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Bacterial Cell Growth Inhibitors Targeting Undecaprenyl Diphosphate Synthase and Undecaprenyl Diphosphate Phosphatase

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Abstract

We synthesized a series of benzoic acids and phenylphosphonic acids and investigated their effects on the growth of *Staphylococcus aureus* and *Bacillus subtilis*. One of the most active compounds (**7**, 5-fluoro-2-(3-(octyloxy)benzamido)benzoic acid, ED_{50} ~ 0.15 μg/mL) acted synergistically with seven antibiotics known to target bacterial cell wall biosynthesis (a fractional inhibitory concentration index, FICI~0.35, on average) but had indifferent effects in combinations with six non cell-wall biosynthesis inhibitors (FICI~1.45, on average). The most active compounds were found to inhibit two enzymes involved in isoprenoid/bacterial cell wall biosynthesis: undecaprenyl diphosphate synthase (UPPS) and undecaprenyl diphophate phosphatase (UPPP), but not farnesyl diphosphate synthase, and there were good correlations between bacterial cell growth inhibition, UPPS inhibition and UPPP inhibition.

Graphical abstract

We synthesized 30 benzoic and phenylphosphonic acids and discovered several that inhibited *Staphylococcus aureus* and *Bacillus subtilis* growth. They acted synergistically with antibiotics that target cell wall biosynthesis but not with other antibiotics. The most potent compounds targeted undecaprenyl diphosphate phosphatase and undecaprenyl diphosphate synthase and there were good correlations between enzyme and cell growth inhibition.

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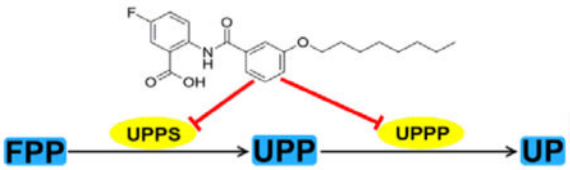
Supporting information for this article is available on the WWW under XXXX; enzyme and cell growth inhibition Tables and graphs; isobolograms; synthesis and characterization; HPLC purity results; ^{1}H NMR spectra of inhibitor compounds.



Keywords

benzoic acids; cell wall biosynthesis; drug discovery; membrane proteins; *Staphylococcus* aureus

Introduction

There is without doubt a pressing need for the development of novel antibiotics having new structures and new targets, to help combat the development of drug resistance.[1] Over the past ~70 years, many antibiotics that target bacterial cell wall biosynthesis, compounds such as penicillin, methicillin and bacitracin, have been discovered and developed and so the enzymes that are used in cell wall biosynthesis are attractive drug targets. A simplified version of bacterial cell wall biosynthesis is shown in Figure 1. Initial steps involve formation of the (C_5) isoprenoids dimethylallyl diphosphate (DMAPP, **1**) and isopentenyl diphosphate (IPP, **2**), formed in either the mevalonate pathway (in e.g. *Staphylococcus aureus*) or the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (in e.g. *E. coli*), with fosmidomycin inhibiting the MEP pathway.[2] DMAPP then condenses, sequentially, with two molecules of IPP to form the (C_{15}) species, farnesyl diphosphate (FPP, **3**) in a reaction catalyzed by farnesyl diphosphate synthase (FPPS). The FPP so produced then condenses with 8 additional IPP molecules to form (C_{55}) undecaprenyl diphosphate (UPP, **4**) in a reaction catalyzed by undecaprenyl diphosphate synthase (UPPS). UPPS is an attractive drug target since it is not used by humans and several inhibitors (e.g. tetramic/tetronic acids; diamidines and benzoic acids) have been reported.[3] UPP is then converted by undecaprenyl diphosphate phosphatase (UPPP) to undecaprenyl phosphate (UP, **5**) which acts as a membrane “anchor” for formation of glycosylated products (Lipid I, Lipid II) which are then converted to peptidoglycan cell wall products, as outlined in Figure 1. Antibiotics such as bacitracin, vancomycin and methicillin target these later stages in cell wall biosynthesis, again as shown in Figure 1. In this work, we sought to find novel benzoic acid inhibitors of UPPS and potentially UPPP since in earlier work,[3b] we had found that (2-(3-(decyloxy)benzamido)-5-nitrobenzoic acid, **6,** Figure 1) inhibited UPPS and we reasoned that similar lipophilic, anionic species might also mimic the UPPP substrate, UPP. We thus synthesized 30 analogs of **6**, **7–36**, primarily benzoic acids but also, four phosphonic acid analogs, and tested them for activity against *Staphylococcus aureus* and *Bacillus subtilis,* as well as against UPPS and UPPP, and in several cases FPPS and a human cell line. In addition, we investigated their possible synergistic activity with a range of known antibiotics that either target, or do not target, bacterial cell wall biosynthesis.

Results and Discussion

In previous work we found that the benzoic acid **6** (Figure 1) was a promising inhibitor of both *E. coli* UPPS (EcUPPS; IC_{50} = 3.0 μM) and *S. aureus* UPPS (SaUPPS; IC_{50} = 0.49 μM). In later work we found that several related lipophilic benzoic acids had activity against gram-positive (but not gram-negative) bacteria with **6** having a 1.3 μM ED_{50} value against *B. subtilis*, but no activity against *E. coli*, leading us to synthesize the 30 compounds whose structures and activities are shown in Table 1. Full synthesis and characterization details are in the Supporting Information.

The rationale for the synthesis of these compounds was first, to investigate a series of compounds (**7–16, 19, 21**) in which we varied the substituent *meta* to the carboxyl group, covering a wide range of Hammett σ_m values (0 for −H, 0.71 for $-NO_2$) which we reasoned would affect the acidity of the carboxyl group, the more electron-withdrawing groups yielding, perhaps, a better analog (carboxylate) of the diphosphate group (in FPP and UPP). We also investigated whether phosphonic acids (**18, 31, 36**) might be better inhibitors than benzoic acids, plus, we investigated a broad range of other benzoic acid ring-substitution patterns, as well as different “side-chains” (corresponding to the octyloxy group in e.g. **7**), in order to probe both enzyme as well as cell activity. We then investigated the cell growth inhibition activity of these compounds against *S. aureus* and *B. subtilis* and ED_{50} values (in μg/mL) are shown in Table 1 (rank ordered by SaUPPS inhibition, as discussed below). We also screened for activity against the yeast *Saccharomyces cerevisiae* and the gram-negative, *E. coli,* but there was no activity with any compound (ED_{50} values > 200 μM). Typical dose-response curves for 3 compounds: benzoic acids with electron withdrawing (**7**) or electron donating (**13**) ring substituents, plus a phosphonic acid analog (**18**), are shown in Figure 2. Dose-response curves for all compounds tested are shown in Supporting Information Figure S1. There were several active compounds amongst the 30 investigated, with the most active being the *m*-trifluoromethoxy analog **11**, with an ED_{50} value of 0.082 μg/mL against *S. aureus*. The most active bacterial cell growth inhibitors were all lipophilic benzoic acids with highly electron-withdrawing ($-NO_2$, $-OCF_3$) ring substituents, Table 1. We screened **11** and three other compounds (**7**, **14**, **18**) against a human cell line (NCI-H460) to assess toxicity finding low activity (a selectivity index of ~1000 for **11,** Figure S2). The presence of electron-donating groups (**13, 14**) decreased anti-bacterial activity, as did the presence of substituents that might hydrogen-bond to water (**19, 22, 27, 29**). The phosphonic acids (**19, 31, 36**) had little or no antibacterial activity although the diethyl phosphonate **21** was active, consistent with the observation that all compounds with additional ionizable groups on the benzoic acid have poor activity.

The question then arises: what might the targets of these inhibitors be? The compounds were designed based on the UPPS inhibition precedent with **6**, but FPPS and UPPP inhibition also seemed possible (with some carboxylic acids being known, potent FPPS inhibitors,[4] binding to an allosteric site). To try and narrow down the possibilities as well as open up potential routes to synergistic combinations, we next investigated whether or not one of the most active compounds, the fluoro-benzoic acid **7**, exhibited synergistic, additive, indifferent or antagonistic activity with a broad range of antibiotics that act either by inhibiting bacterial cell wall biosynthesis, or by other mechanisms. The antibiotics that act by targeting cell wall

biosynthesis were ampicillin, bacitracin, fosmidomycin, carbenicillin, vancomycin, fosfomycin, and cefotaxime. The compounds that do not target bacterial cell wall biosynthesis were kanamycin, tetracycline, sulfamethoxazole, trimethoprim, spectinomycin, and chloramphenicol We determined the fractional inhibitory concentration index (FICI) values for each combination using the FICI formula:[5]

$$FICI = FIC_A + FIC_B = \frac{MIC(AB)}{MIC(A)} + \frac{MIC(BA)}{MIC(B)}$$

where FIC_A, FIC_B are the fractional inhibitory concentrations of drugs A and B, MIC(A) and MIC(B) are the MIC values of drugs A and B acting alone, and MIC(AB) and MIC(BA) are the MIC values of the most effective combination of drug A or B in the presence of drug B or A. Using this method, FICI values of <0.5 indicate synergy, >0.5 and <1.0 indicate additivity, >1 and <2 indicate an indifferent effect, and ≥2 indicates antagonism.[6] In addition, we evaluated isobolograms using the method of Berenbaum.[7] FICI values are shown in Table 2 and representative isobolograms are in Figure 3. All isobolograms are given in SI Figure S3.

As can be seen in Table 2, there are very distinct differences between the FICI values obtained with **7** and the cell-wall biosynthesis inhibitors, and those obtained with **7** and the non cell-wall biosynthesis inhibitors. For *S. aureus*, the mean FICI with a cell wall biosynthesis inhibitor is 0.32, for *B. subtilis*, 0.37. These values represent synergism (i.e. the FICI is <0.5) and suggest that **7** acts in the cell wall biosynthesis pathway. With the non cell-wall biosynthesis inhibitors, the mean FICI for *S. aureus* in 1.42, and for *B. subtilis*, 1.47, meaning indifferent effects, as expected. Clearly then, in both *S. aureus* as well as in *B. subtilis*, **7** is targeting primarily one or more enzymes involved in cell wall biosynthesis and likely candidates that utilize long, lipophilic anionic substrates are UPPS and UPPP, and perhaps, FPPS. We thus next tested all compounds against UPPS (from *S. aureus*), since in earlier work we had found **6** was a UPPS inhibitor, plus, we tested all compounds against a UPP-phosphatase. UPPP is a membrane protein and we used the fusion hybrid of *E. coli* UPPP with *Haloarcula marismortui* bacteriorhodopsin,[8] which is active in detergent-based assays and is inhibited by bacitracin. We also tested the most active compounds against an EcFPPS, but there was no inhibition (up to 200 μM). Typical dose-response curves for SaUPPS and EcUPPP are shown in Figure 4 and include for UPPP, results for the known inhibitor, bacitracin, which has an IC_{50} = 32 μM, as also reported by Chang et al.[9] Full dose response curves for all compounds are in SI Figure S4 and all numerical results are shown in Table 1.

As can be seen in Table 1, the most active cell growth inhibitors are also some of the most potent UPPS and UPPP inhibitors with IC_{50} values as low as 320 nM (for UPPS) and 1.3 μM (for UPPP). For the top 8 most potent SaUPPS inhibitors (**7, 8, 9, 10, 11, 13, 14, 15**) there is a good correlation with reported Hammett σ_m values (R^2=0.79) but this breaks down with bulkier ring substituents. For example, the electron-withdrawing group $(EtO)_2PO$ (σ_m=0.42) has weak activity. We have not been able to obtain X-ray structures of SaUPPS with bound inhibitors, but we have reported the structure of **6** bound to EcUPPS (PDB ID

code 4H2O), which suggests a possible explantion for the results reported here. Specifically, **6** has a decyloxy sidechain and the two molecules that bind to UPPS have relatively solvent exposed nitrobenzoate sidechains. This exposure will have an associated energy penalty. The shorter C_8 sidechain in **8** could improve enzyme inhibition by reducing this solvent exposure, but the effect is small (0.33 μM vs 0.49 μM). However, with another pair of ligands: the fluorobenzoate **7** with a C_8 sidechain and the fluorobenzoate **23** with a C_6 sidechain, we find that the shorter chain species has a 4.9 μM IC_{50} while the longer-chain (C_8) species has a much smaller IC_{50} (IC_{50}=0.32 μM). Taken together, these results indicate that inhibitors with a C_8 side-chain and small, electron-withdrawing ring substituents have close to optimum activity. The bulkier methyl sulfone and the diethyl phosphonate groups then—while being very electron withdrawing—likely contribute to a decrease in protein binding affinity because the added ligand volume is more solvent exposed than with the smaller substituents. This binding mode (as seen with **6** in PDB ID code 4H2O) in which the alkyloxy groups are buried in the interior of UPPS while the benzoates are at or close to the the surface could also explain why adding extra carboxylates or phosphonates reduces activity—the added groups can hydrogen bond with the solvent.

Interestingly, as can be seen in Figure 5a, there is a very good correlation between the pED_{50} (= $-\log_{10}$ ED_{50} [μM]) values for *S. aureus* and *B. subtilis* cell growth inhibition (a Pearson r-value correlation-coefficient, r = 0.90), strongly suggesting that the same targets are involved in both systems. And as shown in Figure 5b, the correlation for *S. aureus* cell growth versus SaUPPS inhibition is also high with an r = 0.85. Figure 5c shows a Pearson r-value correlation-coefficient matrix[10] for all cell and enzyme activities as well as for logD (estimated using the chemicalize.org web portal, http://www.chemaxon.com), from which it can be seen that both UPPS inhibition as well as UPPP inhibition correlate with cell growth inhibition. There are weaker correlations between cell growth inhibition and logD, while the enzyme inhibition results are essentially uncorrelated with logD so, as expected, permeabilty/transport plays a role in overall activity in cell growth inhibition. The enzyme/cell correlations are better than those we discussed in a previous study[11] where we found for 10 different cell/putative enzyme target inhibition assays (three from our group, seven randomly chosen from the literature) that on average the correlation between the enzyme and cell assay results was r~0.55.

What is also apparent from the results shown in Table 1 is that, in general, the IC_{50} values for UPPP inhibition are somewhat larger than the IC_{50} values for UPPS inhibition, suggesting that in cells, UPPS and not UPPP is the primary target. This is, however, likely to be an over-simplification since the enzyme assays were all detergent based; substrate concentrations in cells are not known; UPPP is a membrane protein and inhibitors may accumulate in membranes; and we used an EcUPPP-bacteriorhodopsin fusion in our assay together with FPP, and not UPP. Nevertheless, as can be seen in Table 1, several of the most potent cell growth inhibitors do target both enzymes, and inhibition of two consecutive enzymes in the bacterial cell wall biosynthesis pathway is expected to result in enhanced activity over single target inhibition. Moreover, we find good enzyme inhibition/cell growth correlations, plus, as shown in Figure 3, addition of any of the known cell wall biosynthesis

inhibitors in assays with **7** results in synergistic cell growth inhibition with, on average, FICI values of ~0.35.

Conclusions

The results reported here are of interest since we find that several lipophilic benzoic acids with electron-withdrawing ring substituents have activity against *S. aureus* and *B. subtilis* cell growth. One of the most potent compounds exhibited synergistic activity with numerous known cell wall biosynthesis inhibitors ($FICI_{avg}$~0.35, n=13 different cell/inhibitor combinations), but an indifferent effect ($FICI_{avg}$~1.45, n=12 cell/inhibitor combinations) with non cell-wall biosynthesis antibiotics. We tested all compounds against UPPS and UPPP and in some cases FPPS finding several promising UPPS as well as UPPP inhibitors. The most potent UPPP inhibitor was the trifluoromethoxy analog **11** which was ~40× more active than the known UPPP inhibitor, the antibiotic bacitracin. It also inhibited UPPS and was active against *S. aureus* (ED_{50} ~82 ng/mL). Electron-withdrawing substituents were essential for enzyme/cell inhibition by the benzoic acids, presumably because they make the benzoates much stronger acids (that are fully dissociated), although the presence of multiple acid groups reduced both enzyme and cell activity. The phosphonic acids were worse UPPS/UPPP inhibitors and were mostly inactive in cells. There were good correlations between cell growth inhibition and UPPS inhibition, as well as between cell growth inhibition and UPPP inhibition, but UPPS inhibition was more potent. Overall, the results are of interest since we show for the first time that lipophilic benzoic acids inhibit both UPPP as well as UPPS and are active in cells where they act synergistically with known cell wall biosynthesis inhibitors and as such, they may represent new leads for developing bacterial cell wall biosynthesis inhibitors.

Experimental Section

Chemical Syntheses: General Methods

All chemicals were reagent grade. ^{1}H NMR and ^{13}C NMR spectra were obtained on Varian (Palo Alto, CA) Unity spectrometers at 400 and 500 MHz for ^{1}H. HPLC/MS analyses were performed by using an Agilent LC/MSD Trap XCT Plus system (Agilent Technologies, Santa Clara, CA) with an 1100 series HPLC system including a degasser, an autosampler, a binary pump, and a multiple wavelength detector. Purity was determined by HPLC-MS and structures were characterized by ^{1}H NMR and HRMS. We synthesized 26 benzoic acids and 4 phenylphosphonates using the general methods shown in Scheme 1a, 1b. Full synthesis and characterization detail are in the Supporting Information and details of the synthesis of two representative compounds are shown below.

5-Fluoro-2-(3-(octyloxy)benzamido)benzoic acid (**7**) The scheme used to synthesize **7** is as shown in Scheme 1a. 1-Bromooctane **b** (7.7 g, 40 mmol) was added to a solution of methyl 3-hydroxybenzoate **a** (3.0 g, 20 mmol) in 20 mL DMF followed by addition of potassium carbonate (5.5 g, 20 mmol). The solution was heated to 80°C and stirred for 12 h. After cooling to room temperature, the solution was washed with water (100 mL), then extracted with ethyl acetate (50 mL ×3). The organic layer was dried over Na_2SO_4 and solvent removed under vacuum. The residue was purified by flash chromatography on silica gel

(hexane/EtOAc = 20:1) to give methyl 3-(octyloxy)benzoate **c** as a colorless oil (4.8 g, 90%). Compound **c** (4.0 g, 15 mmol) was dissolved in 20 mL of THF, and aqueous LiOH (1.8 g in 10 mL water) added. The reaction mixture was stirred at room temperature for 12 h, then concentrated under vacuum to remove solvent. The aqueous solution was acidified with HCl to pH 1 upon which a white solid precipitated. The suspension was extracted with ethyl acetate (30 mL ×3). The organic layer was dried over Na_2SO_4 and solvent removed under vacuum. The 3-(octyloxy)benzoic acid **d** was obtained as a white solid (2.5 g, 95%). Compound **d** (2.5 g, 10 mmol) was dissolved in 20 mL of CH_2Cl_2, and 2 mL of oxalyl chloride added. Then, one drop of DMF was added as a catalyst. The reaction mixture was stirred at room temperature for 12 h, then concentrated under vacuum. The residue 3-(octyloxy)benzoyl chloride **e** was obtained as a yellow liquid (3.4 g, 90%). Compound **e** (2.7 g, 10 mmol) was dissolved in 20 mL of CH_2Cl_2, then 2-amino-5-fluorobenzoic acid **f** (1.6 g, 10 mmol) added. After stirring for 5 min, 2 mL of NEt_3 was added. The reaction mixture was stirred at room temperature for 12 h then washed with 2 M HCl (30 mL ×2) and water (30 mL). The residue was concentrated under vacuum to give the final product **g** (**7**) as a light yellow solid (3.0 g, 80%).^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 8.66 (dd, J =5, 9.5 Hz, 1 H), 7.75 (d, J =9.5 Hz, 1 H), 7.54 (t, J =9.5 Hz, 1 H), 7.48 (m, 3 H), 7.19 (d, J =8.0 Hz, 1 H), 4.03 (t, J =6.5 Hz, 2 H), 1.72 (m, 2 H), 1.41 (m, 2 H), 1.26–1.22 (m, 8 H), 0.84 (t, J =7.0 Hz, 3 H). ^{13}C NMR (125 MHz): d 168.8, 164.3, 158.9, 156.9 (d, J = 240 Hz), 137.4, 135.7, 130.1, 122.1 (d, J = 7.6 Hz), 121.0 (d, J = 22.0 Hz), 118.9, 118.7 (d, J = 6.5 Hz), 118.3, 117.0 (d, J = 23.9 Hz), 112.1, 67.6, 31.2, 28.7, 28.6, 28.5, 25.4, 22.0, 13.9. ESI HRMS: m/z $[M+H]^+$ calculated for $C_{22}H_{27}FNO_4^+$: 388.1924, found: 388.1924. Purity of the product determined by HPLC (Phenomenex C6-Phenyl 110A. 100×2 mm, 3 μm, 250 nm, retention time = 8.1 min): 99.7%

2-(N-Methyl-3-(octyloxy)benzamido)phenylphosphonic acid (**31**) The scheme used to synthesize **31** is as shown in Scheme 1b. Compound **e** (540 mg, 2.0 mmol) was dissolved in 20 mL of CH_2Cl_2 and then diethyl(2-aminophenyl)phosphonate **h** (460 mg, 2.0 mmol) was added. After stirring for 5 min, 2 mL of NEt_3 was added. The reaction mixture was stirred at room temperature for 12 h then washed with 2 M HCl (30 mL ×2) and water (30 mL). The residue was concentrated under vacuum to give compound **i** as a white solid (780 mg, 85%). Compound **i** (460 mg, 1 mmol) was dissolved in dry CH_2Cl_2 (15 mL), cooled to 0°C and Me_3SiBr (1.2 mL, 9 mmol) added drop-wise over 30 min. The mixture was then stirred for 2 d at room temperature. The solvent was evaporated and the residue dried in vacuum for approximately 1 h. Then, 20 mL dry methanol was added and the mixture stirred for 20 min at room temperature. The solvent was removed and the residue dried overnight to give the final product **j** (**31**) as a white solid (390 mg, 95%). ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 12.14 (s, 1 H), 8.64 (dd, J =4.5, 8.5 Hz, 1 H), 7.65 (dd, J =7.5, 14.5 Hz, 1 H), 7.59 (d, J =7.5 Hz, 1 H), 7.54 (m, 2 H), 7.44 (t, J =8.0 Hz, 1 H), 7.16 (m, 2 H), 4.03 (t, J =6.5 Hz, 2 H), 1.73 (m, 2 H), 1.42 (m, 2 H), 1.26–1.22 (m, 12 H), 0.85 (t, J =7.0 Hz, 3 H). ESI HRMS: m/z $[M-H]^-$ calculated for $C_{21}H_{27}NO_5P^-$: 404.1627, found: 404.1622. Purity of the product determined by HPLC (Phenomenex C6-Phenyl 110A. 100×2 mm, 3 μm, 250 nm, retention time = 7.3 min): 93.1%.

B. subtilis growth inhibition assay

ED_{50} values for *B. subtilis* cell growth inhibition were determined using a microdilution method. A stationary overnight starter culture of *B. subtilis* (*Bacillus subtilis* subsp. *subtilis* (Ehrenberg) Cohn ATCC 6051) was diluted 1000-fold and grown to an OD_{600} of ~0.3. This log-phase culture was again diluted 500-fold into fresh LB broth to generate the "working solution". 200 μL of working solution was transferred into each well of a 96-well culture plate (Corning 3370). Inhibitors were then added at 1 mM and sequentially diluted 3× to 46 nM, keeping volume and culture broth composition constant. Plates were incubated for 12 hours at 37°C, shaking at 200 RPM, then absorbance at 600 nm was measured to assess bacterial cell growth. ED_{50} values were determined using nonlinear regression whereas minimum inhibitory concentration (MIC) values for each antibiotic and **7** in the synergy assays were calculated by using a Gompertz function in Prism 5 (GraphPad Software, Inc, La Jolla, CA).

S. aureus growth inhibition assay

As with the *B. subtilis* growth inhibition assay, an overnight starter culture of *S. aureus* (Newman strain) was diluted 1000-fold to create a "working solution". Working solutions were transferred into flat-bottom 96-well plates and inhibitors added at 1 mM and sequentially diluted 3× to 46 nM. Plates were incubated at 37°C, shaking at 200 RPM for 24 hours. The OD_{600} was then measured to determine bacterial growth inhibition.

H460 cell toxicity

A broth microdilution method was used to determine the growth inhibition ED_{50} values. Briefly, ~ 10^4 NCI-H460 cells suspended in 100 μL of DMEM supplemented with 10% fetal bovine serum (FBS), 4.5 g/L glucose and L-glutamine and preserved with 1% penicillin–streptomycin were seeded in 96-well plates (Corning Inc., Corning, NY) and incubated at 37 °C in a 5% CO_2 atmosphere. The cells were cultured for 24 h then incubated with different concentrations of compounds for another 24 h. Then, an MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay (ATCC, Manassas, VA) was performed. Data from 4 experiments for each inhibitor were pooled and then fitted to single dose-response curves. The reported values are thus +/− SEM (n=4).

Synergy/Antagonism Assays

In order to investigate possible synergistic interactions between compound **7** and a range of antibiotics, we carried out two-drug combination assays. Bacterial cells were incubated with a 3× gradient of antibiotic typically ranging from 40 μg/mL to 18 ng/mL (200 μg/mL to 90 ng/mL for bacitracin, fosfomycin, and sulfamethoxazole) in the presence half-MIC concentrations of **7**, in addition to a 3× gradient of **7** ranging from 40 μg/mL to 18 ng/mL in the presence of half-MIC concentrations of each antibiotic. New MIC values were calculated by using a Gompertz function in Prism 5 (GraphPad Software, Inc, La Jolla, CA).

Enzyme Inhibition Assays

SaUPPS and EcUPPP were expressed and purified as described previously.[3b, 9] UPPS assays were carried out using a phosphate release assay.[3b] Benzoic acid and phosphonic

acid derivatives were prepared as 10 mM stock solutions in DMSO, and were then serially diluted from 1 mM to 1 nM. Inhibitors were incubated with 25 ng of SaUPPS at room temperature for 10 minutes in a pH 7.5 buffer (50 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, and 0.02% n-dodecyl-β-D-maltopyranoside) before adding "reaction mixture" containing 5 μM FPP, 50 μM IPP, 3 U/mL purine nucleoside phosphorylase, 1 U/mL inorganic phosphatase, and ~600 μM 7-methyl-6-thioguanosine (MESG), again in the same buffer. Reactions were monitored for 15 minutes with the rate of increase in absorbance at 360 nm taken as the rate of UPP synthesis. IC_{50} values were calculated by using Prism 5 (GraphPad Software, Inc, La Jolla, CA). The UPPP inhibition assay was carried out using a malachite-green reagent as described previously.[12] The same 10 mM inhibitor stock solutions and assay buffer as for the SaUPPS assays were used to test for UPPP inhibition. Inhibitors were incubated with 20 nM EcUPPP at room temperature for 15 minutes before adding FPP to 35 μM. Reaction mixtures were incubated at 37°C for 20 minutes, then quenched by adding 30 μL of malachite-green reagent. In this assay, the phosphate released from FPP reacts with ammonium molybdate to form phosphomolybdate (yellow) which then forms a complex (λ_{max} ~ 620 nm) with malachite-green, used to assess phosphatase activity. Phosphate release was measured at 620 nm and quantified based on a phosphate standard curve, and the OD_{620} values used to construct dose-response curves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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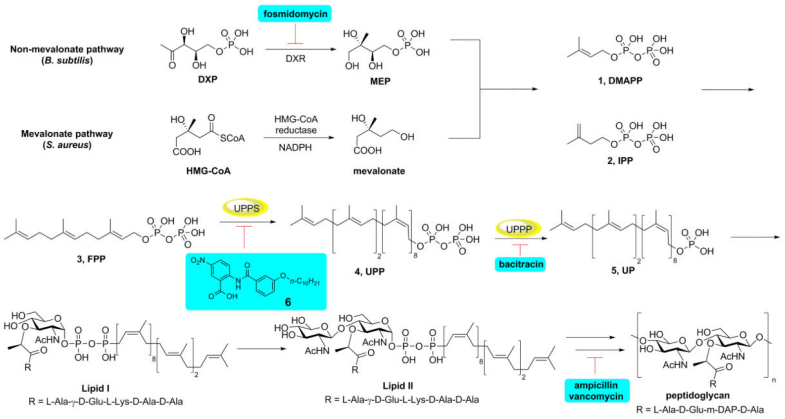


Figure 1.

Schematic outline of cell wall biosynthesis (in most bacteria) delineating the role of isoprenoid biosynthesis in the early stages of peptidoglycan formation, together with the reactions targeted by several compounds discussed in the Text.

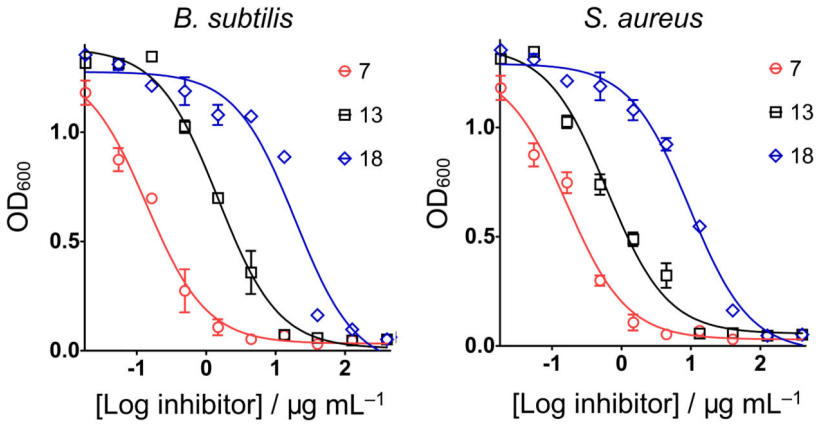


Figure 2.

Representative dose-response curves for three inhibitors (**7**, **13** and **18**) against *B. subtilis* and *S. aureus*. Data points are reported as mean±SD for duplicate experiments.

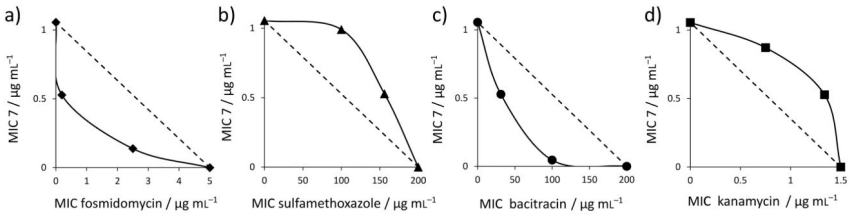


Figure 3.

Representative isobolograms for **7** with antibiotics having known mechanisms of action. a) **7**+fosmidomycin in *B. subtilis* showing synergy (FICI=0.17) of **7** with a cell wall biosynthesis inhibitor (that targets DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase, in the non-mevalonate pathway); b) **7**+sulfamethoxazole in *B. subtilis* showing an indifferent effect (FICI=1.72) of **7** with a nucleic acid biosynthesis inhibitor (that targets dihydropteroate synthase); c) **7**+bacitracin in *S. aureus* showing synergy (FICI=0.20) of **7** with a cell wall biosynthesis inhibitor (that targets UPPP); d) **7**+ kanamycin in *S. aureus* showing an indifferent effect (FICI=1.72) of **7** with a protein biosynthesis inhibitor (that targets ribosome function).

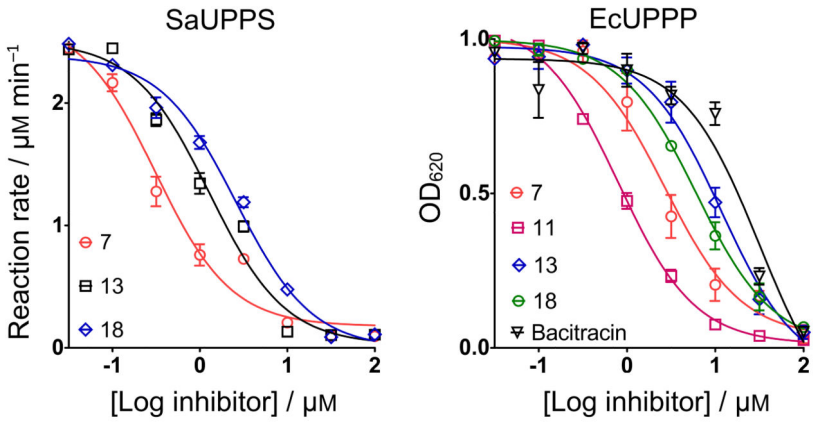


Figure 4.

Dose-response curves of various benzoic acid and phenyl phosphonic acid derivatives against SaUPPS and EcUPPP. The benzoic acids are up to ~40× more potent UPPP inhibitors than bacitracin, a known UPPP inhibitor used as a topical antibiotic. Data points are reported as mean±SD, for duplicate experiments.

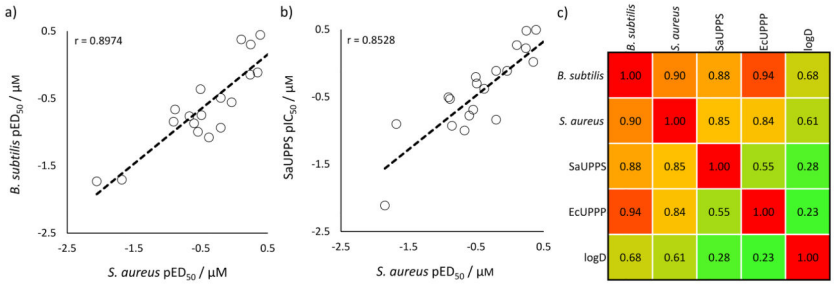
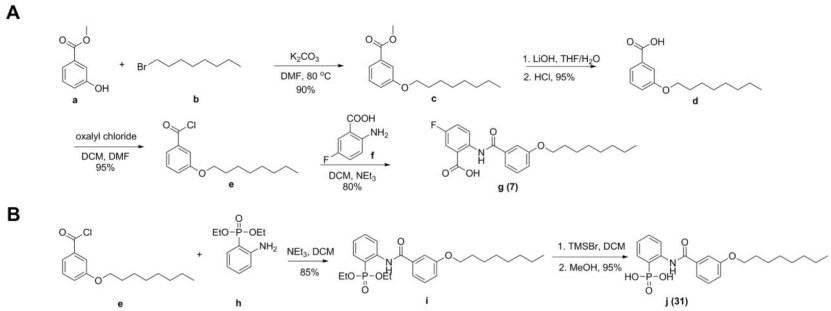


Figure 5.

Correlations between cell growth and enzyme inhibition results. a) Correlation between *S. aureus* and *B. subtilis* cell growth inhibition based on pED_{50} (=$-\log_{10}ED_{50}$ [µM]) results; b) Correlation between *S. aureus* cell growth inhibition and SaUPPS inhibition; c) Pearson r-value correlation matrix/heat map for *S. aureus* cell growth inhibition, *B. subtilis* cell growth inhibition, SaUPPS and EcUPPP enzyme inhibition (all based on pED_{50} or pIC_{50} values), and logD. The Pearson r-values are indicated and red/orange=high correlation, green=low correlation.



Scheme 1.

General synthesis methods. a) for benzoic acids; b) for phenylphosphonates.

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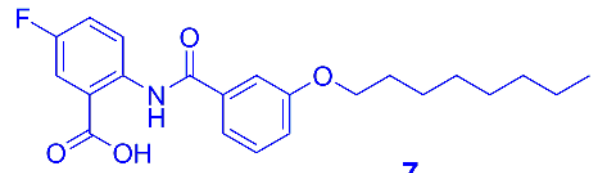
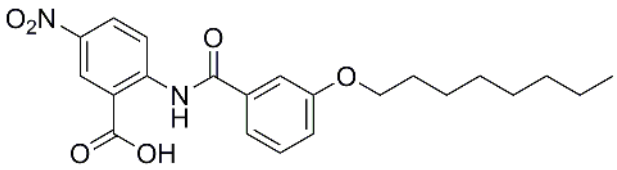
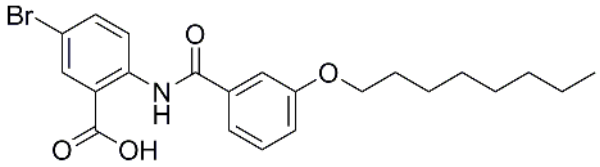
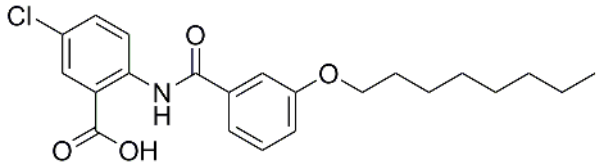
Table 1

ED_{50} values of benzoic acid and phosphonic acid inhibitors against *B. subtilis* and *S. aureus* cell growth (in μg/mL) and IC_{50} values against SaUPPS and EcUPPP enzymes (in μM). Inhibitors discussed in the Text are shown in blue. logD values were estimated using the chemicalize.org server (http://www.chemaxon.com). Standard errors are shown in Table S1.

Compound	logD	*B. subtilis*	*S. aureus*	SaUPPS	EcUPPP
 **7**	3.5	0.14	0.16	0.32	2.7
 **8**	3.2	0.21	0.24	0.33	1.3
 **9**	4.2	0.42	0.79	0.54	4.2
 **10**	4.0	0.56	0.23	0.60	3.0

Compound	logD	*B. subtilis*	*S. aureus*	SaUPPS	EcUPPP
11	4.7	0.21	0.082	0.78	0.83
12	3.7	0.53	0.18	0.96	3.4
13	3.0	1.4	0.42	1.3	11
14	3.1	1.2	0.64	1.3	>200
15	3.3	0.85	1.2	1.6	10

Compound	logD	*B. subtilis*	*S. aureus*	SaUPPS	EcUPPP
16	2.3	2.5	1.4	2.0	9.8
17	2.7	4.1	0.82	2.4	45
18	−1.6	23	11	2.5	6.7
19	3.6	>100	>100	3.0	4.2

Compound	logD	*B. subtilis*	*S. aureus*	SaUPPS	EcUPPP
20	3.2	2.7	3.1	3.2	8.5
21	0.80	2.3	3.9	3.4	>200
22	2.6	>100	>100	4.5	>200
23	2.8	3.6	1.3	4.9	20
24	3.1	>100	>100	4.9	31

Compound	logD	*B. subtilis*	*S. aureus*	SaUPPS	EcUPPP
25	3.1	3.0	1.6	6.0	>200
26	−0.28	3.4	0.6	6.9	>200
27	2.6	>100	>100	7.0	>200
28	0.46	20	18	8.0	>200

Compound	logD	*B. subtilis*	*S. aureus*	SaUPPS	EcUPPP
29	3.1	>100	3.0	8.5	>200
30	2.5	2.7	2.2	10	>200
31	2.1	>100	41	39	6.5
32	5.6	>100	>100	60	>200

Compound	logD	*B. subtilis*	*S. aureus*	SaUPPS	EcUPPP
33	0.79	>100	33	130	>200
34	2.8	20	42	>200	73
35	2.9	>100	>100	>200	>200
36	1.5	>100	>100	>200	7.1
Bacitracin	−2.6	84	145	N.D.	32

Table 2

Combinations of **7** and antibiotics against *B. subtilis* and *S. aureus.* FIC values are reported as mean±SD for duplicate experiments.

	B. subtilis					
	Antibiotic	**MIC antibiotic (μg/mL)**	**FIC antibiotic**	**MIC 7 (μg/mL)**	**FIC 7**	**FIC Index**
Cell Wall Biosynthesis Inhibitors	Fosmidomycin	5	0.039 ± 0.0053	1	0.13 ± 0.056	0.17 ± 0.056
	Carbenicillin	5	0.22 ± 0.073	1	0.15 ± 0.071	0.37 ± 0.10
	Cefotaxime	2.5	0.17 ± 0.026	1	0.14 ± 0.081	0.31 ± 0.085
	Vancomycin	0.5	0.27 ± 0.24	1	0.17 ± 0.063	0.44 ± 0.25
	Fosfomycin	200	0.16 ± 0.15	1	0.063 ± 0.022	0.22 ± 0.15
	Ampicillin	0.5	0.30 ± 0.072	1	0.23 ± 0.084	0.53 ± 0.11
	Bacitracin	200	0.18 ± 0.089	1	0.36 ± 0.14	0.53 ± 0.17
Protein Biosynthesis Inhibitors	Chloramphenicol	0.5	0.75 ± 0.18	1	0.97 ± 0.42	1.72 ± 0.46
	Kanamycin	1.5	0.64 ± 0.14	1	0.71 ± 0.30	1.36 ± 0.33
	Tetracycline	5	0.48 ± 0.047	1	0.57 ± 0.50	1.04 ± 0.50
Nucleic Acid Inhibitors	Sulfamethoxazole	200	0.78 ± 0.21	1	0.94 ± 0.64	1.72 ± 0.67
	Trimethoprim	0.5	0.66 ± 0.13	1	0.85 ± 0.29	1.51 ± 0.32
	S. aureus					
	Antibiotic	MIC antibiotic (μg/mL)	FIC antibiotic	MIC **7** (μg/mL)	FIC **7**	FIC Index
Cell Wall Biosynthesis Inhibitors	Carbenicillin	15	0.23 ± 0.048	1	0.077 ± 0.026	0.30 ± 0.055
	Cefotaxime	2	0.18 ± 0.055	1	0.084 ± 0.031	0.27 ± 0.063
	Vancomycin	1.5	0.14 ± 0.034	1	0.15 ± 0.062	0.29 ± 0.071
	Fosfomycin	200	0.20 ± 0.11	1	0.091 ± 0.034	0.29 ± 0.36
	Ampicillin	0.5	0.41 ± 0.17	1	0.15 ± 0.071	0.56 ± 0.18
	Bacitracin	200	0.16 ± 0.11	1	0.044 ± 0.019	0.20 ± 0.11
Protein Biosynthesis Inhibitors	Spectinomycin	40	0.75 ± 0.43	1	0.81 ± 0.32	1.56 ± 0.54
	Chloramphenicol	5	0.88 ± 0.47	1	0.81 ± 0.31	1.69 ± 0.56
	Kanamycin	1.5	0.89 ± 0.68	1	0.83 ± 0.29	1.72 ± 0.74
	Tetracycline	0.5	0.24 ± 0.11	1	0.51 ± 0.24	0.75 ± 0.26

B. subtilis

	Antibiotic	MIC antibiotic (μg/mL)	FIC antibiotic	MIC 7 (μg/mL)	FIC 7	FIC Index
Nucleic Acid Inhibitors	Sulfamethoxazole	200	0.54 ± 0.13	1	0.98 ± 0.37	1.53 ± 0.39
	Trimethoprim	15	0.69 ± 0.32	1	0.57 ± 0.25	1.26 ± 0.41

Mean FICIs:	*B. subtilis*	inhibitors targeting cell wall biosynthesis	0.37 ± 0.14
		inhibitors targeting nucleic acids and protein biosynthesis	1.47 ± 0.28
	S. aureus	inhibitors targeting cell wall biosynthesis	0.32 ± 0.12
		inhibitors targeting nucleic acids and protein biosynthesis	1.42 ± 0.37