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Synthesis, Bioactivity, and Enzymatic Modification of Antibacterial Thiotetromycin Derivatives

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Abstract

Thiotetronate-containing natural products, including thiolactomycin, thiotetromycin, and thiotetroamide, are potent, broad-spectrum antibacterial compounds that target fatty acid synthesis in bacteria. Natural modifications at the C-5 dialkyl position in this molecular series result in pronounced bioactivity differences. The C-5 acetamide-containing thiotetroamide, which is the more potent antibacterial agent in this family, is biosynthesized from the C-5 ethyl analogue thiotetromycin via a unique two-enzyme process involving the cytochrome P450-amidotransferase enzyme pair TtmP-TtmN. Herein we synthesized a focused library of 17 novel thiotetromycin derivatives differing at the 5-position alkyl substituent to investigate their biological activities and their reactivity towards the hydroxylase TtmP. Although we observed marginal anti-tuberculosis activity, select thiotetromycin analogues showed antibacterial activity against an *Escherichia coli ΔtolC* strain with IC_{50} values in a range of 1.9 – 36 μg/mL. Additional screening efforts highlighted select thiotetronate analogues as inhibitors of the cancer-associated enzyme nicotinamide *N*-methyltransferase (NNMT), with a unique scaffold compared to previously identified NNMT inhibitors. *In vitro* assays further showed that the TtmP P450 was capable of resolving racemic substrate mixtures and had modest promiscuity to hydroxylate derivatives with variable alkyl chains; however triple oxidation to a carboxylic acid remained specific for the natural thiotetromycin substrate. The tendency of TtmP to accept a range of unnatural substrates for hydroxylation makes it an interesting target for P450 engineering towards broader applications.

Graphical Abstract

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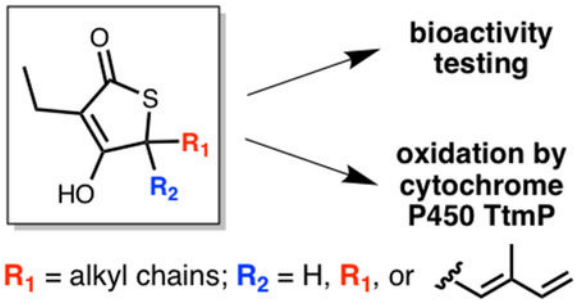
Conflicts of interest

There are no conflicts to declare



Interrogating the bioactivities and enzymatic modification potential of a focused library of novel synthetic thiotetronate compounds.

Introduction

The persistence and global spread of antibiotic-resistant bacteria is a worldwide threat that is becoming more dangerous over time.[1] Misuse and overuse of once life-saving antibiotics drives the evolution of multi-drug resistant strains that dramatically increase the danger of severe bacterial infections. Of particular concern is the pathogenic bacterium *Mycobacterium tuberculosis,* which causes over 8 million new infections and approximately 2 million deaths each year. To combat this trend, the discovery, development and remodeling of drugs against multi-drug-resistant bacterial strains is desperately needed.[2,3]

The type II fatty acid synthase (FAS II) pathway of lipid biosynthesis is an attractive antibacterial target because it involves essential processes for bacterial proliferation that are distinct from the FAS pathway present in mammalian cells.[4] The iterative cycles of condensation, reduction and dehydration in the FAS II pathway have been extensively studied in bacteria and many of their conserved proteins have been previously targeted with antibacterial agents such as cerulenin[5], isoniazid[6] or triclosan[7] (Figure 1).[8,9]

Another class of antibacterial compounds that selectively targets this pathway encompasses the actinomycete-derived thiotetronate antibiotics, including thiolactomycin (**1**)[10,11], thiotetromycin (**2**)[12] and thiotetroamide (**4**).[13] These compounds reversibly inhibit all three variants of β-ketoacyl-ACP-synthases in *Escherichia coli* (FabH, FabB and FabF) with their conserved 5-membered unsaturated thiolactone moieties binding in the malonyl-CoA pocket.[14] First isolated in 1982 from a *Nocardia* species,[10] **1** has potent antimicrobial activity against *M. tuberculosis* in addition to a variety of clinically relevant Gram-positive and Gram-negative pathogens, including *Klebsiella pneumonia* and *Serratia marcescens*.[9] When employed in a mouse model, **1** showed good bioavailability and pharmacological properties with low host toxicity.[15] This family of molecules also inhibit related FAS II enzymes from protozoa parasites including *Plasmodium falciparum*, *African trypanosomiasis* and *Toxoplasma gondii,* showing potential for the treatment of malaria and neglected tropical diseases.[16] These cumulative properties make thiotetronate compounds promising candidates for drug development to combat clinically relevant human infectious diseases.

Other thiotetronate compounds that have been isolated from actinomycete bacteria differ from **1** at the C-3 and/or C-5 alkyl group substituents, including **2** from *Streptomyces* and *Salinispora* genera, and **4** (formerly Tü 3010[13]), containing a primary amide moiety, from *Streptomyces afghaniensis.* Genomics-based discovery approaches have led to the identification of the thiolactomycin biosynthetic gene cluster (*tlm* BGC) which produces both **1** and **2** and the thiotetroamide (*ttm*) BGC, biosynthesizing predominantly **4**.[17–19] We were further interested in the 10-fold improved antibacterial activity of **4** compared to other thiotetronates presumably due to its diagnostic primary amide moiety.[17,20] Installation of this functional group was traced back to three additional genes in the *ttm* BGC, encoding a cytochrome P450 (TtmP), a ferredoxin (TtmO) and a glutamine amidotransferase (TtmN).

The crucial biotransformation towards **4** is a series of oxidations catalyzed by TtmP, which selectively oxidizes the terminal methyl group of **2** three times to synthesize thiotetromycin carboxylic acid (**3**). The carboxylic acid substrate **3** is further transformed into **4** by amidotransferase TtmN in the presence of ATP, L-glutamine and Mg^{2+} (Figure 1). While the ethyl group of **2** is selectively converted to **4** by the enzymatic oxidation-amidation cascade, TtmP shows no reactivity with **1**, which contains a methyl group at the same position.[20] This highlights the unique regiospecificity of TtmP that we were curious to further explore. Because the chemical activation of unreactive C-H bonds is very challenging, the specificity and selectivity of TtmP is a desired property for chemoenzymatic synthesis. P450 enzymes have been employed to catalyze many chemically difficult oxidation reactions[21,22] and their scaffolds have been further elaborated by directed evolution to facilitate a wide variety of diverse chemical transformations.[23–25]

Since the discovery of **1**, there has been considerable synthetic interest in thiotetronate antibiotics with multiple racemic[26,27] and enantiopure[28–31] strategies having been developed. Additional medicinal chemistry studies have generated a variety of thiolactomycin analogues[32–34] in an attempt to interrogate its antimicrobial and antiparasitic bioactivities.[15,32,35] Structure-activity experiments and models of the thiolactomycin-FabB cocrystal suggested that a conjugated and planar side chain is necessary for effective inhibition of the condensing enzyme in *E. coli* by insertion into the active site.[2] Modification of the diene moiety at the 5-position has produced analogues with improved antibacterial potency against *E. coli* and *M. tuberculosis* strains as well as compounds with improved efficacy against the malaria parasite *P. falciparum*.[32,33] Because synthetic analogues have shown increased potency against a variety of clinically-relevant pathogenic organisms, we were interested to investigate the impact of modification of the 5-position alkyl substituent which has yet to be extensively explored. Herein we report the synthesis of a series of novel thiotetronate compounds with C-5 modifications, their antimicrobial activities, and ability to be oxidized by TtmP.

Results and Discussion

Synthesis of Thiotetromycin Analogues

A focused library of thiotetromycin analogues was generated by adapting the strategy employed in the total synthesis of racemic thiolactomycin by Wang and Salvino (Scheme 1).

[26] β-Ketoester **5** was brominated then rearranged in the presence of atmospheric oxygen to the desired regioisomer **6**[36], followed by substitution of the bromine with thioacetate to form **7** in 56% yield over 3 steps. Thioester **7** was subsequently cyclized under basic conditions to provide thiolactone **8**, which was used as the starting material for alkylation reactions to divergently synthesize novel C-5 modified analogues.

The first regioselective alkylation at the 5-position was achieved by generating a di-anionic intermediate with 2 equivalents of base, followed by addition of alkyl halides: ethyl iodide (**a**); 1-iodopropane (**b**); allyl iodide (**c**); 1-iodo-2-methylpropane (**d**); 1-bromobutane (**e**); or 1-bromopentane (**f**). Different amounts of alkylation reagent generated diverse ratios of mono- (**9a-f**) and dialkylated (**10a-f**) products. Following optimization, we determined that three equivalents of alkyl halide were ideal to synthesize both mono- and dialkylated thiotetromycin derivatives at the same time (Figure S1).

To install the diene moiety of the thiotetromycin analogues, we performed a second alkylation reaction at the 5-position of monoalkylated compounds **9a-f** under basic conditions with 3-ethoxymethacrolein.[26] Since the intermediate aldehyde products (**11a-f**) decomposed quickly, we immediately isolated them by silica flash chromatography and modified them by Wittig olefination with methyl phosphonium ylide. This reaction sequence generated racemic thiotetromycin (**12a**) and five novel thiotetromycin analogues (**12b-f**) with yields ranging from 11–23% over the last two steps (Figure S2).

In total, we synthesized 17 novel thiotetronate compounds and the previously synthesized **12a** to initiate our bioactivity and TtmP specificity studies (Figure S3).

Bioactivity Experiments

Antimicrobial activity against *E. coli*.—Because thiotetromycin is known to possess antimicrobial activity against a variety of different bacterial strains, including *M. tuberculosis*, we investigated all thiotetromycin analogues for their antimicrobial activities. To minimize the inherent bacterial resistance to thiotetronate antibiotics via multidrug efflux systems and TolC-dependent type I secretion,[2,37,38] we initially screened our compounds (5 μg) against *E. coli* W3110 Δ*tolC* via spot-on-lawn assays on LB agar plates. If no activity was observed, the compounds were further assessed for antimicrobial activity at higher quantities. This preliminary screen identified **12a** to have the greatest zone of inhibition, while a selection of 5-position dialkylated analogues (**12c**, **12e**, **12f**, **10e**, **10f**) inhibited bacterial growth at 5 μg as well (Figure S4). The remaining 5,5-dialkylated compounds (**10a-d**, **12b**, **12d**) showed clear zones of inhibition at higher quantities (50 μg) whereas all monoalkylated thiotetronates (**9a-f**) only clearly inhibited cell growth at the highest amount tested (100 μg, Figure S5). Because no monoalkylated thiotetromycin analogue provided promising activities, we assume that dialkylation at the 5-position is beneficial for inhibitory interactions between thiotetronate antibiotics and their FAS II protein targets. Because none of the analogues with isobutyl alkyl chains (**9d**, **10d**, **12d**) showed relevant activities, it is possible that restrictions within the active site reduce the ability of the branched substrates to bind. We suggest that for *n*-butyl and *n*-pentyl thiotetronate analogues flexibility of the

substituents is beneficial to overcome these restrictions and explain the observed comparably higher activities.

Comparison of the zones of clearing identified the most active compounds (**12a**, **12c**, **10e** and **10f**) for further IC_{50} testing. Liquid cultures of *E. coli* W3110 Δ*tolC* were grown in the presence of different concentrations of thiotetronate analogues and bacterial growth after 6 hours was assessed spectrophotometrically by monitoring the OD_{600}. Plotting the relative absorbances versus concentrations and fitting the data with SciDavis provided approximate IC_{50} values (Figure 2) Consistent with the spot-on-lawn assays, **12a** was found to be the most potent compound, having comparable antibacterial activity to racemic thiolactomycin against a TolC-deficient *E. coli* strain (MIC = 12 μM or 2.5 μg/mL).[2] Although the conjugated side chain in **1** was previously suggested to be crucial for insertion into the active site of bacterial FAS II enzymes,[2] low IC_{50} values were observed for dibutyl- and dipentyl-thiotetronates (**10e**, **10f** respectively) lacking this moiety. This observation possibly indicates that a different bacterial growth inhibition mechanism is in place for these compounds compared to TLM.

Antimicrobial activity against *M. tuberculosis*.—In collaboration with the Eli Lilly OIDD (Open Innovation Drug Discovery) program, all novel thiotetromycin analogues (**9a-f**, **10a-f**, **12b-f**) were tested against *M. tuberculosis* strain H37Rv. Although **1** shows appreciable antimicrobial activity against this strain (MIC = 3.1 μg/mL),[34] all analogues showed negligible biological activity with bacterial growth inhibition rates varying between −4% and 12% at 20 μM, indicating the compounds are inactive in the tested concentration (Table S1). It is possible that higher concentrations could provide more insight into their relative anti-tuberculosis bioactivity, however these results indicate that modification of the alkyl substituent at the 5-position is not beneficial to enhance bioactivity against *M. tuberculosis*. These findings complement a previous study in which modification of the 3- and 4-position of **1** showed no improvement of potency against the same *M. tuberculosis* H37Rv strain.[34] In general, synthetic modification of the 5-position alkyl moiety in our series of thiotetronate compounds failed to enhance antimicrobial activity and is unlikely to generate better therapeutic leads for this application.

Inhibition of NNMT.—In collaboration with the OIDD, our synthetic analogues were also tested against the metabolic enzyme nicotinamide *N*-methyltransferase (NNMT), which catalyzes the *S*-adenosyl-L-methionine (SAM)-dependent *N*-methylation of nicotinamide. NNMT has been observed to be overexpressed in select cancer cells and upregulation of this gene has been connected with radiation resistance and increased tumour aggressiveness.[39] Either transcriptional downregulation or chemical inhibition of NNMT offers a strategy to sensitise cancer cells to radiation, thus enhancing the effect of therapy.

When incubated with thiotetromycin analogues (**9a-f**, **10a-f**, **12b-f**) at 10 μM, NNMT activity was inhibited by up to 35%. Allyl thiotetromycin **12c** showed the highest inhibition ability, **12d** and **9f** modulated the enzymatic activity by 25% and **9b**, **10d**, **9a** and **10b** showed inhibition effects in the range of 20–22% (Figure S6). There was no obvious structural correlation between the synthetic analogues that showed improved enzyme inhibitory activity; however, it is encouraging that these new thiotetronate analogues show

inhibitory activity towards this important disease target and have a significantly different scaffold than the currently identified pyridine or benzamide-based NNMT inhibitors.[40–43]

Activity of P450 Enzyme TtmP

Improvement of TtmP heme incorporation.—Following bioactivity experiments, we next set out to use our synthetic library to investigate the substrate specificity of TtmP, the enzyme responsible for the oxidation of **2** to **3** in the biosynthesis of **4** (Figure 1b). The bacterial cytochrome P450 TtmP requires a ferredoxin and ferredoxin reductase as partner proteins to regenerate the heme cofactor during the oxidative catalytic cycle.[20] Following previously established methodology, we employed the native *Streptomyces afghaniensis* ferredoxin protein TtmO,[20] however, we used the non-native ferredoxin reductase Bmp10 from *Pseudoalteromonas luteoviolacea* 2ta16,[44] as no ferredoxin reductase was identified within the *ttm* gene cluster.

Expression and purification of all recombinant proteins was initially performed following previously established procedures (Figure S7).[20] However, a significant proportion of heterologously expressed TtmP lacked the necessary heme moiety following addition of precursor δ-aminolevulinic acid (25 mg/L) [20] based on comparison of the protein concentration to the maximal UV absorbance at 424 nm, indicative of heme incorporation into TtmP (Figure S8). As P450 enzymatic catalysis is dependent on the successful incorporation of heme, which may be substoichiometric following recombinant protein overexpression in non-native organisms,[45] we aimed to optimize the incorporation of this cofactor in order to improve TtmP activity. The substitution of the more complete heme precursor hemin (65 mg/L) at the same point of TtmP protein expression improved heme incorporation by 12.8-fold (Figure S8).

Optimization of TtmP assay conditions with racemic thiotetromycin.—As all previously reported TtmP reactions had been performed with enantiopure substrates isolated from bacterial cultures,[20] we decided to optimize reaction conditions for racemic **12a** to further apply them to our non-native substrate analogues. Enzyme concentrations were systematically altered from the previously reported assay conditions and monitored by liquid chromatography mass spectrometry (LCMS), extracting ion chromatograms for: all potential substrate and product masses (black trace); substrate **12a** (red trace); mono-hydroxylated product (blue trace); and carboxylic acid product **3** (green trace) (Figure 3a). The dihydroxylated intermediate after a second TtmP oxidation interconverts with the related aldehyde and both species were only weakly detected by MS. From this analysis, we determined that the highest relative TtmP catalytic activity was achieved at an enzyme ratio of 1:4:1 (TtmP:TtmO:Bmp10) and that higher concentrations of Bmp10 reduced the efficiency of the TtmP catalytic cycle (Figure S9).

We repetitively noticed that around 50% of the substrate remained even following assay optimization, which suggested that TtmP is enantioselective towards only the natural (-)-thiotetromycin substrate (Figure 3b). To interrogate this possibility, we performed a large scale (50 mL) TtmP oxidation of **12a** (100 μM) using identical reaction conditions to those previously described. Following isolation of chemoenzymatically prepared **3** and

comparison to isolated **3** from a Δ*ttmN Streptomyces* strain, circular dichroism (CD) experiments showed features with similar signs for both compounds, supporting our assumption (Figure S11).[20]

TtmP assays with thiotetromycin analogues.—We next applied our optimized assay conditions to investigate the flexibility of TtmP to oxidize our unnatural thiotetronate analogues. Reactions were set up in an analogous fashion to **12a** and were compared to reactions set up in the absence of TtmP to readily identify oxidized products by LCMS. We initially investigated our thiotetromycin analogues (**12b-f**) as they were most structurally similar to native substrate **2.** LCMS analysis showed that TtmP was able to oxidize many of these unnatural analogues to mono-hydroxylated products with an estimated 5–10% conversion based on extracted ion chromatogram intensities; however this was substantially lower than the 50% conversion of racemate **12a** (Figure 4). Propyl (**12b**), allyl (**12c**) and isobutyl thiotetromycin (**12d**) each showed one major mono-hydroxylated peak (blue traces) following incubation with TtmP, while thiotetromycin analogues with longer aliphatic side chains (**12e, 12f**) showed minimal differences from negative controls ran in the absence of TtmP. However, no analogues showed oxidation past mono-hydroxylation, suggesting that the triple oxidation event is specific for the natural substrate **2** and racemate **12a**.

Further investigation of TtmP with 5-monoalkylated (**9a-f**) or 5,5-dialkylated analogues (**10a-f**) lacking the diene moiety of native **2** showed that compounds **10a**, **10b**, **10d** and **10e** were capable of being oxidized to a mono-hydroxylated product(s). However, no potential carboxylic acid products were observed, even with 5,5-diethyl substituted **10a** (Figure 4). None of the 5-monoalkylated analogues (**9a-f**), diallyl (**10c**) or dipentyl thiotetromycin (**10f**) were accepted as substrates for TtmP under the reaction conditions examined (Figure S10). Similar to the trend of the bioactivity experiments, 5,5-disubstitution seems to be necessary for recognition by TtmP. The diene substituent of thiotetromycin analogues appears to be helpful for effective interactions with the enzyme, but not essential since dialkylated analogues **10a**, **10b**, **10d** and **10e** were oxidized as well. It is noticeable that none of the *n*-pentyl containing analogues (**9f, 10f, 12f**) were appreciably accepted as TtmP substrates. This could be due to the longer alkyl chains sterically preventing these molecules from entering the enzyme active site, or potentially adopting a different substrate binding orientation that prevents key C-H bond oxidation. Despite their inactivity with TtmP, *n*-pentyl-containing analogues **10f** and **12f** efficiently inhibited cell growth of *E. coli* W3110 Δ*tolC*, suggesting that the elongated alkyl chain was not deleterious for antibacterial activity.

We next attempted to identify the regioselectivity of TtmP oxidation on non-native substrates. Enzyme assays with selected thiotetromycin analogues producing mono-hydroxylated products (**10a, 12b, 12d**) were successfully scaled up, however, attempts to isolate the assay product compounds led to decomposition during purification. We propose that the TtmP-installed hydroxyl group on the unnatural substrates facilitated an intramolecular ring opening of the thiolactone moiety. The liberated sulfhydryl group can dimerize, oxidize or aggregate to produce a variety of decomposition products (Figure S12). We are currently exploring strategies to stabilize the mono-hydroxylated assay products and

precisely characterize the location of oxidation on the alkyl chain in different thiotetromycin analogues.

Conclusions

A seven-step synthetic strategy was employed using a variety of alkyl halides to divergently generate a library of 17 racemic thiotetromycin analogues in addition to racemic thiotetromycin. While racemic thiotetromycin showed the greatest potency, a selection of thiotetromycin analogues retained antimicrobial activity against *E. coli*. Unfortunately, we did not observe appreciable growth inhibition against *M. tuberculosis*, indicating that modification of the 5-position alkyl substituent is not ideal to generate analogues with improved anti-tuberculosis activity. However, some analogues possessed potentiated activity against the disease target enzyme NNMT with a unique scaffold to previously identified inhibitors.

We were able to improve the relative amount of heme incorporation into TtmP following heterologous protein expression, and successfully optimized enzyme reaction conditions for racemic synthetic substrates *in vitro*. TtmP assays with racemic thiotetromycin improved the enzyme regeneration system and preliminary results suggest that oxidation occurs enantioselectively. Optimization of TtmP heme incorporation and *in vitro* assay conditions with racemic thiotetromycin identified ideal conditions to interrogate our focused thiotetronate library of compounds. The P450 enzyme TtmP showed moderate promiscuity by accepting a range of unnatural substrates for mono-hydroxylation, while only the racemic thiotetromycin was oxidized fully to the carboxylic acid product. These substrate specificity studies identified that chain length is a significant factor for TtmP and that 5,5-dialkylation appears crucial for recognition by the enzyme as none of the monoalkylated analogues were significantly oxidized.

In summary, the observed biological activities show that the derivatization of thiotetromycin provides more options for further drug discovery and development against infectious diseases. The highly selective C-H activation of unnatural substrates by a biosynthetic enzyme makes TtmP a desirable target for enzyme engineering in order to further expand its potential substrate scope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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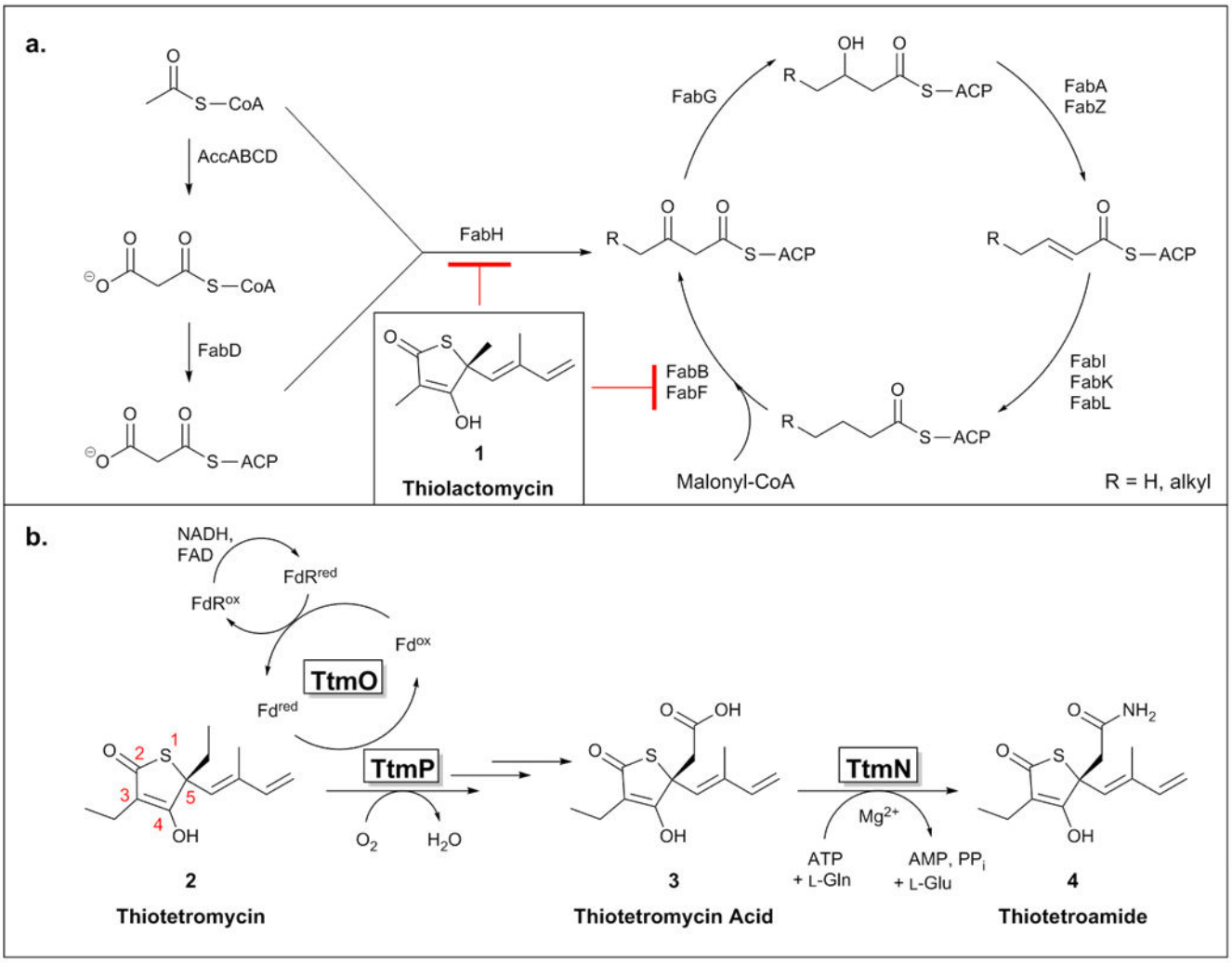


Figure 1.

a) Fatty acid biosynthesis through the type II FAS pathway in *E. coli*. Thiolactomycin (**1**) targets the initial condensation and condensing enzymes FabH, FabB and FabF. b) Oxidation-amidation cascade in the biosynthesis of thiotetroamide (**4**). Cytochrome P450 enzyme TtmP is regenerated via a ferredoxin (TtmO; Fd) and ferredoxin reductase (FdR) to catalyze a triple oxidation of thiotetromycin (**2**) to thiotetromycin acid (**3**), upon which amidotransferase TtmN converts the carboxylic acid to the amide moiety of **4**.[20]

a. **Antibacterial activity of thiotetronates against *E. coli* W3110 Δ*tolC* in spot-on-lawn assays**

	a	b	c	d	e	f
9	100 μg	100 μg	100 μg	100 μg	100 μg	100 μg
10	50 μg	50 μg	50 μg	50 μg	5 μg	5 μg
12	5 μg	50 μg	5 μg	50 μg	5 μg	5 μg

b.

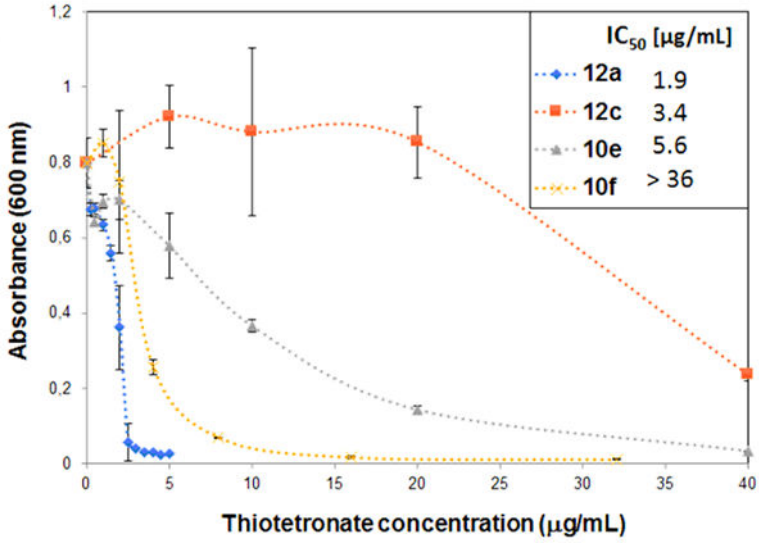


Figure 2.

a) Thiotetromycin analogues classified based on the material amount applied at which a zone of inhibition was observed in spot-on-lawn assays with *E. coli* W3110 Δ*tolC* b) IC_{50} values against *E. coli* W3110 Δ*tolC* for the most promising thiotetromycin analogues based on results from spot-on-lawn bioactivity assays.

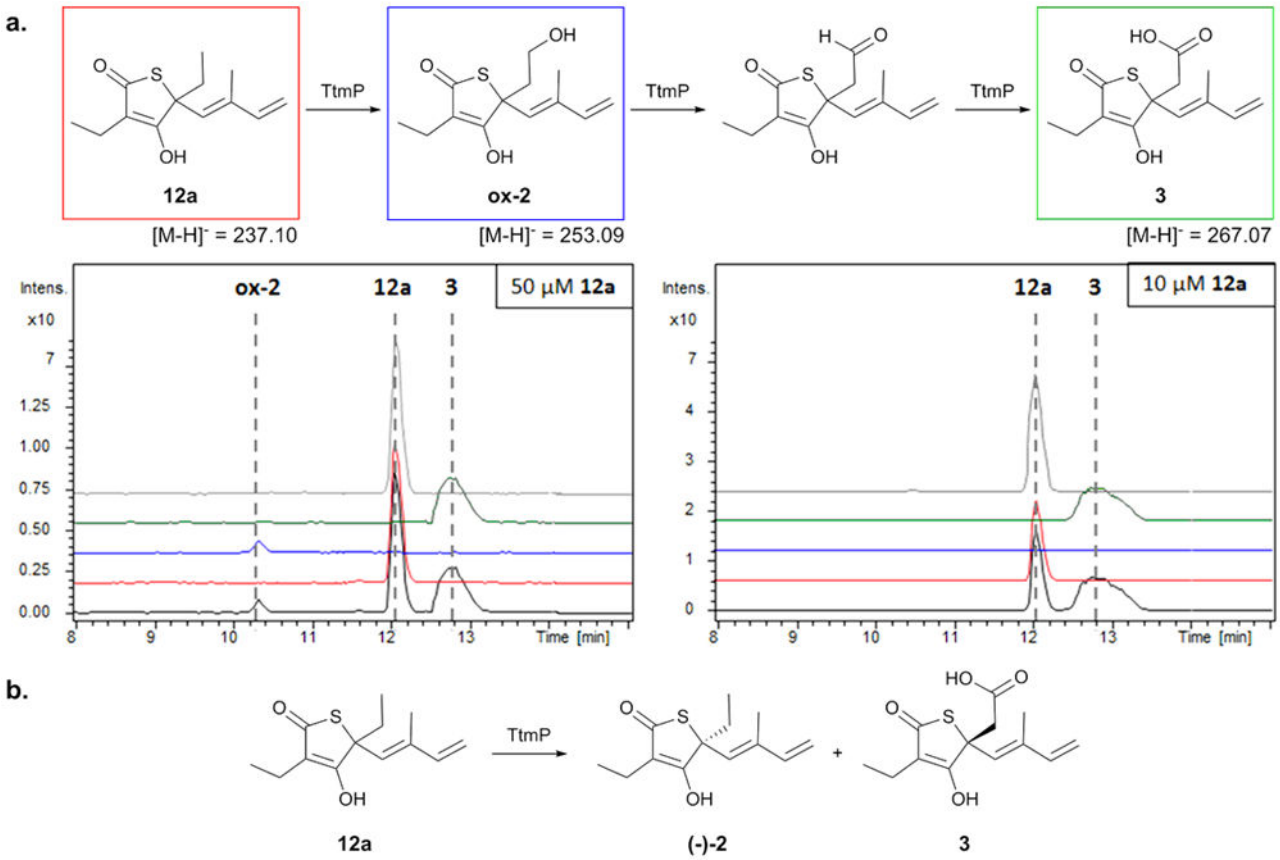


Figure 3.

a) Enzyme assay with TtmP and synthetic racemic thiotetromycin. Negative mode ion chromatogram LCMS traces extracted for: all potential substrate and oxidized products ([M-H]$^-$ = 237.10, 253.09, 267.07 ± 0.5 *m/z*, black); remaining substrate (M-H]$^-$ = 237.10 ± 0.5 *m/z*, red); singly hydroxylated product (M-H]$^-$ = 253.09 ± 0.5 *m/z*, blue); and carboxylic acid product **3** (M-H]$^-$ = 267.07 ± 0.5 *m/z*, green). Assays were compared to negative controls for each compound set up following identical procedures except without the addition of TtmP ([M-H]$^-$ = 237.10, 253.09, 267.07 ± 0.5 *m/z*, grey). b) Conversion of only 50% of the starting material suggests the enantioselective reaction shown.

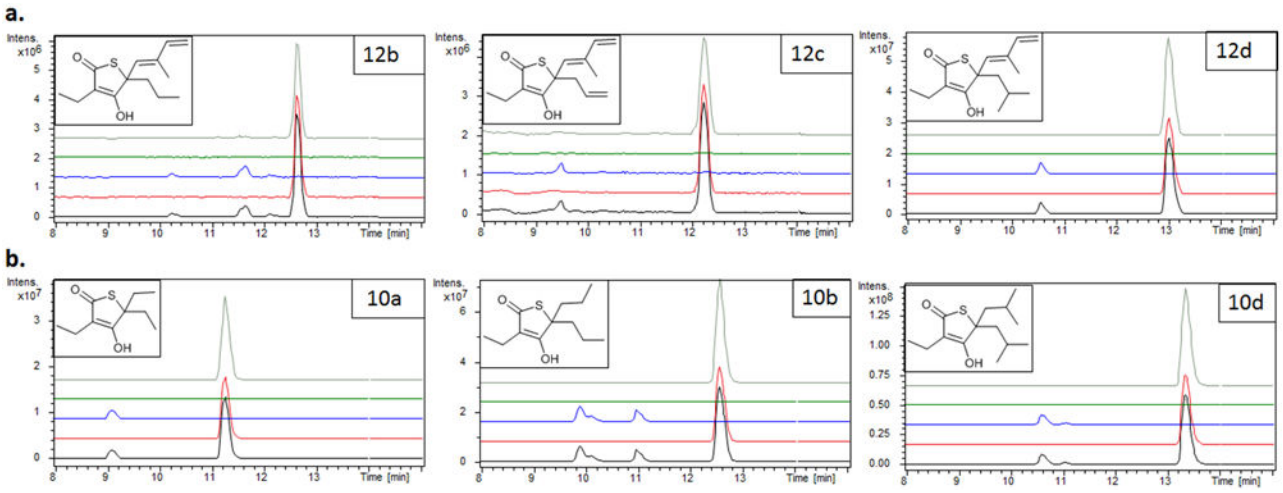
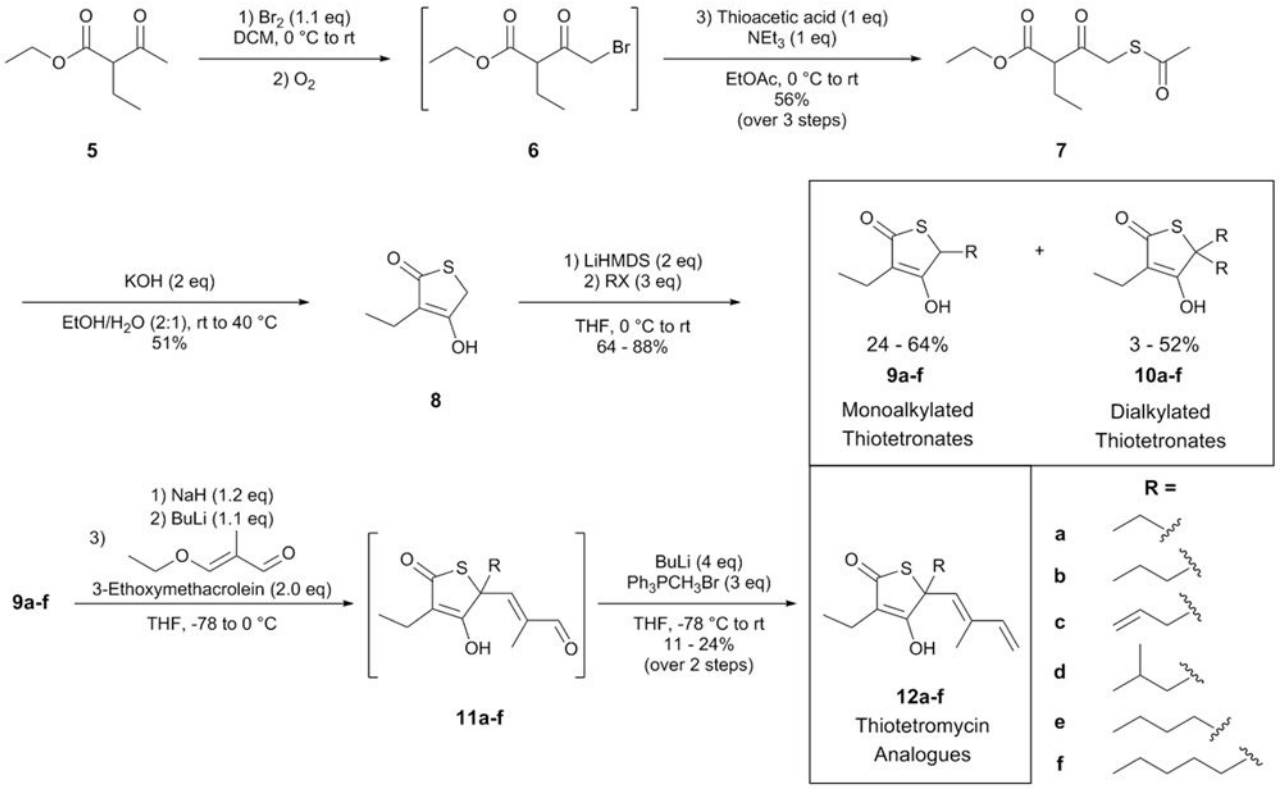


Figure 4.

Enzyme assay with TtmP and a) thiotetromycin analogues or b) 5,5-dialkylated thiotetronate compounds. Negative mode ion chromatogram LCMS traces extracted for: all potential substrate and oxidized products (black); remaining substrate (red); singly hydroxylated product(s) (blue); and carboxylic acid product(s) (green). Assays were compared to negative controls for each compound set up following identical procedures except without the addition of TtmP (grey).



Scheme 1.

Syntheses of 5-monoalkylated (**9a-f**), 5,5-dialkylated (**10a-f**) and thiotetromycin-like (**12a-f**) thiotetronate compounds.