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Selective Inhibitors of Bacterial Phosphopantothenoylcysteine Synthetase

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Phosphopantetheine is a fundamental feature in many biological acyl transfer reactions. The molecule is found embedded within coenzyme A (CoA), as well as on a post-translationally modified, conserved serine of acyl carrier protein (ACP). Both CoA and ACP play essential roles as acyl group donor substrates in several reactions associated with intermediary metabolism and cell membrane assembly in living organisms.[1] Comparative genomics, using phylogenetic profiling, has concluded that the synthesis of phosphopantetheine-containing molecules from common metabolic precursors is fundamentally conserved across all domains of life.[2] Genome-wide transposon mutagenesis studies carried out in *E. coli* have also revealed the essential nature of genes involved in vitamin B_5 metabolism (*coaABCDE*).[3,4] The critical nature of phosphopantetheine-containing molecules to the integrity and viability of cells makes the biosynthetic pathway leading to the production of these compounds an intriguing target for antimicrobial development.

Phosphopantothenoylcysteine synthetase (PPCS) plays a vital role in the conversion of pantothenate (vitamin B_5) to CoA and is responsible for installing the biologically reactive cystamine moiety contained within the phosphopantetheine prosthetic group.[5,6] PPCS activity is essential to every kingdom of life and can be broadly classified by three types: Type I PPCSs are found in a majority of bacteria and archaea, are CTP specific, and are expressed as the C-terminal domain of a bifunctional protein fusion in conjunction with phosphopantothenoylcysteine decarboxylase (PPCDC).[6,7] Type II PPCSs are found mainly in eukaryotes, can use either ATP or CTP to support catalysis, and are expressed as a monofunctional enzyme.[3,8] Type III PPCSs, found in a smaller subset of bacteria, are also expressed monofunctionally and have recently been shown to be CTP specific.[9]

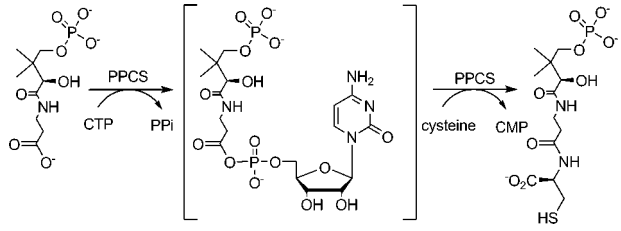

Figure 1. Reaction catalyzed by phosphopantothenoylcysteine synthetase.

Chemically, PPCS catalysis proceeds via an acyl nucleotide activated intermediate, analogous to aminoacyl-tRNA synthetases (Figure 1).[9,10] The topical antibiotic mupirocin inhibits bacterial isoleucyl tRNA synthetase by mimicking binding contacts of the isoleucyl adenylate intermediate.[11] Kinetic and structural studies have shown that the nucleobase binding sites of bacterial PPCS (Types I and III) differ greatly from that of the human Type II enzyme.[8,10,12] We have therefore designed and synthesized several inhibitors which mimic the acyl cytidylate formed during bacterial PPCS catalysis, which utilize exclusively CTP to support catalysis. In the design of our intermediate mimics, we removed the electrophilic carbonyl of the phosphopantothenoyl cytidylate to create stable phosphodiesters **3** and **4**. Alternatively, the carbonyl carbon was left intact in analogues **7** and **8**, and a nonhydrolyzable sulfamate isostere was used in place of the bridging phosphate of the phosphopantothenoyl cytidylate mixed anhydride linkage.

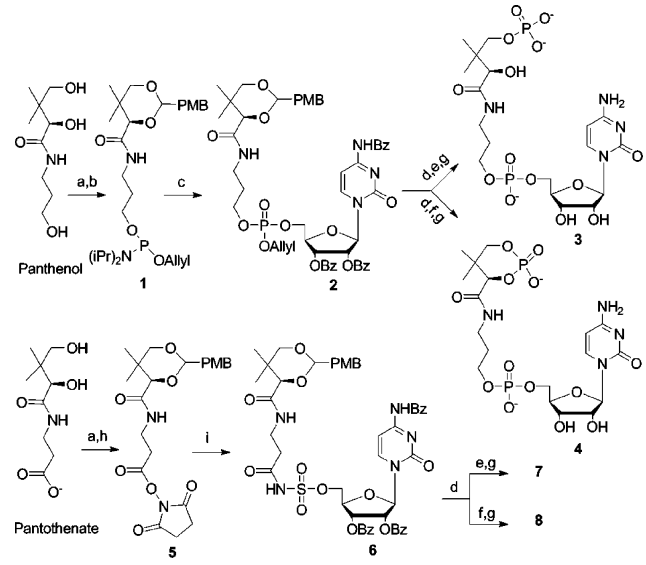

Figure 2. Synthesis of inhibitors: (a) $(MeO)_2CHC_6H_4OMe$, CSA, DMF; (b) Allyl-O-P[N(*i*-Pr_2)]$_2$, 5-(Ethylthio)-1*H*-tetrazole, DCM; (c) 1) 5-(Ethylthio)-1*H*-tetrazole, HO-2′,3′,N^4-tribenzoyl cytidine, CH_3CN; (2) CSO, 0 °C; (d) 80% AcOH; (e) (1) pyridinium HCl, β-cyanoethyl-O-P[N(*i*-Pr_2)]$_2$, CH_3CN, −20 °C; (2) CSO, 0 °C; (f) (1) 5-(Ethylthio)-1*H*-tetrazole, β-cyanoethyl-O-P[N(*i*-Pr_2)]$_2$, CH_3CN; (2) CSO, 0 °C; (g) (1) TMSCl, DBU, CH_3CN; (2) NH_4OH, 55 °C; (h) NHS, DCC, THF; (i) Cs_2CO_3, NH_2SO_2-2′,3′,N^4-tribenzoyl cytidine, DMF.

The synthesis of the phosphodiester mimics begins with the protection of D-panthenol as a *p*-methoxybenzylidene (PMB) acetal, followed by a tetrazole-mediated phosphitylation at the open primary alcohol to give phosphite **1** (Figure 2).[13,14] Phosphite **1** is then coupled in a similar manner to the 5′-hydroxy group of a tribenzoyl protected cytidine, followed by an in situ oxidation using CSO to yield the phosphodiester **2**.[13,14] After removal of the *p*-methoxybenzylidene, the open primary alcohol is phosphitylated using pyridinium HCl as the activator at −20 °C and oxidized *in situ* to give the protected terminal phosphate.[15] However, if tetrazole-mediated phosphitylation of the opened diol was employed at room temperature, subsequent oxidation gave the 1,3-cyclic phosphate. The global deprotection of both the terminal and cyclic phosphate analogues is accomplished in two sequential steps to give the desired products **3** and **4**.[16,17]

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A similar strategy was employed in the synthesis of the sulfamate analogues. D-Pantothenic acid was protected as a PMB acetal and converted to NHS ester **5**. Sulfamoyl tribenzoyl cytidine, obtained by sulfamoyl chloride treatment of tribenzoyl cytidine, was then linked to the activated NHS ester in the presence of Cs_2CO_3.[18−20] Compound **6** was subjected to the aforementioned sequence of PMB deprotection, phosphitylation and oxidation, and global deprotection to generate the sulfamate analogues **7** and **8**.

Phosphodiester **3** proved to be the most potent PPCS inhibitor, showing nanomolar IC_{50} toward both Types I and III bacterial enzymes and 145−1000-fold selectivity for bacteria PPCS over the human enzyme (Table 1). Similar selectivity is seen with compound **4**, which differs from **3** by the cyclization of the terminal phosphate moiety, albeit with a large decrease in potency. Both compounds **7** and **8**, containing the internal sulfamate linkage, display micromolar IC_{50} toward bacterial PPCS with 20−740-fold selectivity for the bacterial enzymes.

Table 1. IC_{50} of Compounds against Types I, II, and III PPCSs[a]

	hsPPCS (II)	efPPCS (III)	spPPCS (III)	ecPPCS (I)
3	10 μM (1)	65 nM (9)	10 nM (2)	68 nM (9)
4	2.7 mM (0.2)	18 μM (4)	13 μM (4)	3.0 μM (0.3)
7	200 μM (11)	2.7 μM (0.2)	3.9 μM (0.2)	270 nM (3)
8	5.9 mM (0.6)	181 μM (7)	279 μM (27)	16 μM (5)

[a] hs = human, ef = *E. faecalis*, sp = *S. pneumoniae*, ec = *E. coli*. Assays were performed in triplicate. Standard error shown in ( ).

Since a full steady state kinetic characterization of efPPCS has recently been performed, this enzyme was used to determine the kinetic mechanism and inhibition constant for the most potent inhibitor, **3**.[9] Upon addition of various concentrations of **3** to the PPCS assay, a pattern of slow-onset inhibition was observed (Figure 3). Subsequent plotting of k_{obs} against inhibitor concentration resulted in data points with a linear relationship. This is indicative of a single-step enzyme inhibition mechanism characterized by slow association and slow dissociation of compound **3** (Figure 3). The slope of the line in Figure 3B gives $k_3^{app} = 1.42 \times 10^4\ M^{-1}\ s^{-1}$, and the *y*-intercept gives $k_4 = 7.02 \times 10^{-4}\ s^{-1}$, giving a $K_i^{app} = 49$ nM. Taking into account the noncompetitive mode of inhibition of our inhibitors, K_i^{app} can be converted to K_i using the Cheng−Prusoff equation (see Supporting Information). Therefore, **3** exhibits a K_i = 24 nM for efPPCS.

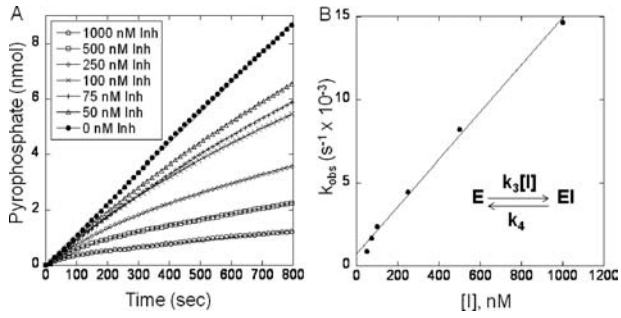

Figure 3. Slow-onset, tight-binding inhibition of *E. faecalis* PPCS by compound **3**. (A) Enzyme reactions (run in triplicate) were initiated by the addition of efPPCS. Concentrations of compound **3** are displayed in the legend. (B) k_{obs} obtained from the fit of the inhibition progress curves is plotted against the concentration of compound **3**.

The compounds reported herein represent the first reported inhibitors of PPCS. While very effective against the isolated enzymes, these compounds exhibit no inhibitory effects against bacterial growth, most likely due to lack of cellular penetration as a result of their physiochemical properties. However, in vitro these compounds show a marked selectivity toward both types of bacterial PPCS, providing a foundation for the possible development of broad spectrum antimicrobial agents. Efforts to cocrystallize these inhibitors with all three types of PPCS are currently being investigated. With these studies we hope to gain insight into the binding determinants of selectivity and potency which could be capitalized upon to design the next generation of inhibitors. Also, previous attempts at obtaining crystal structures of PPCS with substrate L-cysteine bound at the active site have not been successful.[10] Because our compounds mimic the phosphopantothenoyl cytidylate intermediate but are catalytically incompetent, it is possible that we could capture a ternary crystal complex with PPCS, inhibitor, and L-cysteine, which would provide a clear depiction as to the mechanism of PPCS's selectivity for L-cysteine.[21]

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Supporting Information Available: Complete ref 4, synthetic and biochemical experimental procedures, compound spectroscopic characterization, and equations for inhibition constant determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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