CHEMMEDCHEM

DOI: 10.1002/cmdc.200800218

Novel Acyl Phosphate Mimics that Target PlsY, an Essential Acyltransferase in Gram-Positive Bacteria

Kimberly D. Grimes,[a] Ying-Jie Lu,[b] Yong-Mei Zhang,[b] Vicki A. Luna,[c] Julian G. Hurdle,[a] Elizabeth I. Carson,[a] Jianjun Qi,[a] Sucheta Kudrimoti,[a] Charles O. Rock,[b] and Richard E. Lee*[a]

PlsY is a recently discovered acyltransferase that executes an essential step in membrane phospholipid biosynthesis in Gram-positive bacteria. By using a bioisosteric replacement approach to generate substrate-based inhibitors of PlsY as potential novel antibacterial agents, a series of stabilized acyl phosphate mimetics, including acyl phosphonates, acyl α,α-difluoromethyl phosphonates, acyl phosphoramides, reverse amide phosphonates, acyl sulfamates, and acyl sulfamides were designed and synthesized. Several acyl phosphonates, phosphoramides, and sulfamates were identified as inhibitors of PlsY from Streptococcus pneumoniae *and* Bacillus anthracis*. As anticipated, these inhibitors were competitive inhibitors with respect to the acyl phosphate substrate. Antimicrobial testing showed the inhibitors to have generally weak activity against Gram-positive bacteria with the exception of some acyl phosphonates, reverse amide phosphonates, and acyl sulfamates, which had potent activity against multiple strains of* B. anthracis.

Introduction

The widespread occurrence of resistance to current antibiotics by Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) and macrolide-resistant *Streptococcus pneumoniae*, exemplifies the urgent need for the development of new antimicrobials to combat the growing menace of complicated infections.[1] The sequencing of bacterial genomes has identified several new drug targets with the promise of introducing novel classes of antibiotics with different modes of action to overcome the problems of bacterial resistance to current therapies.[2] Among these attractive targets are the unique enzymes that are involved in the biosynthesis of lipids in bacteria.[3–5]

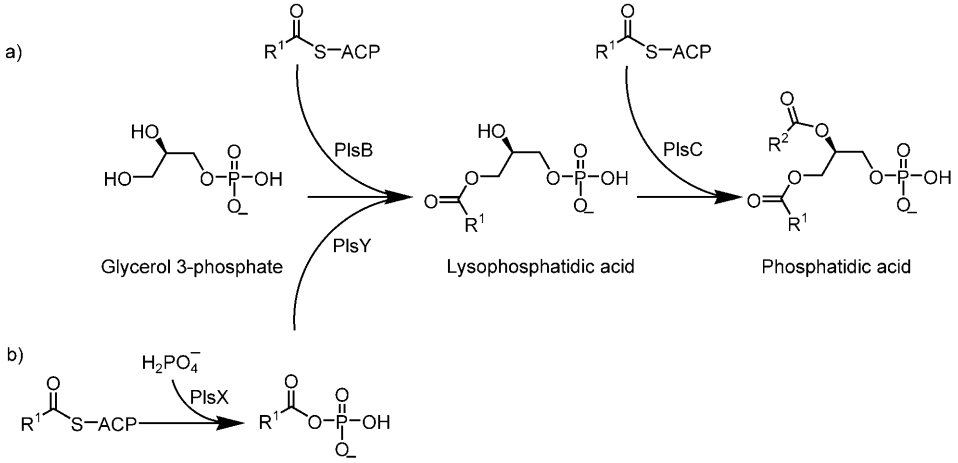


Figure 1. Phosphatidic acid formation: a) Pathway in *E. coli*. b) Pathway in *S. pneumoniae*.

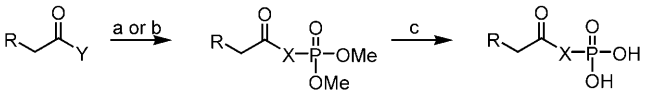
Phospholipids are abundant and essential membrane components in all bacterial species. They form the structural elements of the cell membrane, and inhibition of their biosynthesis results in cell death.[6] Phosphatidic acid is a key intermediate in the biosynthesis of bacterial membrane phospholipids and is formed by the acylation of *sn*-glycerol-3-phosphate (G3P). In *E. coli*, phosphatidic acid biosynthesis is initiated by the PlsB acyltransferase that transfers a fatty acid from the acylated acyl carrier protein (ACP) to the 1-position of G3P (Figure 1 a). Because PlsB homologues exist in mammals, the first step in bacterial phospholipid biosynthesis was typically disfavored as a target for antibiotic drug discovery.[7] However, PlsB is not universally expressed in bacteria. Recently, an alter-

[a] *Dr. K. D. Grimes, Dr. J. G. Hurdle, Dr. E. I. Carson, Dr. J. Qi, Dr. S. Kudrimoti, Dr. R. E. Lee*
Department of Pharmaceutical Sciences
University of Tennessee Health Science Center
847 Monroe Ave, Memphis, TN 38163 (USA)
Fax: (+ 901) 448-6828
E-mail: relee@utmem.edu

[b] *Dr. Y.-J. Lu, Dr. Y.-M. Zhang, Dr. C. O. Rock*
Department of Infectious Diseases, St. Jude Children's Research Hospital
332 N. Lauderdale, Memphis, TN 38105 (USA)

[c] *Dr. V. A. Luna*
Center for Biological Defense, University of South Florida
3602 Spectrum Blvd, Tampa FL 33612 (USA)

native two-step pathway was discovered that is required for G3P acylation in Gram-positive pathogens (Figure 1b).[8] The first step is catalyzed by PlsX, a phosphate:acyl-ACP acyltransferase that produces an acylphosphate (acyl-PO_4) intermediate. The second step is catalyzed by PlsY, an acyl-PO_4:G3P acyltransferase that transfers the acyl group from acyl-PO_4 to the 1-position of G3P. PlsY is an integral transmembrane protein with a highly conserved set of active-site residues that face the interior of the cell.[9] Depletion of the *plsY* gene product results in the blockade of phospholipids biosynthesis in Gram-positive bacteria and cessation of growth.[10] There are no mammalian homologues of the PlsX/PlsY pathway, and this makes it an attractive target for antibacterial drug discovery.



1a R=$CH_3(CH_2)_{13}$, Y=OMe
1b R=$CH_3(CH_2)_5CH{=}CH(CH_2)_8$, Y=Cl
1c R=$CH_3(CH_2)_5$, Y=OMe
1d R=CH_3CH_2, Y=OMe
1e R=$CH_3(CH_2)_9$, Y=OMe
1f R=$Cy(CH_2)_3$, Y=Cl
1g R=$Ph(CH_2)_3$, Y=Cl
1h R=$PhO(CH_2)_9$, Y=Cl
1i R=$CH_3(CH_2)_7CH{=}CH(CH_2)_6$, Y=OMe
1j R=$CH_2{=}CH(CH_2)_7$, Y=Cl

2a-h X= CH_2
3a,e,i,j X= CF_2

4a-h X = CH_2
5a,e,i,j X = CF_2

Scheme 1. *Reagents and conditions*: a) dimethyl methylphosphonate, BuLi, THF, −78 °C, b) diethyl (difluoromethane) phosphonate, LDA, THF, −78 °C, c) TMSBr, CH_2Cl_2, then 95% EtOH.

The PlsX/PlsY pathway is the only route to membrane phospholipids in Firmicutes, which include all most clinically important Gram-positive bacteria.[10] In this study, we focused on designing novel inhibitors of this pathway through the synthesis of stabilized homologues of the acyl-PO_4 reactive intermediate by using a bioisosteric replacement strategy for the highly labile acyl-PO_4 group. This approach is supported by the knowledge that acyl phosphonates, acyl sulfamates, and acyl sulfamides have been used successfully to design inhibitors of other acyl phosphate-using enzymes including aminoacyl-tRNA synthetases and nonribosomal peptide synthetases.[11–17] This work evaluates the study of six acyl-PO_4 bioisosteric head groups combined with a variety of acyl chains (Figure 2); a preliminary structure–activity relationship (SAR) for PlsY inhibition and antibacterial activity has been developed.

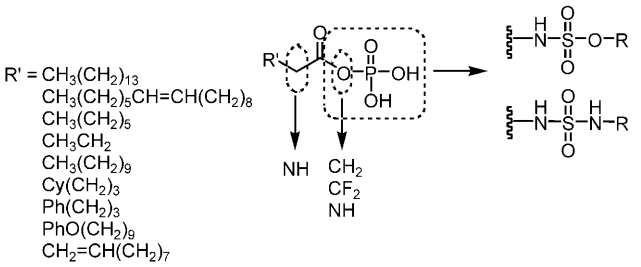
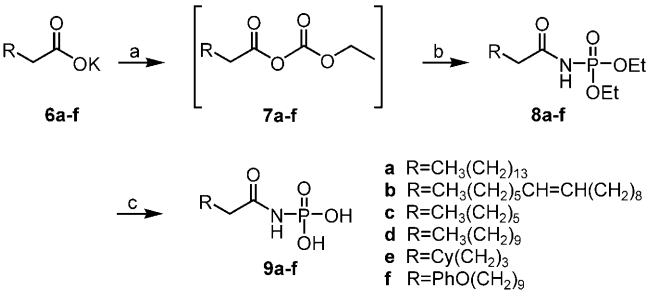


Figure 2. Design of acyl phosphate substrate mimics

Synthesis

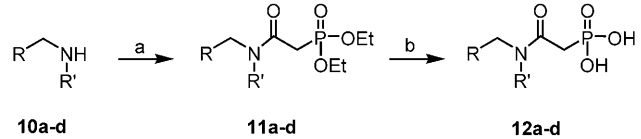
Acyl phosphonate derivatives were synthesized by reacting lithiated dimethyl methane phosphonate with the appropriate esters or acid chlorides **1** to yield the corresponding phosphonate esters in good yields **2a–h** (Scheme 1).[18] Diethyl α,α-difluoromethyl phosphonate esters **3a,e,i,j** were synthesized in an analogous manner from lithiated diethyl α,α-difluoromethyl phosphonate.[19] All the phosphonate esters were cleaved with bromotrimethylsilane (TMSBr), to yield the desired phosphonic acids **4a–h, 5a,e,i,j**. Acyl phosphoramides were prepared by starting from the corresponding fatty acid potassium salts **6a–f**. Acylation of **6a–f** with ethylchloroformate yielded the anyhydrides **7a–f**, which were subsequently reacted with the lithium salt of diethyl phosphoramidate to give acyl phosphoramide esters **8a–f**.[20] Then, TMSBr-mediated cleavage of the phosphoramide esters yielded the target acyl phosphoramides **9a–f** (Scheme 2). Reverse amide phosphonates were synthesized by



a R=$CH_3(CH_2)_{13}$
b R=$CH_3(CH_2)_5CH{=}CH(CH_2)_8$
c R=$CH_3(CH_2)_5$
d R=$CH_3(CH_2)_9$
e R=$Cy(CH_2)_3$
f R=$PhO(CH_2)_9$

Scheme 2. *Reagents and conditions*: a) ethylcholoroformate, CH_2Cl_2; b) diethyl phosphoramidate, BuLi, THF, −78 °C; c) TMSBr, CH_2Cl_2, then 95% EtOH.

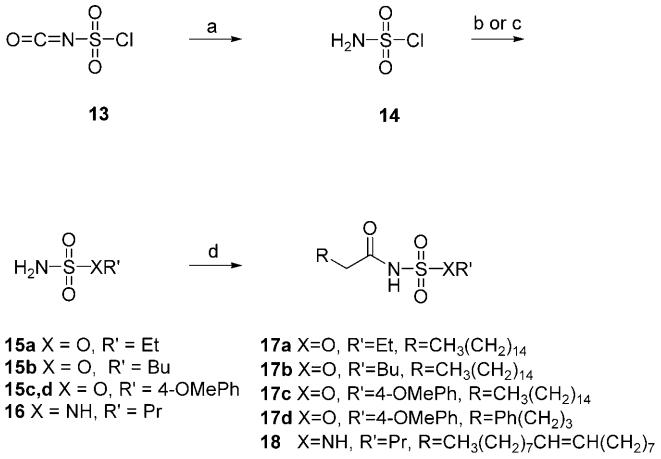
coupling various amines **10a–d** with diethylphosphonoacetic acid by using 1-hydroxybenzotriazole (HOBt) and *N*-(3-*d*imethylaminopropyl)-*N*′-ethylcarbodiimide (EDCI) to form the corresponding phosphonate esters **11a–d**,[21] which were then deprotected with TMSBr to generate the target phosphonates **12a–d** (Scheme 3).



a R=$CH_3(CH_2)_{13}$, R'=H
b R=$CH_3(CH_2)_5CH{=}CH(CH_2)_8$, R'=H
c R=$CH_3(CH_2)_9$, R'=CH_3
d R=$CH_3(CH_2)_9$, R'=H

Scheme 3. *Reagents and conditions*: a) diethylphosphonoacetic acid, HOBt, EDCI, CH_2Cl_2/DMF, RT, 16 h; b) TMSBr, CH_2Cl_2, then 95% EtOH.

The acyl sulfamates and acyl sulfamides were synthesized by starting from the aminosulfonyl chloride **14**, according to the protocol of Appel and Berger.[22] Aminosulfonyl chloride **14** was treated with various alcohols or propylamine to generate the intermediate sulfamates **15a–d**[23] or sulfamide **16**.[24] The sulfamates and sulfamide were then acylated with selected acid chlorides in the presence of triethylamine and catalytic 4-(dimethylamino)pyridine (DMAP) to form the desired acyl sulfamates **17a–d** and acyl sulfamide **18** (Scheme 4).



Scheme 4. *Reagents and conditions*: a) HCO_2H, neat; b) R′OH, DMA; c) $R'NH_2$, neat, NaOH; d) RCOCl, DMAP, NEt_3, CH_2Cl_2/DMF.

Results and Discussion

PlsY inhibition

The synthesized acyl-PO_4 bioisosteres were tested for inhibition of PlsY in two representative Gram-positive pathogens: *S. pneumoniae* (*Sp*PlsY) and *B. anthracis* (*Ba*PlsY1; Table 1). Compounds from all six of the bioisosteric head group series showed varying degrees of PlsY inhibition. The most potent inhibitors in each class corresponded to the long-chain derivatives that had the saturated C_{16} and unsaturated $C_{18\Delta11}$ alkyl chains. The most-active compounds for *Sp*PlsY were acyl phosphoramide **9b** ($IC_{50}=11$ μM, C_{16}), α,α-difluoromethyl phosphonates **5a** ($IC_{50}=39$ μM, C_{16}) and **5i** ($IC_{50}=20$ μM, $C_{18\Delta9}$), and ketophosphonate **4b** ($IC_{50}=48$ μM, $C_{18\Delta11}$). The most active compounds for *Ba*PlsY were the α,α-difluoromethyl phosphonates **5a** ($IC_{50}=50$ μM, C_{16}) and **5e** ($IC_{50}=25$ μM, C_{12}). The long-chain reverse amide phosphonates **12a–c** had moderate activity against both PlsYs. The acyl sulfamates **17a–c** displayed good activity against *Sp*PlsY ($IC_{50}=85$ μM, 70 μM, and 60 μM, respectively) and *Ba*PlsY ($IC_{50}=65$ μM, 50 μM, and 35 μM, respectively). Acyl sulfamide **18** was considerably less potent (*Ba* $IC_{50}=200$ μM, $C_{18\Delta9}$) when compared to the related acyl sulfamates **17a** and **17b** against both PlsYs. The lack of significant activity for sulfamate **18** could be related to its poor aqueous solubility, a property that limited the further exploration of this class of inhibitors.

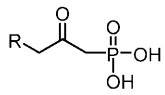
To validate the mechanism of inhibition, a kinetic analysis of the representative acyl-PO_4 bioisosteres **9a** and **9b** was performed against *Sp*PlsY (Figure 3). As anticipated for substrate mimics, these compounds were shown to act as competitive inhibitors of the acyl-PO_4 binding to PlsY and were noncompetitive with respect to the binding of the other substrate, *sn*-glycerol-3-phosphate.

Antimicrobial activity

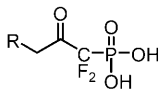
To explore whether our substrate mimics possessed antimicrobial activity, the compound series was tested against a representative panel of Gram-positive bacteria that consisted of *S. pneumoniae*, methicillin-resistant *S. aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, and *B. anthracis* (Table 1). Activity was weak to moderate against most of the Gram-positive species that were tested except for the *B. anthracis* Sterne strain, which was potently inhibited by compounds in the acyl phosphonate and reverse amide phosphonate series. Acyl sulfamate **17c** displayed considerable antibacterial activity against *B. anthracis* and *B. subtilis* with some activity against *S. pneumoniae*. The most-active compounds against *B. anthracis* were acyl phosphonates **4a** ($MIC=1.56\ \mu g\,mL^{-1}$) and **4b** ($MIC=0.05\ \mu g\,mL^{-1}$), reverse amide phosphonates **12a** ($MIC=0.1\ \mu g\,mL^{-1}$), **12b** ($MIC=0.1\ \mu g\,mL^{-1}$), and **12c** ($MIC=3.13\ \mu g\,mL^{-1}$), and acyl sulfamate **17c** ($MIC=3.13\ \mu g\,mL^{-1}$). The inactivity of the acyl phosphoramides might be due to spontaneous or enzymatic-mediated hydrolysis of the phosphoramide bond, or due to poor penetration. Poor bacterial penetration and increased acidity might explain the inactivity of the α,α-difluoromethyl phosphonate series when compared to the similar acyl phosphonates because the electron-withdrawing α,α-difluoro substitution increases the charge on the phosphonate head group, a property that is associated with decreased uptake in bacteria.[25] Though most of the bioisosteres were inactive against the other Gram-positive pathogens, the acyl sulfamate series, and most notably **17c**, did show good *Sp*PlsY inhibition ($IC_{50}=60$ μM) and antibacterial activity against *S. pneumoniae* ($MIC=12.5\ \mu g\,mL^{-1}$), which might warrant further investigation. This series shows that the introduction of a functional group adjacent to the binding phosphate isostere is tolerated and can be exploited in the future design of inhibitors with more drug-like properties. This study raises a question regarding the lack of general antimicrobial activity against Gram-positive bacteria of these compounds despite inhibition of the target enzyme in vitro. Two likely explanations might account for this observation: (i) the enzyme inhibition is still not adequate to cause an antibacterial effect or (ii) that these compounds are limited by bacteria-dependant cellular penetration by an active drug efflux from multidrug efflux pumps or other inactivation processes.

Further specificity studies showed that none of the compounds exhibited antimicrobial activity against the Gram-negative species, *E. coli* and *P. aeruginosa* (data not shown). Cytotoxicity against mammalian cells was also evaluated for the most-active compounds with activity against *B. anthracis* (Table 1). Compounds **4a**, **4b**, **12a**, **12b**, and **17c** only exhibited cytotoxicity at high concentrations, producing high selectivity indices (cytotoxicity IC_{50}/*B. anthracis* MIC) that ranged from 2200–

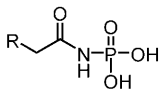
Table 1. Activity Data



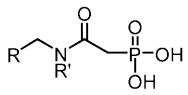
4a-h



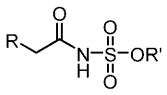
5a,e,i,j



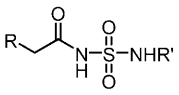
9a-f



12a-d



17a-d



18

Cmpd	R	R′	*Sp*PlsY [%][a]	*Sp*IC$_{50}$ [μM][b]	*Ba*PlsY1 [%][c]	*Ba*IC$_{50}$ [μM][d]	MIC [μg mL^{-1}][e] SP	MRSA	EF	BS	BA	Toxicity[f] [μg mL^{-1}]
4a	$CH_3(CH_2)_{13}$	–	28	nd[g]	67	90	200	100	12.5	25	1.56	205
4b	$CH_3(CH_2)_5CH{=}CH(CH_2)_8$	–	98	48	40	nd	200	100	12.5	25	0.05	235
4c	$CH_3(CH_2)_5$	–	0	nd	0	nd	>200	200	>200	>200	>200	nd
4d	CH_3CH_2	–	18	nd	0	nd	200	>200	>200	>200	100	nd
4e	$CH_3(CH_2)_9$	–	38	nd	28	nd	25	100	25	>200	50	114
4f	$Cy(CH_2)_3$	–	9	nd	nd	nd	>200	>200	>200	25	200	nd
4g	$Ph(CH_2)_3$	–	6	nd	0	nd	>200	>200	>200	>200	>200	nd
4h	$PhO(CH_2)_9$	–	48	nd	55	200	50	100	50	>200	>200	34
5a	$CH_3(CH_2)_{13}$	–	99	39	91	50	>200	100	100	200	200	nd
5e	$CH_3(CH_2)_9$	–	48	nd	100	25	>200	>200	100	>200	200	nd
5i	$CH_3(CH_2)_7CH{=}CH(CH_2)_6$	–	100	20	68	140	>200	>200	6.25	>200	25	nd
5j	$CH_2{=}CH(CH_2)_7$	–	14	nd	29	nd	>200	>200	>200	200	>200	nd
9a	$CH_3(CH_2)_{13}$	–	75	82	nd	nd	100	200	200	>200	>200	nd
9b	$CH_3(CH_2)_5CH{=}CH(CH_2)_8$	–	93	11	nd	nd	>200	200	200	100	100	46
9c	$CH_3(CH_2)_5$	–	0	nd	nd	nd	>200	>200	>200	>200	>200	nd
9d	$CH_3(CH_2)_9$	–	30	nd	nd	nd	100	200	200	>200	>200	nd
9e	$Cy(CH_2)_3$	–	18	nd	nd	nd	200	>200	>200	>200	>200	nd
9f	$PhO(CH_2)_9$	–	47	nd	nd	nd	100	100	100	>200	>200	nd
12a	$CH_3(CH_2)_{13}$	H	42	nd	27	nd	200	50	>200	50	0.1	220
12b	$CH_3(CH_2)_7CH{=}CH(CH_2)_8$	H	49	nd	62	80	>200	100	50	200	0.1	485
12c	$CH_3(CH_2)_9$	CH_3	64	150	23	nd	>200	200	200	>200	3.13	35
12d	$CH_3(CH_2)_5$	H	0	nd	8	nd	>200	>200	>200	>200	100	43
17a	$CH_3(CH_2)_{13}$	Et	98	85	99	65	12.5	>200	>200	>200	>200	nd
17b	$CH_3(CH_2)_{13}$	Bu	100	70	99	50	25	>200	>200	>200	>200	nd
17c	$CH_3(CH_2)_{13}$	4-OMePh	100	60	100	35	12.5	>200	>200	3.12	3.12	nd
17d	$Ph(CH_2)_3$	4-OMePh	12	nd	13	nd	200	>200	>200	200	200	nd
18	$CH_3(CH_2)_7CH{=}CH(CH_2)_6$	Pr	24	nd	56	200	>200	>200	>200	>200	>200	nd

[a] Percent inhibition of *S. pneumoniae* PlsY using 200 μM of inhibitor and 50 μM of palmitoyl phosphate (16:0). [b] IC_{50} values were only determined on compounds for which there was greater than 50% inhibition in *Sp*PlsY. [c] Percent inhibition of *B. anthracis* PlsY using 200 μM of inhibitor and 50 μM of acyl phosphate (16:0). [d] IC_{50} values were only determined on compounds for which there was greater than 50% inhibition in *Ba*PlsY1. [e] Whole-cell minimum inhibitory concentration of the following species: SP = *S. pneumoniae* DAW30, MRSA = methicillin-resistant *S. aureus* ATCC33591, EF = *E. faecalis* ATCC33186, BS = *B. subtilis* ATCC 23857, and BA = *B. anthracis* Sterne 34F2. [f] Cytotoxicity: doxycycline (IC_{50} = 212 μg mL^{-1}) was used as a control, the Vero monkey kidney cell line (CCL-81) was used. [g] nd = not determined.

4850. These results further support the use of these substrate mimics as scaffolds for the design of more drug-like antibiotics with potentially safe cytotoxicity profiles.

The MIC activity of our PlsY-targeted inhibitors against the *B. anthracis* Sterne strain was interesting in view of their relatively poor activity against the other tested Gram-positive organisms. This raised concern as to whether the significant bacterial inhibition in *B. anthracis* resulted from greater permeability due to the structural cellular changes that cause this vaccine strain to be attenuated, and in particular due to the loss of the pXO2 plasmid that produces a protective capsule.[26] To address this hypothesis, the most-active compounds were tested against a panel of attenuated (capsule-deficient) and virulent (capsule-positive) strains of *B. anthracis* (Table 2). Results showed that the acyl phosphonates **4a,b** (MIC = 0.012–3.13 μg mL^{-1}) were also highly active against all strains except for the capsule-deficient lab strain (N105-2-9); whereas the reverse amide phosphonates **12a–c** (MIC = 0.024–6.25 μg mL^{-1}) and acyl sulfamate **17c** (MIC = 0.78-3.13 μg mL^{-1}) maintained good activity across all strains (Table 2). In addition to demonstrating that our substrate mimics were active against clinically relevant virulent strains of *B. anthracis*, these results revealed that the antimicrobial activity was independent of capsule status. We cannot currently rule out the possibility, however, of there being some unique additional antibacterial effects outside of PlsY inhibition to explain the specific activity against *B. anthracis* of our substrate mimics. The inhibitors were tested and shown to have comparable activity against *Bacillus cereus*, a close relative of *B. anthracis* (Table 2). This in part supports the notion of a unique mode of action against *B. anthracis* and the closely related bacterium, *B. cereus*. Through BLAST searches against the genomes of sensitive Bacilli, it was found that *B. anthracis* is unique in that it carries three *plsY* homologues, while *B. cereus* carries two *plsY* homologues. In contrast, *B. subtilus*, which only contains one PlsY, is not inhibited by this compounds series. This finding is unusual because most bacteria

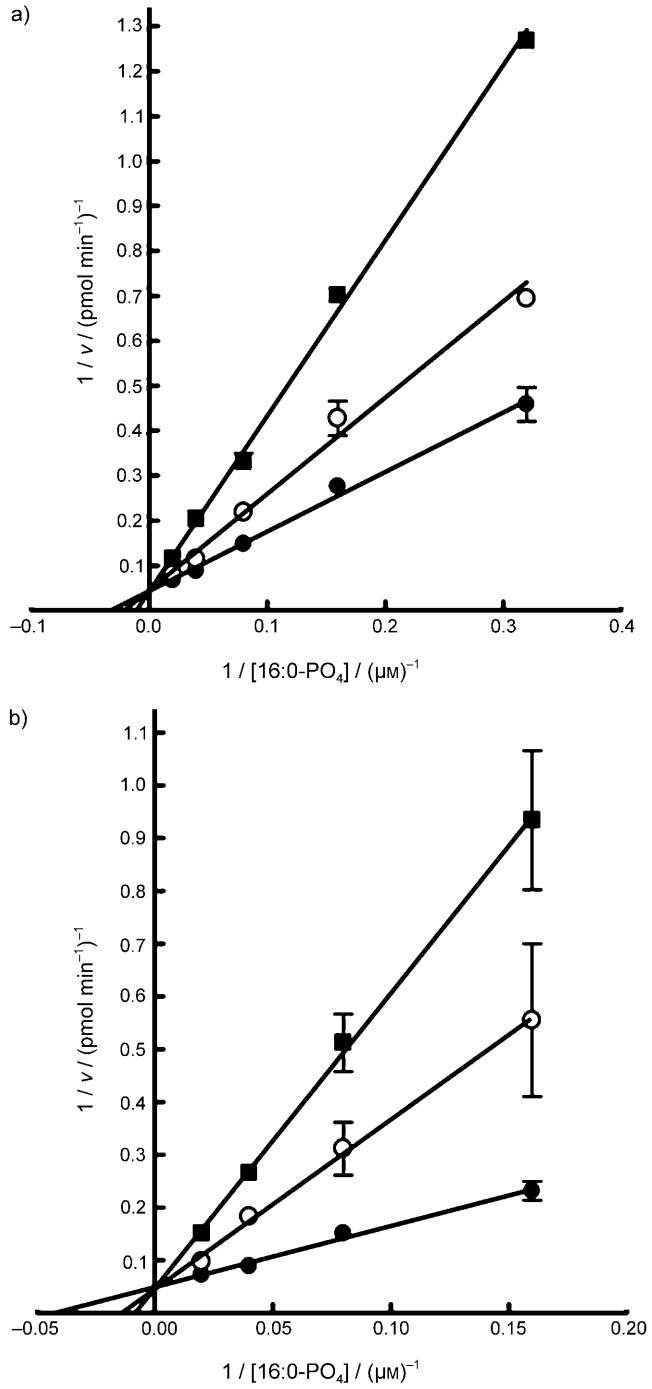


Figure 3. Double reciprocal plots for acyl phosphoramides at PlsY: a) compound **9a** (0, 25, and 200 μM) and b) compound **9b** (0, 6.25, and 25 μM).

(>99%) possess a single copy of *plsY*. Regarding the three PlsYs in *B. anthracis*, *Ba*PlsY1 is most similar to *Sp*PlsY and was chosen to study enzyme inhibition in the current study. Experiments are currently ongoing to determine if the sensitivity of *B. anthracis* to our inhibitors arises from a unique property of one or more of these alternate PlsY proteins, and to evaluate the potential for the development of this series as selective inhibitors of *B. anthracis*.

Conclusions

Novel inhibitors of the Gram-positive PlsX/PlsY pathway to phosphatidic acid have been discovered. Substrate mimics that incorporate six acyl-PO_4 bioisosteric head groups were synthesized, and based on the preliminary SAR, longer-chain analogues that match the substrate were the most active in each series. Kinetic enzyme studies showed that these compounds competitively inhibit PlsY with respect to their counterpart substrate analogue, although further studies are required to validate PlsY as the intracellular target. Nevertheless, compounds **4a–b** and **12a–c** show promise as early leads in the development of potential novel antibacterial agents against *B. anthracis* with good antimicrobial activity and low cytotoxicity. Broader antibacterial activity and strong enzyme inhibition of acyl sulfamate **17c** suggests that the synthesis of a wider panel of acyl sulfamates might be warranted towards the development of a novel class of antibiotics against Gram-positive bacteria.

Experimental Section

All reagents and anhydrous solvents were purchased from Sigma–Aldrich. All the reagent-grade solvents that were used for chromatography were purchased from Fisher Scientific (Suwanee, GA, USA) and flash column chromatography silica cartridges were obtained from Biotage Inc. (Lake Forest, VA, USA). The reactions were monitored by thin-layer chromatography (TLC) on precoated Merck 60 F254 silica gel plates and visualized by using UV light (254 nm) and iodine staining. A Biotage FLASH column chromatography system was used to purify the reaction mixtures. All ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA-500 spectrometer. Chemical shifts (δ) are reported in ppm relative to the residual solvent peak or internal standard (tetramethylsilane), and coupling constants (*J*) are reported in hertz (Hz). Mass spectra were recorded on a Bruker Esquire LC–MS by using ESI. The yields that are quoted were unoptimized. Purity of the final products were analyzed by using a Waters Acquity UPLC-PDA-ELSD-MS. UPLC separations were performed by using an Acquity UPLC 2.1×50 mm BEH C18 column (1.7 um, Waters, Milford, MA, USA) at 50 °C, and a 1.0 mL min^{-1} flow rate. The PDA was set to acquire UV data from 210–400 nm throughout the run. UPLC1: Gradient: solvent A (10 mM NH_4Cl in H_2O) and solvent B (10 mM NH_4Cl in MeCN): 0–0.2 min 10–30% B, 0.20–1.40 min 30–95% B (linear gradient), 1.40–1.70 min 95% B, 1.70–1.75 min 10%B. UPLC2: Gradient: solvent A (10 mM NH_4Cl in H_2O) and solvent B (10 mM NH_4Cl in MeCN): 0–0.2 min 10–30% B, 0.20–1.40 min 30–95% B (linear gradient), 1.40–2.70 min 95% B, 2.70–2.75 min 10% B.

General procedure for synthesis of acyl phosphonate esters (2a–h). A 1.6 M solution of *n*BuLi in hexanes (3.5 equiv) was added to THF at −78 °C. After stirring for 30 min, dimethyl methanephosphonate (3.5 equiv) was added dropwise to the mixture over 30 min to generate the carbanion. After the resulting suspension was stirred for an additional 30 min, various esters or acid chlorides (1.0 equiv) in THF were added dropwise over 30 min. The resulting suspension was allowed to stir until the temperature rose to −20 °C. The reaction was quenched with glacial AcOH (3 mL) until mildly acidic (pH 5). The mixture was extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and then concentrated in vacuo. Flash column chromatography (0–100%,

Table 2. Antimicrobial activity against a panel of *B. anthracis* and *B. cereus* strains.

Cmpd	MIC_{90} [$\mu g\,mL^{-1}$][a]											
	BA (Ames/FL)	BA (Ames/IA)	BA (Davis)	BA (Kruger)	BA NR32 (V770)	BA NR33 (107)	BA NR34 (116)	BA N105 (2–9)[b]	BA N105 (2–11)	BA N105 (2–12)	BA (Vollum)	BC (1056)
4a	0.195	0.39	0.097	0.012	0.097	0.097	0.195	50	0.049	0.049	0.097	0.05
4b	3.13	0.39	0.195	0.097	0.097	0.097	0.024	50	0.049	0.049	0.097	0.2
12a	0.78	6.25	1.56	0.195	1.56	0.39	0.049	0.097	0.097	0.049	1.56	3.13
12b	0.78	3.13	1.56	3.13	3.13	0.78	0.39	3.13	0.097	0.097	0.39	0.8
12c	0.049	0.78	1.56	0.097	0.049	0.049	0.049	3.13	0.097	0.097	0.097	0.024
17c	3.13	3.13	1.56	3.13	3.13	3.13	1.56	0.78	1.56	3.13	1.56	3.13

[a] Whole-cell minimum inhibitory activity against various strains of *B. anthracis* (BA) and *B. cereus* (BC). Penicillin (MIC = 0.008–0.03 $\mu g\,mL^{-1}$) and Ciprofloxacin (MIC = 0.03–0.06 $\mu g\,mL^{-1}$) were used as controls; [b] Capsule-deficient (attenuated) strain.

petroleum ether/EtOAc) yielded the corresponding acyl phosphonate esters. Synthesis of dimethyl 2-oxoheptadecylphosphonate (**2a)** is given as representative example.

Dimethyl 2-oxoheptadecylphosphonate (**2a**). *n*BuLi (32.0 mmol), dimethyl methanephosphonate (32.0 mmol), methylpalmitate (9.24 mmol) and THF (45 mL) were used to synthesize **2a** as a white solid (89%); mp: 47–49 °C. TLC: $R_f = 0.5$ (EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 0.90$ (t, $J = 6.5$ Hz, 3H), 1.27–1.33 (br s, 24H), 1.60 (m, 2H), 2.63 (t, $J = 7.0$ Hz, 2H), 3.11 (d, $J = 23.0$ Hz, 2H), 3.80 (s, 3H), 3.82 ppm (s, 3H); MS (ESI): *m/z*: 385.9 $[M+Na]^+$, 361.3 $[M-H]^-$.

General procedure for synthesis of acyl α,α-difluoromethyl phosphonate esters (3a, e, i, j): A freshly prepared solution of LDA (2 equiv) in THF was cooled to −78 °C for 30 min, before a solution of diethyl α,α-difluoromethyl phosphonate (2.0 equiv) in THF (1 mL) was added dropwise to the mixture over 10 min to generate the carbanion. After the resulting suspension was stirred for an additional 45 min, various esters or acid chlorides (1.0 equiv) in THF (5 mL) were added dropwise over 10 min. The resulting suspension was allowed to stir for 2.5 h. After the reaction was complete, it was quenched with glacial AcOH (2 mL) followed by sat. NH_4Cl. The mixture was allowed to warm to RT and extracted with $CHCl_3$. The organic extracts were washed with brine, dried over Na_2SO_4, and then concentrated in vacuo. Flash column chromatography (petroleum ether/EtOAc, 0–100%) yielded the desired acyl α,α-difluoromethyl phosphonate esters. Synthesis of diethyl 1,1-difluoro-2-oxoheptadecylphosphonic acid (**3a)** is given as representative example.

Diethyl 1,1-difluoro-2-oxoheptadecylphosphonic acid (3a): *n*BuLi (4.25 mmol), iPr$_2$NH (4.25 mmol), diethyl α,α-difluromethyl phosphonate (4.25 mmol), methylpalmitate (2.13 mmol) and THF (30 mL) were used to synthesize **3a** as a colorless oil (55%). TLC: $R_f = 0.5$ (petroleum ether/EtOA, 8:2); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta =$ 0.89 (t, $J = 7.0$ Hz, 3H), 1.25 (br s, 24H), 1.39 (t, $J = 7.0$ Hz, 6H), 1.63 (m, 2H), 2.77 (t, $J = 7.5$ Hz, 2H), 4.31 ppm (m, 4H); MS (ESI): *m/z*: 449.4 $[M+Na]^+$, 425.1 $[M-H]^-$.

General procedure for synthesis of acyl phosphoramidates (8a–f): Various esters or acids (1.0 equiv) were treated with KOH (1.0 equiv) in H_2O/MeCN (1:1). The reaction was stirred and heated at 110 °C until dry, then subjected to high vacuum for 16 h. CH_2Cl_2 (25 mL) was added to the flask. Ethylchloroformate (1.0 equiv) was added to the resulting suspension, which was stirred for 7 h, then chilled to −78 °C. Pretreatment of diethyl phosphoramidate (3.2 equiv) with 1.6 M *n*BuLi (3.2 equiv) to generate the anion was performed before slow addition to the activated acyl species. The reaction was allowed to stir until the temperature rose to 25 °C. The mixture was quenched with glacial AcOH (3 mL) until mildly acidic (pH 5), then extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and then concentrated in vacuo. Flash column chromatography (petroleum ether/EtOAc, 0–100%) yielded the desired acyl phosphoramide esters. Synthesis of diethyl 1-oxohexadecylphosphoramidate (**8a)** is given as representative example.

Diethyl 1-oxohexadecylphosphoramidate (8a): Methylpalmitate (10.0 mmol), MeCN/H_2O (1:1, 20 mL), KOH (10.0 mmol), ethyl chloroformate (10.0 mmol), *n*BuLi (35.0 mmol), and diethyl phosphoramidate (35.0 mmol) were used to synthesize **8a** as a white solid (25%), mp: 53–56 °C. TLC: $R_f = 0.6$ (EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 0.90$ (t, $J = 6.5$ Hz, 3H), 1.27 (br s, 24H) 1.38 (t, $J =$ 7.0 Hz, 6H), 1.65 (m, 2H), 2.36 (t, $J = 7.0$ Hz, 2H), 4.22 (m, 4H), 8.34 ppm (s, 1H); MS (ESI): *m/z*: 390.1 $[M-H]^-$.

General procedure for synthesis of acyl reverse amide phosphonates (11a–d): The appropriate amine (1.0 equiv) was added dropwise to a stirring solution of HOBt (1.0 equiv), diethylphosphonoacetic acid (1.0 equiv), and EDCI (1.0 equiv) in CH_2Cl_2/DMF (1:1). The reaction was allowed to stir at RT for 16 h, then the mixture was extracted with $CHCl_3$. The organic layer was washed with $NaHCO_3$, H_2O, and brine, then dried over Na_2SO_4, and concentrated in vacuo. Flash column chromatography (petroleum ether/EtOAc, 0–100%) yielded the desired reverse amide phosphonate ester. Synthesis of diethyl (tetradecylcarbamoyl) methylphosphonic acid (**11a**) is given as representative example.

Diethyl (tetradecylcarbamoyl)methylphosphonic acid (11a): HOBt (5.0 mmol), EDCI (5.0 mmol), diethylphosphonoacetic acid (5.0 mmol), CH_2Cl_2/DMF (50 mL), and tetradecylamine (5.0 mmol) were used to synthesize **11a** as a yellow solid (29%), mp: 32–34 °C. TLC: $R_f = 0.3$ (EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 0.84$ (t, $J =$ 6.5 Hz, 3H), 1.22 (br s, 22H) 1.31 (t, $J = 7.0$ Hz, 6H), 1.47 (m, 2H), 2.82 (d, $J = 20.5$ Hz, 2H), 3.21 (q, $J = 6.5$ Hz, 2H), 4.11 (m, 4H), 6.91 ppm (br t, 1H); MS (ESI): *m/z*: 390.1 $[M-H]^-$, 414.4 $[M+Na]^+$.

General procedure for ester deprotection (4a–h, 5a,e,i,j, 9a–d, and 12a–d): Each ester (1.0 equiv) was treated with CH_2Cl_2 (2 mL). The flask was flushed several times with argon, then TMSBr (10.0 equiv) was added slowly to the mixture. The resulting solution was allowed to stir 16 h at RT. Excess TMSBr was removed in vacuo. 95% EtOH (5 mL) was added to the reaction flask, and the solution was allowed to stir for 1 h, after which the solvent was removed in vacuo. The residue was triturated several times with Et_2O and filtered.

2-Oxoheptadecylphosphonic acid (4a): Dimethyl 2-oxoheptadecylphosphonate (2.76 mmol), TMSBr (27.6 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **4a** as a white solid (89% overall), mp: 91–93 °C. UPLC1: t_R = 1.18 min, purity: > 99%; ^{1}H NMR ([D$_6$]DMSO, 500 MHz): δ = 0.86 (t, J = 6.0 Hz, 3H), 1.25 (brs, 24H), 1.43 (m, 2H), 2.57 (t, J = 7.0 Hz, 2H), 2.92 (d, J = 22.5 Hz, 2H); ^{13}C NMR ([D$_6$]DMSO, 500 MHz): δ = 14.4, 22.6, 23.4, 29.0, 29.2, 29.4, 29.5, 29.6, 31.8, 43.1, 45.1, 46.1. 203.9 ppm; MS (ESI): m/z: 333.4 $[M-H]^-$.

(*Z*)-2-Oxononadec-12-enylphosphonic acid (4b): (*Z*)-Dimethyl 2-oxononadec-12-enylphosphonate (0.5 mmol), TMSBr (5.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **4b** as a light-yellow waxy solid (77% overall). UPLC1: t_R = 1.25 min, purity: 96%;^{1}H NMR (CD_3OD, 500 MHz): δ = 0.93 (t, J = 7.0 Hz, 3H), 1.33 (brs, 20H), 1.58 (m, 2H), 2.05 (q, J = 8.5 Hz, 4H), 2.67 (t, J = 7.5 Hz, 2H), 3.09 (d, J = 22.5 Hz, 2H,), 5.36 ppm (m, 2H); ^{13}C NMR (CD_3OD, 500 MHz): δ = 13.1, 22.3, 23.1, 26.8, 28.7, 28.9, 29.2, 29.4, 29.5, 31.5, 39.0, 43.3, 129.5, 204.1 ppm; MS (ESI): m/z: 359.2 $[M-H]^-$.

2-Oxononylphosphonic acid (4c): Dimethyl 2-oxononylphosphonate (0.4 mmol), TMSBr (4.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **4c** as a light-yellow oil (14% overall). UPLC1: t_R = 0.37 min, purity: 98%; ^{1}H NMR (CD_3OD, 500 MHz): δ = 0.92 (t, J = 7.0 Hz, 3H), 1.32 (brs, 8H), 1.58 (m, 2H), 2.67 (t, J = 7.0 Hz, 2H), 3.08 ppm (d, J = 20 Hz, 2H); ^{13}C NMR (CD_3OD, 500 MHz): δ = 13.0, 22.3, 23.1, 28.7, 28.8, 31.5, 43.2, 204.1 ppm; MS (ESI): m/z: 223.1 $[M+H]^+$, 221.2 $[M-H]^-$.

2-Oxopentylphosphonic acid (4d): Dimethyl 2-oxopentylphosphonate (2.3 mmol), TMSBr (23.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **4d** as a yellow oil (76% overall). UPLC1: t_R = 0.15 min, purity: > 99%;^{1}H NMR ($CDCl_3$, 500 MHz): δ = 0.91 (t, J = 7.5 Hz, 3H), 1.59 (m, 2H), 2.62 (t, J = 7.0 Hz, 2H), 3.20 ppm (d, J = 22.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 500 MHz): δ = 13.0, 16.6, 31.6, 43.2, 204.0; MS (ESI): m/z: 167.0 $[M+H]^+$, 164.7 $[M-H]^-$.

2-Oxotridecylphosphonic acid (4e): Dimethyl 2-oxotridecylphosphonate (3.0 mmol), TMSBr (30.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **4e** as a white solid (73% overall), mp: 81–84 °C. UPLC1: t_R = 0.73 min, purity: > 99%; ^{1}H NMR (CD_3OD, 500 MHz): δ = 0.92 (t, J = 6.0 Hz, 3H), 1.31 (brs, 16H), 1.57 (m, 2H), 2.67 (t, J = 7.0 Hz, 2H), 3.09 ppm (d, J = 22.5 Hz, 2H); ^{13}C NMR (CD_3OD, 500 MHz): δ = 13.1, 22.4, 23.1, 28.7, 29.1, 29.2, 29.3, 29.4, 31.7, 43.2, 204.1 ppm; MS (ESI): m/z: 279.2 $[M+H]^+$, 277.0 $[M-H]^-$.

6-Cyclohexyl-2-oxohexylphosphonic acid (4f): Dimethyl 6-cyclohexyl-2-oxohexylphosphonate (2.7 mmol), TMSBr (27.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **4f** as a light-yellow oil (69% overall). UPLC1: t_R = 0.51 min, purity: > 99%; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 0.90 (m, 2H), 1.12–1.36 (m, 6H), 1.53- 1.68 (m, 10H), 2.69 (t, J = 7.0 Hz, 2H), 3.06 ppm (d, J = 23.0 Hz, 2H); ^{13}C NMR ($CDCl_3$, 500 MHz): δ = 23.7, 26.2, 26.3, 26.7, 33.3, 37.2, 37.4, 44.2, 204.6 ppm; MS (ESI): m/z: 260.8 $[M-H]^-$.

2-Oxo-6-phenylhexylphosphonic acid (4g): Dimethyl 2-oxo-6-phenylhexylphosphonate (2.2 mmol), TMSBr (22.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **4g** as a light-yellow oil (62% overall). UPLC1: t_R = 0.36 min, purity: > 99%; ^{1}H NMR (CD_3OD, 500 MHz): δ = 1.56 (m, 4H), 2.58 (m, 4H), 3.13 (d, J = 22.5 Hz, 2H), 7.14 (t, J = 8.0 Hz, 3H), 7.24 ppm (t, J = 8.0 Hz, 2H); ^{13}C NMR (CD_3OD, 500 MHz): δ = 23.1, 30.6, 35.6, 44.2, 47.3, 125.8, 128.4, 142.1, 205.0 ppm; MS (ESI): m/z: 257.0 $[M+H]^+$, 254.8 $[M-H]^-$.

2-Oxo-12-phenoxydodecylphosphonic acid (4h): Dimethyl 2-oxo-12-phenoxydodecylphosphonate (2.7 mmol), TMSBr (27.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **4h** as a white solid (66% overall), mp: 93–95 °C. UPLC1: t_R = 0.78 min, purity: > 99%; ^{1}H NMR (CD_3OD, 500 MHz): δ = 1.34 (brs, 10H), 1.49 (m, 2H), 1.58 (m, 2H), 1.78 (m, 2H), 2.67 (t, J = 7.0 Hz, 2H), 3.09 (d, J = 22.5 Hz, 2H), 3.97 (t, J = 6.5 Hz, 2H), 6.91 (d, J = 8.0 Hz, 2H), 6.91 (t, J = 7.5 Hz, 1H), 7.26 ppm (t, J = 7.0 Hz, 2H); ^{13}C NMR (CD_3OD, 500 MHz): δ = 23.1, 25.8, 28.7, 29.1, 29.2, 29.3, 43.3, 67.5, 114.1, 120.1, 129.0, 159.2, 204.1 ppm; MS (ESI): m/z: 355.0 $[M-H]^-$.

1,1-Difluoro-2-oxoheptadecylphosphonic acid (5a): Diethyl 1,1-difluoro-2-oxoheptadecylphosphonic acid (0.1 mmol), TMSBr (1.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **5a** as a white solid (52% overall), mp: 45–48 °C. UPLC1: t_R = 1.19 min, purity: 93%; ^{1}H NMR ([D$_6$]DMSO, 500 MHz): δ = 0.87 (t, J = 6.5 Hz, 3H), 1.25 (brs, 24H), 1.51 (m, 2H), 2.76 ppm (t, J = 7.0 Hz, 2H); ^{13}C NMR ([D$_6$]DMSO, 500 MHz): δ = 13.2, 22.3, 28.6, 29.1, 29.2, 29.3, 29.4, 199.8 ppm; MS (ESI): m/z: 371.3 $[M+H]^+$, 369.1 $[M-H]^-$.

1,1-Difluoro-2-oxotridecylphosphonic Acid (5e): Diethyl 1,1-difluoro-2-oxotridecylphosphonic acid (0.1 mmol), TMSBr (1.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **5e** as a colorless oil (66% overall). ^{1}H NMR ([D$_6$]DMSO, 500 MHz): δ = 0.92 (t, J = 7.0 Hz, 3H), 1.31 (brs, 16H), 1.62 (m, 2H), 2.85 ppm (t, J = 7.0 Hz; 2H); ^{13}C NMR ([D$_6$]DMSO, 500 MHz): δ = 13.1, 22.4, 28.7, 29.1, 29.1, 29.2, 29.3, 31.7, 37.4, 110.0 ppm; MS (ESI): m/z: 312.9 $[M-H]^-$.

(*Z*)-1,1-Difluoro-2-oxononadec-10-enylphosphonic acid (5i): Diethyl (*Z*)-1,1-difluoro-2-oxononadec-10-enylphosphonic acid (0.1 mmol), TMSBr (1.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **5i** as a colorless oil (74% overall). UPLC1: t_R = 0.70 min, purity: 96%; ^{1}H NMR ([D$_6$]DMSO, 500 MHz): δ = 0.92 (t, J = 6.5 Hz, 3H), 1.34 (brd, 20H), 1.62 (m, 2H), 2.05 (q, J = 5.0 Hz, 4H), 2.86 (t, J = 7.0 Hz, 2H), 5.37 ppm (m, 2H); ^{13}C NMR ([D$_6$]DMSO, 500 MHz): δ = 13.1, 22.4, 24.7, 26.7, 28.7, 28.8, 28.9, 29.0, 29.4, 31.7, 37.4, 129.5, 156.0 ppm; MS (ESI), m/z: 395.0 $[M-H]^-$.

1,1-Difluoro-2-oxododec-11-enylphosphonic acid (5j): Diethyl 1,1-difluoro-2-oxododec-11-enylphosphonic acid (1.0 mmol), TMSBr (10.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **5j** as a colorless oil (37% overall). UPLC1: t_R = 0.51 min, purity: > 90%; ^{1}H NMR (CD_3OD, 500 MHz): δ = 1.27 (brs, 10H), 1.59 (m, 2H), 2.03 (q, J = 5.0 Hz; 2H), 2.78 (t, J = 7.0 Hz; 2H), 4.96 (dd, J = 25 Hz, 15 Hz, 2H), 5.80 ppm (m, 1H); ^{13}C NMR (CD_3OD, 500 MHz): δ = 22.4, 28.6, 28.7, 28.8, 29.0, 29.1, 33.5, 37.4, 113.3, 138.7 ppm; MS (ESI): m/z: 296.9 $[M-H]^-$.

1-Oxohexadecylphosphoramidic acid (9a): Diethyl 1-oxohexadecylphosphoramidate (1.0 mmol), TMSBr (1.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **9a** as a white solid (24% overall), mp: 56–58 °C. ^{1}H NMR ([D$_6$]DMSO, 500 MHz): δ = 0.87 (t, J = 6.5 Hz, 3H), 1.25 (brs, 24H), 1.47 (m, 2H), 2.18 (t, J = 7.0 Hz, 2H), 8.98 ppm (d, J = 9.0 Hz, 1H); ^{13}C NMR ([D$_6$]DMSO, 500 MHz): δ = 13.0, 22.3, 26.6, 28.9, 29.0, 29.3, 29.3, 29.4, 31.7, 35.6, 36.6, 39.4, 110.0, 166.7 ppm; MS (ESI): m/z: 334.3 $[M-H]^-$.

(*Z*)-1-Oxooctadec-11-enylphosphoramidic acid (9b): Diethyl (*Z*)-1-oxooctadec-11-enylphosphoramidate (0.1 mmol), TMSBr (1.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **9b** as a white solid (19% overall), mp: 76–80 °C. ^{1}H NMR ([D$_6$]DMSO, 500 MHz): δ = 0.93 (t, J = 7.0 Hz, 3H), 1.33 (brs, 20H), 1.64 (m, 2H), 2.05 (q, J = 9.0 Hz, 4H), 2.32 (t, J = 7.0 Hz, 2H), 5.37 ppm (m, 2H); ^{13}C NMR ([D$_6$]DMSO, 500 MHz): δ = 13.1, 22.3, 24.9, 26.8, 28.6, 28.9, 29.0, 29.1, 29.2, 29.5, 31.5, 129.5, 176.5 ppm; MS (ESI): m/z: 360.1 $[M-H]^-$.

1-Oxooctylphosphoramidic acid (9c): Diethyl 1-oxooctylphosphoramidate (1.5 mmol), TMSBr (15.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **9c** as a white solid (32% overall), mp: 75–77 °C. ^{1}H NMR (CD_3OD, 500 MHz): δ = 0.93 (t, J = 7.0 Hz, 3H), 1.35 (brs, 8H), 1.64 (m, 2H), 2.32 ppm (brs, 2H); ^{13}C NMR (CD_3OD, 500 MHz):

δ=13.1, 22.3, 24.8, 28.8, 31.5, 176.8 ppm; MS (ESI): *m/z*: 221.9 $[M-H]^-$, 224.0 $[M+H]^+$.

1-Oxododecylphosphoramidic acid (9d): Diethyl 1-oxododecylphosphoramidate (0.6 mmol), TMSBr (6.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **9d** as a white solid (10% overall). ^{1}H NMR (CD_3OD, 500 MHz): δ=0.92 (t, *J*=6.5 Hz, 3H), 1.32 (brs, 16H), 1.63 (m, 2H), 2.31 ppm (t, *J*=7.5 Hz, 2H); ^{13}C NMR (CD_3OD, 500 MHz): δ=13.0, 22.3, 24.8, 28.8, 29.0, 29.2, 29.3, 31.7, 168.6 ppm; MS (ESI): *m/z*: 277.9 $[M-H]^-$, 280.1 $[M+Na]^+$.

5-Cyclohexyl-1-oxopentylphosphoramidic acid (9e): Diethyl 5-cyclohexyl-1-oxopentylphosphoramidate (0.6 mmol), TMSBr (6.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **9e** as a white solid (35% overall). ^{1}H NMR (CD_3OD, 500 MHz): δ=0.90 (m, 2H), 1.23 (m, 6H), 1.37 (m, 2H), 1.61 (m, 2H), 1.73 (m, 2H), 2.32 ppm (t, *J*=7.5 Hz, 2H); ^{13}C NMR (CD_3OD, 500 MHz): δ=25.1, 26.1, 26.4, 33.2, 37.0, 37.5, 176.7 ppm; MS (ESI): *m/z*: 264.1 $[M+H]^+$, 261.9 $[M-H]^-$.

1-Oxo-11-phenoxyundecylphosphoramidic acid (9f): Diethyl 1-oxo-11-phenoxyundecylphosphoramidate (0.5 mmol), TMSBr (5.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **9f** as a white solid (15% overall), mp: 100–103 °C. ^{1}H NMR (CD_3OD, 500 MHz): δ=1.35 (brs, 10H), 1.49 (m, 2H), 1.63 (m, 2H), 1.77 (m, 2H), 2.32 (brt, *J*=7.5 Hz, 2H), 3.96 (t, *J*=7.0 Hz, 2H), 6.91 (t, *J*=8.0 Hz, 3H), 7.26 ppm (t, *J*=7.5 Hz, 2H); ^{13}C NMR (CD_3OD, 500 MHz): δ=24.8, 25.8, 28.8, 29.0, 29.1, 29.3, 67.5, 114.1, 120.1, 129.0, 159.2, 176.6 ppm; MS (ESI): *m/z*: 358.3 $[M+H]^+$, 356.0 $[M-H]^-$.

(Tetradecylcarbamoyl)methylphosphonic acid (12a): Diethyl (tetradecylcarbamoyl) methylphosphonic acid (1.0 mmol), TMSBr (30.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **12a** as a white solid (10% overall), mp: 133–135 °C. UPLC1: t_R=1.75 min, purity: >99%; ^{1}H NMR ([D$_6$]DMSO, 500 MHz): δ=0.87 (t, *J*=6.5 Hz, 3H), 1.25 (brs, 22H), 1.38 (m, 2H), 2.57 (d, *J*=20.5 Hz, 2H), 3.03 (q, *J*=6.0 Hz, 2H), 7.74 ppm (t, *J*=5.5 Hz, 1H); ^{13}C NMR ([D$_6$]DMSO, 500 MHz): δ=13.0, 22.3, 26.6, 28.8, 29.0, 29.2, 29.4, 31.7, 39.4 ppm; MS (ESI): *m/z*: 334.1 $[M-H]^-$, 336.3 $[M+H]^+$.

((*Z*)-Octadec-9-enylcarbamoyl)methylphosphonic acid (12b): Diethyl ((*Z*)-octadec-9-enylcarbamoyl)methylphosphonic acid (0.1 mmol), TMSBr (3.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **12b** as a white solid (17% overall), mp: 111–114 °C. UPLC1: t_R=1.32 min, purity: 96%; ^{1}H NMR (CD_3OD, 500 MHz): δ=0.92 (t, *J*=6.5 Hz, 3H), 1.33 (brd, 22H), 1.54 (m, 2H), 2.05 (m, 4H), 2.81 (d, *J*=21.0 Hz, 2H), 3.21 (t, *J*=7.5 Hz, 2H), 5.37 ppm (m, 2H); ^{13}C NMR (CD_3OD, 500 MHz): δ=13.01, 22.4, 26.6, 26.8, 28.9, 29.0, 29.1, 29.2, 29.4, 29.5, 31.7, 38.9, 39.5, 129.5, 166.7 ppm; MS (ESI): *m/z*: 388.0 $[M-H]^-$.

(*N*-Dodecyl-*N*-methylcarbamoyl)methylphosphonic acid (12c): Diethyl (*N*-dodecyl-*N*-methylcarbamoyl)methylphosphonic acid (0.1 mmol), TMSBr (3.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **12c** as a white solid (10% overall), mp: 80–83 °C. UPLC1: t_R=0.83 min, purity: >99%; ^{1}H NMR (CD_3OD, 500 MHz): δ=0.92 (t, *J*=6.5 Hz; 3H), 1.31 (brs, 18H), 1.62 (m, 2H), 3.06 (d, *J*=22.5 Hz, 2H), 3.06 (brd, 3H), 3.44 ppm (dt, *J*=7.5 Hz, 29.5 Hz, 2H); ^{13}C NMR (CD_3OD, 500 MHz): δ=13.1, 22.3, 26.5, 26.7, 27.9, 29.1, 29.4, 31.7, 33.0, 36.0, 51.1, 167.4 ppm; MS (ESI): *m/z*: 320.0 $[M-H]^-$, 322.3 $[M+H]^+$.

(Heptylcarbamoyl)methylphosphonic acid (12d): Diethyl (heptylcarbamoyl)methylphosphonic acid (0.1 mmol), TMSBr (3.0 mmol) and CH_2Cl_2 (2 mL) were used to synthesize **12d** as a white solid (28% overall), mp: 137–140 °C. UPLC1: t_R=0.35 min, purity: >99%; ^{1}H NMR (CD_3OD, 500 MHz): δ=0.93 (t, *J*=6.0 Hz, 3H), 1.34 (brm, 8H) 1.54 (m, 2H), 2.81 (d, *J*=21.0 Hz, 2H), 3.21 ppm (t, *J*=7.0 Hz, 2H,); ^{13}C NMR (CD_3OD, 500 MHz): δ=13.0, 22.3, 26.5, 28.7, 28.9, 31.5, 35.6, 39.4, 166.7 ppm; MS (ESI): *m/z*: 235.8 $[M-H]^-$, 238.0 $[M+H]^+$.

General procedure for synthesis of acyl sulfamates (17a–d): Anhydrous formic acid (99%, 2.0 equiv) was added dropwise to chlorosulfonyl isocyanate (2.0 equiv) at 0 °C under argon. The mixture was allowed to rise to RT and stirred until gas evolution stopped (~2 h). The desired alcohol (1.0 equiv) in DMA (30 mL) was added dropwise to the resulting sulfamoyl chloride at 0 °C under argon. The mixture was allowed to stir at 0 °C for 10 min, then allowed to warm to RT and stirred an additional 3 h. The mixture was then poured into cold brine (100 mL) and extracted with EtOAc. The combined extracts were washed with H_2O and brine, then dried over Na_2SO_4 and concentrated in vacuo. The residue was purified via flash chromatography in hexane/EtOAc to afford the desired sulfamate ester (**15**). Acid chlorides were generated by adding oxalyl chloride (2.0 equiv) and DMF (2 drops) to appropriate acids (1.0 equiv) in THF (15 mL) at 0 °C under argon. After gas evolution stopped (~20 min) the mixture was warmed to RT and allowed to stir for 2 h. The resulting mixture was concentrated in vacuo then dissolved in CH_2Cl_2/DMF (1:1, 5 mL) and added dropwise to a mixture of previously prepared sulfamate ester, **15** (1.0 equiv), DMAP (cat., 10% mol), Et_3N (3.0 equiv) in CH_2Cl_2 /DMF (1:1, 15 mL). The mixture was allowed to stir for 16 h under argon. The mixture was diluted with CH_2Cl_2 and washed with 1 N HCl, H_2O, and brine then dried over Na_2SO_4 and concentrated in vacuo. The residue was purified via flash chromatography (hexane/EtOAc, 0–100%) to afford the desired acyl sulfamate.

Ethyl palmitoylsulfamate (17a): Chlorosulfonyl isocyanate (40.0 mmol), formic acid (40.0 mmol), EtOH (20.0 mmol), DMA (30 mL), palmitic acid (6.0 mmol), oxalylchloride (12.0 mmol), Et_3N (18.0 mmol), and DMAP (0.6 mmol) were used to synthesize **17a** as a white solid (25%), mp: 85–87 °C. TLC: R_f=0.5 ($CHCl_3$/MeOH, 8:2); UPLC1: t_R=1.57 min, purity: >99%; ^{1}H NMR ($CDCl_3$, 500 MHz): δ=0.88 (t, *J*=6.0 Hz, 3H,), 1.26 (brs, 24H), 1.43 (t, *J*=7.0 Hz, 3H), 1.66 (m, 2H), 2.39 (t, *J*=7.5 Hz, 2H), 4.48 (q, *J*=8.0 Hz, 2H), 8.39 ppm (s, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz): δ=14.1, 14.7, 22.7, 24.4, 29.0, 29.3, 29.4, 29.6, 29.7, 31.9, 35.9, 70.8, 171.3 ppm; MS (ESI): *m/z*: 386.3 $[M+Na]^+$, 362.1 $[M-H]^-$.

Butyl palmitoylsulfamate (17b): Chlorosulfonyl isocyanate (40.0 mmol), formic acid (40.0 mmol), butanol (20.0 mmol), DMA (30 mL), palmitic acid (6.0 mmol), oxalylchloride (12.0 mmol), Et_3N (18.0 mmol), and DMAP (0.6 mmol) were used to synthesize **17b** as a white solid (46%), mp: 74–76 °C. TLC: R_f=0.5 ($CHCl_3$/MeOH, 8:2); UPLC1: t_R=1.74 min, purity: >99%; ^{1}H NMR ($CDCl_3$, 500 MHz): δ=0.88 (t, *J*=6.5 Hz, 3H), 0.95 (t, *J*=7.5 Hz, 3H), 1.26 (brs, 24H), 1.44 (m, 2H), 1.65 (m, 2H), 1.75 (m, 2H), 2.39 (t, *J*=7.5 Hz, 2H), 4.40(t, *J*=6.5 Hz, 2H), 8.18 ppm (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ=13.4, 14.1, 18.6, 22.7, 24.4, 29.0, 29.3, 29.4, 29.6, 29.7, 30.8, 31.9, 35.9, 74.4, 171.4 ppm; MS (ESI): *m/z*: 414.4 $[M+Na]^+$, 390.1 $[M-H]^-$.

4-Methoxyphenyl palmitoylsulfamate (17c): Chlorosulfonyl isocyanate (40.0 mmol), formic acid (40.0 mmol), 4-methoxyphenol (20.0 mmol), DMA (30 mL), palmitic acid (6.0 mmol), oxalylchloride (12.0 mmol), Et_3N (18.0 mmol), and DMAP (0.6 mmol) were used to synthesize **17c** as a white solid (10%), mp: 74–76 °C. TLC R_f=0.9 (EtOAc). UPLC1: t_R=1.58 min, purity: > 99%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ=0.90 (t, *J*=6.5 Hz, 3H), 1.27 (brs, 24H), 1.64 (m, 2H), 2.38 (t, *J*=7.0 Hz, 2H), 3.81(s, 3H), 6.89 (d, *J*=9.0 Hz, 2H),

7.21 ppm (d, $J=9.0$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 500 MHz): $\delta=14.1$, 22.7, 24.5, 29.0, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 36.2, 55.6, 114.18, 122.3, 143.2, 158.7, 171.4 ppm; MS (ESI): m/z: 464.2 $[M+Na]^+$, 440.0 $[M-H]^-$.

4-Methoxyphenyl 5-phenylpentanoylsulfamate (17 d): Chlorosulfonyl isocyanate (40.0 mmol), formic acid (40.0 mmol), 4-methoxyphenol (20.0 mmol), DMA (30 mL), 5-phenylpentanoic acid (6.0 mmol), oxalylchloride (12.0 mmol), Et_3N (18.0 mmol), and DMAP (0.6 mmol) were used to synthesize **17 d** as a colorless waxy solid (10%). TLC: $R_f=0.8$ (EtOAc); UPLC1: $t_R=0.61$ min, purity: >99%; ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta=1.60$ (br s, 4H), 2.32 (t, $J=6.0$ Hz, 2H), 2.57 (t, $J=7.0$ Hz, 2H), 3.73 (s, 3H), 6.81 (d, $J=9.0$ Hz, 2H), 7.22 ppm (m, 7H); ^{13}C NMR ($CDCl_3$, 500 MHz): $\delta=24.2$, 30.7, 35.5, 36.5, 55.6, 114.8, 122.9, 125.9, 128.4, 141.9, 143.4, 158.6, 171.3 ppm; MS (ESI), m/z: 386.1 $[M+Na]^+$, 361.9 $[M-H]^-$.

Synthesis of acyl sulfamide (18): Anhydrous formic acid (99%, 40.0 mmol, 2.0 equiv) was added dropwise to chlorosulfonyl isocyanate (40.0 mmol, 2.0 equiv) at 0 °C under argon. The mixture was allowed to rise to RT and stirred until gas evolution stopped (~2 h). Propylamine (20.0 mmol, 2.0 equiv) was added dropwise to the resulting sulfamoyl chloride at 0 °C under argon. The mixture was allowed to stir at 0 °C for 30 min, and then warmed to RT. NaOH (5 mL) was added to the mixture, and the solution was stirred for 1 h. The mixture was then extracted with EtOAc. The combined extracts were washed with brine, then dried over Na_2SO_4 and concentrated in vacuo to afford the propylsulfamide ester (**16**). The acid chloride was generated by adding oxalyl chloride (12.0 mmol, 2.0 equiv) and DMF (0.6 mmol, 10% mol) to oleic acid (6.0 mmol, 1.0 equiv) in THF (15 mL) at 0 °C under argon. After gas evolution stopped (~20 min) the mixture was warmed to RT and allowed to stir for 2 h. The resulting mixture was concentrated in vacuo then dissolved in CH_2Cl_2/DMF (1:1, 5 mL) and added dropwise to a mixture of previously prepared propylsulfamide ester, **16** (6.0 mmol, 1.0 equiv), DMAP (0.6 mmol, 10 mol%), Et_3N (18.0 mmol, 3.0 equiv) in CH_2Cl_2 /DMF (1:1, 15 mL). The mixture was allowed to stir for 16 h under argon. The mixture was diluted with CH_2Cl_2 and washed with 1 N HCl, H_2O, and brine then dried over Na_2SO_4 and concentrated in vacuo. The residue was purified via flash chromatography (hexane/EtOAc, 0–100%) to afford oleoyl *N*-propylsulfamide **18** as a waxy solid (8%). $R_f=0.7$ ($CHCl_3$/MeOH, 8:2); UPLC1: $t_R=2.01$ min, purity: >99%; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta=0.88$ (t, $J=6.5$ Hz, 3H), 0.94 (t, $J=7.0$ Hz, 3H), 1.28 (br s, 20H), 1.59 (m, 4H), 2.00 (m, 4H), 2.62 (t, $J=7.5$ Hz, 2H), 2.95 (t, $J=6.5$ Hz, 2H), 5.34 ppm (m, 2H); ^{13}C NMR ($CDCl_3$, 500 MHz): $\delta=11.3$, 14.1, 22.5, 22.7, 27.2, 29.2, 29.3, 29.5, 29.7, 29.8, 31.9, 45.6, 129.6, 130.1, 174.8 ppm; MS (ESI): m/z: 401.1 $[M-H]^-$.

Membrane preparation and assay of acyltransferase activity: *E. coli* membranes overexpressing either *S. pneumonia* PlsY (SPR0755) or *B. anthracis* PlsY1 (BAS3399) were purified, and acyltransferase activity was measured as described previously.[8] Briefly, the reaction buffer (pH 7.4) contained 100 mM Tris–HCl, 150 mM NaCl, 1 mg mL^{-1} BSA, 5 mM Na_3VO_4, 100 μM [U-^{14}C]G3P (8 mCi mmol^{-1}), inhibitor (200 μ) and 4 μ purified membranes. 16:0-PO_4 (final concentration 50 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^{-1}) prior to scintillation counting. The K_M for 16:0-PO_4 was obtained by varying the concentration of 16:0-PO_4 from 3.125–50 μM at a fixed [^{14}C]G3P concentration of 200 μM.

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 M7 A7 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^{-1} 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^{-1}. Bacterial inoculum was prepared by streaking a −80 °C stock bacterial culture onto an MH agar plate, which was incubated overnight at 37 °C. 2–3 colonies were picked from the plate and used to establish a bacterial inoculum at an optical density of $\lambda_{600}=0.1$ in MH broth, which was further diluted to an OD$\lambda_{600}=0.001$. An aliquot of culture (100 μL) was then added to each well of the 96-well plate to give an OD$\lambda_{600}=0.0005$, which corresponded to about 10^5 CFU mL^{-1}, and final antibiotic concentrations that ranged from 200–0.0125 μg mL^{-1}. The 96-well plates were incubated overnight at 37 °C, and the MIC was recorded as the lowest concentration of drug that inhibited visible bacterial growth.

Cytotoxicity Assay: Vero monkey epithelial cells (ATCC CCL-81) were cultured in Dulbecco's Modified Eagle's medium that was supplemented with 10% fetal bovine serum and maintained in a humidified incubator (37 °C, 5% CO_2). Cells were dislodged with a cell scraper, collected by centrifugation, resuspended in fresh medium at ~106 cells mL^{-1}, dispensed into 96-well microtiter plates (100 μL well^{-1}) and incubated for 18 h at 37 °C. Twofold serial dilutions of test compounds (400–0.2 μg mL^{-1}) in DMEM with FBS were subsequently added, and the cells were incubated for another 72 h. The cytopathic effects of compounds were evaluated colorimetrically by using the MTT Cell Proliferation Assay (ATCC). IC_{50} values were obtained from the dose–response curves, which were plotted as percentage activity versus $\log_{10}$ concentration.

Acknowledgements

We thank the National Institutes of Health Grant GM 34496 (CR), and the American Lebanese and Syrian Associated Charities for their financial support. We would also like to thank Dr. Thomas Webb and his lab members at St. Jude Children's Research Hospital for their assistance with obtaining UPLC analytical data.

Keywords: acyltransferase · antibiotics · *Bacillus anthracis* · phospholipids · PlsY

[1] P. M. Shah, *Clin. Microbiol. Infect.* **2005**, *11*, Suppl 3, 36–42.
[2] A. Coates, Y. Hu, R. Bax, C. Page, *Nat. Rev. Drug Discov.* **2002**, *1*, 895–910.
[3] R. J. Heath, S. W. White, C. O. Rock, *Appl. Microbiol. Biotechnol.* **2002**, *58*, 695–703.
[4] R. J. Heath, S. W. White, C. O. Rock, *Prog. Lipid Res.* **2001**, *40*, 467–497.
[5] Y. M. Zhang, S. W. White, C. O. Rock, *J. Biol. Chem.* **2006**, *281*, 17541–17544.
[6] J. E. Cronan, *Annu. Rev. Microbiol.* **2003**, 57, 203–224.
[7] Y. M. Zhang, C. O. Rock, *J. Lipid Res.* **2008**, *49*, 1867–1874.
[8] Y.-J. Lu, Y.-M. Zhang, K. D. Grimes, J. Qi, R. E. Lee, C. O. Rock, *Mol. Cell* **2006**, *23*, 765–772.
[9] Y.-J. Lu, F. Zhang, K. D. Grimes, R. E. Lee, C. O. Rock, *J. Biol. Chem.* **2007**, *282*, 11339–11346.
[10] L. Paoletti, Y. J. Lu, G. E. Schujman, D. de Mendoza, C. O. Rock, *J. Bacteriol.* **2007**, *189*, 5816–5824.

[11] J. G. Hurdle, A. J. O'Neill, I. Chopra, *Antimicrob. Agents Chemother.* **2005**, *49*, 4821–4833.
[12] J. Pohlmann, H. Brötz-Oesterhelt, *Curr. Drug Targets* **2004**, *4*, 261–272.
[13] J. Y. Winum, A. Scozzafava, J. L. Montero, C. T. Supuran, *Med. Res. Rev.* **2005**, *25*, 186–228.
[14] C. Qiao, A. Gupte, H. I. Boshoff, D. J. Wilson, E. M. Bennett, R. V. Somu, C. E. Barry III, C. C. Aldrich, *J. Med. Chem.* **2007**, *50*, 6080–6094.
[15] R. V. Somu, H. Boshoff, C. Qiao, E. M. Bennett, C. E. Barry III, C. C. Aldrich, *J. Med. Chem.* **2006**, *49*, 31–34.
[16] R. V. Somu, D. J. Wilson, E. M. Bennett, H. I. Boshoff, L. Celia, B. J. Beck, C. E. Barry III, C. C. Aldrich, *J. Med. Chem.* **2006**, *49*, 7623–7635.
[17] J. Vannada, E. M. Bennett, D. J. Wilson, H. I. Boshoff, C. E. Barry III, C. C. Aldrich, *Org. Lett.* **2006**, *8*, 4707–4710.
[18] K.-C. Tang, J. K. Coward, *J. Org. Chem.* **1983**, *48*, 5001–5006.
[19] D. B. Berkowitz, M. Eggen, Q. Shen, R. K. Shoemaker, *J. Org. Chem.* **1996**, *61*, 4666–4675.
[20] L. A. Adams, R. J. Cox, J. S. Gibson, M. B. Mayo-Martín, M. Walter, W. Whittingham, *Chem. Commun. (Camb)* **2002**, *18*, 2004–2005.
[21] M. C. Schulbach, S. Mahapatra, M. Macchia, S. Barontini, C. Papi, F. Minutolo, S. Bertini, P. J. Brennan, D. C. Crick, *J. Biol. Chem.* **2001**, *276*, 11624–11630.
[22] R. Appel, G. Berger, *Chem. Ber.* **1959**, *92*, 1339–1341.
[23] C. Fruit, P. Muller, *Helv. Chim. Acta* **2004**, *87*, 1607–1615.
[24] C.-H. Lee, H. Kohn, *J. Org. Chem.* **1990**, *55*, 6098–6104.
[25] C. S. Rye, J. B. Baell, *Curr. Med. Chem.* **2005**, *12*, 3127–3141.
[26] V. A. Luna, D. S. King, K. K. Peak, F. Reeves, L. Heberlein-Larson, W. Veguilla, L. Heller, K. E. Duncan, A. C. Cannons, P. Amuso, J. Cattani, *J. Clin. Microbiol.* **2006**, *44*, 2367–2377.

Received: July 11, 2008
Revised: August 20, 2008
Published online on November 17, 2008

