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KAHA Ligations that form Aspartyl Aldehyde Residues as Synthetic Handles for Protein Modification and Purification

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Supporting Information Placeholder

ABSTRACT: Aldehydes are widely recognized as valuable synthetic handles for the chemoselective manipulation of peptides and proteins. In this report, we show that peptides and small proteins containing the aspartic acid semi-aldehyde (Asa) side-chain can be easily prepared by a chemoselective amide-forming ligation that results in the formation of the Asa residue at the ligation site. This strategy employs the α-keto-acid–hydroxylamine (KAHA) ligation in combination with a new isoxazolidine monomer that forms a side-chain aldehyde upon ligation. This monomer is easily prepared on a preparative scale by a catalytic, enantioselective approach and is readily introduced onto the *N*-terminus of a peptide segment by solid phase peptide synthesis. The ligated product can be further functionalized by bioorthogonal reactions between the aldehyde residue and alkoxyamines or hydrazides. We demonstrated that glucagon aldehyde, an unprotected 29-mer peptide prepared by KAHA ligation, can be site-specifically and chemoselectively modified with biotin, dyes, aliphatic oximes and hydroxylamines. We further describe a simple and high recovery one-step purification process based on the capture of a 29-mer glucagon aldehyde and a 76-mer ubiquitin aldehyde by an alkoxyamine-functionalized polyethyleneglycol resin. The peptide or protein was released from the resin by addition of a hydroxylamine to provide the corresponding oximes.

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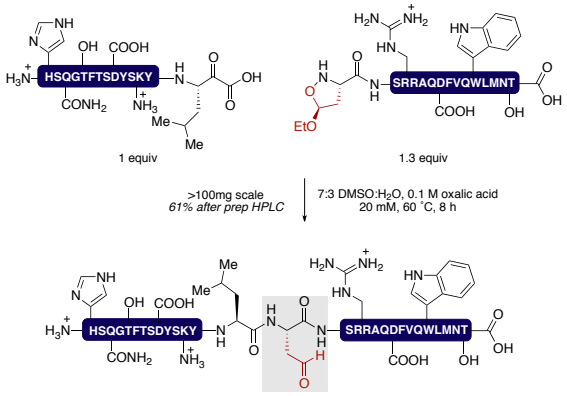
INTRODUCTION

Aldehydes present in biological molecules including peptides, proteins, and carbohydrates provide a convenient handle for site-specific conjugation.[1,2] By undergoing rapid, chemoselective reactions with a variety of functional groups, especially hydroxylamines and hydrazines, aldehydes are ideal for the attachment of dyes and tags.[3,4] Their unique chemical reactivity and ability to undergo reversible reactions also makes them well-suited for affinity chromatography and catch-and-release purification processes. Due to their high reactivity, however, aldehydes must be introduced at a late stage of a chemical synthesis by post-assembly chemical modification, either by a specific chemical reaction or by unmasking of a protected form.[5,6,7,8,9] Several studies have been devoted to the synthesis of peptide aldehydes in solid phase peptide synthesis (SPPS),[10] or to the use of *N*-terminal peptide aldehydes.[11,12,13] Bertozzi has exploited the formylglycine-generating enzyme (FGE) consensus as a genetically encoded aldehyde tag for site- specific protein modification.[14] The enzyme oxidizes a cysteine residue within a ~13 amino acid consensus sequence to form an aldehyde-bearing formylglycine residue.

In this report, we introduce a new isoxazolidine monomer for the α-ketoacid–hydroxylamine (KAHA) amide forming ligation that reveals an aspartic semi-aldehyde side-chain upon reaction of two unprotected peptide segments. The ligation product possesses a single aldehyde functional group that can be readily modified by standard oxime or hydrazone formation, transformed to hydroxylamines for subsequent KAHA ligations, or used for catch-and-release purification of the ligation products from the reaction mixtures without preparative HPLC.

The basis for this work is our recent identification of 5-oxaproline as a chemically stable, yet highly reactive partner for the KAHA ligation of large, unprotected peptide segments.[15] Protein synthesis by KAHA ligation with 5-oxaproline offers the advantages of easily prepared starting materials and ligations under acidic aqueous conditions that are well-suited for solubilizing peptide segments. Its primary disadvantage is the formation of a non-canonical homoserine residue at the ligation site. This is normally an innocuous mutation of most amino acids in terms of tertiary structure and biochemical activity. The use of the 5-oxaproline residue also leads to the formation of esters as the initial ligation products; base induced rearrangements form the native peptide bond.[16]

Scheme 1. Ketoacid–hydroxylamine ligation to form the peptide aspartic semi-aldehyde (Asa) residues



As part of ongoing efforts to design new stable hydroxylamines that afford other unnatural or natural residues at the ligation site, we considered the possibility of using KAHA ligation to reveal an aldehyde upon N–O bond cleavage. In the context of the synthesis of short β[3]-peptides, we have described isoxazolidine monomers that form ketones upon ligation,[17] but it was not clear if a corresponding design for an aldehyde-forming substrate would be sufficiently stable to peptide cleavage, HPLC purification and ligation. Furthermore, the increased steric hindrance could interfere with the ligation of longer peptides. As documented below, these fears proved unfounded and a suitable monomer could be readily prepared, incorporated into peptide segments, and used for KAHA ligation of unprotected segments to give peptides bearing a single aspartic acid semi-aldehyde residue (Asa) at the ligation site.

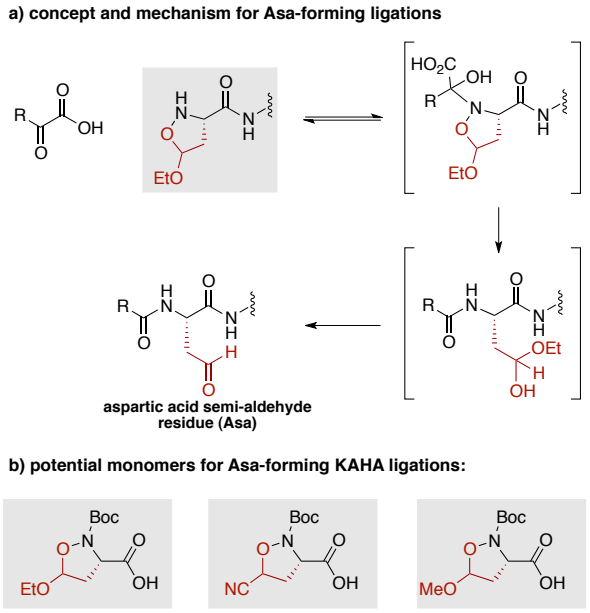


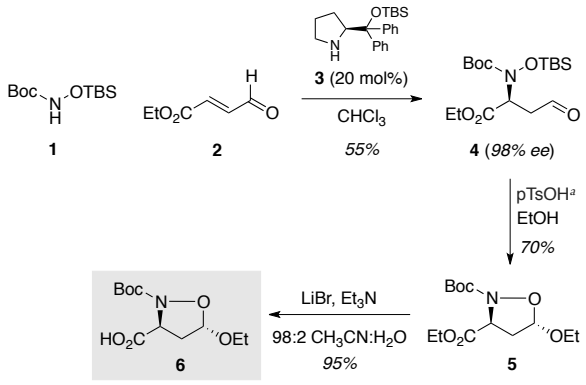
Figure 1. Monomer design

Previous work on *(S)*-aspartic semi-aldehyde residues[18] was based on the introduction of a masked aldehyde linker,[19] or the incorporation of a Weinreb amide as a precursor of an aldehyde on the side-chain of aspartyl residues.[20] There has also been evidence of Asa in natural proteins arising from post-translational enzymatic reduction of aspartic acid.[21] However, to the best of our knowledge, there is no viable approach to the incorporation of Asa into longer peptides or proteins by either synthetic or enzymatic methods. Our study describes the first method for the late stage introduction of the aldehyde function in the γ-position (Asa), eliminating the risk of epimerization.

RESULTS AND DISCUSSION

Monomer synthesis. Substituted isoxazolidine **6** was readily prepared in an enantiomerically pure form through a short, optimized sequence. Based on the work of MacMillan[22] and Cordova[23] on organocatalytic asymmetric conjugate additions to α,β-unsaturated aldehydes, we established an operationally simple route to the synthesis of the enantioenriched isoxazolidine **6** (Scheme 2) on a gram scale. An alternative route using an L-gulose derived chiral auxiliary for nitrone cyclization with ethyl vinyl ether was also feasible.[24,25]

Scheme 2. Synthesis of the enantiopure *(S)*-isoxazolidine



[a]pTsOH = *para*-toluenesulfonic acid

The key step in this route was the intramolecular acetalization of aldehyde **4** in acidic ethanol to give cyclic adduct **5**. Although the reaction was complete in several hours, some Boc deprotection occurred and the reaction progress needed to be monitored carefully. Ester hydrolysis of **5** with triethylamine and lithium bromide provided *(S)*-isoxazolidine **6**, ready for use in SPPS. The relative and absolute configuration of **6** was assigned by X-ray crystallographic analysis of an amide derivative (Figure 2). No epimerization was observed in the cyclization or the hydrolysis step (See Supporting Information for epimerization study).

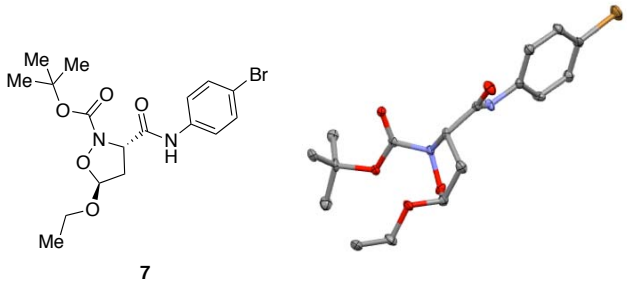
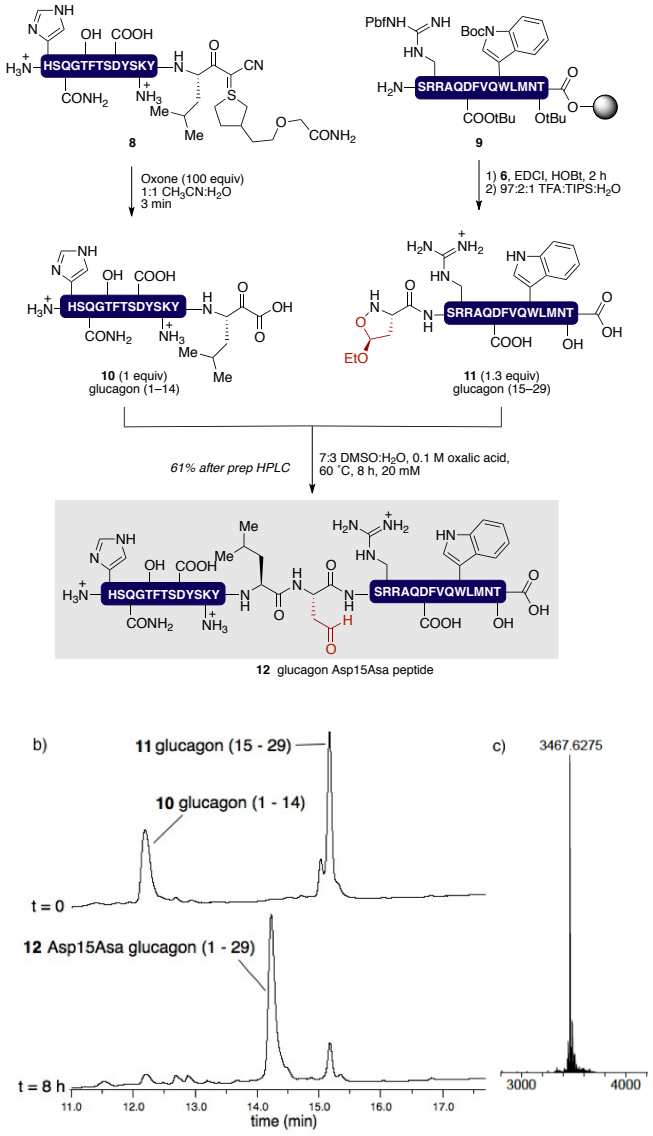


Figure 2. X-Ray crystallographic analysis of **7**

Solid Phase Peptide Synthesis and Asa-Forming Ligations. In order to demonstrate the utility of this monomer for peptide aldehyde formation, we applied it to the chemical synthesis of glucagon, a 29 amino acid peptide hormone. We chose Leu (residue 14) and Asp (residue 15) as the ligation site. This disconnection required two peptide segments of around 15 residues each. In the ligated peptide, Asp (residue 15) would be mutated to aspartic semi-aldehyde (Asp15Asa).

The isoxazolidine segment of glucagon (15–29) **11** was prepared on a resin bearing a Wang linker by automated Fmoc SPPS, followed by manual coupling of the isoxazolidine monomer **6** onto protected peptide **9**. Side-chain deprotection and cleavage of the peptide from the resin and purification by HPLC proceeded without difficulty to yield glucagon (15–29) **11** (Scheme 3). The glucagon (1–14)-α-ketoacid segment **10** was synthesized in good yield by using one of our established procedures for the Fmoc SPPS of peptide α-ketoacids.[26]

Scheme 3. Preparation of glucagon aldehyde by Asa-forming KAHA ligation.



a) Synthesis of glucagon α-ketoacid (1–14) **10** and glucagon (15–29) **11** and their ligation to form glucagon Asp15Asa peptide. b) Analytical RP-HPLC traces of the glucagon aldehyde forming ligation. c) HRMS of glucagon aldehyde **12**.

With the two peptide segments in hand, we proceeded with milligram scale reactions using the standard ligation conditions established with the 5-oxaproline substrates. No deviation from our standard conditions for KAHA ligation[15] was necessary and the reactions proceeded cleanly to give a single ligation product. Purification of the ligation mixture by preparative HPLC afforded glucagon Asp15Asa **12** in 61% yield. This demonstrates that the substituted isoxazolidine monomer is stable towards the ligation conditions, tolerates acidic deprotection and cleavage conditions, and that both the starting peptide segment and the Asa-containing ligation products, can be purified by preparative HPLC. The rate of the ligation was comparable to an experiment performed using the glucagon segment bearing a 5-oxaproline residue. In contrast to 5-oxaproline, we observed only the expected amide products; no esters or other products arising from trapping of the putative nitrilium intermediate were observed.[16]

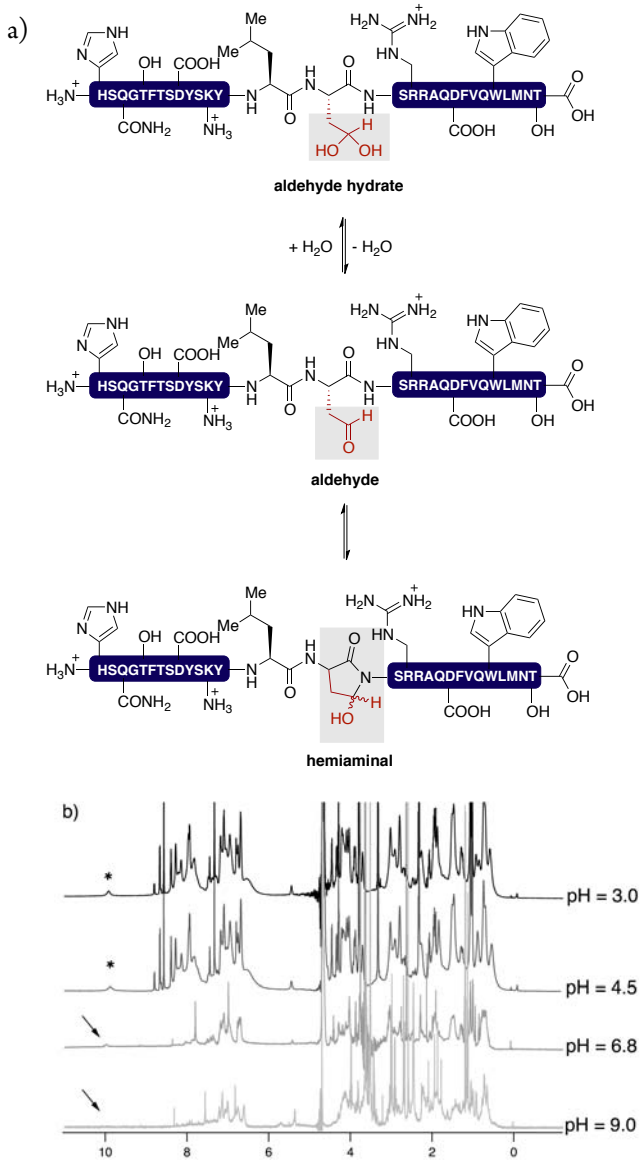


Figure 3. a) pH dependent hydration and hemiaminal formation of a peptide containing aspartic semi-aldehyde side-chain. b) ^{1}H NMR spectra (600 MHz with presaturation, 300 K, KH_2PO_4/H_3PO_4 buffer, H_2O/D_2O 95:5 for pH 3 and 4.5. The pH was raised by addition of solid Na_2CO_3). The asterisks * mark the aldehyde peak resulting from the linear aldehyde glucagon-CHO. The measurement at acidic pH showed the presence of the aldehyde peak along with the hydrate form. Subsequent raising of the pH to 6.6 and 9.0 showed almost no aldehyde signal left (marked by black arrow).

Characterization of the Asa residue. High-resolution mass spectrometry (MALDI-HRMS) measurement confirmed the exact mass of the glucagon peptide with the Asp15Asa mutation. It appears likely that *(S)*-Asa residue exists primarily as the hydrate in solution.[27] Reversible cyclization of peptide aldehydes into hemiaminals as a pH-dependant equilibrium has already been documented and studied by ^{1}H NMR by Geyer.[28] The work of Omura[29] also described the formation of a five-membered ring hemiaminal by the side-chain of an L-aspartic semi-aldehyde with the backbone amide.

To establish whether or not the Asa side-chain in our synthetic glucagon was present in the aldehyde form or as a hemiaminal with a backbone amide bond or another side-chain functional group, we performed NMR studies over a range of pH values between 3 and 9. These measurements showed that a significant proportion of the hydrate form of the aldehyde was present under acidic conditions. Under basic conditions, the hemiaminal was predominant (Figure 3). These ^{1}H NMR studies are consistent with the observations by Geyer.[28]

Chemoselective conjugations to the Asa side-chain. With the aldehyde group installed, we evaluated chemoselective conjugations with aminooxy- or hydrazide-functionalized molecules for site-specific modification. Reaction partners included fluorescent imaging probe BODIPY-hydroxylamine, affinity probes such as biotin hydrazide, and conjugations with aliphatic hydroxylamines (Table 1). The conjugation reactions with aminoxy-functionalized molecules were carried out in aqueous buffers under slightly acidic pH, as formation of oximes suffers from slow reaction kinetics at a pH > 6.[30] Benzyl derived hydroxylamine proved to be more reactive than hydroxylamine or *O*-methylhydroxylamine (Table 1, entry 1,5). The use of aniline based catalysts[31] (aniline, *m*-phenylene diamine, 3,5-diaminobenzoic acid) only slightly improved the outcome in our case. BODIPY-hydroxylamine was successfully conjugated with glucagon Asp15Asa in 12 hours at 37 °C, affording fluorescent product **14** in good yield.[4] The fluorescence spectra of **14** with excitation and emission wavelengths is shown in Figure 4.

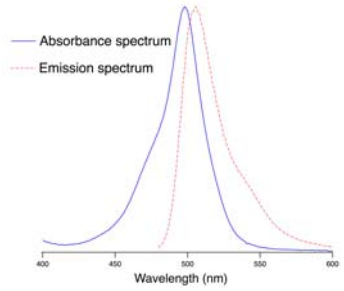


Figure 4. Absorbance and excitation spectrum of BODIPY-dye-functionalized peptide **14**

We also incorporated an affinity tag by the use of biotin hydrazide. The reaction is known to proceed slowly at acidic pH due to protonation of the hydrazide moiety, but to provide the hydrazone in good yield at pH > 6.[32] Hydrazide reagents are known to have modest reaction kinetics because of the presence of electron-withdrawing groups adjacent to the nucleophilic nitrogen moiety, accounting for the moderate yield of our conjugation reaction (Table 1, entry 2).

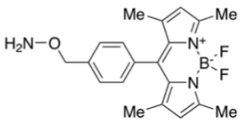
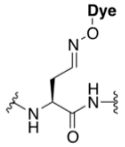
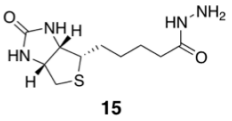
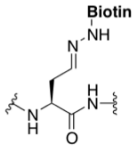
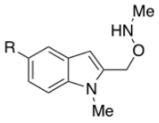
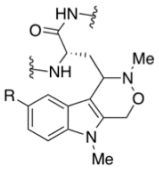
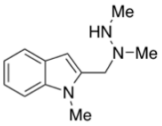
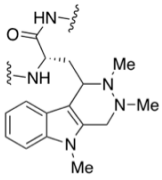
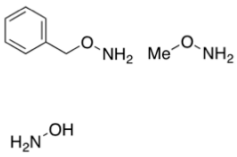
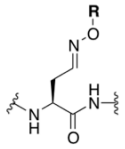
We also sought to generate hydrolytically stable reaction products based on the recently reported Pictet-Spengler ligation with aminoxy nucleophiles.[33] We designed two reaction partners for this transformation, aminoxy-functionalized indoles **17** and **18**. We found that they reacted smoothly with glucagon aldehyde to yield the stable conjugates at a slightly acidic pH in high yield (Table 1, entry 3) when a ten-fold excess of reagent was employed. With this result in hand, we aimed for generating a stable product near neutral pH, by using the hydrazino-Pictet-Spengler (HIPS) ligation.[34] Hydrazine-Pictet-Spengler products are known to exhibit a good stability and reactivity near neutral pH compared to the oxime-linked conjugate. We prepared indole **21** that generated a hydrolytically stable reaction product upon ligation with glucagon Asp15Asa **12** (Table 1, entry 4).

Finally, we also used simple hydroxylamines to form oximes (Table 1, entry 5), which were easily prepared and stable to HPLC analysis and purification.

Conversion of aldehydes to hydroxylamines and further ligation. The oxime products prepared from glucagon aldehyde **12** and the simple hydroxylamines shown in Table 1, entry 5 could be easily reduced to the corresponding hydroxylamines with $NaCNBH_3$. The hydroxylamine side-chain was suitable for KAHA ligations with α-ketoacids (Scheme 4) in good yield, provided an excess of the ketoacid was employed.[35] We also found that the more stable, easily handled *O*-Me alkoxyamine could be ligated with *N*-methyliminodiacetyl (MIDA) acyl boronates (Scheme 4). [36]

Although only simple α-ketoacids and MIDA acyl boronates were employed in this study, the high reactivity and chemoselectivity of these ligations offer a promising route to the late-stage functionalization of unprotected peptides to form stable, biocompatible amide bonds.[37] Given the known instability of oximes and hydrazines, this two-step approach may prove to be better suited for many peptide modifications.

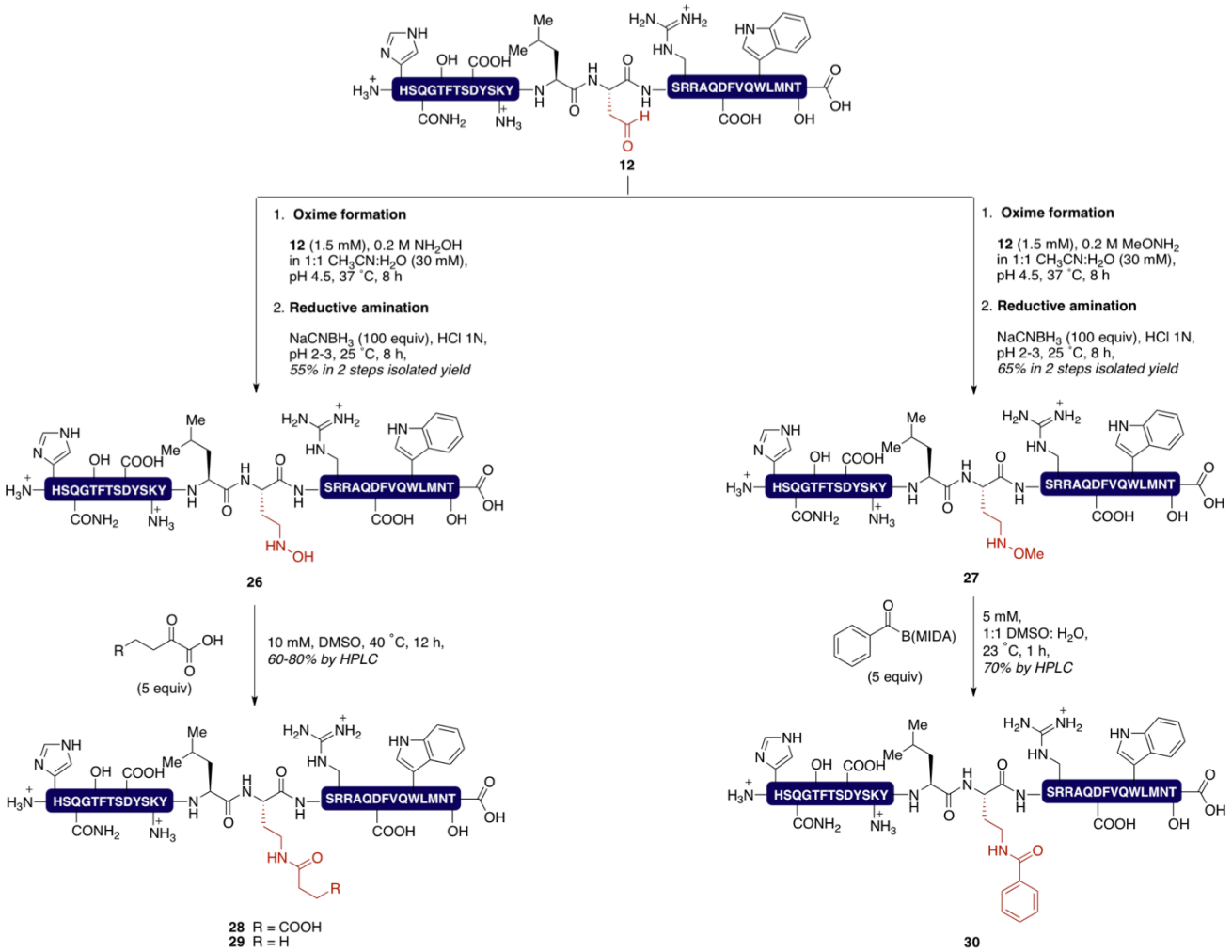
Table 1. Examples of aldehyde conjugations and conditions[a]

	Aldehyde modifier	Structure	Product	Reagent concentration	Conditions
1	BODIPY hydroxylamine dye	 **13**	 **14**	50 mM	**12** (5 mM), 5:1 sodium acetate buffer:CH_3CN, pH 4.5, 37 °C, 6 h 78% after prep HPLC (2.6 mg product obtained)
2	Biotin hydrazide	 **15**	 **16**	50 mM	**12** (2.5 mM), 5:1 sodium phosphate buffer:CH_3CN, pH 6.6, 40 °C, 8 h 47% after prep HPLC (0.5 mg product obtained)
3	Aminoxy-indole	 **17** R = H **18** R = OMe	 **19** R = H **20** R = OMe	25 mM	**12** (2.5 mM), 5:1 sodium acetate buffer:CH_3CN, pH 4.5, 40 °C, 6 h 70% after prep HPLC (~1 mg of each product obtained)
4	Hydrazino-indole	 **21**	 **22**	25 mM	**12** (2.5 mM), 5:1 citric acid-sodium dihydrogen phosphate buffer:CH_3CN, pH 6, 40 °C, 6 h 70% after prep HPLC (~0.8 mg of product obtained)
5	Other hydroxylamines		 **23** R = Bn **24** R = Me **25** R = H	30-50 mM	**12** (1.5–2.5 mM), 5:1 sodium acetate buffer:CH_3CN, pH 4.5, 37 °C, 6–14 h 45–77% after prep HPLC (1–2 mg of products obtained)

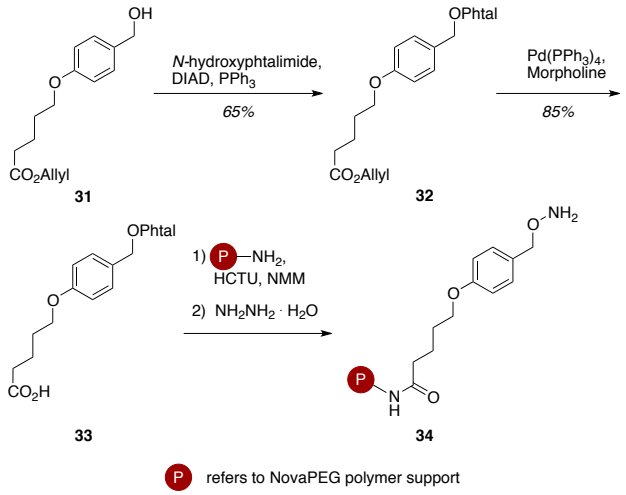
[a] Conjugation experiments were conducted on 1–3 mg of glucagon Asp15Asa **12**. Yields refer to isolated yields of pure product following preparative HPLC.

Catch and release purification of ligation products. Reversible, covalent reactions of aldehydes also makes possible affinity based purification strategies. A key advantage of the Asa-forming ligations is the ability to easily separate the ligation products from any unreacted starting materials by affinity purification. The aspartic semi-aldehyde residue provides a perfect handle for immobilization with hydroxylamines or other covalent capture methods. We designed a solid supported alkoxyamine that would selectively "catch" the peptide aldehyde and would allow removal of the impurities, side-products and unreacted starting materials. To achieve this we required a resin compatible with organic solvents, but which also possessed excellent swelling properties in an aqueous environment under which the unprotected peptide would be handled. In order to "release" the trapped peptide, a small aminoxy group would be used to exchange the bound oxime for a soluble one.

Scheme 4. Chemoselective reactions for hydroxylamine side-chain



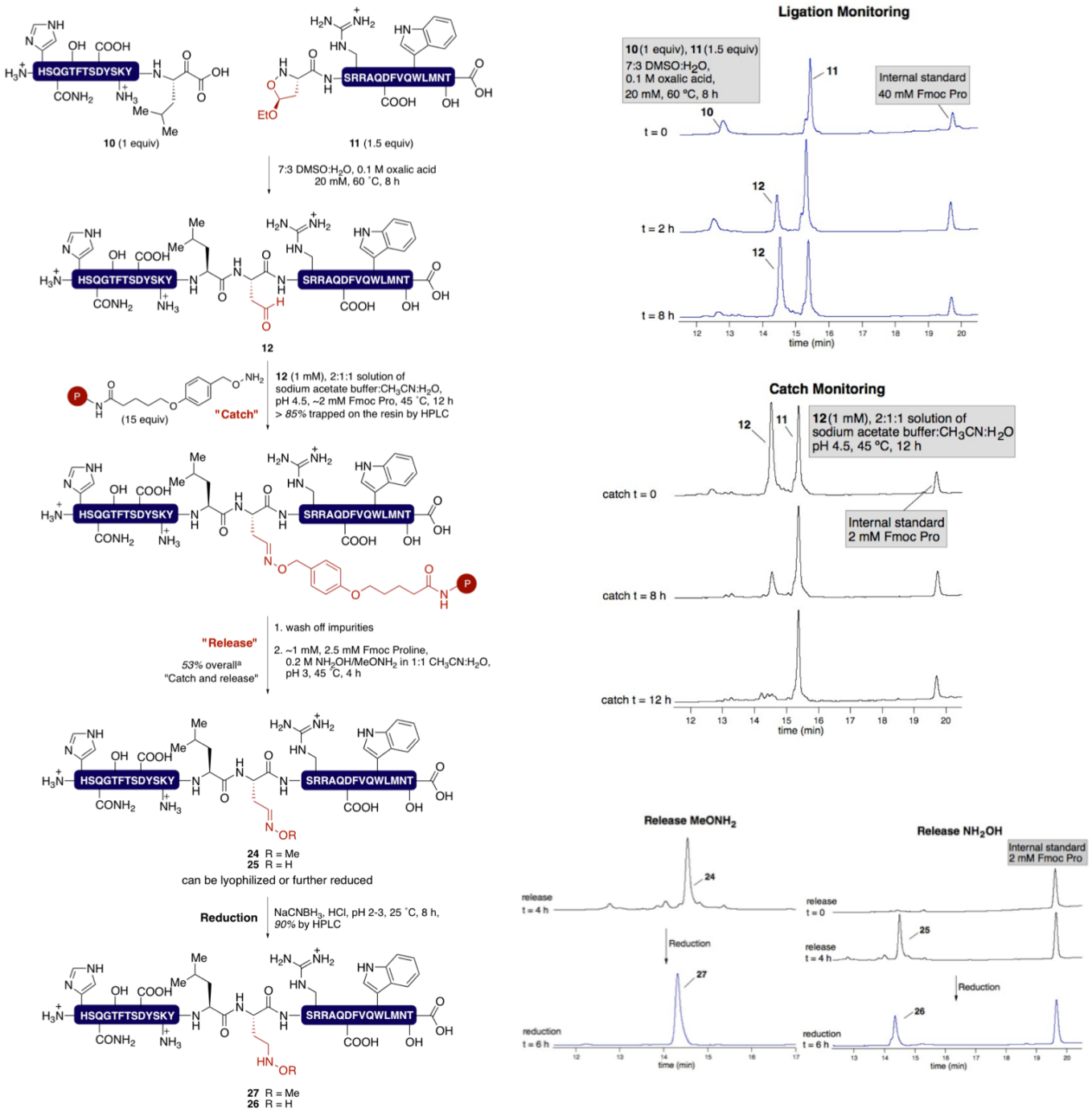
Scheme 5. Preparation of the hydroxylamine "Catch" resin



We began by evaluating resins compatible with a mixture of H_2O/CH_3CN. Initially, we tested amino PEGA resin, but found it was difficult to handle the wet beads. The use of an amino NovaPEG resin improved the outcome due to its superior mechanical properties; the resin beads are free flowing in the dry state. We synthesized the Wang-type linker[38] **33** via a Mitsunobu[39] reaction of alcohol **31** and *N*-hydroxyphtalimide, followed by allyl deprotection (Scheme 5). Loading **33** onto NovaPEG resin via amide coupling, followed by hydrazinolysis gave stable, storable resin **34**.

To demonstrate the efficiency and utility of the catch-and-release strategy,[40] we examined its application to the synthesis of glucagon aldehyde from the two corresponding peptide segments. For this purpose, we performed a ligation between the two peptide segments of glucagon and, after aldehyde product formation, incubated the ligation mixture with the resin under gentle shaking at 45 °C (Scheme 6). We were pleased to see that the hydroxylamine resin quantitatively and selectively trapped the glucagon aldehyde over 12 hours at slightly acidic pH. Small amounts of decomposition products or unreacted peptide aldehyde were observed, but all traces were removed by washing the resin containing the bound peptide aldehyde. The peptide was released from the resin by treatment with a solution of *O*-

Scheme 6. Monitoring of the catch-and-release purification of Asp15Asa glucagon peptide



methylhydroxylamine or hydroxylamine at pH 3 for 4 hours to afford, after filtration, oximes **24** or **25**. Additionally, the peptide could be recovered as oxime **25** by cleavage with a standard TFA:TIPS:H_2O cocktail. However, after this treatment the hydroxylamine resin cannot be recovered or reused.

The solution of the released peptide can either be lyophilized to afford the corresponding oximes without further purification or reduced by $NaCNBH_3$ in the same pot. Reduction was achieved in several hours, providing the desired hydroxylamine peptides in high purity with a good overall yield after preparative HPLC (Scheme 6). It is noteworthy that the freshly generated hydroxylamines can be further used in chemoselective reactions, such as those shown in Scheme 4.

To further demonstrate the selectivity and applicability to otherwise extremely challenging purification tasks, we mixed glucagon (1-29) acid (no Asa modification, no mutation) **35** with glucagon Asp15Asa **12**. Using the same conditions as previously stated, we incubated this mixture for 8 h together with the hydroxylamine resin and evaluated the specificity of our purification method. As expected, only glucagon aldehyde was captured on the resin, while the second peptide remained in solution. The degree of purification achieved by this method is

significant (Figure 5), as from our knowledge mixtures of this type are nearly impossible to separate by HPLC.

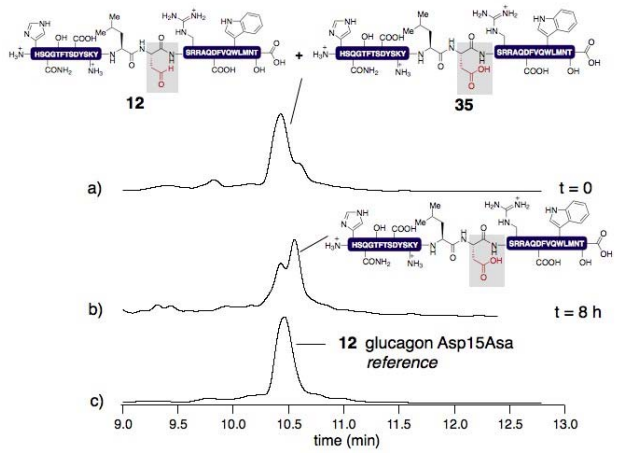


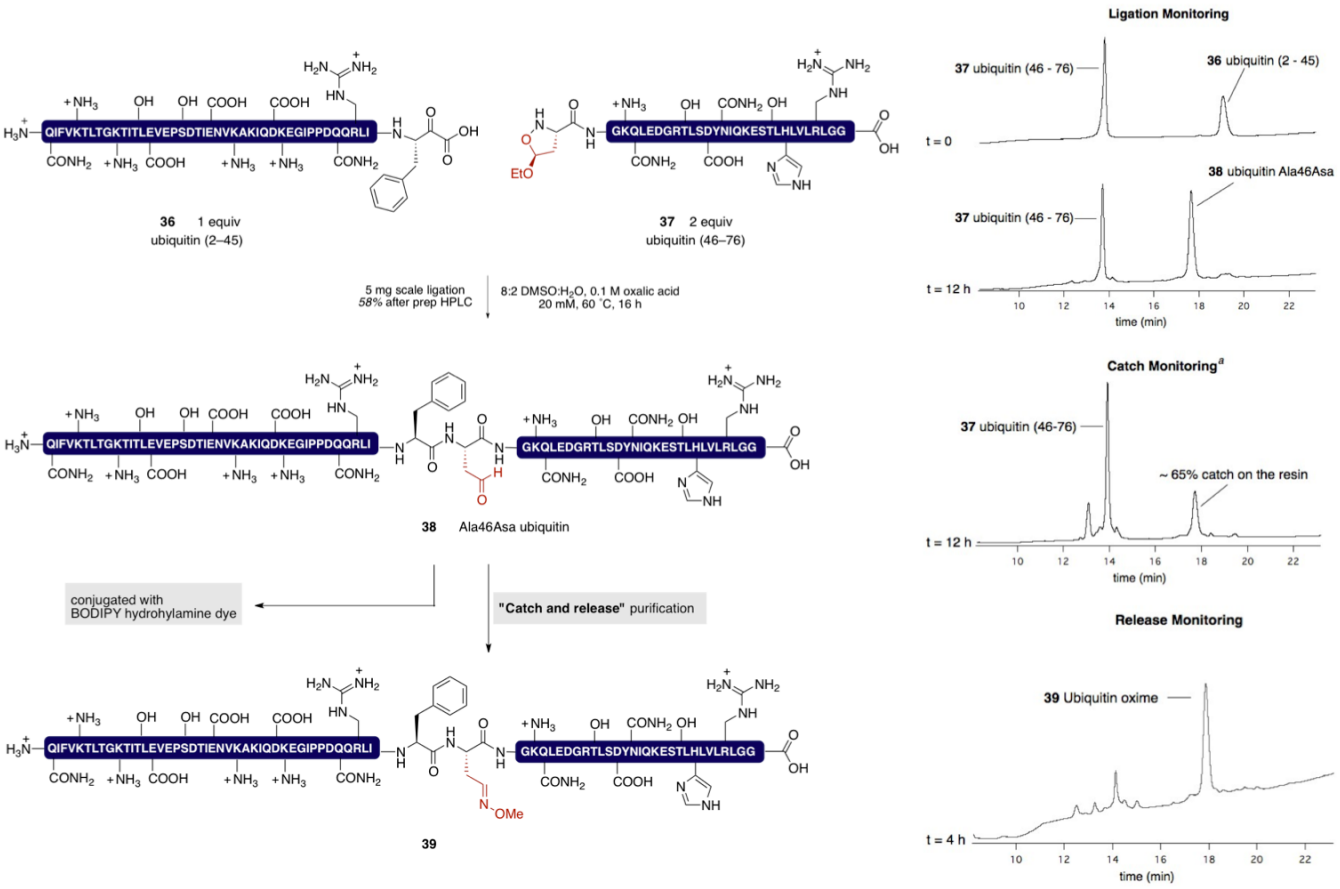
Figure 5. Analytical RP-HPLC traces at 220 nm of the supernatant of mixed glucagon aldehyde and acid at different stages within the same HPLC monitoring conditions. (a) Initial conditions prior to capture; (b) 8 h capture; (c) reference peak of aldehyde.

Synthesis and catch-and-release purification of ubiquitin Ala46Asa. The Asa-forming ligations are also suitable for improving synthetic access and purification of protein targets. We successfully incorporated the aldehyde functional group into ubiquitin[41] Ala46Asa. We chose a ligation site between Phe (residue 45) and Ala (residue 46, mutated to Asa).

The isoxazolidine segment of ubiquitin (46–76) **37** was prepared by automated Fmoc SPPS followed by manual coupling of the isoxazolidine monomer **6**. Side-chain deprotection, cleavage from resin and purification provided ubiquitin (46–76) **37** (Scheme 7). The ubiquitin (2–45)-α-ketoacid segment **36** was synthesized in a good yield by using a linker for preparing α-ketoacids.[42] With the two peptide segments in hand, the milligram scale ligation proceeded cleanly to the desired product **38**. Purification of the ligation mixture by preparative HPLC afforded ubiquitin Ala46Asa in 58% yield. This demonstrated that our isoxazolidine monomer could be applied to a small protein and the aspartic semi-aldehyde residue could be introduced successfully into ubiquitin.

We evaluated the efficiency of the catch-and-release purification strategy for ubiquitin Ala46Asa (Scheme 7). The hydroxylamine resin captured 65% of ubiquitin aldehyde when the resin loading was 0.3 mmol/g. However, by decreasing the loading of the hydroxylamine resin to ~0.1 mmol/g, the catch efficiency increased (see Suporting Information for analytical

Scheme 7. Synthesis and Catch-and-Release Purification of ubiquitin Ala46Asa



[a]Catch monitoring of ubiquitin Ala46Asa is only 65% if using a resin loading of 0.3 mmol/g. When using a resin loading of ~0.1 mmol/g, the catch efficiency increased (see Supporting Information)

HPLC traces when using 0.1 mmol/g resin loading). The release with *O*-methylhydroxylamine proceeded well, and the supernatant was lyophilized to afford the ubiquitin protein in one chemical ligation, without any HPLC purification.

Finally, we successfully conjugated ubiquitin Ala46Asa with BODIPY hydroxylamine dye following the same reaction conditions as Table 1, entry 1 to give the desired product in 50% isolated yield (see Supporting Information for experimental details).

CONCLUSION

In summary, the facile generation of peptide aldehydes was made possible by a chiral isoxazolidine monomer that undergoes KAHA ligation with *C*-terminal peptide α-ketoacids to form the aspartic semi-aldehyde (Asa) residue at the ligation site. We have succeeded in installing an aldehyde motif[43] into a therapeutically relevant peptide and a small protein by KAHA ligation with easily prepared segments. We successfully showed that we could introduce site-selective modifications with a variety of modifiers and hope that this new route to aldehyde-containing peptides complement biochemical techniques to incorporate synthetic handles into peptides. The ability to prepare a panel of site-specific modifications of proteins will be useful in the preparation of a wide array of post-translational modified proteins,[44] or in the formation of peptide-conjugated dendrimers for drug delivery applications.[45] In addition, we developed a simple purification method for the aldehyde peptide through a catch-and-release strategy. This method is inexpensive, effective and could be highly attractive due to the facile elimination of impurities.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures and spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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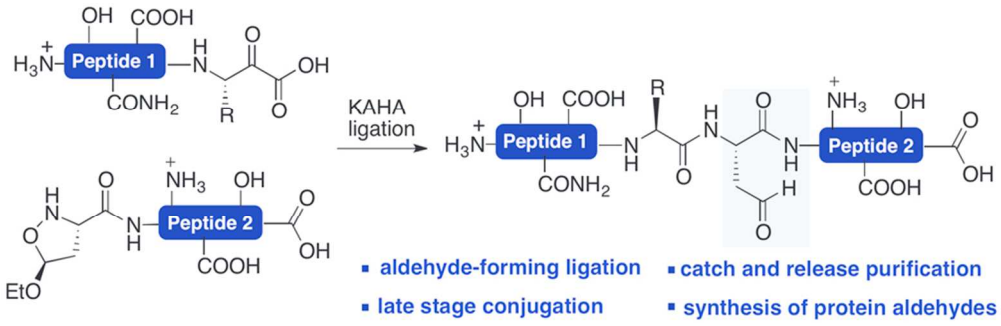

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