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Acyl-sulfamates target the essential glycerol-phosphate acyltransferase (PlsY) in Gram-positive bacteria

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ABSTRACT

PlsY is the essential first step in membrane phospholipid synthesis of Gram-positive pathogens. PlsY catalyzes the transfer of the fatty acid from acyl-phosphate to the 1-position of glycerol-3-phosphate to form the first intermediate in membrane biogenesis. A series of non-metabolizable, acyl-sulfamate analogs of the acyl-phosphate PlsY substrate were prepared and evaluated as inhibitors of *Staphylococcus aureus* PlsY and for their Gram-positive antibacterial activities. From this series phenyl (8-phenyloctanoyl) sulfamate had the best overall profile, selectively inhibiting *S. aureus* phospholipid biosynthesis and causing the accumulation of both long-chain fatty acids and acyl–acyl carrier protein intermediates demonstrating that PlsY was the primary cellular target. *Bacillus anthracis* was unique in being more potently inhibited by long chain acyl-sulfamates than other bacterial species. However, it is shown that *Bacillus anthracis* PlsY is not more sensitive to the acyl-sulfamates than *S. aureus* PlsY. Metabolic profiling showed that *B. anthracis* growth inhibition by the acyl-sulfamates was not specific for lipid synthesis illustrating that the amphipathic acyl-sulfamates can also have off-target effects in Gram-positive bacteria. Nonetheless, this study further advances PlsY as a druggable target for the development of novel antibacterial therapeutics, through the discovery and validation of the probe compound phenyl (8-phenyloctanoyl) sulfamate as a *S. aureus* PlsY inhibitor.

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1. Introduction

The creeping rise in antibacterial drug resistance combined with the failure of current discovery programs to produce new antibacterial targets and lead compounds to meet this growing threat is of great concern to the whole medical community.[1] This comes at a time when increasingly virulent and resistant strains of bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) lineage USA300 are becoming dominant in our clinics causing much morbidity and mortality. Current efforts at developing new Gram-positive antibacterial agents are largely focused around standard antibacterial classes including β-lactams, oxazolidinones and fluoroquinolones for which class specific resistance mechanisms preexist.[2] Thus, there is an urgent need to develop new therapeutic targets that are essential for cell viability and suitable for therapeutic intervention.

One such alternative target is the recently discovered PlsX and PlsY mediated phospholipid biosynthetic pathway.[3] PlsX catalyzes the formation of acyl-PO_4 from the acyl–acyl carrier protein (ACP) end-products of fatty acid biosynthesis. PlsY transfers the acyl group from acyl-PO_4 to the 1-position of glycerol-PO_4 to form the first intermediate in membrane phospholipid synthesis. Targeting this pathway appears desirable because it is essential in all significant Gram-positive human pathogens[3] and disrupting bacterial membrane biogenesis is a proven strategy for the development of potent antibacterials.[4] Furthermore, humans synthesize the phospholipids using acyl-CoA-dependent glycerol-PO_4 acyltransferases and PlsY homologs are not found in mammalian genomes. Despite these clear advantages, targeting PlsY presents challenges. PlsY is a small (23 kDa) integral membrane protein with 5 transmembrane helicies.[5] Although the residues critical for catalysis are known, structural information is not available and the enzyme is most active in its native membrane environment. Thus, our first approach to inhibitor design was to synthesize five classes of nonhydrolyzable acyl-PO_4-based bioisosteric inhibitors of PlsY.[6]

Abbreviations: ACP, acyl carrier protein; PlsY, acyl-phosphate:glycerol-3-phosphate acyltransferase; PlsX, acyl-phosphate:ACP transacylase; acyl-PO_4, acyl-phosphate; glycerol-PO_4, *sn*-glycerol-3-phosphate; SAR, structure–activity relationship.

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† These authors contributed equally to the work.

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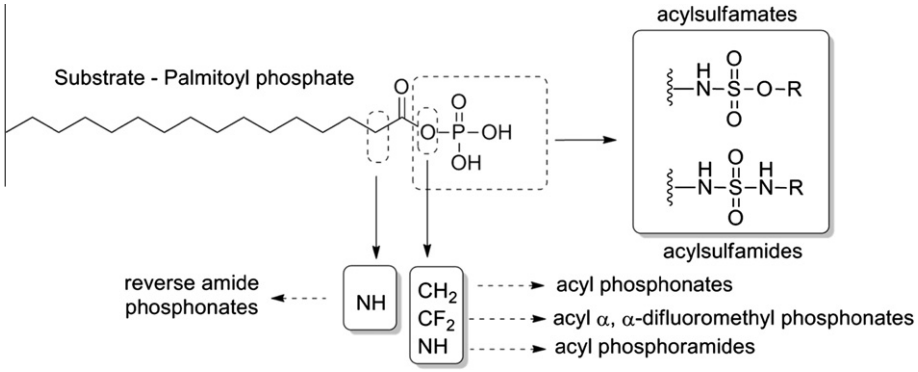
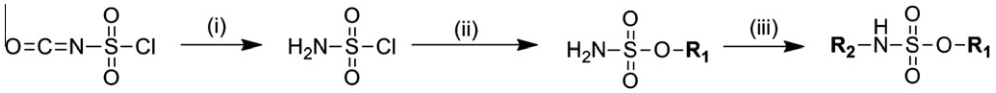


Figure 1. Substrate based inhibitors of PlsY. Palmitoyl-phosphate is a PlsY substrate. Non-hydrolyzable substrate mimics of PlsY were generated by bioisosteric replacement of the phosphate head group. The acyl-sulfamate class was expanded upon in this study.



Scheme 1. Overall synthetic scheme for preparing acyl-sulfamates. (i) HCOOH, neat (ii) R_1OH, DMA (iii) R_2COCl, DMAP, NEt_3, DCM:DMF.

These chemical classes included acyl-phosphonates, acyl-phosphoramides, reverse amide phosphonates, acyl-sulfamates and acyl-sulfamides (Fig. 1). The most active inhibitors from each series generally contained a long aliphatic chain that mimicked the acyl-PO_4 substrate of PlsY. These PlsY inhibitors displayed weak to moderate antimicrobial activity against a representative panel of Gram-positive bacteria, with the exception of *Bacillus anthracis*, for which potent antibacterial activity was observed.

This study expands the SAR and mechanism of action within the acyl-sulfamate class (Fig. 1), the most potent of the bioisosteric PlsY inhibitors.[6] The acyl-sulfamate scaffold was modified at the R_1 (head) and R_2 (tail) positions (Scheme 1) to determine the effect of substitutions on the potency against PlsY and antimicrobial activity. Biochemical analysis of acyl-sulfamate treated cells confirmed that these molecules target the PlsY pathway for phospholipid synthesis, although these amphipathic molecules can also exhibit off-target activities, which need to be carefully monitored to gain a clear understanding of antibacterial structure–activity relationships.

2. Results and discussion

2.1. Chemistry

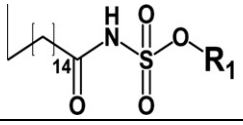
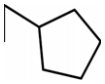
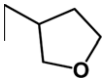
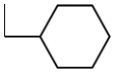
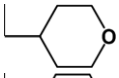
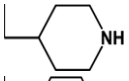
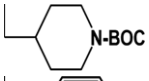
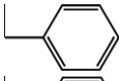
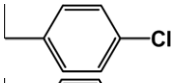
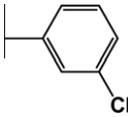
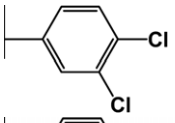
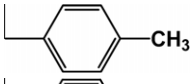
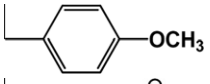
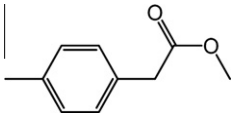
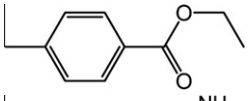
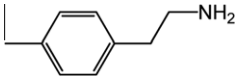
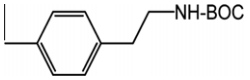
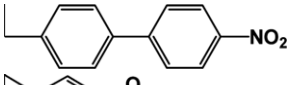
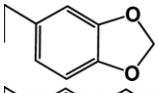
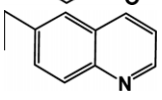
The acyl-sulfamates were synthesized as described previously.[6] Briefly, chlorosulfonyl isocyanate was treated with neat formic acid to generate sulfamoyl chloride that was reacted with various alcohols to synthesize a series of sulfamates introducing the R_1 substitution. Acylation of the sulfamates with selected acyl chlorides provided the desired acylsulfamates introducing the R_2 acyl tail (Scheme 1).

2.2. SAR of acyl-sulfamates against *S. aureus* PlsY

The SAR at the acyl-PO_4 R_1 head site (Scheme 1) was explored by the synthesis of compounds **1**–**21** (Table 1) that maintained a palmitoyl (C16) chain at the R_2 tail position to mimic the natural substrate. The potency of these acyl-sulfamates as PlsY inhibitors was influenced by the size of the R_1 substituent with the larger six membered and bicyclic analogs being generally more potent than the smaller furan (**3**), cyclopentane (**2**) and methyl (**1**) analogs. The potency was also influenced by the hydrophobicity of the R_1 substituent. The more hydrophobic cyclohexane analog **4** was six-fold more potent than the 4-oxygen containing pyran analog **5** and at least twenty-fold more potent than the 4-NH containing piperidine analog **6**. The considerable loss of potency of **6** suggested that a free amine was not tolerated at this site due to a site specific interaction and a potentially strong desolvation penalty of this cation. This was further corroborated by the significant restoration of potency upon protection of the 4-NH of piperidine (**6**) with a Boc-group (**7**). Replacing the saturated and comparatively flexible cyclohexane with an aromatic phenyl group (**8**) led to a minor loss in potency; nevertheless, the availability of various substituted phenols made it the better option for further exploring this site. A 4-Cl substituent on the phenyl ring (**9**) proved to be somewhat better than the unsubstituted **8**. The 3, 4-dichloro analog **11** and the 3-chloro analog **10** were less potent than **9** suggesting that the 4-position on the phenyl was the preferred site for further exploration. We replaced the 4-Cl with substituents of increasing size including methyl (**12**), hydroxymethyl (**13**) methyl propionate (**14**), ethyl acetate (**15**) and 4-(4-NO_2) biphenyl (**18**). Despite the substantial increase in size at the 4-position, the potency was only two-fold or less compared to **9**. The only 4-substitutent from our series that did show a significant loss of potency was the 4-ethylamine analog **16**. This loss was attributed to the presence of the free amine also found in **16** rather than the size because potency was restored in the Boc-protected analog **17**. This trend was similar to the preceding amine containing pair (**6** and **7**). We also synthesized bicyclic analogs **19**–**21** and found that the benzodioxole (**19**) and quinoline (**20**) derivatives had potencies similar to most of the 4-substituted phenols while the benzofuranyl 3-sulfamate (**21**) was four-fold less potent.

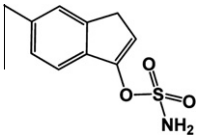
The SAR at R_2 acyl tail position (Scheme 1) was explored by synthesizing derivatives **22–28** that incorporated a phenyl or 4-OCH_3 phenyl at R_1 head (Table 2). The myristoyl (C14) derivative **22** was slightly better than the C16 derivative **13** while further decreasing the chain length to C11 in **23** led to a threefold decrease in potency. Interestingly, a terminal phenyl on the C11 aliphatic chain (**24**) at R_2 was well-tolerated and the compound was equipotent to the palmitoyl analog **8**. Further decreasing the chain length between

Table 1
SAR at the R_1 position

No.		IC_{50}[a] (μM)	MIC[b] (μg/ml)					
		SA-PIsY	SA	SP	EF	BS	BA	
							Median[c]	Range[d]
1	CH_3	80	>200	>200	>200	>200	>100	>100
2		>100	>200	>200	>200	>200	>100	>100
3		45	25	>200	>200	>200	1.56	0.195–6.25
4		5	>200	100	>200	>200	6.25	0.78–12.5
5		30	>200	>200	>200	>200	3.13	0.39–6.25
6		>100	>200	>200	>200	>200	>100	>100
7		15	>200	>200	>200	>200	3.13	0.78–3.13
8		8	>200	>200	50	50	3.13	0.78–6.25
9		6	>200	100	>200	>200	6.25	1.56–12.5
10		14	>200	>200	100	100	3.13	0.78–6.25
11		9	>200	>200	100	100	12.5	6.25–12.5
12		12	>200	>200	200	200	6.25	0.78–12.5
13		8	12.5	12.5	3.12	3.12	3.13	0.78–3.13
14		11	25	0.78	>200	>200	3.13	0.39–12.5
15		10	6.25	1.56	>200	>200	3.13	0.78–3.13
16		>100	>200	>200	>200	>200	>100	>100
17		22	>200	3.13	>200	>200	1.56	0.78–3.13
18		11	100	0.78	>200	>200	1.56	1.56–3.13
19		12	100	3.13	>200	>200	>100	>100
20		9	>200	1.56	200	200	3.13	0.78–3.13

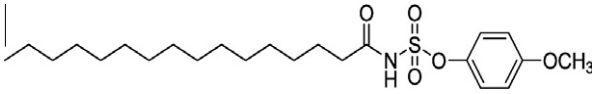
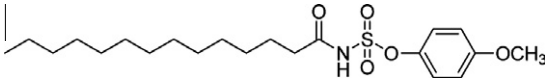
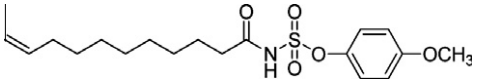
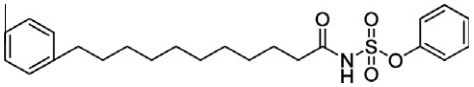
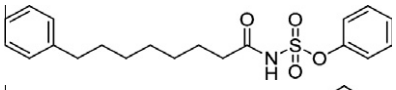
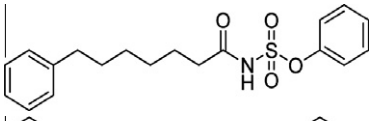
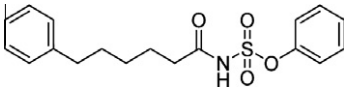
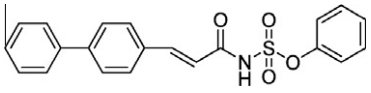
(*continued on next page*)

Table 1 (*continued*)

No.		IC_{50}[a] (μM)	MIC[b] (μg/ml)					
		SA-PIsY	SA	SP	EF	BS	BA	
							Median[c]	Range[d]
21		41	25	3.13	100	100	3.13	1.56–12.5

[a] IC_{50} against *S. aureus* PlsY.
[b] MIC: Whole-cell minimum inhibitory concentration of the following species: SA, *S. aureus* ATCC 29213; SP, *S. pneumoniae* R6; EF, *E. faecalis* ATCC 33186; BS, *B. subtilis* ATCC 23857.
[c] Median: The median MIC value against 22 virulent strains of *B. anthracis* (BA).
[d] Range: The highest and lowest MIC values recorded against the 22 *B. anthracis* strains.

Table 2
SAR at the R_2 position

No.		IC_{50}[a] (μM)	MIC[b] (μg/ml)					
		SA-PIsY	SA	SP	EF	BS	BA	
							Median[c]	Range[d]
13		8	12.5	12.5	12.5	3.12	3.13	0.78–3.13
22		6	>200	>200	6.25	>200	0.78	0.39–1.56
23		27	12.5	200	1.6	6.25	12.5	6.25–25
24		8	6.25	200	12.5	100–200	3.13	3.13
25		25	12.5	100	100	>200	6.25	1.56–6.25
26		76	50	50	100	>200	12.5	12.5
27		100	100	50	50	>200	100	>100
28		>100	25	100	50	>200	100	25–100

[a] IC_{50} against *S. aureus* PlsY.
[b] MIC: Whole-cell minimum inhibitory concentration of the following species: SA, *S. aureus* ATCC 29213; SP, *S. pneumoniae* R6; EF, *E. faecalis* ATCC 33186; BS, *B. subtilis* ATCC 23857.
[c] Median: The median MIC value against 22 virulent strains of *B. anthracis* (BA).
[d] Range: The highest and lowest MIC values recorded against the 22 *B. anthracis* strains.

the terminal phenyl and the head group to C8 (**25**) led to a three-fold decrease in potency while the shorter C7 (**26**) and C6 (**27**) analogs and the cinnamic acid analog (**28**) were at least ten-fold less potent than **24**. Thus, for the compound series **24**–**28**, the potency was directly related to the alkyl chain length at R_2.

2.3. Antimicrobial activity

The synthesized compounds were screened against a representative panel of Gram-positive bacteria including 22 virulent strains of *B. anthracis* (Tables 1 and 2). Palmitoyl (C16) analogs that were modified at the R_1 position (**1**–**21**) and found to be inactive against *S. aureus* PlsY (**1**, **2**, **6** and **16**) were, in general, inactive against our panel of Gram-positive bacteria (Table 1). In contrast to their *S. aureus* PlsY inhibitory activity, most of the palmitoyl analogs were inactive against *S. aureus* in the whole cell assay (Table 1) indicating that additional factors including permeability may be influencing the whole cell activity of these compounds. Exceptions in this case were compounds **15** and **13**, which had low MIC of 6.25 and 12.5 μg/ml, respectively against *S. aureus*. Interestingly, most of the palmitoyl analogs were highly active against *E. faecalis* and *B. anthracis*; however, the MIC values of these compounds were

considerably lower than the corresponding PlsY IC_{50} suggesting that this class of molecules can affect bacterial growth by a non-PlsY mechanism (Table 1). Most of these analogs were generally inactive against *S. pneumoniae* and *B. subtilis* except for **13** which displayed good activity against both (Table 1).

For compounds incorporating a 4-OCH_3 phenyl at the R_1 position, shortening the chain length at R_2 from C16 (**13**) to C14 (**22**) led to significant loss of activity against *S. pneumoniae*, *S. aureus* and *B. subtilis* but caused a slight improvement in activity against *E. feacalis* and *B. anthracis* (Table 2). Further decreasing the chain length to C11 (**23**) led to significant restoration of the *S. aureus* and *B. subtilis* activity, further improvement in the *E. faecalis* activity and a modest decreasing in the *B. anthracis* activity. Thus **23** displayed a broad antimicrobial profile with good activity against multiple species in our panel of Gram-positive bacteria.

For compounds **24–27** that incorporated a phenyl group at the R_1 position and a terminal phenyl group on the R_2 acyl chain, decreasing the chain length between the head group and the terminal phenyl at R_2 led to a proportional decrease in the *S. aureus* inhibitory activity, in similar fashion to their PlsY inhibitory activity (Table 2). The MIC values of **24–27** against *S. aureus* are comparable to their PlsY IC_{50}. A similar trend of increasing MIC with decreasing chain length in **24–27** is also observed for *B. anthracis* (Table 2). Compounds **24–27** were generally inactive against *S. pneumoniae* and *B. subtilis*. The cinnamic acid analog **28** showed moderate to weak activity against *S. aureus* and *E. faecalis* but was generally inactive against the other species (Table 2).

2.4. Target validation

Whether the antibacterial effects of the acyl-sulfamates were due to PlsY inhibition was addressed in a series of metabolic labeling and structural studies. The phenyl (8-phenyloctanoyl) sulfamate **25** was selected for this purpose based on its reasonable PlsY inhibition, good antimicrobial activity against *S. aureus* and *B. anthracis* and comparatively lower molecular weight. Two genetic systems provided the template for assessing the physiological changes that occur after PlsY inhibition. One is in *Bacillus subtilis* where the *plsY* gene was silenced[7] and the second is the *gpsA* mutant of *S. aureus* that conditionally deprives the cells of the glycerol-PO_4 for PlsY.[8,9] Lipid synthesis inhibitors do not cause cell lysis or immediate growth cessation. Rather, bacteria continue to grow for a few generations until the imbalance between membrane lipid and protein synthesis becomes intolerable and the cells cease proliferation.[10] *S. aureus* cell growth did not immediately cease following the addition of **25**, but slowed down over approximately 2 h before stopping completely when treated with 25 μM or greater of **25** (Fig. 2A). This gradual stop to *S. aureus* cell growth was reminiscent of the *gpsA* mutants deprived of glycerol and PlsY knockdown cells.[8,9] Metabolic labeling in the presence and absence of 50 μM **25** was used to assess its effect on the four major pathways of macromolecular biosynthesis (Fig. 2B). Compound **25** selectively inhibited lipid biosynthesis compared to the other major macromolecules. Accordingly, **25** caused a concentration dependent decrease in [^{14}C] acetate incorporation into fatty acids and membrane phospholipids (Fig. 2C). In the *Bacillus subtilis* PlsY conditional gene knockout model, knocking out PlsY causes accumulation of intracellular free fatty acids.[7] An increase in ^{14}C-labeled free fatty acids was also observed in **25** treated cells (not shown), consistent with PlsY inhibition as the in vivo mode of action for **25** against *S. aureus*.

Another metabolic defect associated with a PlsY inhibitor was an increase in the average chain-length of fatty acids in treated cells. The chain-length of membrane fatty acids was determined by the competition between the elongation condensing enzyme (FabF) and the glycerol-PO_4 acyltransferase.[11] If the acyltransferase

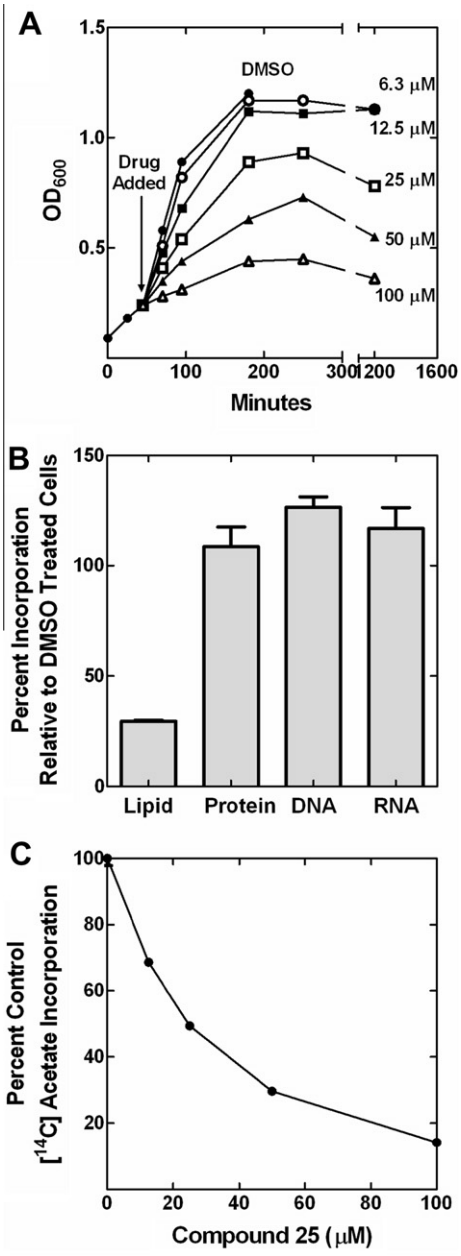


Figure 2. Effect of **25** on the growth and lipid synthesis in *S. aureus* strain RN4220. (A) Growth curves of *S. aureus* strain RN4220 treated with 0 (●), 6.25 (○), 12.5 (■), 25 (□), 50 (▲), or 100 μM (△) of **25**. (B) The effect of **25** on the major biosynthetic pathways was measured in strain RN4220 treated with 50 μM **25** relative to untreated cells. Metabolic labeling with [^{14}C]acetate was used for lipid biosynthesis, a ^{3}H-labeled amino acid mixture was used to measure protein biosynthesis, [^{3}H]thymidine incorporation measured DNA biosynthesis, and [^{3}H]uracil measured stable RNA biosynthesis. (C) The rates of lipid biosynthesis in strain RN4220 treated with 0, 12.5, 25, 50, or 100 μM of **25** were measured by the incorporation of [^{14}C]acetate into the lipid fraction. [^{14}C]Acetate incorporation in the absence of drug was 194,000 cpm per 5×10^9 cells.

was inhibited, then FabF has a competitive advantage and the acyl-ACP end-product undergo additional rounds of elongation leading to an increase in the average chain length in the membrane phospholipids. The major fatty acids in control (DMSO-treated) strain RN4220 were 15:0 and 17:0 with only small amounts of 19:0 and 20:0 chain lengths (Table 3). Treatment with increasing amounts of **25** led to a progressive increase in the chain length of fatty acids produced by the cells. For example, 21-carbon fatty acids were not detected in untreated strain RN4220, but comprised

Table 3
Effect of compound **25** on fatty acid composition

Fatty Acid[a]	DMSO	Compound 25	
		25 μM	50 μM
15:0[b]	43%[c]	37%	32%
16:0	5%	5%	6%
17:0	25%	10%	8%
18:0	11%	9%	12%
19:0	9%	18%	14%
20:0	6%	9%	13%
21:0	n.d.[d]	12%	14%

[a] *S. aureus* strain RN4220 was treated with the indicated concentration of **25** for 2.5 h and the fatty acid composition analyzed as described in the experimental section.
[b] Fatty acid nomenclature X:Y denotes that the fatty acid has X number of total carbons and Y number of double bonds.
[c] Percent values are weight percent from the average of duplicate measures, rounded to the nearest percent.
[d] Not detected, means <1%.

14% of the total fatty acids in *S. aureus* treated with 50 μM **25**. The appearance of these longer chain lengths correlated with a decrease in the 15-carbon fatty acids. Phosphatidylglycerol (PtdGro) was the major membrane phospholipid in *S. aureus*, and mass spectrometry was used to determine how the distribution of PtdGro molecular species was impacted by treatment with **25**. In untreated cells, the major molecular species was the 32-carbon PtdGro consisting of 17:0 in the 1-position and 15:0 in the 2-position (Fig. 3A).[10] In cells treated with 25 μM **25**, the principle molecular species contained 34 carbons, and in cells treated with 50 μM **25**, 35-carbon PtdGro was the most abundant species (Fig. 3B). A 36-carbon molecular species was a major component in cells treated with 50 μM **25**, but was barely detected in untreated cells (Fig. 3C). An increase in the average chain-length of fatty acids in membrane phospholipids was a hallmark feature that points to the acyl-sulfamates targeting PlsY in vivo.

A blockade at the glycerol-PO_4 acyltransferase step should also lead to the accumulation of long chain acyl-ACP, which continues to be produced by the fatty acid biosynthetic pathway, but fails to be utilized due to the block in phospholipid synthesis.[12] Therefore, we examined the composition of the ACP pool in *S. aureus* strain RN4220 treated with various concentrations of **25** (Fig. 4). Acyl-ACPs can be separated by urea gel electrophoresis based on the hydrophobicity of the attached acyl chain[13], and were detected by western blotting using anti-ACP antibody.[14] The ACP pool in untreated cells was primarily non-esterified ACP. Treatment with **25** led to a significant increase in the level of long-chain acyl-ACP in the cells, indicating a block in the utilization of the end products of fatty acid synthesis as acyltransferase substrates. These measurements of the intracellular ACP pool composition strongly support the conclusion that PlsY was a target for **25** in *S. aureus*.

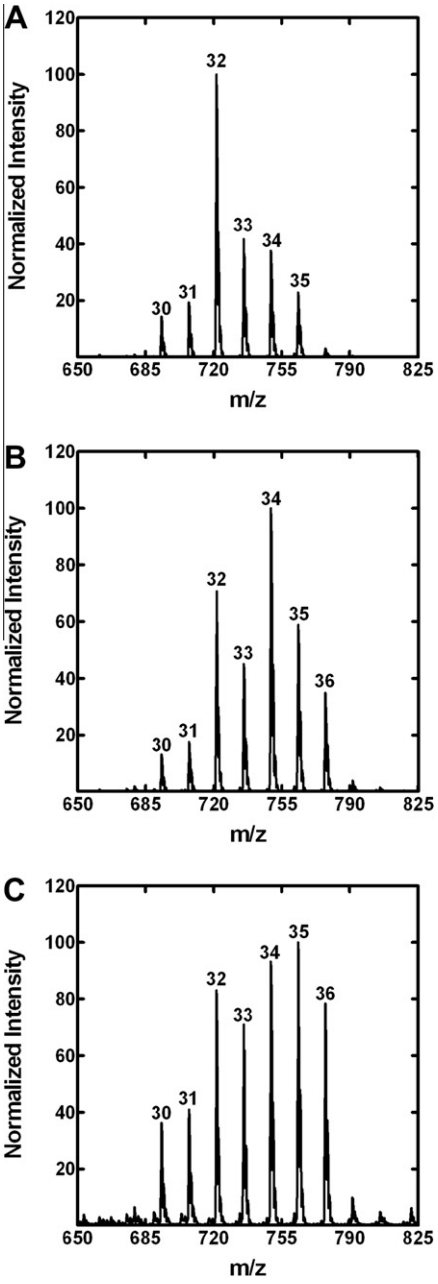


Figure 3. Effect of **25** on the molecular species distribution in the major membrane phospholipid of *S. aureus*, phosphatidylglyerol (PtdGro). *S. aureus* strain RN4220 was grown in Luria-Bertini broth and treated with DMSO as a control (A), or either 25 μM (B) or (C) 50 μM **25** for 2.5 h. The distribution of PtdGro molecular species was determined by mass spectrometry as described under the Experimental Section. The numbers above the peaks correspond to the total number of carbons in the two acyl chains of the PtdGro molecular species at that mass.

2.5. Mode of action in *B. anthracis*

There are three PlsY homologs in *B. anthracis*. BaPlsY1 and BaPlsY2 have reasonable homology to the SaPlsY (~43–45% identity). BaPlsY3 has a much lower homology (28% identity). We reconstituted the enzymatic activity of all three BaPlsY. BaPlsY3 has no detected acyltransferase activity. BaPlsY2 has lower acyltransferase activity, while BaPlsY1 has the most acyltransferase activity. Based on bioinformatics predication and enzyme activity from reconstitution, we believe that BaPlsY1 is the major PlsY in Bacillus anthracis, and therefore choose that for compound activity testing. Many PlsY inhibitors had more potent antibacterial activity against *B. anthracis* compared to other Gram-positive bacteria (Tables 1 and 2). These lower MICs in *B. anthracis* suggested that either the PlsY of this organism was more sensitive to the acyl-sulfamates than the *S. aureus* enzyme, or that the acyl-sulfamates also have an off-target effect on *B. anthracis* growth. The first possibility was tested by cloning and expressing the *B. anthracis* PlsY1, and determining the IC_{50} for the prototypical acyl-sulfamate, **25** (Fig. 5A). The 2-fold increase in the potency of **25** against *B. anthracis* PlsY1 was about the same as the difference between the MIC for *S. aureus* and the average MIC for the wild-type *B. anthracis* strain panel (Table 2). However, the effect of **25** on the growth of *B. anthracis* Sterne was also not characteristic of lipid synthesis inhibitors (Fig. 5B). Compound **25** addition caused

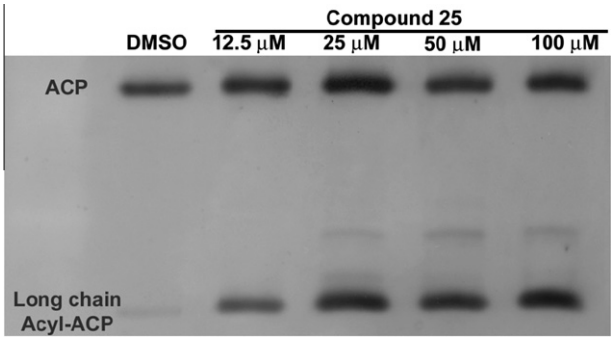


Figure 4. The effect of **25** on the intracellular acyl-ACP pool. *S. aureus* strain RN4220 was treated with the indicated concentrations of **25** for 2.5 h, cell extracts were prepared and the proteins fractionated on 15% polyacrylamide gels containing 2 M urea to separate the ACP species based on the length of the attached fatty acid chain. ACP species were detected using anti-ACP antibody to visualize the different acylated ACP species.

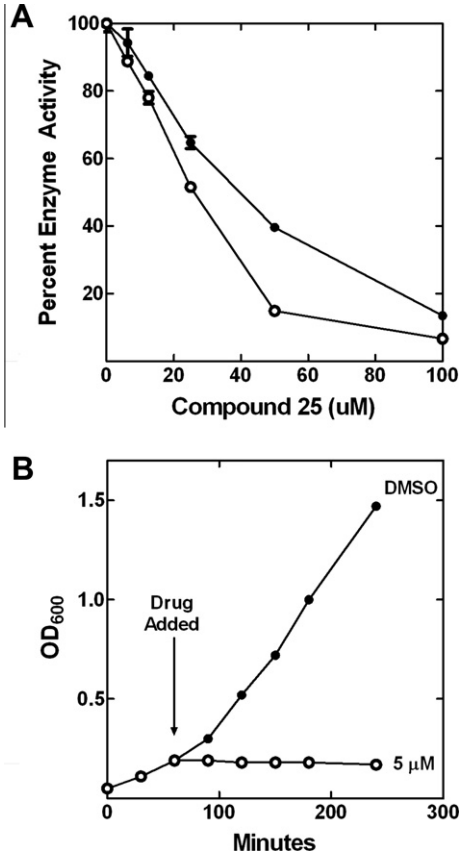


Figure 5. Effect of **25** on *B. anthracis* PlsY1 enzyme activity and growth. (A) IC_{50} for **25** using either *S. aureus* PlsY (●) of *B. anthracis* PlsY1 (○) as the enzyme source. (B) Growth of *B. anthracis* Sterne in the absence (●, DMSO-treated control) or presence (○) of 5 μM **25**.

an abrupt cessation of cell growth that was more indicative of a membrane disruptive agent as opposed to specific inhibition of membrane biogenesis. Furthermore, the acyl-sulfamates inhibited protein, DNA and RNA synthesis in *B. anthracis* Sterne (not shown) compared to their selective effect on lipid synthesis observed in *S. aureus*. An important caveat to these experiments was that we cannot determine if the sensitivity of *B. anthracis* Sterne vaccine strain is due to the loss of the pXO2 plasmid that produces a protective capsule, which may allow the acyl-sulfamates to target the cell membrane. Nonetheless, these data suggest that the acyl-sulfamates may have off-target effects on bacterial membrane function in some bacteria.

3. Conclusions

As part of our SAR study, a series of acyl-sulfamates with modifications at the R_1 head and R_2 acyl tail positions were synthesized and evaluated as *S. aureus* PlsY inhibitors and for Gram-positive antibacterial activities. At the R_1 position, a wide range of substituents were tolerated (**1**–**21**) with the exception of substituents containing a free amine (**6**, **16**) that led to significant loss of PlsY activity. At R_2, the acyl chain could be shortened from C16 to C11 with minimal loss of PlsY activity (**13** vs **22**, **23**) and compounds incorporating a terminal phenyl on the acyl chain (**24**–**27**) displayed a direct correlation between chain length and PlsY inhibitory activity. The acyl-sulfamates, typified by **25**, possessed antibacterial activity through the inhibition of PlsY in vivo. Compound **25** caused a slow inhibition of *S. aureus* growth that was characteristic of other lipid biosynthesis inhibitors. Compound **25** selectively inhibited lipid synthesis compared to other major branches of macromolecular biosynthesis, caused an increase in the average chain-length of membrane phospholipid fatty acids and triggered the accumulation of long-chain acyl-ACP end-products of fatty acid synthesis. All of these metabolic effects are consistent with PlsY inhibition as the ultimate cellular target. A potential drawback to this series of compounds was revealed by the analysis of the acyl-sulfamate series against *B. anthracis* Sterne. In this system, the amphipathic acyl-sulfamate possessed significant off-target effects on *B. anthracis* metabolism and caused the immediate cessation of bacterial growth that is not observed in *S. aureus*. Nonetheless, the analysis of the acyl-sulfamate class further advances PlsY as a druggable target for the development of novel antibacterial therapeutics. Future development of the acyl-sulfamate compound series will include finding suitable molecular replacements for the long aliphatic chain at position R_2 (Scheme 1) that will retain PlsY activity and have improved drug-like properties and less off-target activity.

4. Experimental section

4.1. General synthetic methods

All reagents and solvents were purchased from commercial sources. All reactions were performed under inert atmosphere. The final reaction mixtures were purified on Isolera Flash Purification System using high performance SNAP columns from Biotage. The solvent system used for all purifications was hexane/ethyl acetate (5–100% gradient). The purity and mass of the synthesized compounds were determined on a Waters ACQUITY UPLC-PDA-ELSD-MS system using a C18 reverse phase column and 0.1% formic acid/water—0.1% formic acid/acetonitrile binary solvent system. All synthesized compounds were at least 95% pure. The structures of the synthesized compounds were confirmed by ^1H NMR which was recorded on a 400 Mhz Varian AVANCE 400-FT NMR.

4.2. General procedure for the synthesis of palmitoylsulfamates (1–21)

Formic acid (98%, 2 equiv) was added drop-wise to chlorosulfonyl isocyanate (2 equiv) at 0 °C. The mixture was allowed to rise to room temperature and stirred until the gas evolution stopped (~2 h). The desired alcohol (1 equiv) in dimethylacetamide (DMA) was added drop-wise to the resulting sulfamoyl chloride at 0 °C. The mixture was stirred at 0 °C for 10 min and then at room temperature for 3 h. The mixture was then poured into cold brine

and extracted with EtOAc. The combined extracts were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo* to provide the desired sulfamate ester which was used directly in the next step.

To a mixture of the obtained sulfamate ester (1 equiv), triethylamine (3 equiv) and *N*,*N*-dimethylpyridin-4-amine [DMAP] (0.1 equiv) in anhydrous DCM:DMF (1:1) at 0 °C were added palmitoyl chloride (1 equiv). The mixture was stirred at 0 °C for 10 min and then at room temperature overnight. The reaction mixture was diluted with DCM and washed with 2.5% HCl, water and brine, dried over Na_2SO_4 and concentrated in vacuo. The crude mixture was separated by column chromatography as described above.

4.3.1. Methyl palmitoylsulfamate (1)

Synthesized with methanol. Yield = 17%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (br s, 1H), 4.06 (s, 3H), 2.39 (t, J = 7.5 Hz, 2H), 1.66 (p, J = 7.5 Hz, 2H), 1.25 (br s, 24H), 0.88 (t, J = 6.8 Hz, 3H). MS-ESI m/z = 348.22 $[M-H]^-$

4.3.2. Cyclopentyl palmitoylsulfamate (2)

Synthesized with cyclopentanol. Yield = 9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (br s, 1H), 5.38 (m, 1H), 2.41 (t, J = 7.3 Hz, 2H), 2.10–1.75 (m, 6H), 1.74–1.58 (m, 4H), 1.27 (br s, 24H), 0.90 (t, J = 5.9 Hz, 3H). MS-ESI m/z = 402.48 $[M-H]^-$.

4.3.3 Tetrahydrofuran-3-yl palmitoylsulfamate (3)

Synthesized with tetrahydrofuran-3-ol. Yield = 50%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (br s, 1H), 5.59 (t, J = 5.0 Hz, 1H), 4.10–3.79 (m, 5H), 2.36 (t, J = 7.5 Hz, 2H), 2.30–2.16 (m, 2H), 1.74–1.61 (m, 3H), 1.26 (br s, 21H), 0.88 (t, J = 6.8 Hz, 3H). MS-ESI m/z = 404.31 $[M-H]^-$.

4.3.4. Cyclohexyl palmitoylsulfamate (4)

Synthesized with cyclohexanol. Yield = 75%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (br s, 1H), 4.90 (dt, J = 8.9, 4.8 Hz, 2H), 2.38 (t, J = 6.9, 6.4 Hz, 3H), 2.03–1.93 (m, 2H), 1.83–1.73 (m, 2H), 1.73–1.47 (m, 6H), 1.47–1.15 (m, 24H), 0.88 (t, J = 6.8 Hz, 3H). MS-ESI m/z = 416.29 $[M-H]^-$.

4.3.5. Tetrahydro-2*H*-pyran-4-yl palmitoylsulfamate (5)

Synthesized with tetrahydro-2*H*-pyran-4-ol. Yield = 63%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (br s, 1H), 5.22–5.13 (m, 1H), 4.02–3.90 (m, 2H), 3.59 (ddd, J = 11.7, 8.5, 3.2 Hz, 2H), 2.38 (t, J = 7.5 Hz, 2H), 213–2.04 (m, 2H), 1.97–1.86 (m, 2H), 1.68 (p, J = 7.3 Hz, 2H), 1.28 (s, 24H), 0.90 (t, J = 6.6 Hz, 3H). MS-ESI m/z = 418.43 $[M-H]^-$.

4.3.6. Piperidin-4-yl palmitoylsulfamate (6)

Compound (**7**) was stirred in a 10% solution of Trifluoroacetic acid in DCM for 30 min and the solvent evaporated to afford (**6**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.16–5.04 (m, 1H), 3.34–3.16 (m, 4H), 2.35–2.24 (m, 1H), 2.24–2.06 (m, 3H), 1.62 (p, J = 7.9 Hz, 2H), 1.25 (br s, 24H), 0.88 (t, J = 5.7 Hz, 2H). MS-ESI m/z = 417.42 $[M-H]^-$.

4.3.7. *tert*-butyl 4-(*N*-palmitoylsulfamoyloxy)piperidine-1-carboxylate (7)

Synthesized with *t*-butyl 4-hydroxypiperidine-1-carboxylate. Yield = 38%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.49 (br s, 1H), 5.21–5.06 (m, 1H), 3.72–3.61 (m, 2H), 3.42–3.32 (m, 4.6 Hz, 2H), 2.39 (t, J = 6.5 Hz, 2H), 2.03–1.92 (m, 2H), 1.92–1.80 (m, 2H), 1.75–1.59 (m, 2H), 1.48 (s, 9H), 1.28 (br s, 24H), 0.90 (t, J = 5.9 Hz, 3H). MS-ESI m/z = 517.38 $[M-H]^-$.

4.3.8. Phenyl palmitoylsulfamate (8)

Synthesized with phenol. Yield = 70%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (br s, 1H), 7.47–7.27 (m, 5H), 2.40 (t, J = 7.5 Hz, 2H), 1.75–1.46 (m, 2H), 1.26 (br s, 24H), 0.88 (t, J = 6.3 Hz, 3H). MS-ESI m/z = 410.73 $[M-H]^-$.

4.3.9. 4-chlorophenyl palmitoylsulfamate (9)

Synthesized with 4-chlorophenol. Yield = 63%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (br s, 1H), 7.38 (d, 8.3 Hz, 2H), 7.25 (d, J = 9.1 Hz, 2H), 2.40 (t, J = 7.4 Hz, 2H), 1.64 (q, J = 7.3 Hz, 2H), 1.26 (br s, 24H), 0.88 (t, J = 6.7 Hz, 3H). MS-ESI m/z = 444.55 $[M-H]^-$.

4.3.10. 3-chlorophenyl palmitoylsulfamate (10)

Synthesized with 3-chlorophenol. Yield = 33%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.30 (m, 3H), 7.21 (dt, J = 6.8, 2.3 Hz, 1H), 2.39 (t, J = 7.4 Hz, 2H), 1.79–1.50 (m, 2H), 1.25 (br s, 24H), 0.88 (t, J = 6.7 Hz, 3H). MS-ESI m/z = 444.20 $[M-H]^-$.

4.3.11. 3,4-dichlorophenyl palmitoylsulfamate (11)

Synthesized with 3,4-dichlorophenol. Yield = 32%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (d, J = 7.5 Hz, 1H), 7.44 (d, J = 7.4 Hz, 1H), 7.20 (d, J = 7.18 Hz, 1H), 2.39 (t, J = 7.4 Hz, 1H), 1.78–1.46 (m, 1H), 1.25 (br s, 24H), 0.88 (t, 6.5 Hz, 3H). MS-ESI m/z = 478.16 $[M-H]^-$.

4.3.12. *p*-Tolyl palmitoylsulfamate (12)

Synthesized with *p*-cresol. Yield = 65%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18 (q, J = 8.7 Hz, 4H), 2.50–2. 23 (m, 3H), 1.69–1.57 (m, 2H), 1.26 (br s, 24H), 0.88 (t, J = 6.8 Hz, 3H). MS-ESI m/z = 424.29 $[M-H]^-$.

4.3.13. 4-Methoxyphenyl palmitoylsulfamate (13)

Synthesized with 4-methoxyphenol. Yield = 85%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 (d, J = 9.2 Hz, 2H), 6.90 (d, J = 9.2 Hz, 2H), 3.81 (s, 2H), 2.40 (t, J = 7.5 Hz, 2H), 1.65 (dt, J = 14.6, 7.4 Hz, 2H), 1.26 (br s, 24H), 0.88 (t, J = 6.8 Hz, 3H). MS-ESI m/z = 440.23 $[M-H]^-$.

4.3.14. Methyl 2-(4-(*N*-palmitoylsulfamoyloxy)phenyl)acetate (14)

Synthesized with methyl 2-(4-hydroxyphenol)acetate. Yield = 41%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (d, J = 8.7 Hz, 2H), 7.24 (d, J = 8.7 Hz, 2H), 3.71 (s, 3H), 3.64 (s, 2H), 2.39 (t, J = 7.5 Hz, 2H), 1.65 (dt, J = 14.9, 7.4 Hz, 2H), 1.26 (br s, 24H), 0.88 (t, J = 6.8 Hz, 2H). MS-ESI m/z = 482.32 $[M-H]^-$.

4.3.15. Ethyl 4-(*N*-palmitoylsulfamoyloxy)benzoate (15)

Synthesized with ethyl 4-hydroxybenzoate. Yield = 51%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, J = 8.8 Hz, 2H), 7.36 (d, J = 8.8 Hz, 2H), 4.39 (q, J = 7.1 Hz, 2H), 2.40 (t, J = 7.4 Hz, 2H), 1.69–1.60 (m, 2H), 1.40 (t, J = 7.1 Hz, 3H), 1.25 (br s, 24H), 0.88 (t, J = 5.8 Hz, 3H). MS-ESI m/z = 482.42 $[M-H]^-$.

4.3.16. 4-(2-Aminoethyl)phenyl palmitoylsulfamate (16)

Compound (**17**) was stirred in neat Trifluoroacetic acid (TFA) for 12 h. The TFA was evaporated and the crude mixture dissolved in minimal amount of acetone. The product was precipitated by addition of water and then filtered and dried under vacuum to provide (**16**) in 37% yield. ^{1}H NMR (400 MHz, DMSO) δ 7.76 (br s, 2H), 7.18 (d, J = 8.0 Hz, 2H), 7.11 (d, J = 8.3 Hz, 2H), 3.13–2.92 (m, 2H), 2.89–2.71 (m, 2H), 1.95 (t, J = 7.4 Hz, 3H), 1.48–1.34 (m, 4H), 1.24 (s, 24H), 0.86 (t, J = 6.6 Hz, 3H). MS-ESI m/z = 453.17 $[M-H]^-$.

4.3.17. 4-(2-(*tert*-Butoxycarbonylamino)ethyl)phenyl palmitoylsulfamate (17)

Synthesized with t-butyl 4-hydroxyphenethylcarbamate. Yield = 54%. ^{1}H NMR (400 MHz, DMSO) δ 7.30 (d, J = 8.5 Hz, 2H), 7.15 (d, J = 8.5 Hz, 2H), 3.13 (q, J = 6.5 Hz, 2H), 2.71 (t, J = 7.5 Hz, 2H), 2.26 (t, J = 7.2 Hz, 2H), 1.49 (dt, J = 12.5, 5.7 Hz, 2H), 1.24 (br s, 24H), 0.86 (t, J = 6.5 Hz 3H). MS-ESI m/z = 553.29 $[M-H]^-$.

4.3.18. 4′-Nitrobiphenyl-4-yl palmitoylsulfamate (18)

Synthesized with 4'-nitrobiphenyl-4-ol. Yield = 65%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (d, J = 8.8 Hz, 2H), 7.68 (dd, J = 17.4, 8.7 Hz, 4H), 7.44 (d, J = 8.7 Hz, 2H), 2.43 (t, J = 7.5 Hz, 2H), 1.86–1.62 (m, 2H), 1.36–1.16 (m, 24H), 0.88 (t, J = 6.7 Hz, 3H). MS-ESI m/z = 531.24 $[M-H]^-$.

4.3.19. Benzo[*d*][1,3]dioxol-5-yl palmitoylsulfamate (19)

Synthesized with Benzo[*d*][1,3]dioxol-5-ol. Yield = 62%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.87–6.65 (m, 3H), 6.02 (s, 2H), 2.41 (t, J = 7.4 Hz, 2H), 1.67–1.62 (m, 2H), 1.25 (br s, 24H), 0.88 (t, J = 5.9 Hz, 3H). MS-ESI m/z = 454.32 $[M-H]^-$.

4.3.20. Quinolin-6-yl palmitoylsulfamate (20)

Synthesized with quinolin-6-ol. Yield = 21%. ^{1}H NMR (400 MHz, DMSO) δ 8.88 (dd, J = 4.2, 1.7 Hz, 1H), 8.38 (dd, J = 8.4, 1.6 Hz, 1H), 8.02 (d, J = 9.1 Hz, 1H), 7.79 (d, J = 2.6 Hz, 1H), 7.61 (dd, J = 9.1, 2.6 Hz, 1H), 7.54 (dd, J = 8.3, 4.2 Hz, 1H), 2.07 (t, J = 7.3 Hz, 2H), 1.40 (p, J = 7.1 Hz, 2H), 1.33–1.06 (m, 24H), 0.86 (t, J = 6.8 Hz, 3H). MS-ESI m/z = 461.30 $[M-H]^-$.

4.3.21. 3-(Sulfamoyloxy)benzofuran-6-yl palmitoylsulfamate (21)

Synthesized with 6-hydroxybenzofuran-3(2H)-one. Yield = 2%. ^{1}H NMR (400 MHz, DMSO) δ 8.25 (br s, 1H), 8.06 (s, 1H), 7.65 (d, J = 8.6 Hz, 1H), 7.58 (d, J = 1.7 Hz, 1H), 7.28 (dd, J = 8.6, 1.9 Hz, 1H), 2.20 (t, J = 7.1 Hz, 2H), 1.50–1.41 (m, 2H), 1.31–1.13 (m, 24H), 0.86 (t, J = 6.8 Hz, 3H). MS-ESI m/z = 545.23 $[M-H]^-$.

4.4. General procedure for the synthesis of 22–28

To a solution of the desired carboxylic acid (1 equiv) in anhydrous DCM containing 2 drops of DMF was added oxalyl chloride (1 equiv) at 0 °C. The mixture was stirred at 0 °C for 15 min and then at room temperature for 30 min. The solution was then added to a mixture of DMAP (0.1 equiv), triethylamine (3 equiv) and either 4-methoxyphenyl sulfamate (1 equiv) (**22–23**) or Phenyl sulfamate (**24–28**) in anhydrous DCM/DMF (1:1). After stirring overnight, the reaction mixture was extracted with 2.5% HCl, H_2O and brine and dried with Na_2SO_4. The organic layer was evaporated under vacuum and the crude mixture separated by column chromatography as described above.

4.4.1. 4-Methoxyphenyl tetradecanoylsulfamate (22)

Synthesized with Myristic acid. Yield = 61%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (br, 1H), 7.20 (d, J = 9.1 Hz, 2H), 6.89 (d, J = 9.1 Hz, 2H), 3.81 (s, 3H), 2.40 (t, J = 7.5 Hz, 2H), 1.69–1.59 (m, 2H), 1.26 (br s, 20H), 0.88 (t, J = 6.7 Hz, 3H). MS-ESI m/z = 412.25 $[M-H]^-$.

4.4.2. 4-Methoxyphenyl undec-10-enoylsulfamate (23)

Synthesized with undec-10-enoic acid. Yield = (62%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23–7.16 (m, 2H), 6.90 (dd, J = 2.12, 7.01 Hz, 2H), 5.81 (ddt, J = 6.66, 10.24, 16.89 Hz, 1H), 5.08–4.82 (m, 2H), 3.81 (s, 3H), 2.54–2.33 (m, 2H), 2.04 (q, J = 6.74 Hz, 2H), 1.77–1.46 (m, 5H), 1.46–1.11 (m, 12H). MS-ESI m/z = 367.94 $[M-H]^-$.

4.4.3. Phenyl (11-phenylundecanoyl) sulfamate (24)

Synthesized with 11-phenylundecanoic acid. Yield = 31%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43–7.32 (m, 2H), 7.32–7.20 (m, 5H), 7.19–7.11 (m, 3H), 2.78–2.15 (m, 3H), 1.72–1.39 (m, 5H), 1.17 (dd, J = 14.56, 42.81 Hz, 8H), 0.94–0.65 (m, 2H). MS-ESI m/z = 416.22 $[M-H]^-$.

4.4.4. Phenyl (8-phenyloctanoyl) sulfamate (25)

Synthesized with 8-phenyloctanoic acid. Yield = 40%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42–7.35 (m, 2H), 7.35–7.29 (m, 1H), 7.29–7.26 (m, 4H), 7.20–7.12 (m, 3H), 2.58 (td, J = 8.0, 3.6 Hz, 2H), 2.34 (td, J = 7.6, 3.0 Hz, 2H), 1.59 (br s, 4H), 1.29 (br s, 6H). MS-ESI m/z = 374.09 $[M-H]^-$.

4.4.5. Phenyl (7-phenylheptanoyl) sulfamate (26)

Synthesized with 7-phenylheptanoic acid. Yield = 64%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45–7.37 (m, 2H), 7.33 (t, J = 7.4 Hz, 2H), 7.28 (d, J = 5.4 Hz, 3H), 7.17 (t, J = 8.2 Hz, 3H), 2.59 (t, J = 7.7 Hz, 2H), 2.34 (dt, J = 15.2, 7.5 Hz, 2H), 1.62 (q, J = 8.5, 7.9 Hz, 4H), 1.34 (dq, J = 7.3, 4.4, 3.6 Hz, 4H). MS-ESI m/z = 360.13 $[M-H]^-$.

4.4.6. Phenyl (6-phenylhexanoyl) sulfamate (27)

Synthesized with 6-phenylhexanoic acid. Yield = 74%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (dt, J = 8.1, 4.1 Hz, 2H), 7.35–7.30 (m, 2H), 7.30–7.26 (m, 3H), 7.16 (t, J = 6.0 Hz, 3H), 2.59 (t, J = 7.5 Hz, 2H), 2.43–2.23 (m, 2H), 1.62 (dq, J = 14.8, 7.5 Hz, 4H), 1.46–1.28 (m, 2H). MS-ESI m/z = 346.11 $[M-H]^-$.

4.4.7. (*E*)-Phenyl 3-(biphenyl-4-yl)acryloylsulfamate (28)

Synthesized with 4-phenylcinnamic acid. Yield = 11%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, J = 15.6 Hz, 1H), 7.63–7.60 (m, 6H), 7.46 (q, J = 8.3, 7.8 Hz, 2H), 7.39 (t, J = 6.7 Hz, 3H), 7.37–7.30 (m, 3H), 6.56 (d, J = 15.6 Hz, 1H). MS-ESI m/z = 378.13 $[M-H]^-$.

4.5. Membrane preparation and PlsY assay

PlsY is an intrinsic membrane protein and was expressed and assayed in a native membrane environment. *E. coli* strain FB23281 (Δ*plsY*) was used as the host for plasmids expressing either the *S. aureus* PlsY (SA1187) or *B. anthracis* PlsY1 (BAS3399). *B. anthracis* has 3 PlsY homologs, and PlsY1 was the most similar to the single prototypical single PlsY proteins expressed in Gram-positive bacteria. Membranes were purified and acyltransferase activity was measured as described previously.[5] Briefly, the reaction buffer (pH 7.4) contained 100 mM Tris–HCl, 150 mM NaCl, 1 mg/mL BSA, 5 mM Na_3VO_4, 100 μM [U-^{14}C]glycerol-PO_4 (8 mCi/mmol), inhibitor (200 μM) and 4 μg purified membranes. 16:0-PO_4 (final concentration 100 μM) was added to start the reaction. Reactions were terminated after incubation at 37 °C for 20 min by aliquotting the reaction mixture (20 μL) onto a Whatman 3 mm cellulose filter disc. Filter discs were washed in 10%, 5%, and 1% ice-cold trichloroacetic acid (20 min, 20 mL/disk) to remove unreacted glycerol-PO_4 prior to scintillation counting.

4.6. MIC determinations

The MIC of each test compound was determined by the microbroth dilution method in Mueller–Hinton (MH) media according to the Clinical Laboratory Standards Institute (CLSI) document M7A7 for testing of the antibiotic susceptibility of aerobic bacteria. For growth of *S. pneumoniae* and *S. pyogenes*, MH broth was supplemented with 5% lysed horse blood from BD Diagnostic Systems (Loveton Circle, Sparks, MD, USA). All test compounds were dissolved in DMSO at a concentration of 10 mg/mL and stored at −80 °C. Twofold serial dilutions of test compound were prepared in MH broth in 96-well plates to give drug concentrations that

ranged from 400–0.025 μg/mL. Bacterial inoculums were prepared by streaking at −80 °C stock bacterial culture onto an MH agar plate, which was incubated overnight at 37 °C. A single colony is picked into 10 ml of MH broth and incubated in 37 °C shaking incubator. The culture is grown to mid-log phase (OD_{600} = 0.5) and then diluted to OD_{600} = 0.001. An aliquot of culture (100 μL) was then added to each well of the 96-well plate to give an OD_{600} = 0.0005, which corresponded to about 105 CFU/mL, and final antibiotic concentrations that ranged from 200–0.0125 μg/mL. The 96-well plates were incubated overnight at 37 °C, and the MIC was recorded as the lowest concentration of drug that inhibited 90% of visible bacterial growth. The MIC for *B. anthracis* was determined as previously described.[15,16]

4.7. Acetate labeling

[^{14}C]Acetate incorporation experiments were conducted to measure lipid metabolism activity in *S. aureus* or *B. anthracis*. For both bacteria, the culture was grown overnight and the starter culture was back diluted to an initial OD_{600} of 0.1 and grown until OD_{600} of 0.5. The culture was split into 10 mL aliquots and each aliquot was incubated with the appropriate concentration of inhibitors or DMSO (control) for 15 min and the OD_{600} of the culture was collected. [^{14}C]Acetate (10 μCi) was added to each aliquot for 30 min. The cells are harvested by centrifugation and washed 4 times with phosphate-buffered saline. Pelleted cells are then lysed to harvest for lipids using the method of Bligh and Dyer.[17] The radioactivity from the lipid extraction was counted on a LS6500 Multipurpose Scintillation Counter and normalized to the OD_{600} of the cell culture. All measurements were made in duplicate and the averages with standard error were reported. The radioactive lipid extract was also separated on Silica Gel G layers developed with $CHCl_3$/methanol/acetic acid (98:2:1) to separate the different lipid species. The radioactivity of the plate was counted using the Bioscan imaging system.

4.8. Pathway Labeling

The method for protein, DNA, and RNA pathway labeling was similar to acetate labeling experiments. Cells were grown to OD_{600} of 0.5 before being split to 10 mL aliquots and incubated with appropriate inhibitors for 15 min. Then, either a ^{3}H-labled amino acid mix (10 μCi) for protein pathway metabolism, [^{3}H]thymidine (10 μCi) for DNA pathway metabolism, or [^{3}H]uracil (10 μCi) for stable RNA synthesis was added to the culture and grown for 30 min. The labeled cells are then collected via vacuum filtration through 0.45 μm HA filters, and the radioactivity was counted and normalized to the OD_{600} of the culture. The cell density adjusted activity of the metabolic pathways from cells treated with inhibitor is divided by the metabolic activity of the untreated cells to determine the effect of the inhibitor on the metabolic pathways. Duplicated measures were made with averages and standard error were reported.

4.9. ACP immunoblotting

Cultures of *S. aureus* RN4220 cells were grown to OD_{600} = 0.5, and then split into 50 mL aliquots. Appropriate concentrations of inhibitors were added to the aliquots and grown at 37 °C for 30 min and the OD_{600} was collected. Cells were harvested, extracted and immunoblotted to determine the ACP species as described previously.[10] The amount of supernatant loaded is adjusted to OD_{600} such that a similar amount of total protein is loaded for each drug treated sample.

4.10. Gas chromatography and mass spectrometry

S. aureus was grown in Luria–Bertani broth at 37 °C with rigorous shaking (225 rpm). Cells (250 mL) were grown to OD_{600} of 0.5, aliquoted to 50 mL cultures, and treated with no inhibitor, 25 μM, or 50 μM of **25** for 2.5 hours. Cells were washed with PBS and harvested for lipids using the method of Bligh and Dyer.[17] Gas chromatography was performed on *S. aureus* strain RN4220 as described.[10] The PtdGro molecular species were also analyzed by mass spectrometry as described previously.[10]

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