precipitate appeared. After filtration, the solid was washed with cold water, and 49 mg of a white solid was recovered.

^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 12.47 (br s, 1H), 10.19 (s, 1H), 9.38 (s, 1H), 8.90 (s, 1H), 8.51 (s, 1H), 7.83 (d, J = 8.5 Hz, 2H), 7.69 (d, J = 8.5 Hz, 2H), and 2.08 (s, 3H) ppm. ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 175.82 (Cq−S), 169.25 (Cq═O), 154.03 (N═CH), 142.68 (C_{arom}q), 129.68 (2CH), 127.93 (C_{arom}q), 119.12 (2CH), and 24.58 (CH_3) ppm. ^{1}H/^{15}N-HMQC, NMR 2D (50.7 MHz, DMSO-d_6) δ (ppm): 164 (CH═N−N), 136 (CONH), 102 (C−NH_2), −94 (CH═N−N).

MS (ESI^-): m/z 267 $[M - H]^-$. HRMS (ESI^-) for $C_{10}H_{11}N_4O_3S$): calcd., 276.0552; found, 267.0541.

The filtrate of TAZ−sulfinic acid was poured into cold diethyl ether (20 mL) for precipitation. After filtration, the yellow solid was washed with cold ether and dried under vacuum, and 50 mg of the yellow solid was recovered. The ^{1}H NMR spectrum revealed the presence of two major products, amidine **4** (described in the next section) and TAZ−sulfonic acid (data below).

^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 12.88 (br s, 1H), 10.22 (s, 1H), 9.73 (s, NH_2), 8.57 (s, 1H), 7.88 (d, J = 8.7 Hz, 2H), 7.71 (d, J = 8.7 Hz, 2H), and 2.08 (s, 3H) ppm.

MS (ESI^-): m/z 283 $[M - H]^-$.

Synthesis of the Amidine 4. A 30% H_2O_2 aqueous solution (110 μL) was added into a solution of TAZ−SO_2H (15 mg, 0.07 mol) in 3 mL of acetic acid.

This mixture was stirred for 2 h at room temperature. Then, 10 mL of diethyl ether was added for precipitation. The solvent was removed, and the solid was washed with diethyl ether. Later, the isolated solid was analyzed by NMR and mass spectrometry.

^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.24 (s, NHCO, 1H), 9.62 (d, J = 6 Hz, NH, 1H), 9.44 (d, J = 12 Hz, NH, 1H), 8.33 (s, PhCH═N, 1H), 8.24 (dd, J = 6.0 and 12.0 Hz, N═CH−NH_2, 2H), 7.87 (d, J = 7.5 Hz, CH arom, 2H), 7.71 (d, J = 7.5, CH arom, 2H), and 2.08 (s, CH_3, 3H) ppm. ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm): 169.31 (C═O), 153.96 (PhCH═N), 151.55 (N−CH−NH_2), 143.03 ($\underline{C}_{arom}$q−NHCO), 129.88 (2CH), 127.38 ($\underline{C}_{arom}$q−CHN), 119.16 (2CH), and 24.62 (CH_3) ppm. ^{1}H/^{15}N-HMQC, NMR 2D (60.8 MHz, DMSO-d_6) δ (ppm): 171 (CH═$\underline{N}$−$\underline{N}$), 136 (CONH), 112 (C−NH_2), −94 (CH═$\underline{N}$−$\underline{N}$).

MS (DCI/NH_3): m/z 205 $[M + H]^+$.

Synthesis of the Azine 5. A solution of TAZ−SO_2H in DMSO-d_6 was left at room temperature for several days (10 days), which was further analyzed as described below.

^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.20 (s, NHCO, 1H), 8.62 (d, J = 6 Hz, NH, 1H), 7.80 (d, J = 8.7 Hz, CH arom, 2H), 7.70 (d, J = 8.7, CH arom, 2H) and 2.09 (s, CH_3, 3H) ppm. ^{13}C NMR (75.5 MHz, DMSO-d_6) δ (ppm): 161.09 (C═O), 142.46 ($\underline{C}_{arom}$q−NHCO), 129.58 (2CH), 128.93 ($\underline{C}_{arom}$q−CHN), 119.27 (2CH), and 24.61 (CH_3) ppm. ^{1}H/^{15}N-HMQC, NMR 2D (60.8 MHz, DMSO-d_6) δ (ppm): 171 (CH═$\underline{N}$−$\underline{N}$), 136 (CONH), 112 (C−NH_2), −94 (CH═$\underline{N}$−$\underline{N}$). MS (DCI/NH_3): m/z 323 $[M + H]^+$.

Reactivity of TAZ−Sulfinic Acid (TAZ−SO_2H) with Nucleophiles. TAZ−SO_2H (16 mg, 0.06 mmol) was suspended in 2 mL of absolute ethanol (25 °C). Then, a 40% methylamine aqueous solution (10 μL, d = 0.89 g cm^{-3}) or 2-methoxyethylamine (7.0 μL, 0.08 mmol, d = 0.872 g cm^{-3}) was added into the suspension. After addition of the nucleophile, the TAZ−SO_2H acid dissolved entirely within a few seconds. This mixture was stirred for 10 min. For the reaction with 2-methoxyethylamine, the solvent was evaporated under reduced pressure and the obtained solid (14 mg) was analyzed by mass spectrometry. For the reaction with methylamine, the mixture was then poured into diethyl ether, and the mixture was allowed to stand at 4 °C for 30 min until a white precipitate appeared. After filtration, 14 mg of a white solid was obtained and analyzed by mass spectrometry.

8a and **9a**, Nu═H_2NCHCH_2OCH_3: MS (ESI^+) m/z 278 ($M_{guanidine}$ + H)$^+$, 263 ($M_{amidine\ substituted}$ + H)$^+$, 205 ($M_{amidine\ 4}$ + H)$^+$.

8b and **9b** Nu═CH_3NH_2: MS (ESI^+) m/z 234 ($M_{guanidine}$ + H)$^+$, 219 ($M_{amidine\ substituted}$ + H)$^+$, 205 ($M_{amidine\ 4}$ + H)$^+$.

Reactivity of the Amidine 4 Toward H_2NCHCH_2OCH_3. Amidine **4** (13 mg, 0.06 mmol) was dissolved in 2 mL of absolute ethanol (25 °C). Then, 2-methoxyethylamine (7.0 μL, 0.08 mmol, d = 0.872 g cm^{-3}) was added. This mixture was stirred for 10 min, and the solvent was removed under reduced pressure. The solid residue **9a** was analyzed by ^{1}H NMR and MS.

^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.13 (s, NHCO, 1H), 8.18 (s, CH, 1H), 8.08 (s, CH, 1H), CH_{arom}, 7.58 (m, J = 3.4 Hz, CH_{arom}, 4H), 3.47 (t, J = 6.0 Hz, CH_2, 2H) 3,27 (s, OCH_3, 3H), 2.88 (t, J = 6.0 Hz, CH_2, 2H), and 2.06 (s, C═OCH_3, 3H) ppm.

MS (ESI^+): m/z 263 $[M + H]^+$.

ISO Oxidation by H_2O_2. To a solution of isoxyl (31 mg, 0.078 mmol) in a mixture of 3.5 mL of acetone and 0.15 mL of acetic acid, 80 μL (0.71 mmol) of a 30% H_2O_2 aqueous solution was added. This mixture was stirred for 1 h at room temperature. Then, this suspension was centrifugated and the solvent removed under low pressure. The solid recovered was dried under vacuum. Analysis by TLC showed formation of several products. The raw solid was applied onto a

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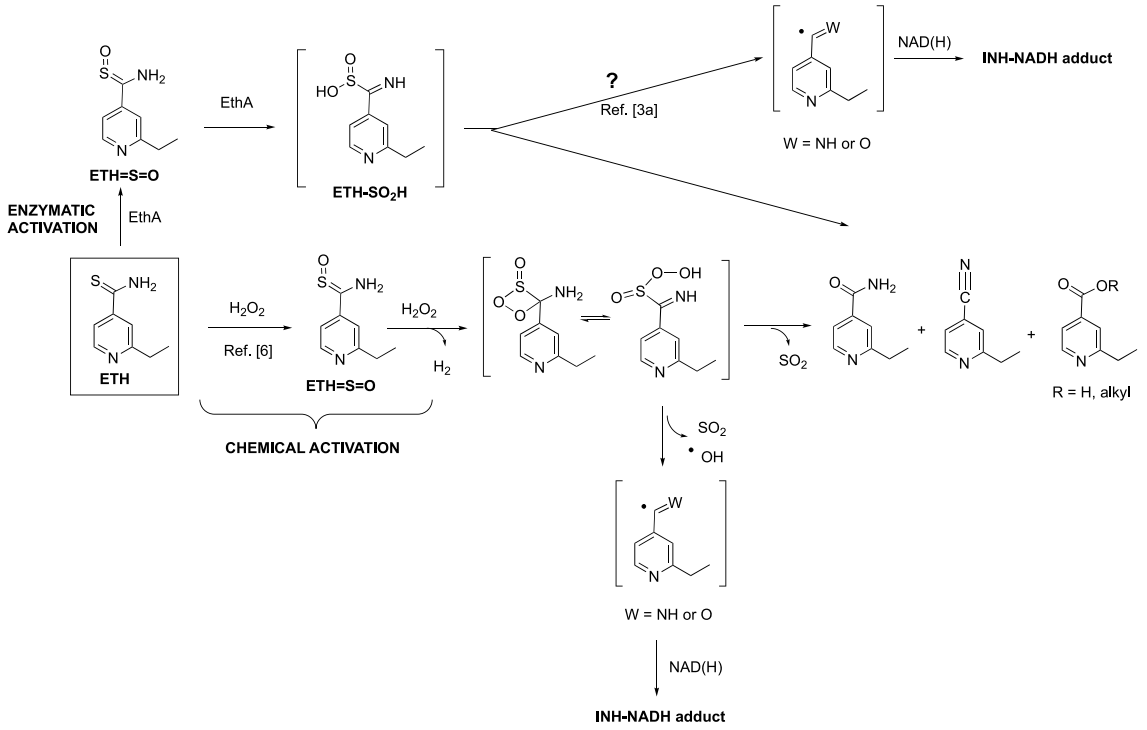
Scheme 1. Proposed Reactivity of Ethiomamide (ETH) in *Mtb*



chromatographic column of SiO_2 (hexane/ethyl acetate 5% → 25% then CH_2Cl_2 and acetone), and three fractions were obtained, whose NMR spectra are shown in the Supporting Information.

3. RESULTS AND DISCUSSION

3.1. Ethionamide. Ethionamide (ETH) and prothionamide (PTA) (Figure 1) are both thioamide-based prodrugs that, after oxidative enzymatic activation by EthA, act similarly to isoniazid via an isonicotinoyl-type radical intermediate.[9] The radical generated from ETH, which was detected by EPR,[10] reacts covalently with the cofactor NAD(H), giving rise to an adduct (ETH−NADH), which then inhibits InhA, an essential enzyme for *Mtb*.[4] Due to the close structural homology between ETH and PTA and their identical mechanism of action, only ETH was investigated in this work.

It is assumed that oxidation of ETH by the EthA enzyme first generates the corresponding *S*-oxide (ETH−SO) as an intermediate, which then undergoes further oxidation to the corresponding sulfinic acid (ETH−SO_2H; Scheme 1). This latter species was proposed as the direct precursor of the harmful 2-ethylisonicotinoyl radical (or iminoyl radical).[3a] However, this proposition is still fragile, since ETH−SO_2H has never been formally isolated nor detected. Moreover, the hypothesis that an aroyl radical could be formed by homolytic cleavage of the C−S bond from the putative ETH−SO_2H is poorly supported in the literature. We recently proposed that the aroyl radical can be formed without involvement of a sulfinic acid precursor via a mechanism initiated by a more conventional homolytic cleavage of a peroxide bond embedded in a 1,2-dioxetane cyclic intermediate.[10] Through this cyclic intermediate, the observed formation of desulfurized nontoxic metabolites such as the corresponding amide, acid/esters, and nitrile could also be explained (Scheme 1).

Analogously to other well-studied thiocarbonyl compounds,[2b] ETH is also a substrate for the mammalian FMO enzyme,[5a−c] being first converted to ETH−SO and then to the presumed ETH−SO_2H, even though this second S oxygenation seems to be slower.[5c] The toxicity of ETH could thus be attributed to this FMO metabolization step, converting ETH into the putative sulfinic acid (ETH−SO_2H), which has been assumed to react directly as a nonradical acylating agent with cellular proteins and lipids.[11] While there is experimental evidence for the presence of covalent enzymatic adducts, that of the sulfinic acid species has not been validated. To the best of our knowledge, no study has ever been carried out on the chemical reactivity of the *S*-oxide ETH derivative (ETH−SO) with amino nucleophiles and its possible involvement in toxic pathways.

ETH−SO was previously described,[3d,10,12] proving to be sufficiently stable to be isolated. We recently reported the X-ray crystal structure of ETH−SO,[10] in which only one tautomer, the amino−sulfine form (ETH═S═O and not the imino−sulfenic acid form ETH−S−OH), was identified as a single stereoisomer (*Z*). Beyond the structure of ETH−SO in the solid state, we were interested in learning more about the tautomeric equilibrium in aqueous buffered solution as a model of the biological media. To address this question, careful characterization of ETH−SO by Raman spectroscopy was performed. The experimental Raman spectra of ETH−SO in the solid state or dissolved (saturated) in 0.1 mol L^{-1} phosphate buffer solution (pH 7.4) were recorded (Figure 2) and compared with the DFT-calculated ones (DFT = density functional theory) for both the sulfine (ETH═S═O) and the sulfenic acid (ETH−S−OH) tautomeric forms (Figure 2). The results indicated that the experimental Raman spectra of ETH−SO in either the solid or the solution state match much closer with the theoretical spectrum of ETH═S═O than that of ETH−S−OH (Figure 2). These results are in agreement with our previous theoretical calculations of the difference of energy ($\Delta\Delta G$) between ETH═S═O and ETH−SOH, showing that ETH═S═O was more stable than ETH−S−OH.[13,14] It can be therefore assumed that in vivo ETH−SO is found essentially under a single tautomeric form, (*Z*)-ETH═S═O.

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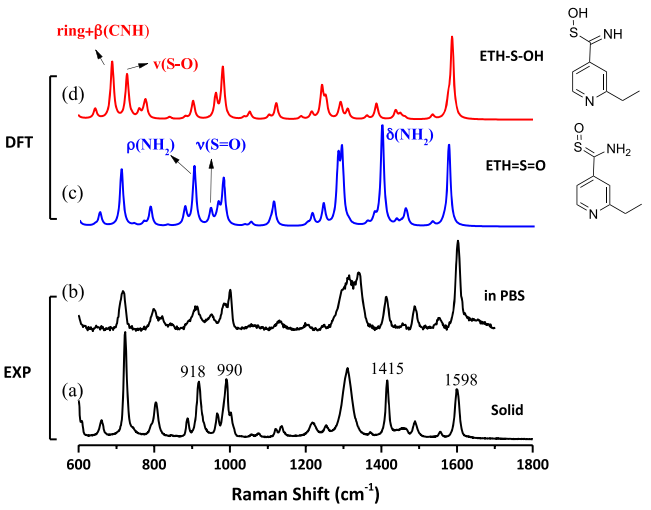


Figure 2. Experimental normal Raman spectrum (λ_0 = 785 nm) of (a) solid ETH−SO and (b) saturated ETH−SO solution in 0.1 M PBS at pH 7.4, and DFT-calculated Raman spectra of ETH═S═O (c) and ETH−S−OH (d) tautomers at the B3LYP/6-311++G(d,p) theory level.

With the ETH═S═O sample in hand, its reactivity with amino nucleophiles was investigated in order to estimate its possible interactions with biomolecules causing direct toxicity. The reaction of ETH═S═O with methylamine (1.5 equiv) was carried out under mild conditions and analyzed by mass spectrometry (MS). The mass spectrum (DCI/CH_4) showed peaks at m/z = 164.1 (100%) and 180.1 (50%), which were, respectively, assigned to the $[M + H^+]$ and $[M + CH_5^+]$ fragments of the amidine structure **1**; alternatively, the same peaks could be assigned to the $[M]^+$ and $[M + CH_4]^+$ fragments of the amide structure **1′** (Scheme 2).

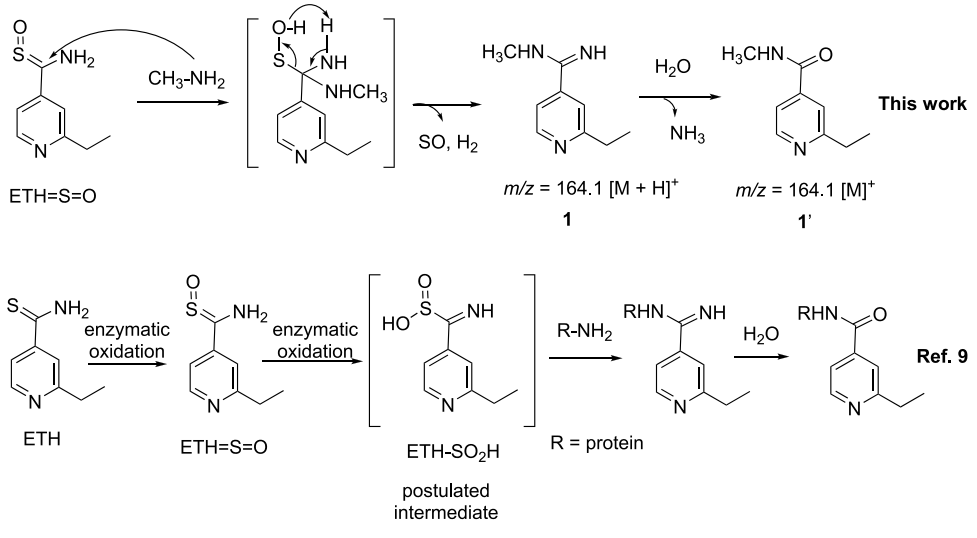
As ETH−SO is able to react with an amine, it can be assumed that it can also react with protein amino groups in vivo and can manifest a toxic potential similar to that reported for the putative ETH−SO_2H. Since ETH−SO_2H could never be isolated, it was not possible to study the corresponding reaction of ETH−SO_2H with methylamine. While the "acylation" (imidoylation) potential of the putative sulfinic acid ETH−SO_2H toward nucleophiles can only be indirectly suggested, that of the corresponding sulfine was unambiguously confirmed using ETH−SO as a direct precursor.

3.2. Thioacetazone (TAZ). TAZ is a thiourea-type antitubercular compound (MIC = 0.1−0.5 μg/mL; 0.4−2.1 μM)[15] proposed to be also activated by EthA. Indeed, clinical cases of cross resistance between ETH and TAZ have been described,[16] and mutations of the EthA enzyme have been shown to confer TAZ resistance.[17]

Enzymatic oxidation of thioacetazone by a recombinant EthA enzyme was reported by Ortiz de Montellano and co-worker[3c] to yield TAZ−SO_2H and carbodiimide **2** as major and minor products, respectively. These products were separated by HPLC, and their elution times and UV spectra were compared with those of synthetic standards (Scheme 3). Albeit not detected, the monooxygenated TAZ derivative (TAZ−SO) was assumed to be the unstable precursor of both TAZ−SO_2H and carbodiimide **2**. Later in 2009, François et al.[5b] reported the detection of TAZ−SO, as well as TAZ−SO_2H and carbodiimide **2**, which were observed using either EthA or *Sf*9 cell microsomes containing heterologously expressed human FMO1 or FMO3. While for ETH it is postulated that the active and toxic metabolites are both derived from two oxidation steps, for TAZ it has been postulated that the monooxygenated TAZ derivative (TAZ−SO) in its sulfenic acid tautomeric form (TAZ−S−OH) is the actual active and toxic metabolite of this drug. It is noteworthy to mention that our previous work on the calculation of the difference in free enthalpy ($\Delta\Delta G$) between TAZ═S═O (sulfine) and TAZ−S−OH (sulfenic acid) indicated that TAZ−S−OH is more stable than TAZ═S═O by 4.2 kcal/mol, in contrast to ETH, for which the sulfine form was the most stable.[13] Furthermore, it is curious to note that oxidative enzymatic activation of TAZ by EthA is not found to give the putative TAZ−NADH adduct, while TAZ−SO_2H is still formed.[3c] There are interesting reactivity particularities where the dioxygenated metabolite of TAZ (TAZ−SO_2H) does not behave as ETH (ETH−NADH formation) at all.

Jackson et al.[18] proposed that the TAZ−SOH tautomer reacts as an electrophilic agent with the Cys61 residue of the protein HadA (dehydratase enzyme of the FAS-I type II fatty acid synthase−elongation cycle), thus forming a covalent disulfide bond. As a result, it modifies the protein structure and makes it

Scheme 2. Reactivity of Amines with ETH Oxidation Products



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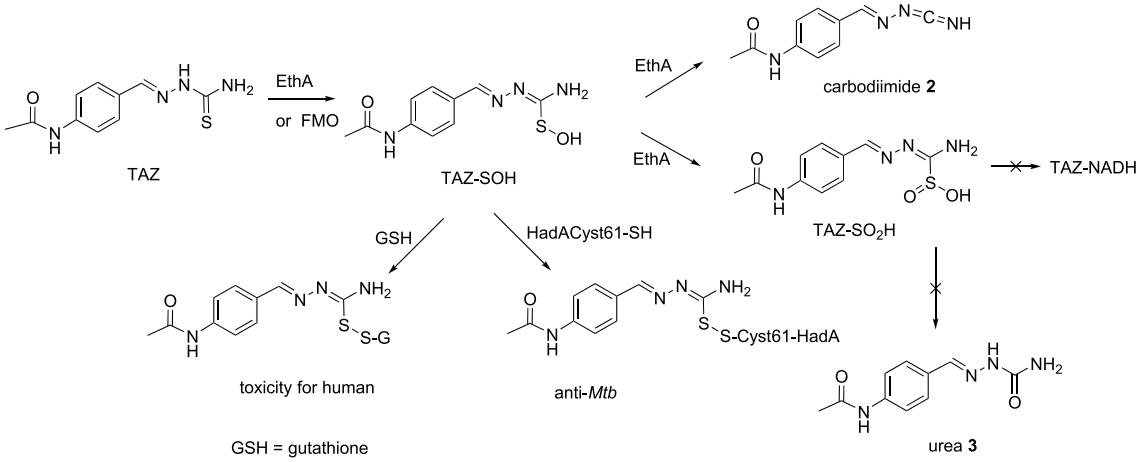
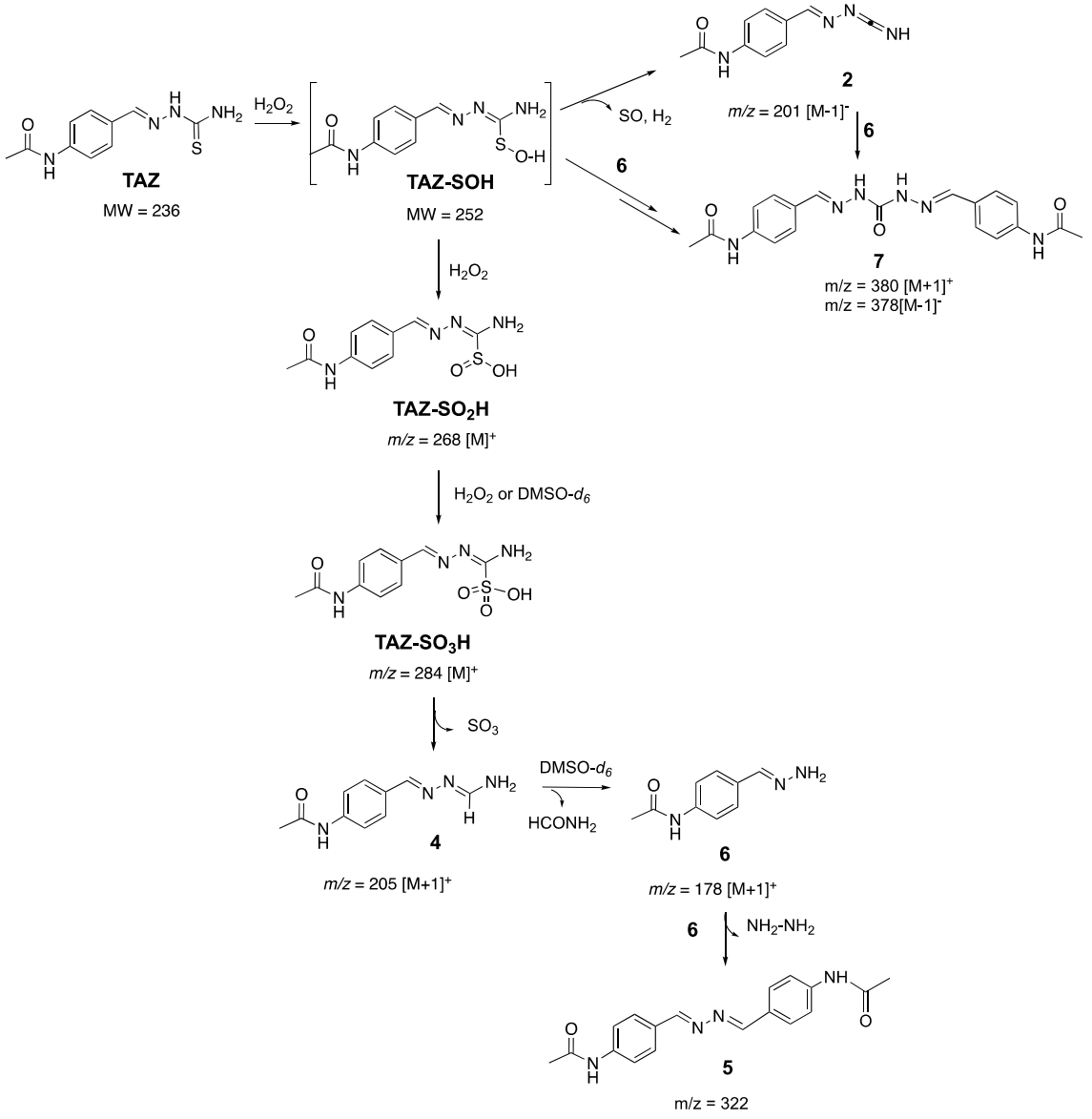
Scheme 3. Proposed Reactivity of Thioacetazone (TAZ) in *Mtb*



Scheme 4. Oxidative Decomposition Pathways of Thioacetazone (TAZ)



inactive, thus interrupting the mycolic acid biosynthesis. Qian and Ortiz de Montellano[3c] also proposed that TAZ−SOH (with an electrophilic sulfur atom) could also react with the sulfur atoms of mycothiols (small molecules acting as antioxidant) by a

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similar reaction (S−S bond formation) in *Mtb*,, thus provoking an oxidative stress in the mycobacterium and accounting for TAZ antimycobacterial activity. These authors demonstrated that TAZ−SOH could also be formed by the action of human FMOs and then interact with glutathione (potent antioxidant molecule in the liver). This process is proposed to explain, at least in part, the hepatotoxicity of TAZ. In contrast to what is reported for ETH, no toxic (for *Mtb* and human) effect associated with TAZ−SO_2H has been reported.

In summary, the ETH and TAZ reactivities toward oxidizing enzymes are very different. If, on one hand, TAZ−SOH can react as an electrophile or be further oxidized to TAZ−SO_2H, on the other hand, the latter compound does not evolve to the NADH−drug adduct as putative ETH−SO_2H is supposed to do. TAZ−SO_2H does not lead, via a desulfurization step, to the urea **3** compound: an equivalent pathway, found in the literature, to explain formation of the ETH amide derivative from ETH−SO_2H (Scheme 3).[19]

The chemical oxidation of TAZ by hydrogen peroxide[3c] was first studied by Qian and Ortiz de Montellano, who reported that the reaction of TAZ with H_2O_2 (20 equiv) in acetic acid gives TAZ−SO_2H in 20% yield after precipitation in water. This low yield might be due to (i) incomplete conversion at the first and/or second oxidation steps (in spite of the 20 equiv of H_2O_2), (ii) loss of TAZ−SO_2H during the purification step, or (iii) formation of other water-soluble products that were not isolated and ended up discarded (as carbodiimide **2** and other products). The same conditions were thus implemented, and in our hands, TAZ−SO_2H was obtained in 39% yield using cold water for precipitation and washing. The filtrate was then poured into diethyl ether, and a second solid was formed and recovered.

The ^{1}H NMR data of the main product (Figure S1A) were in agreement with those previously reported for TAZ−SO_2H.[3c] When compared to TAZ, the NH_2 signals of TAZ−SO_2H were shifted downfield from 7.90 and 8.13 ppm to 8.90 and 9.38 ppm. In addition, the absence of a singlet signal at 10.08 ppm, assigned to the NNH moiety in TAZ, along with the appearance of a new broad signal at 12.47 ppm, attributable to a O*H* group, suggested the formation of TAZ−SO_2H (Figure S1A). Analysis of the TAZ oxidation product was completed by ^{13}C NMR, 2D ^{1}H−^{15}N NMR, and high-resolution mass spectrometry. NMR spectra were recorded, in deuterated DMSO, immediately after sample preparation, which were consistent with the proposed structure of TAZ−SO_2H (Figures S1B and S2), in particular the ^{13}C signal of the Ar−C̲H═N unit (154.0 vs 142.5 ppm for TAZ). The 2D ^{1}H−^{15}N HMQC spectrum shows the presence of four different nonequivalent nitrogen atoms at 164, 136, 102, and −94 ppm corresponding to ArCH═N̲−N═, H_3CCON̲H−Ar, N̲H_2C═N, and −N̲═C(SO_2H)−NH_2, respectively. Due to the low solubility of TAZ−SO_2H in water, the Raman spectrum was not collected, but further assignment of this species was achieved by the presence of a peak at m/z = 267 $[M - H]^-$ in the negative mode of the ES-MS spectrum (Figure S3). Nevertheless, an additional peak at m/z = 283 $[M - H]^-$ (16 higher than TAZ−SO_2H) was observed, suggesting the presence of the sulfonic acid TAZ−SO_3H, which was not observed by neither ^{1}H nor ^{13}C NMR spectroscopy. The latter species could be a result of the oxidation of TAZ−SO_2H by dissolved dioxygen and/or by DMSO used as solvent in the preparation of MS samples (the samples were analyzed only a few days later). On the other hand, monitoring a sample of TAZ−SO_2H in DMSO-d_6 by ^{1}H NMR over 72 h showed that TAZ−SO_2H happens to be unstable, slowly converting to a new substance, which, after analysis by ^{1}H NMR, ^{13}C NMR, and MS spectroscopies (Figures S4−S6), was assigned to the desulfurized amidine structure **4** (Scheme 4). In the ^{13}C NMR spectrum (Figure S4, spectrum B), indeed, the disappearance of the N_2C−S signal of TAZ−SO_2H at 175.8 ppm and concomitant appearance of a signal at 151.6, assignable to a N_2C−H motif, suggest the extrusion of SO_2. In the ^{1}H NMR spectrum (Figure S4, spectrum A), a doublet of doublets at 8.25 ppm integrating for one proton (and corresponding to the ^{13}CH signal at 151.6 ppm) indicates two couplings with two differentiable ^{1}H nuclei such as the syn and anti NH protons of the amidine H(N)C═NH_2 bond. In the 2D ^{1}H−^{15}N NMR spectrum (Figure S6), the ^{15}N signal at 112 ppm correlates with two hydrogens at 9.60 and 9.45 ppm, confirming the presence of an NH_2 group. All of these data are consistent with the amidine structure **4**, which was confirmed by MS analysis showing a peak at m/z = 205, which corresponds to the molecular weight ion plus one ($[M + 1]^+$) (Scheme 3).

In deuterated ethanol [CD_3CD_2OD] solution, the decomposition of TAZ−SO_2H was found to be much slower than that in DMSO solution, suggesting that DMSO can play a role as an oxidizing agent. Moreover, no O-acylation product of ethanol was observed, supporting the weak acylating power of TAZ−SO_2H itself.

Altogether, these results suggest that TAZ−SO_2H might decompose to the amidine product **4** through a transient sulfonic intermediate TAZ−SO_3H, detected by MS and resulting from a further oxidation by DMSO and/or dioxygen. To determine whether further oxidation of TAZ−SO_2H is indeed required for the formation of the amidine **4**, TAZ−SO_2H was treated with H_2O_2 (1 equiv) in acetic acid medium at ca. 0 °C for just 10 min (instead of the initial 70 h in the absence of H_2O_2). ^{1}H NMR analysis, in DMSO-d_6 solution, indicated that TAZ−SO_2H was completely consumed and quantitatively converted to the amidine **4**, thus confirming that TAZ−SO_2H oxidation is a prerequisite for the formation of **4**.

The DMSO-d_6 solution of **4**, in contact with air for ca. 96 h, yielded single crystals suitable for X-ray diffraction analysis. Surprisingly, the cystallographic structure (Table S1) indicated the formation of a new product, azine **5** (Figure 3 and Scheme 3). MS and ^{1}H NMR spectra of the sample (Figures S7 and S8) are also consistent with the structure of **5**. According to the ^{1}H NMR results, the acetamidophenyl portion is conserved accompanied by another single signal (singlet) at 8.62 ppm. The formation of the symmetrical azine **5** might proceed by hydrolysis of the amidine **4**, yielding the hydrazone **6**, which would then undergo an autocondensation process (Scheme 4).

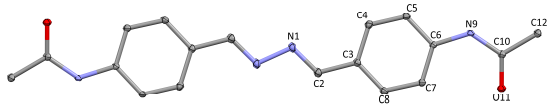

Figure 3. X-ray crystal structure (ORTEP plot) of the symmetrical azine **5**. H atoms were omitted for clarity.

Beyond TAZ−SO_2H, isolated in 39% yield, treatment of TAZ with H_2O_2 also led to a second solid collected from the ether fraction (see above). ^{1}H NMR analysis indicated that this solid consists of two main compounds identified as the above-described amidine **4** (sharp singlet C*H*═N at 8.32 ppm) and the likely sulfonic acid TAZ−SO_3H (a broad singlet of SO_3H at 12.88 ppm, sharp singlet of C*H*═N at 8.57 ppm, and singlet of NH_2 at 9.73 ppm) (Figure S9). These species would have been produced by oxidation of the remaining TAZ−SO_2H (not

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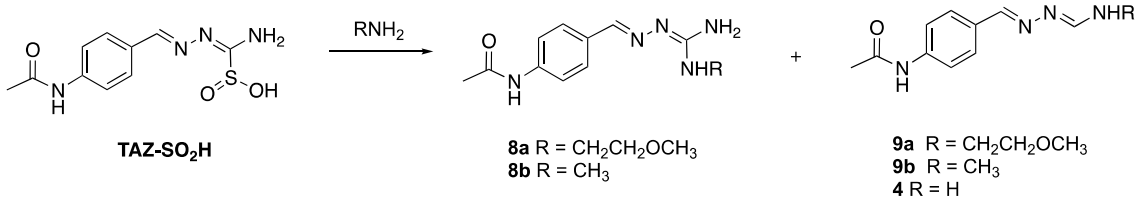
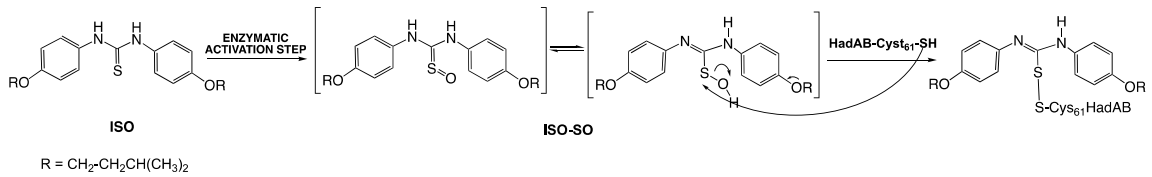
Scheme 5. Reaction of the Thioacetazone Sulfinic Derivative with Amines



Scheme 6. : Proposed Molecular Mechanism of ISO Interaction with the *Mtb* Target, HadAB Enzyme (FAS-II Cycle)



precipitated after water addition) and/or from additional in situ oxidation of TAZ−SO_2H forming a more hydrosoluble compound (TAZ−SO_3H). In addition, ESI-MS analysis showed peaks at m/z = 283 $[M - 1]^-$ and 201 $[M - 1]^-$ in the negative mode and at m/z = 205 $[M + 1]^+$ in the positive mode (Figure S10), which are consistent with TAZ−SO_3H, carbodiimide **2** (minor), and amidine **4** (major), respectively. These results show that the conversion of TAZ is complete, but the low yield in sulfinic acid (TAZ−SO_2H) may be due to its limited solubility in EtOH/H_2O. This may allow its overoxidation, generating a more hydrosoluble sulfonic counterpart TAZ−SO_3H, gradually decomposing to the amidine **4**.

Attempts to oxidize TAZ using a smaller amount of H_2O_2 (1−2 equiv instead of 20 equiv) did not allow detection of the sulfine intermediate TAZ−SO or TAZ−S−O−H tautomer on the way to TAZ−SO_2H. Even though TAZ is not completely consumed, the amidine **4** (produced from TAZ−SO_2H) could be evidenced by MS analysis (m/z = 205 $[M + 1]^+$) along with the hydrazone **6** (m/z = 178 $[M + 1]^+$). The latter species would be generated through oxidation of TAZ−SO_2H to TAZ−SO_3H followed by hydrolytic release of SO_3 and formamide HC(O)-NH_2 (Scheme 4). In addition to TAZ−SO_2H, ES-MS analysis in the negative mode suggests the formation of two other compounds: the carbodiimide **2** (m/z = 201) and the urea **7** (m/z = 378), which would result from the condensation of **2** with the hydrazone **6**.

Finally, these observations can be interpreted by assuming that oxidation of the sulfenic intermediate TAZ−SOH occurs much faster than oxidation of TAZ into TAZ−SOH.

Reaction of TAZ−SO_2H with Nucleophiles. With the goal of evaluating the potential toxicity of TAZ−SO_2H, its reactivity with nucleophiles was investigated in the same way as it was performed previously for ETH−SO. Methylamine (1.5 equiv) or 2-methoxyethylamine (1.5 equiv) was thus added to a hydroalcoholic suspension of TAZ−SO_2H: after 10 min, the reaction medium was concentrated to dryness and the residue analyzed by MS. From the methoxyethylamine treatment, peaks at m/z = 278.1 $[M + 1]^+$ and 263.2 $[M - 15]^+$ could be assigned to the guanidine **8a** and amidine **9a**, respectively. Likewise, from methylamine treatment, peaks at m/z = 234.1 $[M + 1]^+$ and 219.1 $[M - 15]^+$ were assigned to the guanidine **8b** and amidine **9b**, respectively. In both cases, the peak of the amidine **4** at m/z = 205.1 was also observed. Therefore, while the guanidines **8a** and **8b** arise from the reaction of TAZ−SO_2H with R−NH_2 (R = Me, $MeOCH_2CH_2$), the amidine **9** could be generated by exchange of the amino group with the amidine **4** resulting from the decomposition of TAZ−SO_2H via extrusion of SO_2 (Scheme 5). Indeed, the reaction of methoxyethylamine with **4**, independently prepared by overoxidation of TAZ−SO_2H (see above), was shown to produce amidine **9a** according to MS (m/z = 263.2 $[M + 1]^+$).

In summary, TAZ−SO_2H reacts with amines, as an electrophile or as an acid, giving rise to distinct compounds. As for ETH═S═O, it is assumed that TAZ−SO_2H and the carbodiimide **2** are both able to react with the amino residues of the amino acid side chains of the proteins.

3.3. Isoxyl. As for thioacetazone, isoxyl (ISO) is also a thiourea-based prodrug known for its bacteriostatic activity against *Mtb* (MIC = 1−10 μg/mL, 2.5−25 μM) that was used as an antituberculosis agent in the 1960s.[20] Jackson et al. demonstrated that EthA-mediated oxidation of ISO is required for its antimycobacterial action.[3b,21] It is reported that this drug acts by inhibition of oleic and mycolic acid biosynthesis in *Mtb*.[18,21,22] While the target for the inhibition of oleic acid biosynthesis is the stearoyl-coenzyme A (Δ9) desaturase enzyme (DesA3), that of mycolic acid synthesis is a dehydratase enzyme (HadAB) belonging to the FAS-II pathway. These authors thus proposed that several macromolecular cellular targets might be attacked by different metabolites of ISO. Once using purified EthA for in vitro oxidation of ISO, Jackson et al. confirmed the existence of several such metabolites: the reaction was not complete, but the main metabolites were detected by MS. These compounds were later identified (see below) as the urea derivative **10** (m/z = 385 $[M + 1]^+$, equivalent to the amide metabolite of the ETH thioamide) (Schemes 7 and 1) and the desulfurized amidine **11** (m/z = 369 $[M + 1]^+$, equivalent of **4** in the TAZ series) (Schemes 7 and 4). Two minor LC unresolved products evidenced by LC-MS at m/z = 417 were assigned to ISO−oxide isomers (MW = 416). Two unassigned signals at m/z = 474 and 546 were attributed to experimental artifacts by these authors. In 2015, the same authors using a whole-cell system demonstrated that the sulfenic acid form (ISO−SOH) of the ISO oxide metabolite was able to react with a cysteine residue (Cys61) of the HadAB dimer, thus forming a disulfide covalent bond (Scheme 6) and showing that ISO−SOH, just as for TAZ−SOH in the TAZ series, is an active metabolite of that prodrug.

Jackson's experimental results are in agreement with our previous computational data, which showed that the two tautomers of ISO oxide, ISO═S═O (sulfine form) and

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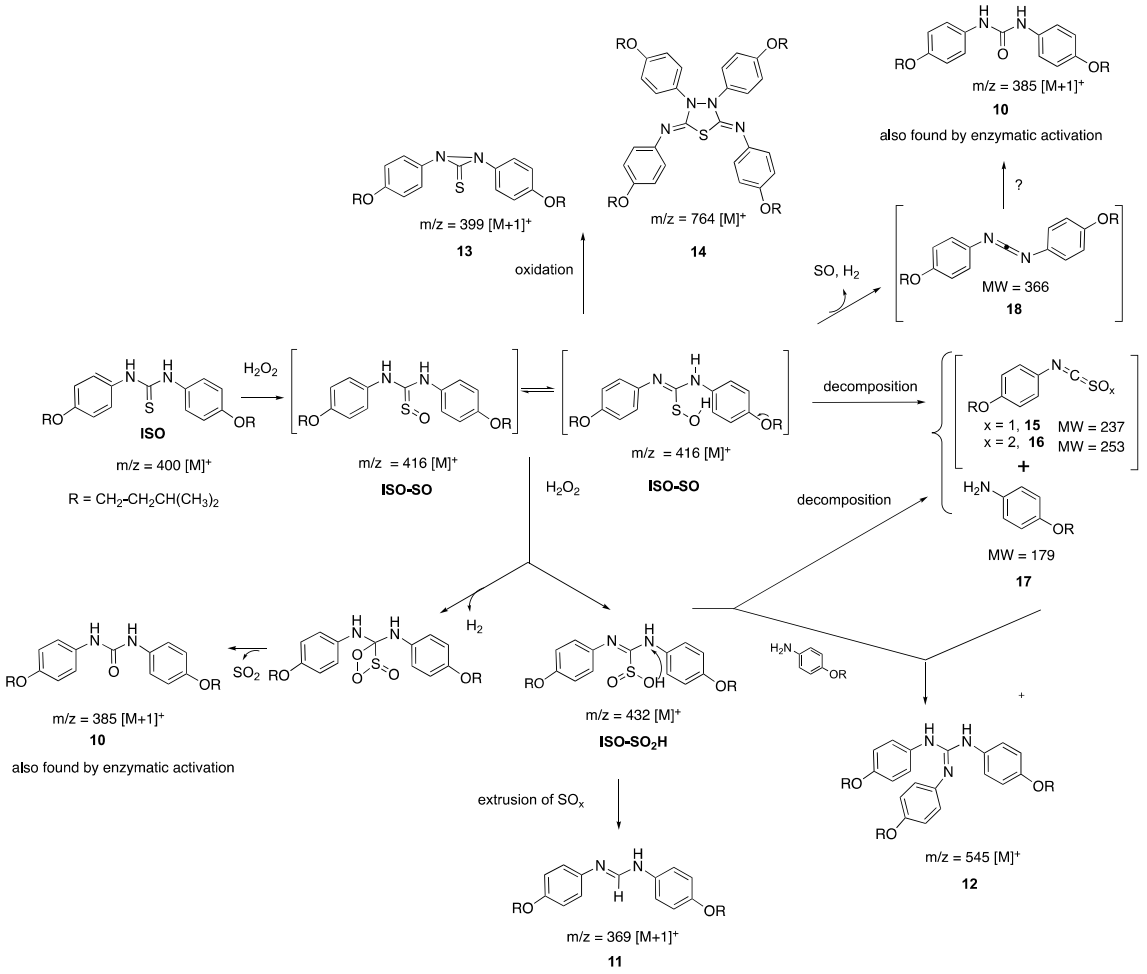
Scheme 7. Oxidative Decomposition Pathways of Isoxyl (ISO)



ISO−SOH (sulfenic acid form),[13] have similar energies and might be simultaneously present, interconverting in solution. Indeed, the two unresolved ISO oxide isomers detected by LC-MS can be assigned to these tautomeric species.

Chemical activation of ISO was envisaged using the biomimetic system (H_2O_2) validated in the ETH and TAZ series (see above). Treatment of ISO in an acetic acid:acetone solution (0.045:1 for solubility purposes) with 10 equiv of H_2O_2 at 0 °C for 1 h was observed to generate a complex mixture of products according to TLC analysis. Column chromatography of this mixture on silica gel yielded three fractions, each consisting of several products. The most polar fraction showed two main TLC spots corresponding to two MS peaks at m/z = 369 and 546 (Figure S12). The latter was also previously detected by Jackson et al. but assigned to an artifact. The ^{1}H NMR spectrum in the aromatic region (Figure S11) showed two pairs of doublets in a 2.2:1 ratio: a major pair of signals at 7.44 (d, J = 8.9 Hz) and 7.00 (d, J = 8.9 Hz) ppm and a minor pair at 7.25 (d, J = 8.8 Hz) and 6.85 (d, J = 8.9 Hz) ppm. In addition, a singlet signal at 8.93 ppm characteristic of a C*H* amidine proton and with consistent hydrogen integration was attributed to the major product, which can be assigned to the pseudosymmetrical amidine structure **11** (MW = 368 consistent with the MS peak at m/z = 369 $[M + 1]^+$). Though not very informative, ^{1}H NMR data of the minor product are in line with a truly symmetrical structure with a C*H* chemical shift different from that of a thiourea motif (Figure S11): for this minor compound, the guaninide structure **12** would be consistent with the other MS peak at m/z = 546 ($[MH]^+$). MS analysis of the less polar fraction showed two major peaks at m/z 399 (100%) and 765 (90%) (Figure S12). The corresponding ^{1}H NMR spectrum showed two sets of aromatic protons, a major set at 7.34 (d, J = 9.0 Hz) and 7.00 (d, J = 9.0 Hz) ppm and a minor set at 7.15 (d, J = 8.9 Hz) and 6.93 (d, J = 8.8 Hz) ppm, without any signal above 7.5 ppm, ruling out the presence of a thiourea motif (ISO shows NH protons at 8.66 ppm) (Figure S11). Altogether, these data led us to propose the thione **13** (MW = 398) and thiadiazoline diimine **14** (MW = 764) as putative oxidation products of ISO (Scheme 6). While the formation of **13** formally corresponds to a dehydrogenative cyclization of ISO, the formation of a 1,3,4-thiadiazole ring in **14** may result from an autocondensation of the ISO monoxide (ISO−SO) followed by an oxidative ring closure, a process previously reported from other aromatic thioureas.[23] Finally, TLC analysis of the intermediate chromatographic fraction suggested a complex mixture. The ^{1}H NMR spectrum, recorded just after the purification step, albeit poorly informative, indicated the presence of a largely predominant compound (Figure S11). Nevertheless, a ^{1}H signal at 7.9 ppm correlating with the ^{13}C signal of the aromatic CH centers in the ^{1}H−^{13}C HMBC spectrum could be tentatively assigned to a mobile NH proton, while a very broad ^{1}H signal at 10.3 ppm could correspond to a strongly acidic S−O−*H* proton. MS analysis, performed a few days later (Figure S12), showed three main peaks at m/z 546 (65%), 385 (35%), and 369 (100%),

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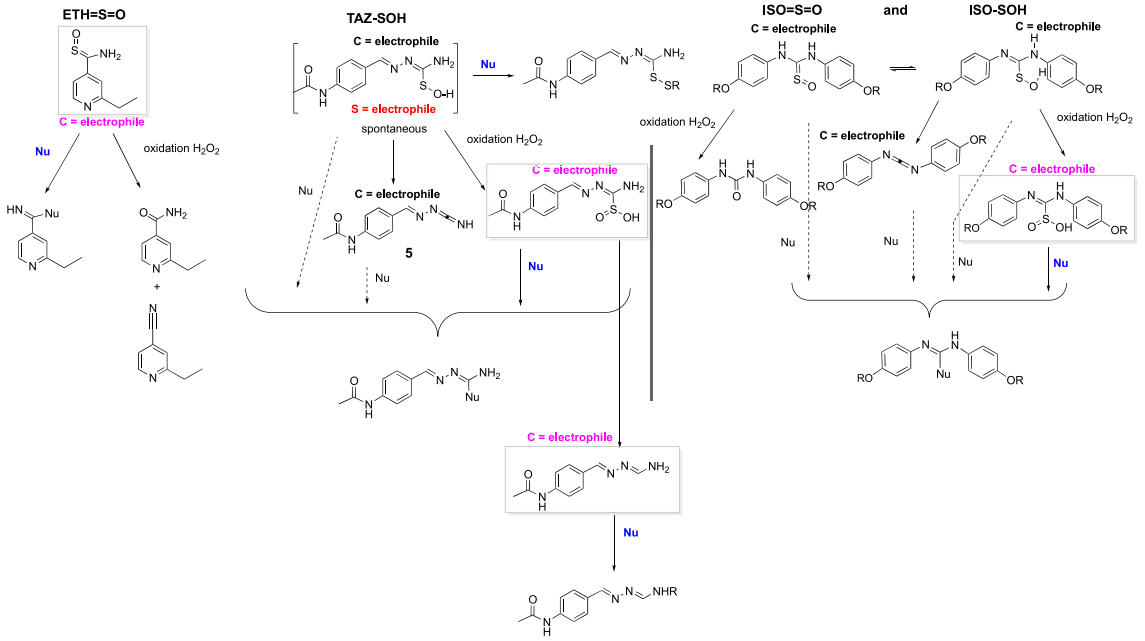
Scheme 8. Overview of the Electrophilic Reactivity of the Oxidative Metabolites of the ETH, TAZ, and ISO Prodrugs, Highlighting Various Possible Modes of Their Toxic Effects



none of them corresponding to the molecular mass of an ISO−SO_nH species (n = 1, 2, or 3) but assigned to those of **12** [M + 1]$^+$, **10** [M + 1]$^+$, and **11** [M + 1]$^+$, respectively, instead. As **12** and **11** were not observed in the ^{1}H NMR spectrum, it can be suggested that these two compounds emerged from an unstable intermediate in the sample waiting for MS analysis. The formation of the urea **10** (m/z = 385) and amidine **11** (m/z = 369) suggests a reactivity of ISO similar to that of ETH and TAZ, respectively. Notably, the urea analogue was not detected in the TAZ series, while the amidine analogue was not detected in the ETH series.

We also assumed that by an alternative reaction, the ISO−SO_2H compound can break down into the dioxyisothiocyanate **16** and aniline **17**. Indeed, the ISO sulfinic acid can protonate the aniline nitrogen, making it an ammonium leaving group, thus leading to the formation of compounds **16** and **17**. The ISO−SO compound, in its turn, could also be the precursor of oxyisothiocyanate **15** and thiadiazole **14**. Ortiz de Montellano et al. also proposed ISO−SOH as the precursor of carbodiimide **18** (Scheme 7). The strongly nucleophilic *p*-alkoxyaniline **17**, assumed to be formed in situ, could react with electrophiles occurring in the medium, such as the carbodiimide **18** and ISO−SO_xH (x = 1 or 2) to give the isolated guanidine **12**. As for the urea **10**, it can be produced by H_2O_2-mediated oxidation of the sulfine ISO═S═O to a dioxetane intermediate, undergoing spontaneous extrusion of SO_2, as in the ETH series (Scheme 1). At the very outset, the urea **10** might also be produced by hydrolysis of the carbodiimide **18**, but this route appears to be less relevant: in the TAZ series; indeed, the carbodimide **2** (Scheme 3) was not found to give the corresponding urea.

In an attempt to detect the ISO−SO_x species, ISO was treated in the same conditions with only 1 equiv of H_2O_2 for 20 min. ^{1}H NMR analysis showed that only a very small amount of ISO was oxidized: besides the major ^{1}H NMR signals of ISO, two minor pairs of signals in the aromatic region were found at 7.43 (d) and 7.29 (d) ppm and 7.01 (d) and 6.86 (d) ppm. MS peaks at m/z 401 (100%), 417 (10%), and 433 (10%) were identified as those of ISO [M + 1], ISO oxide [M + 1], and ISO−SO_2H [M + 1], respectively. The peak of the guanidine **12** was also observed at m/z = 546 (30%). The peak at m/z = 474 reported by Ortiz de Montellano et al. was not observed in our conditions, but it might be produced a posteriori and interpreted as resulting from a fragmentation of the ether motif of **12** with loss of isopentane (546 − 72 = 474).

The failure to isolate the ISO oxide and ISO sulfinic acid prevented any test reactions with amines as performed in the ETH and TAZ series. However, ISO−SO_xH (x = 1, 2) and the carbodiimide **18** are electrophilic species obviously capable of reacting with nucleophiles present in the medium: in vivo, biological nucleophiles such as proteins would compete with the aniline **17** and be altered while initiating toxic effects.

4. CONCLUSION

These results show that the biomimetic oxidative reactivities of the thiocarbonyl antitubercular prodrugs ETH, TAZ, and ISO are distinct and dependent on the equilibrium of the *S*-oxidized tautomeric forms (sufine vs sulfenic acid forms). They also underline that these primary metabolites evolve differently toward other reactive metabolites such as *S*-dioxides (sulfinic acids), *S*-trioxide (sulfonic acid), or carbodiimide. All of these species have a high toxicity potential, not only for the *Mtb* pathogen but also for the human host. Overall, the ways by which thiocarbonyl-type prodrugs will exert their therapeutic and/or toxic action sharply depend on the following factors: (i) the structural environment of the thioamide or thiourea group, (ii) the location where the oxidative steps occur (pathogen or host), and (iii) the nature of the local biochemical environment (e.g., S- or N-nucleophilic). Scheme 8 summarizes the oxidative metabolites of the ETH, TAZ, and ISO prodrugs having a demonstrated reactivity with nucleophiles and thus a strong toxic potential.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.1c00164.

NMR and mass spectra of TAZ−SO_2H, TAZ−SO_3H, **4**, **5**, and products formed upon oxidation of isoxyl by H_2O_2; crystal data of the azine **5** (PDF)

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Notes

The authors declare no competing financial interest.

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