Discovery and Application of a Lysine 5-Hydroxylase for Bioorthogonal Chemistry

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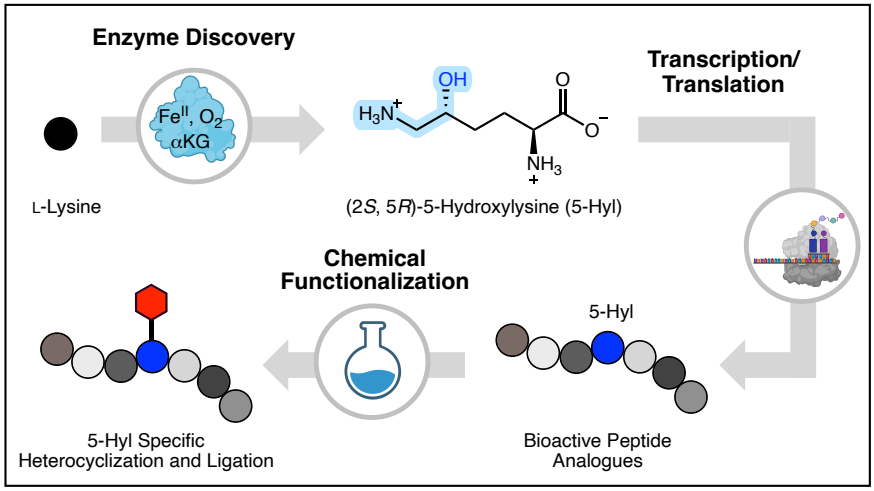

ABSTRACT: The selective functionalization of unactivated C(sp^3)–H bonds remains an ongoing challenge in synthetic organic chemistry. In this context, biocatalysis provides an attractive strategy to perform such chemistry under mild reaction conditions. We now report the discovery of K5H, the first enzyme that catalyzes the one-step conversion of free L-lysine into enantiopure (2*S*, 5*R*)-5-hydroxylysine (5-Hyl), producing a β-amino alcohol motif on the lysine side-chain. As chiral β-amino alcohols are versatile synthetic motifs found in natural products, pharmaceuticals, ligands, and other complex molecules, we demonstrate that this noncanonical amino acid can be incorporated into several pharmaceutically-relevant peptides in place of lysine through a tandem one-pot biocatalytic cascade using in vitro transcription/translation. Indeed, we show that the introduction of a single hydroxyl group adjacent to the ε-amine on lysine serves as a selective handle for downstream bioorthogonal chemistry such as heterocyclization, ligation to various payloads, and formation of branched peptides. Taken together, the discovery and characterization of K5H provides a modular genetically-encoded platform to tune the structure and properties of diverse bioactive peptides via biocatalytic transformations of unactivated C(sp^3)–H bonds.

MAIN TEXT

The most common structural component of organic molecules is the C(sp^3)–H bond, yet the relatively inert nature of these bonds and the abundance of similarly reactive sites renders regiocontrolled and selective C–H functionalization challenging.[1] Enzymes have evolved to exhibit unparalleled selectivity and activity under mild conditions, making them powerful C–H functionalization catalysts that can accommodate substrates with high functional group density.[2] Amino acids provide a particularly interesting substrate class due to the broad applications of synthetic and biosynthetic noncanonical amino acids (ncAAs) that can serve as chiral building blocks, pharmacophores, ligands, catalysts, spectroscopic probes, and tools for chemical biology (**Figure 1A**).[3]

While a number of different transformations are possible on amino acids, especially at more activated or sp^2-hybridized sites, we are specifically interested in enzymes that can introduce synthetic handles onto unreactive C(sp^3) hydrocarbon side-chains.[4] Towards this effort, we recently reported the discovery of the BesD family of radical halogenases, which selectively chlorinate L-lysine, L-ornithine, and various aliphatic L-amino acids using a nonheme Fe^{II}/α-ketoglutarate (Fe/αKG) active site and molecular oxygen.[5] Interestingly, enzymes of the Fe/αKG superfamily appear to be an evolutionarily privileged class for the modification of amino acid C(sp^3)–H bonds, carrying out a range of reactions including hydroxylation, halogenation, desaturation, epimerization, and epoxidation.[6] However, the sequence conservation between individual families is quite low, which can make new sequences and reactions difficult to identify.

We thus set out to explore the BesD family with the goal of discovering new biocatalytic transformations to produce useful ncAAs. Using a BLAST search of the UniProt protein sequence database, we identified S3ZC11 as a promising sequence with 30% sequence identity to BesD and 32% sequence identity to Hydrox, a previously characterized BesD homolog with L-lysine C4 hydroxylation activity (**Figure S1**).[7] Sequence alignment to other BesD family members and an AlphaFold 2 model show that S3ZC11 contains the distinguishing facial triad motif for non-halogenase activity while retaining residues involved in amino acid recognition, in particular R80 and H139, suggesting that it would accept an amino acid substrate but would carry out a different reaction (**Figure 1B, Figure S1**).

To determine the activity of this enzyme, S3ZC11 was cloned for heterologous expression in *Escherichia coli* and purified by affinity chromatography (**Table S1, Figure S2**). The purified enzyme was added to a reaction mixture containing the 20 proteinogenic amino acids, and the reaction was analyzed by LC-MS (**Figure S3**). Gratifyingly, we only observed depletion of L-lysine and the formation of a product consistent with lysine hydroxylation. Isolation of the product followed by NMR analysis and comparison to an enantiopure chemical standard revealed that S3ZC11 selectively produces (2*S*, 5*R*)-5-hydroxylysine (5-Hyl; **1**) (**Figure 1C, Figure S4**).[8] Steady state kinetic characterization showed that S3ZC11 catalyzes this reaction with a k_{cat} of 25 ± 1 min^{-1}, K_M of 1.3 ± 0.2 mM, and k_{cat}/K_M of 20 ± 3 $mM^{-1}min^{-1}$ (**Figure S5**), which are within the range of values observed for similar enzymes reacting with their native substrates.[5b] On the basis of this observed substrate- and site-selectivity, we henceforth refer to this enzyme as K5H.

Although several lysine hydroxylases have been reported to modify L-lysine at C3 and C4, K5H is unique in its ability to achieve the site- and stereoselective hydroxylation of free L-lysine at the C5 position.[9] Notably, the C5 selectivity of K5H for yields a β-amino alcohol on the side-chain, which is an important substructure of natural products, pharmaceuticals, agrochemicals, and as chiral ligands as well as a useful handle for downstream reactions.[10] Furthermore, lysine 5-hydroxylation has been identified as a prominent post-translational modification (PTM) most commonly found in collagen and collagen-like proteins.[11] Despite these examples of lysine hydroxylases in biology, K5H has low sequence identity to all previously identified lysine hydroxylases protein hydroxylases, with the exception of Hydrox (**Figure S1**). Given its biological significance, several multi-step syntheses of (2*S*, 5*R*)-5-hydroxylysine have been reported since the first racemic synthesis in 1950,[12] involving chiral (i) HPLC resolution, (ii) resolving agents, (iii) auxiliaries, (iv) catalysis, and (v) substrate-directed approaches to obtain enantiopure material.[8, 13] In

comparison, K5H provides a one-step synthesis of the naturally-occurring diasteromer of 5-Hyl directly from L-lysine without protecting groups. Indeed, incubation of L-lysine with K5H (2.5 mol%) in pH 7.5 HEPES buffer for 24 h at room temperature resulted in essentially quantitative conversion (>99%) to a single diastereomer of 5-Hyl as determined by NMR (**Figure 2C; Figure S4**).

We next sought to utilize K5H to access 5-Hyl for the production of bioactive peptide analogues. Peptide and protein biologics constitute a rapidly growing class of molecules used to treat a range of human health conditions.[14] As such, there is great interest in developing methods to access new amino acids,[3a, 3d] modify peptide architectures (e.g., macrocyclization or stapling),[15] and append valuable payloads via bioconjugation strategies.[16] Although ncAAs can be incorporated through solid- or solution-phase peptide synthesis, ribosomal synthesis offers an advantage in that the products are genetically encoded to allow for efficient engineering or evolution of large compound libraries. Our approach was to use cell-free gene expression[17] to test whether 5-Hyl could be accommodated by the translational machinery. To this end, K5H was used to generate **1** *in situ* from L-Lys, which was then directly transferred into in vitro transcription/translation (IVTT) using PURExpress with the addition of the remaining 19 canonical amino acids and a plasmid encoding for a linear analogue of the peptide hormone oxytocin (**Figure 2A**).[18] 5-Hyl was successfully incorporated into the lysine site of the oxytocin analogue sequence as shown by high-resolution LC-MS and MS/MS fragmentation (**Figure 2C; Figure S6**). This strategy could be generalized to seven additional peptides with variable lengths, amino acid compositions, secondary structures, and bioactivities, providing high levels (89% to essentially quantitative) of 5-Hyl incorporation (**Table 1; Figure S7**). Notably, in instances when multiple lysines were present in the sequence, each residue was replaced with 5-Hyl (**Table 1**, entries 7 and 8). These results demonstrate that this platform can be used to produce a wide range of other valuable lysine-containing peptides or proteins, such as cell-penetrating peptides for drug delivery, antibody-drug conjugates, and biomaterials.[19]

With several 5-Hyl-containing peptides in hand, we turned our attention to characterizing the compatibility of unprotected 5-Hyl peptides with downstream chemical reactions (**Figure 3**). First, we demonstrated that the formylated-methionine that is introduced via IVTT through the start codon can be easily removed post-translationally upon treatment with cyanogen bromide in formic acid, exemplified by converting **2b** into **3** (**Figure S8**).[20] Furthermore, 5-Hyl can also still engage in typical reactivity through the ε-amine.[21] Incubation of the 5-Hyl oxytocin analogue **2b** with biotin *N*-hydroxysuccinimide ester in sodium phosphate buffer pH 7.5 resulted in full consumption of the starting material and production of the corresponding biotinylated peptide **4** (**Figure 3; Figure S9**).

We then set out to examine applications of the unique β-amino alcohol moiety of these 5-Hyl-containing peptides. The 5-hydroxyl group of 5-Hyl provides a second nucleophile for 5-membered ring formation that can generate new heterocycles or assist in downstream bioorthogonal chemistry. Inspired by a method developed by Raj and co-workers to cleave peptide backbones at serine and threonine residues,[28] **2b** was treated with *N*,*N*'-disuccinimidyl carbonate (DSC), *N*,*N*'-diisopropylethylamine (DIEA), and 4-dimethylaminopyridine (DMAP) in H_2O/DMF, resulting in full consumption of starting material and formation of a oxazolidinone heterocycle (**5**), a common motifs in pharmaceuticals and chiral auxiliaries (**Figure 3; Figure S10**).[29] To our knowledge, oxazolidinone formation of this nature on the side-chain of a peptide has not been previously reported. In addition to being key structural motifs in pharmaceuticals and chiral auxiliaries, oxazolidinones can also serve as precursors to oxazoline or oxazole rings, other pharmaceutically-relevant heterocycles,[30] via additional dehydration. Furthermore, the oxazolidinone can serve as a protecting group for the reactive ε-amine of Hyl, or provide a handle for further derivatizations through pH-controlled *N*-functionalizations.[31]

The hydroxyl group of **1** also serves as a minimal handle for chemical ligation, promoting *N*-acylation of the ε-amine through a neighboring group effect, differentiating 5-Hyl from Lys sidechains. Similarly, *N*-terminal serine/threonines contain a β-amino alcohol that has been used for the ligation of peptides.[32] These

N-termini preferentially react with an *O*-functionalized salicylaldehyde to form an imine, which then cyclizes upon attack from the vicinal hydroxyl group (**7**, **Figure 4A**). Spontaneous *O*-to-*N* acyl transfer occurs to afford an *N*-acylated *N*,*O*-benzylidene acetal (**8**). This intermediate can then be cleaved upon treatment with acid to reveal a backbone amide. If 5-Hyl were to display similar reactivity, it would allow us to site-selectively ligate various functional payloads at 5-Hyl positions. To test this hypothesis, we reacted **2b** with *O*-benzoyl salicylaldehyde **6a** and observed near quantitative (94%) consumption of the starting material and formation of the *N*,*O*-benzylidene acetal intermediate **8a**. Subsequent treatment of the reaction mixture with TFA resulted in quantitative conversion to the desired *N*-functionalized peptide **9a** (**Figure 4B; Figure S11**). Notably, no reaction occurs when using the analogous lysine-containing peptide, demonstrating the selectivity of this reaction for 5-Hyl residues (**Figure S11**).

This ligation reaction can be applied to the transfer of functional payloads, which are accessed in a one-step coupling reaction with salicylaldehyde to form the ester. Here, we show that amino acid-containing substrates can be easily prepared to yield branched peptides bearing Fmoc-protected Phe (**9b**) or Fmoc-protected *p*-benzoyl-L-phenylalanine (Bpa; **9c**), a photocrosslinking ncAA (**Figure 4A; Figure S11**).[33] This method could be extended to ligate peptides or proteins bearing an *O*-salicylaldehyde ester at a side-chain or on the *C*-terminus. We anticipate that additional small-molecules, amino acids (canonical or noncanonical), peptides, proteins, antibodies, probes, dyes,[34] or other payloads of interest could be bioconjugated selectively to 5-Hyl in this manner.

Taken together, we have shown that the bioinformatics-guided discovery of K5H — the first free L-lysine 5-hydroxylase — enables the efficient synthesis of diverse, value-added products under mild and sustainable biocatalytic conditions. This enables direct access to an enantiopure β-amino alcohol-containing ncAA in one-step without the need for protecting groups or chiral separation. In addition to its use as a chiral ligand, auxiliary, or building block,[35] 5-Hyl can be incorporated directly into peptides by IVTT, providing a modular, genetically-encoded method to produce peptides or proteins containing the synthetically versatile chiral β-amino alcohol motif as a chemical handle for downstream reactions.

As noted, peptides comprise a unique class of pharmaceuticals, with more than 80 peptide drugs approved to treat a range of ailments including diabetes, cancer, HIV, and bacterial infections.[14] Though there are over 7,000 naturally occurring peptides that have been identified, these compounds are not often suitable as therapeutics due to their poor chemical and physical stability and low membrane permeability. However, the introduction of ncAAs has proven beneficial for tuning the three-dimension structure, functionality, and properties of drug scaffolds.[3a, 3e, 3f, 3j] We show that the 5-Hyl residue provides unique side-chain reactivity via the β-amino alcohol motif, where a simple hydroxyl group introduces differential reactivity as compared to native Lys residues. This ncAA therefore enables the selective formation of various heterocycles or ligation of different chemical functionalities, providing many opportunities to further tune the structure and properties of bioactive peptides through bioconjugation.

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NOTE: The authors declare no competing financial interest.

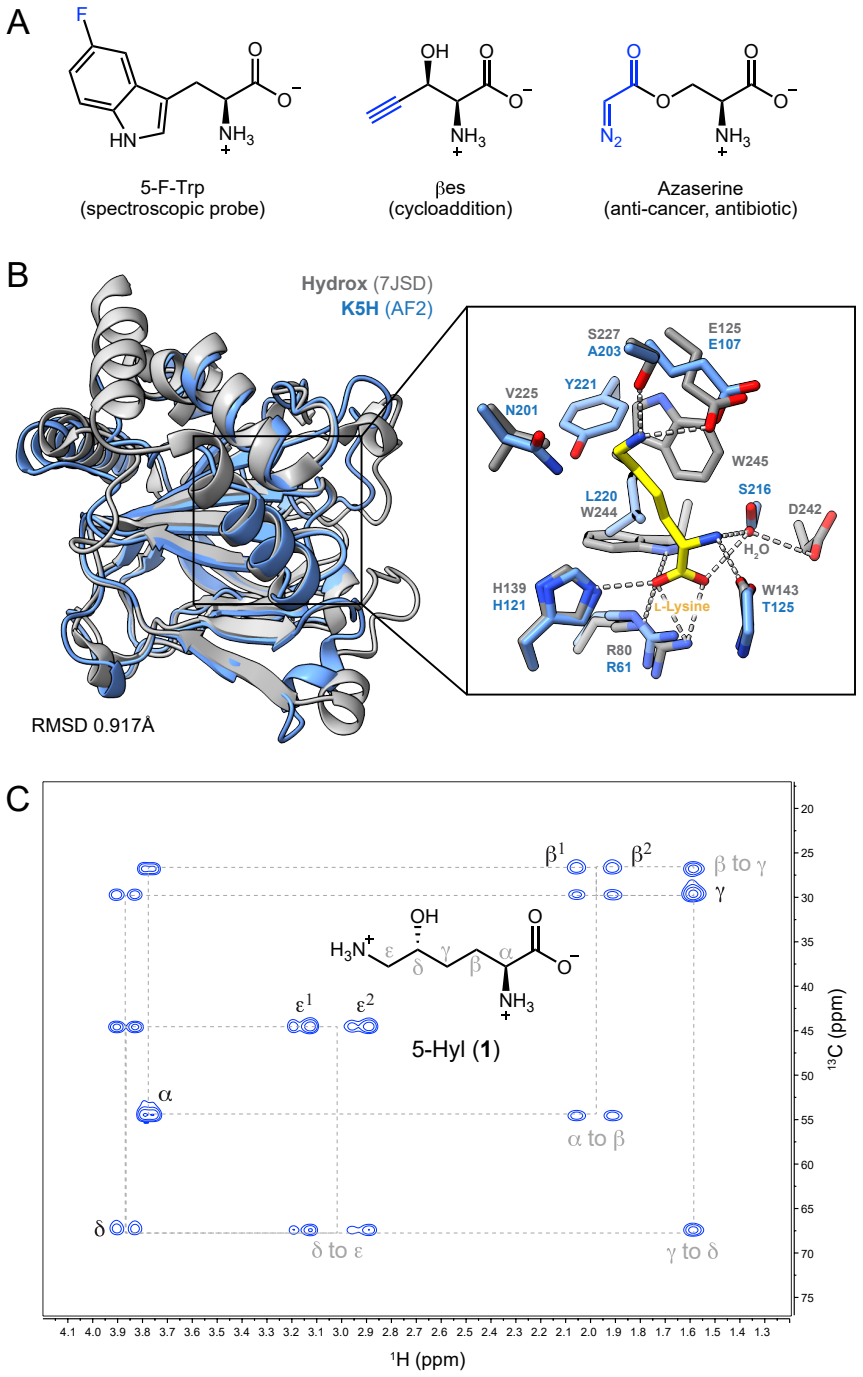

Figure 1. Noncanonical amino acids as valuable chiral building blocks, featuring 5-hydroxylysine produced from the newly discovered and characterized Fe/αKG-dependent enzyme, K5H. (A) Examples of naturally-produced and synthetic noncanonical amino acids. (B) Overlaid crystal structure of Hydrox (gray, PDB 7JSD) and AlphaFold 2 model of K5H (blue) reveals high overall structural similarity. Hydrox residues R80 and H139 involved in recognizing the substrate α-carboxylate are conserved in K5H, whereas a water in Hydrox that hydrogen bonds the α-amine may be substituted by residue S216 in K5H. (C) HCCH COSY (700 MHz, D_2O) of the purified product obtained from incubating K5H with $^{13}C_6$, $^{15}N_2$-L-lysine·2HCl.

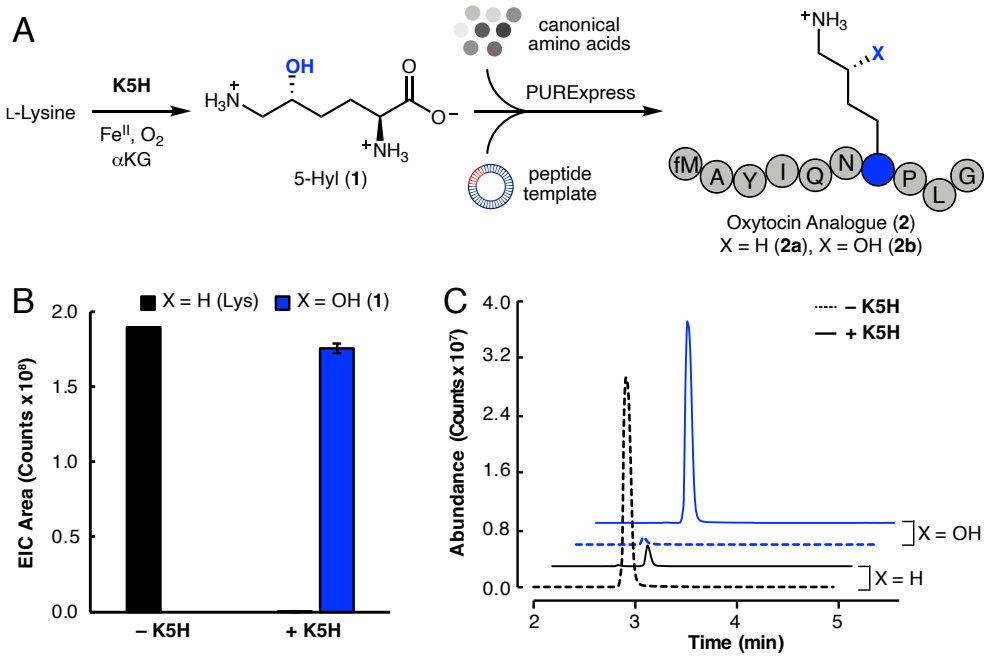

Figure 2. Biocatalytic cascade enables the synthesis of 5-Hyl-containing peptide **2b**. (A) Workflow for *in situ* biocatalytic production of 5-Hyl followed by *in vitro* transcription/translation (IVTT) to access an analogue of the peptide hormone oxytocin. (B) LC-MS analysis of Lys (black, m/z = 147.1128) and 5-Hyl (**1**; blue, m/z = 163.1077) produced by K5H. The reaction was allowed to proceed for 24 h at room temperature before being filtered through a 10-kDa MWCO spin column to remove the protein for LC-MS analysis. The average of three technical replicates is shown. (C) Extracted ion chromatograms of peptides obtained from IVTT including (solid line) or excluding (dashed line) K5H from the initial biocatalysis step to produce Lys- (**2a**; black, m/z = 1162.5926) or 5-Hyl-containing peptide (**2b**; blue, m/z = 1178.5874).

Table 1. Biologically active peptides synthesized via biocatalytic cascade using K5H to produce 5-Hyl in situ followed by IVTT. All Lys residues (bold) were successfully replaced with 5-Hyl. 5-Hyl incorporation was calculated by dividing the extracted ion counts (EIC) for 5-Hyl-containing peptides by that of the total peptides (Lys- and 5-Hyl-peptides) produced when K5H is added to the reaction mixture prior to IVTT.

Entry	Sequence[a]	Biological Activity	5-Hyl Incorporation (%)
1	AYIQN**K**PLG	Oxytocin analogue; hormone	97
2[b]	CR**K**RLDRNC	Anticancer[22]	95
3	GL**K**AGVIAV	Anticancer[23]	93
4[b]	**K**CCYSL	Anticancer[22]	97
5[c]	PLYEN**K**PRRPYIL	Neurotensin; neuropeptide[24]	89
6[c]	PA**K**SNGGSN	Serum thymic factor; hormone[25]	86
7	YGGFLR**K**YP**K**	α-Neoendorphin; opioid[26]	>99
8	SGLD**K**DYL**K**PDD	Anticancer[27]	98

[a] Peptides synthesized via IVTT begin with formylated methionine (fM). [b] Quenched with DTT (25 mM) to reduce cysteine disulfide bonds. [c] The native peptide sequence contains an *N*-terminal pyroglutamic acid residue; however, proline was used as a surrogate for IVTT.

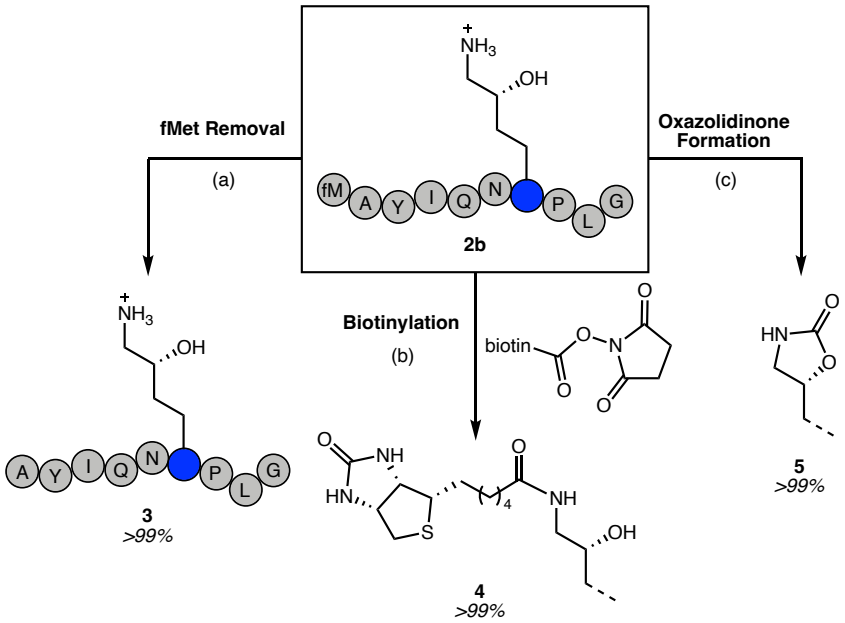

Figure 3. Examples of downstream chemical functionalizations available to 5-Hyl-containing peptides obtained via IVTT. Reaction conditions: (a) CNBr, 88% formic acid, MeCN/H_2O, rt, dark, 12 h. (b) Sodium phosphate buffer pH 7.5, 37°C, 2 h. (c) DSC, DMAP, DIEA, DMF, 37°C, 12 h. Percentages depicted indicate consumption of starting material as determined by LC-MS. See Supporting Information for additional reaction details.

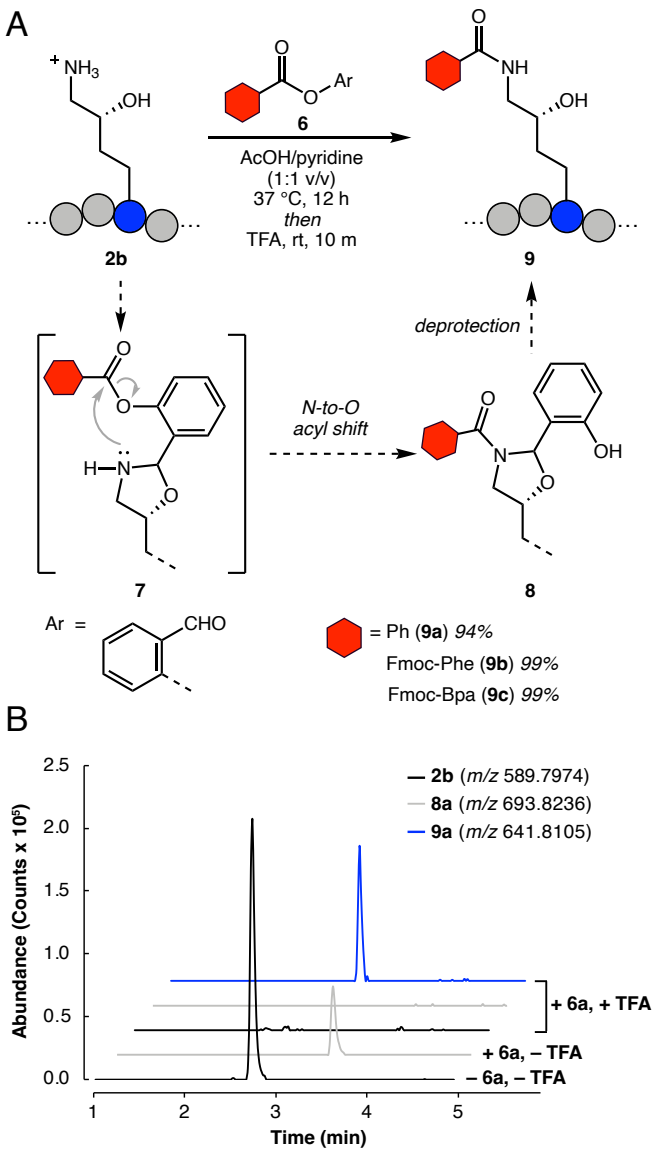

Figure 4. Chemoselective chemical ligation of 5-Hyl-containing peptides. (A) Using the β-amino alcohol motif, *O*-salicylaldehyde esters **6** couple with 5-Hyl-peptide **2b**, proceeding through an *N*,*O*-benzylidene acetal intermediates **7** and **8**, which can be cleaved with acid to afford side-chain modified peptides **9**. Percentages depicted indicate consumption of starting material as determined by LC-MS. (B) Extracted ion chromatograms of the unmodified peptide (**2b**, black), acetal intermediate (**8a**, gray), and benzoylated peptide (**9a**, blue) throughout the reaction. See Supporting Information for additional reaction details.

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