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β -Keto and β -hydroxyphosphonate analogs of biotin-5'-AMP are inhibitors of holocarboxylase synthetase

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ABSTRACT

Holocarboxylase synthetase (HLCS) catalyzes the covalent attachment of biotin to cytoplasmic and mitochondrial carboxylases, nuclear histones, and over a hundred human proteins.

Nonhydrolyzable ketophosphonate (β -ketoP) and hydroxyphosphonate (β -hydroxyP) analogs of biotin-5'-AMP inhibit holocarboxylase synthetase (HLCS) with IC_{50} values of 39.7 μ M and 203.7 μ M. By comparison, an IC_{50} value of 7 μ M was observed with the previously reported biotinol-5'-AMP. The K_i values, 3.4 μ M and 17.3 μ M, respectively, are consistent with the IC_{50} results, and close to the K_i obtained for biotinol-5'-AMP (7 μ M). The β -ketoP and β -hydroxyP molecules are competitive inhibitors of HLCS while biotinol-5'-AMP inhibited HLCS by a mixed mechanism.

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Holocarboxylase synthetase (HLCS) is the sole enzyme in the human proteome capable of catalyzing the covalent attachment of biotin to lysine residues.¹ HLCS localizes in the cytoplasm, mitochondria, and cell nuclei.^{1,2} HLCS catalyzes biotinylation of five carboxylases in mitochondria and cytoplasm, which play key roles in gluconeogenesis, fatty acid metabolism, and leucine metabolism.³ In addition, a recent mass spectrometry screen identified 108 novel biotinylated proteins; heat shock proteins and enzymes from glycolysis are overrepresented among these proteins.⁴ HLCS orchestrates the assembly of a multiprotein gene repression complex in human chromatin, partially mediated through HLCS-dependent methylation of the histone methyltransferase EHMT1 and the nuclear receptor co-repressor N-CoR.⁵ Consistent with the importance of HLCS in intermediary metabolism and cell function, no living HLCS null person has ever been reported, and persons with HLCS mutations require lifelong treatment with pharmacological doses of biotin.⁶

HLCS-dependent biotinylation involves basically two steps (Fig. 1). In the first, activation of biotin by ATP generates the mixed anhydride biotin-5'-adenosine monophosphate (Bio-5'-AMP) (1).

In the second step, the phosphate anhydride serves as an acylating agent for a target lysine in carboxylases (2), histones (3), and other proteins,^{3,4,7} covalently linking biotin to the substrate via an amide bond.

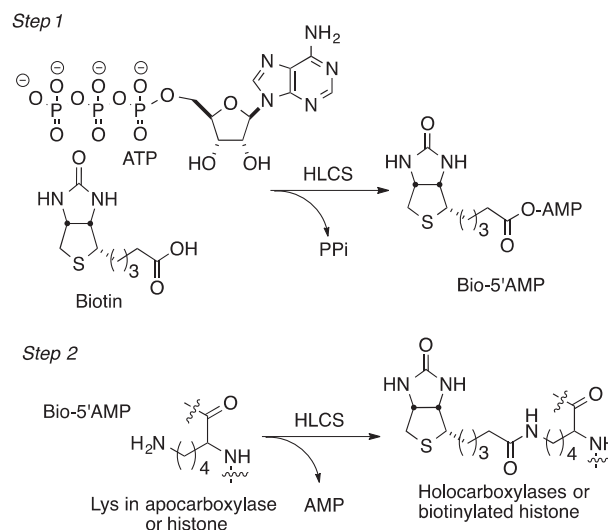


Figure 1. HLCS catalysis.

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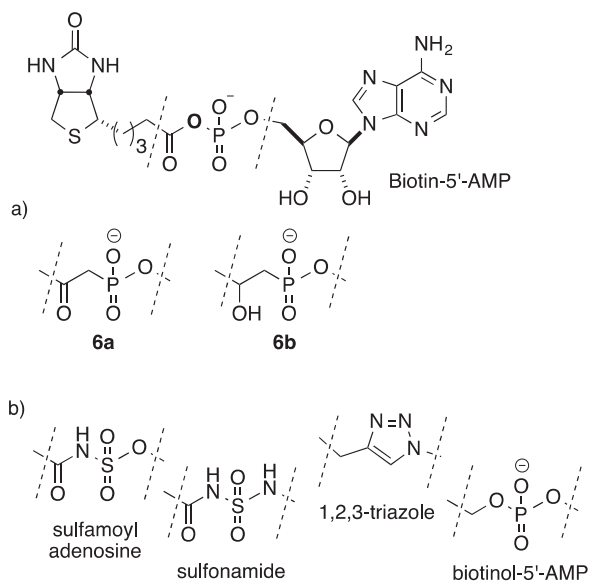
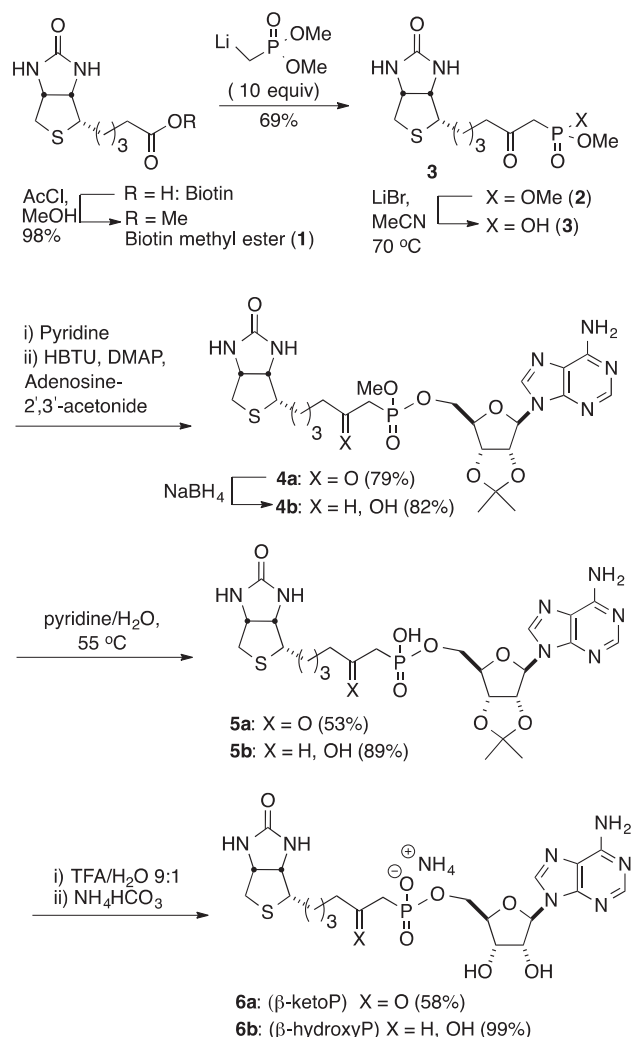


Figure 2. (a) Chemical structure of biotin-5'-AMP and ketophosphonate (**6a**) and hydroxyphosphonate (**6b**) analogs; (b) reported sulfamoyl, sulfonamide, triazole, and phosphate inhibitors.

The objective of this study was to develop a new class of synthetic HLCS inhibitors which could potentially be targeted to distinct cellular structures. Such an inhibitor would be a useful analytical tool in studies of HLCS-dependent biotinylation events in the cytoplasm, mitochondria, and nucleus. We based these studies on analogs of biotin-5'-AMP, namely biotin β -ketophosphonate-5'-AMP (β -ketoP) and biotin β -hydroxyphosphonate-5'-AMP (β -hydroxyP), that substitute a hydrolytically stable phosphonate for the acyl phosphate found in biotin-5'-AMP (Fig. 2a). The use of phosphonates as unreactive isosteres of phosphates is well established.⁸ For example, nonhydrolyzable aminoacyl analogs of aspartyl adenylate exhibit potent inhibitory activity against *Escherichia coli* aspartyl-tRNA synthetase.⁹ There is precedence for the efficacy of structurally analogous compounds sulfamides, sulfonamides, and 1,2,3-triazoles in the inhibition of a microbial biotin protein ligase, the HLCS ortholog BirA (Fig. 2b).^{10–13} We also investigated biotinol-5'-AMP, a known phosphate analog of biotin-5'-AMP which replaces the carbonyl oxygen with a methylene (CH_2).^{10,14}

Inhibitor synthesis: The central element of the synthesis is the formation of a protected version of a biotin ketophosphonate (**4a**) via condensation of a biotin-derived ketophosphonic acid (**3**) with a protected adenosine (Scheme 1). The synthesis begins with biotin methyl ester (**1**), prepared via the acid-catalyzed esterification of biotin.¹⁵ Reaction with the carbanion derived from methyl phosphonate was anticipated to offer a convenient route to a precursor of the desired phosphonates. However, reaction of ester **1** with the lithiated methylphosphonate, generated using lithium bis(trimethylsilyl)amide (LiHMDS) or *n*-butyl lithium (*n*-BuLi), resulted in poor yields. Fortunately, reaction of the ester with a large excess of the lithiated phosphonate, followed by quenching with deionized water to minimize demethylation of the phosphonate diester product, produced a 69% yield of dimethyl β -keto-phosphonate **2**.¹⁶ Selective monodemethylation with lithium bromide provides a good yield of mono ester **3**. The pyridinium salt of **3** underwent coupling with 2',3'-isopropylideneadenosine (*i*-PrA) in the presence of *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) to provide a mixed phosphonate diester (**4a**) in 79% yield for two steps.^{17,18}

No coupling was observed if *N,N'*-dicyclohexylcarbodiimide (DCC) was substituted for HBTU. The acidic methylene of the



Scheme 1. Synthesis of ketophosphonate (β -ketoP) and hydroxyphosphonate (β -hydroxyP) analogs of biotin-5'-AMP.

ketone phosphonate was observed (NMR) to readily undergo H/D exchange upon dissolution in d_4 -MeOH.

Reduction of the ketone with NaBH₄ resulted in formation of the corresponding alcohol (**4b**) as a mixture of diastereomers at the newly formed stereocenter. Stirring the ketone diester (**4a**) or the alcohol diester (**4b**) in pyridine/water resulted in selective demethylation to afford monoesters **5a** or **5b**, respectively. Removal of the acetonide protecting group from the sugar followed by neutralization with ammonium bicarbonate provided the target biotin β -ketophosphonate (β -ketoP) **6a** in 58% yield for two steps. The same procedure, when applied to alcohol **5b**, furnished biotin β -hydroxyphosphonate (β -hydroxyP) **6b** in 99% yield.

Inhibition of HLCS: Biotin β -ketophosphonate-5'-AMP (β -ketoP, **6a**) inhibited HLCS in a dose-dependent manner. The polypeptide p67 is a substrate for biotinylation by HLCS.¹⁹ When recombinant p67 was incubated with recombinant HLCS and 20 μM biotin in the presence of 50–500 μM β -ketoBP, HLCS inhibition was maximal at the highest inhibitor concentration tested. For example, the inhibition at 500 μM β -ketoBP was $81.3\% \pm 11.1\%$ ($P < 0.01$; $n = 4$) compared with vehicle control (Fig. 3A). Data are presented as mean \pm SD of 4 replicates. Incubation of p67 in the absence of HLCS produced no detectable signal (lane 6 in Fig. 3B). Under the conditions used here, IC₅₀ and K_i for β -ketoBP equaled $39.7 \pm 1.9 \mu\text{M}$ and $2.0 \pm 0.1 \mu\text{M}$, respectively, see Supplementary materials for details regarding assays and calculations. When tested under identical

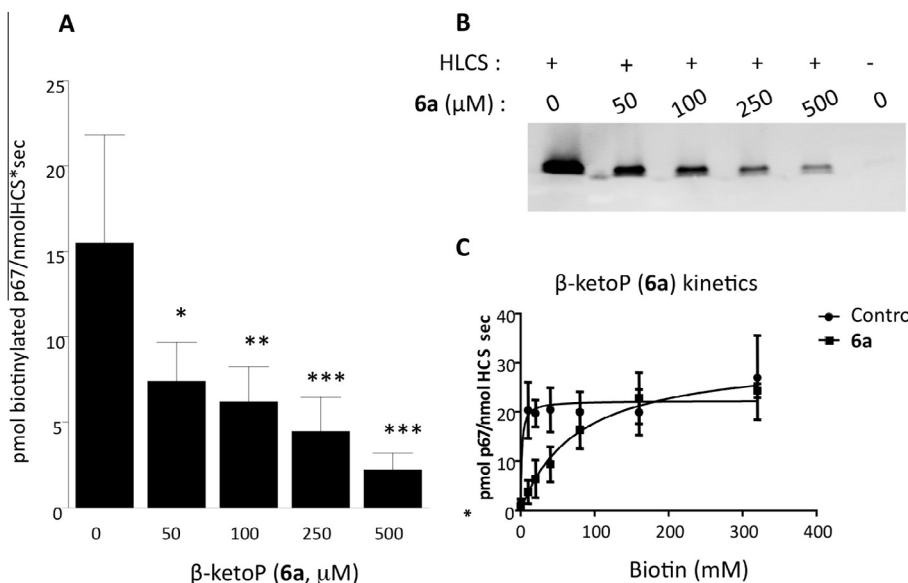


Figure 3. Inhibition of HLCS by biotin β -ketophosphonate-5'-AMP. (A) HLCS activity was quantified using infrared absorbance in the presence of 20 μ M biotin and various concentrations of biotin β -ketophosphonate-5'-AMP (One way-ANOVA; * $P < 0.05$; $N = 4$; Dunnett's multiple t -test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (B) Representative gel, depicting biotinylation of p67 in the presence of various concentrations of biotin β -ketophosphonate-5'-AMP. Lane 6 shows p67 incubated in the absence of HLCS. (C) Competitive inhibition of HLCS by biotin β -ketophosphonate-5'-AMP.

conditions, β -hydroxyP inhibited HLCS activity by $67.7 \pm 10.0\%$ ($P = 0.0001$; $n = 4$) at concentrations of 500 μ M inhibitor. The IC_{50} and K_i values calculated under these conditions were $203.7 \pm 3.7 \mu$ M and $10.4 \pm 0.2 \mu$ M, respectively. β -KetoP is a competitive inhibitor of HLCS, based on competition studies with biotin. In these studies, the concentration of the inhibitor was held constant at 250 μ M while that of biotin was varied from 0 to 320 μ M; a second curve was generated in the absence of inhibitor (Fig. 3C). The apparent V_{max} was similar for incubations with and without inhibitor [31.5 ± 1.6 vs 22.3 ± 1.51 pmol biotinylated p67/(nmol HLCS \times s); $n = 4$] whereas the apparent K_m for biotin was increased by the addition of inhibitor (77.9 ± 10.3 vs $1.6 \pm 1.8 \mu$ M biotin; $N = 4$). β -HydroxyP (**6b**) also acts as a competitive inhibitor as evidenced by similar apparent V_{max} values with and without inhibitor [20.6 ± 1.8 vs 22.6 ± 1.4 pmol biotinylated p67/nmol HLCS \times s); $n = 4$] whereas reactions incubated with inhibitor increased the apparent K_m for biotin (82.5 ± 20.4 vs $1.9 \pm 1.8 \mu$ M biotin; $n = 4$). As a negative control we conducted incubations with a biotin ketophosphonic acid (compound **3** in Scheme 1). This substrate incorporates an electrophilic carbonyl carbon beta to a charged phosphonate but lacks the adenosyl fragment hypothesized as essential for mediating HLCS inhibition. Consistent with this theory, the ketophosphonic acid compound did not inhibit HLCS (data not shown).

The results were compared against assays conducted with biotinol-AMP, a known phosphate analog of biotin-5'-AMP which has previously been employed for inhibition of BirA (biotin protein ligase).^{10,11} Biotinol-AMP reduces HLCS activity by $98.01 \pm 0.1\%$ at concentrations of 500 μ M and has an IC_{50} value and K_i of $8.8 \pm 3.6 \mu$ M and 754 ± 303 nM, respectively. When reactions incubated with biotinol-AMP were challenged with increasing amounts of up to 320 μ M biotin, the apparent V_{max} decreased compared to reactions without inhibitor (22.6 ± 1.4 vs 5.9 ± 1.4 ; $n = 4$) and K_m increased (1.9 ± 1.8 vs 146 ± 79 ; $n = 4$) indicating the biotinol-AMP most likely acts as a mixed inhibitor.

There remains limited knowledge regarding the structure of HLCS and the mechanism of catalysis.^{11,13b,20} Similarly, little is known about the basis for selectivity between the classic carboxylase targets of HLCS and novel targets in chromatin and other proteins. The

objective of this study was to develop a synthetic HLCS inhibitor capable of penetrating cell membranes and which could potentially be targeted to distinct cellular structures. Such an inhibitor would be a useful analytical tool in studies of carboxylase biotinylation in cytoplasm and mitochondria, studies of chromatin protein biotinylation and HLCS-dependent formation of multiprotein gene repression complexes in nuclei, and studies of newly discovered species of biotinylated proteins throughout the cell.

Consistent with the importance of HLCS in intermediary metabolism and epigenetics, no living HLCS null individual has ever been reported, suggesting embryonic lethality. HLCS knockdown in *Drosophila melanogaster* ($\sim 30\%$ residual activity) produces phenotypes such as decreased life span and reduced heat resistance.²¹ Mutations and single nucleotide polymorphisms have been identified and characterized in the human HLCS gene; these mutations cause a substantial decrease in HLCS activity, aberrant gene regulation and metabolic abnormalities.^{6,22} Unless diagnosed and treated at an early stage, homozygous severe HLCS deficiency is characteristically fatal.²³ Three independent cancer and patent databases correlate HLCS loss or mutation with an increase in detected tumors.²⁴

Several classes of biotin-5'-AMP analogs have been applied to study the function of biotin protein ligases (BPLs), exemplified by HLCS as well as BirA, an enzyme catalyzing biotinylation of acyl carrier protein in prokaryotes.^{10,13,14} BirA from *E. coli* has 21% sequence similarity to HLCS.²⁵ Biotinol-5'-AMP, a phosphate ester lacking the acyl carbonyl of biotin-5'-AMP, binds tightly to the *Escherichia coli* biotin repressor ($K_D = 1.5 \pm 0.2$ nM)^{11,20} and inhibits biotin transfer to the acceptor protein.^{10,14} Biotinol-5'-AMP also binds tightly to *Staphylococcus aureus* BPL ($K_i = 0.03 \pm 0.01 \mu$ M). The activity of this analog would appear to suggest the key role of the phosphate moiety and the relative lack of importance of hydrogen-bonding interactions to the acyl carbonyl of biotin-5'-AMP. However, other work has demonstrated that a sulfamoyl-containing bisubstrate analog, replacing the acyl phosphate with an acyl sulfonamide, strongly binds *Mycobacteria tuberculosis* BPL (MtBPL); a co-crystal revealing multiple hydrogen-bonding interactions between the protein and the acyl sulfonamide.¹² More recently, a biotin-5'-AMP analog replacing the acyl phosphate with

a 1,2,3-triazole was found to bind tightly to BPL and exhibit >1100-fold selectivity for the *S. aureus* BPL over the human homologue.¹³ This suggests the possibility of designing potent inhibitors of bacterial BPL. However, no similar approach has been used to study the function of HLCS or human BPL.

A model of the HLCS/biotin-5'-AMP complex as well as the crystal structure of biotin-5'-AMP with BPL from *Pyrococcus horikoshii* OT3 (pdb:1wqw) suggests the importance of hydrogen bonding involving the carbonyl and phosphonate oxygen (Fig. S1).^{13b,22b} The β -ketophosphonate and β -hydroxyphosphonate analogs introduced here maintain the natural charge state of biotin-AMP and place a basic oxygen atom beta to the phosphonate group. However, in contrast to the BirA inhibitors described above, the keto-phosphonate (β -ketoP, **6**) incorporates an electrophilic carbon at the location of the original acyl group in biotin-5'-AMP. Although the reduced activity of the new inhibitors compared with biotin-AMP suggests that preservation of an electrophilic center (C=O) or hydrogen bonding group (CHOH) beta to phosphonate is of limited importance in inhibitor design, we note that the 1,2,3-triazole analogs completely lacking a carbonyl group show no inhibition toward human BPL. It is also possible that conformational differences between the acyl phosphate of biotin-5'-AMP and the phosphonate of **6a** and **6b** might also contribute to the reduced binding observed.

In conclusion, we have described a new class of inhibitors of holocarboxylase synthetase HLCS based upon replacement of the ester of biotin-5'-AMP with a ketone or a secondary alcohol. The analogs produce significant levels of inhibition with isolated enzyme. Efficacy of the new inhibitors in vivo has not been tested and further investigations are warranted.

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Supplementary data

Supplementary data (synthetic protocols, spectral listings, and assay conditions) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2014.11.010>.

These data include MOL files and InChIKeys of the most important compounds described in this article.

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