Tyrosine-Selective Bioconjugation with Iminoxyl Radicals

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Abstract

A novel Tyr-selective protein bioconjugation using the water-soluble persistent iminoxyl radical is described. The conjugation proceeded with high Tyr-selectivity and short reaction time under biocompatible conditions (room temperature in buffered media under air). The stability of the conjugates was tunable depending on the steric hindrance of iminoxyl. The presence of sodium ascorbate and/or light irradiation promoted traceless deconjugation, restoring the native Tyr structure. The method is applied to the synthesis of a protein-dye conjugate and further derivatization to azobenzene-modified peptides.

Main Text

Engineering protein structures to impart artificial functions to proteins is widely explored, advancing various research fields such as organic synthesis using chemically-modified enzymes, biocompatible materials, and diagnostics and therapeutics.[1] Bioconjugation reactions targeting proteinogenic amino acids[2] can straightforwardly access to engineered proteins from native proteins without relying on genetic manipulation/expansion. In recent years, bioconjugation reactions targeting low-reactive proteinogenic amino acids (e.g. Tyr[3] , Trp[4], Met[5]) have been developed, complementing the methods targeting non-proteinogenic amino acids containing biorthogonal functional groups, which are introduced by genetic expansion, or highly reactive proteinogenic amino acids, such as Lys and Cys, which exist comprehensively on the protein surface. A plausible advantage of bioconjugation targeting low-reactive proteinogenic amino acids is high homogeneity of the products without costly genetic manipulations[6].

Meanwhile, dissociative bioorthogonal reactions, the reverse processes of bioorthogonal conjugation reactions, promise to expand repertoires of bioorthogonal chemistry.[7] Dissociative bioorthogonal reactions have been applied to conditional/spatiotemporal regulation of biomolecules[8], pull down study of medicinal targets[9], and drug delivery[10]. Proteinogenic amino acid-targeting dissociative reactions of Cys/Lys/Ser side chains are utilized for targeted covalent inhibitors[11].

Tyrosine (Tyr) is a typical low-reactive proteinogenic amino acid of moderate natural abundance in primary protein sequences (3-4 %)[12] and surface exposure[13]. Thus, Tyr is an attractive target residue for realizing site-selective bioconjugation. Tyr-selective bioconjugations have been reported.[3] There still remains room for improvement, however, especially for harsh reaction conditions, use of toxic reagents, or cross-reactivity to other amino acid residues. Recently emerging electrochemical activations have a great potential for overcoming the issues.[4b, 14] Furthermore, Tyr plays multiple critical roles in protein functions[15], such as controlling cellular signal transduction through post-translational modifications, constituting a hotspot for protein-protein interactions[16], acting as a mediator of electron transfer processes[17] and maintaining integrity of protein structures through characteristic hydrogen bonding, π-interaction, and redox capabilities. Therefore, artificial modulations of Tyr residues may affect protein functions. Considering those characteristics, it will be ideal if the conjugation and deconjugation states can be controlled on demand under biocompatible conditions. Recently, Ball reported an interesting example of a reversible, Tyr-selective bioconjugation/deconjugation protocol through rhodium-π arene complex formation and dithiothreitol (DTT)-mediated decomplexation [3h].

In this study, we developed a Tyr-selective protein bioconjugation using iminoxyl radicals. The conjugation proceeded with high Tyr-selectivity and short reaction time under biocompatible conditions (room temperature in buffered media under air). The stability of the conjugates was tunable depending on the structure of the iminoxyl radicals. The conjugates with *t*Bu-substituted iminoxyl radicals were deconjugated cleanly by treating with an external trigger (a reducing agent and visible light), restoring the native Tyr structure in a traceless manner.

Sterically hindered, persistent iminoxyls[18,19] are easily accessible from oxime precursors through single electron oxidation. The adduct formation of iminoxyls with phenol derivatives was reported previously[20]. However, the synthetic utility of such persistent iminoxyls is still underrepresented. On the basis of our previous studies for uses of *N*-oxyl radicals in transformations of multifunctional substrates including proteins[21], we expected that iminoxyls are tolerant with aqueous media and proteinogenic functional groups. Thus, we started to explore Tyr-selective bioconjugation with persistent iminoxyls.

Because previously-reported iminoxyls were insoluble in water and used in organic solvents, we designed a water-soluble, sterically congested iminoxyl precursor oxime **3a**, bearing a tertiary ammonium cation moiety. The reaction conditions were optimized using **3a** and a water-soluble coumarin-conjugated peptide **1a**. The yield of product **2aa** was determined by HPLC (Table 1). To minimize undesired oxidation of substrate **1a** during the oxidative activation of **3a**, the iminoxyl was generated by premixing **3a** (10 equiv) and CAN (4 equiv) in a separate vessel for 120 secs in 20 mM PBS buffer. The addition of the thus-generated iminoxyl solution to **1a** produced **2aa** in 86% yield after 5 min (entry 1). To prevent decomposition of the iminoxyl via dimerization[19a], shortening the

premixing time resulted in increased yield (93-94%, entries 2 and 3). When 100 mM phosphate buffer (PB) was used, yield of **2aa** significantly decreased (68%, entry 4). When 100 mM citrate buffer (CB) was used instead of PB, however, yield of **2aa** increased to 92% (entry 5). Yield decreased according to the increased pH (entries 6-8), indicating that weakly acidic CB is effective for this reaction.

Next, amino acid compatibility was investigated (Table 2). Mixtures of **1a** and a control peptide containing an oxidation-sensitive His, Lys, Ser, Met, or Trp residue, but not containing a Tyr residue, were subjected to the optimized reaction conditions, and recovery of the control peptides was evaluated by HPLC analysis. In all the entries, conjugate **2aa** was obtained in high yield, while control peptides were recovered unchanged in greater than 90% yield, indicating high Tyr selectivity of the reaction. A Met-containing control substrate (Bz-Met-OH) was oxidized at the Met side chain. This cross-reactivity was, however, effectively suppressed by adding 100 equiv oxidant-scavenger, thiourea. Thiourea was not detrimental to the desired iminoxyl conjugation, but effectively suppressed undesired oxidation of the Met side chain (entry 4).

During the course of the optimization studies, we noticed that the product underwent deconjugation in a traceless manner depending on the conditions, restoring the native Tyr structure. Thus, we next studied this reverse reaction by changing the oxime precursor structures and reaction conditions (Figure 1). Conjugate **2ab** with greater steric hindrance was more prone to the deconjugation than **2aa** (Figure 1, chart (a/b) *vs* (c/d)). Because the bond dissociation energy (BDE) of the oxime O-H bond decreased according to the steric bulkiness of the substituents[22], the deconjugation reaction was facile as the iminoxyl was bulkier and more stable. The deconjugation reaction was markedly accelerated by adding 100 equiv sodium ascorbate (Na asc) and/or irradiating visible light (white LED), indicating that the reductive process promoted by photo-induced electron transfer would be beneficial for the deconjugation reaction.

The generality of peptide substrates was surveyed using the optimal protocol for the conjugation reaction (Table 3). Yield was measured by HPLC at 272 nm, where the absorbance coefficient of the Tyr side chain does not change before and after the reaction (see SI). Reactions with the iminoxyl derived from **3a** or **3b** to various peptides produced the corresponding conjugates in high yield. Yield of **3b**-conjugates was generally lower than that of **3a**-conjugates, probably due to the steric hindrance and partial deconjugation during the reactions with **3b**. The reaction with neurotensin (**1g**), which contains two Tyr residues, produced a single HPLC peak of the conjugate, suggesting that only one of the two Tyr residues preferentially reacted (entry 6). If Tyr was present at the *N*-terminus (entries 7 and 8), the reaction produced multiple HPLC peaks showing the same *m/z* value corresponding to the target conjugate. This was due to the intramolecular 1,4-addition of the terminal NH_2 group to the dienone structure (confirmed by the reaction with small molecule, see SI).

The generality of protein substrates was also examined (Table 4). The reactivity of proteins was not sufficient, however, and the optimized conditions for peptide substrates was not applicable to

proteins (entry 1'). Thus, we re-examined the reaction conditions for proteins, and found that the reactivity was significantly improved by conducting the reaction in 100 mM CB (pH 3.0). Judged from LC/MS analysis, the reaction proceeded cleanly under the conditions in the presence of thiourea, except for myoglobin (**4d**: entry 4) (entries 1-8). The reactions of widely used biologics drugs, insulin (**4k**, entry 9) and trastuzumab (**4l**, entry 10), proceeded with good conversion, suggesting the applicability of this method to synthesize novel bioconjugate drugs[20]. The traceless reversibility at protein level was also confirmed by the addition of Na asc to **5hb** (α-chymotrypsinogen (**4h**)-**3b** conjugate) (Figure 2).

This reaction was further applied to late-stage functionalization[23] of peptides and proteins. Conjugation between lysozyme (**4a**) and compound **3c** bearing a coumarin dye afforded mono-conjugate **5ac** in 40% yield (Figure 3). The conjugate can be further derivatized under biocompatible conditions. Thus, condensation of conjugate **2ba** with arylhydrazine[24] in a buffered aqueous media in the presence of Kool's catalyst[25] produced azobenzene structure **6**, which may be an attractive motif in the field of photopharmacology (Figure 4) [26, 27].

In conclusion, we developed a Tyr-selective bioconjugation reaction by identifying the water-soluble persistent iminoxyls, which proceeded under biocompatible conditions. Further, the conjugate derived from sterically hindered iminoxyl bearing a *t*Bu substituent was labile in the presence of sodium ascorbate and light irradiation, restoring the native Tyr side chain through traceless deconjugation. Efficiency of the deconjugation was dependent on the steric factor of the iminoxyl precursor. The reaction was applicable to late-stage functionalization of peptides and proteins: synthesis of a protein-dye conjugate and further derivatization of the conjugate to azobenzene-modified peptides were demonstrated.

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Keywords

Reversibility • Bioconjugation • Protein • Organoradical • Tyrosine

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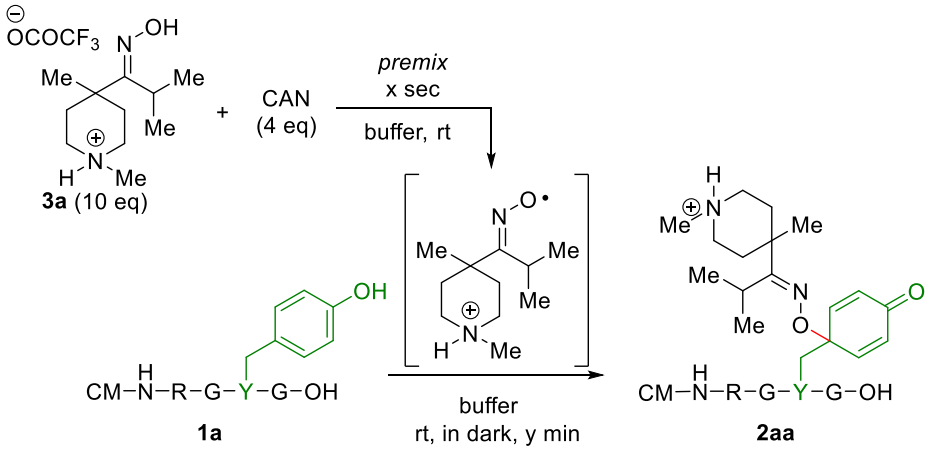
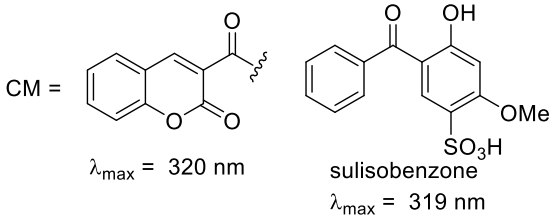
Table 1. Optimization of Tyr-selective Bioconjugation Using Water-Soluble Iminoxyl



entry	premix (x sec)	buffer	$[\mathbf{1a}]_{init}$ (mM)	reaction (y min)	Yield (%)[a]
1	120	20 mM PBS (pH 6.0)	0.5	5	86
2	60	20 mM PBS (pH 6.0)	0.5	5	93
3	30	20 mM PBS (pH 6.0)	0.5	5	94
4	30	100 mM PB (pH 6.0)	0.5	5	68
5	30	100 mM CB (pH 6.0)	0.5	30	92
6	30	100 mM CB (pH 6.0)	0.33	30	90
7	30	100 mM CB (pH 6.7)	0.33	30	83
8	30	100 mM CB (pH 7.4)	0.33	30	75

[a] HPLC yield was determined with sulisobenzone as the internal standard at 320 nm.



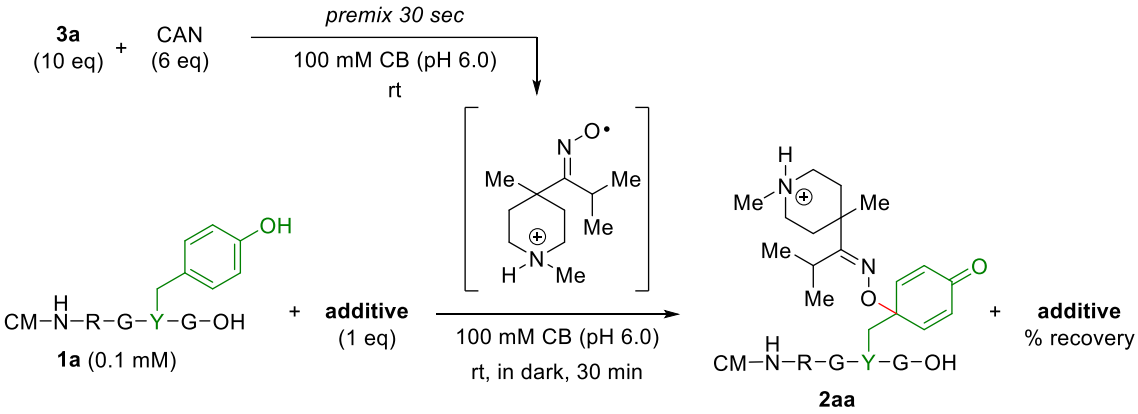
λ_{max} = 320 nm

sulisobenzone

λ_{max} = 319 nm

CAN = $(NH_4)_2[Ce(NO_3)_6]$
PBS = phosphate-buffered saline
PB = phosphate buffer
CB = citrate buffer

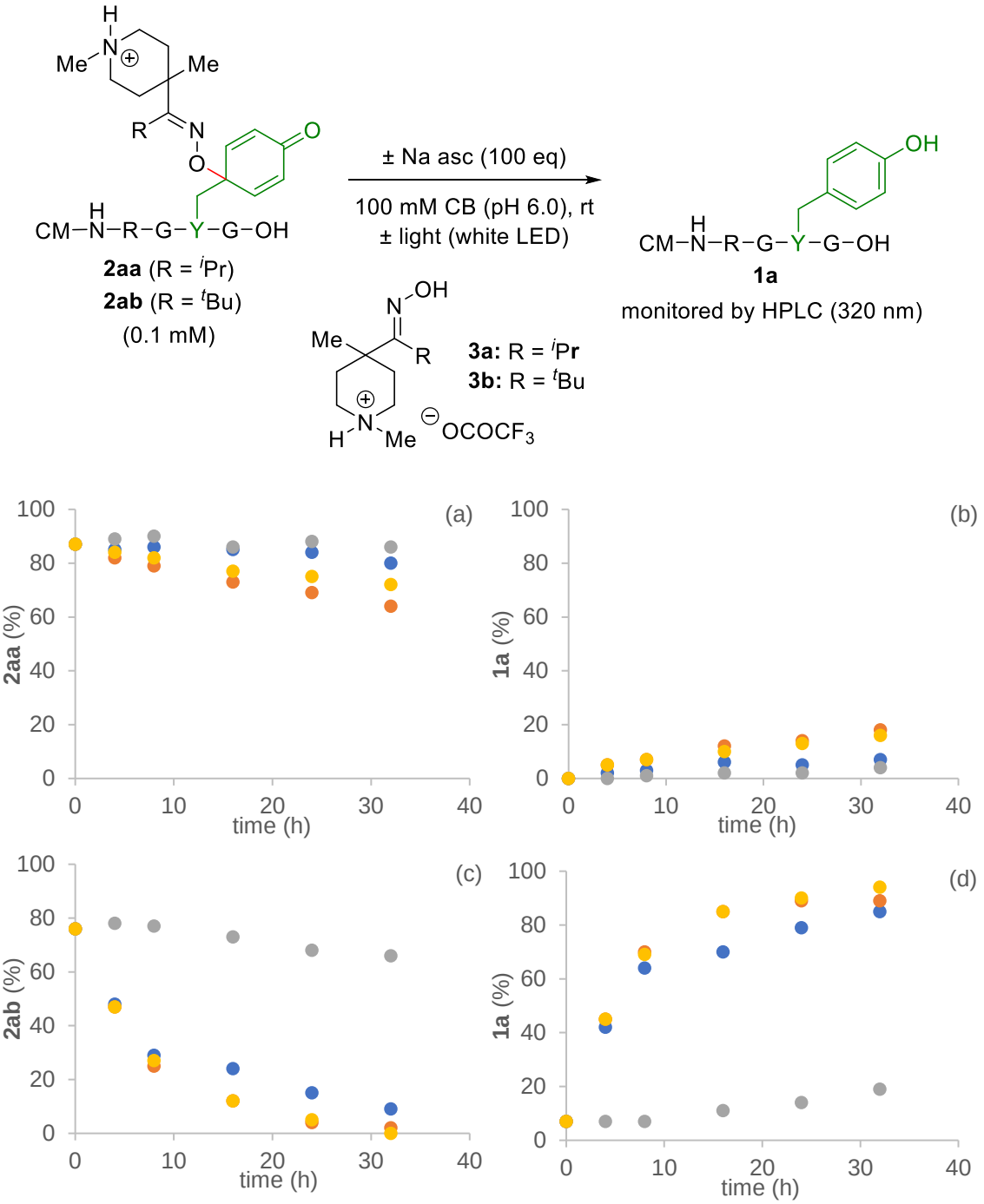
Table 2. Amino Acid Compatibility



entry	additive	yield of **2aa** (%)[a]	recovered additive (%)[a]
1[b]	CM–N–H–G–OH	90	>99
2	CM–N–K–G–OH	90	>99
3	CM–N–S–G–OH	91	>99
4[c]	Bz–N–M–OH	85	93[d]
5	CM–N–R–G–W–G–OH	89	91

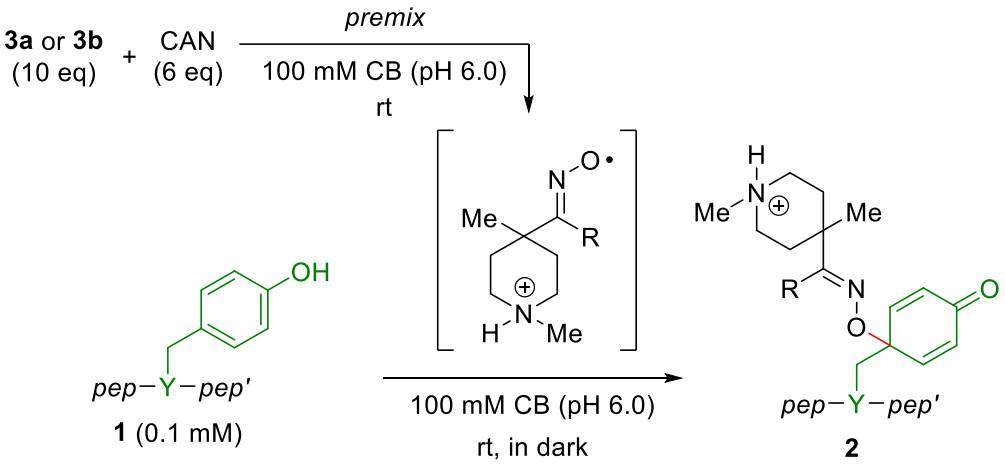
[a] HPLC yield was determined with sulisobenzone as the internal standard at 320 nm.
[b] Reaction was performed in 0.5% DMSO/CB.
[c] Thiourea (100 eq) was added into the reaction batch.
[d] HPLC yield was determined with sulisobenzone as the internal standard at 254 nm.

Figure 1. Time Course Study of Traceless Deconjugation



Time course study of (a) remained **2aa**, (b) regenerated **1a** from **2aa**, (c) remained **2ab**, (d) regenerated **1a** from **2ab**. Orange: Na asc (+), light (+); Blue: Na asc (+), light (–); Yellow: Na asc (–), light (+); Gray: Na asc (–), light (–).

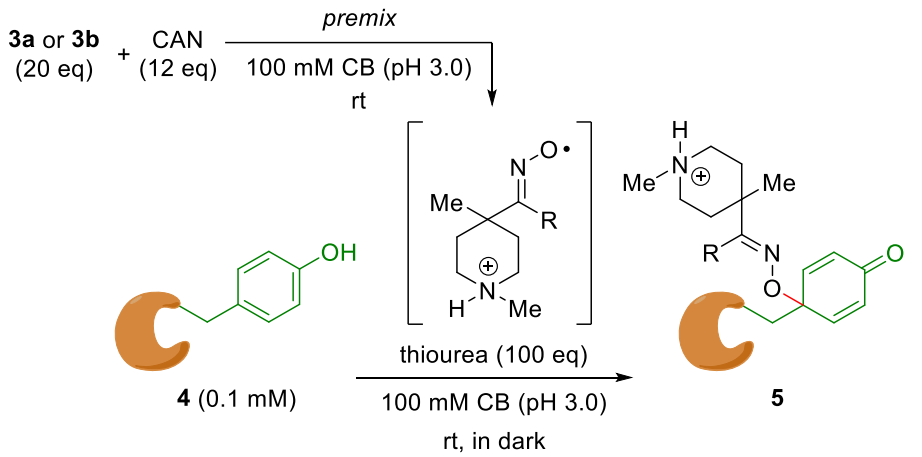
Table 3. Tyr-selective Iminoxyl Bioconjugation to Peptides



entry	substrate	yield (%)[a] using **3a**[c]	yield (%)[a] using **3b**[d]
1	Leuprorelin Acetate (**1b**) Pyr-HWS**Y**$_D$LLRP-NHEt·AcOH	90	69
2	Angiotensin I (**1c**) DRV**Y**IHPFHL	97	71
3	Bivalirudin, TFA salt (**1d**) $_D$FPRPGGGGNGDFEEIPEE**Y**L·TFA	93	82
4	Angiotensin IV (**1e**) V**Y**IHPF	87	83
5	Oxytocin (**1f**) C**Y**IQNCPLG-NH_2, cyclic S-S	64	67
6	Neurotensin (**1g**) Pyr-L**Y**ENKPRRP**Y**IL	79[b]	58[b]
7	Leu-Enkephalin (**1h**) **Y**GGFL	>99	>99
8	Kisspeptin-10 (**1i**) **Y**NWNSFGLRF-NH_2	34	>99

[a] HPLC yield was determined at 272 nm.
[b] Yield of mono-bioconjugate.
[c] Premix 30 sec, reaction 1 h [d] Premix 10 min, reaction 2 h

Table 4. Tyr-selective Iminoxyl Bioconjugation to Proteins



entry	substrate	conversion (%)[a] using **3a**[b]	conversion (%)[a] using **3b**[c]
1 1'[d]	Lysozyme (**4a**) (129AA, Y x 3)	53% (+ 1-3 mod) 6%	52% (+ 1 mod)
2	Concanavalin A (**4b**) (237AA, Y x 7)	68% (+ 1-2 mod)	42% (+ 1 mod)
3	Carbonic Anhydrase II (**4c**) (259AA, Y x 8)	52% (+ 1-3 mod)	57% (+ 1-2 mod)
4	Myoglobin (**4d**) (153AA, Y x 2)	5% (+ 1 mod)	10% (+ 1 mod)
5	α-Lactoalbumin (**4e**) (123AA, Y x 4)	73% (+ 1-3 mod)	62% (+ 1-2 mod)
6	Streptavidin (**4f**) (159AA, Y x 6)	>99% (+ 1-2 mod)	47% (+ 1 mod)
7	Ribonuclease (**4g**) (124AA, Y x 6)	75% (+ 1-2 mod)	29% (+ 1-2 mod)
8	α-Chymotrypsinogen A (4**h**) (245AA, Y x 4)	90% (+ 1-2 mod)	60% (+ 1 mod)
9	Human Insulin (**4i**) (51AA, Y x 4)	58% (+ 1-4 mod)	33% (+ 1 mod)
10	Trastuzumab (**4j**) (HC: 449AA, Y x 21 LC: 214AA, Y x 10)	ND (+ 0-8 mod)	ND (+ 1-7 mod)

[a] Conversion was determined by integral of deconvoluted MS peak.
[b] Premix 30 s, reaction 1 h [c] Premix 10 min, reaction 2 h
[d] Reaction was conducted in 100mM CB (pH 6.0).
AA = amino acid, HC= heavy chain, LC=light chain, ND = not determined.

Figure 2. Traceless Deconjugation of Protein Conjugate

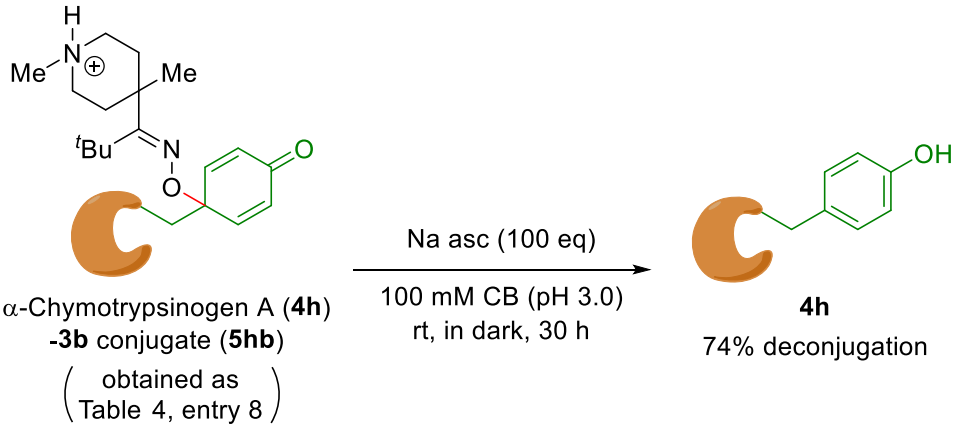


Figure 3. Synthesis of Protein-Dye Conjugate

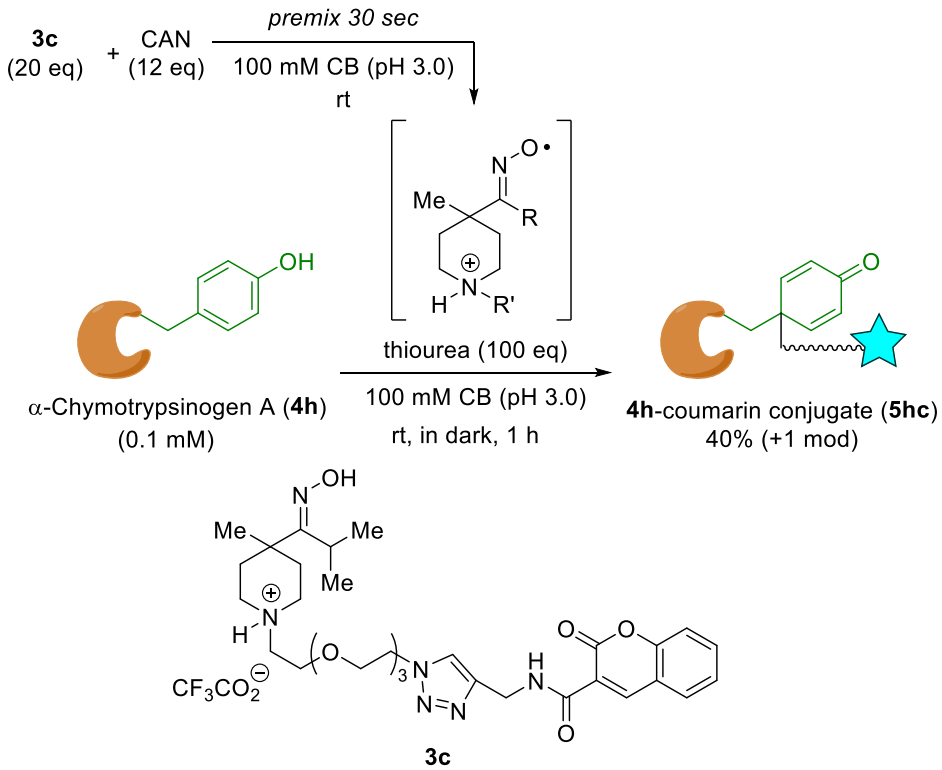
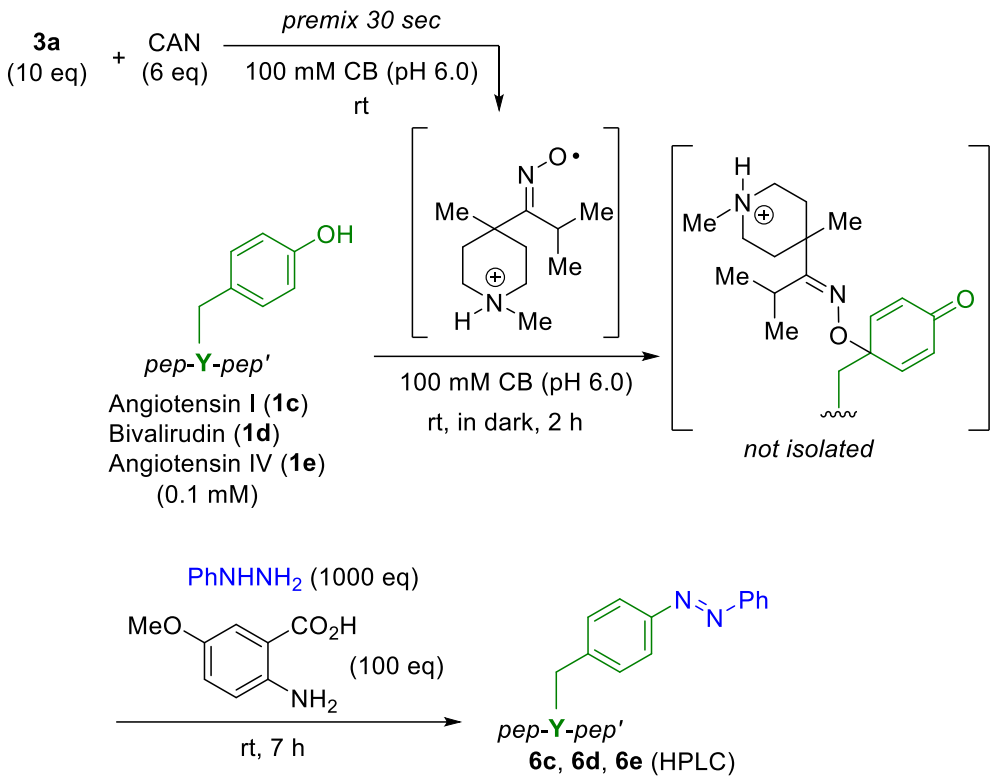
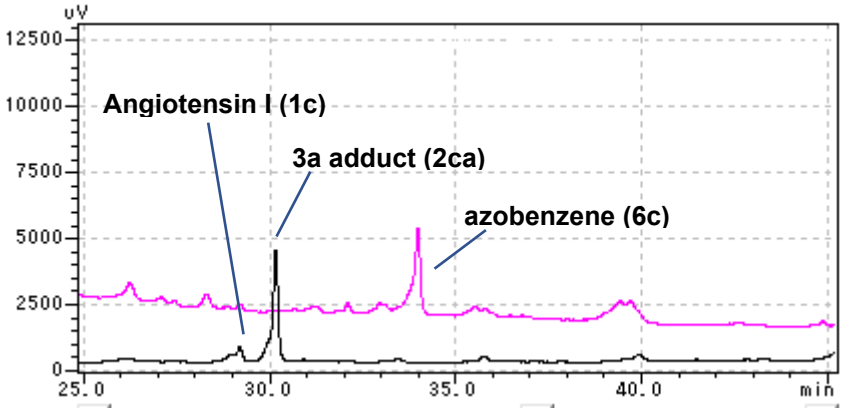


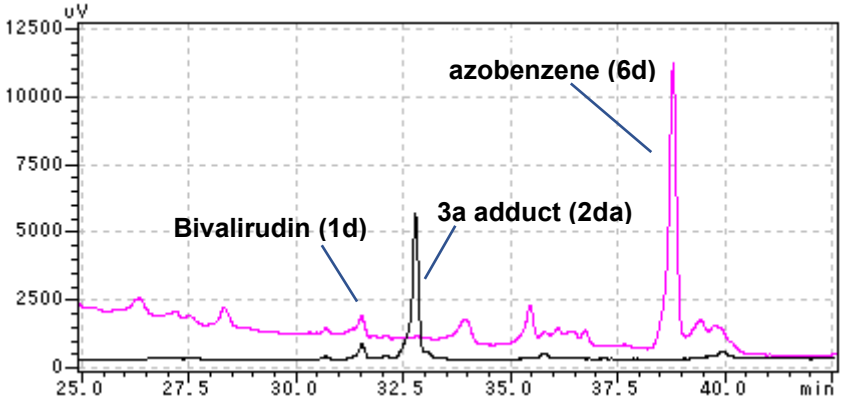
Figure 4. Late-Stage Transformation of Tyr side chain to Azobenzene



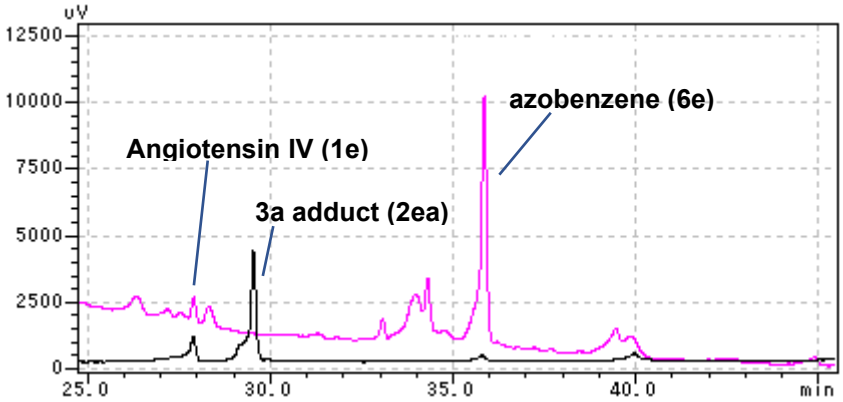
(a)



(b)

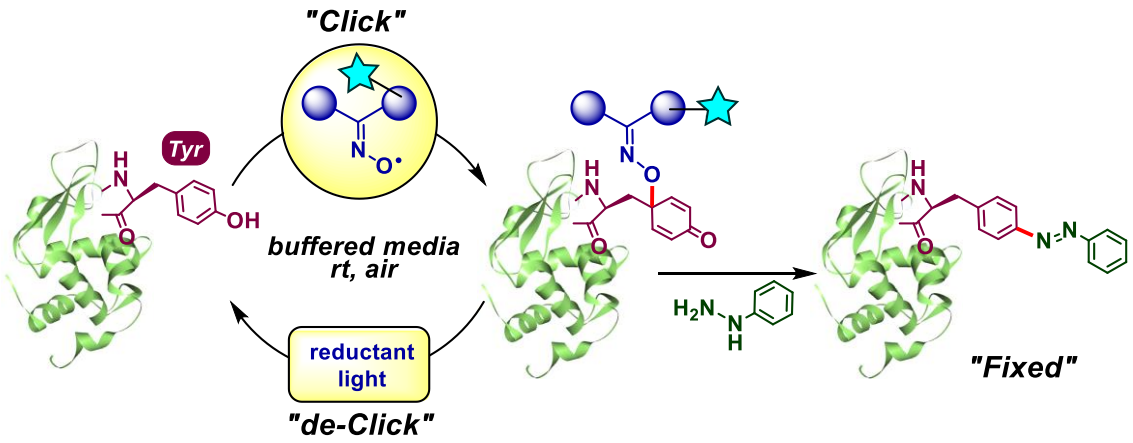


(c)



HPLC charts for the conversion to azobenzene (272 nm). (a) Angiotensin I (**1c**), (b) Bivalirudin (**1d**), (c) Angiotensin IV (**1e**). Black: After oxime addition; Magenta: At 7 h.

TOC


"Click"
Tyr
buffered media
rt, air
reductant
light
"de-Click"
"Fixed"