Protein Modification at Tyrosine with Iminoxyl Radicals

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ABSTRACT: Post-translational modifications (PTMs) of proteins are a biological mechanism for reversibly controlling protein function. Synthetic protein modifications (SPMs) at specific canonical amino acids can mimic PTMs. However, reversible SPMs at hydrophobic amino acid residues in proteins are especially limited. Here we report a tyrosine (Tyr)-selective SPM utilizing persistent iminoxyl radicals, which are readily generated from sterically hindered oximes via single electron oxidation. The reactivity of iminoxyl radicals with Tyr was dependent on the steric and electronic demands of oximes; isopropyl methyl piperidinium oxime **1f** formed stable adducts, whereas the reaction of *tert*-butyl methyl piperidinium oxime **1o** was reversible. The difference in reversibility between **1f** and **1o**, differentiated only by one methyl group, is due to the stability of iminoxyl radicals, which is partly dictated by the bond dissociation energy of oxime O–H groups. The Tyr-selective modifications with **1f** and **1o** proceeded under physiologically-relevant, mild conditions. Specifically, the stable Tyr-modification with **1f** introduced functional small molecules, including an azobenzene photoswitch, to proteins, whereas the reversible modification of Tyr with **1o** switched protein function on and off in an enzyme and in a monoclonal antibody by modification and deconjugation processes.

Introduction

Life emerges from dynamic chemical networks of biomolecules.[1,2] Together with the one-way information flow from DNA to RNA and proteins (the central dogma), the biochemical order of life is also governed by reversible, enzymatic post-translational modifications (PTMs) of biomacromolecules.[3] Lysine acylation/deacylation[4,5] and serine/threonine/tyrosine phosphorylation/dephosphorylation[6–8] are typical examples of reversible PTMs at protein levels. Inspired by nature's PTMs, methodologies for synthetic protein modifications (SPMs) are emerging, aimed at creating novel biomaterials, therapeutics, and diagnoses through *in vitro* reactions[9–13] and understanding and intervening into biological mechanisms through *in vivo* reactions.[14,15] Although nucleophilic amino acids, such as Lys and Cys, have been the main SPM sites, recent endeavors are expanding to target chemically more-inert amino acids Tyr,[16,17] Trp,[18] Met,[19–21] His,[22–24] and Ser.[25] This new trend in synthetic organic chemistry will vastly broaden both accessible chemical space and the potential functions of protein-based molecules with high structural homogeneity.

Reversible SPMs, mimicking biochemical PTMs, however, remain less explored. Examples of reversible SPMs have been mostly limited to Michael addition/retro-Michael reaction[26–34] or alkylation/dealkylation at Cys[35-37]; nevertheless, this chemistry has recently expanded to His[38] (Figure 1a). The use of a pH-sensitive linker[39,40] and dynamic imine formation[41–47] at Lys have been demonstrated more recently, the latter of which was extended to *N*-terminal Cys modifications[48,49] (Figure 1b). Reversible SPMs targeting hydrophobic, more-inert amino acid residues are much rarer. The hypervalent iodine (III) reagent developed by Gaunt selectively reacted with Met, and the sulfonium products of this forward reaction regenerated Met by reductive treatment with tris(2-carboxyethyl)phosphine (TCEP) (Figure 1c).[20] Ball developed a unique conjugation method at Tyr involving η^6-arene complex formation of the Tyr phenol with a cationic rhodium(III) species.[50] This complex was deconjugated by treatment with dithiothreitol (DTT) or hydrogen peroxide (Figure 1d). These seminal studies on more-inert amino acids, however, did not address whether protein function was recovered after deconjugation. Reversible SPMs will enable on-demand control of protein function. However, molecular design principles accommodating both stability and cleavability for protein conjugates are still limited. Much of the bond cleavage chemistry is not compatible with mild, near-physiological, conditions (neutral pH at ambient temperature in aqueous media) and functional group-enriched circumstances.[51–55]

Tyrosine (Tyr) exists in low abundance in protein primary sequences (~3.0%),[56] but is moderately exposed on protein surface due to amphiphilicity of the phenol functional group. Surface Tyr is often involved in protein functions in a variety of ways, as a target of PTM phosphorylation as well as a crucial functional motif for enzyme–substrate or protein–protein interactions (PPIs).[57] Therefore, Tyr-selective SPMs can dramatically affect protein functions. There are several examples for Tyr-selective SPMs complementary to enzymatic methodologies[58–62]: e.g. Barbas[63,64] reported *ortho-C*-functionalization of the Tyr phenol with triazolinediones, which was later advanced further to enzymatic and electrochemical Tyr SPMs by Nakamura[65–69] and Gouin[70], respectively. Lei[71] and MacMillan[72] also developed electrochemical and photochemical *ortho-C*-functionalization for Tyr SPMs, respectively, using phenothiazine derivatives. For *O*-functionalization, Francis[73] and Lee/Kim[74] reported a palladium-complex-mediated allylic substitution and sulfur fluoride exchange chemistry (SuFEx), respectively. As described above, Ball[50] developed a reversible Tyr modification (Figure 1d). Herein, we describe a Tyr-selective SPM using persistent iminoxyl radicals (Figure 1e), enabling dynamic control of protein function through reversible covalent bond formation and cleavage. The steric and electronic demands of iminoxyl radicals influence their thermodynamic stability and reversibility, constituting a molecular principle for the stability and cleavability of protein conjugates.

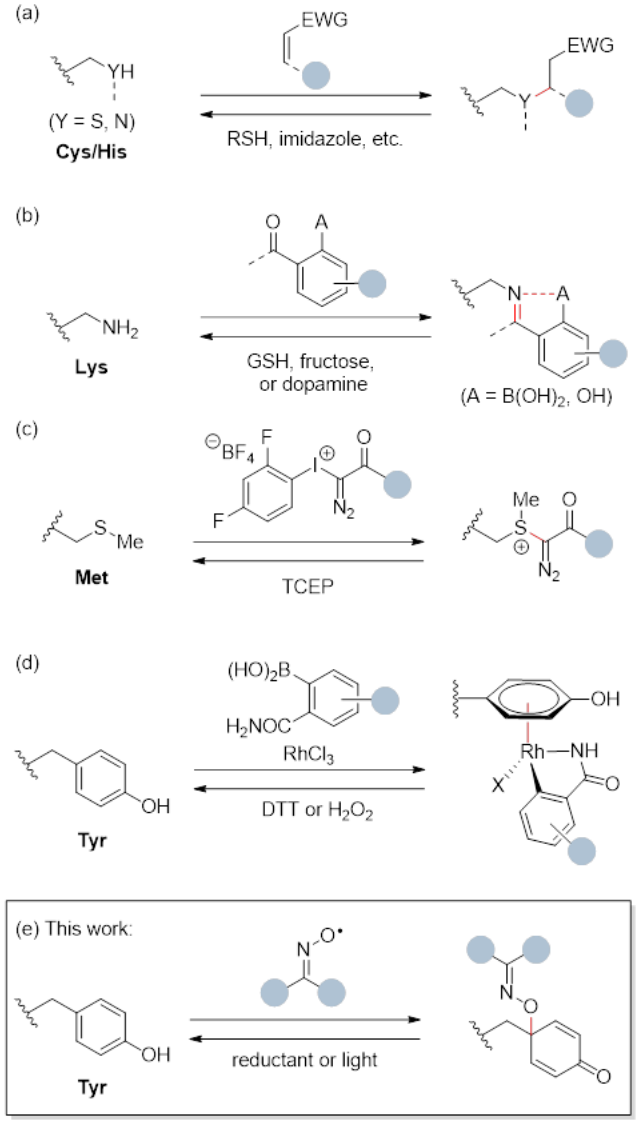
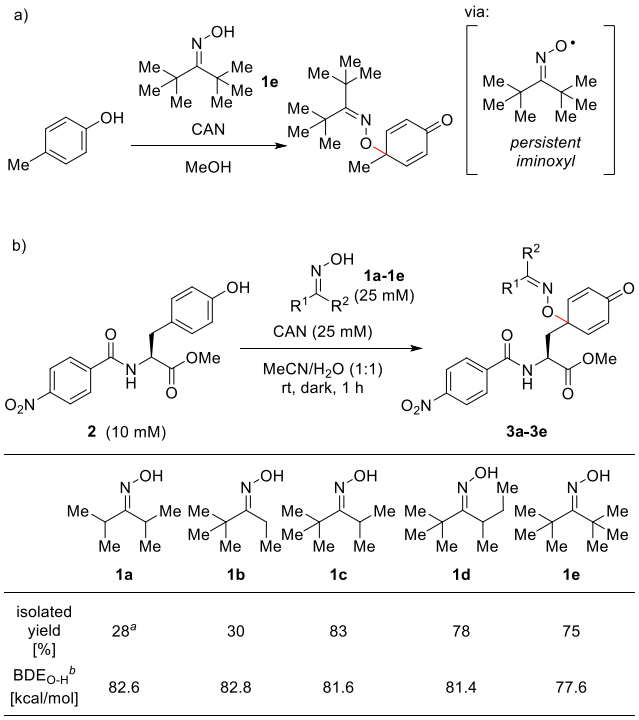


Figure 1. Representative precedents for reversible synthetic protein modifications (SPMs).

Results

Optimization of Tyr-Selective Modification with Iminoxyl Radicals. Identifying orthogonal reactivity of aminoxyl radicals to water and various polar functional groups,[75–78] we previously developed a Trp-selective SPM using a sterically less-hindered and electronically activated persistent aminoxyl radical, keto-ABNO.[78] We are also interested in properties and reactivity of iminoxyl radicals, an alternative group of persistent *N*-oxyl radicals unexplored in SPM.[79] Mendenhall and coworkers reported a conjugation reaction between *p*-cresol and di-*tert*-butyl iminoxyl radical, which was generated *in situ* via one-electron oxidation of oxime **1e** with ceric ammonium nitrate (CAN; Figure 2a).[80] Based on this report and our research experiences, we began to develop a novel SPM platform using iminoxyl radicals at the Tyr phenol.



	1a	1b	1c	1d	1e
isolated yield [%]	28[a]	30	83	78	75
BDE_{O-H}[b] [kcal/mol]	82.6	82.8	81.6	81.4	77.6

Figure 2. Effects of oximes' steric and electronic factors on reactivity. (a) Oxidative coupling between oxime **1e** and *p*-cresol through di-*t*Bu iminoxyl radical, reported by Mendenhall.[80] (b) Comparison of oximes. Reaction conditions: substrate 2 (0.5 mmol, 10 mM), oxime **1** (1.25 mmol, 25 mM), and CAN (1.25 mmol, 25 mM) in MeCN/H_2O (1:1) at room temperature in the dark for 1 h. [a] Reaction time was 0.5 h. [b] BDE_{O-H} (for *E*-isomer of **1b**, **1c**, and **1d**) was calculated using DFT [(RO)B3LYP/6-311+G(2d, 2p)//(U)B3LYP/6-31G(d)].[84]

We first examined oxidative coupling of oximes **1** bearing systematically altered substituents with *N*-*p*-nitrobenzoyl-$_L$-tyrosine methyl ester (**2**) in an acetonitrile (MeCN)/H_2O mixed solvent (Figure 2b). When oximes **1a**–**1e**, CAN, and **2** were reacted in the dark, corresponding oxime adducts **3a**–**3e** were obtained. All the oxime adducts were stable enough for purification by silica gel chromatography. Steric and electronic factors of oximes correlated with yield of the products; sterically less hindered oximes **1a** and **1b** resulted in lower yield than bulkier oximes **1c**–**1e**, with intermediate-size oxime **1c** affording the highest yield (83%). The observed tendency is likely due to the balance between stability and reactivity of iminoxyl radicals,[81–83] which are in part dictated by the bond dissociation energy (BDE_{O-H}) of oximes' O–H bonds.[84] The conversion of oximes to iminoxyl radicals is facile for **1a**–**1e**, due to the great oxidation potential of CAN. Sterically less demanding iminoxyl radicals **1a** and **1b** are, however, prone to deactivation through self-dimerization.[81,82,85] Therefore, the concentration of iminoxyl radicals is higher according to the steric demands. Due to steric repulsion, however, the reactivity of iminoxyl radicals with the phenol group is lesser according to the steric demands (**1c** > **1d** > **1e**). Higher stability of sterically more demanding iminoxyl radicals due to the increased hyperconjugation by electron-donating methyl groups, which reflects to smaller BDE values, also contribute to the observed reactivity order.

To apply the reaction to peptides and proteins, we then further optimized the oxime structure using water-soluble peptide **4** containing a coumarin dye at the *N*-terminal as a model

substrate, in an organic solvent-free, neutral buffered medium at 37 °C (Figure 3). Yield of adduct **5** was quantitatively evaluated by HPLC. The reaction of **1c** with **4** proceeded only in moderate yield (38%), likely due to the low water-solubility of **1c**. Thus, we examined water-soluble oximes bearing polar functional groups[86] (**1f**–**1o**, Figure 3, also see Figure S2 for more comprehensive screening results). Among the examined, **1f** containing a methyl piperidinium and isopropyl (*i*Pr) substituents exhibited the highest yield (83%). When the piperidinium group was used as a secondary alkyl substituent (**1g**), yield was lower (44%). Maintaining the methyl piperidinium substituent, we investigated the steric effects of the other substituent (**1h**–**1o**). The reactivity dependence on steric factors was consistent with the bell-shape tendency observed in the simpler system (Figure 2b). Other substitution patterns did not produce superior yields (Figure S2). Overall, for high-yielding Tyr-modification, oxime reagents must fulfil the following three structural requirements: (1) presence of a polar functional group to confer water-solubility, (2) substituent combination to be *tert*-butyl (*t*Bu) and *i*Pr groups or their equivalents, and (3) non-conjugated oximes.

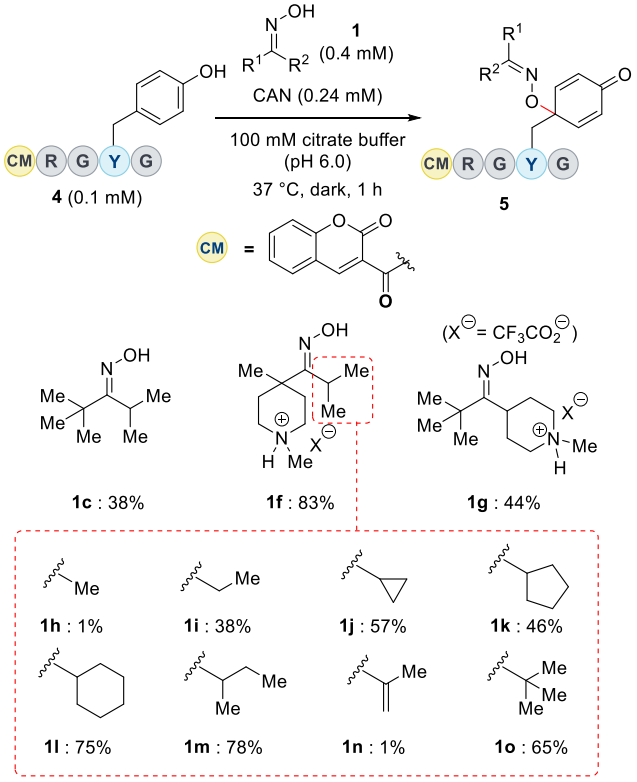
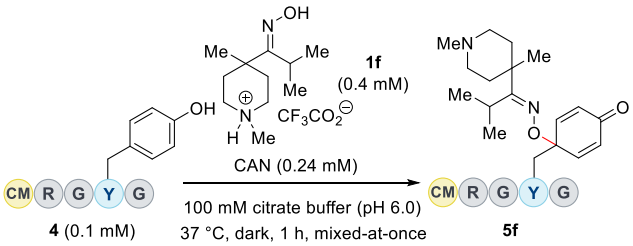


Figure 3. Optimization of oxime structure for Tyr-selective modification in buffered media. Reaction conditions: peptide **4** (0.1 mM), oxime **1** (0.4 mM), and CAN (0.24 mM) in 100 mM citrate buffer (pH 6.0) at 37 °C in the dark for 1 h. HPLC yield was determined from the peak area of **5** at 320 nm detection using sulisobenzone as an internal standard.

Using oxime **1f**, we next optimized the reaction conditions (Figure 4). When oxidants other than CAN were used, the reaction did not proceed smoothly and resulted in low yield (< 57%, entry 2; Figure S3). Under white LED irradiation (entry 3) or at temperatures lower than 37 °C (entry 4, 5), yield diminished, especially in the former case (5%). The reaction using increased reagent amounts [**1f** (1 mM, 10 equiv to **4**) and CAN (0.6 mM)] in a 100 mM citrate buffer (pH 6.0) afforded product **5** in higher yield (90%, entry 6). Use of buffers with higher pH (pH 6.7–8.0) or buffers other than citrate buffer diminished yield (57–83%, entry 7–9, 11, 12). When unbuffered water was used as a solvent, the reaction medium reached pH ~ 4 due to the acidity of CAN, although the reaction proceeded smoothly (87%, entry 10). Because direct exposure to CAN was detrimental to some proteins (Figure S4), we identified a premixed procedure of **1f** and CAN taking advantage of persistent nature of the iminoxyl radical. Thus, **4** was added after complete consumption of CAN by premixing with **1f** for 30 seconds, producing comparable yield (85%, entry 13) to the mixing-at-once conditions (entry 6).

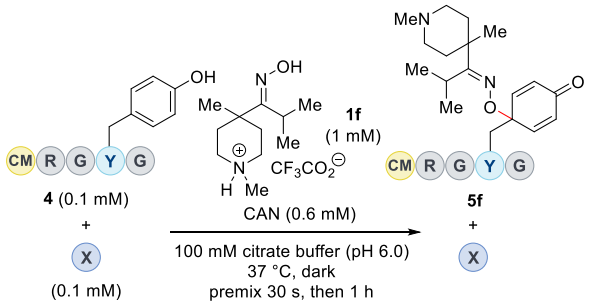


entry	deviation from above conditions	yield (%)[a] 5f	4
1	none	83	10
2	other oxidants[b]	< 57	> 17
3	white LED	5	93
4	room temperature	76	16
5	0 °C	39	51
6	citrate buffer (pH 6.0)	90	2
7	citrate buffer (pH 6.7)	83	1
8	citrate buffer (pH 7.4)	81	3
9	citrate buffer (pH 8.0)	80	8
10	H_2O[c]	87	3
11	100 mM phosphate buffer (pH 6.0)	57	36
12	100 mM Bis-Tris buffer (pH 6.0)	57	33
13	premix **1f** and CAN for 30 s	85	1

(oxime (1 mM), CAN (0.6 mM))

Figure 4. Optimization of reaction conditions. Standard reaction conditions (entry 1): peptide **4** (0.1 mM), oxime **1f** (0.4 mM), CAN (0.24 mM) in 100 mM citrate buffer (pH 6.0), 37 °C, dark, 1 h. [a] Determined by the peak area of HPLC chart (320 nm) using sulisobenzone as an internal standard. [b] Yield of **5f**: TEMPO$^+$BF4$^-$; 57%, $NaIO_4$, $NaIO_3$, $K_2S_2O_8$, $Cu(OAc)_2$, $K_3[Fe(CN)_6]$, $KMnO_4$, $NaNO_2$, H_2O_2, NaClO; 0%. [c] The reaction solution was acidic (pH ~ 4).

Under the optimized conditions (Figure 4, entry 13), we examined the amino acid compatibility by the reaction of **4** in the presence of an equimolar competitive amino acid **X** (Figure 5). The Tyr-modification of **4** proceeded smoothly in the presence of amino acids with polar side chains (Lys, Ser), aromatic side chains (His, Phe, Trp), and Cys dimer, with all amino acids recovered in high rates (entry 1–5, 7, Figure S5-S9, S11). When Met was added as **X**, however, competitive oxidation of the thioether group proceeded and the recovery rate of Met was low (26%). Addition of thiourea as an orthogonal reductant to iminoxyl radicals dramatically improved the recovery rate of Met (>99%, entry 6 in Figure 5, Figure S10). These results showcase high Tyr-selectivity of the iminoxyl radical-mediated modification.



entry	X	yield (%)[a]	intact X (%)[b]
1	Bz-His-OH	95	> 99
2	Bz-Lys-OH	95	> 99
3	Bz-Ser-OH	88	97
4	Bz-Phe-OH	92	> 99
5	Bz-Trp-OH	89	88
6	Bz-Met-OH	96[c] (96[d])	> 99[c] (26[d])
7	$(Bz\text{-}Cys\text{-}OH)_2$	96	> 99

Figure 5. Amino acid compatibility of Tyr-selective modification. Reaction conditions: peptide **4** (0.1 mM), amino acid **X** (0.1 mM), oxime **1f** (1 mM), CAN (0.6 mM) in 100 mM citrate buffer (pH 6.0, < 1% DMSO), 37 °C, dark, premix **1f** and CAN for 30 s, then conjugation reaction for 1 h. [a] Determined by the HPLC peak area (320 nm) using sulisobenzone as an internal standard. [b] Determined by the HPLC peak area (254 nm) using sulisobenzone as internal standard. [c] Thiourea (10 mM) was added. [d] Without thiourea.

Tyr-Selective Peptide- and Protein-Modification with 1f-Derived Iminoxyl Radicals. We applied the optimized conditions using oxime **1f** to biorelevant peptides containing Tyr (Figure 6). The yield was calculated by HPLC peak areas at the isosbestic point (272 nm) of Tyr and Tyr-oxime adduct (Figure S12). The reaction proceeded in high efficiency with leuprorelin, angiotensin I, bivalirudin, angiotensin IV, neurotensin, and oxytocin bearing macrocyclic disulfide. Modifications of Leu-enkephalin and kisspeptin-10, which contain Tyr at the *N*-terminal, produced multiple peaks with mass values consistent with the desired products in HPLC charts (Figure S13-S20). This is due to the formation of cyclized products through a successive intramolecular conjugate addition of the *N*-terminal amino group to the cyclohexadienone moiety, generated after oxime addition (the structure in the bracket in Figure 6). As confirmed by LC-MS analysis, yield of peptide modifications with **1f** was generally high.

We next applied the reaction with **1f** to proteins (Figure 7). Lysozyme (14 kDa), insulin (5.8 kDa), concanavalin A (26 kDa), α-chymotrypsinogen A (26 kDa), ribonuclease A (14 kDa), α-lactalbumin (14 kDa), and α-chymotrypsin (25 kDa) were modified in good yield. Tyr-selectivity was confirmed by tryptic digestion followed by LC-MS/MS analysis of the conjugates (Figure S21-S27). The reaction was also applicable to anti-HER2 antibody trastuzumab (14.8 kDa) (Figure S28). Extent of modification (yield and modification number) were substrate-dependent, reflecting the reactivity and accessibility of Tyr residues. Retention of the higher order structures of proteins after modification was confirmed by circular dichroism (CD) spectroscopy (Figure S21-S27).

Having established the foundation for Tyr-selective peptide- and protein-modifications with the iminoxyl radical derived from oxime **1f**, we then applied this method to protein functionalization using **1f**-based oximes attached with functional molecules (**1p**–**1u**, Figure 8). The nitrogen atom in the methyl piperidinium moiety of **1f** was suitable for attaching various functional molecules without changing the reactivity of the iminoxyl radical. Alkyne (**1p**) and azide (**1q**) groups were facilely introduced on ribonuclease A using this method in 74% and 87% yield, respectively, which can act as handles for bioorthogonal modifications. As an example, the ribonuclease A-**1q** adduct was tagged with fluorescein by strain-promoted azide-alkyne cycloaddition (Figure 8a-(ii), 8b, see also Figure S35-S38).[87] The linkage was sufficiently stable to the reductive treatment with DTT and heating at 95 °C for 30 min, conditions required for detection by SDS-PAGE. Oximes attached to anticancer agent SN-38 (**1r**), *N*-acetylglucosamine (β-$_D$-GlcNAc, **1s**), desthiobiotin (**1t**), and biotin (**1u**) were successfully conjugated to proteins in useful yield without damaging the payloads (Figure S29-S33, S35), demonstrating the high functional group tolerance. The binding ability of biotin with streptavidin beads remained after the protein conjugation, as demonstrated by the mock-purification experiment of biotin-modified ribonuclease A from a mixture with cell lysate (Figure 8c). Furthermore, the cyclohexadienone moiety generated from the Tyr side chain after modification can be an alternative handle for another type of biorthogonal transformation, hydrazone formation. Thus, treatment of the ribonuclease A-**1f** adduct with *p*-(HO_2C)-C_6H_4-$NHNH_2$ in the presence of 4-methoxyanthranilic acid[88] produced an azobenzene through elimination of the oxime (Figure 8a-(iii), Figure S39-S40).[89,90] This transformation can be a new approach to introducing an azobenzene switch for the photodynamic control of protein functions,[91] at the position closest to proteins' main chains possible without relying on genetic manipulation.

Deconjugation. When di-*t*Bu oxime adduct **3e** was left on the bench at room temperature, we unexpectedly found that the starting tyrosine derivative **2** was regenerated. Therefore, we investigated the stability of oxime adducts **3a**–**3e** in a $CDCl_3$ solution stored in the dark at room temperature (Figure 9, S41). Adduct **3a** decomposed rapidly ($t_{1/2}$ ~ 5 h) into unidentified products without regenerating **2**. While adducts **3b**, **3c**, and **3d** containing a primary or secondary alkyl group as one of the oxime substituents were sufficiently stable, di-*t*Bu oxime adduct **3e** deconjugated over time ($t_{1/2}$ ~ 110 h) and cleanly regenerated **2** as a dominant product. Furthermore, the deconjugation process of **3e** accelerated under room lighting. The stability of adducts **3b**–**3e** is again correlated to the $BDE_{O\text{-}H}$ values[84] of oximes (Figure 2b), which dictate the stability of iminoxyl radicals. We assume that the deconjugation reaction begins with thermally- or photochemically-promoted homolytic cleavage of the *ipso*-C–O bond of **3e**, followed by reduction of the resulting phenoxy radical.

We also confirmed that adduct **5o** generated from peptide **4** and water-soluble oxime **1o** (Figure 3), which contains a comparable steric demand to **1e**, was successfully deconjugated by the treatment with penicillamine or light irradiation in a buffered medium in a much shorter time (4 h), while adduct **5f** generated from **4** and sterically less demanding **1f** was sufficiently stable (Figure S42). In peptide and protein modifications (i.e., forward reaction), oxime **1o** generally produced comparable to slightly lower yield than **1f**, except for concanavalin A (Figure 6, 7). To investigate the generality of the deconjugation reaction, we evaluated regeneration of the original peptide substrates from their oxime adducts with **1o** (Figure 10, S43-S50). Oxime **1o**-adducts derived from leuprorelin, angiotensin I, bivalirudin,

angiotensin IV, and neurotensin regenerated the original unmodified peptides through deconjugation by penicillamine in high yield. The **1o**-adduct derived from oxytocin, however, did not afford the original peptide. Cleavage of the disulfide bond with penicillamine competed in this case, producing multiple products of unidentified structures. Deconjugation from Leu-enkephalin-**1o** and kisspeptin-10-**1o** adducts did not proceed at all. Those adducts contained stable cyclized structures at the *N*-terminal Tyr (see Figure 6), and the adducts were recovered in nearly quantitative yield.

The Tyr-selective and reversible protein modification by the iminoxyl radical derived from oxime **1o** enables ON/OFF chemical switching of protein functions. We here demonstrate two such examples, switching enzymatic activity and antibody-antigen binding on and off (Figure 11). Preparing α-chymotrypsin-**1o** adduct, the enzymatic activity for hydrolysis of Bz-Tyr-OEt to Bz-Tyr-OH was assessed (see SI). The modified α-chymotrypsin showed a decreased activity by 42% compared to α-chymotrypsin: the initial reaction rate constants (k_0) were 0.051 mM•min^{-1} for the modified enzyme and 0.12 mM•min^{-1} for unmodified α-chymotrypsin (Figure 11b). We identified Tyr228 existing in the substrate-binding pocket as a modification site, based on tryptic digestion and LC-MS/MS analysis (Figure S27).[92,93] Changes in chemical and physical properties of the substrate binding site may be a reason for the observed smaller kinetics after oxime conjugation. Then, treating the adduct with penicillamine at 37 °C promoted deconjugation. After 6 h, quantitative regeneration of intact α-chymotrypsin was observed by LC-MS (Figure 11a, S51). The enzymatic activity recovered to a comparable level (k_0 = 0.11 mM•min^{-1}) to that of unmodified α-chymotrypsin after deconjugation.

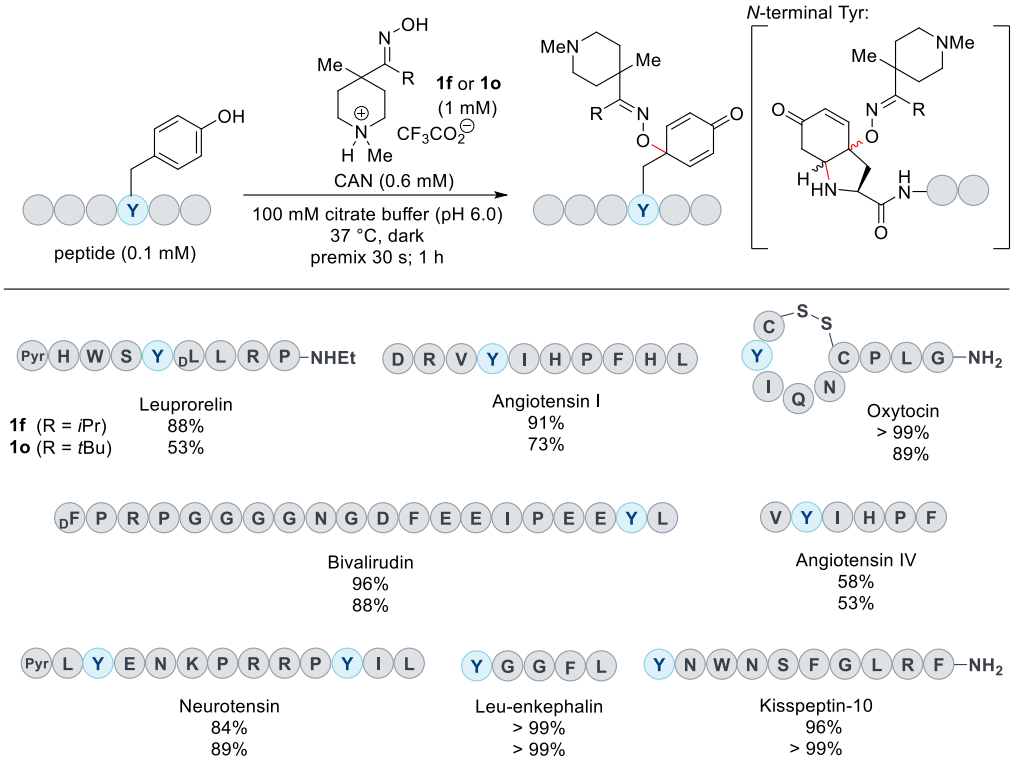


Figure 6. Tyr-selective modification of biorelevant peptides. Reaction conditions: peptide (0.1 mM), oxime **1f** or **1o** (1 mM), CAN (0.6 mM) in 100 mM citrate buffer (pH 6.0), 37 °C, dark, premix **1f** or **1o** and CAN for 30 s, then conjugation reaction for 1 h. Yield was determined by the HPLC peak area (272 nm).

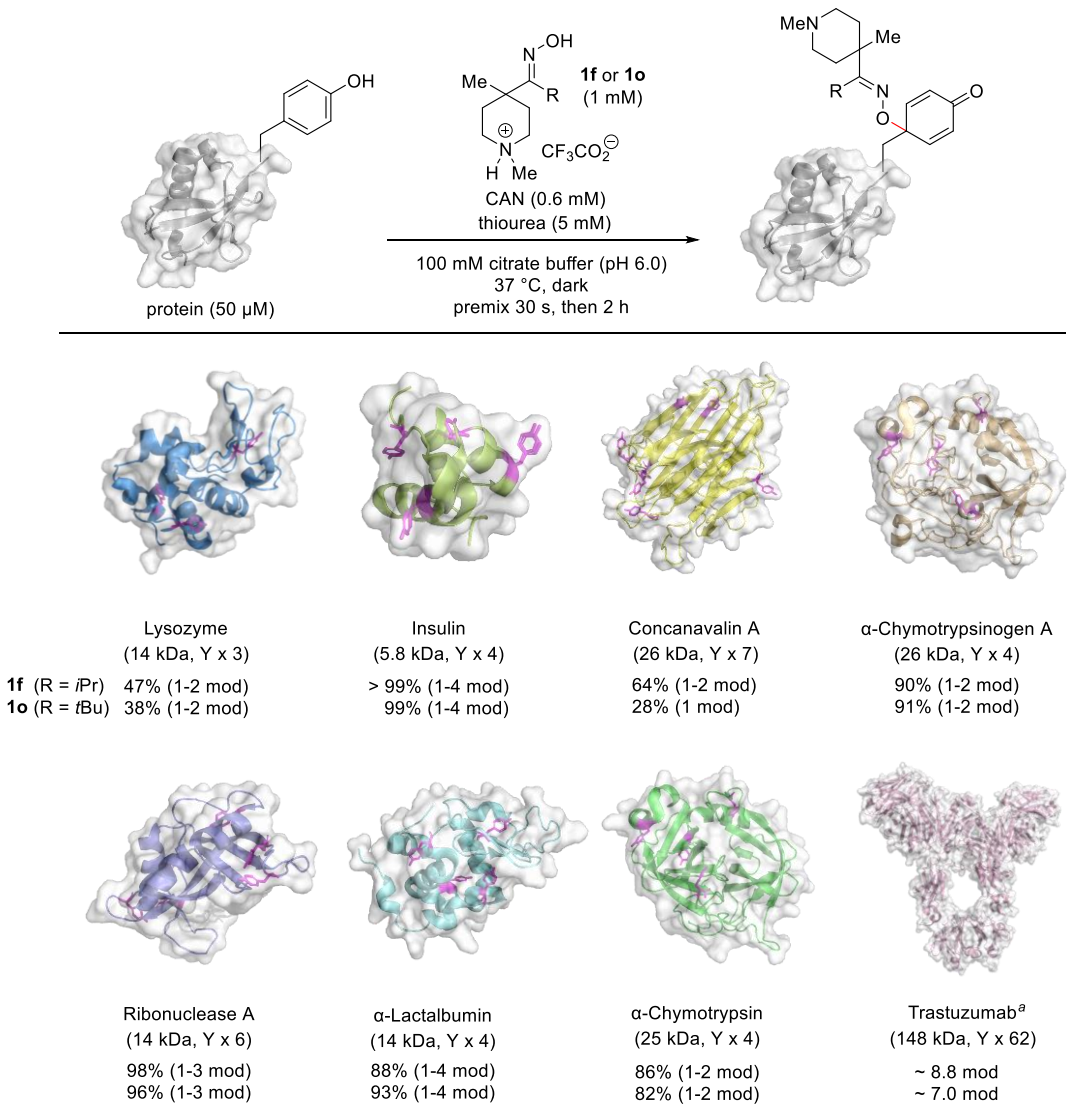


Figure 7. Tyr-selective modification of proteins. Reaction conditions: protein (50 μM), oxime **1f** or **1o** (1 mM), CAN (0.6 mM), thiourea (5 mM) in 100 mM citrate buffer (pH 6.0), 37 °C, dark, premix **1f** or **1o** and CAN for 30 s then conjugation reaction for 2 h. Yield was determined by the relative intensity of mass spectrum of corresponding species. Modification number (mod) was determined from LC-MS. [a] Trastuzumab (40 μM), oxime (2 mM), CAN (1.2 mM), thiourea (10 mM).

We further examined switching the binding of an antibody, trastuzumab, with HER2 antigen on and off. Tyr residues frequently exist on the surface of the complementarity determining region (CDR) of the antibody and play important roles in antigen-antibody interactions.[94,95] Therefore, Tyr modification should markedly affect the interactions. Thus, trastuzumab-**1o** adduct [modification number (mod) = ~ 5.2] was prepared and the HER2 binding activity was assessed by ELISA assay. The affinity of trastuzumab markedly diminished by one-eighth after modification (Figure 11d): EC_{50} values for unmodified trastuzumab and trastuzumab-**1o** adduct were 0.37 nM and 2.8 nM, respectively. Treating the adduct with penicillamine, deconjugation proceeded in 15 h (mod = ~ 1.9, Figure 11c). The binding affinity was recovered by the deconjugation reaction (EC_{50}= 0.63 nM: Figure 11d). These results, at least partly, demonstrate that protein functions can be reversibly controlled by Tyr-oxime conjugation and deconjugation under mild, physiologically-relevant conditions.

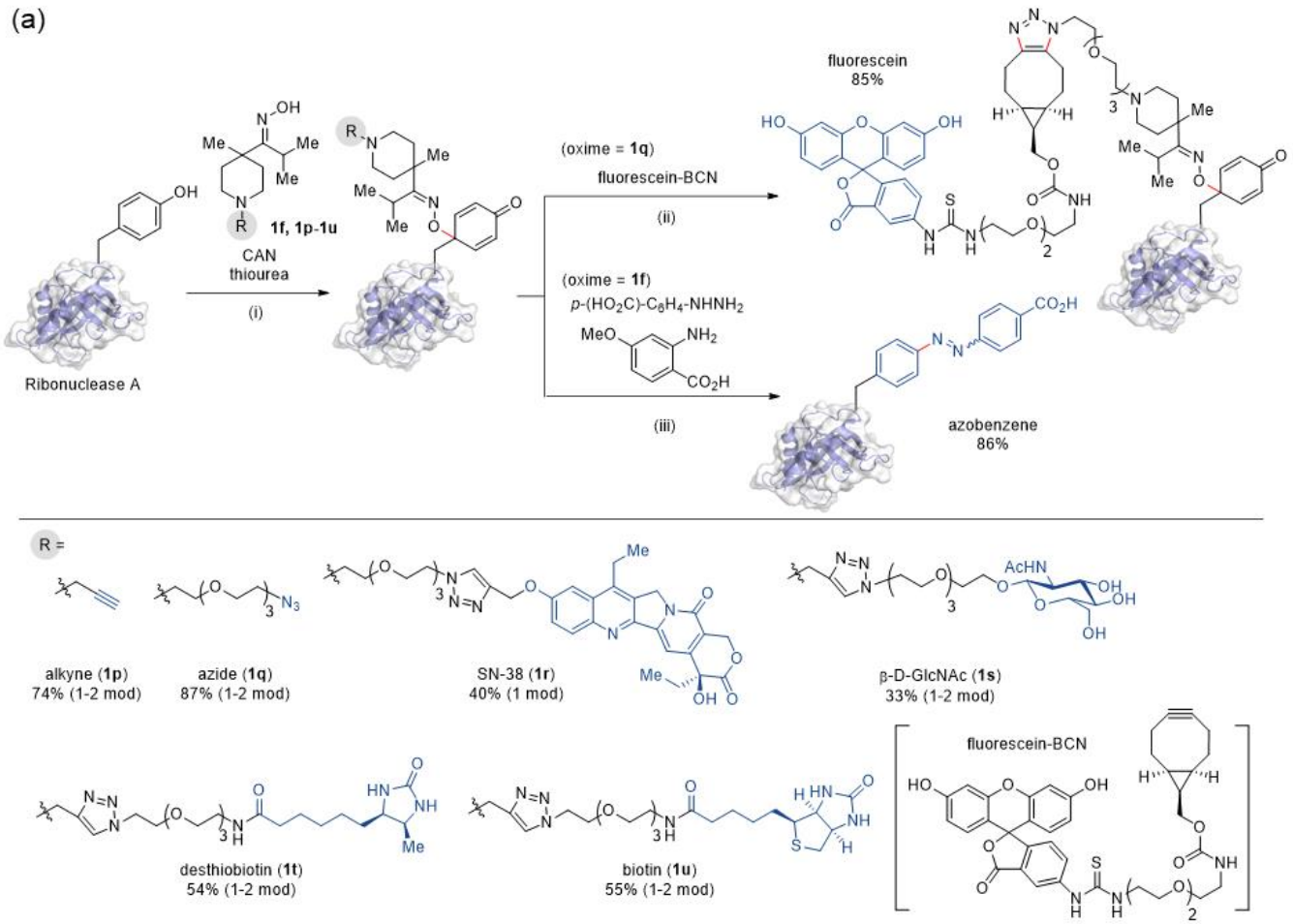


Figure 8. Functionalization of ribonuclease A (RNase A) by Tyr-selective modification. (a) Reaction conditions: (i) RNase A (50 μM), oxime **1** (1 mM), CAN (0.6 mM), thiourea (5 mM) in 100 mM citrate buffer (pH 6.0), 37 °C, dark, premix of **1f**, **1p**–**1u** and CAN for 30 s then conjugation for 2 h. (ii) Modified RNase A (25 μM), fluorescein-BCN (0.5 mM) in 1x PBS (pH 7.4), rt, dark, 12 h. (iii) Modified RNase A (25 μM), *p*-(HO_2C)-C_6H_4-$NHNH_2$·HCl (25 mM), 4-methoxyanthranic acid (2.5 mM) in 100 mM citrate buffer (pH 5.0), rt, 21 h. Yield was determined by the relative intensity of mass spectra of corresponding species. (b) SDS-PAGE of RNase A functionalized with fluorescein. CBB staining (up) and fluorescence detection (down). Lane 1: unmodified RNase A, lane 2: RNase A-**1q** adduct, lane 3: RNase A-**1q** adduct treated with fluorescein-BCN. (c) SDS-PAGE of pull-down experiments. Biotinylated RNase A (RNase A-**1u** adduct) was pulled down from its mixture with HeLa S3 cell lysate using streptavidin beads. The band at 13 kDa corresponds to streptavidin released from the beads by boiling. Lane 1: RNase A-**1u** adduct, lane 2: cell lysate, lane 3: a mixture of cell lysate and RNase A-**1u** adduct used for the pull down experiment, lane 4: elution from streptavidin beads after pull down from the mixture of cell lysate and RNase A-**1u** adduct, wash with CRB (50 mM Tris-HCl (pH 7.5), 0.3% TritonX-100, 300 mM NaCl; 3 times), and boiling, lane 5: elution from streptavidin beads treated with cell lysate without RNase A-**1u** adduct, wash with CRB (3 times), and boiling, lane 6: elution from streptavidin beads treated with RNase A-**1u** adduct without cell lysate, wash with CRB (3 times), and boiling.

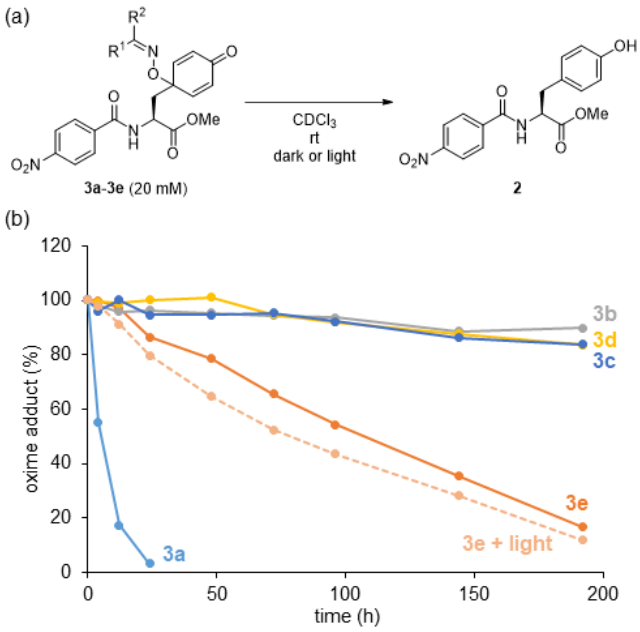
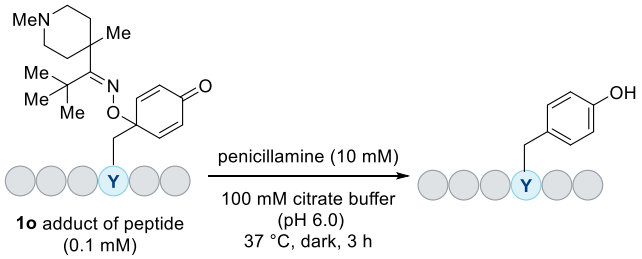


Figure 9. Stability of oxime adducts **3**. The stability was monitored by ^{1}H NMR using 1,1,2,2-tetrachloroethane as an internal standard.



entry	peptide	yield (%)[a]
1	Leuprorelin	> 99
2	Angiotensin I	> 99
3	Oxytocin	0
4	Bivalirudin	> 99[b]
5	Angiotensin IV	74[b]
6	Neurotensin	91
7	Leu-enkephalin	0
8	Kisspeptin-10	0

Figure 10. Deconjugation of peptide-**1o** adducts. Reaction conditions: modified peptide (0.1 mM), penicillamine (10 mM) in 100 mM citrate buffer (pH 6.0), 37 °C, dark, 3 h. [a] Yield was determined by the HPLC peak area (272 nm). [b] Reaction time: 6 h.

Discussion

Most radical reactions are thermodynamically downhill and thus irreversible due to high reactivity of radicals. Here we found that the reaction between sterically hindered, persistent iminoxyl radicals and the phenol functional group of Tyr can be reversible. The reversibility is tunable by modifying the steric and electronic demands of iminoxyl radicals and reaction conditions. Specifically, substitution of a hydrogen atom to a methyl group at the α-position of iminoxyl radicals had profound effects on their reactivity and reversibility (Figure 2b, 9): an Et-substituted iminoxyl radical (from oxime **1b**) was too unstable to be used in conjugation reactions, an *i*Pr-substituted iminoxyl radical (from **1c**) afforded stable conjugates in high yield, and a *t*Bu-substituted iminoxyl radical (from **1e**) furnished reversibility. This tendency can be attributed to stabilization of the electrophilic radical by facile hyperconjugation of alkyl groups.[81] Steric factors also contribute to the prevention of deactivation of iminoxyl radicals through dimerization and facile homolytic cleavage of the *ipso*-C–O bond in the dearomatized conjugation products. These properties are partly dictated by $BDE_{O\text{-}H}$.

The reversibility of the reaction allowed for on-demand caging and decaging of tyrosine residues, which are often crucial for various protein functions. As showcases, we demonstrated reversible regulation of enzymatic activity and antibody-antigen interactions. One of the mechanisms for controlling protein functions in living systems is reversible PTMs mediated by enzymes. There is no example, however, that can mimic reversible PTMs by abiotic chemical reactions under physiological conditions, either in cells or test tubes. Although there are many hurdles before realizing abiotic reversible PTMs intervening into the chemical order of living systems, our reaction may be a step forward to such a research area. Further studies are needed, especially for developing a more biocompatible "on-site" oxidation method for activation of the oxime precursors under physiological conditions. A more realistic application in the shorter term may be decaging functions of biologics or small-molecular drugs depending on local redox states in living organisms. It is known that cancer cells in progressive solid tumors experience hypoxia.[96] As we demonstrated in this paper, the caged antigen-binding ability of an antibody can be restored by reductive decaging with a thiol. Our method may be useful for selective activation of anticancer biologics or cytotoxic drugs under a local reducing, hypoxic environment. Prodrugging antibodies is an emerging research area, which can promise new theranostics.[97] Current antibody prodrugging methods, however, have mostly relied on elaborate protein engineering. There are few chemical methods for prodrugged antibodies without altering their primary sequences. A representative is Lys-caging with photolabile protecting groups[98–100] and phosphatase decaging at Cys.[101] Due to the requirement of UV irradiation for decaging and randomness and low abundance of Lys in the CDR region,[93] however, the extent of functional ON/OFF switching has been moderate. The reversible Tyr modification developed here may produce prodrugged antibodies with sharp responses to the local redox environment.

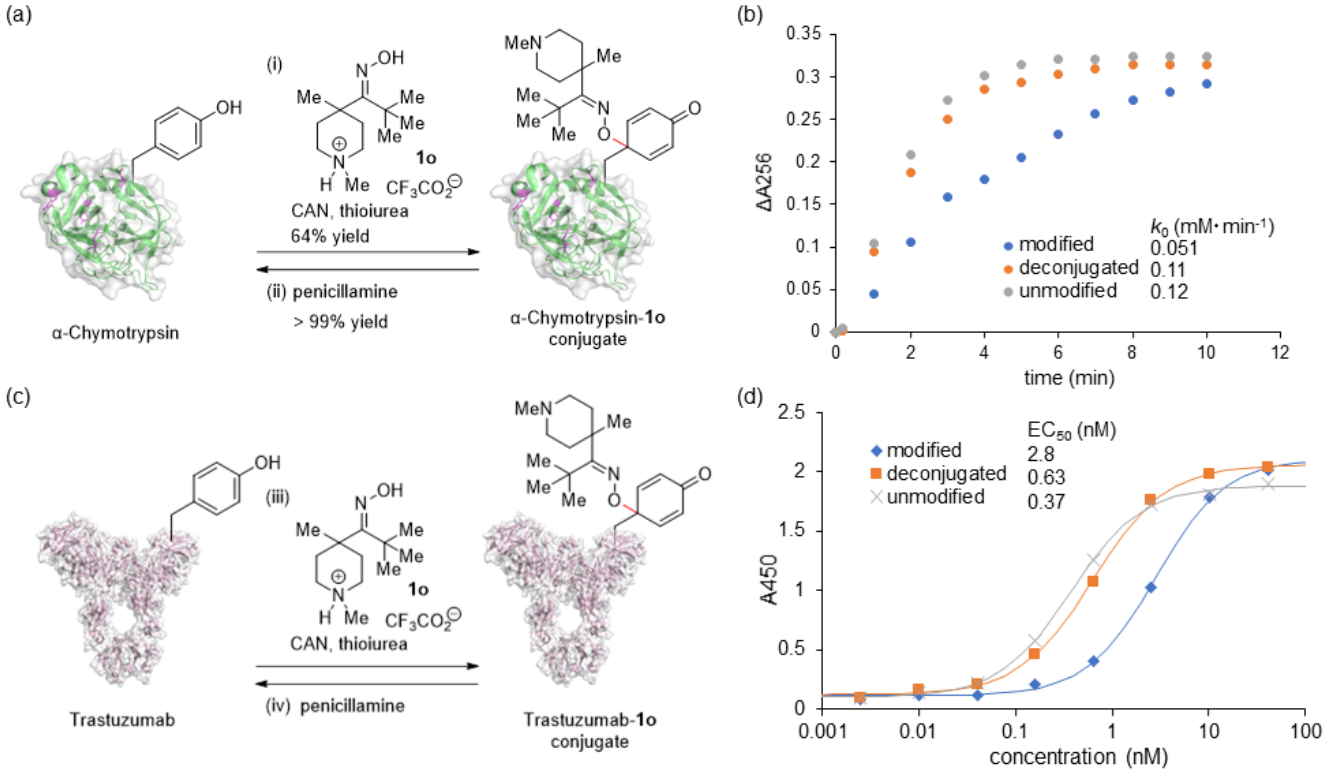


Figure 11. ON/OFF switching of protein functions by reversible Tyr modification. (a) Conjugation of α-chymotrypsin with oxime **1o** and deconjugation of the adduct. Reaction conditions: (i) α-Chymotrypsin (50 μM), oxime **1o** (1 mM), CAN (0.6 mM), thiourea (5 mM) in 100 mM citrate buffer (pH 6.0), 37 °C, dark, premix **1o** and CAN for 30 s, then reaction for 2 h. (ii) α-Chymotrypsin-**1o** adduct (12.5 μM), penicillamine (1.25 mM), 37 °C, 6 h. Yield was determined by the relative intensity of mass spectra of corresponding species. (b) Comparison of enzymatic activity by hydrolytic rate of Bz-Tyr-OEt to Bz-Tyr-OH. The reaction progress was monitored by the increase of absorbance at 256 nm (ΔA256). k_0 (mM•min^{-1}) was calculated based on the initial reaction rate. (c) Conjugation of trastuzumab with oxime **1o** and deconjugation of the adduct. Reaction conditions: (iii) Trastuzumab (50 μM), oxime **1o** (2.5 mM), CAN (1.5 mM), thiourea (12.5 mM) in 100 mM citrate buffer (pH 6.0), 37 °C, dark, premix **1o** and CAN for 30 s, then reaction for 1 h. (iv) Trastuzumab-**1o** adduct (10 μM), penicillamine (1 mM), dark, 37 °C, 15 h. (d) Comparison of binding affinity with fixed HER2 determined by ELISA (see SI for experimental details).

Conclusion

In conclusion, we developed a Tyr-selective peptide- and protein-modification with persistent iminoxyl radicals, conveniently generated from oxime precursors by pretreatment with CAN. The reaction proceeded in high yield under physiologically-relevant buffered conditions. Using water-soluble oxime **1f** or **1o** with a slight structural difference, the adduct was either stable or cleanly deconjugated by treatment with a thiol reductant: the reaction using oxime **1f** enabled conjugation of various functional molecules to proteins by the forward reaction, whereas the reversible reaction using oxime **1o** could switch protein function on and off. The protein-oxime **1f** adduct contains a reactive cyclohexadienone functional group, which acts as a handle for introducing azobenzene functionality to proteins. Further application of this method in biological research is currently ongoing.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures, characterization data, computational procedure, and data. The Supporting Information is available free of charge at https://pubs.acs.org.

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Author Contributions

K.M., K.O., and M.K. conceived and designed the study. K.M.. T.I., Y.S., and T.T. carried out all experimental work and collected the data. K.S. carried out the computational work. K. M., T.T., K.O. and M.K. analyzed the data. K.M., K.O., and M.K. co-wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

PTM, post-translational modification; SPM, synthetic protein modification; GSH, glutathione; TCEP, tris(2-carboxyethyl)phosphine; DTT, dithiothreitol; SuFEX, sulfur fluoride exchange; CAN, ceric ammonium nitrate; BDE, bond-dissociation energy; Et, ethyl; *i*Pr, isopropyl; *t*Bu, *tert*-butyl; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; HER2, human EGFR-related 2; CD, circular dichroism; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; RNase A, ribonuclease A; PBS, phosphate buffer saline; BCN, bicyclo[6.1.0]nonyne; CBB, coomassie brilliant blue, CRB, cell resuspension buffer; CDR, complementarity determining region; ELISA, enzyme-linked immunosorbent assay.

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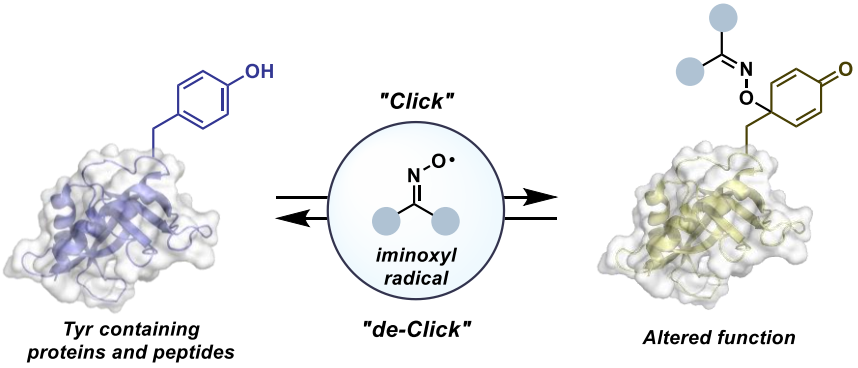

- Tyr-selective
- Oxime structure-dependent reversibility
- Mild reaction conditions
- High functional group tolerance