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**Xanthates: metabolism by flavoprotein-containing  
monooxygenases (FMO) and antimycobacterial  
activity\***

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**Running title:** Xanthates, FMO and antimycobacterial activity

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**ABBREVIATIONS:** EtaA, mycobacterial flavin-dependent monooxygenase; ETH, ethionamide; FMO, flavoprotein monooxygenase; INH, isoniazid; Mtb, *M. tuberculosis*; PRO, prothionamide.

## **ABSTRACT**

Ethionamide (ETH) plays a central role in the treatment of tuberculosis in patients resistant to the first-line drugs. The ETH, thioamide, and thiourea class of antituberculosis agents are pro-drugs that are oxidatively converted to their active **S**-oxides by the flavin monooxygenase EtaA of *M. tuberculosis*, thus initiating the chain of reactions that result in inhibition of mycolic acid biosynthesis and cell lysis. ~~As shown here, several xanthates are oxidized by purified EtaA to S-oxide metabolites (perxanthates) implicated in their antimycobacterial activity. This process is analogous to that responsible for activation of ETH. As part of a search for new lead candidates, we report here that several xanthates are oxidized by purified EtaA to S-oxide metabolites (perxanthates) that are implicated in the antimycobacterial activity of these compounds. This process, which is analogous to that responsible for activation of ETH, is also catalyzed by human flavin monooxygenase FMO3.~~ EtaA was not inhibited in a time-dependent manner during the reaction. Xanthates with longer alkyl chains were oxidized more efficiently. EtaA oxidized octyl-xanthate ( $K_m = 5 \mu\text{M}$ ;  $V_{\text{max}} = 1.023 \text{ nmolP/min}$ ;  $k_{\text{cat}} = 5.2 \text{ molP/min/mole}$ ) more efficiently than ETH ( $194 \mu\text{M}$ ;  $1.46 \text{ nmolP/min}$ ;  $7.73 \text{ nmolP/min/mole}$ , respectively). Furthermore, the *in vitro* antimycobacterial activity of four xanthates against *M. tuberculosis* H37Hv was higher (MIC around  $1 \mu\text{M}$ ) than that of ETH ( $12 \mu\text{M}$ ).

## Introduction

The increasing resistance of pathogenic bacteria and viruses against the available antibiotic and chemotherapeutic agents is one of the major problems of modern healthcare. At the top of the list of diseases affected by this resistance are AIDS, influenza, and tuberculosis. The genetic variability of *Mycobacterium* underlies the current treatment consisting of a cocktail of several “first line” drugs. In the case of resistance to this treatment, therapy resorts to “second-line” drugs. The available antituberculosis drugs, as well as those under development, attack *M. tuberculosis* at several target points, including the bacterial cell wall. Isoniazid (INH), ethionamide (ETH), and prothionamide (PRO) inhibit the enoyl-acyl carrier protein (ACP) reductase InhA, a key enzyme in cell wall assembly. All three of these agents are prodrugs that must be activated to become effective (Banerjee et al., 1994; Johnsson et al., 1995). These three drugs specifically disrupt mycolic acid synthesis by forming adducts with NADH that inhibit InhA, thus blocking the synthesis of mycolic acids, key factors in cell envelope permeability, host immunomodulation and persistence of *M. tuberculosis* (Mtb) (Daffé et al., 2017).

Isoniazid is activated by the catalase-peroxidase hemoprotein KatG (Zhang et al., 1992). However, ethionamide, a second line drug, is a sulfur-containing compound that must be activated to its **S**-oxide by the mycobacterial flavin-dependent monooxygenase (EtaA) to exert its bacteriostatic action (Baulard et al., 2000). Flavoprotein monooxygenases (FMOs) belong to a large group of flavin oxygenases

found in species ranging from microorganisms to mammals. These enzymes are characterized by the NADPH-dependent reduction of an enzyme-bound flavin, leading to formation of the active oxidizing species, a C4a-flavin (hydro)peroxide (Phillips and Shephard, 2017).

Human FMO enzymes perform *N*- or *S*-oxygenation of nucleophilic heteroatom-containing small exogenous and endogenous molecules and are grouped into five classes depending on their sequence, structure, organ distribution, genetic control, and substrate specificity. FMO1 is expressed in human fetal liver and adult human kidney and intestine; FMO4 is expressed in kidney and brain; and the highly polymorphic enzyme FMO2 is expressed in the lung. Today, 26% of individuals of African descent and 5% of Hispanics carry at least one allele coding for FMO2.1, which has a very high activity for *S*-oxidation of thioureas and thus increases the risk for lung toxicity in tuberculosis patients treated with ETH (Krueger et al., 2002). The primary flavin dependent monooxygenase in human liver is FMO3, which participates in the oxidation of a number of nitrogen and sulfur-containing compounds (Cashman and Zhang, 2006).

Classical, so-called Baeyer-Villiger monooxygenases (BVMOs) generate reactive (hydro) peroxide intermediates that carry out both nucleophilic and electrophilic oxygenations. In contrast to classical Baeyer-Villiger reactions, when the BVMO EtaA oxidizes sulfur-containing substrates, its C4a-adduct is most likely reacting as a hydroperoxide rather than as a peroxide (Ballou, 2013). The heterologous expression and purification of EtaA has made the enzyme

available for testing the metabolism of heteroatom-containing compounds under in vitro conditions (Vannelli et al., 2002). Studies of the reactivity of xanthates with oxygen in the presence of metals have shown that the primary oxidation products with hydrogen peroxide are the corresponding sulphines or perxanthates (Silvester et al., 2002).

Xanthates are salts of different derivatives of dithiocarbonic acid that have a large spectrum of biological effects. These include antiviral, anticancer, and chelating effects, as well as the inhibition of several enzymes of importance in intercellular signaling processes (Adibhatla et al., 2012). In 1956 Ivanov et al. published data that different xanthate derivatives have an antibacterial action against Mtb due to their oxidation to toxic metabolites (Ivanov, 1956).

Here we present the results from the study of metabolism of several xanthates by EtaA and FMO3 to **S**-oxygenated metabolites, and compare the nature and reactivity-antimycobacterial properties of the resulting metabolites with those from ETH. The results suggest that this metabolic activation is essential for their antimycobacterial action.

## Experimental procedures

**Materials.** Potassium salts of various [derivatives of dithiocarbonic acid](#) (xanthates,  $\text{ROCS}_2\text{K}$ ), where R corresponds to alkyl groups with C1, C2, C3, C3iso, 2-methyl-C3, C4, C5, C8, C10 and C12 carbon atoms, or a cyclohexene ring; for example, C3 = propylxanthate =  $\text{CH}_3\text{CH}_2\text{CH}_2\text{COS}_2\text{K}^+$ , were synthesized as previously described (Rao, 1971). Ethionamide (ETH), O-tricyclo[5.2.1.0<sup>2,6</sup>]dec-9-yl-dithiocarbonate (D609), NADPH, bovine serum albumin (BSA), superoxide dismutase, catalase and all other chemical reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, United States). Human liver FMO3 was obtained from BD Biosciences (San Jose, CA). Purified mycobacterial flavin monooxygenase (EtaA) was kindly provided by Dr. Clinton Nishida (UCSF, San Francisco, CA).

**Spectrophotometric analyses.** The metabolism of various xanthates was studied spectrophotometrically. Stock solutions of various xanthates were prepared in water; C10 and C12 were dissolved in DMSO; ETH was dissolved in acetonitrile. The final organic solvent concentration was held to 0.1% (v/v).

### 1. Xanthates, ETH, and hydrogen peroxide:

Spectral changes of xanthates and ETH in 0.1 M phosphate buffer, pH = 7.4, were determined after incubation with 10 mM hydrogen peroxide.

### 2. Xanthate, ETH, and EtaA:

The final reaction medium contained EtaA (400 nM), bovine serum albumin (BSA, 0.1 mg/mL), superoxide dismutase (100 U/mL), catalase



(100 U/mL), KCl (100 mM), NADPH (200  $\mu$ M) and different concentrations of xanthates in HEPES buffer (50 mM, pH = 7.5). When used, the NADPH regenerating system consisted of glucose-6-phosphate (2.5 mM) and glucose-6-phosphate dehydrogenase (2 U/mL). After 2 min pre-incubation at 37 °C the reaction was initiated by adding the enzyme to the sample cuvette and an equal volume of water to the reference cuvette in a Varian Cary 300 Bio dual-beam spectrophotometer (Varian, Inc., Palo Alto, CA).

Spectral scans between 250 and 400 nm were recorded every 30 s at a scan rate of 1515 nm/min and a spectral bandwidth of 4 nm. A baseline of the reaction mixture in the two cuvettes was collected prior to addition of the enzyme. The rates of substrate consumption and product formation were determined at 301 ( $\epsilon = 17,600 \text{ M}^{-1}\text{cm}^{-1}$ ) and 347 ( $\epsilon = 10,400 \text{ M}^{-1}\text{cm}^{-1}$ ) nm, respectively (Hao et al., 2000).

Single-wavelength kinetic studies were conducted at either 321 or 347 nm for the observation of either NADPH ( $\epsilon = 4,752 \text{ M}^{-1}\text{cm}^{-1}$ ) consumption or product formation, respectively.

### 3. *Xanthates and FMO3*:

The same experimental protocol as above was used to follow the spectral changes between 250 and 400 nm after incubation of xanthates with FMO3. For kinetic studies, both the reference and sample cuvettes were charged with a 500  $\mu$ l volume of buffer containing the desired xanthate at a 200  $\mu$ M final concentration. The reaction was started by adding NADPH (200  $\mu$ M) and enzyme protein

(50  $\mu\text{g}$ , 60 pmoles FAD) to the sample cuvette and its progress followed by recording the absorption changes at 347 nm for 5 min at 37 °C.

**Antimycobacterial activity of xanthates and ETH.** The *in vitro* antimycobacterial activity of C2, C5, C8, D609 and ETH was determined through the proportional method of Canetti (**Canetti et al., 1969**) towards reference strain *M. tuberculosis* H37Rv. Each compound was tested at seven concentrations - 10  $\mu\text{g}/\text{ml}$ , 5  $\mu\text{g}/\text{ml}$ , 2  $\mu\text{g}/\text{ml}$ , 1  $\mu\text{g}/\text{ml}$ , 0.5  $\mu\text{g}/\text{ml}$ , 0.2  $\mu\text{g}/\text{ml}$  and 0.1  $\mu\text{g}/\text{ml}$  in DMSO. Tubes with Löwenstein-Jensen medium (5 ml) containing tested compounds and those without them (controls) were inoculated with 0.1 ml suspension of *M. tuberculosis* H37Rv ( $10^5$  cells/ml) and incubated for 45 days at 37 °C. The ratio between the number of colonies of *M. tuberculosis* grown in medium containing compounds and the number of colonies in control medium were calculated and expressed as percentage of inhibition. The MIC is defined as the minimum concentration of compound (in  $\mu\text{M}$ ) required to inhibit bacterial growth completely (0% growth).

**Statistics.** Non-linear and linear regression analyses to calculate the enzyme kinetic parameters were done using GraphPad Prism 6.0 software. Paired t-test was applied for statistical comparison of the enzyme activity data.

## Results

### 1. Oxidation of xanthates and ETH by hydrogen peroxide.

Hydrogen peroxide oxidizes xanthates and ETH to single metabolites: perxanthate or sulfine ( $\lambda_{\max}$  at 347 nm) and ethionamide-**S**-oxide ( $\lambda_{\max}$  around 350 nm), respectively (Fig. 1 [aA](#), [bB](#)).

The sharper increase of perxanthate over time compared to that of ETH-**S**-oxide was not only due to the higher extinction coefficient of the former -  $\epsilon = 10,400 \text{ M}^{-1}\text{cm}^{-1}$  (Hao et al., 2000) versus  $\epsilon = 6,000 \text{ M}^{-1}\text{cm}^{-1}$  (Vannelli et al., 2002). The rate of oxidation of C5-xanthate by  $\text{H}_2\text{O}_2$  to its metabolite was more than two times faster (2.34 nmoles/min) compared to that of ETH (1.0 nmoles/min). It is important to note that the oxidation of ETH to ETH-SO by  $\text{H}_2\text{O}_2$  occurs without the assistance of any cofactor such as  $\text{NAD}^+$  (Laborde et al., 2016).

### 2. Metabolism of xanthates by EtaA.

*Scanning Kinetics.* Initial metabolism studies with an NADPH regenerating system and EtaA enabled the direct observation and comparison of product formation and substrate consumption rates. These data for a sampling of xanthate substrates are presented in Table 1 and Fig. [2a2A](#).

The substrate consumption (decrease of absorption at 301 nm) and product formation (increase of absorption at 347 nm) rates after incubation with EtaA correlate well, indicating conversion of the substrate to a single product, the corresponding perxanthate. The presence of a clear isosbestic point at 321 nm (Fig. [2Ab](#)) supports the conclusion that the substrate is converted to a single product. It is to be

noted that with the longer straight chain alkyl-xanthates (i.e. decyl and dodecyl-xanthates) there was a discrepancy between the higher rates of substrate depletion and the lower rates of product formation. This discrepancy could be due to aggregation and subsequent precipitation of the product of the longer substrates in the aqueous solution. Xanthates bearing cyclic or branched side-chains (2-methyl-C3, Cyclo and D609) were metabolized by EtaA at significantly slower rate.

**Steady-State Kinetics.** The dependence of the C8 oxidation rate on the substrate concentration follows the Michaelis-Menten equation. Fig. 3 presents the best fit through the data-set obtained and the approximate kinetic values are based on that fit. The maximum rate of oxidation of C8-xanthate by EtaA was practically the same as that previously obtained for ETH ( $V_{\max}=1.46$  nmolP/min and  $7.73$  molP/min/molEe, respectively). It is important to note that the  $k_{\text{cat}}$  values for the oxidation of most substrates by EtaA are nearly the same. This is due to the fact that the rate limiting step in the FMO catalytic cycle is the release of water from the enzyme with the concomitant dehydration of the C4a-flavin hydroxide (Beaty and Ballou, 1981).

However, the  $K_m$  value shows that C8 has a much higher affinity for EtaA than ETH ( $K_m=5$   $\mu\text{M}$  versus  $194$   $\mu\text{M}$ , respectively) (Vannelli et al., 2002). Stopped flow studies show that the  $K_m$  values for **S**-oxygenatable substrates depend largely on their reactivity with the hydroxyperoxyflavin rather than on their interaction with enzyme protein. The determining factor is the nucleophilicity of the sulfur atom (Poulsen and Ziegler, 1995), which in all tested xanthates is

significantly higher than that of ETH. The higher reactivity of the xanthates was consistent also with the higher rate of oxidation by hydrogen peroxide.

### 3. Metabolism of xanthates by human isozyme FMO3.

Similar results were also obtained by examining the metabolism of xanthates to **S**-oxidative products (perxanthates) by human liver FMO3 (Fig. 4).

The results from tracking the rate of formation of the perxanthate metabolites from different xanthates by FMO3 are listed in Table 2.

The results show that the rate of **S**-oxidation increases with increasing number of carbon atoms in the alkyl chain substituent in incubations of xanthates with EtaA and FMO3. Thus, C8 was oxidized most rapidly to its perxanthate by FMO3. Again, the derivatives with cyclic or branched side-chains substituents (D609, C3iso and 2-methyl-C3) were oxidized at a slower rate. Surprisingly the two xanthates with longest alkyl chain substituents (C10 and C12), which are insoluble in water (stock solution dissolved in DMSO), were oxidized by FMO3 much faster than the corresponding water soluble xanthates. This difference in the kinetic properties of the two flavin monooxygenases with substrates of different lipophilicities may be due to the fact that insect cell supersomes containing FMO3 were employed for these studies. Although the concentration of DMSO in the samples was kept low (less than 1%), an increase of the catalytic activity is possible in supersomes due to the presence of a detergent, as previously reported (Reddy et al., 2010).

#### 4. Antimycobacterial activity of different xanthates and ETH.

The results on the antimycobacterial activity of selected xanthates relative to that of ETH are presented in Table 3.

The xanthates with short to medium alkyl chain substituents had MIC values ten times lower than that of ETH, whereas the MIC value of D609, compound with a cyclic substituent, was comparable to that of ETH.

As a whole, some of the xanthate analogues tested in this study showed a moderate level of bacteriostatic antimycobacterial activity, in most cases comparable or even higher than that of the reference drug ethambutol (Jadaun et al., 2007).

## Discussion

The present results show that the xanthates are primarily or exclusively oxidized by EtaA to the corresponding sulfines or perxanthates, which have a maximum absorption peak at 347 nm. The calculated kinetic parameters for production of these metabolites were much better faster than those for oxidation of ETH by the same enzyme. The xanthate metabolites produced by EtaA appeared to be identical to those obtained upon incubation with hydrogen peroxide. The spectral properties of the xanthates suggest that they may be useful substrates to determine FMO activity in biological samples if the SOD and catalase activity in these samples is inhibited. The ready synthetic access to xanthate derivatives may provide access to compounds with higher anti-mycobacterial activities.

There is a large group of thiourea prodrugs with potent bacteriostatic anti-mycobacterial activity that must be oxidized by EtaA to exert their action. Thus, thioacetazone (THZ) is oxidized into its active form, 2-ethyl-4-amidopyridine. It has a relatively low affinity ( $K_m = 131 \pm 29 \mu\text{M}$ ) for EtaA (Qian and Ortiz de Montellano, 2006; Alahari et al., 2007). Isoxyl (thiocarlide; 4,4'-diisoamyloxydiphenylthiourea) is likewise activated by EthAEtaA, although its anti-mycobacterial activity differs in that it inhibits the synthesis of oleic acid as well as that of mycolic acid (Phetsuksiri et al., 2003). Other compounds with thioamide and thiourea groups, including thiobenzamide, isothionicotinamide, and prothionamide are oxidized by EtaA and human FMO enzymes (Vannelli et al., 2002; Nishida and Ortiz de Montellano, 2011).

The spectroscopic results in the oxidation of xanthates by EtaA and the published data on the possible sulfur-oxidized metabolites implicate the following scheme in the metabolism of xanthates by EtaA (Fig. 5).

Thus the killing efficacy of different thioamide and thiourea compounds against Mtb supports the idea that it is may be possible to improve their clinical application through dosage reduction, thus minimizing side effects and improving patient compliance. This approach can in principle be achieved by manipulating EtaA efficiency:

- a. By increasing EtaA activity by boosting the enzyme gene expression.
- b. By compounds with higher affinity for EtaA. Thus, as decreasing the dose achieves the same efficacy at reduced toxicity.

In the context of a comparison of anti-bacterial effects of ETH and alkyl chain xanthates derivatives it should be considered that the actual active ETH metabolite is not ETH-SO, but the so called ETH\* (Hanouille et al., 2006), a metabolite with still unknown molecular characteristics which accumulates within the bacterial cells whilst the other ETH derivatives were exclusively found in the extracellular milieu. Whether the physicochemical characteristics of the peraxanthate have allowed more efficient penetration into the bacterial cell and therefore greater antibacterial activity, this remains to be demonstrated.

In conclusion, the xanthates examined exhibit high affinity for FMOs that oxidized them to the corresponding **S**-oxides (perxanthates), apparently without altering the activating enzyme activity. They could be used as test substrates for rapid and selective determination of FMO activity in various biological media. Xanthates have antimycobacterial



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activity against *M. tuberculosis* H37Rv comparable to that of ETH. The synthesis of further xanthate derivatives would appear to be a promising route to compounds with higher antimycobacterial activity.

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## Authorship contributions

*Participated in research design:* Yanev, Valcheva, Ortiz de Montellano

*Conducted experiments:* Yanev, Stoyanova, Valcheva

*Performed data analysis:* Yanev, Valcheva

*Wrote or contributed to the writing of the manuscript:* Yanev, Valcheva, Ortiz de Montellano

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## Figures Legends

**Fig. 1.** Spectral changes of **aA**. C5-xanthate (50  $\mu$ M) and **bB**. ETH (200  $\mu$ M) in 0.1 M phosphate buffer, pH 7.4, by 10 mM hydrogen peroxide.

**Fig. 2. aA.** Spectroscopic characteristics of metabolism of octyl xanthate (80  $\mu$ M) by EtaA measured over 20 min; **bB.** Changes in absorption over time at 301 (blue), 321 (black), and 347 (red) nm.

**Fig. 3.** Steady-state kinetics of the oxidation of octyl xanthate by EtaA. The red line represents the best nonlinear curve-fit ( $r^2= 0.9749$ ). The Michaelis-Menten parameters are represented as means  $\pm$  SD from three different experiments.

**Fig. 4. A.** Spectroscopic characteristics of cyclohexyl xanthate (80  $\mu$ M) after incubation with human FMO3; **B.** Rate of metabolism of 2-methylpropyl xanthate (80  $\mu$ M) on incubation with human FMO3.

**Fig. 5. [Structure of ethionamide and Proposed-proposed](#)** scheme of S-oxidation of xanthates by EtaA to perxanthates. [A similar reaction is mediated by human FMO3.](#)

**Table 1.** Metabolism of different xanthates (100  $\mu$ M) by EtaA (400 nM); rates of decrease of substrate at 301 nm and increase of product at 347 nm are presented. Values are the means  $\pm$  SD from three different experiments.

| Xanthate        | $V_{301\text{-nm}}$<br>(nmoles/min) | $V_{347\text{-nm}}$<br>(nmoles/m) |
|-----------------|-------------------------------------|-----------------------------------|
| C3              | - 1.27 $\pm$ 0.07                   | 0.97 $\pm$<br>0.077               |
| 2-methyl-<br>C3 | - 0.38 $\pm$<br>0.06                | 0.22 $\pm$<br>0.012               |
| C4              | - 1.57 $\pm$ 0.12                   | 0.93 $\pm$<br>0.065               |
| C5              | - 1.53 $\pm$ 0.09                   | 0.74 $\pm$<br>0.052               |
| C8              | - 1.35 $\pm$ 0.08                   | 0.90 $\pm$<br>0.072               |
| Cyclo           | - 0.8 $\pm$ 0.07                    | 0.71 $\pm$<br>0.064               |
| C10             | - 2.47 $\pm$ 0.18                   | 0.49 $\pm$<br>0.110               |
| C12             | - 1.51 $\pm$ 0.10                   | 0.03 $\pm$<br>0.007               |
| D609            | - 0.89 $\pm$ 0.08                   | 0.58 $\pm$<br>0.040               |



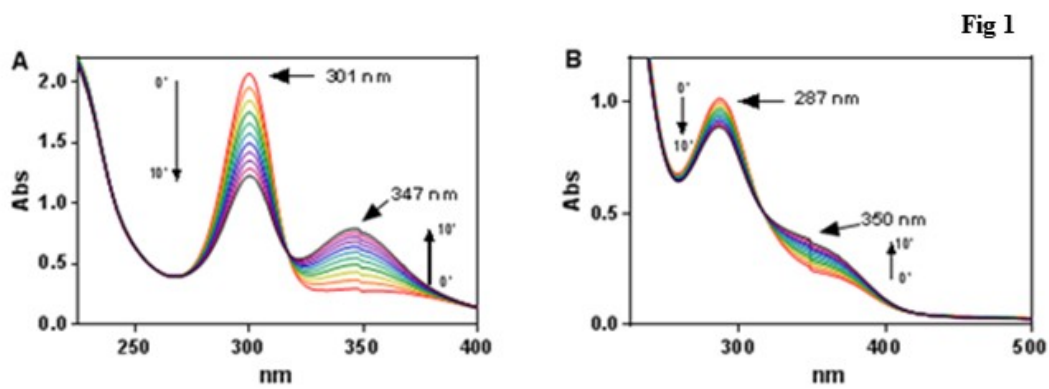
**Table 2.** Rate of formation of perxanthate metabolites at 347 nm from different xanthates (200  $\mu$ M final concentration) after incubation with FMO3 (50  $\mu$ g, 60 pmoles FAD) in the presence of NADPH (200  $\mu$ M).

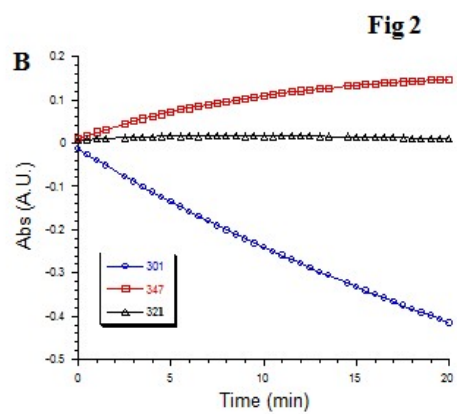
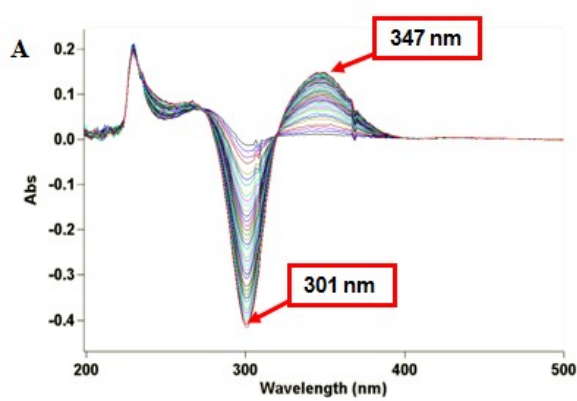
Values are means of three different experiments.

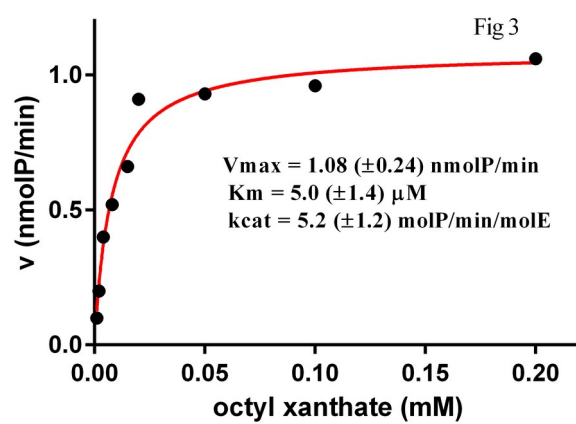
| Xanthates   | V<br>(nmolP/min) | Linear regression<br>( $r^2$ ) |
|-------------|------------------|--------------------------------|
| C1          | 0.34             | 0.847                          |
| C2          | 0.44             | 0.958                          |
| C3          | 0.78             | 0.911                          |
| C3iso       | 0.46             | 0.891                          |
| 2-Methyl-C3 | 0.38             | 0.803                          |
| C4          | 0.78             | 0.998                          |
| C5          | 0.84             | 0.895                          |
| C8          | 0.95             | 0.860                          |
| C10         | 2.54             | 0.988                          |
| C12         | 3.43             | 0.985                          |
| Cyclo       | 0.56             | 0.835                          |
| D609        | 0.38             | 0.812                          |

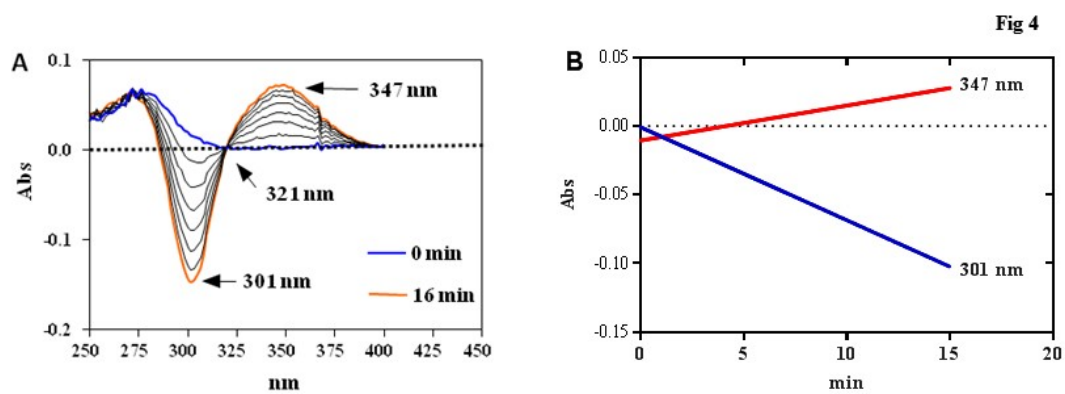
**Table 3.** Antimycobacterial activity of selected xanthates and ETH as measured by the proportional method.

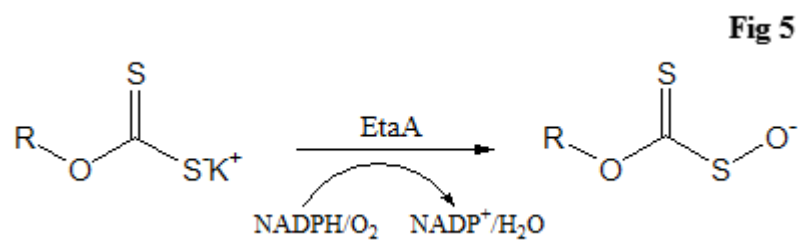
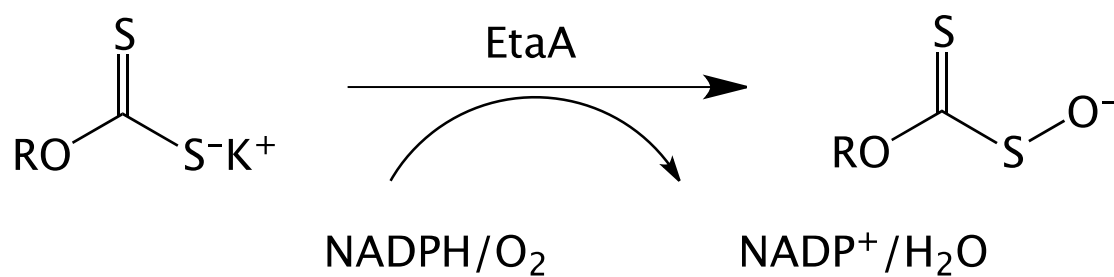
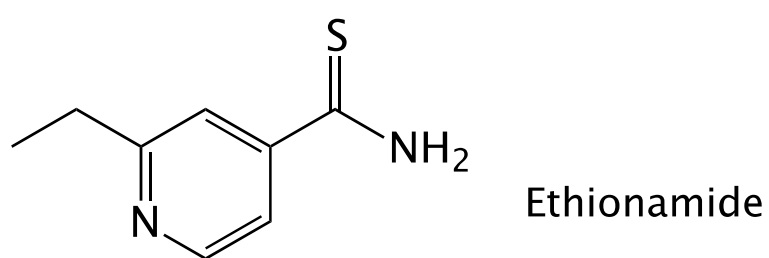
| <b>Compound</b>      | <b>MIC (<math>\mu\text{M}</math>)</b> |
|----------------------|---------------------------------------|
| <b>C2 - xanthate</b> | <b>1.25</b>                           |
| <b>C5 - xanthate</b> | <b>0.99</b>                           |
| <b>C8 - xanthate</b> | <b>2.05</b>                           |
| <b>D609</b>          | <b>18.8</b>                           |
| <b>Ethionamide</b>   | <b>12.0</b>                           |









**Fig 5**

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