Published in final edited form as:

ChemMedChem. 2008 December ; 3(12): 1936–1945. doi:10.1002/cmdc.200.

Novel Acylphosphate 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]

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

^[b] Dr. Y. Lu, Dr. Y. Zhang, Dr. C. O. Rock, Department of Infectious Diseases, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105

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

Abstract

PlsY is a recently discovered acyltransferase that executes an essential step in membrane phospholipid biosynthesis in Gram-positive bacteria. Using a bioisosteric replacement approach to generate substrate-based inhibitors of PlsY as potential novel antibacterial agents, a series of stabilized acylphosphate mimetics, including acylphosphonates, acyl α,α -difluoromethyl phosphonates, acyl phosphoramides, reverse amide phosphonates, acylsulfamates and acylsulfamides 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 acylphosphate substrate. Antimicrobial testing showed the inhibitors to have generally weak anti Gram-positive activity with the exception of some acyl phosphonates, reverse amide phosphonates, and acylsulfamates that had potent activity against multiple strains of *Bacillus anthracis*.

Keywords

PlsY; acyltransferase; phospholipid; *B. anthracis*; antibiotic

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 involved in the biosynthesis of lipids in bacteria.[3–5]

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 1a). Since PlsB homologs exist in mammals, the first step in bacterial phospholipid biosynthesis was typically disfavoured as a target for antibiotic drug discovery.[7] However, PlsB is not universally expressed in bacteria. Recently, an alternative 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, which produces an acylphosphate (acyl-PO₄) intermediate. The second step is catalyzed by PlsY, an acyl-PO₄:G3P acyltransferase that transfers the acyl group from acyl-PO₄ to the 1-position of G3P. PlsY is an integral transmembrane protein with a highly conserved set of active site residues facing 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 homologs of the PlsX/PlsY pathway making this an attractive target for antibacterial drug discovery.

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 homologs of the acyl-PO₄ reactive intermediate utilizing a bioisosteric replacement strategy for the highly labile acyl-PO₄ group. This approach is supported by the knowledge that acylphosphonates, acylsulfamates, and acylsulfamides have been used successfully to design inhibitors of other acylphosphate utilizing enzymes including aminoacyl-tRNA synthetases and non-ribosomal peptide synthetases.[11–17] This work evaluates the study of six acyl-PO₄ bioisosteric head groups combined with a variety of acyl chains (Figure 2) to develop a preliminary structure activity relationship (SAR) for PlsY inhibition and anti-bacterial activity.

Synthesis

Acylphosphonate 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 starting from the corresponding fatty acid potassium salts **6a–f**. Acylation of **6a–f** with ethylchloroformate yielded the anhydrides **7a–f**, which were subsequently reacted with the lithium salt of diethyl phosphoramidate to give acylphosphoramidate esters **8a–f**. [20] Then, TMSBr mediated cleavage of the phosphoramidate esters yielded the target acylphosphoramides **9a–f** (Scheme 2). Reverse amide phosphonates were synthesized by coupling various amines **10a–d** with diethylphosphonoacetic acid using 1-hydroxybenzotriazole (HOBt) and N-(3-dimethylaminopropyl)-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).

The acylsulfamates and acylsulfamides were synthesized 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 acylsulfamates **17a–d** and acylsulfamide **18** (Scheme 4).

Results and Discussion

PlsY inhibition

The synthesized acyl-PO₄ bioisosteres were tested for inhibition of PlsY in two representative Gram-positive pathogens: *Streptococcus pneumoniae* (SpPlsY) and *Bacillus anthracis* (BaPlsY) (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 having the saturated C₁₆ and unsaturated C₁₈ 11 alkyl chains. The most active compounds for SpPlsY were acylphosphoramidate **9b** (IC₅₀ 11 μM, C₁₆), α,α-difluoromethyl phosphonates **5a** (IC₅₀ 39 μM, C₁₆) and **5i** (IC₅₀ 20 μM, C_{18Δ9}), and ketophosphonate **4b** (IC₅₀ 48 μM, C_{18Δ11}). The most active compounds for BaPlsY were the α,α-difluoromethyl phosphonates **5a** (IC₅₀ 50 μM, C₁₆) and **5e** (IC₅₀ 25 μM, C₁₂). The long chain reverse amide phosphonates **12a–c** had moderate activity against both PlsYs. The acylsulfamates **17a–c** displayed good activity against SpPlsY (IC₅₀ 85 μM, 70 μM, and 60 μM, respectively) and BaPlsY (IC₅₀ 65 μM, 50 μM, and 35 μM, respectively). Acylsulfamide **18** (BaIC₅₀ 200 μM, C_{18Δ9}) was considerably less potent when compared to the related acylsulfamates **17a** and **17b** against both PlsYs. This could be related to their poor aqueous solubility, a property that limited their further exploration.

To validate the mechanism of inhibition, a kinetic analysis of the representative acyl-PO₄ bioisosteres **9a** and **9b** was performed against SpPlsY (Figure 3). As anticipated for substrate mimics, these compounds were shown to act as competitive inhibitors of the acyl-PO₄ binding to PlsY and were non-competitive 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 consisting of *Streptococcus pneumoniae*, Methicillin resistant *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, and *Bacillus anthracis* (Table 1). Activity was weak to moderate against most Gram-positive species tested except for the *B. anthracis* Sterne strain, which was potently inhibited by compounds in the acylphosphonate and reverse amide phosphonate series. Acylsulfamate **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 acylphosphonates **4a** (MIC 1.56 μg/mL) and **4b** (MIC 0.05 μg/mL), reverse amide phosphonates **12a** (MIC 0.1 μg/mL), **12b** (MIC 0.1 μg/mL), and **12c** (MIC 3.13 μg/mL), and acylsulfamate **17c** (MIC 3.13 μg/mL). The inactivity of the acylphosphoramidates may be due to spontaneous or enzymatic mediated hydrolysis of the phosphoramidate bond, or due to poor penetration. Poor bacterial penetration and increased acidity may explain the inactivity of the α,α-difluoromethyl phosphonate series when compared to the similar acylphosphonates; as the electron withdrawing α,α-difluoro substitution increases the charge on the phosphonate head group; a property associated with decreased uptake in bacteria. [25] Though most of the bioisosteres were inactive against the other Gram-positive pathogens the acylsulfamate series, most notably **17c**, did show good SpPlsY inhibition (IC₅₀ 60 μM) and anti *S. pneumoniae* activity (MIC 12.5 μg/mL), which may warrant further investigation. This series shows that introduction of a functional group adjacent to the binding phosphate isostere is tolerated and can be exploited in future design of inhibitors with more drug-like properties. This study raises a question regarding the lack of general anti Gram-positive activity of these compounds despite inhibition of the target enzyme *in vitro*. Two likely explanations may explain this observation:

(i) the enzyme inhibition is still not adequate to see an anti-bacterial effect or (ii) that these compounds are limited by bacterial dependant cellular penetration by active drug efflux from multidrug efflux pumps or other inactivation processes.

Further specificity studies showed that none of the compounds exhibited antimicrobial activity against Gram-negative species, *E. coli* and *P. aeruginosa* (data not shown). Cytotoxicity against mammalian cells was also evaluated for the most active compounds with anti-*B. anthracis* activity (Table 1). Compounds **4a**, **4b**, **12a**, **12b** and **17c** only exhibited cytotoxicity at high concentrations, producing high selectivity indices (Cytotoxicity IC₅₀/*B. anthracis* MIC) ranging from 2200–4850. 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 acylphosphonates **4a,b** (MIC 0.012 – 3.13 µg/mL) 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) and acylsulfamate **17c** (MIC 0.78 – 3.13 µg/mL) 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. However, we cannot currently rule out the possibility of there being some unique additional antibacterial effects outside of PlsY inhibition to explain the specific anti-anthraxis activity of our substrate mimics. Thus, the activity of the inhibitors was also tested against *B. cereus*, a close relative of *B. anthracis*, which was shown to be sensitive to these compounds (Table 2) and in part supports the notion of a unique mode of action against *B. anthracis* and its closely related *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* homologs, while *B. cereus* carries two *plsY* homologs, while *B. subtilis* carries one *plsY* homolog. In contrast, *B. subtilis* is insensitive to these compounds which only contains one PlsY. This finding is unusual because most bacteria (>99%) possess a single copy of *plsY*. Regarding the three PlsYs in *B. anthracis*, BaPlsY1 is most similar to SpPlsY 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 multiple of these alternate PlsY proteins and to evaluate the potential for the development of this series as selective inhibitors of *B. anthracis*.

Conclusion

Novel inhibitors of the Gram-positive PlsX/PlsY pathway to phosphatidic acid have been discovered. Substrate mimics incorporating six acyl-PO₄ bioisosteric head groups were synthesized and based on the preliminary SAR, longer chain analogues matching 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 analog, 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 anti-*B. anthracis* agents with good antimicrobial activity and low cytotoxicity. Broader antibacterial activity and strong enzyme inhibition of acylsulfamate **17c** suggests that the synthesis of a

wider panel of acylsulfamates may be warranted towards the development of a novel class of anti Gram-positive agents.

Experimental Section

All reagents and anhydrous solvents were purchased from Sigma-Aldrich. All the reagent-grade solvents used for chromatography were purchased from Fisher Scientific (Suwanee, GA) and flash column chromatography silica cartridges were obtained from Biotage Inc. (Lake Forest, VA). The reactions were monitored by thin-layer chromatography (TLC) on pre-coated Merck 60 F254 silica gel plates and visualized using UV light (254 nm) and Iodine staining. A Biotage FLASH column chromatography system was used to purify the reaction mixtures. All ^1H 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 LCMS using ESI. The yields quoted are unoptimized. Purity of the final products were analyzed by a Waters Acquity UPLC-PDA-ELSD-MS. UPLC separations were performed using an Acquity UPLC 2.1×50 mm BEH C18 column (1.7 μm , Waters) at 50°C , and a 1.0 mL/min flow rate. The PDA was set to acquire UV data from 210–400 nm throughout the run. UPLC1: Gradient: solvent A (10 mM ammonium acetate in water) and solvent B (10 mM ammonium acetate in acetonitrile): 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 ammonium acetate in water) and solvent B (10 mM ammonium acetate in acetonitrile): 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 acylphosphonate esters (2a–h)

A 1.6M solution of *n*-Butyl lithium (3.5 eq) in hexanes was added to THF at -78°C . After stirring for 30min, dimethyl methanephosphonate (3.5 eq) was added drop-wise to the mixture over 30 min generating the carbanion. After the resulting suspension was stirred for an additional 30 min, various esters or acid chlorides (1.0 eq) in THF were added drop-wise over 30 min. The resulting suspension was allowed to stir until the temperature rose to -20°C . The reaction mix was quenched with glacial acetic acid (3 mL) until mildly acidic ($\text{pH} = 5$). The reaction mix was extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 , and then concentrated *in vacuo*. Flash column purification (0–100%, Petroleum Ether/Ethyl Acetate) yielded the corresponding acylphosphonate esters. Synthesis of Dimethyl 2-oxoheptadecylphosphonate (2a) is given as representative example.

Dimethyl 2-oxoheptadecylphosphonate (2a)

Using the above general procedure, *n*-butyl lithium (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%), $\text{mp} = 47\text{--}49^\circ\text{C}$. TLC $R_f = 0.5$ (Ethyl Acetate). ^1H NMR (CDCl_3 , 500MHz): δ 0.90 (t, 3H, $J = 6.5$ Hz), 1.27–1.33 (bs, 24H), 1.60 (m, 2H), 2.63 (t, 2H, $J = 7.0$ Hz), 3.11 (d, 2H, $J = 23.0$ Hz), 3.80 (s, 3H), 3.82 (s, 3H). MS(ESI), $m/z = 385.9$ [$\text{M} + \text{Na}$] $^+$, 361.3 [$\text{M} - \text{H}$] $^-$.

General procedure for synthesis of acyl α,α -difluoromethyl phosphonate esters (3a,e,i,j)

A freshly prepared solution of LDA (2.0 eq) was added to THF (20 mL) at -78°C . After stirring for 30 min, diethyl α,α -difluoromethyl phosphonate (2.0 eq) in THF (1 mL) was added drop-wise 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 eq) in THF (5 mL) were added drop-wise over 10 min. The resulting suspension was allowed to stir for 2.5h. After the reaction was complete, it was quenched with glacial acetic acid (2 mL) followed by saturated

ammonium chloride. The mixture was allowed to warm to room temperature and extracted with chloroform. The organic extracts were washed with brine, dried over Na₂SO₄, and then concentrated *in vacuo*. Flash column purification (0–100%, Petroleum Ether/Ethyl Acetate) yielded the desired acyl $\alpha\alpha$, -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**)

Using the above general procedure, *n*-butyl lithium (4.25 mmol), diisopropylamine (4.25 mmol), diethyl $\alpha\alpha$, -difluoromethyl 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 (8:2, Pet Ether:EtOAc). ¹H NMR (CDCl₃, 500MHz): δ 0.89 (t, 3H, *J* = 7.0 Hz), 1.25 (bs, 24H), 1.39 (t, 6H, *J* = 7.0 Hz), 1.63 (m, 2H), 2.77 (t, 2H, *J* = 7.5 Hz), 4.31 (m, 4H). MS(ESI), *m/z* = 449.4 [M+Na]⁺, 425.1 [M-H][−].

General procedure for synthesis of acylphosphoramidates (**8a–f**)

Various esters or acids (1.0 eq) were treated with KOH (1.0 eq) in H₂O:ACN (1:1). The reaction mixture was stirred and heated at 110°C until dry, then subjected to high vacuum for 16h. DCM (25 mL) was added to the flask. To the resulting suspension was added ethylchloroformate (1.0 eq) which was stirred for 7h, then chilled to −78°C. Pretreatment of diethyl phosphoramidate (3.2 eq) with 1.6M *n*-butyl lithium (3.2 eq) 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 reaction mixture was quenched with glacial acetic acid (3 mL) until mildly acidic (pH = 5). The reaction mix was extracted with ethyl acetate. The organic layer was washed with brine, dried over Na₂SO₄, and then concentrated *in vacuo*. Flash column purification (0–100%, Petroleum Ether/Ethyl Acetate) yielded the desired acylphosphoramidate esters. Synthesis of Diethyl 1-oxohexadecylphosphoramidate (**8a**) is given as representative example.

Diethyl 1-oxohexadecylphosphoramidate (**8a**)

Using the above general procedure, methylpalmitate (10.0 mmol), ACN:H₂O (20 mL), KOH (10.0 mmol), ethyl chloroformate (10.0 mmol), *n*-butyl lithium (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 (Ethyl Acetate). ¹H NMR (CDCl₃, 500MHz): δ 0.90 (t, 3H, *J* = 6.5 Hz), 1.27 (bs, 24H), 1.38 (t, 6H, *J* = 7.0 Hz), 1.65 (m, 2H), 2.36 (t, 2H, *J* = 7.0 Hz), 4.22 (m, 4H), 8.34 (s, 1H). MS(ESI), *m/z* = 390.1 [M-H][−].

General procedure for synthesis of acyl reverse amide phosphonates (**11a–d**)

To DCM/DMF (1:1) was added HOBt (1.0 eq), diethylphosphonoacetic acid (1.0 eq), and EDCI (1.0 eq). The mixture was allowed to stir for 30 min. Drop-wise various amines (1.0 eq) were added. The reaction mixture was allowed to stir at room temperature for 16h. The mixture was extracted with chloroform. The organic layer was washed with NaHCO₃, water, and brine, then dried over Na₂SO₄, and concentrated *in vacuo*. Flash column purification (0–100%, Petroleum Ether/Ethyl Acetate) yielded the desired reverse amide phosphonate esters. Synthesis of Diethyl (Tetradecylcarbamoyl) methylphosphonic acid (**11a**) is given as representative example.

Diethyl tetradecylcarbamoyl)methylphosphonic acid (**11a**)

Using the above general procedure, HOBt (5.0 mmol), EDCI (5.0 mmol), diethylphosphonoacetic acid (5.0 mmol), DCM/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 (Ethyl Acetate). ¹H NMR (CDCl₃, 500MHz): δ 0.84 (t, 3H, *J* = 6.5 Hz), 1.22 (bs, 22H), 1.31 (t, 6H,

$J = 7.0$ Hz), 1.47 (m, 2H), 2.82 (d, 2H, $J = 20.5$ Hz), 3.21 (q, 2H, $J = 6.5$ Hz), 4.11 (m, 4H), 6.91 (bt, 1H) MS(ESI), $m/z = 390.1$ $[M-H]^-$, $m/z = 414.4$ $[M+Na]^+$.

General procedure for ester deprotection (4a–h, 5a,e,i,j, 9a–d, and 12a–d)

Each ester (1.0 eq) was treated with 2 ml of DCM. The flask was flushed several times with Argon. Bromotrimethylsilane (10.0 eq) was added slowly to the mixture. The resulting solution was allowed to stir 16h at ambient temperature. Excess TMSBr was removed *in vacuo*. 95% Ethanol (5 mL) was added to the reaction flask and allowed to stir for 1h after which the solvent was removed *in vacuo*. The residue was triturated several times with diethyl ether and filtered.

2-oxoheptadecylphosphonic acid (4a)

Using the above general procedure, dimethyl 2-oxoheptadecylphosphonate (2.76 mmol), TMSBr (27.6 mmol) and DCM (2 mL) were used to synthesize **4a** as a white solid (89% overall), mp = 91–93°C. 1H NMR (d_6 -DMSO, 500MHz): δ 0.86 (t, 3H, $J = 6.0$ Hz), 1.25 (bs, 24H), 1.43 (m, 2H), 2.57 (t, 2H, $J = 7.0$ Hz), 2.92 (d, 2H, $J = 22.5$ Hz). ^{13}C NMR (d_6 -DMSO, 500MHz): 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. MS (ESI), $m/z = 333.4$ $[M-H]^-$. UPLC1: t_R 1.18 min, Purity > 99%.

(Z)-2-oxononadec-12-enylphosphonic acid (4b)

Using the above general procedure, (Z)-dimethyl 2-oxononadec-12-enylphosphonate (0.5 mmol), TMSBr (5.0 mmol) and DCM (2 mL) were used to synthesize **4b** as a light yellow waxy solid (77% overall). 1H NMR (CD_3OD , 500MHz): δ 0.93 (t, 3H, $J = 7.0$ Hz), 1.33 (bs, 20H), 1.58 (m, 2H), 2.05 (q, 4H, $J = 8.5$ Hz), 2.67 (t, 2H, $J = 7.5$ Hz), 3.09 (d, 2H, $J = 22.5$ Hz), 5.36 (m, 2H). ^{13}C NMR (CD_3OD , 500MHz): 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. MS(ESI), $m/z = 359.2$ $[M-H]^-$. UPLC1: t_R 1.25 min, Purity 96%.

2-oxononylphosphonic acid (4c)

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

2-oxopentylphosphonic acid (4d)

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

2-oxotridecylphosphonic acid (4e)

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

6-cyclohexyl-2-oxohexylphosphonic acid (4f)

Using the above general procedure, Dimethyl 6-cyclohexyl-2-oxohexylphosphonate (2.7 mmol), TMSBr (27.0 mmol) and DCM (2 mL) were used to synthesize **4f** as a light yellow oil (69% overall). ¹H NMR (CDCl₃, 500MHz): δ 0.90 (m, 2H), 1.12–1.36 (m, 6H), 1.53–1.68 (m, 10H), 2.69 (t, 2H, *J* = 7.0 Hz), 3.06 (d, 2H, *J* = 23.0 Hz). ¹³C NMR (CDCl₃, 500MHz): δ 23.7, 26.2, 26.3, 26.7, 33.3, 37.2, 37.4, 44.2, 204.6. MS(ESI), *m/z* = 260.8 [M–H][–]. UPLC1: *t*_R 0.51 min, Purity > 99%.

2-oxo-6-phenylhexylphosphonic acid (4g)

Using the above general procedure, Dimethyl 2-oxo-6-phenylhexylphosphonate (2.2 mmol), TMSBr (22.0 mmol) and DCM (2 mL) were used to synthesize **4g** as a light yellow oil (62% overall). ¹H NMR (CD₃OD, 500MHz): δ 1.56 (m, 4H), 2.58 (m, 4H), 3.13 (d, 2H, *J* = 22.5 Hz), 7.14 (t, 3H, *J* = 8.0 Hz), 7.24 (t, 2H, *J* = 8.0 Hz). ¹³C NMR (CD₃OD, 500MHz): δ 23.1, 30.6, 35.6, 44.2, 47.3, 125.8, 128.4, 142.1, 205.0. MS(ESI), *m/z* = 257.0 [M+H]⁺, 254.8 [M–H][–]. UPLC1: *t*_R 0.36 min, Purity > 99%.

2-oxo-12-phenoxydodecylphosphonic acid (4h)

Using the above general procedure, Dimethyl 2-oxo-12-phenoxydodecylphosphonate (2.7 mmol), TMSBr (27.0 mmol) and DCM (2 mL) were used to synthesize **4h** as a white solid (66% overall), mp = 93–95°C. ¹H NMR (CD₃OD, 500MHz): δ 1.34 (bs, 10H), 1.49 (m, 2H), 1.58 (m, 2H), 1.78 (m, 2H), 2.67 (t, 2H, *J* = 7.0 Hz), 3.09 (d, 2H, *J* = 22.5 Hz), 3.97 (t, 2H, *J* = 6.5 Hz), 6.91 (d, 2H, *J* = 8.0 Hz), 6.91 (t, 1H, *J* = 7.5 Hz), 7.26 (t, 2H, *J* = 7.0 Hz). ¹³C NMR (CD₃OD, 500MHz): 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. MS(ESI), *m/z* = 355.0 [M–H][–]. UPLC1: *t*_R 0.78 min, Purity > 99%.

1,1-difluoro-2-oxoheptadecylphosphonic acid (5a)

Using the above general procedure, Diethyl 1,1-difluoro-2-oxoheptadecylphosphonic acid (0.1 mmol), TMSBr (1.0 mmol) and DCM (2 mL) were used to synthesize **5a** as a white solid (52% overall), mp = 45–48°C. ¹H NMR (d₆-DMSO, 500MHz): δ 0.87 (t, 3H, *J* = 6.5 Hz), 1.25 (bs, 24H), 1.51 (m, 2H), 2.76 (t, 2H, *J* = 7.0 Hz). ¹³C NMR (d₆-DMSO, 500MHz): δ 13.2, 22.3, 28.6, 29.1, 29.2, 29.3, 29.4, 199.8. MS(ESI), *m/z* = 371.3 [M+H]⁺, 369.1 [M–H][–]. UPLC1: *t*_R 1.19 min, Purity 93%.

1,1-difluoro-2-oxotridecylphosphonic acid (5e)

Using the above general procedure, Diethyl 1,1-difluoro-2-oxotridecylphosphonic acid (0.1 mmol), TMSBr (1.0 mmol) and DCM (2 mL) were used to synthesize **5e** as a colorless oil (66% overall). ¹H NMR (d₆-DMSO, 500MHz): δ 0.92 (t, 3H, *J* = 7.0 Hz), 1.31 (bs, 16H), 1.62 (m, 2H), 2.85 (t, 2H, *J* = 7.0 Hz). ¹³C NMR (d₆-DMSO, 500MHz): δ 13.1, 22.4, 28.7, 29.1, 29.1, 29.2, 29.3, 31.7, 37.4, 110.0. MS(ESI), *m/z* = 312.9 [M–H][–].

(Z)-1,1-difluoro-2-oxononadec-10-enylphosphonic acid (5i)

Using the above general procedure, Diethyl (Z)-1,1-difluoro-2-oxononadec-10-enylphosphonic acid (0.1 mmol), TMSBr (1.0 mmol) and DCM (2 mL) were used to synthesize **5i** as a colorless oil (74% overall). ¹H NMR (d₆-DMSO, 500MHz): δ 0.92 (t, 3H, *J* = 6.5 Hz), 1.34 (bd, 20H), 1.62 (m, 2H), 2.05 (q, 4H, *J* = 5.0 Hz), 2.86 (t, 2H, *J* = 7.0 Hz), 5.37 (m, 2H). ¹³C NMR (d₆-DMSO, 500MHz): 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. MS(ESI), *m/z* = 395.0 [M–H][–]. UPLC1: *t*_R 0.70 min, Purity 96%.

1,1-difluoro-2-oxododec-11-enylphosphonic acid (5j)

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

1-oxohexadecylphosphoramidic acid (9a)

Using the above general procedure, (Diethyl) 1-oxohexadecylphosphoramidate (1.0 mmol), TMSBr (1.0 mmol) and DCM (2 mL) were used to synthesize **9a** as a white solid (24% overall), mp = 56–58°C. ^1H NMR (d_6 -DMSO, 500MHz): δ 0.87 (t, 3H, $J = 6.5$ Hz), 1.25 (bs, 24H), 1.47 (m, 2H), 2.18 (t, 2H, $J = 7.0$ Hz), 8.98 (d, 1H, $J = 9.0$ Hz). 13.0, 22.3, 26.6, 28.9, 29.0, ^{13}C NMR (d_6 -DMSO, 500MHz): δ 29.3, 29.3, 29.4, 31.7, 35.6, 36.6, 39.4, 110.0, 166.7. MS(ESI), $m/z = 334.3$ $[\text{M}-\text{H}]^-$.

(Z)-1-oxooctadec-11-enylphosphoramidic acid (9b)

Using the above general procedure, (Diethyl) (Z)-1-oxooctadec-11-enylphosphoramidate (0.1 mmol), TMSBr (1.0 mmol) and DCM (2 mL) were used to synthesize **9b** as a white solid (19% overall), mp = 76–80°C. ^1H NMR (d_6 -DMSO, 500MHz): δ 0.93 (t, 3H, $J = 7.0$ Hz), 1.33 (bs, 20H), 1.64 (m, 2H), 2.05 (q, 4H, $J = 9.0$ Hz), 2.32 (t, 2H, $J = 7.0$ Hz), 5.37 (m, 2H). ^{13}C NMR (d_6 -DMSO, 500MHz): 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. MS (ESI), $m/z = 360.1$ $[\text{M}-\text{H}]^-$.

1-oxooctylphosphoramidic acid (9c)

Using the above general procedure, (Diethyl) 1-oxooctylphosphoramidate (1.5 mmol), TMSBr (15.0 mmol) and DCM (2 mL) were used to synthesize **9c** as a white solid (32% overall), mp = 75–77°C. ^1H NMR (CD_3OD , 500MHz): δ 0.93 (t, 3H, $J = 7.0$ Hz), 1.35 (bs, 8H), 1.64 (m, 2H), 2.32 (bs, 2H); ^{13}C NMR (CD_3OD , 500MHz): δ 13.1, 22.3, 24.8, 28.8, 31.5, 176.8. MS (ESI), $m/z = 221.9$ $[\text{M}-\text{H}]^-$, 224.0 $[\text{M}+\text{H}]^+$.

1-oxododecylphosphoramidic acid (9d)

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

5-cyclohexyl-1-oxopentylphosphoramidic acid (9e)

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

1-oxo-11-phenoxyundecylphosphoramidic acid (9f)

Using the above general procedure, (Diethyl) 1-oxo-11-phenoxyundecylphosphoramidate (0.5 mmol), TMSBr (5.0 mmol) and DCM (2 mL) were used to synthesize **9f** as a white solid (15% overall), mp = 100–103°C. ^1H NMR (CD_3OD , 500MHz): δ 1.35 (bs, 10H), 1.49 (m, 2H), 1.63 (m, 2H), 1.77 (m, 2H), 2.32 (bt, 2H, $J = 7.5$ Hz), 3.96 (t, 2H, $J = 7.0$ Hz), 6.91 (t, 3H, 8.0 Hz),

7.26 (t, 2H, $J = 7.5$ Hz). ^{13}C NMR (CD_3OD , 500MHz): 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. MS(ESI), $m/z = 358.3$ $[\text{M}+\text{H}]^+$, 356.0 $[\text{M}-\text{H}]^-$.

(Tetradecylcarbamoyl)methylphosphonic acid (**12a**)

Using the above general procedure, Diethyl (Tetradecylcarbamoyl) methylphosphonic acid (1.0 mmol), TMSBr (30.0 mmol) and DCM (2 mL) were used to synthesize **12a** as a white solid (10% overall), mp = 133–135°C. ^1H NMR (d_6 -DMSO, 500MHz): δ 0.87 (t, 3H, $J = 6.5$ Hz), 1.25 (bs, 22H), 1.38 (m, 2H), 2.57 (d, 2H, $J = 20.5$ Hz), 3.03 (q, 2H, $J = 6.0$ Hz), 7.74 (t, 1H, $J = 5.5$ Hz). ^{13}C NMR (d_6 -DMSO, 500MHz): δ 13.0, 22.3, 26.6, 28.8, 29.0, 29.2, 29.4, 31.7, 39.4. MS(ESI), $m/z = 334.1$ $[\text{M}-\text{H}]^-$, $m/z = 336.3$ $[\text{M}+\text{H}]^+$. UPLC1: t_R 1.75 min, Purity > 99%.

((Z)-octadec-9-enylcarbamoyl)methylphosphonic acid (**12b**)

Using the above general procedure, Diethyl ((Z)-octadec-9-enylcarbamoyl)methylphosphonic acid (0.1 mmol), TMSBr (3.0 mmol) and DCM (2 mL) were used to synthesize **12b** as a white solid (17% overall), mp = 111–114°C. ^1H NMR (CD_3OD , 500MHz): δ 0.92 (t, 3H, $J = 6.5$ Hz), 1.33 (bd, 22H), 1.54 (m, 2H), 2.05 (m, 4H), 2.81 (d, 2H, $J = 21.0$ Hz), 3.21 (t, 2H, $J = 7.5$ Hz), 5.37 (m, 2H). ^{13}C NMR (CD_3OD , 500MHz): 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. MS(ESI), $m/z = 388.0$ $[\text{M}-\text{H}]^-$. UPLC1: t_R 1.32 min, Purity 96%.

(N-dodecyl-N-methylcarbamoyl)methylphosphonic acid (**12c**)

Using the above general procedure, Diethyl (N-dodecyl-N-methylcarbamoyl) methylphosphonic acid (0.1 mmol), TMSBr (3.0 mmol) and DCM (2 mL) were used to synthesize **12c** as a white solid (10% overall), mp = 80–83°C. ^1H NMR (CD_3OD , 500MHz): δ 0.92 (t, 3H, $J = 6.5$ Hz), 1.31 (bs, 18H), 1.62 (m, 2H), 3.06 (d, 2H, $J = 22.5$ Hz), 3.06 (bd, 3H), 3.44 (dt, 2H, $J = 7.5$ Hz, 29.5 Hz). ^{13}C NMR (CD_3OD , 500MHz): 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. MS(ESI), $m/z = 320.0$ $[\text{M}-\text{H}]^-$, $m/z = 322.3$ $[\text{M}+\text{H}]^+$. UPLC1: t_R 0.83 min, Purity > 99%.

(Heptylcarbamoyl)methylphosphonic acid (**12d**)

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

General procedure for synthesis of acylsulfamates (**17a–d**)

Anhydrous formic acid (99%, 2.0 eq) was added drop-wise to chlorosulfonyl isocyanate (2.0 eq) at 0°C under argon. The mixture was allowed to rise to room temperature and stirred until gas evolution stopped (~2h). The desired alcohol (1.0 eq) in DMA (30 mL) was added drop-wise 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 room temperature and stirred an additional 3h. The mixture was then poured into cold brine (100 mL) and extracted with ethyl acetate. The combined extracts were washed with water 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 eq) and DMF (2 drops) to appropriate acids (1.0 eq) in THF (15 mL) at 0°C under argon. After gas evolution stopped (~20 min) the mixture was warmed to room temperature and allowed to stir for 2h. The resulting mixture was concentrated *in vacuo* then dissolved in DCM/DMF (1:1,

5 mL) and added drop-wise to a mixture of previously prepared sulfamate ester, **15** (1.0 eq), DMAP (cat., 10% mol), triethylamine (3.0 eq) in DCM/DMF (1:1, 15 mL). The mixture was allowed to stir for 16h under argon. The mixture was diluted with DCM and washed with 1N HCl, water, and brine then dried over Na₂SO₄ and concentrated *in vacuo*. The residue was purified via flash chromatography (0–100%, Hexane/Ethyl Acetate) to afford the desired acylsulfamate.

Ethyl palmitoylsulfamate (17a)

Using the above general procedure, chlorosulfonyl isocyanate (40.0 mmol), formic acid (40.0 mmol), ethanol (20.0 mmol), DMA (30 mL), palmitic acid (6.0 mmol), oxalylchloride (12.0 mmol), triethylamine (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 (8:2, CHCl₃:MeOH). ¹H NMR (CDCl₃, 500MHz): δ 0.88 (t, 3H, *J* = 6.0 Hz), 1.26 (bs, 24H), 1.43 (t, 3H, *J* = 7.0 Hz), 1.66 (m, 2H), 2.39 (t, 2H, *J* = 7.5 Hz), 4.48 (q, 2H, *J* = 8.0 Hz), 8.39 (s, 1H). ¹³C NMR (CDCl₃, 500MHz): 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. MS(ESI), *m/z* = 386.3 [M+Na]⁺, 362.1 [M-H][−]. UPLC1: *t*_R 1.57 min, Purity > 99%.

Butyl palmitoylsulfamate (17b)

Using the above general procedure, 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), triethylamine (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 (8:2, CHCl₃:MeOH). ¹H NMR (CDCl₃, 500MHz): δ 0.88 (t, 3H, *J* = 6.5 Hz), 0.95 (t, 3H, *J* = 7.5 Hz), 1.26 (bs, 24H), 1.44 (m, 2H), 1.65 (m, 2H), 1.75 (m, 2H), 2.39 (t, 2H, *J* = 7.5 Hz), 4.40 (t, 2H, *J* = 6.5 Hz), 8.18 (s, 1H). ¹³C NMR (CDCl₃, 500MHz): 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. MS(ESI), *m/z* = 414.4 [M+Na]⁺, 390.1 [M-H][−]. UPLC1: *t*_R 1.74 min, Purity > 99%.

4-methoxyphenyl palmitoylsulfamate (17c)

Using the above general procedure, 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), triethylamine (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 (Ethyl Acetate). ¹H NMR (CDCl₃, 300MHz): δ 0.90 (t, 3H, *J* = 6.5 Hz), 1.27 (bs, 24H), 1.64 (m, 2H), 2.38 (t, 2H, *J* = 7.0 Hz), 3.81 (s, 3H), 6.89 (d, 2H, *J* = 9.0 Hz), 7.21 (d, 2H, *J* = 9.0 Hz). ¹³C NMR (CDCl₃, 500MHz): δ 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. MS(ESI), *m/z* = 464.2 [M+Na]⁺, 440.0 [M-H][−]. UPLC1: *t*_R 1.58 min, Purity > 99%.

4-methoxyphenyl 5-phenylpentanoylsulfamate (17d)

Using the above general procedure, 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), triethylamine (18.0 mmol), and DMAP (0.6 mmol) were used to synthesize **17d** as a colorless waxy solid (10%). TLC *R*_f = 0.8 (Ethyl Acetate). ¹H NMR (CDCl₃, 300MHz): δ 1.60 (bs, 4H), 2.32 (t, 2H, *J* = 6.0 Hz), 2.57 (t, 2H, *J* = 7.0 Hz), 3.73 (s, 3H), 6.81 (d, 2H, *J* = 9.0 Hz), 7.22 (m, 7H). ¹³C NMR (CDCl₃, 500MHz): δ 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. MS(ESI), *m/z* = 386.1 [M+Na]⁺, 361.9 [M-H][−]. UPLC1: *t*_R 0.61 min, Purity > 99%.

Synthesis of acylsulfamide (18)

Anhydrous formic acid (99%, 40.0 mmol, 2.0 eq) was added drop-wise to chlorosulfonyl isocyanate (40.0 mmol, 2.0 eq) at 0°C under argon. The mixture was allowed to rise to room temperature and stirred until gas evolution stopped (~2h). Propylamine (20.0 mmol, 2.0 eq) was added drop-wise 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 room temperature. NaOH (5 mL) was added to the mixture and stirred for 1h. The mixture was then extracted with Ethyl Acetate. The combined extracts were washed with brine, then dried over Na₂SO₄ and concentrated *in vacuo* to afford the propylsulfamide ester (**16**). The acid chloride was generated by adding oxalyl chloride (12.0 mmol, 2.0 eq) and DMF (0.6 mmol, 10% mol) to oleic acid (6.0 mmol, 1.0 eq) in THF (15 mL) at 0°C under argon. After gas evolution stopped (~20 min) the mixture was warmed to room temperature and allowed to stir for 2h. The resulting mixture was concentrated *in vacuo* then dissolved in DCM/DMF (1:1, 5 mL) and added drop-wise to a mixture of previously prepared propylsulfamide ester, **16** (6.0 mmol, 1.0 eq), DMAP (0.6 mmol, 10% mol), triethylamine (18.0 mmol, 3.0 eq) in DCM/DMF (1:1, 15 mL). The mixture was allowed to stir for 16h under argon. The mixture was diluted with DCM and washed with 1N HCl, water, and brine then dried over Na₂SO₄ and concentrated *in vacuo*. The residue was purified via flash chromatography (0–100%, Hexane/Ethyl Acetate) to afford oleoyl N-propylsulfamide **18** as a waxy solid (8%). *R_f* = 0.7 (8:2, CHCl₃:MeOH) ¹H NMR (CDCl₃, 500MHz): δ 0.88 (t, 3H, *J* = 6.5 Hz), 0.94 (t, 3H, *J* = 7.0 Hz), 1.28 (bs, 20H), 1.59 (m, 4H), 2.00 (m, 4H), 2.62 (t, 2H, *J* = 7.5 Hz), 2.95 (t, 2H, *J* = 6.5 Hz), 5.34 (m, 2H). ¹³C NMR (CDCl₃, 500MHz): δ 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. MS(ESI), *m/z* = 401.1 [M-H][−]. UPLC1: *t_R* 2.01 min, Purity > 99%.

Membrane Preparation and Assay of Acyltransferase Activity

Membranes of *E. Coli* 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 contained 100 mM Tris-HCL, pH 7.4, 150mM NaCl, 1 mg/mL BSA, 5 mM Na₃VO₄, 200 μM inhibitor and purified membranes. 16:0-PO₄ was added to 50 μM to start the reaction. Reactions were terminated after incubation at 37°C for 20 min by pipetting 20 μl of the reaction mixture onto a Whatman 3MM cellulose filter disc. Filter discs were washed in 10%, 5%, and 1% ice-cold trichloroacetic acid (20min, 20 ml/disk) prior to scintillation counting. The *K_M* for 16:0-PO₄ was obtained by varying the concentration of 16:0-PO₄ from 3.125 to 50 μM at a fixed [¹⁴C]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. All test compounds were dissolved in DMSO at a concentration of 10 mg/mL and stored at −80°C. Two fold serial dilutions of test compound were prepared in MH broth in 96-well plates to give drug concentrations ranging from 400 to 0.025 μg/ml. 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 λ₆₀₀=0.1 in MH broth, which was further diluted to an OD_{λ600}= 0.001. 100 μl of culture was then added to each well of the 96-well plate resulting in an OD_{λ600}= 0.0005, which corresponds to about 10⁵ CFU/ml, and final antibiotic concentrations ranging from 200 to 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 visible bacterial growth.

Cytotoxicity Assay

Vero monkey epithelial cells (ATCC CCL-81) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and maintained in a humidified incubator (37°C, 5% CO₂). Cells were dislodged with a cell scraper, collected by centrifugation, resuspended in fresh medium at ~106 cells/mL, dispensed into 96-well microtiter plates (100 µl/well) and incubated for 18 h at 37°C. Two-fold serial dilutions of test compounds (400-0.2 µg/mL) in DMEM with FBS were subsequently added and cells incubated for another 72 hours. The cytopathic effects of compounds were evaluated colorimetrically using the MTT Cell Proliferation Assay (ATCC). IC₅₀ data were obtained from dose response curves plotted as percentage activity versus log₁₀ concentration.

Acknowledgments

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.

References

1. Shah PM. Clinical microbiology and infectious diseases 2005;11 Suppl 3:36–42.
2. Coates A, Hu Y, Bax R, Page C. Nature reviews: drug discovery 2002;1:895–910.
3. Heath RJ, White SW, Rock CO. Applied microbiology and biotechnology 2002;58(6):695–703. [PubMed: 12021787]
4. Heath RJ, White SW, Rock CO. Progress in lipid research 2001;40(6):467–497. [PubMed: 11591436]
5. Zhang YM, White SW, Rock CO. The journal of biological chemistry 2006;281(26):17541–17544. [PubMed: 16648134]
6. Cronan JE. Annual reviews in microbiology 2003;57:203–224.
7. Zhang YM, Rock CO. Journal of lipid research. 2008[Epub ahead of print]
8. Lu YJ, Zhang YM, Grimes KD, Qi J, Lee RE, Rock CO. Molecular cell 2006;23:765–772. [PubMed: 16949372]
9. Lu YJ, Zhang F, Grimes KD, Lee RE, Rock CO. Journal of biological chemistry 2007;282(15):11339–11346. [PubMed: 17308305]
10. Paoletti L, Lu YJ, Schujman GE, de Mendoza D, Rock CO. Journal of bacteriology 2007;189(16):5816–5824. [PubMed: 17557823]
11. Hurdle JG, O'Neill AJ, Chopra I. Antimicrobial agents and chemotherapy 2005;49(12):4821–4833. [PubMed: 16304142]
12. Pohlmann J, Brotz-Oesterhelt H. Current drug targets 2004;4(4):261–272. [PubMed: 15578968]
13. Winum JY, Scozzafava A, Montero JL, Supuran CT. Medicinal research reviews 2005;25(2):186–228. [PubMed: 15478125]
14. Qiao C, Gupte A, Boshoff HI, Wilson DJ, Bennett EM, Somu RV, Barry CE 3rd, Aldrich CC. Journal of medicinal chemistry 2007;50(24):6080–6094. [PubMed: 17967002]
15. Somu RV, Boshoff H, Qiao C, Bennett EM, Barry CE 3rd, Aldrich CC. Journal of medicinal chemistry 2006;49(1):31–34. [PubMed: 16392788]
16. Somu RV, Wilson DJ, Bennett EM, Boshoff HI, Celia L, Beck BJ, Barry CE 3rd, Aldrich CC. Journal of medicinal chemistry 2006;49(26):7623–7635. [PubMed: 17181146]
17. Vannada J, Bennett EM, Wilson DJ, Boshoff HI, Barry CE 3rd, Aldrich CC. Organic letters 2006;8(21):4707–4710. [PubMed: 17020283]
18. Tang K-C, Coward JK. Journal of organic chemistry 1983;48:5001–5006.
19. Berkowitz DB, Eggen M, Shen Q, Shoemaker RK. Journal of organic chemistry 1996;61:4666–4675. [PubMed: 11667395]
20. Adams LA, Cox RJ, Gibson JS, Mayo-Martin MB, Walter M, Whittingham W. Chemical communication 2002;18:2004–2205.

21. Schulbach MC, Mahapatra S, Macchia M, Barontini S, Papi C, Minutolo F, Bertini S, Brennan PJ, Crick DC. *Journal of biological chemistry* 2001;276(15):11624–11630. [PubMed: 11152452]
22. Appel R, Berger G. *Chemische Berichte* 1959;91:1339–1341.
23. Fruit C, Muller P. *Helvetica Chimica Acta* 2004;87:1607–1615.
24. Lee CH, Kohn H. *Journal of organic chemistry* 1990;55:6098–6104.
25. Rye CS, Baell JB. *Current medicinal chemistry* 2005;12(26):3127–3141. [PubMed: 16375706]
26. Luna VA, King DS, Peak KK, Reeves F, Heberlein-Larson L, Veguilla W, Heller L, Duncan KE, Cannons AC, Amuso P, Cattani J. *Journal of clinical microbiology* 2006;44(7):2367–2377. [PubMed: 16825351]

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

Figure 2.
Design of Acylphosphate Substrate Mimics

(a)

(b)

Figure 3.

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

a, R = CH₃(CH₂)₁₃, Y = OMe; **b**, R = CH₃(CH₂)₅CH=CH(CH₂)₈, Y = Cl; **c**, R = CH₃(CH₂)₆, Y = OMe; **d**, R = CH₃CH₂, Y = OMe; **e**, R = CH₃(CH₂)₉, Y = OMe; **f**, R = Cy(CH₂)₃, Y = Cl; **g**, R = Ph(CH₂)₃, Y = Cl; **h**, R = PhO(CH₂)₉, Y = Cl; **i**, R = CH₃(CH₂)₇CH=CH(CH₂)₆, Y = OMe; **j**, R = CH₂=CH(CH₂)₇, Y = Cl; **2a-h**, X = CH₂; **3a,e,i,j**, X = CF₂; **4a-h**, X = CH₂; **5a,e,i,j**, X = CF₂

Scheme 1.

Reagents and Conditions: (i) Dimethyl methylphosphonate, BuLi, THF, -78°C, (ii) Diethyl (difluoromethane) phosphonate, LDA, THF, -78°C, (iii) TMSBr, DCM, then 95% EtOH.

a, R = CH₃(CH₂)₁₃; **b**, R = CH₃(CH₂)₅CH=CH(CH₂)₈; **c**, R = CH₃(CH₂)₆; **d**, R = CH₃(CH₂)₉; **e**, R = Cy(CH₂)₃; **f**, R = PhO(CH₂)₉

Scheme 2.

Reagents and Conditions: (i) Ethylchloroformate, DCM; (ii) Diethyl phosphoramidate, BuLi, THF, -78°C, (iii) TMSBr, DCM, then 95% EtOH.

Scheme 3.

Reagents and Conditions: (i) Diethylphosphonoacetic acid, HOBt, EDCI, DCM:DMF, rt, 16hrs; (ii) TMSBr, DCM, then 95% EtOH.

Reagents and Conditions: (i) HCO_2H , neat; (ii) $\text{R}'\text{OH}$, DMA; (iii) $\text{R}'\text{NH}_2$, neat, NaOH ; (iv) RCOCl , DMAP, NEt_3 , $\text{DCM}:\text{DMF}$.

Table 1

Activity Data

compound	R	R'	MIC (µg/ml) ^a										Cytox ^f (µg/ml)											
			5a-e,i,j					18a,b																
			4a-h	9a-f	12a-d	17a-d	SP	MRSA	EF	BS	BA													
			SpPlsY %inhibition ^a	SpIC ₅₀ (µM) ^b	BaPlsY 1 %inhibition ^c	BaIC ₅₀ (µM) ^d																		
4a	CH ₃ (CH ₂) ₁₃		28	nd ^g	67	90	200	100	12.5	25	1.56	205												
4b	CH ₃ (CH ₂) ₅ CH=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 ₃ CH 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) ₇ CH=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) ₅ CH=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) ₇ CH=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												

compound	R	R'	4a-h		5a,e,i,j		9a-f		12a-d		17a-d		18a,b					MIC(μg/ml) ^d					Cytos ^f (μg/ml)
			SpPlsY	%inhibition ^a	SpIC ₅₀ (μM) ^b	BaPlsY	1 %inhibition ^c	BaIC ₅₀ (μM) ^d	SP	MRSA	EF	BS	BA										
17b	CH ₃ (CH ₂) ₁₃	Bu	100	100	70	99	100	50	25	>200	>200	>200	>200	>200	>200	>200	>200	>200	nd				
17c	CH ₃ (CH ₂) ₁₃	4-OMeph	100	100	60	100	35	12.5	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	nd				
17d	Ph(CH ₂) ₃	4-OMeph	12	13	nd	13	200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	nd				
18	CH ₃ (CH ₂) ₇ CH=CH(CH ₂) ₆	pr	24	56	nd	56	200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	nd				

^a % Inhibition of *S. pneumoniae* PlsY @ 200μM inhibitor and 50μM acyl (16:0) phosphate.

^b IC₅₀ were only determined on compounds for which there was greater than 50% inhibition at SpPlsY.

^c % Inhibition of *B. anthracis* PlsY @ 200μM inhibitor and 50μM acyl (16:0) phosphate.

^d IC₅₀ were only determined on compounds for which there was greater than 50% inhibition at BaPlsY1.

^e Whole-cell Minimum Inhibitory Concentration of the following species: SP-*Streptococcus pneumoniae* DAW30, MRSA-Medihillin resistant *Staphylococcus aureus* ATCC33591, EF-*Enterococcus faecalis* ATCC33186, BS-*Bacillus subtilis* ATCC 23857, and BA-*Bacillus anthracis* Sterne 34F2.

^f Doxycycline (IC₅₀ = 212μg/mL) was used as a control. Assays were performed using the Vero monkey kidney cell line (CCL-81).

^g nd = not determined.

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

Compound	MIC ₉₀ (µg/mL) ^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)	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	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	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	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	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	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	3.13

^aWhole cell minimum inhibitory activity against various strains of *Bacillus anthracis* (BA) and *Bacillus cereus* (BC). Penicillin (MIC = 0.008–0.03 µg/ml) and Ciprofloxacin (MIC = 0.03–0.06 µg/ml) were used as controls.

^bcapsule-deficient (attenuated) strain.