Structure−Activity Relationships at the 5-Position of Thiolactomycin: An Intact (5*R*)-Isoprene Unit Is Required for Activity against the Condensing Enzymes from *Mycobacterium tuberculosis* and *Escherichia coli*

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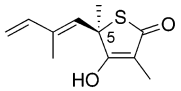
Received August 18, 2005

Thiolactomycin inhibits bacterial cell growth through inhibition of the β-ketoacyl-ACP synthase activity of type II fatty acid synthases. The effect of modifications of the 5-position isoprenoid side chain on both IC_{50} and MIC were determined. Synthesis and screening of a structurally diverse set of 5-position analogues revealed very little tolerance for substitution in purified enzyme assays, but a few analogues retained MIC, presumably through another target. Even subtle modifications such as reducing one or both double bonds of the diene were not tolerated. The only permissible structural modifications were removal of the isoprene methyl group or addition of a methyl group to the terminus. Cocrystallization of these two inhibitors with the condensing enzyme from *Escherichia coli* revealed that they retained the TLM binding mode at the active site with reduced affinity. These results suggest a strict requirement for a conjugated, planar side chain inserting within the condensing enzyme active site.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is one of the most deadly infectious diseases, infecting more than 8 million and killing more than 2 million people each year.[1] Multidrug-resistant strains of TB and co-infection with HIV have made treating TB even more difficult. The most widely used TB therapy is a combination of isoniazid, rifampicin, pyrazinamide, and ethambutol.[2,3] Even such four-drug regimens still require treatment times of at least 6 months, decreasing TB patient compliance. New TB therapies are desperately needed that will shorten the treatment period and combat multidrug-resistant TB.

Cell wall biosynthesis is a proven broad-spectrum antibacterial drug target that has been historically very efficacious.[4] In the treatment of TB in particular two of the four agents that constitute front-line therapy (isoniazid and ethambutol) target different aspects of cell wall biosynthesis. Although recent analysis suggests that interrupting cell wall synthesis may have little potential to significantly shorten the duration of therapy, new agents active against cell wall targets may offer valuable therapeutic options for the management of cases of drug-resistant disease.[5] Thiolactomycin (TLM, **1**) is a thiotetronic acid-containing natural product that inhibits bacterial and plant type II fatty acid synthases (FASII), which provide essential building blocks for bacterial cell walls.[6−8] Although fatty acid synthases are also present in higher eukaryotes, these type I systems are structurally distinct, and the presence of a 3-position methyl group on thiotetronic acids has been shown to confer high selectivity for type II over type I systems.[9] TLM exerts its effect via inhibition of the β-ketoacyl-ACP synthases (KAS), key condensing enzymes involved in chain elongation in FASII.[10−12] The core thiotetronic acid structure is believed to mimic the transition state adopted by the thiomalonate intermediate in chain elongation. But there are more subtle specific interactions, since even closely related condensing enzymes such as FabH, which initiates chain formation from acetyl-CoA and malonyl-ACP, are only weakly inhibited by TLM and are not physiologically relevant targets for the antibiotic.[13−15] In Mtb, for example, TLM inhibits KasA and KasB, two KAS enzymes that are components of the specialized FASII system involved in synthesis of the very long-chain meromycolic acids (ca. C_{50}), but is only a weak inhibitor of the initiating condensing enzyme mtFabH.[11,16] TLM has only modest activity against laboratory strains of Mtb, but it retains this activity against several strains of Mtb that are resistant to other drugs.[11,17]



Thiolactomycin (TLM, **1**)

TLM has several characteristics that make it an attractive lead molecule for TB.[18] It has low toxicity in mice,[19] low molecular weight (MW = 210 g/mol), high water solubility, and drug-appropriate lipophilicity (log P = 3). TLM is well absorbed orally and has shown chemotherapeutic efficacy against *Serratia marcescens* and *Klebsiella pneumoniae* in a mouse infection model.[19] Thus, derivatization of TLM to improve potency for various pathogens has been the focus of many recent investiga-

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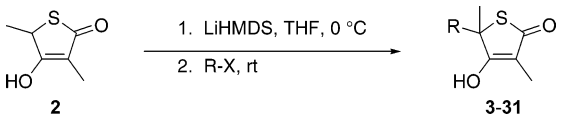
[▽] Current address: Imperial College London, London UK SW7 2AZ.

10.1021/jm050825p CCC: $33.50
Published on Web 12/13/2005

Scheme 1. Synthesis of Simple 5-Position Analogues



tions. For reasons of synthetic accessibility, the 5-position of the molecule has received the most attention. Many groups have reported a wide variety of substitutions at the 5-position and evaluated whole-cell activity against plants, malaria, trypanosomes, and livestock pathogens, or have reported activity in complex, partially purified fatty acid synthase systems with varying degrees of success.[10,20,21] 5-Position analogues have been reported with improvement in MIC against whole cells of Mtb over TLM, but no attempt was made to measure IC_{50} against the relevant condensing enzymes and to correlate it to MIC.[11] Other reports have shown that 5-biphenyl or 5-acetylenic analogues of TLM were able to inhibit the related Mtb FabH; however, no Mtb MIC values were provided, and FabH is not essential in Mtb, suggesting these would not be expected to have whole-cell potency.[16,22,23] Because nearly all of these studies fail to report IC_{50} values against the relevant purified condensing enzymes, evaluation of the resulting structure−activity relationship (SAR) has been impossible. Moreover, the presence of multiple condensing enzymes, including both type I and type II in addition to condensing enzymes present to produce essential polyketide components of the cell envelope of Mtb, has made it especially difficult to interpret the wide variety of structures that have been produced.

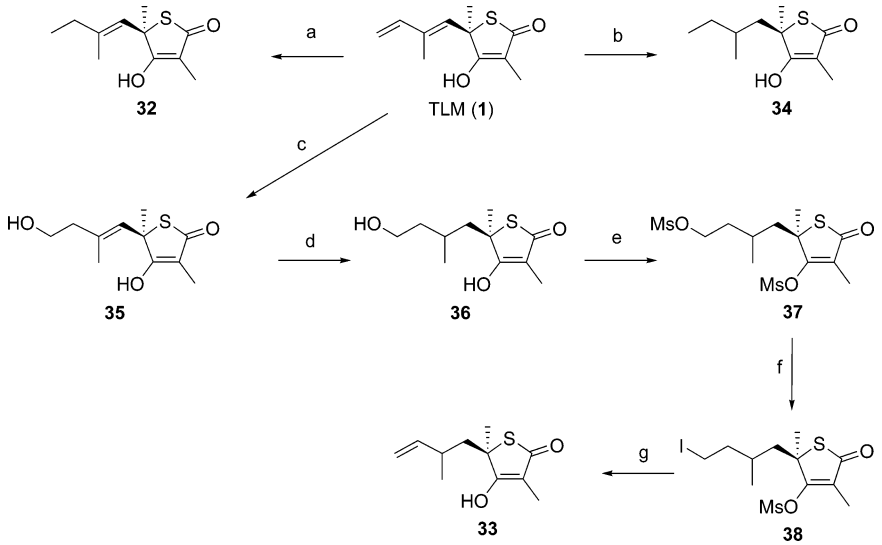
The *Escherichia coli* model system has been instrumental in elucidating the mechanism of action of TLM. The FabB condensing enzyme appears to be the relevant physiological target since cells that overexpress FabB, but not FabH, are resistant to TLM.[13] Likewise, cells of Mtb that overexpress KasA acquire TLM resistance.[24] In addition, selection of drug-resistant *E. coli* has shown that the F390V mutation in FabB confers TLM resistance.[15] The cocrystal structure of TLM bound to *E. coli* FabB provides a detailed view of how this molecule interacts with the active site.[12] TLM binds to the malonyl-ACP side of the active site with the 2-position carbonyl oxygen of TLM forming tight hydrogen bonds with His-298 and His-333. The 4-position hydroxyl group forms hydrogen bonds with a series of water molecules which in turn interact with the backbone of the polypeptide chain. These water molecules lie within the portion of FabB that forms the binding site for acyl carrier protein-bound phosphopantetheine. The sulfur atom of the thiolactone does not make any specific contacts but lies adjacent to the active site cysteine. The isoprene side chain of TLM slips into a narrow lipophilic pocket between two peptide bonds, and this conjugated aliphatic chain interacts with the peptide via tight van der Waals interactions. A hydrophobic pocket opens up beyond the isoprene binding slot, an observation that led to an early conclusion that this binding site is not "optimally" filled by TLM and that hydrophobic 5-position analogues may improve the binding affinity. To attempt to improve the binding of the TLM isoprene side chain, we began to explore the SAR of the 5-position of TLM for the condensing enzymes of the type II FAS system.

Chemistry

Synthetic routes to both racemic and chiral TLM have been reported.[25−27] 3,5-Dimethylthiotetronic acid (**2**) was prepared using the method of Wang and Salvino.[27] Using a method similar to that previously reported,[28] the dianion of **2** was generated using LiHMDS at 0 °C (Scheme 1). Subsequent addition of alkyl or aryl halides gave racemic 5-substituted TLM derivatives (**3**−**31**) in moderate to good yields (30−90%). In cases in which the isolated purity was below 80% (by LC/MS), the compound was purified by flash chromatography. In general, bulky halides or alkyl and aryl chlorides afforded lower yields. The compounds were made in parallel using the Radley's carousel reaction system. In all, 77 compounds were prepared by this method and were examined for biological activity. Compounds **3**−**31** are a representative set. (For a complete list, see Supporting Information.)

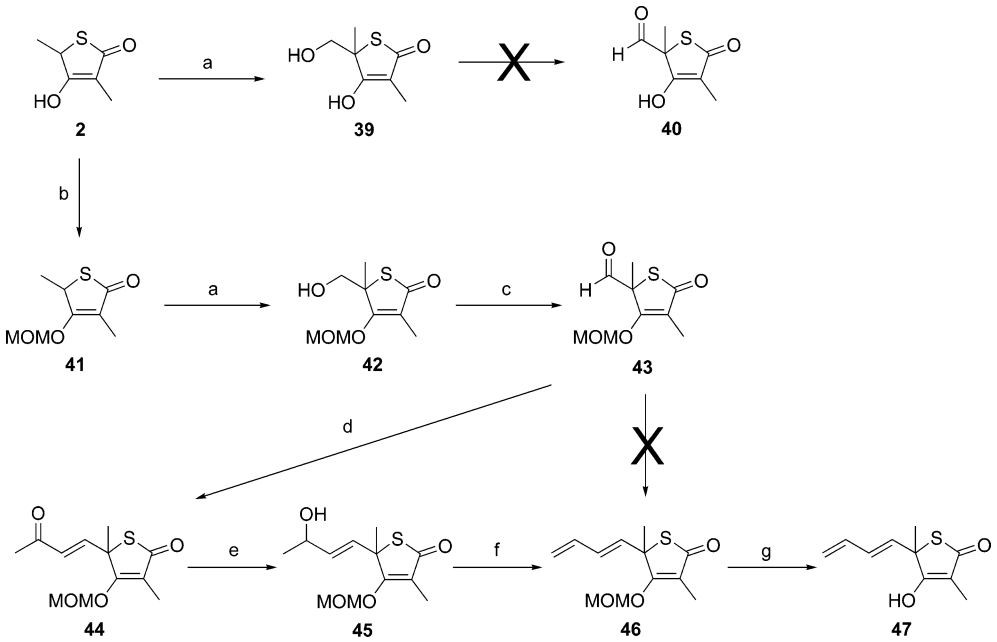
To investigate the relative importance of each double bond in the isoprenoid side chain, compounds **32** and **33**, where each double bond was reduced individually, as well as compound **34**, the fully saturated analogue, were prepared. Chiral compounds **32**−**34** were prepared from fermentation-derived TLM, preserving the stereochemistry of the 5-position.

Scheme 2. Synthesis of Analogues to Examine Importance of Isoprene Double Bonds in TLM, **32**−**34**[a]



[a] Reagents and conditions: (a) $NH_2NH_2{\cdot}H_2O$ (39 equiv), 30% H_2O_2 (aq), EtOH, 0 °C → rt, 1.5 h; (b) $NH_2NH_2{\cdot}H_2O$ (500 equiv), 30% H_2O_2 (aq), EtOH, 0 °C → rt, overnight; (c) i) 9-BBN, THF, rt, 6 h; ii) 3 M NaOH (aq), 30% H_2O_2 (aq), 0 °C → rt, 10 min; (d) $NH_2NH_2{\cdot}H_2O$ (935 equiv), 30% H_2O_2 (aq), EtOH, 0 °C → rt, overnight; (e) MsCl, Et_3N, CH_2Cl_2, rt, 3 h; (f) NaI, acetone, reflux, 4 h; (g) *t*-BuOK in THF, CH_2Cl_2, rt, 30 min.

Scheme 3. Syntheses of 5-Alkylidene Analogues and Diolefinic Desmethyl-TLM[a]



[a] Reagents and conditions: (a) (i) LiHMDS, THF, 0 °C; (ii) paraformaldehyde, 0 °C → rt; (b) MOMCl, DIPEA, CH_2Cl_2, rt; (c) Dess−Martin periodinane, CH_2Cl_2, rt; (d) $AcCH_2P(=O)(OEt)_2$, DIPEA, LiCl, CH_3CN, rt; (e) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, MeOH, rt; (f) Burgess reagent, toluene, rt; (g) $NaHSO_4 \cdot SiO_2$, CH_2Cl_2, rt.

While catalytic hydrogenation attempts using either 5% Pd/C or 5% Rh/Al_2O_3 gave only trace amounts of **32** or **34**, an excess of diimide[29] from $NH_2NH_2 \cdot H_2O$ and 30% H_2O_2 (aq) generated these two compounds readily from TLM (Scheme 2). To obtain compound **33**, the terminal double bond of the TLM isoprene side chain was first masked. Hydroboration of TLM with 9-BBN and subsequent oxidation were used to produce terminal alcohol **35** in 88% yield.[30] The internal double bond of **35** was then reduced with an excess of diimide, resulting in compound **36**. Mesylation using MsCl and Et_3N then furnished dimesylate **37** in modest yield.[31] Unfortunately, attempted elimination of mesylate **37** with DBU in toluene under reflux did not afford the desired terminal double bond of **33** but instead generated two compounds having the same molecular weight as **33** but that were far less polar than the desired target molecule.[32] Functional group transformation from mesylate **37** to iodide was successful using NaI in acetone to afford compound **38** in 66% yield.[33] Dehydroiodination and C4-mesylate deprotection of **38** were accomplished in one pot using an excess of *t*-BuOK (20 equiv) in CH_2Cl_2 to give compound **33** in 50% yield.[34] Use of fewer equivalents or slow addition of *t*-BuOK led to the same nonpolar compounds as above, most likely by intramolecular nucleophilic attack of the demesylated C4-oxygen onto the 5-position side chain. Several other elimination attempts such as DBU, TBAF, and *t*-BuOK in THF gave similar results. The diastereomers of compounds **33** and **36**−**38** were not separated.

To investigate the importance of the methyl branch on the isoprene side chain, the simple butadiene analogue of TLM (i.e., desmethyl-TLM, **47**) was prepared (Scheme 3). The racemic 5-position aldehyde was selected as the initial step from 3,5-dimethylthiotetronic acid **2**, anticipating that simple olefination would afford the desired butadiene. However, direct formylation of **2** was unsuccessful; therefore, the dianion of **2** was treated with paraformaldehyde to afford the 5-hydroxymethyl compound **39**.[35] Unfortunately, several oxidation conditions (PCC, PDC, TPAP/NMO, Dess−Martin periodinane, and Swern oxidation) were unsuccessful at converting **39** to aldehyde **40**. Unsuccessful oxidation attempts of a primary alcohol at the 5-position of either tetronic or thiotetronic acid without first protecting the 4-hydroxyl group have been previously reported.[26,36] Protection of the 4-hydroxyl group of **2** by treatment with MOMCl and DIPEA provided compound **41**.[37] 5-Hydroxymethylation of **41** then furnished **42** in 78% yield.[35] Oxidation of **42** using Dess−Martin periodinane gave the desired aldehyde **43** (43% from **2**).[38] When **43** was subjected to Horner olefination conditions using diethyl allylphosphonate,[39] the reaction generated **41** as the major product via deformylation, in addition to trace amounts of the 5-diene analogue **46**.

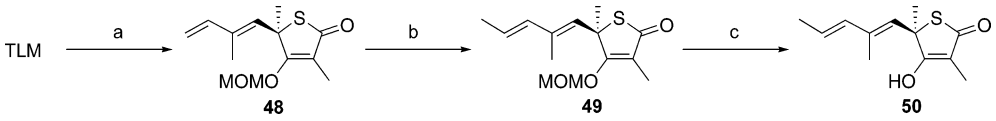
From aldehyde **43**, a Horner-Wadsworth-Emmons reaction with a stabilized ylide diethyl 2-oxopropylphosphonate, gave *trans*-enone **44** in 48% yield in addition to the deformylation product **41** (18%).[39,40] The ketone of **44** was then successfully reduced with $NaBH_4$ and $CeCl_3 \cdot 7H_2O$ to afford allyl alcohol **45** in 64% yield.[41] Subsequent dehydration of **45** in the presence of the Burgess reagent[42] furnished butadiene **46**. A variety of conditions were employed to deprotect compound **46**, most yielding only nonpolar products.[32] The most successful condition was the very mild $NaHSO_4 \cdot SiO_2$[43] at room temperature. These conditions furnished the desired deprotected product **47** in 75% yield from compound **46**.

Finally, we prepared analogue **50**, containing an additional methyl group at the terminus of the isoprenoid side chain of TLM (Scheme 4). The 4-position hydroxyl group of TLM was first protected using MOMCl to give compound **48** in nearly quantitative yield. Olefin cross-metathesis,[44] using Grubbs' second generation catalyst in the presence of *trans*-2-butene, furnished (1*E*,3*E*)-diene **49** in good yield. The optimal deprotection condition for compound **49** was found to be polymer-bound TsOH stirred with silica gel at room temperature.[45] This condition yielded desired compound **50** in 75% yield.

Results and Discussion

Effect of 5-Position Stereochemistry on Biological Activity of TLM. Because many of the 5-substituted TLM analogues

Scheme 4. Synthesis of Compound **50**[a]



[a] Reagents and conditions: (a) MOMCl, DIPEA, CH_2Cl_2, rt; (b) Grubbs' II catalyst, *trans*-2-butene, sealed tube, CH_2Cl_2, 45 °C; (c) silica gel, polymer-bound TsOH, CH_2Cl_2, rt.

to be evaluated were racemic, it was important to establish whether the presence of the opposite enantiomer of this series of molecules would antagonize activity of chiral TLM, isolated from fermentation of *Nocardia* sp. 2-200. Therefore, we evaluated the activity of racemic TLM (Sigma-Aldrich) in our biological assays. The IC_{50} of racemic TLM against *E. coli* FabB was 20 μM, exactly 2-fold higher than that of TLM (Figure 1A). This result indicates that the enantiomer of TLM is inactive and nonantagonistic, consistent with the crystal structure of the FabB−TLM complex in which the shape and hydrogen bonding network of the TLM binding pocket is only able to accommodate the natural stereoisomer. Similarly, the MIC of racemic TLM against *E. coli* strain ANS1 was 12 μM, 2-fold higher than that of TLM (Figure 1B). These results suggested that racemic TLM analogues would likely result in only an apparent 2-fold decrease in potency compared with TLM itself. Since our goal was to obtain enhanced inhibition of Mtb KasA and KasB, the activity of TLM was also measured in a KasA (or KasB) extension assay using palmitoyl-ACP (from *E. coli*) with [^{14}C]-malonyl-CoA in the presence of FabD and reduced ACP. Reaction products were reduced with $NaBH_4$ and organic soluble radioactivity then provided a measure of enzyme activity. Using this assay, the IC_{50} of TLM for Mtb KasA and KasB was found to be 4 μM and 6 μM, respectively (Figure 1C). TLM showed little selectivity between the enzymes.

Biological Activity of Structurally Diverse 5-Position Analogues of TLM. The biological assay results for compounds **3**−**31** are shown in Table 1. Enzyme inhibition assays were performed at 100 μM against *E. coli* FabB/H and Mtb KasA/B and FabH. When no inhibition was seen at 100 μM, the compound was not tested further for enzyme inhibitory activity. When the compound showed greater than 30% inhibition of the enzyme, a discrete IC_{50} was measured as shown in Figure 1. Inhibition between 0 and 30% was recorded as an IC_{50} value of >100 μM. The compounds were also evaluated in MIC assays against *E. coli* strain ANS1 and Mtb strain H37Rv.

As a set, the simple 5-alkyl, alkenyl, and aryl substituted thiotetronic acid analogues lacked significant activity in the condensing enzyme assays. Straight-chain alkyl groups showed very modest activity against *E.* coli and Mtb FabH enzymes with longer aliphatic chains correlated with improved activity. A relationship between chain length and FabH activity was shown in compounds **4**−**9**. Increasing chain length resulted in improved activity against Mtb FabH, with the maximal inhibition by compound **7** (C_{12}). Longer chain lengths led to less activity against FabH. These compounds, however, were inactive against FabB, KasA, and KasB and did not have enhanced MIC values against Mtb. Likewise, they showed only modest MICs against *E.* coli. These data are consistent with the report that in Mtb FabH is not essential.[23] In some cases, growth inhibition toward an organism (**7**, *E. coli*) did not necessarily translate into inhibition of a known condensing enzyme.

One striking observation was that compound **14,** corresponding to a simple saturated version of the racemic form of TLM completely lacked activity. We were surprised to note that this comparatively simple modification abolished activity against the

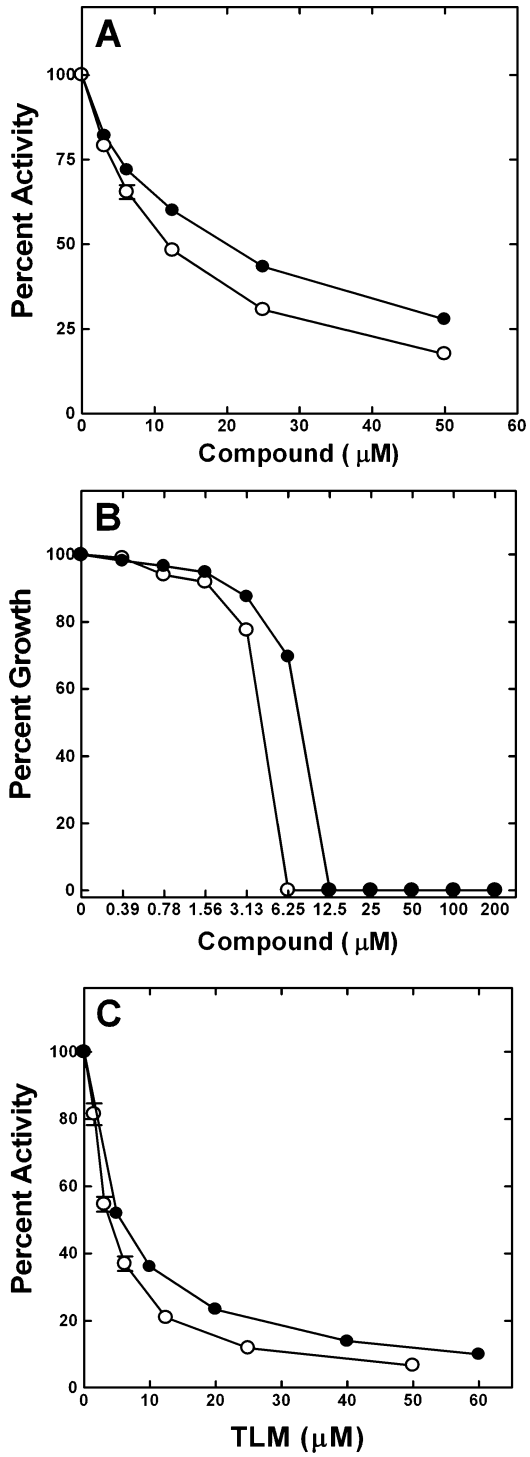
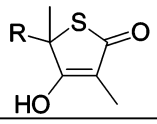
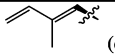
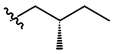
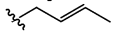
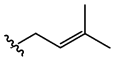
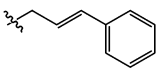
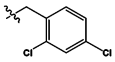
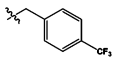
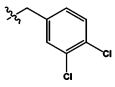


Figure 1. (A) IC_{50} of TLM (open circles) and racemic TLM (closed circles) at *E. coli* FabB. (B) MIC of TLM (open circles) and racemic TLM (closed circles) in *E. coli* strain ANS1. (C) IC_{50} of TLM against *M. tuberculosis* KasA (open circles) and KasB (closed circles).

Table 1. 5-Position TLM Derivatives

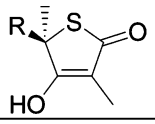


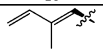
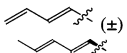
		IC$_{50}$ (μM)[a]					MIC (μM)[b]	
compd	R	ec FabB	ec FabH	mt FabH	mt sKasB	mt sKasA	*E. coli* ANS1	*M. tuberculosis* H37Rv
TLM (1)	 (chiral)	12	-	87	6	4	6	62.5
2	-H	na	na	na	na	na	>1000	>1000
3	-(CH$_2$)$_4$CH$_3$	na	na	na	na	na	>1000	500
4	-(CH$_2$)$_5$CH$_3$	na	na	na	na	na	>1000	1000
5	-(CH$_2$)$_7$CH$_3$	na	>100	>100	na	na	200	>1000
6	-(CH$_2$)$_9$CH$_3$	na	100	>100	na	na	100	500
7	-(CH$_2$)$_{11}$CH$_3$	na	100	50	na	na	25	1000
8	-(CH$_2$)$_{12}$CH$_3$	>100	100	80	na	na	>200	500
9	-(CH$_2$)$_{13}$CH$_3$	>100	100	80	na	na	200	1000
10		na	na	na	na	na	>1000	>1000
11		na	na	na	na	na	>1000	1000
12		na	na	na	na	na	>1000	>1000
13		na	na	>100	na	na	>1000	>1000
14		na	>100	na	na	na	>1000	500
15		na	>100	na	na	na	>1000	>1000
16		na	na	na	na	na	>1000	>1000
17		na	na	>100	na	na	>1000	1000
18	-CH$_2$CH=CH$_2$	na	na	na	na	na	>1000	>1000
19		na	na	na	na	na	>1000	>1000
20		na	na	na	na	>100	>1000	>1000
21		na	na	na	na	na	>1000	>1000
22	-(CH$_2$)$_3$CH=CH$_2$	na	na	na	na	>100	>1000	>1000
23		na	na	na	na	na	>1000	>1000
24	-(CH$_2$)$_4$CH=CH$_2$	na	na	na	na	>100	>1000	1000
25	-geranyl	na	100	na	na	na	>1000	>1000
26	-farnesyl	na	>100	na	> 100	na	200	>1000
27		na	na	na	na	na	>1000	>1000
28		na	na	na	na	na	>1000	>1000
29		na	na	>100	na	na	150	500
30		na	na	>100	na	na	>1000	1000
31		na	>100	>100	na	na	>1000	500

[a] Where a discrete value is shown, the IC$_{50}$ was measured for that compound. Where a value of >100 μM is shown, the inhibition of the condensing enzyme was less than 30% at 100 μM. "na" indicates that no inhibition occurred at 100 μM. "–" indicates that the value was not determined. Each value is the average of duplicates. [b] MIC values are the average of triplicates.

condensing enzymes and failed to show any observable MIC against the intact organisms relative to that of TLM given the apparent permissive nature of tolerated substitutions at this position reported by other authors. Similar results were found

Table 2. Analogues of TLM Examining Structural Features of the Isoprene Side Chain



		IC_{50} (μM)[a]			MIC (μM)[b]		IC_{50}(μM)[a]
compd	R	ec FabB	mt KasA	mt KasB	*E. coli* ANS1	*M. tuberculosis* H37Rv	Human FASI
TLM (1)		12	4	6	6	62.5	100[9]
Racemic TLM (51)		20	-	-	12	125	143[9]
32		na	na	na	>200	>1000	na
33		na	na	na	>200	>1000	na
34		na	na	na	>200	>1000	na
47	 (±)	500	25	400	200	1000	na
50		500	50	400	200	1000	na

[a] Where a discrete value is shown, the IC_{50} was measured for that compound. Where a value of >100 μM is shown, the inhibition of the condensing enzyme was less than 30% at 100 μM. "na" indicates that no inhibition occurred at 100 μM. "−" indicates that the value was not determined. Each value is the average of duplicates. [b] MIC values are the average of triplicates.

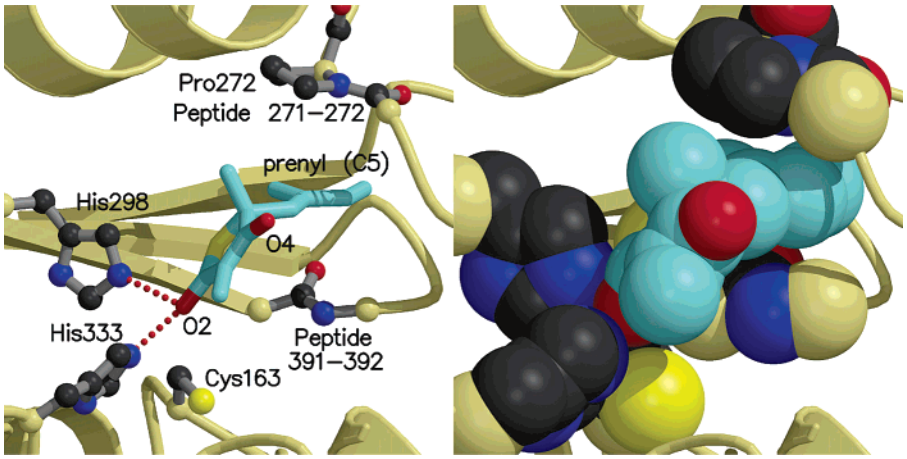


Figure 2. Detail of TLM−FabB cocrystal highlighting placement of isoprene side chain. Left panel: TLM binds on the malonyl-ACP side of the active site and forms two strong hydrogen bonds with both active site histidines. The isoprene side chain inserts between the peptide bond of residues 271−272 and 391−392. Right panel: Space filling model of TLM in the same orientation as shown at left. The isoprene chain interacts with the peptide backbone through van der Waals interactions that leave no room for nonplanar or bulky substituents at the 5-position.

with racemic compound **23**, where the proximal double bond was reduced. The geranyl-substituted analogue **25** had low activity in all assays employed, which was also contrary to prior reports.[11]

Because of the lack of activity displayed by compounds **3**−**31**, it became clear that the intact isoprene side chain of TLM was absolutely critical to its activity. Our focus then shifted to determining the precise structural features within the isoprene unit that were responsible for the binding of TLM.

The Relative Importance of Each Olefin as a Binding Determinant of the Isoprene Side Chain. Using chiral, fermentation-derived TLM allowed us to systematically explore the contributions of each double bond in the TLM side chain independently using compounds **32**−**34**. Racemic **47** allowed us to explore the contribution of the methyl branch in the isoprenoid side chain. The results of *E. coli* and *M. tuberculosis* β-ketoacyl-ACP synthase activity assays and MIC determinations are shown in Table 2. Surprisingly, reduction of either double bond, independently or both together, completely ablated both enzyme inhibition and whole-cell growth-inhibitory activity. Therefore, the presence of two conjugated double bonds adjacent to the thiolactone ring appears to be a formal requirement of this mode of binding to the condensing enzyme active site. Compound **47**, corresponding to the racemic desmethyl analogue of TLM, maintained full activity toward KasA (the measured IC_{50} is for the racemate, so presumably the correct enantiomer has an IC_{50} of half this concentration). Despite this potent activity against KasA, this molecule was totally inactive against FabB and KasB and lacked whole-cell MIC activity against both organisms as well.

To extend our understanding of the structural requirements for binding beyond the essentiality of the conjugated diene, we also examined the effect of elongation of the side chain by addition of an extra methyl group to the terminus of the isoprenoid unit. The FabB−TLM cocrystal structure suggested that this should be tolerated because the binding pocket extends beyond the TLM side chain toward the interior of the protein.[12] Compound **50**, methyl-TLM, retains activity as an inhibitor of KasA only (Table 2). Compound **50** showed no activity against FabB or KasB, and, similar to desmethyl-TLM, did not show inhibition of growth of either organism.

The compounds in Table 2 were also examined for their inhibitory effects against human FASI isolated from breast

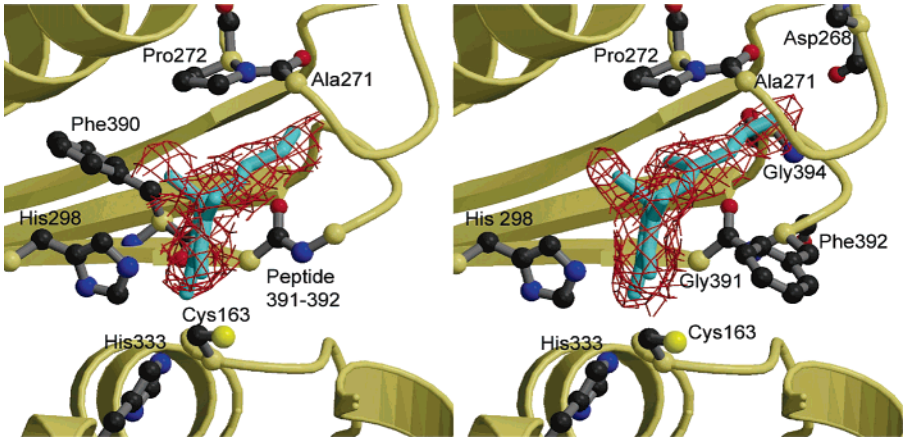


Figure 3. Compound **47**−FabB (left, PDB code 2AQB) and **50**−FabB (right, PDB code 2AQ7) cocrystal complexes. Simulated annealing omit maps are shown in red for each ligand, and the docked ligands are shown in cyan. Both compounds bind to the active site in a similar way to TLM, and this is emphasized by preserving the orientation of the active site that is shown for the TLM−FabB complex in Figure 2. The maps are contoured at 2.5 σ.

Table 3. Data Measurement and Refinement Statistics for **47**−FabB and **50**−FabB Cocrystal Complexes

crystal details	**47**−FabB	**50**−FabB
space group	$P2_12_12_1$	$P2_12_12_1$
cell: $\alpha,\beta, \gamma = 90.0°$	$a = 59.10$, $b = 139.19$, $c = 212.33$	$a = 59.12$, $b = 139.00$, $c = 212.46$
processing		
wavelength (Å)	1.54169	0.97926
resolution limit (Å)	2.2	2.3
reflections (unique)	612,686 (90,238)	519,111 (75,836)
% complete (last shell)	99.5 (98)	96.5 (87.2)
I/σ (last shell)	18.6 (5)	6.0 (4.6)
% R_{merge} (last shell)	7.7 (18.2)	9.2 (15.2)
refinement		
resolution (Å)	40−2.2	40−2.3
no. reflections	90,238	75,757
% R_{work} (% R_{free})	19.6 (22.5)	18.4 (22.8)
rmsd	bonds 0.008 Å, angles 1.2°	bonds 0.008 Å, angles 1.2°
no. protein residues	1615 of 1624	1615 of 1624
no. other molecules	537 waters, 3 of compound **47**	538 waters, 3 of compound **50**
mean B factor (Å^2)	15.5	18.2

cancer cell line ZR-75-1 and found to be inactive. These results correlate well with the recent report that the presence of a 3-position methyl group is a key structural determinant of the observed specificity for FASII compared with FASI.[9]

Molecular Modeling of TLM and Its Analogues. We employed a virtual docking strategy using the FlexX module within the Tripos drug discovery software suite to gain insights into how the shape and double-bond arrangement of the isoprenoid side chain might affect TLM binding affinity. To verify the validity of the approach, FlexX was first used to dock TLM into the FabB active site. FlexX was not able to dock TLM into the native FabB structure, but it did succeed in docking TLM into the FabB−TLM complex structure from which TLM had been removed. This implies that a small but significant conformational change occurs within the FabB active site upon TLM binding[12] that is absolutely required for FlexX to reproduce the observed binding. Analogues lacking one or both double bonds (**32**, **33**, **34**) and desmethyl TLM (**47**) were docked to the FabB structure from which TLM had been extracted. ChemScore was used[46] to rank the resulting solutions because it placed TLM at the top of the rankings when used as an internal control. In general, none of the analogues docked with scores comparable to that of native TLM. To achieve a realistic docking result, it was necessary to lock the thiolactone ring within the active site according to the crystal structure. Generally, variants that contained the branch methyl group in the isoprene side chain were ranked higher than those lacking it, and the presence or absence of the isoprene double bonds were of secondary importance. Since this result is contrary to the observed biochemical outcome, it is apparent that FlexX significantly underestimates the importance of the van der Waals interactions of the conjugated diene system.

Examination of the Binding Mode of Desmethyl- and Methyl-TLM with *E. coli* FabB. From the IC_{50} values of the compounds shown in Table 2, compounds **47** and **50** displayed a clear selectivity for KasA over KasB and FabB. To attempt to understand these differences and to verify that **47** and **50** bind to the active site in a similar mode as TLM, ligand binding was directly visualized using X-ray crystallography. Since KasA crystals remain elusive despite considerable effort, *E. coli* FabB was used for these studies even though the IC_{50} values suggested that this might be problematic. Cocrystallization was performed using a 3-fold molar excess of ligand, and data were collected for **47** and **50** at 2.2 and 2.3 Å, respectively. The structures were solved by molecular replacement and refined, and pertinent statistics for both FabB complexes are shown in Table 3. Electron density for both compounds was present in the cocrystal structures (Figure 3) but was significantly lower than the surrounding protein, indicating partial occupancy, as was expected from the IC_{50} values. To avoid model bias, the electron densities shown in Figure 3 correspond to simulated annealing omit maps in which the compounds were not included in the refinement. Despite the poor ligand electron densities, the placement of **47** and **50** in each active site was straightforward and unambiguous. By maintaining a constant ligand temperature factor during refinement corresponding to the average value of the active site residues, it was possible to estimate the ligand occupancies as 60% for **47** and 75% for **50**. These two values are consistent with the relative quality of the ligand densities where that of **50** is clearly superior. The concentration of each compound which allowed for cocrystallization was only 2-fold above the IC_{50} value, but attempts to use higher concentrations to increase occupancy compromised crystallization.

Compounds **47** and **50** dock into the active site in the same orientation as TLM. For comparison, the **47** and **50** active-site complexes are shown in Figure 3 (left and right panels, respectively) in the same orientation as the TLM complex shown

in Figure 2. Although FabB does not undergo any large conformational changes upon binding TLM, His298 and Phe392 have altered positions to accommodate the inhibitor,[12] and this is also the case with compounds **47** and **50**. The 50-fold loss in activity toward FabB of **47** and **50** relative to TLM is not easily understood from structural comparisons alone. However, two points are worth noting. Compound **47** does not contain the branch methyl group of TLM, and it may bind more weakly than TLM due to the loss of van der Waals interactions with residues Phe390 and Pro272. In the case of compound **50**, the problem may be steric in nature. The extra methyl group extending distally from the isoprene group sits in a small cavity bounded by the backbone carbonyl oxygens of Asp268 and Ala271, and the amide nitrogen of Gly394. Using the SegMod algorithm,[47] we have constructed homology models of KasA and KasB based on the FabB structure to identify any obvious differences in the active sites of the Mtb enzymes. The active sites are extremely similar, and the molecular basis for the selectivity of **47** and **50** for KasA is not immediately apparent.

Conclusion

This work suggests a conceptual shift is necessary for evaluating 5-position substitutions in the development of more effective TLM analogues for bacterial condensing enzymes. Far from being permissive to replacement and substitution, the isoprenoid side chain appears to be absolutely required to maintain activity against KasA, KasB, and FabB. Unfortunately, this side chain also provides one of the most serious obstacles to the exploration of analogues of this structural class because of the difficult synthetic accessibility of this moiety. Despite the appearance of several reports in the literature suggesting that significant substitutions in this position would be tolerated, we found that selective removal of either double bond of TLM resulted in a dramatic drop in the ability of this molecule to inhibit the elongation catalyzed by the condensing enzyme reaction and the resulting growth-inhibitory activity of the analogue.

Derivatives that had improved activity against whole cells were obtained, but the effect on whole cells did not correlate with inhibition of enzyme activity, suggesting that the mode of action of these molecules does not involve these specific condensing enzymes. Compound **7**, for example, had an MIC against *E. coli* of 25 μM, but has no activity against FabB, the known TLM target. We observe weak activity of compound **7** against FabH, but the IC_{50} of 100 μM is higher than the MIC, ruling out FabH as the likely cellular target unless significant accumulation occurs in vivo. Furthermore, compound **7** has a lower IC_{50} against Mtb FabH compared with TLM; however, **7** does not inhibit Mtb in vivo, a result congruent with the nonessentiality of this gene.[23] These data demonstrate that the activity of such TLM analogues against Mtb cannot be casually ascribed to inhibition of the mycolic acid biosynthetic pathway without solid biochemical corroboration. This result is perhaps not too surprising since TLM is thought to act as a transition-state mimic for the β-ketoacyl-ACP synthase elongation enzymes, and there are no fewer than six essential enzymes that catalyze this reaction in Mtb.[23] Analogues of TLM with appreciable Mtb MICs and without KasA or KasB activity may well inhibit another condensation enzyme, or they may interact with a different target altogether. This complexity dramatically reduces the utility of developing an SAR based upon simple whole-cell activity for any analogues of this class of molecules.

Although space-filling models of the active site suggested that larger hydrophobic molecules may fit in the cavity beyond the slot occupied by the isoprene unit, our data show that the presence of the planar, conjugated diene structure of the side chain within that slot is critical for high affinity binding. The narrow opening occupied by this side chain in the active site favors a planar geometry that predetermines the position of the entire side chain relative to the thiolactone ring. The cocrystal structure clearly reveals the rigidity of the pocket and the extremely tight fit of the isoprenoid unit (Figure 2). It is clear that the presence of a conjugated diene at the 5-position of TLM, structurally and stereochemically, cannot be altered while retaining binding mode and activity against the elongation condensing enzymes.

One intriguing finding from this study is the differential activity of compounds **47** and **50** against various condensing enzymes. From these two examples, it would appear that inhibition of mtKasA alone is not sufficient for killing the whole organism, despite strong genetic evidence that this enzyme is essential for viability. The most likely possibility is that these two compounds are effluxed or metabolized more efficiently. The disparity between the IC_{50} of TLM (4 μM) and the MIC (63 μM) suggests that penetration, metabolism, or efflux play a role in determining the susceptibility of Mtb to this agent. The other possibility is that KasB compensates for the loss of elongating activity of KasA. This possibility seems less likely since the biological substrates for these two enzymes are very different, KasA operating to extend palmitoyl-CoA to products that are approximately C_{40}, while KasB extends the products of KasA to products of an average size of C_{54}.[48] Both enzymes are capable, however, of extension of shorter acyl-CoAs, so this possibility cannot be excluded.

These results support the notion that TLM analogues must be evaluated with respect to both their activity in terms of IC_{50} against a specific condensing enzyme as well as in terms of growth-inhibitory potential to avoid confusing SAR resulting from effects on multiple enzyme targets. The tight fit of the isoprenoid side chain to a narrow groove adjacent to the enzyme active site of KasA, KasB, and FabB drastically limits the ability to simplify TLM to a simple thiotetronic acid skeleton upon which variation may be induced. Because of the synthetic complexity introduced by the chiral conjugated diene at the 5-position, future efforts at semisynthetic analogues at the 4-position enol, which extends in the direction of the binding tunnel for the phosphopantetheine prosthetic group, may be a profitable area for exploration.

Experimental Section

Anhydrous solvents and reagents were purchased from Sigma-Aldrich and used as received unless otherwise stated. Melting points were obtained on an Electrothermal 9100 apparatus and are uncorrected. LC/MS analysis was conducted on an Agilent 1100 series HPLC with attached Agilent quadrupole mass analyzer model G1946 D SL with electrospray ionization in positive ion mode. LC chromatography used a Phenomenex Luna $C_{18}(2)$ column (2 $\times$ 50 mm, 3 μm) with a water/acetonitrile (each with 0.1% (v/v) formic acid) gradient using a flow rate of 0.3 mL/min. UV detection was with an Agilent Diode Array Detector model G1315A spectrometer at 270 and 310 nm. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded at 300 and 75.5 MHz, respectively, on a Varian Gemini spectrometer, using TMS or the solvent peak as an internal standard. Column chromatography was conducted using either silica gel (Geduran 60, 40-63 μm) or prepacked RediSep columns (Teledyne Isco, Inc., Lincoln, NE) on an Isco CombiFlash Optix10 instrument. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA). HRMS analyses were performed at the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT).

General Procedure for Compounds 3−31. At 0 °C and under an argon atmosphere, LiHMDS (1 M in THF, 4 mmol) was added to a stirring solution of 3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (**2**) (0.25 g, 1.73 mmol) in anhydrous THF (6 mL). The reaction mixture was allowed to stir at 0 °C for 30 min during which time the mixture turned from clear to opaque yellow. The alkyl halide (1.73 mmol) in anhydrous THF (10 mL) was added, and the reaction mixture was allowed to come to room temperature overnight. Saturated NH_4Cl (aq) solution was added to quench the reaction, and the mixture was extracted between H_2O and EtOAc (3 × 25 mL). The combined EtOAc layers were dried with anhydrous $MgSO_4$, filtered, and evaporated in vacuo to yield the crude product. Reaction products less than 80% pure (by LC/MS) were purified by column chromatography. All compounds with purity above 80% were characterized by mass spectrometry and ^{1}H NMR and assayed for biological activity. Yields and spectral data are given for selected compounds. Physical and biological data for the entire compound set can be found in Supporting Information.

3,5-Dimethyl-4-hydroxy-5-pentyl-5*H*-thiophen-2-one (3). Yield 61%. ^{1}H NMR (acetone-d_6) δ 0.87 (t, J = 6.9 Hz, 3H), 1.26−1.31 (m, 6H), 1.62 (s, 3H), 1.65 (s, 3H), 1.84−1.88 (m, 2H), 2.92 (bs, 1H). LC/MS: 215.0 (M + 1), 237.1 (M + Na^+).

3,5-Dimethyl-5-hexyl-4-hydroxy-5*H*-thiophen-2-one (4). Yield 42%. ^{1}H NMR ($CDCl_3$) δ 0.85−0.90 (m, 3H), 1.26−1.34 (m, 8H), 1.66 (s, 3H), 1.73 (s, 3H), 1.81−1.87 (m, 2H), 5.68 (bs, 1H). LC/MS: 229.1 (M + 1), 251.0 (M + Na^+).

3,5-Dimethyl-4-hydroxy-5-octyl-5*H*-thiophen-2-one (5). Yield 79%. ^{1}H NMR (DMSO-d_6) δ 0.85 (t, J = 7.2 Hz, 3H), 1.02−1.38 (m, 12H), 1.48 (s, 3H), 1.51 (s, 3H), 1.66−1.74 (m, 2H). LC/MS: 257.1 (M + 1), 279.0 (M + Na^+).

5-Decyl-3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (6). Yield 99%. Mp 70−72 °C. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.3 Hz, 3H), 1.25 (bs, 16H), 1.66 (s, 3H), 1.73 (s, 3H), 1.81−1.87 (m, 2H). LC/MS: 285.1 (M + 1), 307.1 (M + Na^+).

3,5-Dimethyl-5-dodecyl-4-hydroxy-5*H*-thiophen-2-one (7). Yield 72%. ^{1}H NMR (acetone-d_6) δ 0.88 (t, J = 6.3 Hz, 3H), 1.28 (bs, 20H), 1.63 (s, 3H), 1.66 (s, 3H), 1.84−1.91 (m, 2H). GC/MS: 312 (M^+).

3,5-Dimethyl-4-hydroxy-5-tridecyl-5*H*-thiophen-2-one (8). Yield 69%. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.3 Hz, 3H), 1.25−1.33 (m, 22H), 1.66 (s, 3H), 1.73 (s, 3H), 1.81−1.86 (m, 2H). LC/MS: 327.2 (M + 1).

3,5-Dimethyl-4-hydroxy-5-tetradecyl-5*H*-thiophen-2-one (9). Yield 96%. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6 Hz, 3H), 1.25−1.33 (m, 24H), 1.66 (s, 3H), 1.73 (s, 3H), 1.81−1.86 (m, 2H). LC/MS: 341.2 (M + 1).

3,5-Dimethyl-4-hydroxy-5-isobutyl-5*H*-thiophen-2-one (10). Yield 38%. ^{1}H NMR (acetone-d_6) δ 0.86 (d, J = 6.3 Hz, 3H), 0.92 (d, J = 6.6 Hz, 3H), 1.54 (s, 3H), 1.59 (s, 3H), 1.76−1.80 (m, 2H), 2.07−2.09 (m, 1H). LC/MS: 201.1 (M + 1), 223.1 (M + Na^+).

3,5-Dimethyl-5-(2-ethylbutyl)-4-hydroxy-5*H*-thiophen-2-one (11). Yield 45%. ^{1}H NMR ($CDCl_3$) δ 0.77−0.90 (m, 6H), 1.20−1.44 (m, 5H), 1.65 (s, 3H), 1.73 (s, 3H), 1.78−1.82 (m, 2H). LC/MS: 229.1 (M + 1), 251.1 (M + Na^+).

3,5-Dimethyl-4-hydroxy-5-isopropyl-5*H*-thiophen-2-one (12). Yield 61%. ^{1}H NMR (acetone-d_6) δ 0.81 (d, J = 6.6 Hz, 6H), 1.61 (m, 6H), 2.07−2.10 (m, 1H). LC/MS: 187.0 (M + 1), 209.1 (M + Na^+).

3,5-Dimethyl-4-hydroxy-5-(1-methylpropyl)-5*H*-thiophen-2-one (13). Yield 36%. ^{1}H NMR (acetone-d_6) δ 0.82−0.97 (m, 5H), 1.03 (d, J = 6.6 Hz, 3H), 1.67 (s, 3H), 1.69 (d, J = 3.9 Hz, 3H), 1.93−2.01 (m, 1H). LC/MS: 200.9 (M + 1), 223.0 (M + Na^+), 423.1 (2M + Na^+).

3,5-Dimethyl-4-hydroxy-5-((*S*)-2-methylbutyl)-5*H*-thiophen-2-one (14). Yield 29%. ^{1}H NMR ($CDCl_3$) δ 0.81−0.88 (m, 4.5H), 0.96 (d, J = 6.9 Hz, 1.5H), 1.08−1.50 (m, 3H), 1.60 (s, 3H), 1.68−1.80 (m, 1H), 1.74 (s, 3H), 1.86−1.97 (m, 1H). LC/MS: 215.2 (M + 1), 237.1 (M + Na^+).

5-Cyclopropylmethyl-3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (15). Yield 71%. ^{1}H NMR ($CDCl_3$) δ 0.11−0.20 (m, 2H), 0.39−0.54 (m, 2H), 0.75−0.85 (m, 1H), 1.70 (s, 3H), 1.75 (s, 3H), 1.96 (d, J = 6.0 Hz, 1H), 2.01 (d, J = 6.3 Hz, 1H). LC/MS: 199.0 (M + 1), 221.1 (M + Na^+).

5-Cyclopentyl-3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (16). Yield 57% ^{1}H NMR (acetone-d_6) δ 1.49−1.71 (m, 8H), 1.65 (s, 3H), 1.67 (s, 3H), 1.92−1.97 (m, 1H). LC/MS: 213.1 (M + 1), 235.0 (M + Na^+).

5-Cyclohexyl-3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (17). Yield 9%. ^{1}H NMR ($CDCl_3$) δ 1.02−1.36 (m, 4H), 1.43−1.86 (m, 6H), 1.68 (s, 3H), 1.71 (s, 3H), 1.92−2.00 (m, 1H). LC/MS: 227.1 (M + 1), 248.9 (M + Na^+).

5-Allyl-3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (18). Yield 95%. ^{1}H NMR ($CDCl_3$) δ 1.66 (s, 3H), 1.73 (s, 3H), 2.63 (m, 2H), 5.11−5.20 (m, 2H), 5.75 (ddt, J = 17.1, 10.2, 7.2 Hz, 1H). LC/MS: 185.1 (M + 1), 206.9 (M + Na^+).

5-((*E*)-But-2-enyl)-3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (19). Yield 96%. ^{1}H NMR (acetone-d_6) δ 1.58 (s, 3H), 1.61−1.64 (m, 6H), 2.46−2.62 (m, 2H), 5.35−5.45 (m, 1H), 5.50−5.59 (m, 1H). LC/MS: 199.0 (M + 1), 221.1 (M + Na^+).

3,5-Dimethyl-4-hydroxy-5-(3-methylbut-2-enyl)-5*H*-thiophen-2-one (20). Yield 77%. ^{1}H NMR (acetone-d_6) δ 1.61−1.68 (m, 12H), 2.43−2.53 (m, 1H), 2.64−2.71 (m, 1H), 5.11−5.15 (m, 1H). LC/MS: 213.1 (M + 1), 235.0 (M + Na^+).

3,5-Dimethyl-4-hydroxy-5-(2-methylallyl)-5*H*-thiophen-2-one (21). Yield 48%. ^{1}H NMR (acetone-d_6) δ 1.63 (s, 3H), 1.66 (s, 3H), 1.78 (s, 2H), 2.60−2.72 (m, 3H), 4.80 (bs, 1H), 4.88 (bs, 1H). LC/MS: 199.1 (M + 1), 221.0 (M + Na^+).

3,5-Dimethyl-4-hydroxy-5-(pent-4-enyl)-5*H*-thiophen-2-one (22). Yield 77%. ^{1}H NMR (acetone-d_6) δ 1.24−1.34 (m, 2H), 1.64 (s, 3H), 1.67 (s, 3H), 1.85−1.92 (m, 4H), 4.90−4.95 (m, 1H), 4.96−5.03 (m, 1H), 5.79 (ddt, J = 17.1, 10.5, 6.6 Hz, 1H). LC/MS: 213.1 (M + 1).

3,5-Dimethyl-4-hydroxy-5-(2-methylbut-3-enyl)-5*H*-thiophen-2-one (23). To a solution of **2** (500 mg, 3.47 mmol) in THF (25 mL) was added dropwise 1.0 M LiHMDS (7.3 mL, 7.3 mmol) in THF at 0 °C under an argon atmosphere. The reaction mixture was stirred at 0 °C for 45 min. A solution of 2-methyl-3-butenyl methanesulfonate[49,50] (854 mg, 5.20 mmol) and NaI (779 mg, 5.20 mmol) in THF (5 mL) was prepared separately. To this solution, the above prepared dianion solution of **2** was added by cannula at 0 °C. The reaction mixture was stirred at room temperature for 1 h and then heated at 40−50 °C overnight. The reaction mixture was quenched with H_2O, acidified with 1 N HCl (aq), and extracted with EtOAc (1×). The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. The crude mixture was chromatographed on silica gel (EtOAc/hexanes = 1:1) to give the title compound as a colorless oil (60 mg, 0.28 mmol, 8%) in addition to recovered **2** (42 mg, 0.29 mmol). ^{1}H NMR ($CDCl_3$) δ 1.00 (d, J = 6.6 Hz, 1.5H), 1.01 (d, J = 6.6 Hz, 1.5H), 1.66 (s, 1.5H), 1.68 (s, 1.5H), 1.69 (s, 1.5H), 1.76 (s, 1.5H), 1.85−2.09 (m, 2H), 2.25−2.43 (m, 1H), 4.83−4.98 (m, 2H), 5.58−5.82 (m, 1H). LC/MS: 213.1 (M + 1), 235.0 (M + Na^+).

3,5-Dimethyl-5-(hex-5-enyl)-4-hydroxy-5*H*-thiophen-2-one (24). Yield 84%. ^{1}H NMR (acetone-d_6) δ 1.19−1.57 (m, 6H), 1.63 (s, 3H), 1.65 (s, 3H), 1.83−1.95 (m, 2H), 4.90 (m, 1H), 4.98 (m, 1H), 5.79 (ddt, J = 17.4, 10.5, 6.6 Hz, 1H). LC/MS: 227.1 (M + 1), 249.1 (M + Na^+).

3,5-Dimethyl-5-((2*E*,6*E*)-3,7-dimethylocta-2,6-dienyl)-4-hydroxy-5*H*-thiophen-2-one (25). Yield 89%. ^{1}H NMR ($CDCl_3$) δ 1.66 (m, 15H), 1.98−2.12 (m, 4H), 2.51 (dd, J = 13.2, 6.6 Hz, 1H), 2.67 (dd, J = 15.9, 8.1 Hz, 1H), 5.00−5.13 (m, 1H), 5.14−5.20 (m, 1H). LC/MS: 281.1 (M + 1), 303.0 (M + Na^+).

3,5-Dimethyl-4-hydroxy-5-((2*E*,6*E*,10*E*)-3,7,11-trimethyldodeca-2,6,10-trienyl)-5*H*-thiophen-2-one (26). Yield 97%. ^{1}H NMR (acetone-d_6) δ 1.56−1.68 (m, 18H), 1.97−2.09 (m, 10H), 5.08−5.19 (m, 3H). LC/MS: 349.1 (M + 1), 371.2 (M + Na^+).

5-Benzyl-3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (27). Yield 64%. ^{1}H NMR (acetone-d_6) δ 1.60 (s, 3H), 1.67 (s, 3H), 3.20 (s, 2H), 7.22−7.27 (m, 5H). LC/MS: 235.1 (M + 1), 257.0 (M + Na^+).

3,5-Dimethyl-4-hydroxy-5-((*E*)-3-phenylallyl)-5*H*-thiophen-2-one (28). Yield 60%. ^{1}H NMR ($CDCl_3$) δ 1.71 (s, 3H), 1.72 (s, 3H), 2.70−2.85 (m, 2H), 6.15 (dt, J = 15.6, 7.5 Hz, 1H), 6.51 (d, J = 15.6 Hz, 1H), 7.22−7.34 (m, 5H). LC/MS: 261.1 (M + 1), 283.0 (M + Na^+).

5-(2,4-Dichlorobenzyl)-3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (29). Yield 31%. ^{1}H NMR (acetone-d_6) δ 1.65 (s, 3H), 1.77 (s, 3H), 3.30 (d, J = 14.1 Hz, 1H), 3.54 (d, J = 14.1 Hz, 1H), 7.26−7.35 (m, 2H), 7.46 (d, J = 1.8 Hz, 1H). LC/MS: 304.0 (M + 1), 325.9 (M + Na^+).

3,5-Dimethyl-4-hydroxy-5-(4-trifluoromethylbenzyl)-5*H*-thiophen-2-one (30). Yield 69%. ^{1}H NMR ($CDCl_3$) δ 1.58 (s, 3H), 1.72 (s, 3H), 3.18−3.26 (m, 1H), 3.37−3.47 (m, 1H), 7.27−7.36 (m, 2H), 7.49−7.61 (s, 2H). LC/MS: 303.0 (M + 1), 325.0 (M + Na^+).

5-(3,4-Dichlorobenzyl)-3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (31). Yield 39%. ^{1}H NMR (acetone-d_6) δ 1.59 (s, 3H), 1.73 (s, 3H), 3.19 (d, J = 13.5 Hz, 1H), 3.29 (d, J = 13.8 Hz, 1H), 7.23 (dd, J = 8.1, 1.8 Hz, 1H), 7.44−7.46 (m, 2H). LC/MS: 304.0 (M + 1), 325.9 (M + Na^+).

(*R*)-3,5-Dimethyl-4-hydroxy-5-((*E*)-2-methylbut-1-enyl)-5*H*-thiophen-2-one (32). To a solution of TLM (55 mg, 0.26 mmol) in EtOH (3 mL) was added NH_2NH_2·H_2O (0.50 mL, 10 mmol) at 0 °C. To this mixture 30% H_2O_2 (aq) (1.0 mL) was added dropwise at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h and acidified with 6 N HCl (aq) at 0 °C to pH 2. The reaction mixture was diluted with H_2O and extracted with EtOAc (3×). The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. Chromatographic separation on silica gel (EtOAc/hexanes = 1/9 to 1/6) gave the title compound (18 mg, 0.085 mmol, 33%) as a white solid in addition to a 5:1 (in LC/MS) mixture of **32** and **34** (9.3 mg, 0.037 mmol, 14%). $[\alpha]_D^{20}$ = +63 (*c* 0.72, MeOH). mp 103−105 °C. ^{1}H NMR ($CDCl_3$) δ 1.00 (t, J = 7.5 Hz, 3H), 1.60 (d, J = 1.2 Hz, 3H), 1.76 (s, 3H), 1.81 (s, 3H), 2.03 (q, J = 7.5 Hz, 2H), 5.27 (q, J = 1.2 Hz, 1H), 7.56 (bs, 1H). HRMS (ESMS) calcd for $C_{11}H_{16}O_2S$ [M + Na^+] 235.0769, found 235.0765.

(*R*)-3,5-Dimethyl-4-hydroxy-5-(2-methylbutyl)-5*H*-thiophen-2-one (34). To a solution of TLM (52 mg, 0.24 mmol) in EtOH (3 mL) was added NH_2NH_2·H_2O (3.0 mL, 60 mmol) at 0 °C. To this mixture 30% H_2O_2 (aq) (6.0 mL) was added dropwise at 0 °C. The reaction mixture was stirred at room temperature for 7 h. To this reaction mixture was added again NH_2NH_2·H_2O (3.0 mL, 60 mmol) and 30% H_2O_2 (aq) (6.0 mL) at 0 °C. The reaction mixture was stirred at room temperature overnight and acidified with 6 N HCl (aq) at 0 °C to pH = 2. The reaction mixture was diluted with H_2O, and extracted with EtOAc (3×). The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. Chromatographic separation on silica gel (EtOAc/hexanes = 1/9 to 1/7) gave the title compound (10 mg, 0.048 mmol, 20%) as a slightly yellow oil in addition to a 1:1 (in LC/MS) mixture of **32** and **34** (12 mg, 0.028 mmol, 12%). ^{1}H NMR ($CDCl_3$) δ 0.80−0.88 (m, 4.5H), 0.95 (d, J = 6.6 Hz, 1.5H), 1.09−1.50 (m, 3H), 1.66 (s, 3H), 1.69−1.80 (m, 1H), 1.74 (s, 3H), 1.86−1.97 (m, 1H). HRMS (ESMS) calcd for $C_{11}H_{18}O_2S$ [M + H^+] 215.1106, found 215.1103.

(*R*)-3,5-Dimethyl-4-hydroxy-5-((*E*)-4-hydroxy-2-methylbut-1-enyl)-5*H*-thiophen-2-one (35). To a solution of TLM (503 mg, 2.39 mmol) in THF (50 mL) was added dropwise 0.5 M 9-BBN (40.0 mL, 20.0 mmol) in THF for 50 min. The reaction mixture was stirred at room temperature for 6 h. To this mixture, 3 M NaOH (aq) (8.4 mL) and 30% H_2O_2 (aq) (4.2 mL) were added sequentially at 0 °C. The reaction mixture was stirred at room temperature for 10 min and concentrated. Chromatographic separation on silica gel (EtOAc/hexanes = 2/1 to MeOH/EtOAc = 1/10) gave the title compound as a colorless oil (478 mg, 2.10 mmol, 88%). $[\alpha]_D^{20}$ = +70 (*c* 0.56, MeOH). ^{1}H NMR (MeOH-d_4) δ 1.60 (d, J = 0.9 Hz, 3H), 1.66 (s, 3H), 1.73 (s, 3H), 2.22 (t, J = 6.6 Hz, 2H), 3.64 (t, J = 6.6 Hz, 2H), 5.37 (q, J = 1.2 Hz, 1H). ^{13}C NMR (MeOH-d_4) δ 8.0, 16.8, 30.7, 44.7, 57.1, 61.1, 108.6, 127.2, 140.1, 187.6, 198.1. Anal. ($C_{11}H_{16}O_3S$·0.5H_2O) C, H.

(*R*)-3,5-Dimethyl-4-hydroxy-5-(4-hydroxy-2-methylbutyl)-5*H*-thiophen-2-one (36). To a solution of **35** (370 mg, 1.62 mmol) in EtOH (50 mL) was added NH_2NH_2·H_2O (75 mL, 1.5 mol) at room temperature. To this mixture 30% H_2O_2 (aq) (130 mL) was added dropwise at 0 °C for 50 min. The reaction mixture was stirred at room temperature overnight, concentrated to a half, and acidified with 6 N HCl (aq) (90 mL) at 0 °C. The reaction mixture was extracted with EtOAc (3×). The combined organic layers were dried with anhydrous $MgSO_4$ and concentrated. Chromatographic separation on silica gel (EtOAc/hexanes = 2/3 to 1/1)) gave the title compound as a colorless oil (87.3 mg, 0.378 mmol, 23%) in addition to a 1:10 (in LC/MS) mixture of **35** and **36** (98.4 mg, 0.428 mmol, 26.4%). ^{1}H NMR ($CDCl_3$) δ 1.13 (d, J = 6.6 Hz, 3H), 1.37−2.07 (m, 4H), 1.77 (s, 3H), 1.85 (s, 3H), 2.28 (dd, J = 14.7, 5.1 Hz, 1H), 3.75−3.97 (m, 2H). HRMS (ESMS) calcd for $C_{11}H_{18}O_3S$ [M + H^+] 231.1049, found 231.1052.

(*R*)-2,4-dimethyl-2-(4-methanesulfonyloxy-2-methylbutyl)-5-oxo-2,5-dihydrothiophen-3-yl methanesulfonate (37). To a solution of **36** (35.3 mg, 0.153 mmol) and Et_3N (0.10 mL, 0.72 mmol) in CH_2Cl_2 (5 mL) was added MsCl (0.050 mL, 0.65 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 3 h, acidified with 1 N HCl (aq), and extracted with EtOAc (2×). The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. Chromatographic separation on silica gel (EtOAc/hexanes = 3/1) gave the title compound as a colorless oil (25 mg, 0.064 mmol, 42%). ^{1}H NMR ($CDCl_3$) δ 0.98 (d, J = 6.3 Hz, 3H), 1.56−2.10 (m, 5H), 1.69 (s, 3H), 1.93 (s, 3H), 3.01 (s, 3H), 3.38 (s, 3H), 4.18−4.33 (m, 2H). HRMS (ESMS) calcd for $C_{13}H_{22}O_7S_3$ [M + Na^+] 409.0425, found 409.0426.

(*R*)-2,4-dimethyl-2-(4-iodo-2-methylbutyl)-5-oxo-2,5-dihydrothiophen-3-yl methanesulfonate (38). A solution of **37** (36.9 mg, 0.0955 mmol) and NaI (79.0 mg, 0.527 mmol) in acetone (10 mL) was refluxed for 4 h. The reaction mixture was filtered, concentrated, and purified by preparative TLC (EtOAc/hexanes = 1/2) to give the title compound as a slightly yellow oil (26.2 mg, 0.0626 mmol, 65.6%). ^{1}H NMR ($CDCl_3$) δ 0.99 (d, J = 6.3 Hz, 3H), 1.52−1.96 (m, 5H), 1.71 (s, 3H), 1.93 (s, 3H), 3.07−3.15 (m, 1H), 3.20−3.28 (m, 1H), 3.38 (s, 3H). HRMS (ESMS) calcd for $C_{12}H_{19}IO_4S_2$ [M + H^+] 418.9848, found 418.9847.

3,5-Dimethyl-4-hydroxy-5-(2-methylbut-3-enyl)-5*H*-thiophen-2-one (33). To a solution of **38** (20 mg, 0.048 mmol) in CH_2Cl_2 (3 mL) was added 1.0 M *t*-BuOK (1.0 mL, 1.0 mmol) in THF at room temperature. The reaction mixture was stirred at room temperature for 30 min, quenched with 1 N HCl (aq), and extracted with EtOAc (2×). The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. The crude mixture was chromatographed on silica gel (EtOAc/hexanes = 1/2) to give the title compound as a colorless oil (5.0 mg, 0.024 mmol, 50%). ^{1}H NMR ($CDCl_3$) δ 1.00 (d, J = 6.6 Hz, 1.5H), 1.01 (d, J = 6.6 Hz, 1.5H), 1.65 (s, 1.5H), 1.66 (s, 1.5H), 1.67 (s, 1.5H), 1.74 (s, 1.5H), 1.80−2.17 (m, 2H), 2.28−2.43 (m, 1H), 4.82−5.00 (m, 2H), 5.57−5.83 (m, 1H). HRMS (ESMS) calcd for $C_{11}H_{16}O_2S$ [M + H^+] 213.0949, found 213.0946.

3,5-Dimethyl-4-hydroxy-5-hydroxymethyl-5*H*-thiophen-2-one (39). To a solution of **2** (1.0 g, 6.9 mmol) in THF (60 mL) was added 1.0 M LiHMDS (14.6 mL, 14.6 mmol) in THF at 0 °C under an argon atmosphere. The reaction mixture was stirred for 30 min at 0 °C. To this reaction mixture $(CH_2O)_n$ (329 mg, 10.4 mmol) was added at 0 °C. The reaction mixture was stirred overnight at room temperature, quenched with H_2O, acidified with 6 N HCl (aq), and extracted with EtOAc (2×). The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. The crude mixture was chromatographed on silica gel (EtOAc/hexanes = 2/1) to give the title compound as a white solid (360 mg, 2.07 mmol, 30%). mp 136−138 °C. ^{1}H NMR (MeOH-d_4) δ 1.60 (s, 3H), 1.68 (s, 3H), 3.77 (s, 2H). LC/MS: 175.0 (M + 1).

3,5-Dimethyl-4-methoxymethoxy-5*H*-thiophen-2-one (41). To a solution of **2** (5.0 g, 35 mmol) in CH_2Cl_2 (150 mL) was added

DIPEA (7.3 mL, 41 mmol) at room temperature. To this solution MOMCl (3.2 mL, 42 mmol) was added dropwise at 0 °C. The reaction mixture was stirred at 0 °C for 30 min, quenched with water, and concentrated. The aqueous layer was extracted with EtOAc (2×). The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$, filtered, and concentrated. The crude mixture was chromatographed on silica gel (EtOAc/hexanes = 1/2) to give the title compound as a slightly yellow oil (5.5 g, 29 mmol, 84%). ^{1}H NMR ($CDCl_3$) δ 1.59 (d, J = 7.2 Hz, 3H), 1.83 (d, J = 1.2 Hz, 3H), 3.51 (s, 3H), 4.23 (qq, J = 7.2, 1.2 Hz, 1H), 5.12 (d, J = 6.6 Hz, 1H), 5.30 (d, J = 6.3 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 8.8, 20.0, 42.1, 57.2, 95.5, 117.0, 176.2, 196.1. HRMS (ESMS) calcd for $C_8H_{12}O_3S$ [M + Li^+] 195.0662, found 196.0669.

3,5-Dimethyl-5-hydroxymethyl-4-methoxymethoxy-5*H*-thiophen-2-one (42). To a solution of **41** (1.88 g, 9.99 mmol) in THF (50 mL) was added 1.0 M LiHMDS (11.2 mL, 11.2 mmol) in THF at 0 °C under an argon atmosphere. The reaction mixture was stirred for 30 min at 0 °C. To this reaction mixture $(CH_2O)_n$ (377 mg, 11.9 mmol) was added at 0 °C. The reaction mixture was stirred for 30 min at room temperature, quenched with H_2O, and extracted with EtOAc (1×). The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. The crude mixture was chromatographed on silica gel (EtOAc/hexanes = 3/2) to give the title compound as a colorless oil (1.70 g, 7.79 mmol, 78%). ^{1}H NMR ($CDCl_3$) δ 1.55 (s, 3H), 1.86 (s, 3H), 2.82 (bs, 1H), 3.50 (s, 3H), 3.63−3.85 (m, 2H), 5.23 (d, J = 6.3 Hz, 1H), 5.29 (d, J = 6.3 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 9.4, 22.0, 57.3, 59.8, 67.0, 96.2, 114.3, 175.7, 195.5. HRMS (ESMS) calcd for $C_9H_{14}O_4S$ [M + Li^+] 225.0768, found 225.0774. Anal. ($C_9H_{14}O_4S$) C, H.

2,4-Dimethyl-3-methoxymethoxy-5-oxo-2,5-dihydrothiophene-2-carbaldehyde (43). To a solution of **42** (352 mg, 1.61 mmol) in CH_2Cl_2 (10 mL) was added Dess−Martin periodinane (852 mg, 1.95 mmol) at room temperature. The reaction mixture was stirred for 30 min at room temperature, filtered, and concentrated. The crude mixture was chromatographed on silica gel (EtOAc/hexanes = 1/3 to 1/1) to give the title compound as a colorless oil (225 mg, 1.04 mmol, 65%). ^{1}H NMR ($CDCl_3$) δ 1.70 (s, 3H), 1.95 (s, 3H), 3.45 (s, 3H), 5.23 (dd, J = 2.4, 0.6 Hz, 2H), 9.02 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 9.8, 16.7, 57.4, 63.4, 96.3, 118.0, 172.1, 189.6, 193.5. HRMS (ESMS) calcd for $C_9H_{12}O_4S$ [M + Li^+] 223.0611, found 223.0618.

3,5-Dimethyl-4-methoxymethoxy-5-((*E*)-3-oxobut-1-enyl)-5*H*-thiophen-2-one (44). To a solution of LiCl (49 mg, 1.2 mmol) in CH_3CN (5 mL) was added diethyl (2-oxopropyl)phosphonate (0.23 mL, 1.2 mmol) and DIPEA (0.17 mL, 0.97 mmol) at room temperature. To this mixture was added a solution of **43** (209 mg, 0.966 mmol) in CH_3CN (5 mL) at room temperature. The reaction mixture was stirred for 3 h at room temperature and concentrated. The crude mixture was chromatographed on silica gel (EtOAc/hexanes = 1/6) to give the title compound as a colorless oil (120 mg, 0.47 mmol, 48%) in addition to **41** (33 mg, 0.18 mmol, 18%). ^{1}H NMR ($CDCl_3$) δ 1.79 (s, 3H), 1.93 (s, 3H), 2.26 (s, 3H), 3.48 (s, 3H), 5.25 (dd, J = 8.7, 6.3 Hz, 2H), 6.23 (d, J = 15.9 Hz, 1H), 6.75 (d, J = 15.9 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 9.6, 23.4, 27.7, 56.1, 57.5, 96.2, 114.0, 130.0, 146.5, 175.3, 194.2, 198.1. HRMS (ESMS) calcd for $C_{12}H_{16}O_4S$ [M + Na^+] 279.0662, found 279.0670.

3,5-Dimethyl-5-((*E*)-3-hydroxybut-1-enyl)-4-methoxymethoxy-5*H*-thiophen-2-one (45). To a solution of $CeCl_3 \cdot 7H_2O$ (212 mg, 0.569 mmol) in MeOH (5 mL) was added a solution of **44** (80.9 mg, 0.316 mmol) in MeOH (5 mL) at room temperature. To this mixture was added $NaBH_4$ (14.0 mg, 0.363 mmol) at room temperature. The reaction mixture was stirred for 30 min at room temperature, quenched with water and extracted with EtOAc (2×). The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. The crude mixture was chromatographed on silica gel (EtOAc/hexanes = 1/2) to give the title compound, a mixture of diasteromers, as a colorless oil (52.4 mg, 0.203 mmol, 64%). ^{1}H NMR ($CDCl_3$) δ 1.28 (d, J = 6.6 Hz, 3H), 1.69 (bs, 1H), 1.76 (s, 3H), 1.91 (s, 3H), 3.51 (dd, J = 1.8, 0.6 Hz, 3H), 4.34 (quint, J = 5.4 Hz, 1H), 5.25 (m, 2H), 5.79 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 9.47, 9.48, 23.45, 23.48, 24.26, 24.32, 56.7, 57.36, 57.39, 68.2, 96.1, 113.4, 113.6, 130.8, 130.9, 135.17, 135.21, 176.6, 195.4. HRMS (ESMS) calcd for $C_{12}H_{18}O_4S$ [M + Na^+] 281.0818, found 281.0824.

5-((*E*)-Buta-1,3-dienyl)-3,5-dimethyl-4-methoxymethoxy-5*H*-thiophen-2-one (46). To a solution of **45** (46.8 mg, 0.181 mmol) in toluene (5 mL) was added the Burgess reagent (137 mg, 0.558 mmol) at room temperature. The reaction mixture was stirred at room temperature for 16 h, quenched with H_2O, and then extracted with EtOAc (2×). The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. The crude mixture was chromatographed by preparative TLC (EtOAc/hexanes = 1/5) to give the title compound as a colorless oil (7.0 mg, 0.029 mmol, 16%). ^{1}H NMR ($CDCl_3$) δ 1.80 (s, 3H), 1.93 (s, 3H), 3.50 (s, 3H), 5.12−5.15 (m, 1H), 5.21−5.31 (m, 1H), 5.26 (s, 2H), 5.76−5.85 (m, 1H), 6.25−6.40 (m, 2H). HRMS (ESMS) calcd for $C_{12}H_{16}O_3S$ [M + Na^+] 263.0712, found 263.0719.

5-((*E*)-Buta-1,3-dienyl)-3,5-dimethyl-4-hydroxy-5*H*-thiophen-2-one (47). To a solution of **46** (8.6 mg, 0.036 mmol) in CH_2Cl_2 (1 mL) was added $NaHSO_4 \cdot SiO_2$ (50 mg) at room temperature. The reaction mixture was stirred at room temperature for 3 h, filtered, and concentrated. The crude mixture was chromatographed by preparative TLC (MeOH/EtOAc = 1/7) to give the title compound as a colorless oil (5.3 mg, 0.027 mmol, 75%). ^{1}H NMR ($CDCl_3$) δ 1.59 (s, 3H), 1.63 (s, 3H), 5.00 (dd, J = 9.9, 2.1 Hz, 1H), 5.16 (dd, J = 16.2, 2.7 Hz, 1H), 5.79 (d, J = 14.4 Hz, 1H), 6.19−6.40 (m, 2H). HRMS (ESMS) calcd for $C_{10}H_{12}O_2S$ [M + Li^+] 203.0713, found 203.0720.

(*R*)-3,5-Dimethyl-4-methoxymethoxy-5-((*E*)-2-methylbuta-1,3-dienyl)-5*H*-thiophen-2-one (48). To a solution of TLM (80 mg, 0.38 mmol) in CH_2Cl_2 (3 mL) was added DIPEA (0.24 mL, 1.4 mmol) at room temperature. To this solution MOMCl (0.12 mL, 1.6 mmol) was added dropwise at 0 °C. The reaction mixture was stirred at room temperature for 3 h, quenched with water, and extracted with CH_2Cl_2 (2×). The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and concentrated. The crude mixture was chromatographed on silica gel (EtOAc/hexanes = 1/2) to give the title compound as a colorless oil (92 mg, 0.36 mmol, 95%). ^{1}H NMR ($CDCl_3$) δ 1.74 (s, 3H), 1.82 (s, 3H), 1.94 (s, 3H), 3.50 (s, 3H), 5.04 (d, J = 10.8 Hz, 1H), 5.22 (d, J = 17.4 Hz, 1H), 5.28 (s, 2H), 5.63 (s, 1H), 6.29 (dd, J = 17.4, 10.8 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 9.4, 12.3, 30.2, 55.2, 57.3, 96.0, 113.0, 113.5, 131.0, 139.2, 141.1, 177.5, 195.8. HRMS (ESMS) calcd for $C_{13}H_{18}O_3S$ [M + Na^+] 277.0869, found 277.0859.

(*R*)-3,5-Dimethyl-4-methoxymethoxy-5-((1*E*,3*E*)-2-methylpenta-1,3-dienyl)-5*H*-thiophen-2-one (49). To a solution of **48** (44 mg, 0.17 mmol) and Grubbs' II catalyst (9.1 mg, 6 mol %) in CH_2Cl_2 (3 mL) was added *trans*-2-butene (105 mg) at −78 °C in a sealed tube. The reaction mixture was stirred at 45 °C for 1 day. The crude mixture was chromatographed on silica gel (EtOAc/hexanes = 1/10 to 1/4) to give the title compound as a colorless oil (31 mg, 0.12 mmol, 66%). ^{1}H NMR ($CDCl_3$) δ 1.72 (s, 3H), 1.76 (d, J = 6.6 Hz, 3H), 1.94 (s, 3H), 3.51 (s, 3H), 5.28 (s, 2H), 5.48 (s, 1H), 5.72 (dq, J = 15.9, 6.6 Hz, 1H), 6.01 (m, 1H). HRMS (ESMS) calcd for $C_{14}H_{20}O_3S$ [M + Na^+] 291.1025, found 291.1019.

(*R*)-3,5-Dimethyl-4-hydroxy-5-((1*E*,3*E*)-2-methylpenta-1,3-dienyl)-5*H*-thiophen-2-one (50). To a solution of **49** (24 mg, 0.17 mmol) in MeOH (2 mL) was added silica gel (180 mg) and polymer-bound TsOH (46 mg, 0.092 mmol) at room temperature. The reaction mixture was stirred at room temperature overnight. The reaction mixture was filtered and concentrated to give the title compound as a colorless oil (15 mg, 0.067 mmol, 75%). $[\alpha]_D^{20}$ = +117 (*c* 0.59, MeOH). ^{1}H NMR ($CDCl_3$) δ 1.68 (d, J = 1.2 Hz, 3H), 1.69 (s, 3H), 1.77 (dd, J = 6.6, 1.2 Hz, 3H), 1.79 (s, 3H), 5.46 (s, 1H), 5.77 (dq, J = 15.3, 6.6 Hz, 1H), 6.07 (m, 1H). HRMS (ESMS) calcd for $C_{12}H_{16}O_2S$ [M + Na^+] 247.0763, found 247.0772.

Condensing Enzyme Assays. Every compound was tested against five condensing enzymes, FabH and FabB from *E. coli* (ecFabH and ecFabB); FabH, KasA and KasB from *M. tuberculosis* (mtFabH, KasA, and KasB). The compounds were dissolved in 20% DMSO and diluted 10-fold in the reaction to the indicated

concentrations. Enzymatic activity of the reactions treated with the solvent was used as controls.

ecFabH Assay. The coupled assay of FabH, as described previously,[51] contained 25 μM ACP, 1 mM β-mercaptoethanol, 65 μM malonyl-CoA, 45 μM [1-^{14}C]-acetyl-CoA (specific activity 60 μCi/μmol), 1 μg of purified FabD, 0.1 M sodium phosphate buffer, pH 7.0, and 10 ng of FabH protein in a final volume of 40 μL. The FabD protein was present to generate the malonyl-ACP substrate for the reaction. The ACP, β-mercaptoethanol, and buffer were preincubated at 37 °C for 30 min to ensure the complete reduction of ACP. The reaction was initiated by the addition of FabH. After incubation of the sample at 37 °C for 15 min, 35 μL of reaction mixture was removed and dispensed onto a paper filter disk (Whatman No. 3MM filter paper). The disk was washed successively with ice cold 10, 5, and 1% trichloroacetic acid with 20 min for each wash and 20 mL of wash solution per disk. The filter disks were dried and counted for ^{14}C-isotope in 3 mL of scintillation fluid.

mtFabH Assays. The filter disk assay developed for ecFabH[51] was modified to measure the activity of mtFabH with [1-^{14}C]-lauroyl-CoA (specific activity, 55 μCi/μmol). The assays contained 50 μM ACP, 1 mM β-mercaptoethanol, 0.1 M sodium phosphate buffer pH 7.0, 50 μM malonyl-CoA, 12.5 μM [1-^{14}C]-lauroyl-CoA, 1 μg *E. coli* FabD and 40 ng of mtFabH in a final volume of 40 μL. ACP, β-mercaptoethanol, and the buffer was incubated at 37 °C for 30 min to ensure complete reduction of ACP before the remaining components (except mtFabH) were added. The mixture was then aliquoted into the assay tubes, and the reaction was initiated by the addition of mtFabH. After incubation at 37 °C for 40 min, 35 μL of the reaction mixture was removed and deposited on a Whatman DE-81 filter disk. The disks were washed three times (20 min for each wash, 20 mL wash solution for each disk) with ice-cold chloroform/methanol/acetic acid (2:5:3, v/v/v) containing 0.2 M LiCl. The filters were dried and ^{14}C-isotope was counted in 3 mL of scintillation fluid.

ecFabB, KasA and KasB Assay. The condensation assay was essentially the same as described previously.[15] Briefly, condensing enzyme assays contained 45 μM acyl-ACP (myristoyl-ACP for ecFabB, palmitoyl-ACP for KasA and KasB), 50 μM [2-^{14}C]-malonyl-CoA (specific activity, 52 μCi/μmol), 100 μM ACP, 1 μg of FabD and condensing enzyme (25 ng of ecFabB, 500 ng of KasA, or 500 ng of KasB) in a final volume of 20 μL. ACP was reduced by 0.3 mM DTT before the other reaction components were added. The reaction was initiated by the addition of the enzyme. After incubation of the sample at 37 °C for 15 min, the reaction was stopped by adding 0.4 mL of the reducing reagent containing 0.1 M K_2HPO_4, 0.4 M KCl, 30% tetrahydrofuran, and 5 mg/mL $NaBH_4$. The reaction contents were vigorously mixed after the addition of the reducing reagent and incubated at 37 °C for 40 min before being extracted into 0.4 mL toluene. The ^{14}C-isotope in the upper phase was quantitated by scintillation counting.

***M. tuberculosis* MIC Assay.** A stock culture of *M. tuberculosis* H37Rv was grown to OD 0.5 in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80, 0.2% glycerol and albumin/NaCl/glucose (ADC) complex. The culture was diluted 1:1000 in 7H9-based medium before aliquoting 50 μL into each well of a 96-well plate. The inhibitors were dissolved in DMSO to make stock solutions of 50 μmol/mL. Inhibitors were added to the first row of wells of the 96-well plate with 100 μL 7H9-based medium. After pipet mixing and using a multichannel pipet, 50 μL was removed from each well in the first row and added to the second row. 2-Fold dilution in this manner was carried out to give eight dilutions of each inhibitor (1000 μM-7.8 μM). The plates were incubated for 2 weeks at 37 °C and were read macroscopically using an inverted plate reader. Each measurement was made three independent times.

***E. coli* MIC Assay.** Strain ANS1 (*metB1 relA1 spoT1 gyrA216 tolC::*Tn*10* λ^- λ^r F$^-$) was defective in TolC-dependent efflux pumps and was used to determine the minimal inhibitory concentrations.[15] The MICs of the test compounds against *E. coli* strain ANS1 were determined by a broth microdilution method. ANS1 was grown to mid-log phase in 1% tryptone broth and then diluted 30 000-fold in the same medium. A 10 μL aliquot of the diluted cell suspension (3000 to 5000 colony forming units) was used to inoculate each well of a 96-well plate (U-bottom with low evaporation lid) containing 100 μL of tryptone broth with the indicated concentration of inhibitors. The plate was incubated at 37 °C for 20 h before being read with a Fusion Universal Microplate analyzer (Packard, Canada) at 600 nm. The absorbance was normalized to the solvent treated control which was considered as 100%.

Crystallization and Data Measurement. *N*-terminal His-tagged FabB was expressed, purified, and crystallized as previously described.[12] For cocrystallization, 14 mg/mL of protein was incubated with 1 mM of compound **47** or **50,** and drops were set up at 18 °C over a well solution containing 1.9 M ammonium sulfate, 0.1 M HEPES pH 7.5, and 2% PEG 400. For compound **47,** X-ray data were measured with a Bruker-Nonius CCD, integrated with SAINT (Bruker AXS), and scaled using PROSCALE (Bruker AXS). For compound **50,** data were measured at the Southeastern Regional Collaborative Access Team (SER−CAT) beamline 22ID at the Advanced Photon Source, integrated using MOSFLM[52] and scaled using SCALA.[53]

Model Building and Refinement. Both **47**−FabB and **50**−FabB structures were solved by molecular replacement using AMoRe.[54] The protein model from the known TLM−FabB crystal complex (PDB code 1FJ4) was used as the search model. Iterative rounds of model building using O[55] and refinement using REFMAC[56] were performed. CNS-1.1[57] was used for the B-factor and occupancy refinements.

Acknowledgment. We thank Amy Sullivan and Matt Frank for their excellent technical assistance. Fermentation-derived TLM was obtained as a gift from GlaxoSmithKline, for which we are very grateful. This work was supported by NIH Grant GM 34496 (C.O.R.), Cancer Center (CORE) Support Grant CA 21765 (C.O.R., S.W.W.), the American Lebanese Syrian Associated Charities (C.O.R., S.W.W.), an NIH Challenge Grant 1 UC1 AI49520-01 (J.L., K.D., C.E.B.), and the NIAID Division of Intramural Research (C.E.B.).

Supporting Information Available: Physical and biological data for the complete set of analogues plus elemental analyses for key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM050825P