Bioconjugation

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Enzymatic "Click" Ligation: Selective Cysteine Modification in Polypeptides Enabled by Promiscuous Glutathione S-Transferase**

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Post-translational modifications of biomolecules enable the precise placement of various functional moieties on demand in vivo.[1] Synthetic chemists have strived to develop a similar reaction toolkit with chemical orthogonality, selectivity, and reactivity comparable to those of natural systems.[2] In particular, "click" chemistry has produced several powerful transformations that are applicable to a wide range of synthetic settings.[3] Although the efficiency and chemical orthogonality of such "click" processes are remarkable, the regioselectivity of these transformations has been limited and remains a major challenge.[4] The difficulty in selectively differentiating between two chemically identical sites within a macromolecule is routinely surmounted in natural systems by recognition elements, such as binding pockets, directing groups, and allosteric components. Although various enzymes have been engineered to catalyze reactions for protein modification and engineering,[5] they often require a long recognition sequence in polypeptides, and their substrate scope is relatively restricted. In certain cases, enzymes are capable of recognizing and carrying out efficient transformations on more than one specific substrate, a phenomenon known as enzyme promiscuity. Promiscuous enzymes have previously been engineered to catalyze several synthetically important organic transformations.[6]

Synthetic methods for modifying biomolecules provide opportunities for studying protein structure–function relationships as well as creating proteins with new properties and applications.[7] Cysteine (Cys) is often chosen for modification because of the unique reactivity of its thiol group[8] as well as the low abundance of Cys residues in the majority of naturally occurring proteins (ca. 1.7%).[9] Various chemical methods for cysteine modification have been developed, such as alkylation,[10] oxidation,[11] and desulfurization.[12] Recently, we developed an approach to the modification of unprotected peptides that was based on a nucleophilic aromatic substitution (S_NAr) reaction between perfluoroarenes and cysteine residues (Figure 1 A).[13] Although this method satisfies several requirements of "click" chemistry,[14] the insolubility and low reactivity of the perfluoroaromatic reagents in aqueous media restricts the general application of this process to a narrow range of biomolecules. In our search to render this chemistry suitable for aqueous conditions, we turned our attention to glutathione S-transferase (GST).[15] Known as a class of promiscuous enzymes, GST catalyzes conjugation reactions between the Cys residue of glutathione (GSH, γ-Glu-Cys-Gly) and various electrophiles, thus allowing the cell to detoxify xenobiotics in vivo (Figure 1 B).[15a]

Herein we report a novel bioconjugation strategy that combines the previously developed perfluoroarene–cysteine S_NAr "click" reaction with GST enzyme catalysis (Figure 1 C).

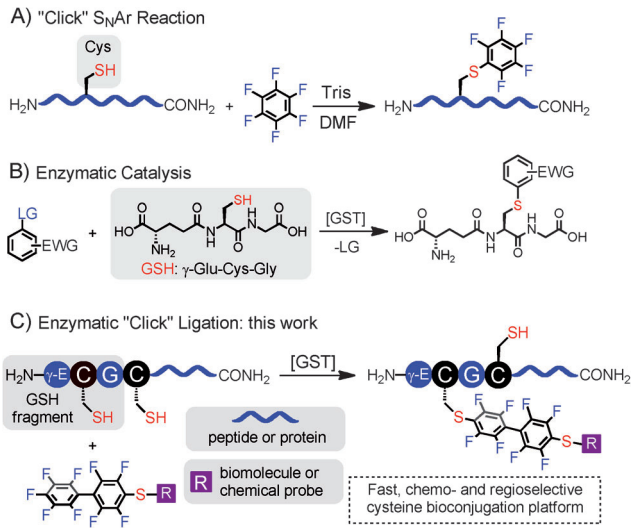

Figure 1. A) Peptide "click" modification through an S_NAr reaction between a cysteine residue and a perfluoroarene. DMF: *N*,*N*-dimethylformamide, Tris: 2-amino-2-hydroxymethylpropane-1,3-diol. B) GST-catalyzed S_NAr reaction. GST catalyzes the conjugation of activated aromatic electrophiles to the cysteine thiol group of GSH. EWG: electron-withdrawing group, LG: leaving group. C) GST-catalyzed cysteine arylation. GST catalyzes the conjugation of probes bearing a 4-mercaptoperfluorobiphenyl moiety (Cys-**III**) to the N-terminal γ-Glu-Cys-Gly sequence of a peptide or a protein. Amino acids are described by their single-letter code; γ-E stands for γ-glutamyl.

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The promiscuous nature of GST facilitates this bioconjugation with polypeptides other than GSH in aqueous media and enables the previously unattainable chemo- and regioselective functionalization of a single cysteine thiol group in the presence of other unprotected cysteine residues and reactive functional groups on the same polypeptide chain. Furthermore, we show that this process can be completed in seconds, thereby providing a new and efficient approach to peptide macrocyclization. This transformation can be carried out over a broad range of temperatures (4–60 °C) and is compatible with the addition of organic cosolvents (up to 20 %).

We hypothesized that the broad range of electrophiles accepted by GST isozymes might be sufficient for members of this enzyme family to mediate reactions between perfluoroaryl electrophiles and peptides containing GSH in an aqueous environment. To maximize the range of suitable electrophiles, we chose a mixture of GST isozymes for screening.[16] We first tested the GST-catalyzed conjugation of GSH to model peptides containing L-pentafluorophenylalanine (residue **I**, Figure 2 A). The treatment of **1** with GSH (2 mg mL^{-1}; ca. 5–10 mol % relative to **1**) at 37 °C in aqueous solution at pH 8.0 for 2 h generated conjugated product **2**, as confirmed by LC–MS analysis (see Figure S6 in the Supporting Information), whereas no product was observed without the enzyme. Nucleophilic residues in model peptide **1**, such as Cys and Lys, were unreactive. This result indicated that the Cys residue of GSH could be selectively modified with pentafluorophenyl-based electrophiles under GST catalysis.

To examine the scope of this reaction, we first tested whether the mixture of GST isozymes could catalyze the conjugation of the L-pentafluorophenylalanine residue to peptides bearing N-terminal GSH (γ-Glu-Cys-Gly). However, a hexapeptide containing an N-terminal glutathione sequence (γ-Glu-Cys-Gly-Gly-Leu-Leu) did not show reactivity towards **1** (see Figure S7). We then hypothesized that an increase in the electrophilicity of the perfluoroaryl moiety might improve the reactivity of the peptide-based substrate sufficiently to allow GST-mediated conjugation with peptides containing an N-terminal GSH sequence. Our previous study showed that a *para*-thioether substituent on the perfluoroaryl moiety can stabilize the negative charge of the S_NAr reaction intermediate, thereby increasing the reaction rate.[13] We evaluated the enzymatic reactivity of peptides containing several *para*-thioether-substituted electrophiles derived from cysteine. Importantly, these peptides showed enhanced reaction rates as compared to the peptide containing L-pentafluorophenylalanine (residue **I**, Figure 2 A; see also Figure S7). Specifically, peptide **3a** containing a perfluorophenyl-modified cysteine residue (Cys-**II**, Figure 2 A,C; see Figure S1 for the synthesis of this peptide) reacted with GSH in the presence of GST at a significantly higher rate as compared to **1**: the GSH-conjugated product was formed in 93 % yield in less than 4 h. Reactions of peptides **3b** and **3c** containing a Cys residue functionalized with a perfluorobiphenyl moiety (Cys-**III**, Figure 2 A,B) and a perfluorobiphenyl sulfide (Cys-**IV**, Figure 2 A), respectively, proceeded with quantitative conversion in less than 30 min (Figure 2 C).

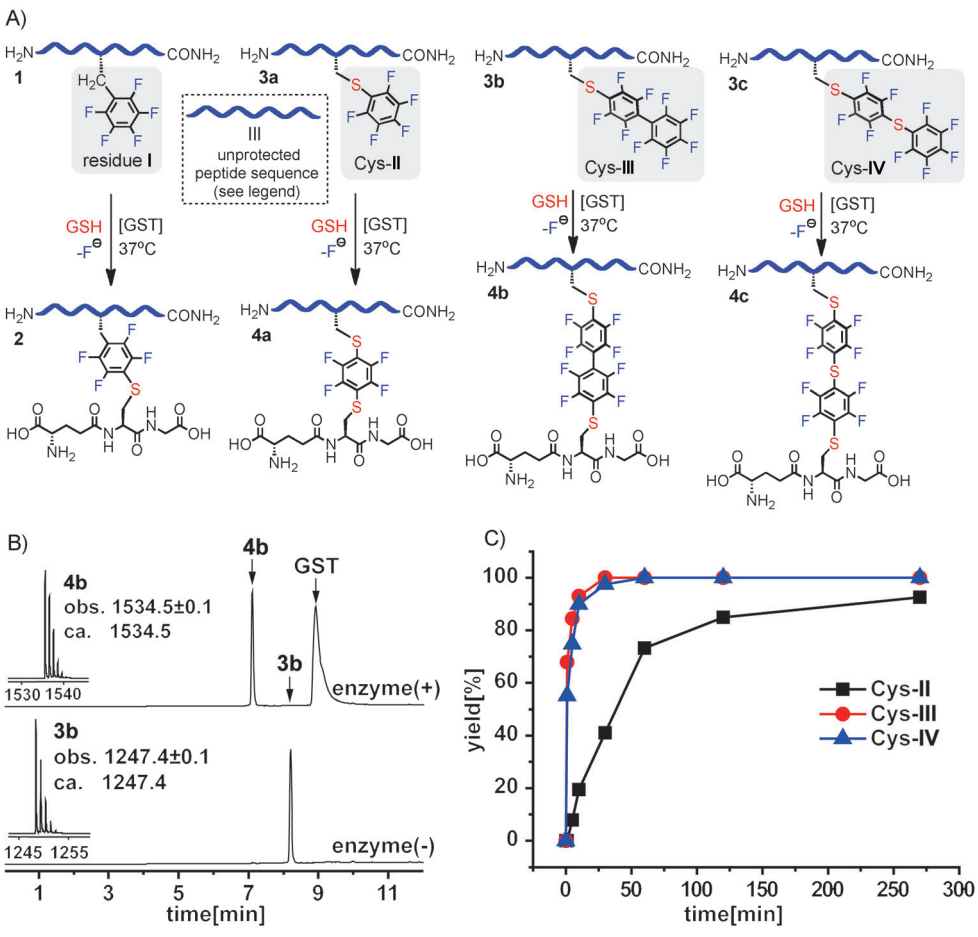
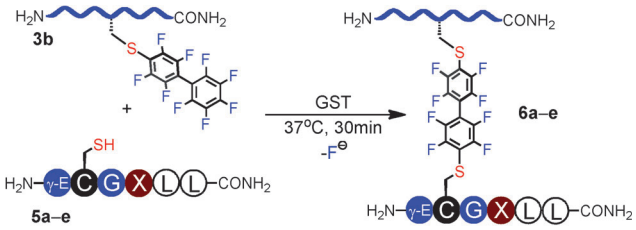


Figure 2. A) GST-catalyzed conjugation of GSH with peptides containing perfluoroaromatic electrophilic residues. Peptide sequence of **1**: NH_2-ITPCNLLF*YYGKKK-$CONH_2$ (F* stands for L-pentafluorophenylalanine); peptide sequences of **3a–c**: H_2N-VTLPSTC*GAS-$CONH_2$ (C* refers to the modified cysteine residue). Reaction conditions: **1** or **3a–c** (1 mM), GSH (1 mM), GST (2 mg mL^{-1}), tris(2-carboxyethyl)phosphane hydrochloride (TCEP·HCl; 20 mM), 0.1 M phosphate buffer, pH 8.0, 37 °C. B) LC–MS analysis of the crude reaction mixture with the peptide containing the Cys-**III** residue after 30 min. MS data are given in Dalton (Da). C) Rates of formation of the GSH-conjugated product with different electrophiles (see Figure S14 for complete LC–MS analysis). Yields were determined by LC–MS analysis (see the Supporting Information).

We extended our investigation to substrates other than GSH with more reactive electrophiles. We focused on ligations between peptides containing the Cys-**III** residue and hexamer peptides with an N-terminal γ-Glu-Cys-Gly sequence. Our study commenced with the synthesis of glycyl-modified GSH-based peptides in which the first amino acid directly after the GSH sequence was varied (Figure 3, **5a–e**).



Entry	X		Yield (%)
1	Gly	**5a**	99
2	Leu	**5b**	86 (99*)
3	His	**5c**	98 (99*)
4	Lys	**5d**	68 (99*)
5	Pro	**5e**	67 (99*)

Figure 3. Conjugation of peptide **3b** containing the Cys-**III** residue (H_2N-VTLPSTC*GAS-$CONH_2$; C* refers to the modified cysteine residue) to peptides with an N-terminal γ-Glu-Cys-Gly sequence featuring various neighboring amino acid residues under GST catalysis. Yields were determined by LC–MS analysis at $\lambda = 280$ nm (see the Supporting Information) after 30 min. Reaction conditions: **3b** (2 mM), **5a–e** (1 mM), GST (2 mg mL^{-1}), TCEP·HCl (20 mM), 0.1 M phosphate buffer, pH 8.0, 37 °C. [*] Yield of the reaction after 120 min.

All reactions proceeded quantitatively within 2 h (Figure 3, entries 1–5), and the reaction with **5a** showed high conversion within 30 min (Figure 3, entry 1). Decreased reaction rates with **5b** and **5e** as compared to **5a** were observed, which suggested that this site may be important for interaction with GST. Changing the second amino acid in the sequence linked to the Gly site to a less bulky residue (see Figure S13F) had no effect on the conjugation rate. Nucleophilic residues (His and Lys; Figure 3, entries 3 and 4) are compatible with the reaction. Surprisingly, the reaction with **5c** proceeded with 98% conversion within 30 min, which suggests that a His residue may favor the reaction.

Whereas the residue C-terminal to the GSH sequence can affect the relative rate of the GST-catalyzed reaction, the observed product yield for reactions with Cys-**III** have thus far been independent of the peptide sequence employed, which further suggests that the electrophilicity of the perfluoroaryl substituent can dominate the GST-catalyzed S_NAr reaction with modified GSH (see above). These results show that the promiscuity associated with GST is adequate to catalyze the selective bioconjugation of two unprotected polypeptide fragments and demonstrate the possibility of enhancing a click reaction through enzymatic catalysis.[17]

This reaction is highly selective for the arylation of Cys thiol groups in GSH sequences, as evidenced by a competition experiment with an exogenous thiol (4-mercaptophenylacetic acid, MPAA) present in large excess (100-fold; see Figure S8). No MPAA-arylated product was generated; the product of GST catalysis was exclusively produced. Furthermore, the treatment of **3b** with another peptide containing a C-terminal hydrazide moiety and an N-terminal γ-Glu-Cys-Gly sequence gave solely the desired S-arylated product, which could be further modified by hydrazone ligation (see Figure S8).[18]

The unique chemo- and regioselectivity of the GST-catalyzed arylation reaction could be exploited to label one Cys residue in the presence of another on a fully unprotected peptide or protein. We first labeled the N-terminal GSH Cys residue with biotin and subsequently modified a separate Cys residue with a fluorophore maleimide. This procedure provides the first example of the orthogonal site-specific labeling of two unprotected Cys residues within the same unprotected peptide or protein. The biotin probe **11** containing the Cys-**III** moiety (Figure 4A; see Figure S3 for the synthesis of **11**) was conjugated to the Cys position of the N-terminal γ-Glu-Cys-Gly fragment in protein **7** (see Figure S4 for the synthesis of **7**). The enzymatic reaction produced the monolabeled product **8**, and the other cysteine residue was then labeled with fluorescein-5-maleimide (**10**) to produce the site-specifically dual-labeled protein species **9** (Figure 4A). By a similar procedure, the biotin and fluorescein probes were regiospecifically attached to a peptide containing two cysteine residues to produce a dual-labeled species (see Figure S10). We prepared an authentic sample of the monolabeled peptide intermediate to confirm its identity (see Figure S16). Importantly, the regiospecific and orthogonal modification of two chemically identical Cys sites was enabled by selective recognition of the N-terminal GSH moiety by GST. This result indicates that the GST-catalyzed arylation could greatly expand the scope of cysteine modification beyond that of previous methods, which necessitate the use of protecting groups or multiple steps for the differential functionalization of two or more cysteine residues.[19]

Cyclic peptides constitute a very important class of medicinally relevant macrocycles.[20] Although various methods have been developed,[21] the synthesis of macrocyclic peptide fragments remains challenging. We found that the GST-catalyzed intramolecular arylation of peptide **12** containing a γ-Glu-Cys-Gly fragment and a Cys-**III** site led to quantitative conversion into the cyclized product **13** in less than 30 s (Figure 4B). In contrast, a control experiment without GST showed no product formation by LC–MS analysis. Furthermore, the cyclization reaction was shown to be dominant even when GSH was added as a competing substrate (see Figure S11). The perfluorinated moiety used in this example can potentially enhance the cell permeability of cyclic peptides, as shown previously.[13]

This GST-mediated transformation is not limited to specific temperatures, solvent conditions, or peptides featuring a γ-Glu-Cys-Gly sequence. For example, the reaction between **3b** and GSH under GST catalysis produced the desired arylated product **4b** at temperatures ranging from 4 to 60 °C (Figure 4C, right-hand graph), as well as in the presence of an organic cosolvent as up to 20% of the reaction medium (Figure 4C, left-hand graph). Additionally, GSH analogues

with the mutation of γ-Glu to a genetically encodable Glu or Asp residue (Glu-Cys-Gly or Asp-Cys-Gly) underwent facile S-arylation with peptide **3b** under the developed conditions of GST catalysis (see Figure S17). Finally, our experiments show that N-terminal Glu-Cys-Gly or Asp-Cys-Gly sequences in peptides (see Figure S12) can also be selectively S-arylated with peptide **3b**, although with lower efficiency as compared to peptides with an N-terminal γ-Glu-Cys-Gly moiety (see Figure S13 A, **5a**). These results are consistent with previous findings that certain GST isozymes are capable of catalyzing the S_NAr reaction between 1,4-dinitrochlorobenzene and glutathione analogues.[22] Together, our observations suggest that the requirement for an N-terminal γ-glutamic acid residue could be eliminated with engineered GST.

In conclusion, we have demonstrated a powerful glutathione S-transferase catalyzed S_NAr "click" process for site-specific cysteine modification. This method allowed us to selectively modify the cysteine residue in an N-terminal γ-Glu-Cys-Gly sequence within peptide and protein chains. The unique chemical orthogonality of the discovered arylation enabled by GST provides a route for the modification of multiple cysteine sites with different chemical probes or biomolecules while avoiding the use of protecting groups and additional synthetic procedures. By showing how a naturally occurring enzyme catalyst improves and significantly broadens the scope of an established "click" synthetic transformation, this development paves the way to the discovery of new enzyme-

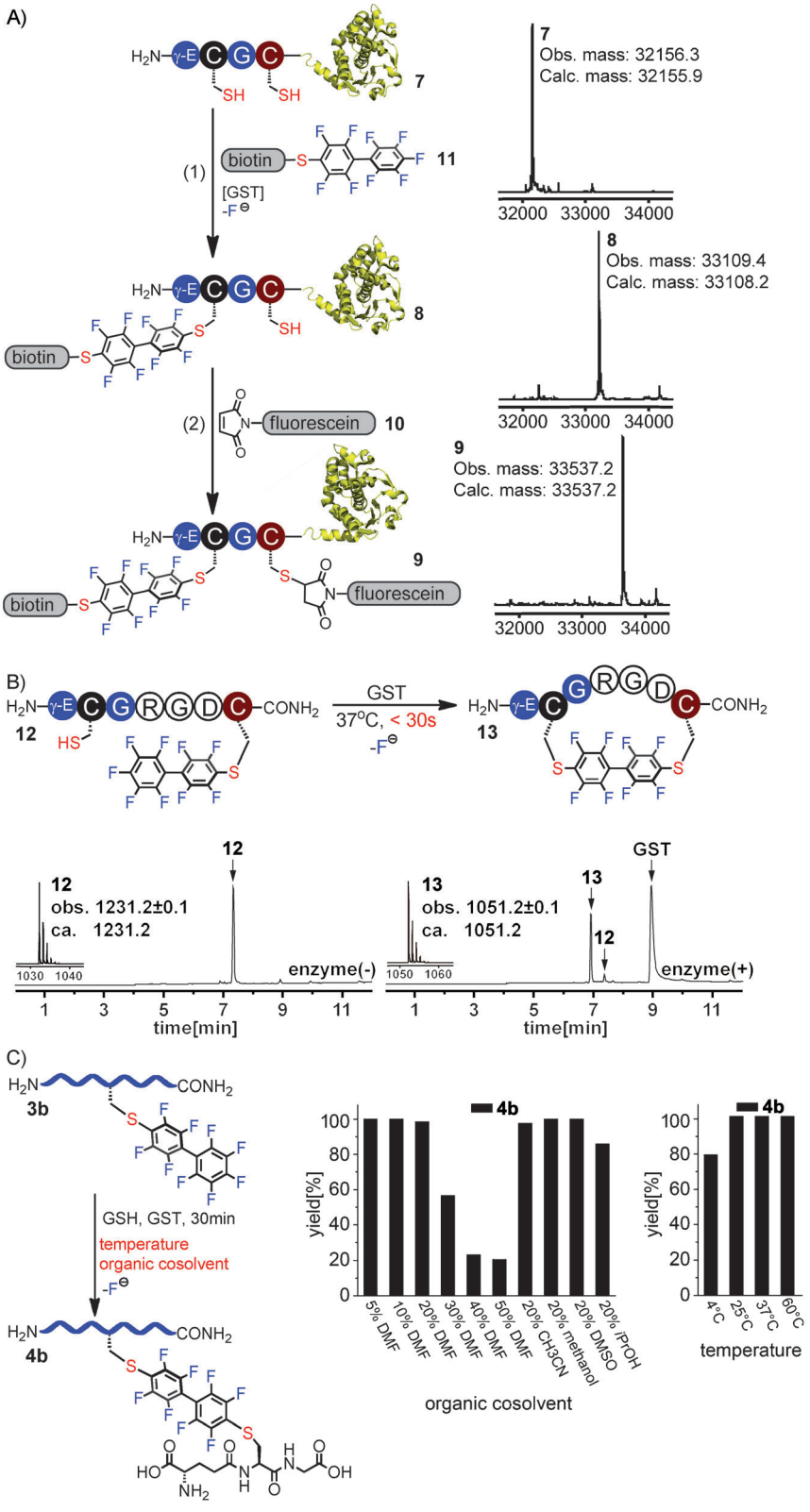


Figure 4. Synthetic utility of GST-catalyzed cysteine modification. LC–MS traces are shown as total ion current. Yields were determined by LC–MS analysis at $\lambda = 280$ nm (see the Supporting Information). MS data are given in Dalton (Da). A) Sequential labeling of protein **7** containing two Cys residues with biotin and fluorescein probes. The crystal structure shown is the N-terminal domain of anthrax toxin lethal factor 1–263 (PDB ID: 1-J7N). Reaction conditions: 1) **11** (0.5 mM), **7** (26 μM), GST (2 mg mL^{-1}), TCEP·HCl (20 mM), 0.1 M phosphate buffer, pH 8.0, 37 °C, 2 h; 2) **8** (13 μM), **10** (100 μM), 0.1 M phosphate buffer, pH 6.0, room temperature, 10 min. B) Peptide macrocyclization catalyzed by GST. Reaction conditions: **12** (1 mM), GST (2 mg mL^{-1}), TCEP·HCl (20 mM), 0.1 M phosphate buffer, pH 8.0, 37 °C. MS data are given in Dalton (Da). C) GST-catalyzed conjugation of GSH to peptide **3b** containing the Cys-**III** residue in various solvent mixtures and at various temperatures. The reaction conditions were the same as those in Figure 2 except for the solvent (left-hand graph, percentages correspond to the volume ratio) and temperature (right-hand graph; see Figure S15 for complete LC–MS analysis). DMSO: dimethyl sulfoxide.

mediated reactivity modes suited for the chemoselective modification of biomolecules.

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