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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 IC50 values of 39.7 μM and 203.7 μM. By comparison, an IC_{50} value of 7 μM was observed with the previously reported biotinol-5'-AMP. The K_i values, 3.4 μM and 17.3 μM, respectively, are consistent with the IC_{50} results, and close to the K_i obtained for biotinol-5'-AMP (7 μM). The β-ketoP and β-hydroxyP molecules are competitive inhibitors of HLCS while biotinol-5'-AMP inhibited HLCS by a mixed mechanism.

Keywords

Biotin-5′-AMP; β-ketophosphonate; β-hydroxyphosphonate; holocarboxylase synthetase, biotinylation

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

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Supplementary Material
Synthetic protocols, spectral listings, and assay conditions.

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.

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 (Figure 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 *E. 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-ketophosphonate **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′-isopropylidineadenosine (*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 ketone phosphonate was observed (NMR) to readily undergo H/D exchange upon dissolution in d_4-MeOH.

Reduction of the ketone with $NaBH_4$ 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 to 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±11.1% ($P<0.01$; n=4) compared with vehicle control (Fig. 3A). Data are presented as mean±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, IC_{50} and K_i for β-ketoBP equaled 39.7±1.9 μM and 2.0 ±0.1 μM, respectively; see Supplementary Materials for details regarding assays and calculations. When tested under identical conditions, β-hydroxyP inhibited HLCS activity by 67.7 ±10.0% ($P=0.0001$; n=4) at concentrations of 500 μM inhibitor. The IC_{50} and K_i values calculated under these conditions were 203.7 ±3.7μM and 10.4±0.2μM, 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 μM while that of biotin was varied from 0-320 μM; 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 *x* s); n=4] whereas the apparent K_m for biotin was increased by the addition of inhibitor (77.9±10.3 vs. 1.6±1.8 μM 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 *x* s); n=4] whereas reactions incubated with inhibitor increased the apparent K_m for biotin (82.5±20.4 vs. 1.9±1.8 μM 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±0.1% at concentrations of 500 μM and has an IC_{50} value and K_i of 8.8±3.6μM and 754±303nM, respectively. When reactions incubated with biotinol-AMP were challenged with increasing amounts of up to 320 μM 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* (~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 ± 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 ± 0.01 μM). 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 (*Mt*BPL); 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 (Figure S1).[13b,26] 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 ketophosphonate (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 biotinol-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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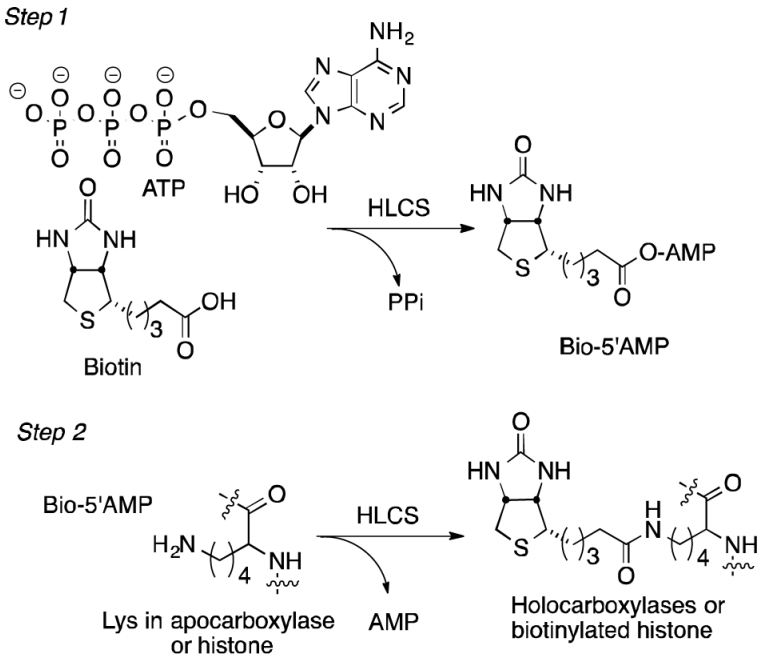


Figure 1.
HLCS catalysis

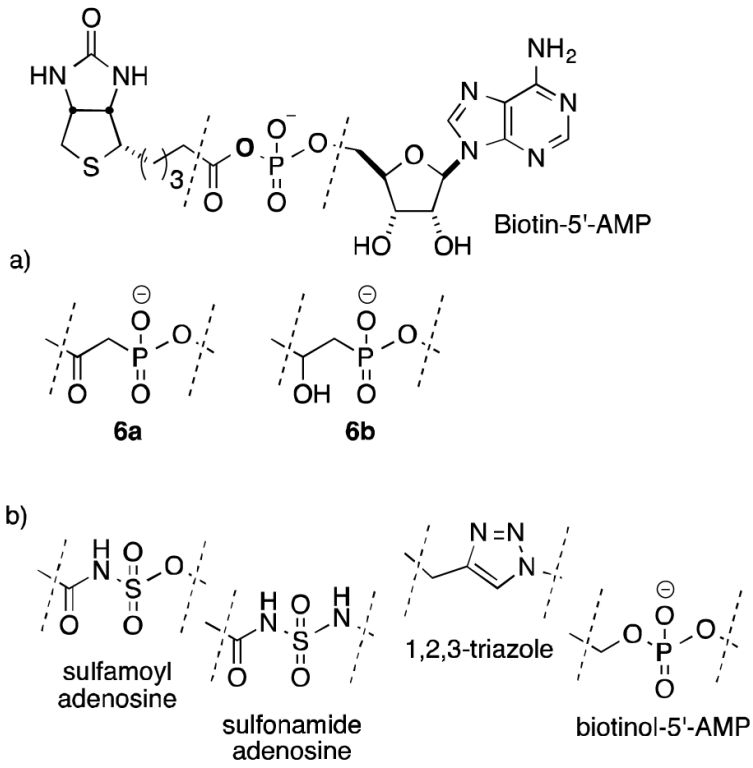
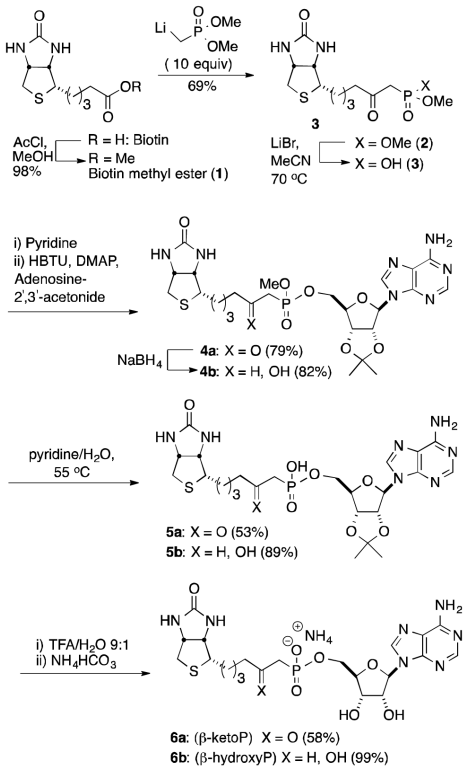


Figure 2.

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



Scheme 1.

Synthesis of ketophosphonate (β-ketoP) and hydroxyphosphonate (β-hydroxyP) analogs of biotin-5′-AMP

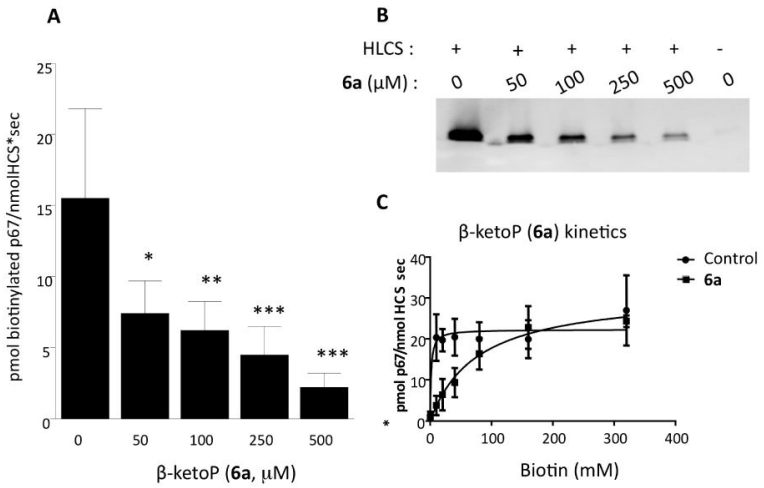


Figure 3.

Inhibition of HLCS by biotin β-ketophosphonate-5′-AMP. (A) HLCS activity was quantified using infrared absorbance in the presence of 20 μM 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.

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

Experimental Procedures. General experimental procedures have been described.[1]

Biotin methyl ester.[2] Acetyl chloride (0.6 mL, 7 mmol) was added dropwise to 4 mL of methanol in a round bottom flask held at 0 °C. After stirring for 15 min., the solution was then added to a room temp suspension of biotin (0.5068 g, 2.1 mmol) in methanol (4 mL) and the reaction was stirred until the biotin was completely consumed (TLC, ~30 min.). Solvent was removed under reduced pressure and the residue was partitioned between sat. $NaHCO_3$ and 5% methanol in dichloromethane. The separated organic layer was dried over $MgSO_4$ and evaporated under vacuum to obtain compound **1** as a white solid (0.5226 g, 98%): $[\alpha]^{25}_D$ = +63 (*c* = 0.15, reagent grade $CHCl_3$); mp 156-157 °C; R_f = 0.59 (10% MeOH in CH_2Cl_2); IR (ZnSe) 3267, 3193, 2927, 2849, 1744, 1702, 1462, 1431 cm-1; ^{1}H NMR (DMSO, 400 MHz): δ 4.31 (dd, *J* = 5.2, 7.7 Hz, 1H), 4.13 (ddd, *J* = 1.8, 4.4, 7.7 Hz, 1H), 3.59 (s, 3H), 3.10 (ddd, *J* = 4.4, 6.1, 8.4 Hz, 1H), 2.83 (dd, *J* = 5.1, 12.4 Hz, 1H), 2.58 (d, *J* = 12.4 Hz, 1H), 2.31 (t, *J* = 7.4 Hz, 3H), 1.61-1.30 (m, 6H); ^{13}C NMR (DMSO, 100 MHz, WS-3-37) δ 173.8, 163.2, 61.5, 59.6, 55.8, 51.7, 33.5, 28.5, 28.4, 24.9.

Dimethyl-2-oxo-6-((3aS,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl) hexylphosphonate (**2**) To a solution of dimethyl methylphosphonate (1.08 mL, 9.9 mmol) in 40 mL anhydrous THF was added dropwise *n*-BuLi (nominally 1.6 M in hexane, 6.25 mL, 9.9 mmol) at -78 °C. The reaction was stirred for 5-10 min and then added to a -78 °C suspension of biotin methyl ester (**1**, 0.2557 g, 0.99 mmol) in 5 mL THF via cannula. The reaction was stirred for 30 min. The cooling bath was removed and the reaction was allowed to warm to room temperature over 20 min. Deionized water (~4.5 mL) was added until the mixture turned clear. The solution was then concentrated and the residue was purified by flash chromatography using a gradient of 5/10 % MeOH in CH_2Cl_2 to afford compound **2** as a white solid (0.2386 g, 69%): $[\alpha]^{25}_D$ = +20 (*c* 0.06, reagent grade CH_2Cl_2); mp. 93-94 °C; R_f= 0.41 (10% MeOH/ CH_2Cl_2); IR (ZnSe) 3248, 2923, 2852, 1697, 1680, 1463, 1253, 1030, 815 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz, WS-2-51A-31P): δ 6.53 (s, 1H), 5.65 (s, 1H), 4.48 (dd, *J* = 5.2, 7.7 Hz, 1H), 4.28 (ddd, *J* = 1.0, 4.7, 7.4 Hz, 1H), 3.78 (d, *J* = 11.2 Hz, 3H), 3.77 (d, ^3J(H,P) = 11.2 Hz, 3H), 3.17 (dd, ^2J(H,P) = 22.8, ^2J(H,H) = 13.8 Hz, 1H), 3.10 (dd, ^2J(H,P) = 22.8, ^2J(H,H) = 13.8 Hz, 1H), 3.12 (dt, *J* = 4.8, 7.31H), 2.88 (dd, *J* = 4.8, 12.9 Hz, 1H), 2.72 (d, *J* = 12.9 Hz, 1H), 2.64 (dt, *J* = 2.8, 7.1 Hz, 2H), 1.75-1.61 (m, 4H), 1.40 (q, *J* = 7.6 Hz, 2H); ^{13}C NMR($CDCl_3$, 100 MHz) δ 202.3 (d, ^2J (C,P)=

6.0 Hz),164.1, 61.8, 60.1, 55.7, 53.2 (d, ^{2}J(C,P)= 6.0 Hz) 53.1 (d, ^{2}J(C,P) = 7.0 Hz) 43.8, 41.0 (d, ^{1}J(C,P) = 127.0 Hz) 40.6, 28.3, 28.0, 23.4; ^{31}P NMR ($CDCl_3$, 162MHz) δ 23.2; HRESI-MS: calcd for $^{12}C_{13}{}^{1}H_{23}{}^{23}Na^{14}N_2{}^{16}O_5{}^{35}PS$ $[M+Na]^+$: 373.0963; found: 373.0955.

Methyl hydrogen 2-oxo-6-((6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl) hexylphosphonate (**3**). A suspension of the dimethyl ketophosphonate (**2**, 0.1474 g, 0.42 mmol) and LiBr (0.073 g, 0.84 mmol) in anhydrous acetonitrile (8.4 mL) was stirred at 60 ^{o}C overnight. The resulting solution, containing a white precipitate, was concentrated and the residue was purified by flash chromatography (MeCN: H_2O = 8:1 to 7:1) to afford compound **3** as a white solid (0.121g, 86 %); $[\alpha]^{25}_{D}$ = +130 (*c* = 0.075, MeOH); mp 226^{o}C (decomp); R_f= 0.51 (50% MeOH/ CH_2Cl_2); IR (ZnSe) 3343, 2931, 2852, 2470, 1665, 1460, 1205, 1042 cm-1; ^{1}H NMR (MeOD, 400 MHz): δ .4.50 (dd, *J* = 4.3, 7.9 Hz, 1H), 4.33 (dd, *J* = 4.5, 7.9 Hz, 1H), 3.60 (d, ^{3}J (H,P) = 11.0 Hz, 3H), 3.22 (ddd, *J* = 4.6, 6.0, 10.5 Hz, 1H), 2.96 (d, ^{2}J (H,P)= 21.7 Hz, 2H), 2.94 (dd, *J* = 4.8, 12.9 Hz, 1H), 2.73 (dt, *J* = 2.7, 7.4 Hz, 2H), 2.71 (d, *J* = 13.0 Hz, 1H), 1.80-1.71 (m, 1H), 1.66-1.54 (m, 3H), 1.48-1.40 (m, 2H); ^{13}C NMR (MeOD, 75 MHz) δ 207.1 (d, ^{2}J (C,P) = 5.0 Hz), 164.7, 61.9, 60.2, 55.6, 50.8 (d, ^{2}J (C,P) = 6.0 Hz), 43.54 (d, ^{1}J (C,P) = 115.0 Hz), 42.44, 39.65, 28.17, 28.13, 23.05; ^{31}P NMR (MeOD, 162MHz) δ 14.32; HRESI-MS: calcd for $^{12}C_{12}{}^{1}H_{20}{}^{23}Na_2{}^{14}N_2{}^{16}O_5PS$: $[M-H+2Na]^+$: 381.0626; found: 381.0625.

((3aR,4R,6R,6aR)-6-(6-Amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl methyl 2-oxo-6-((3aS,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)hexylphosphonate (**4a**). The ketophosphonic acid (**3**, 98.8 mg, 0.29 mmol) was co-evaporated with pyridine (2 x 2 mL) to give the pyridinium salt. A solution of the salt, 2′, 3′-isopropylideneadenosine (0.3549 g, 1.15 mmol), *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) (0.3493 mg, 0.92 mmol) and DMAP (40.1 mg, 0.33 mmol) in anhydrous DMF (2.0 mL) was stirred at room temperature for 4 h. The mixture was then partitioned between 5% MeOH/CH_2Cl_2 and sat. aq. NH_4Cl. The organic layer was dried over Na_2SO_4 and the residue obtained upon concentration in vacuum was purified by flash column chromatography with 10% MeOH in dichloromethane. R_f= 0.44 (20% MeOH/ CH_2Cl_2); the compound was used for the next reaction without further purification.

((3aR,4R,6R,6aR)-6-(6-Amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl methyl 2-hydroxy-6-((2RS, 3aS,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)hexylphosphonate (**4b**). To a 0 ºC solution of the protected β-ketophosphonate **3** (0.1900 g, 0.30 mmol) in 4:1 isopropanol:CH_2Cl_2 (10 mL) was added $NaBH_4$ (0.0460 g, 1.2 mmol). The reaction was warmed up to rt and stirred until no starting material remained (TLC ~ 30 min). The reaction was then cooled to 0 ºC on ice bath and quenched with 15 mL of sat. $NaHCO_3$. The solution was stirred for 30 min and then extracted with CH_2Cl_2 30mLx3. The organic layers were combined and dried over Na_2SO_4. The residue obtained upon concentration was purified by flash chromatography over silica gel (10% and 15% MeOH in CH_2Cl_2) to furnish a mixture of four diastereoisomers as a white solid (0.1563 g, 82%): mp 30-40 ºC; R_f = 0.39 (WS-3-58) (15% MeOH/CH_2Cl_2); IR (ZnSe) 3271, 2929, 2849, 1692, 1596, 1212, 1023 cm^{-1}; ^{1}H NMR (MeOD, 600 MHz, WS-5-61_7May2012): ^{1}H NMR (MeOD, 600 MHz): δ 8.305/8.296 (two overlapping d, J = 4.8 Hz, total 1H, H-8), 8.258/8.256 (two overlapping singlet, total 1H, H-2), 6.25 (d, J = 6.0Hz, total 1H, H-1′), 5.51 (m, 1H, H-2′), 5.16 (m, 1H, H-3), 4.52-4.47 (m, 2H), 4.34-4.21 (m, 3H), 3.83 (br m, 1H), 3.69-3.65 (m, 3H), 3.20 (m, 1H), 2.95-2.92 (m, 1H), 2.72 (d, J = 12 Hz, total 1H), 1.96-1.87 (m, 2H), 1.76-1.66 (br m, 1H), 1.62 (overlapping signal, s, 3H), 1.62-1.53 (overlapping signal, m, 1H), 1.41 (overlapping signal, s, 3H), 1.50-1.25 (br m, 6H); ^{13}C NMR (MeOD, 150 MHz) δ 164.8, 156.0, 152.7, 148.9, 140.4-140.3 (3 lines), 119.2, 114.2-114.15 (3 lines), 90.6-90.4 (4 lines), 85.5-85.3 (6 lines), 84.0-83.8 (4 lines), 81.3-81.2 (3 lines), 65.9-65.8 (4 lines), 65.14-65.06 (3 lines), 62.03/62.01, 60.2, 55.7, 53.42, 51.9-51.6 (4 lines), 39.7, 37.9/37.8, 32.9/32.8, 28.7-28.3 (5 lines), 25.0-24.9 (3 lines), 24.15; ^{31}P NMR (MeOD, 243MHz) δ 32.84, 32.70, 32.63; HRESI-MS: calcd for $^{12}C_{25}{}^{1}H_{38}{}^{14}N_7{}^{16}O_8PSNa$: $[M+Na]^+$:650.2138; found: 650.2114.

((3aR,4R,6R,6aR)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl hydrogen (2-hydroxy-6-((3aS,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)hexyl)phosphonate (**5b**): A solution of methyl ketophosphonate (**4b**, 23 mg, 37 μmol) in 2 mL of 9:1 pyridine/water was stirred at 60ºC for 3 days. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography using 2:1:1 of *i*PrOH: CH_3CN: 50mM aq. NH_4CO_3 to afford compound **5** as a white solid (20 mg, 89 %): mp 154-170 ºC; R_f= 0.77 (2:1:1 *i*PrOH: CH_3CN: 50mM aq NH_4CO_3); IR (ZnSe) 3275 (br), 2932, 2860, 1678, 1602, 1208, 1054 cm-1; ^{1}H NMR (MeOD, 300 MHz): δ 8.46/8.45 (two apparent s, total 1H), 8.247/8.244 (two overlapping s, total 1H), 6.23 (d, J = 3.0 Hz, 1H), 5.40 (dd, J = 3.0, 6.0 Hz, 1H), 5.15/5.13 (two overlapping dd, J = 2.4, 5.7 Hz, 1H), 4.52-4.46 (bm, 2H), 4.31/4.29 (two overlapping dd, J = 4.4, 7.8 Hz, 1H), 4.13-3.97 (m, 2H), 3.78 (bm, 1H),

3.85-3.80 (br m, 1H), 3.36 (s, 3H), 3.16 (m, 1H), 2.91 (dt, J = 4.8, 12.3 Hz, 1H), 2.69 (d, J = 12.6 Hz, 1H), 1.62 (apparent s, 3H, overlapping signal), 1.41 (apparent s, 3H, overlapping signal), 1.75-1.25 (m, 10H); ^{13}C NMR (MeOD, 75 MHz) δ 164.8, 155.9, 152.5, 149.1, 140.08/140.01 (two apparent s), 118.9/118.8 (two overlapping s), 113.86/113.75 (two apparent singlet), 90.6/90.4 (two apparent singlet), 85.7 (d, ^3J (C,P) = 7.5 Hz), 84.4/84.2 (two apparent s), 81.9/81.7 (two apparent s), 67.15/67.11 (two overlapping d, C*H*OH, ^2J (C,P) = 3.0 Hz), 63.70 (two apparent d, CH_2 OP, ^2J (C,P) = 5.3 Hz), 61.98/61.96 (two overlapping s), 60.2, 55.72/55.70 (two overlapping s), 39.7, 37.57/37.41 (two overlapping d, CH_2 CHOH, ^3J (C,P) = 11.3 Hz), 34.2/34.0 (two overlappling d, CH_2 P, ^1J (C,P) = 131.3 Hz), 28.69/28.67 (two overlapping s), 28.32/28.29 (two overlapping s), 26.11, 25.06/24.99 (two overlapping s), 24.14; ^{31}P NMR (MeOD, 162MHz) δ 26.49/26.45 (two overlapping s); HRESI-MS: calcd for $^{12}C_{21}{}^{1}H_{35}{}^{14}N_7{}^{16}O_8Na_2PS$: $[M+2Na]^+$:658.1801; found = 658.1795.

ammonium ((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl (2-hydroxy-6-((3aS,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)hexyl)phosphonate (**6b**): A solution of the ketophosphonate (**5b**, 17.8 mg, 0.03 mmol) in 1 mL of 9/1 TFA/water was stirred at room temperature for 10 min. The solvent was removed under vacuum and the residue was washed with CH_2Cl_2. The residue was treated with 50 mM aq. ammonium bicarbonate (0.57 mL) and the solution was evaporated to dryness. The residue was washed with methanol to afford a white solid (17 mg, 99%): R_f= 0.50 (8:1:1 *i*PrOH: CH_3CN: 50 mM aq NH_4OH); ^{1}H NMR (D_2O, 600 MHz, WS-5-75): δ 8.55/8.54 (two overlapping s, 1H, H-8), 8.39 (s, 1H, H-2), 6.12/6.11 (two overlapping d, J = 4.8 Hz, 1H, H-1′), 4.70 (m overlapping with D_2O, H-2′), 4.50-4.43 (m, 2H, CH_2C*H*-NH overlapping with H-3′), 4.32 (br s, 1H, H-4′), 4.27 (ddd, J = 4.8, 7.8, 19.8 Hz, 1H, CHC*H*-NH) 4.20-4.09 (m, 2H, H-5′), 3.81 (br s, 1H, C*H*-OH), 3.15/3.10 (two apparent pentet, J = 4.8 Hz, total 1H, S-C*H*), 2.86/2.83 (two overlapping dd, J = 4.8, 13.2 Hz, 1H, SC*H*H), 2.64/2.62 (two overlapping d, J = 12.6 Hz, 1H, SCH*H*), 1.95-1.75 (m, 2H, CH_2P), 1.60-1.00 (m, 8H); ^{13}C NMR (D_2O, 150 MHz, WS-5-75) δ 165.31/165.29 (two apparent s), 163.2-162.5 (4 lines), 150.0, 148.43/148.37 (two apparent s), 144.73/144.69 (two apparent s), 142.6/142.5 (two apparent s), 119.2, 118.8/118.7 (two apparent s), 117.3, 115.4, 113.4, 88.5/88.3 (two apparent s), 83.9-83.7 (4 lines), 74.44/74.33 (two s), 70.0/69.9 (two s), 67.2-67.0 (3 lines), 63.44/63.30 (two d, ^2J (C,P) = 5.3 Hz, *C*H_2OP), 62.07/62.04 (two apparent s), 60.26/60.23 (two apparent s), 55.47/55.41 (two apparent s), 39.69, 36.84/36.78 (two overlapping d, ^2J (C,P) = 4.8 Hz, $CH_2CH_2CH_2$*C*H_2CHOH), 33.76/33.58 (two overlapping d, ^1J (C,P) = 131 Hz, *C*H_2P), 28.45/28.21 (two s, *C*H_2CH_2CHOH), 27.93/27.85 (two

s, SCH*C*H$_2$), 24.65/24.47 (two s, SCHCH$_2$*C*H$_2$); ^{31}P NMR(D$_2$O, 162 MHz) δ 29.87, 29.33; HRESI-MS (WS-5-75): calcd for $^{12}C_{21}{}^{1}H_{33}{}^{14}N_7{}^{16}O_8PS$: $[M+H]^+$:574.1849; found: 574.1835.

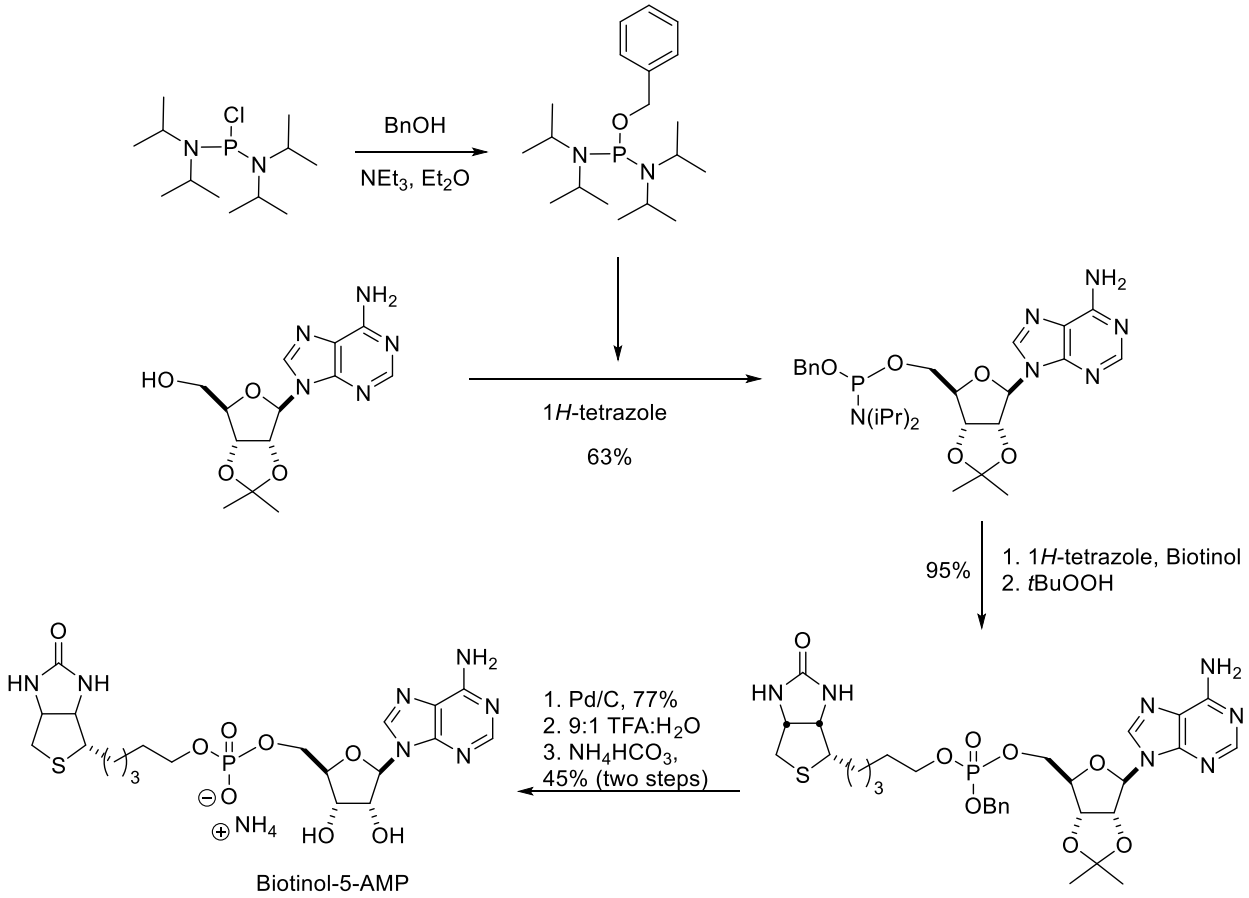
((3aR,4R,6R,6aR)-6-(6-Amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl hydrogen 2-oxo-6-((3aS,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)hexylphosphonate (5a). A solution of methyl ketophosphonate (**4a**, 19.8 mg, 0.032 mmol) in 3 mL of 9:1 pyridine/water was stirred at 50 ºC for 16 hr. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (MeCN:H$_2$O from 8:1 to 7:1) to afford compound **5a** as a white solid (10.1 mg, 53 %): $[\alpha]^{25}_D$ = -14 (*c* = 0.25, MeOH); mp. 184-186 ºC; R_f = 0.38 (50% MeOH/ CH_2Cl_2); IR (ZnSe) 3317, 3182, 2993, 2931, 2858, 1695, 1645, 1600, 1248, 1211, 1022, 728 cm-1; ^{1}H NMR (MeOD, 400 MHz): δ 8.50 (s, 1H), 8.24 (s, 1H), 6.24 (d, *J* = 3.4 Hz, 1H), 5.36 (dd, *J* = 3.4, 6.1 Hz, 1H), 5.15 (dd, *J* = 2.0, 6.1 Hz, 1H), 4.50 (bs, 1H), 4.47 (dd, *J* = 4.4, 7.8 Hz, 1H), 4.29 (dd, *J* = 4.4, 7.8 Hz, 1H), 4.11 (ddd, *J* = 3.7, 11.2, ^3J(H,P) = 6.4 Hz, 1H), 4.05 (ddd, *J* = 3.9, 11.2, ^3J(H,P) = 4.0 Hz, 1H) , 3.15 (ddd, *J* = 4.5, 6.2, 10.7 Hz, 1H), 2.95 (d, ^2J(H,P) = 21.4 Hz, 2H), 2.91 (dd, *J* = 5.0, 12.7 Hz, 1H), 2.69 (d, *J* = 12.7 Hz, 1H), 2.66 (t, *J* = 7.1 Hz, 2H), 1.75-1.35 (m, 12H); ^{13}C NMR (MeOD, 100 MHz) δ 206.8, 164.8, 156.0, 152.6, 149.2, 140.0, 118.8, 113.8, 90.3, 85.3 (d, ^3J (C,P) = 8.0 Hz), 84.3, 81.8, 64.4 (d, ^2J (C,P) = 5.0 Hz), 61.8, 60.19, 55.49, 44.4 (apparent singlet, CH_2-P comfirm by HSQC), 42.5, 39.68, 28.06 (2C), 26.14, 24.17, 22.93; ^{31}P NMR (MeOH, 162MHz, WS-3-66-Feb12-11) δ 13.25; HRESI-MS (WS-3-67): calcd for $^{12}C_{24}{}^{1}H_{33}{}^{23}Na_2{}^{14}N_7{}^{16}O_8PS$: $[M-H+2Na]^+$: 656.1644; found: 656.1635.

((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl hydrogen 2-oxo-6-((3aS,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl) hexylphosphonate (**β-ketoP, 6a**) A solution of the ketophosphonate (**5a**, 40 mg, 0.065 mmol) in 1 mL of 9/1 TFA/water was stirred at room temperature for 15 min. The solvent was removed under vacuum and the residue was precipitated and washed with MeOH to obtain compound **6** as a white solid. Ammonium bicarbonate (NH_4HCO_3, 3.0 mg, 0.038mmol) was added to the white solid followed by 1 mL of DI water. The resulting solution was evaporated to furnish the ammonium salt of β-ketoP (**6a**) in 22.4 mg (58% for 2 steps): $[\alpha]^{25}_D$ = +40 (*c* = 0.07, H_2O); m 160-170 ºC with color change; IR (ZnSe) 3326, 3191, 2934, 2856, 1663, 1651, 1599, 1464, 1201, 1034 cm^{-1}; ^{1}H NMR (D$_2$O, 400 MHz, WS-3-60): δ 8.50 (s, 1H), 8.33 (s, 1H), 6.07 (d, *J* = 4.8 Hz, 1H), 4.61 (t, *J* = 4.8 Hz, 1H), 4.41 (dd, *J* = 4.8, 8.0 Hz, 1H), 4.38 (t, *J* = 4.8, 8.0 Hz, 1H),

4.27 (bs, 1H), 4.19 (dd, J = 4.4, 7.6 Hz, 1H), 4.1-4.0 (bm, 2H), 3.10-2.85 (m, 3H), 2.77 (dd, J = 4.8, 12.8 Hz, 1H), 2.56 (d, J = 13.2 Hz, 1H), 2.50 (t, J = 7.2 Hz, 2H), 1.7-0.9 (m, 12H); ^{13}C NMR (D_2O, 100 MHz) δ 210.0 (d, ^2J (C,P) = 6 Hz), 165.2, 149.9, 148.4, 144.7, 142.4, 118.6, 88.0, 84.1 (d, ^3J (C,P) = 8.0 Hz), 74.5, 70.1, 63.6 (d, ^2J (C,P) = 5.0 Hz), 61.9, 60.2, 55.2, 44 (see no peak but confirm by HSQC from WS-3-66-Mar12), 43.1, 39.7, 27.9, 27.7, 22.7; ^{31}P NMR(D_2O, 162MHz, WS-3-66-Feb12-11) δ 15.15; HRESI-MS (WS-3-60): calcd for $^{12}C_{21}{}^{1}H_{31}{}^{14}N_7{}^{16}O_8PS$: $[M+H]^+$:572.1692; found: 572.1710 and calcd for $^{12}C_{21}{}^{1}H_{30}{}^{14}N_7{}^{16}O_8PSNa$: $[M+Na]^+$:594.1512; found: 594.1523. The ^{13}C signal at 44 ppm, assigned as -CO**C**H_2PO_3- by HSQC) was not observed due to deuterium exchange and subsequent splitting of the 13C signal by both D and ^{31}P. Acquisition of ^{13}C spectra in aprotic solvents was hindered by limited substrate solubility.

Synthesis of Biotinol-5′-AMP.

The synthesis of Biotinol-5′-AMP was previously reported by the coupling reaction between 5′-AMP and biotinol. Scheme S1 describes an alternative synthesis procedure adapted from reported literature reported (Iwashita, M.; Makide, K.; Nonomura, T.; Misumi, Y.; Otani, Y.; Ishida, M.; Taguchi, R.; Tsujimoto, M.; Aoki, J.; Arai, H.; Ohwada, T., *J Med Chem* **2009,** *52*, 5837); our approach employs the phosphoramidite method to construct the phosphate diester moiety.



Scheme S1. The synthesis of Biotinol-5′-AMP

Benzyl 2′,3′-*O*-isopropylidineadenosine-5′-*N*,*N*-diisopropylphosphoamidite was prepared using a reported procedure (Iwashita, 2009-see above). Benzyl bis(diisopropylamino)phosphite (0.340 g, 1.00 mmol, 1equiv, prepared using a reported procedure,[2] and 2′,3′-*O*-isopropylidineadenosine (0.300 g, 1.00 mmol, 1 equiv) were dissolved in 6 mL of distilled CH_2Cl_2 under an atmosphere of N_2. To this mixture, a solution of 1*H*-tetrazole (0.07 g, 1 mmol, 1 equiv) in 2mL THF was added, resulting after a few minutes in a cloudy solution which was stirred for 2 h. The reaction was then quenched with 5 mL of sat. aq $NaHCO_3$ and extracted with CH_2Cl_2 (5 mL x 2). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography using 6:4:1 EtOAc:Hex:NEt_3 to afford a low-melting white solid (0.344 g, 63% yield): mp. 25-35 ^oC; R_f= 0.29 (40% EA in Hex); IR (ZnSe, cm^{-1},WS-7-97) 3321, 3168, 2967, 2926, 2866, 1644, 1597,

1202, 1077, 1023, 975, 734 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz, WS-7-97-col): δ 8.34 (s, 1H, H-8), 8.15/8.08 (two s, total 1H, H-2), 7.40-7.22 (m, 5H), 6.20/6.17 (two d, J = 2.8 Hz, total 1H, H-2′), 5.61 (br s, 2H, NH_2), 5.29/5.20 (two dd, J = 6.0/6.4, 2.8/2.8 Hz, total 1H, H-1′), 5.02/4.98 (two d, J = 6.4, 2.4 Hz, total 1H, H-3′), 4.78-4.61 (m 2H, H-5′), 4.53 (m 1H, H-4′), 3.96-3.73 (m, 2H, CH_2Ph), 3.66-3.52 (m, 2H, CH(CH_3)$_2$), 1.65 (s, 3H), 1.40/1.39 (two s, 3H), 1.19 (d, J = 7.2 Hz, 3H), 1.17 (d, J = 7.2 Hz, 3H), 1.13 (d, J = 6.8 Hz, 3H), 1.11 (d, J = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, WS-7-97-col) δ 155.4, 153.2, 149.66/149.50 (two s), 139.47/139.43 (two s), 139.16/139.09 (two s), 128.31/128.26 (two s), 127.41/127.34 (two s), 127.01/127.00 (two s), 120.14/119.93 (two s), 114.15/114.06 (two s), 91.48/91.16 (two s), 86.36/86.27/86.18 (three s), 84.75/84.71 (two s), 81.83/81.80 (two s), 65.68-65.39 (four lines), 65.63-63.26 (four lines), 43.08-42.94 (four lines), 27.20/27.18 (two s), 25.32, 24.61-24.49 (five lines); HRESI-MS (WS-7-97): calcd for $^{12}C_{26}{}^{1}H_{37}{}^{14}N_6{}^{16}O_5PNa$: $[M+Na]^+$:567.2461; found: 567.2477.

Benzyl 2′,3′-*O*-isopropylidineadenosine-5′-biotinyl triphosphate: Benzyl 2′,3′-*O*-isopropylidineadenosine-5′-*N*,*N*-diisopropylphosphoamidite (0.450 g, 0.83 mmol, 1.2 equiv) and biotinol (0.1585 g, 0.69 mmol, 1 equiv) were dissolved in 3.5 mL of distilled CH_2Cl_2 at rt under an atmosphere of N_2. To this mixture, a solution of 1*H*-tetrazole (0.106 g, 1.38 mmol, 2.2 equiv) in 3.5 mL THF was added, resulting after a few minutes in a cloudy solution. The reaction was stirred for 45 min afterwhich was added 0.25 mL (1.38 mmol, 2.2 equiv) of *t*-butyl hydroperoxide (TBHP) in decane. The reaction mixture was stirred for an additional 2 h at rt and then quenched with 15 mL of sat. aq. $NaHCO_3$. The mixture was partitioned with 10% MeOH in CH_2Cl_2 (10 mL x 3). The combined organic layers were dried over Na_2SO_4. The solvent was evaporated and the residue was purified by column chromatography using a step gradient of 19:1:0.2 to 9:1:0.1 of CH_2Cl_2:MeOH:NEt_3 to afford a white solid (0.4512 g, 95% yield): mp. 80-100 ^oC; R_f = 0.38 (10% MeOH in CH_3Cl_2); IR (ZnSe, cm^{-1},WS-7-97) 3319, 3203, 2938, 2852, 1688, 1642, 1594, 1252, 1009, 752 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz, WS-7-97-col): δ 8.233/8.226 (two overlapping s, total 1H), 7.96 (s, 1H), 7.87 (s, 1H), 7.72 (s, 1H), 7.45-7.29 (m, 5H), 6.08 (m, 1H), 5.45 (m, 1H), 5.30 (m, 1H), 5.16-4.92 (m, 3H), 4.53 (m, 1H), 4.48-4.35 (m, 2H), 4.29 (m, 1H), 4.15 (m, 1H), 3.96-3.77 (m, 2H), 3.17 (m, 1H), 2.94 (m, 1H), 2.76 (d, J = 12.8 Hz, 1H), 5.61 (br s, 2H, NH_2), 1.62 (s, 3H, overlapping signal), 1.34 (s, 3H, overlapping signal), 1.80-1.10 (m, 8H); ^{13}C NMR ($CDCl_3$, 100 MHz, WS-7-97-col) δ 164.33 (two s), 156.45/156.38 (two s), 152.69, 148.74/148.69 (two s), 139.68/139.54 (two s), 128.61/128.57 (two s), 127.99/127.89 (two s), 120.2, 114. 37, 91.45/91.26 (two s), 85.68/85.59 (two s), 69.37/69.32 (two s), 67.98/67.92 (two s), 66.73/66.67 (two s), 59.87/59.81 (two s), 45.95, 40.82/40.76 (two s), 29.69/29.62/29.11/29.03/28.96 (four s), 27.09, 25.83, 25.31; ^{31}P

NMR($CDCl_3$, 100MHz) δ -0.55, -0.78; HRESI-MS (WS-7-99): calcd for $^{12}C_{30}{}^{1}H_{40}{}^{14}N_7{}^{16}O_8PSNa$: $[M+Na]^+$:712.2294; found: 712.2280.

2′,3′-*O*-isopropylidineadenosine-5′-biotinyl phosphate: A suspension of benzyl 2′,3′-*O*-isopropylidineadenosine-5′-biotinyl triphosphate (0.150 g, 0.220 mmol) and 10% Pd/C (0.047 g, 0.002 mmol, 0.2 equiv) in anhydrous MeOH (2 mL) was placed in a vial under an atmosphere of H_2 and stirred at rt for 5 h. The mixture was then filtered through a plug of Celite, which was washed with MeOH. The filtrate was then evaporated under vacuum and the residue was purified by column chromatography using 5:1:1 of iPrOH:CH_3CN:50mM aq. NH_4HCO_3 to afford white solid (0.101 g, 77%): mp. 110-122 ºC; ^{1}H NMR (D_2O, 400 MHz, WS-7-97-col): δ 8.29 (s, 1H), 8.12 (s, 1H), 6.15 (d, J = 3.2 Hz, 1H), 5.36 (dd, J = 6.0, 3.2 Hz, 1H), 5.13 (dd, J = 6.0, 2.0 Hz,1H), 4.56 (br s, 1H), 4.46 (dd, J = 8.0, 4.8 Hz, 1H), 4.20 (dd, J = 8.0, 4.4 Hz, 1H), 4.05-3.88 (m, 2H), 3.47 (m. 1H), 2.99 (m, 1H), 2.81 (dd, J = 13.2, 4.8 Hz, 1H), 2.63 (d, J = 13.2 Hz, 1H), 1.59 (s, 3H,), 1.38 (s, 3H, overlapping signal), 1.45-1.15 (m, 4H), 1.06-0.85 (m, 4H); ^{13}C NMR (MeOD, 100 MHz, WS-7-97-col) δ 164.78, 156.02, 152.66, 149.13, 140.05, 118.86, 113.85, 90.29, 85.58/85.49 (two s), 84.19, 81.84, 65.25/65.20/65.13 (three s), 62.00, 60.20, 55.72, 53.46, 46.31, 39.70, 30.13/30.06 (two s), 28.52/28.36 (two s), 26.16, 25.42, 24.17, 7.81; ^{31}P NMR($CDCl_3$, 100MHz) δ 1.50; HRESI-MS (WS-7-99): calcd for $^{12}C_{23}{}^{1}H_{35}{}^{14}N_7{}^{16}O_8PS$: $[M+H]^+$:600.2005; found: 600.1987. This molecule has been previously reported (reference?) without characterization data.

Biotinol adenylate: A solution of 2′,3′-*O*-isopropylidineadenosine-5′-biotinyl phosphate (0.190 g, 0.317 mmol) in 1 mL of 9/1 TFA/water was stirred at room temperature for 5 min. The solvent was removed under vacuum and the residue was neutralized with about 1.5 mL (1.5 equiv) of 1M aq. NH_4HCO_3. The resulting solution was evaporated to dryness and the residue was purified by column chromatography using 5:1:1 of *i*PrOH:CH_3CN:50mM aq NH_4HCO_3 to afford a white solid (0.079 g, 45%): ^{1}H NMR (D_2O, 400 MHz, WS-8-2): δ 8.41 (s, 1H, H-8), 8.12 (s, 1H, H-2), 6.05 (d, J = 5.6 Hz, 1H, H-1′), 4.75 (m, 1H, overlapping with D_2O, H-2′), 4.50-4.40 (m, 2H, H-3′ overlapping with CH_2CH-NH), 4.30 (m, 1H H-4′), 4.17 (dd, J = 8.0, 4.8 Hz, 1H, CHC*H*-NH), 4.04 (m, 1H, H-5′), 3.64 (m, 2H, CH_2CH_2OP), 2.97 (m, 1H, S-C*H*), 2.79 (dd, J = 13, 5.2 Hz, 1H, SC*H*H), 2.60 (d, J = 13 Hz, 1H, SCH*H*), 1.45-1.30 (m, 3H), 1.30-1.15 (m, 1H), 1.15-0.93 (m, 4H); ^{13}C NMR (D_2O, 100 MHz, WS-7-97-col) δ 165.24, 155.03, 152.13, 149.08, 139.94, 118.63, 86.98, 83.94/83.85 (two s or a doublet due to coupling with P), 73.97, 70.32, 66.15/66.10 (two s or a doublet due to coupling with P), 64.69/64.64 (two s or a doublet due to coupling with P), 61.89. 60.14, 55.23, 53.86, 39.63, 29.38/29.32 (two s), 27.86, 27.70, 24.60; ^{31}P NMR ($CDCl_3$,

100MHz) δ 1.38; HRESI-MS (WS-6-14): calcd for $^{12}C_{20}{}^{1}H_{31}{}^{14}N_{7}{}^{16}O_{8}PS$: $[M+H]^+$:560.1692; found: 560.1683.

HLCS activity. This assay is based on the HLCS dependent biotinylation of the p67 polypeptide.[3] Briefly, recombinant biotin-free p67 and human HLCS and were prepared as described. The polypeptide p67 comprises the 67 C-terminal amino acids in PCC, including the biotin-binding site K694; p67 is a well-established substrate for biotinylation by HLCS.[3] Briefly, 30 nM HLCS was incubated with 2 μM p67 in 75 mM Tris-acetate (pH 7.5), 45 mM $MgCl_2$, 7.5 mM ATP, 0.3 mM DTT, and 20 μM biotin in the presence of 0.05 to 0.5 mM biotin β-ketophosphonate-5′-AMP (50 μl final volume) at 37ºC for 2 h; controls were incubated in the absence of inhibitor. Reactions were stopped by the addition of 50 μl of Tricine loading dye (Invitrogen) and heating at 95ºC for 10 min. Proteins were run on 16% Tricine gels and transferred to polyvinylidene blots. Biotinylated p67 was probed using anti-biotin and anti-goat conjugated secondary antibody, and was quantified using an Odyssey infrared imaging system.[4]

Competitive inhibition assays

These experiments use a similar approach to HLCS activity assays with some modifications. Briefly, 30 nM HLCS was incubated with 2 μM p67 and cofactors as described previously. Reactions were incubated either in the presence or absence of 250 μM inhibitor and challenged with increasing amounts of biotin ranging from 0-360 μM. Reactions were stopped by addition of 50 μl Tricine loading dye (Invitrogen) and heating at 95ºC for 10 min, and proteins were analyzed by Western blot analysis as described previously. Data presented in the paper reflects mean±SD of 4 replicates.

IC_{50} and K_i Calculation

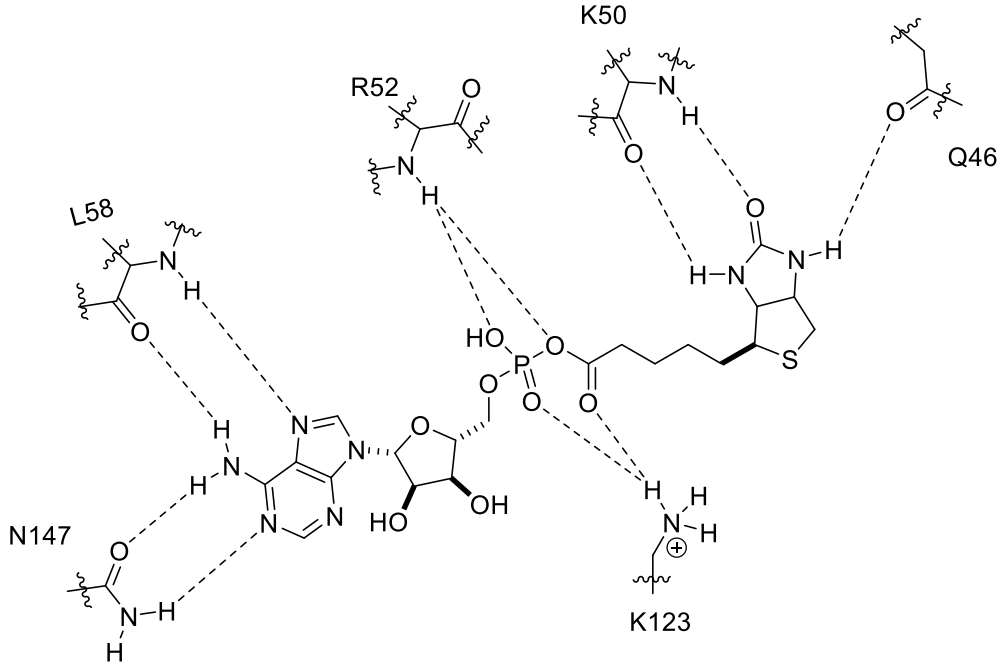
The Michaelis-Menten constant (K_m) and IC_{50} values were calculated using nonlinear regression analysis in Graph Pad Prism 6.0 software. The K_m was estimated by plotting substrate concentration vs. velocity obtained from enzyme activity and competition assays, and fitting the curve to the Michaelis-Menten equation. The half-maximal inhibitory concentration (IC_{50}) was calculated by plotting the logarithm of the concentration of substrate vs. binding and the curve

generated was fit to the competition binding equation, One-site-Fit-logIC_{50}. K_i was calculated from IC_{50} using an online-based tool.[5]

Statistics

One-way ANOVA was used to determine whether differences among treatment groups were significantly different. Dunnett's multiple comparisons t-test was used to determine if differences between the control and individual treatment groups were statistically significant. Statistical analysis was performed in Graph Pad Prism 6.0.

Figure S1. Schematic view of polar interaction between biotin-5′-AMP and wild-type HCS. The structure of three-dimensional (3D) model of wild-type HCS bound with biotin-5′-AMP was obtained using the crystal structure of Biotin-protein ligase from pyrococcus horikoshii OT3 (pdb code: 1wqw) as the structural template. The experimental basis has been described.[6]



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