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Anthranilate 4*H*-oxazol-5-ones: novel small molecule antibacterial acyl carrier protein synthase (AcpS) inhibitors

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Abstract—D-optimal design and Projection to Latent Structures (PLS) analysis were used to optimize screening hit **5** (*B. subtilis* AcpS IC_{50}: 15 μM, *B. subtilis* MIC: >200 μM) into a series of 4*H*-oxazol-5-one, small molecule, antibacterial, AcpS inhibitors. Specifically, **15**, **16** and **18** show μM or sub-μM AcpS inhibition (IC_{50}s: **15**: 1.1 μM, **16**: 1.5 μM, **18**: 0.27 μM) and moderate antibacterial activity (MICs: 12.5–50 μM) against *B. subtilis*, *E. faecalis ATCC*, *E. faecalis VRE* and *S. pneumo*$^+$.

1. Introduction

Rapidly developing resistance of known bacterial strains to currently prescribed pharmaceutical agents has stimulated a vigorous search for novel mechanisms to kill bacteria.[1,2] Strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), penicillin-resistant *Streptococcus pneumoniae* (PRSP) and multiple drug-resistant *Enterobacteriaceae klebsiella* (MDR ESBL) do not respond to current clinical drug therapies including quinolones and β-lactams.

In order to overcome bacterial resistance to currently prescribed antibiotics, we have searched for other cellular processes that would serve as good antibacterial targets. One such process is the conversion of apo-acyl carrier protein (apo-Acp) to holo-acyl carrier protein (holo-Acp) which is catalyzed by acyl carrier protein synthase (AcpS), an enzyme that catalyzes the transfer of the 4′-phosphopantetheinyl group of coenzyme A (CoA) to a serine residue of apo-Acp. The resulting holo-Acp accepts acetyl and malonyl groups from acetyl-CoA and malonyl-CoA respectively and shuttles these acyl groups between enzymes in the fatty acid biosynthesis pathway.[3] AcpS is required for the biosynthesis of important components of membrane lipids and bacterial lipopolysaccharides, and the essentiality of this enzyme was determined genetically in *Streptococcus pneumoniae* (*S. pneumo*)[4] and *Escherichia coli* (*E. coli*).[5] The crystal structures of *S. pneumo*[6] and *Bacillus subtilis* (*B. subtilis*)[7] AcpS have been determined but no small molecule inhibitors of AcpS have previously been reported.

In this letter, we disclose the discovery of a series of small molecule, antibacterial, anthranilate 4*H*-oxazol-5-one AcpS inhibitors **1**. In addition, we will show how multivariate analysis (MVA) and experimental design were used to rapidly optimize the biological activity of these molecules.

2. Chemistry

Anthranilate 4*H*-oxazol-5-ones **1** were prepared according to literature procedures. Thus, acid chlorides **2** were converted to hippuric acids **3**,[8] and cyclized to

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the corresponding 4*H*-oxazol-5-ones **4** with triethyl orthoformate and Ac_2O.[9] The target anthranilate 4*H*-oxazol-5-ones **1** were prepared by heating **3** with anthranilic acids in EtOH.[10]

3. Biology

AcpS inhibitory activity was determined using a *B. subtilis* GST-Acp-HTRF assay.[11] The in vitro determination of the minimal inhibitory concentrations (MICs) against aerobic bacteria was performed by the microdilution broth method as recommended by the National Committee for Clinical Laboratory Standards.[12]

4. Results and discussion

4-Cl Phenyl 4*H*-oxazol-5-one **5** was identified as an inhibitor of *B. subtilis* AcpS in a high-throughput screen (AcpS IC_{50}: 15 μM) (Fig. 1). Unfortunately this compound had no MIC activity against several strains of bacteria. Compound **5** showed a low PAMPA permeability (0.05×10^{-6} cm/s)[13] and a low AlogD (0.43) suggesting that this compound may not enter the bacterial cell to reach its target. Molecular modeling of **5** into the active site of *B. subtilis* AcpS showed that variation of the R^1, R^2 and R^3 positions could lead to optimized contacts in the active site, thereby increasing binding affinity. These modeling constraints were used to select monomers for an initial synthetic array using 4 acid chlorides and 14 anthranilic acids (chosen using a D-optimal design[14] of the t[1], t[2] and t[3] scores of the PCA of a 27 anthranilic acid candidate set) containing variations of the R^1, and R^2 substituents (Fig. 1).

A set of 42 (**Set 1**) oxazolones was synthesized using the procedure outlined in Scheme 1 (Fig. 1). AcpS inhibitory and MIC data are presented for selected compounds in Table 1. MVA[15] was used to summarize/correlate the calculated and the measured molecular physicochemical properties with in vitro AcpS potency and MIC activity for **Set 1**. PLS models were generated

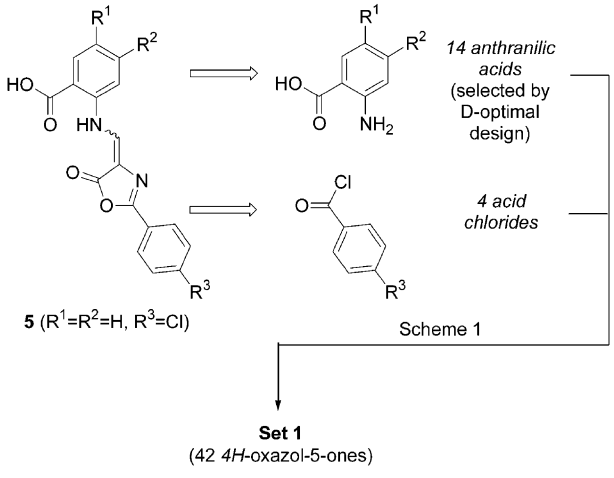
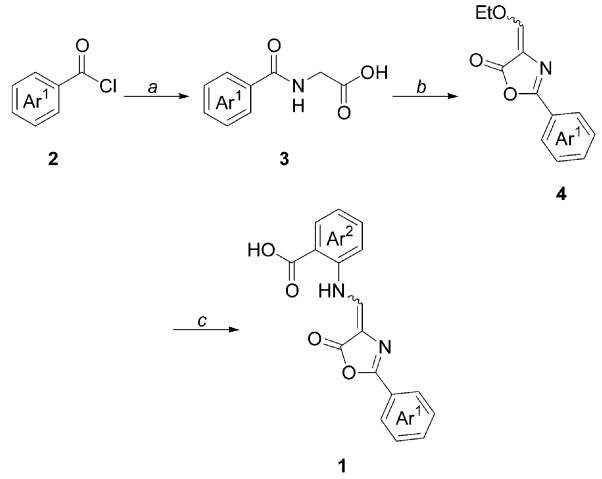


Figure 1. Retrosynthetic analysis of compound **5**, monomer selection and synthesis of 4*H*-oxazol-5-one **Set 1**.



Scheme 1. Reagents and conditions: (a) Glycine, NaOH, H_2O, 23 °C; (b) $(EtO)_3CH$, Ac_2O, 95 °C; (c) Anthranilic acid ($H_2N(Ar^2)CO_2H$), EtOH, 80 °C.

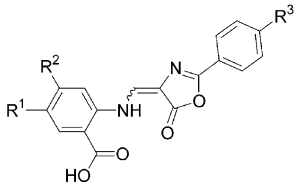
using calculated MW, hydrogen bond donor (HBD) count, hydrogen bond acceptor (HBA) count, daylight clogP and cMR, polar surface area (PSA),[16] and measured σ values[17] and atom counts for the whole molecule as well as the R^1, R^2 and R^3 substituents,

AcpS inhibition data (IC_{50}) and MIC data (*B. subtilis*, *E. faecalis ATCC*, *E. faecalis VRE* and *S. pneumo*$^+$) using SIMCA-P.[18] Two PLS models were generated to understand the effects of simultaneously varying the R^1, R^2 and R^3 substituents: a model that correlates physicochemical properties with AcpS inhibitory activity (A: 2, R^2X: 0.50, R^2Y: 0.71, Q^2(cum): 0.63, Fig. 2), and a model that correlates physicochemical properties AcpS inhibitory activity and MIC activity A: 2, R^2X: 0.50, R^2Y: 0.44, Q^2(cum): 0.29, Fig. 3).

Figure 2 shows AcpS inhibitory activity is primarily correlated with increasing R^3clogP, R^3sigma and R^3MW. This effect is seen in comparing several compounds in Table 1: compare **10** (R^3 = Cl) to **11** (R^3 = CF_3) and **12** (R^3 = OMe) to **13** (R^3 = CF_3). Figure 2 also shows that AcpS inhibition can be increased by decreasing the values of the R^2cMR and R^2clogP variables. The R^1PSA, R^1HBD, R^1HBA variables are on an orthogonal axis to the R^2, R^3 and AcpS inhibitory activity variables and thus have a minimal effect. The goodness of prediction of the model is satisfactory as the Q^2(cum) term is above 0.5 (Q^2(cum) = 0.63).

The PLS model in Figure 3 is even more informative as it correlates increasing AcpS inhibition and MIC activity with increasing R^3clogP, R^3sigma and R^3MW and decreasing values of the R^1 and R^2 variables (primarily the w*c[1] loading). AcpS inhibitory activity and MIC activity appear to be correlated— the correlation is strongest for the *B. subtilis MIC* activity, and there is correlation between increasing AcpS inhibition/MIC activity and increasing molecular clogP and MW. Importantly, analysis of these two PLS models indicates that more potent antibacterial AcpS inhibitors can be synthesized via adjustment of the R^3 substituent noted

Table 1. In vitro AcpS inhibitory data and minimum inhibitory concentration (MIC) data for selected **Set 1** oxazolones



Compd				In vitro AcpS inhibition	MIC data (μM)[b]			
	R^1	R^2	R^3	IC_{50} (μM)[a]	*B. subtilis*	*E. faecalis ATCC*	*E. faecalis VRE*	*S. pneumo*$^+$
5	H	H	Cl	15.0	>200	>200	>200	>200
6	H	H	H	>20	—	—	—	—
7	F	H	Cl	2.1	>200	100	100	200
8	Br	H	Cl	3.4	>200	100	200	100
9	I	H	Cl	1.3	200	25	50	12.5
10	Br	Cl	Cl	>20	>200	>200	—	—
11	Br	Cl	CF_3	2.0	>200	100	50	100
12	F	H	OMe	>20	>200	>200	—	—
13	F	H	CF_3	1.9	200	100	200	100
14	Me	H	CF_3	0.83	100	>200	100	100

[a] IC_{50} values were determined once over a range of 7 concentrations. Standard error for each run is ±10%.
[b] Compounds were tested 1× against each bacterial strain. Standard error for each run is ±1 dilution.

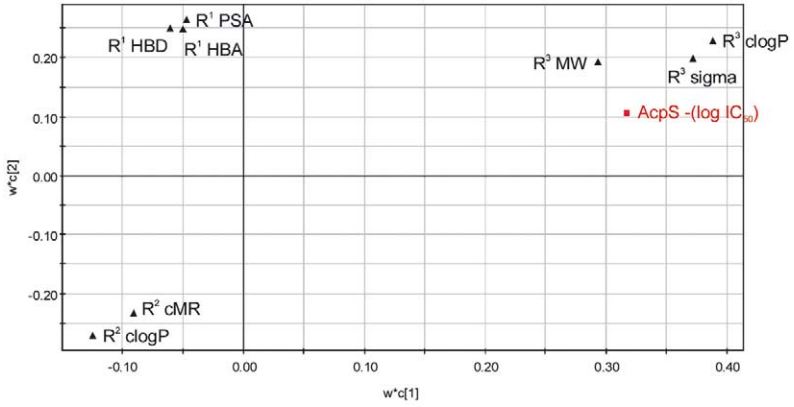


Figure 2. PLS loadings (w*c[1] vs w*c[2]) of **Set 1**: physicochemical properties (X matrix), AcpS inhibitory data (Y matrix) (A: 2, R^2X: 0.50, R^2Y: 0.71, Q^2(cum): 0.63). Model shows that AcpS inhibitory activity primarily increases with increasing R^3clogP, R^3sigma and R^3MW values and decreasing R^2clogP and R^2cMR values. R^1HBD, R^1HBA and R^1PSA have little effect.

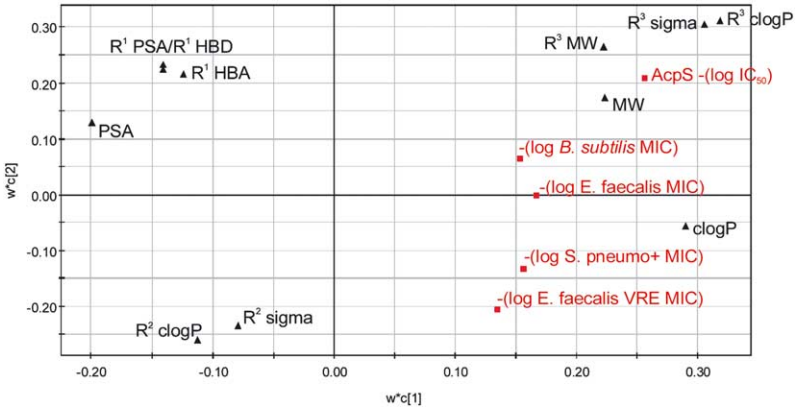
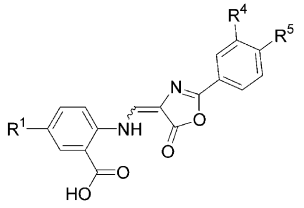


Figure 3. PLS loadings (w*c[1] vs w*c[2]) of **Set 1**: physicochemical properties (X matrix), AcpS inhibitory data and bacterial MIC data (Y matrix) (A: 2, R^2X: 0.50, R^2Y: 0.44, Q^2(cum): 0.29). Model shows that AcpS inhibitory activity and MIC activity primarily increases with increasing R^3clogP, R^3sigma and R^3MW values and with decreasing values for the R^1 and R^2 variables. AcpS inhibitory activity and MIC activity also increase with increasing molecular clogP and MW.

Table 2. In vitro AcpS inhibitory data and minimum inhibitory concentration (MIC) data for selected **Set 2** oxazolones



Compd				In vitro AcpS inhibition	MIC data[b]			
	R^1	R^4	R^5	IC_{50} (μM)[a]	*B. subtilis* (μM)[b]	*E. faecalis ATCC*	*E. faecalis VRE*	*S. pneumo*$^+$
15	Cl	F	CF_3	1.1	50	50	50	12.5
16	Br	F	CF_3	1.5	50	25	25	12.5
17	NAc	F	CF_3	0.13	>200	>200	>200	200
18	Me	F	CF_3	0.27	25	100	100	25
19	Cl	OCF_3	H	20	50	100	50	25
20	Br	OCF_3	H	15	25	50	25	12.5
21	Me	OCF_3	H	4.4	25	200	100	25

[a] IC_{50} values were determined 1× over a range of 7 concentrations. Standard error for each run is ±10%.
[b] Compounds were tested once against each bacterial strain. Standard error for each run is ±1 dilution.

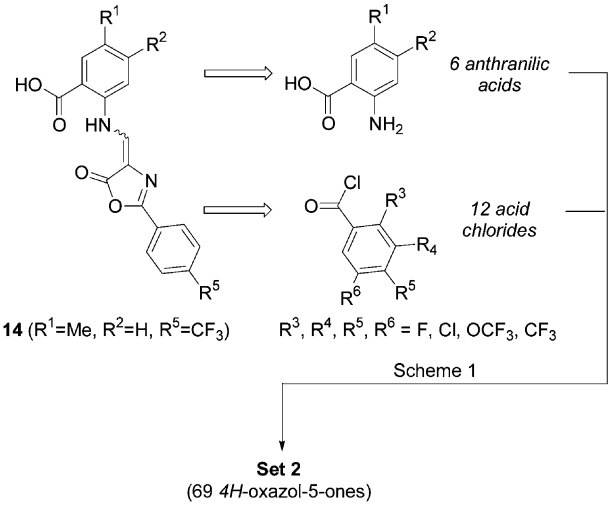


Figure 4. Retrosynthetic analysis of compound **14**, monomer selection and synthesis of 4*H*-oxazol-5-one **Set 2**.

above and by increasing overall molecular lipophilicity/MW. This conclusion was then used as the new starting point for a new library synthesis.

A second set of oxazolones (**Set 2**) was then synthesized as shown in Figure 4. Acid chlorides were used where the R^5 substituent (R^3 substituent in **Set 1**) was set to F, Cl, OCF_3 and CF_3, and anthranilic acids were used that gave active compounds in **Set 1**. AcpS inhibitory and MIC data are presented for selected **Set 2** compounds in Table 2. Potent antibacterial AcpS inhibitors were obtained where $R^4 = F$ and $R^5 = CF_3$ as seen in **15**, **16** and **18**. Compound **18**, having an AcpS $IC_{50} = 0.27$ μM and a *B. subtilis* MIC = 25 μM, is the most potent compound in terms of both AcpS inhibition and *B. subtilis* MIC activity. Compound **17** is the most potent AcpS inhibitor obtained, but it fails to show any MIC activity. A second series of antibacterial AcpS inhibitors emerged with the substitution pattern of $R^4 = OCF_3$ and $R^5 = H$ (**19**–**21**). These compounds show similar MIC activity as **15**, **16** and **18** but have less potent enzymatic activity. These two groups of compounds do show the same trend in biological activity-AcpS IC_{50}: $R^1 = Cl$. $R^1 = Br$ $> R^1 = Me$; Overall MIC activity: $R^1 = Me > R^1 = Cl$. $R^1 = Br$.

Thus we have shown how MVA and experimental design can be used to rapidly optimize both in vitro AcpS inhibitory/antibacterial activity to produce a series of small molecule AcpS inhibitors represented by compound **18**. MVA is a particularly powerful as it effectively summarizes variables into simpler linear combinations that are readily interpretable. It also allows one to interpret the effect of simultaneously varying multiple positions within a molecule. Additional studies on other small molecule AcpS inhibitors will be forthcoming.

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11. Enzyme activity was measured using a homogeneous time-resolved fluorescence resonance energy transfer (HTRF®) assay. Assays were run in 384 well plates and consisted of compound, 15 ng/mL AcpS, 1.9 μM biotin-CoA (prepared by reacting equimolar amounts of CoA and biocytin maleimide (Molecular Probes, Eugene, OR)), and 1.25 μg/mL GST-ACP in 50 mM Tris·HCl, pH 8.0, 7.5 mM $MgCl_2$, 3.75 mM DTT, 0.0375% Tween-20, and 37.5 μg/mL BSA in a total volume 20 μL. After 3 h at room temperature, the reaction was terminated by adding 60 μL of the stop/detection mix (82 ng/mL europium cryptate conjugate of GST monoclonal antibody (Packard, Meriden, CT), 28.7 μg/mL streptavidin–allophycocyanin conjugate (ProZyme, San Leandro, CA), 410 mM KF, 4.1 mM EDTA in 0.5×PBS). The mixture was incubated overnight before reading in a Wallac Victor™ plate reader (excitation at 340 nm, emission at 665 nm).
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