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Improved Synthesis of Biotinol-5′-AMP: Implications for Antibacterial Discovery

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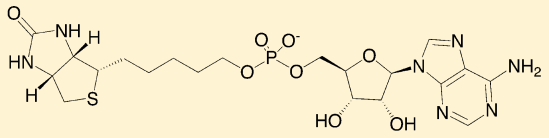
Supporting Information

ABSTRACT: An improved synthesis of biotinol-5′-AMP, an acyl-AMP mimic of the natural reaction intermediate of biotin protein ligase (BPL), is reported. This compound was shown to be a pan inhibitor of BPLs from a series of clinically important bacteria, particularly *Staphylococcus aureus* and *Mycobacterium tuberculosis*, and kinetic analysis revealed it to be competitive against the substrate biotin. Biotinol-5′-AMP also exhibits antibacterial activity against a panel of clinical isolates of *S. aureus* and *M. tuberculosis* with MIC values of 1−8 and 0.5−2.5 μg/mL, respectively, while being devoid of cytotoxicity to human HepG2 cells.



Staphylococcus aureus	K_i 18 nM	MIC 1 - 4 μg/ml
Mycobacteria tuberculosis	K_i 52 nM	MIC 0.5 - 2.5 μg/ml

KEYWORDS: *Antibiotics, enzyme inhibitors, biotin protein ligase, chemical synthesis, drug design*

Adenylate-forming enzymes play a central role in many key metabolic pathways such as ribosomal and nonribosomal peptide synthesis, fatty acid synthesis, and enzyme regulation. As such they are of significant interest as potential drug targets.[1,2] These enzymes function by activating a carboxylate metabolite on reaction with ATP to form an acyl-AMP intermediate, which then reacts with a nucleophile to generate the desired product (Figure 1). The acyl-AMP reaction intermediates possess a high binding affinity for the enzyme, often 2−3 orders of magnitude greater than the carboxylic acid or ATP substrates.[3,4]

The inhibition of biotinyl-5′-AMP **1**, one such intermediate produced by the biotin protein ligase (BPL) catalyzed reaction of biotin and ATP, has attracted recent interest as a potential new class of antibiotic.[5,6] An important approach to these inhibitors involves replacing the hydrolytically unstable acyl phosphate linker of **1** with a more stable bioisostere, as in biotinol-5′-AMP **2**.[6,7] This phosphodiester mimic is a potent inhibitor of BPLs from *S. aureus*, *Escherichia coli*, and *Homo sapiens*.[6,8] However, its antibacterial properties have not been investigated. Other acyl phosphate bioisosteres used in this context include, sulfonyl and 1,2,3-triazole groups, among others.[5,6,9−25] Importantly, acyl-AMP mimics (containing sulfonmyl and 1,2,3-triazole isosteres, see **3** and **4** in Figure 1) have been shown to be potent agents against *M. tuberculosis* and *S. aureus*, respectively.[26] Given the central importance of biotinol-5′-AMP **2** as a BPL inhibitor, we now report an expedient method for its synthesis and an investigation of its activity profile against clinically significant bacteria.

Biotinol-5′-AMP **2** is reportedly prepared according to a modified "phosphodiester" procedure as shown in Scheme 1.[7] However, in our hands this procedure gave an unsatisfactory overall yield of 9% from adenosine **5**. The key limitation with this procedure was a DCC mediated coupling of **6** and **8** to give phosphodiester **9**, with subsequent *in situ* deprotection of the acetonide group to give **2**. An overall yield of 15% was obtained over these two steps. Attempts to optimize the DCC coupling step using other solvents, particularly pyridine or DMF, or longer reaction times (up to 48 h) failed to improve the yield. The use of other acids for the deprotection (20−60% acetic acid instead of TFA) similarly failed to improve the efficiency of this sequence. Thus, an alternative approach to biotinol-5′-AMP **2** was investigated that would avoid the problematic DCC coupling and hemiacetal deprotection steps, see Scheme 2. This methodology was then used to prepare sufficient quantities of **2** for antimicrobial studies. A very recent report, investigating biotinol-5′-AMP **2** and its phosphite analogues as human BPL

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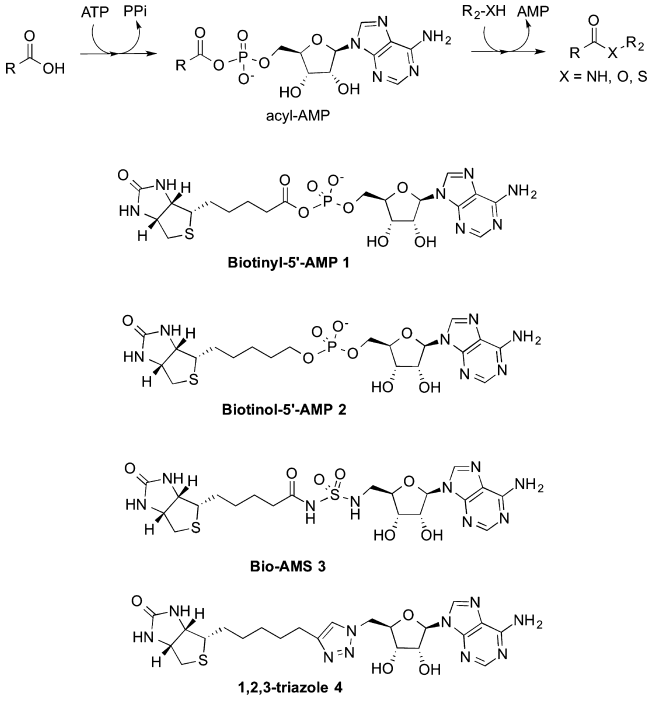
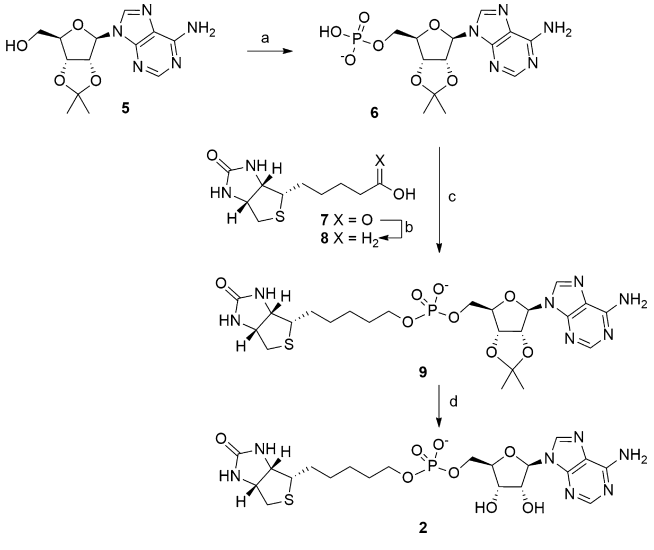


Figure 1. General catalytic mechanism of adenylate forming enzymes (top). Chemical structures are shown for the acyl-AMP intermediate of BPL biotinyl-5′-AMP (**1**) and its phosphodiester mimic biotinol-5′-AMP (**2**), acyl-sulfamide biotin adenosine monosulfamide (Bio-AMS) (**3**), and 1,2,3-triazole (**4**).

Scheme 1[a]

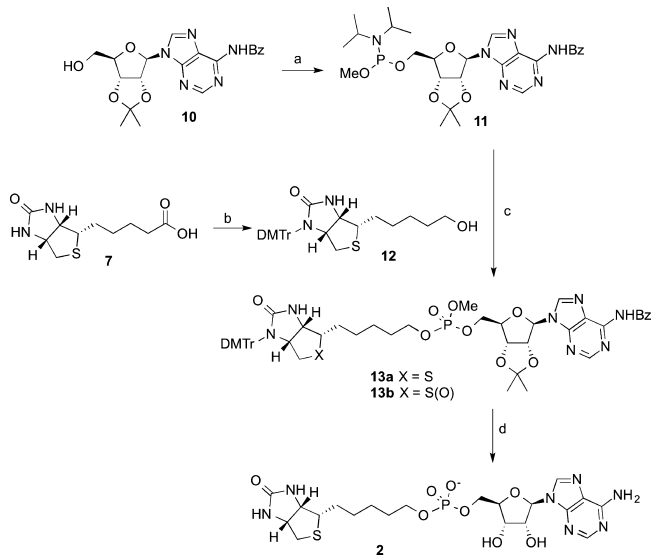


[a]Reagents and conditions: (a) (i) $POCl_3$, $PO(Et)_3$; (ii) TEAB buffer; (b) (i) $SOCl_2$, MeOH; (ii) $LiAlH_4$, THF; (c) DCC, py; (d) 60% $AcOH_{(aq)}$.

inhibitors, implemented a similar strategy to Scheme 2 for the synthesis of biotinol-5′-AMP **2**.[25]

The alternative "phosphoramidate" synthesis commenced with the conversion of *N*-benzoyl protected adenosine **10** to the corresponding phosphoramidate **11** in 67% yield.[27] Biotinol **12** was synthesized as previously reported.[28] Specifically, biotin **7** was esterified under acidic conditions to the corresponding methyl ester, followed by selective *N*-tritylation of the ureido ring of biotin methyl ester and a subsequent reduction to give

Scheme 2[a]



[a]Reagents and conditions: (a) $ClP(OMe)N(iPr)_2$, Et_3N, DCM; (b) (i) $SOCl_2$, MeOH; (ii) DMTrCl, DCM; (iii) $LiAlH_4$, THF; (c) (i) 5-ETT, MeCN; (ii) tBuOOH; (d) (i) 10% TFA, DCM; (ii) NH_3OH, MeOH/H2O; (iii) NaI, H_2O.

biotinol **12**. The phosphoramidate **11** was then coupled to biotinol **12** using 5-(ethylthio)-1*H*-tetrazole, with oxidation of the resulting phosphite with *tert*-butyl hydrogen peroxide (TBHP) and immediate quenching with sodium metabisulfite to give phosphotriester **13a** in 59%. Attempts at coupling **11** with **12** without the addition of bisulfate gave predominately the overoxidized sulfone analogue **13b**, as confirmed by mass spectrometry, see Supporting Information. The formation of **13b** was judged to be irreversible, as reduction of **13b** to **13a** would have resulted in epimerization of the biotin alkyl chain.[29] A sequential treatment of phosphotriester **13a** with TFA, NH_3OH, and NaI followed by purification of the crude product by reverse phase HPLC gave **2** in >95% purity. This sequence gave biotinol-5′-AMP **2** with an overall 2-fold improvement in yield starting from adenosine **10** compared to the earlier phosphodiester synthesis.

The inhibitory activity of the newly synthesized biotinol-5′-AMP **2** was investigated against a panel of BPLs using an *in vitro* biotinylation assay that measures the incorporation of radiolabeled biotin onto an acceptor protein.[6,30] The activity of each enzyme was determined in the presence of varying concentrations of biotinol-5′-AMP **2**. The apparent inhibition constants (K_i^{app}) were calculated using the Morrison equation for tight binding inhibitors as most of the K_i^{app} values were within 10-fold of the concentration of BPL employed in the assay. Biotinol-5′-AMP **2** inhibited all five clinically relevant microbial BPLs with the greatest potency against *S. aureus* (K_i^{app} = 18 nM) and *M. tuberculosis* (K_i^{app} = 52 nM). It also inhibited human BPL with a K_i^{app} of 182 nM. Thus, biotinol-5′-AMP **2** is a pan BPL inhibitor, presumably since its structure closely resembles that of the natural reaction intermediate biotinyl-5′-AMP **1**, which is common to all BPLs.

It has previously been established that biotin and ATP bind to BPL in an ordered manner, with biotin binding first.[6,31] This suggests that biotinol-5′-AMP **2** would be a competitive inhibitor against biotin. In support, our published X-ray crystal structures of *S. aureus* BPL in complex with biotin [PDB

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3RKY][32] and biotinol-5′-AMP **2** [PDB 4DQ2][6] demonstrate that both compounds occupy the same binding pocket. The activity of *S. aureus* BPL was measured in the presence of varying concentrations of both inhibitor and biotin in order to define the kinetics of inhibition.[33] Lineweaver−Burk double-reciprocal plots of 1/enzyme velocity versus 1/biotin concentration gave four straight lines that had a common *y*-intercept (Figure 2). The data demonstrated that increasing

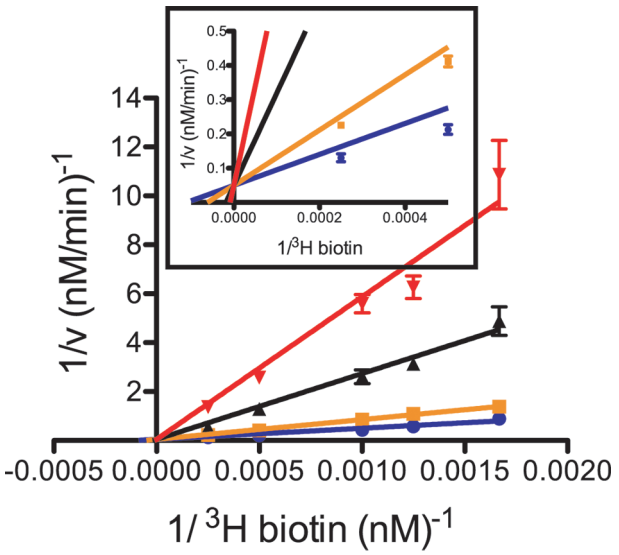

Figure 2. Kinetic analysis of inhibitor binding. *S. aureus* BPL activity was measured in the presence of inhibitor and varying concentrations of biotin. Lineweaver−Burk curves are shown for ^{3}H-biotin. The insert is an expanded view close to the origin showing the curves intersecting the *y*-axis. The concentrations of **2** employed in the assays were 0 (blue curves), 20 nM (orange curves), 100 nM (black curves), and 200 nM (red curves). Data points are the mean ± SEM for three replicates.

concentrations of inhibitor were accompanied by increases in the K_M for biotin, while the V_{max} remained unchanged. This supports the proposal that biotinol-5′-AMP **2** is a competitive inhibitor of biotin. Accordingly, the K_i^{app} were extrapolated for each species of BPL using the apparent K_M values for biotin (Table 1).[34]

Table 1. *In Vitro* Biotinylation Assay Results for Biotin and Inhibition Constants for Biotinol-5′-AMP 2

biotin protein ligase	K_M biotin (μM)	K_i^{app} biotinol-5′-AMP **2** (nM)
Escherichia coli	0.3	87
Staphylococcus aureus	1.0	18
Klebsiella pneumoniae	2.0	482
Acinetobacter calcoaceticus	0.3	461
Mycobacterium tuberculosis	0.5	52
Homo sapiens	1.1	182

Finally, the antimicrobial activity of biotinol-5′-AMP **2** was assessed against a library of clinically important bacteria using a microdilution broth assay. The resulting minimal inhibitory concentrations (MIC) are shown in Table 2. Encouragingly, biotinol-5′-AMP **2** was effective against staphylococci with minimal inhibitory concentrations varying from 1−8 μg/mL, but not enterococci nor *E. coli*. The difference in antibacterial activity observed here may be due to differences in compound

Table 2. *In Vitro* Susceptibility Assay of Biotinol-5′-AMP 2 against a Series of Bacterial Strains

bacterium class	strains	resistance	MIC (μg/mL)
M. tuberculosis	H37Rv		2.5
M. tuberculosis	#37892293		2.5
M. tuberculosis	#3688023		0.5
M. tuberculosis	BCG (SI)	pyrazinamide	resistant
M. tuberculosis	Who-185	rifampicin	2.5
M. tuberculosis	Who-1841	isoniazid	0.5
S. aureus (n = 8)	MSSA		2−4
S. aureus (n = 8)	MRSA	methicillin	2−8
Coagulase negative staphylococci (n = 7)			1−4
Escherichia coli	ATCC 25922		>128
Enterococcus faecalis (n = 3)			>128
Enterococcus faecium (n = 5)			>128
Homo sapiens, HepG2 cell line	HB8065		>200

uptake between the different species. Likewise, *M. tuberculosis* was sensitive to biotinol-5′-AMP **2**, including strains resistant to front line antibiotics. In particular, biotinol-5′-AMP inhibited growth of WHO-1841 at 0.5 μg/mL, a strain resistant to the fatty acid biosynthesis inhibitor isoniazid. The notable exception was the pyrazinamide resistant strain BCG (SI) that was also insensitive to biotinol-5′-AMP **2** for reasons that are not clear. It is noteworthy that, although biotinol-5′-AMP **2** inhibited human BPL *in vitro*, the compound showed no cell cytotoxicity (CC_{50}) against the human liver cell line HepG2 at concentrations as high as 200 μg/mL, providing a therapeutic index (CC_{50}/MIC) of >25−400-fold, based upon the MICs for *S. aureus* and *M. tuberculosis*, respectively. The observed MIC values of biotinol-5′-AMP **2** against *M. tuberculosis* (0.5−2.5 μg/mL) are comparable with acyl-AMP mimic bio-AMS **3** (0.1−0.5 μg/mL).[5] Interestingly, while both **2** and **3** are active against *M. tuberculosis*, only **2** is cytotoxic over Gram-positive *S. aureus*.

In summary, an improved synthesis of biotinol-5′-AMP **2** and its subsequent biological characterization are reported. The new approach gives an improved yield of 29%. Enzyme inhibition and *in vitro* antibacterial susceptibility assays revealed that biotinol-5′-AMP **2** is a prime candidate for further development as a new class of antimicrobial agent. While this compound is a pan BPL inhibitor, it possesses selective antimicrobial inhibitory activity toward Gram-positive microbes over Gram-negative and importantly human cell lines. Significantly, MIC values between 0.5−2.5 μg/mL were obtained against resistant strains of *M. tuberculosis*.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Biological assays, synthetic procedures, and data for selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. Medicinal chemistry was performed by W.T. and A.D.A., biochemical assays were performed by A.S.P., B.N., M.Y.Y., T.P.S., and G.W. under the guidance of S.W.P., M.C.J.W., and G.W.B., antibacterial susceptibility assays were performed by R.L., J.M.B., and J.D.T., and cell culture assays were performed by S.W.P.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BPL, biotin protein ligase; MIC, minimal inhibitory concentration

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