Sulfoxide-mediated Cys-Trp-selective bioconjugation that enables protein labeling and peptide heterodimerization

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ABSTRACT: A method was developed that enables the magnesium chloride ($MgCl_2$)-activated *S*-acetamidomethyl cysteine sulfoxide (Cys(Acm)(O)) to induce the sp2(C−H) sulfenylation of the indole ring of Trp residues. The reaction operates under mild acidic conditions using acetic acid or an ionic liquid in a highly Trp-selective manner to give the Trp-sulfenylated products. Other than Trp, all other proteinogenic amino acids are unreactive to the sulfenylation conditions. We demonstrated the successful application of this reaction to a variety of peptides, including lysozyme protein. Furthermore, we achieved the Trp-modification of a monoclonal antibody (trastuzumab) by a $MgCl_2$-mediated reaction in an acidic ionic liquid. The resulting modified antibody exhibited antibody performance comparable to the parent protein. The presence of an amide moiety in the Acm group contributes to the difference in chemical behavior between *S*-Acm and *S*-*p*-methoxybenzyl (MBzl)-protected cysteine sulfoxide. This is because the *S*-Acm sulfoxide is converted to S-chlorocysteine responsible for Trp-sulfenylation under less acidic conditions than those required for the reaction of *S*-MBzl sulfoxide. Based on this rationale, we prepared a linker possessing *S*-Acm and *S*-MBzl oxide moieties and subjected the linker to hetero dimerization of DNA-binding MYC and MAX peptides containing a Trp handle. The one-pot/stepwise Cys-Trp conjugation between the linker and DNA-binding peptides allowed the generation of a heterodimeric MYC/MAX DNA binder.

INTRODUCTION

Residue-specific reactions that enable the site-selective incorporation of artificial units into peptides/proteins have advanced research relevant to a variety of biological and biomedical disciplines that involve peptide/protein molecules.[1] Such reactions include bioconjugations specific to cysteine (Cys) and tryptophan (Trp), which are both low in relative abundance in proteins. Typically, general bioconjugation reactions on Cys utilize alkylation of the nucleophilic thiol[2] or metal-catalyzed coupling of the thiol with aryl halides.[3] Umpolung strategies of the nucleophilic thiol have also become an alternative coupling format.[4] In terms of Trp-modification, a variety of indole-selective reactions have been utilized that include transition metal-,[5] metal carbenoid-,[6] or radical-mediated reaction.[7,8] The C−H sulfenylation of indole with electrophilic sulfenyl chlorides,[9] such as *S*-chlorocysteine or its congener, represents an inter-residue selective conjugation between Trp and Cys residues, that requires the oxidative umpolung to the *S*-chlorocysteine using harmful chlorination agents such as sulfuryl chloride (SO_2Cl_2).[10] Accordingly, these reactions are not compatible with the presence of Trp. Recently, we have developed inter-residue selective reactions between Cys and Trp residues. In these reactions, the *S*-*p*-methoxybenzyl or *S*-acetamidomethyl cysteine sulfoxide (Cys(MBzl)(O) or Cys(Acm)(O))[11,12] moieties are converted to the corresponding *S*-chlorocysteine under acidic conditions using trifluoroacetic acid (TFA) as solvent in the presence of guanidine hydrochloride (Gn·HCl) or diisopropylamine hydrochloride (DA·HCl). The resulting electrophilic species participate in aromatic electrophilic substitution (S_EAr) reactions with indole to achieve the C−H sulfenylation of the Trp (Figure 1).[12] Here, Gn·HCl or DA·HCl is indispensable for the reaction, because they function as cation scavengers (amine part) and chloride anion source. TFA activates the sulfoxide to a sulfonium cation and assists in liberating the protecting group (***P***) as a cation from the resulting sulfonium intermediate. The use of sulfoxide enables the oxidant-free/acid-mediated

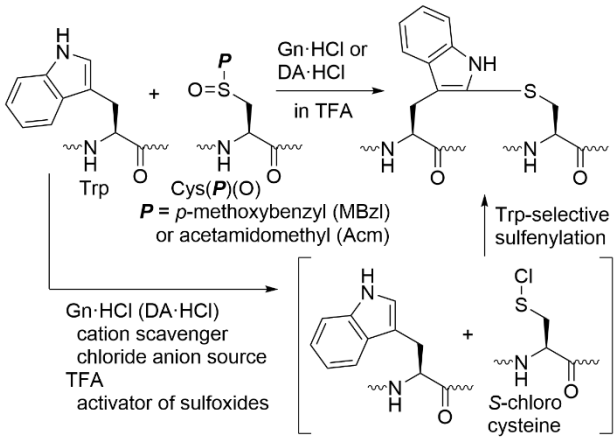


Figure 1. C−H Sulfenylation of Trp using acid-activated *S*-protected cysteine sulfoxide in the presence of guanidine hydrochloride (Gn·HCl) or diisopropylamine hydrochloride (DA·HCl)).

conversion to the *S*-chlorocysteine without affecting the indole ring of Trp. Consequently, this format allows intramolecular or intermolecular Cys-Trp linking in peptides. However, the use of TFA as a protein denaturant prevents the method from being applied to proteins. In our current work, we have achieved an alternative sulfoxide-mediated Cys-Trp conjugating condition that is applicable to a wide variety of proteins. Our investigation began by examining whether *S*-MBzl or *S*-Acm[13] groups allow formation of *S*-chlorocysteine from the corresponding sulfoxide under less acidic conditions than TFA. The generation of *S*-chlorocysteine proceeds with the *S*-protection being liberated as a cation. Removal of MBzl generally requires stronger acidity than TFA, which indicates that Cys(MBzl)(O) is not a suitable derivative for sulfenylation of Trp in proteins. A critical consideration for *S*-protection came from the unique chemical character of the *S*-Acm group[13] that is widely used in peptide synthesis. The Acm group remains intact under highly acidic conditions. This is probably due to the formation of dicationic species resulting from dual protonation on the sulfur and amide of the *S*-Acm moiety. In contrast, the coordination of the sulfur atom to metals (Hg, Tl, Pd etc)[13,14] or iodine[15] under less acidic conditions results in Acm deprotection through the formation of monocationic metalated or halogenated sulfonium species (Figure 2a). This prompted us to hypothesize that coordination of the sulfoxide oxygen of Cys(Acm)(O) with an appropriate metal chloride under less acidic conditions

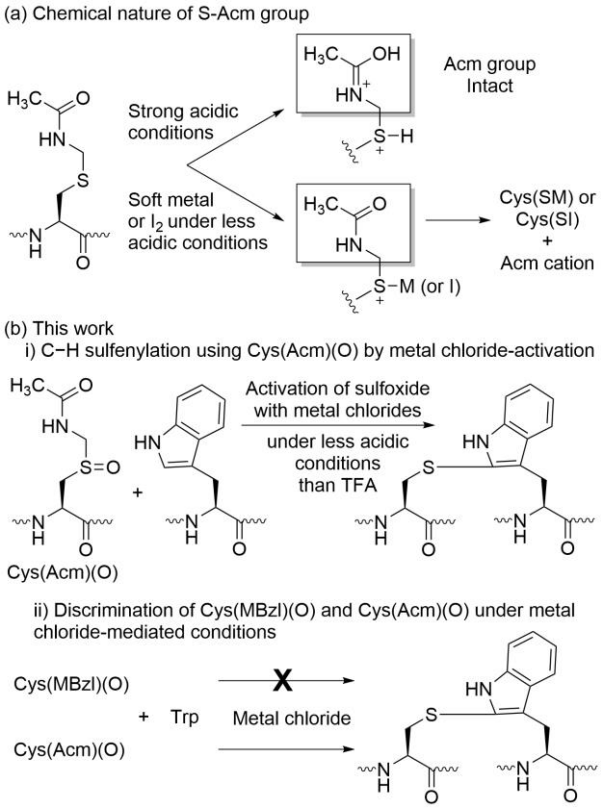


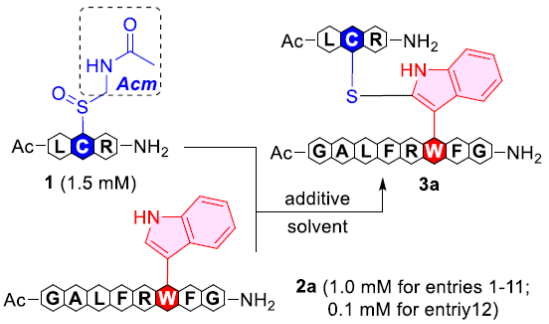
Figure 2. Envisioned application of *S*-acetamidomethylcysteine sulfoxide (Cys(Acm)(O)) to the C−H sulfenylation of Trp inspired by the unique chemical nature of Cys(Acm).

might result in forming *S*-chlorocysteine via the oxymetalated sulfonium monocationic intermediate, which could easily release the Acm cation (Figure 2b(i)). Herein we describe the development of a Trp-selective modification that proceedes under protein-friendly conditions, which is enabled by metal chloride-activated Cys(Acm)(O). This methodology has practical application to a wide variety of peptides/proteins. In addition, by taking notice of the difference in the chemical behavior between *S*-Acm and *S*-MBzl oxides in the presence of a metal chloride (Figure 2b(ii)), we were able to create a linker that enabled the one-pot/stepwise heterodimerization of Trp-containing peptides.

RESULTS and DISCUSSION

Initially, we evaluated the utility of using metal chlorides for forming thioether linkages between Trp and Cys using the model peptides (Ac-**L**-Cys(Acm)(O)-**R**-NH_2 (**1**) and Ac-**GALFR**-Trp-**FG**-NH_2 (**2a**)). We employed AcOH as solvent because this solubilizes a variety of peptides (Table 1, Figures 3 and S5). The requisite substrate peptide **1** was easily obtainable by oxidation of the precursor peptide (Ac-**L**-Cys(Acm)-**R**-NH_2) using $NaIO_4$ in MeCN/H_2O (Figure S3). Incubation of a mixture of **1** (1.5 mM) and **2a** (1.0 mM) in AcOH at 25 °C for 15 h in the absence of chloride anion failed to give Cys-Trp-linked peptide **3a** with both substrates remaining intact (Table 1, entry 1, Figure S5(A)). Addition of tetra-*n*-butylammonium chloride ((*n*Bu)$_4$NCl) resulted in the slight formation of the desired **3a** in 6% conversion yield (entry 2, Figure S5(B)). The use of lithium chloride (LiCl) or calcium chloride ($CaCl_2$) improved the reaction outcome, such that the desired **3a** was obtained over in 40% conversion yield (entries 3 and 4, Figures S5(C and D)). Further improvement resulted from the use of magnesium chloride ($MgCl_2$). The reaction of **1** (1.5 mM) with **2a** (1.0 mM) in AcOH in the presence of 30 mM $MgCl_2$ at 25 °C for 15 h proceeded in over 95% conversion (entry 5, Figure S5(E)). The use of other solvent systems (AcOH/H_2O, hexafluoroisopropanol (HFIP) and trifluoroethanol (TFE)) failed to give **3a** (entries 6−8, Figures S5(F and G)). The use of HFIP led to the formation of $MgCl_2$-precipitated mixtures. The presence of a large volume of water resulted in decomposition of the transiently formed *S*-chlorocysteine to yield cysteine sulfenic acid (Cys-S-OH),[16] which self-dimerized to give peptides S2 (Figure S5(F)). Addition of a small amount of TFA (0.1% TFA in AcOH) accelerated the linking reaction (entry 9, Figures 3(a) and S5(H)) to afford **3a** in 60% isolated yield (Figure S6). Here, $MgCl_2$ serves as an indispensable Lewis acid, no desired reaction being observed in the absence of $MgCl_2$ (entry 10, Figure. S5(I)). Replacement of TFA with sodium trifluoroacetate significantly decreased the reaction efficacy (entries 9 vs. 11, Figure S5(J)). This indicates that TFA functions as a crucial acidic accelerator for the reaction, although its precise role remains unclear. Further application of the $MgCl_2$-mediated Cys-Trp linking reaction to a range of proteins requires widening the available solvent combinations that are appropriate to protein modifications. Although the most ideal solvent for proteins is typically aqueous, the system that we developed doesn't work well under aqueous condi-

Table 1. Examination of metal salts for forming the Cys-Trp linkage



entry	additive[a]	solvent (conditions)	conversion (%)[b]
1[c]	none	AcOH (25 °C, 15 h)	0
2[c]	(nBu)$_4$NCl	AcOH (25 °C, 15 h)	6
3[c]	LiCl	AcOH (25 °C, 15 h)	46
4[c]	$CaCl_2$	AcOH (25 °C, 15 h)	43
5[c]	$MgCl_2$	AcOH (25 °C, 15 h)	>95
6[c]	$MgCl_2$	50% AcOH in H_2O (25 °C, 15 h)	ND[e]
7[c]	$MgCl_2$	HFIP (25 °C, 15 h)	ND[f]
8[c]	$MgCl_2$	TFE (25 °C, 15 h)	ND
9[c]	$MgCl_2$	0.1% TFA in AcOH (25 °C, 6 h)	>95
10[c]	none	0.1% TFA in AcOH (25 °C, 6 h)	0
11[c]	$MgCl_2$	0.1% Na·TFA in AcOH (25 °C, 6 h)	34
12[c]	$MgCl_2$	0.1% TFA in BMPy·OTf (50 °C, 12 h)	73
13[c]	$MgCl_2$	0.1% TFA 1% H_2O in BMPy·OTf (50 °C, 12 h)	55
14[d]	$MgCl_2$	0.1% TFA 1% H_2O in BMPy·OTf (50 °C, 12 h)	93

[a]Additives (30 mM) were added except for LiCl (60 mM). [b]Conversion (%) were determined by HPLC analysis with UV detection at 220 nm and calculated using the equation: percent formation = 100 [integ. **3a**/(integ. **2a** + **3a**)], where integ. = integration of peak area of the UV absorption. [c]Substrates **1** (1.5 mM) and **2a** (1.0 mM) were reacted. [d]Substrates **1** (1.5 mM) and **2a** (0.1 mM) were reacted. [e]Substrate **1** was decomposed. [f]$MgCl_2$ hardly dissolved in HFIP.

tions (entry 6). Recently, ionic liquids have become a potential choice as an alternate to water in protein chemistry, and bioconjugation using an ionic liquid (1-butyl-1-methylpyrolidinium trifluoromethanesulfonate (BMPy·OTf)) has been reported.[17,18] In light of this, we next examined the applicability of $MgCl_2$-mediated Trp sulfenylation under ionic acid conditions. We initially attempted reacting **1** (1.5 mM) with **2a** (1.0 mM) in BMPy·OTf in the presence of 30 mM $MgCl_2$ and 0.1% TFA at 50 °C. However, the reaction mixture became high viscous and achieving complete dissolution of the materials was highly time-consuming. None-the-less, we were able to obtain the desired product **3a** in moderate yield after reacting for 12 h (entry 12, Figure S5(K)).

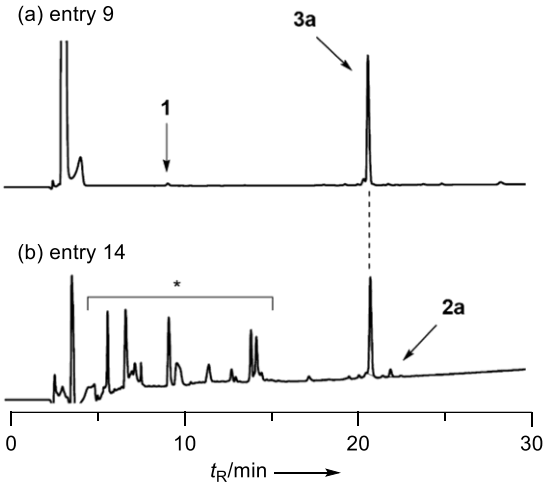
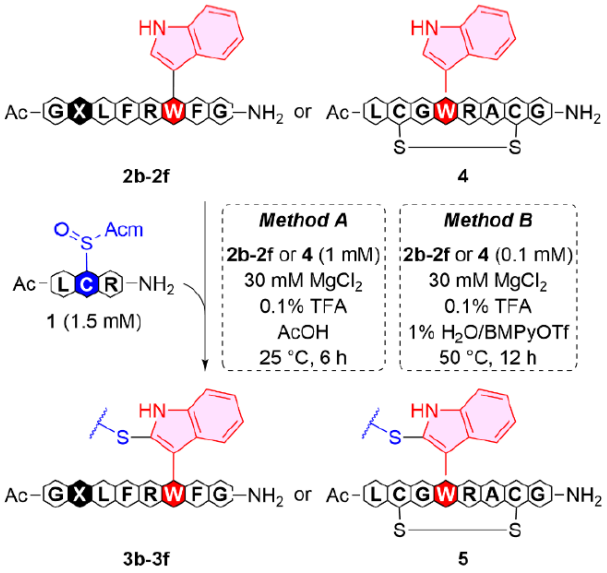


Figure 3. HPLC analyses of the C−H sulfenylation of **2a** with the Cys(Acm)(O) peptide **1** in AcOH ((a) entry 9 in Table 1) or in BMPy·OTf ((b) entry 14 in Table 1). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 65% over 30 min. UV detection at 220 nm. *materials derived from the use of excess amount of **1** in the BMPy·OTf system, see SI (Figure S7).

Addition of H_2O (1%) provided a clear solution; however, conversion to product **3a** decreased in yield to 55%. This was probably due to the decomposition of *S*-chlorocysteine species in the presence of H_2O (entry 13, Figure S5(L)). Using an excess of **1** (1.5 mM) relative to **2a** (0.1 mM) led to the almost complete consumption of **2a**, affording **3a** in high yield (entry 14, Figure. 3(b) and S5(M)). Early eluting HPLC peaks observed in entry 14 resulted from excessively large Cys(Acm)(O) peptide **1** as shown in Figure S7. Trp-sulfenylation at the 2-position of the indole was confirmed by the reaction of Ac-Trp-OMe (**S3**) with **1** followed by NMR analyses (Figures S8−10).

Having established Trp-sulfenylation systems accessible using $MgCl_2$-activated Cys(Acm)(O), we next explored applying this methodology to a variety of peptide substrates, including model peptides (Ac-**G**-Xaa-**LFR**-Trp-**FG**-NH_2: Xaa = Lys (**2b**), Tyr (**2c**), Met (**2d**), His (**2e**), Ser (**2f**) and disulfide form of Ac-**L**-Cys-**G**-Trp-**RA**-Cys-**G**-NH_2 (**4**)) and biologically active peptides (Kisspeptin-10[19] (**6a**), α-MSH[20] (**6b**), Octreotide[21] (**6c**) and Daptomycin[22] (**6d**)). Each reaction of **2b-f**, or **4** (1.0 mmol) with **1** (1.5 mM) in AcOH performed in the presence of 30 mM $MgCl_2$ and 0.1% TFA at 25 °C went to completion after 6 h to afford the desired products **3b-f**, or **5** in quantitative yield (>95%) (Table 2 Method A, entries 1-6 (Figures 4(a) for **2c** and S11 for **2b**−**f**, **4**)). Trp selective sulfenylation was confirmed by trypsin digestion of each product followed by peptide mapping (Figure S12). As compared to reactions run in AcOH, the sulfenylation of **2b-f** (0.1 mmol) with **1** (1.5 mM) in 1% H_2O/BMPy·OTf containing 30 mM $MgCl_2$ and 0.1% TFA at 50 °C exhibited decreased reaction efficiency to give **3b-f** in moderate yield (73−85%), with the exception of the disulfide-containing

Table 2. Substrate versatility



entry	Trp-containing peptide	Method	conversion (%)[a]
1	**2b** (**X** = Lys)	A	**3b** (>95)
2	**2c** (**X** = Tyr)	A	**3c** (>95)
3	**2d** (**X** = Met)	A	**3d** (>95)
4	**2e** (**X** = His)	A	**3e** (>95)
5	**2f** (**X** = Ser)	A	**3f** (>95)
6	**4**	A	**3g** (>95)
7	**2b** (**X** = Lys)	B	**3b** (82), **2b** (18)
8	**2c** (**X** = Tyr)	B	**3c** (81), **2c** (19)
9	**2d** (**X** = Met)	B	**3d** (85), **2d** (15)
10	**2e** (**X** = His)	B	**3e** (73), **2e** (27)
11	**2f** (**X** = Ser)	B	**3f** (81), **2f** (19)
12	**4**	B	**3g** (>95)

[a]Conversion (%) were determined by HPLC analysis with UV detection at 220 nm and calculated using the equation: percent formation = 100 [integ. **3a-3f** or **5** / (integ. **2a-2f** or **4** + **3a-3f** or **5**)], where integ. = integration of peak area of the UV absorption.

substrate **4** (Table 2 Method B, entries 7−12, Figures 4 (b and c) for **2c** and **4**, and Figure S13 for **2b**−**f**, **4**). Attempted sulfenylation of biologically active peptides **6** in AcOH solvent encountered difficulties related to peptide solubility, with reactions containing **6a**, **6b**, or **6d** affording a suspension of incompletely dissolved solids. The suspensions became homogeneous solutions upon adding 2% BMPy·OTf at 25 °C. However, the increase in concentration of BMPy·OTf led to a decrease in the conversion efficacy (Figure S14). The sulfenylation of **6c** (1.0 mM) with **1** (1.5 mM) in 0.1% TFA/AcOH in the presence of 30 mM $MgCl_2$ at 25 °C for 6 h (Condition A) gave the modified Octreotide (**7c**) in 94% yield (Figure S15(A)). As mentioned above, addition of BMPy·OTf, decreased the reaction efficiency. Therefore, sulfenylation of **6a**, **b** or **d** (1.0 mM) with **1** (1.5 mM) was performed with the reaction temperature elevated to 37 °C from 25 °C in 2% BMPy·OTf/AcOH

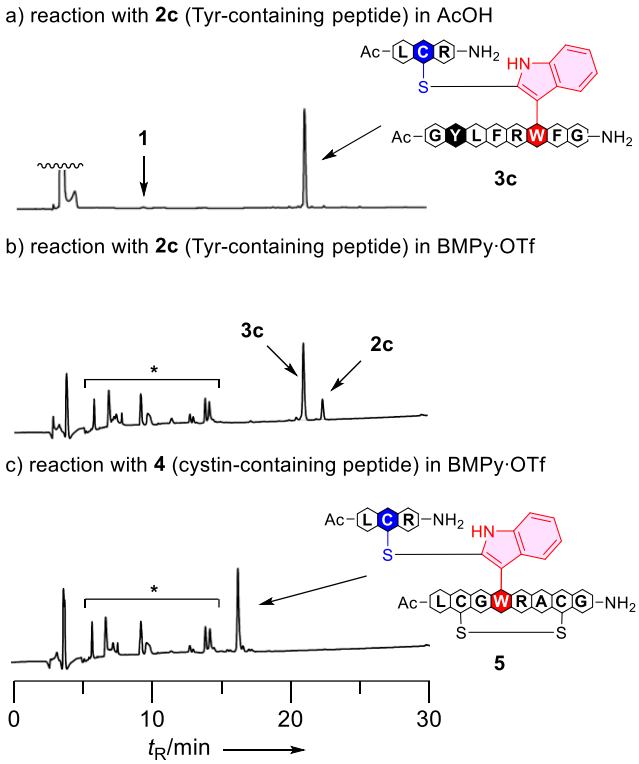


Figure 4. Representative examples of Trp-selectivity in peptides containing amino acids possibly susceptible to *S*-chlorocysteine species. (a) Reaction of **1** (1.5 mM) with Tyr-containing peptide **2c** (1.0 mM) in AcOH. (b) Reaction of **1** (1.5 mM) with Tyr-containing peptide **2c** (0.1 mM) in BMPy·OTf. (c) Reaction of **1** (1.5 mM) with Tyr-containing peptide **2c** (0.1 mM) in BMPy·OTf. *materials derived from the use of excess amount of **1** in the BMPy·OTf system, see SI (Figure S7).

in the presence of both 0.1% TFA and 30 mM $MgCl_2$ (Condition B). Under these conditions the corresponding Trp-modified peptides **7a**, **7b** and **7d** were obtained in quantitative conversion yields after 6 h (Figure 5 (inside the red frame) and Figures S15(A, B and D)). Having confirmed the versatility of the reactions against a variety of substrates, we next evaluated applying the Cys(Acm)(O) modifier agents to substrates possessing an alkyne or biotin unit (Biotin-Gly-miniPEG-Phe-miniPEG-Cys(Acm)(O)-NH_2 (**8**) or Ac-propargyl-Gly-miniPEG-Phe-miniPEG-Cys(Acm)(O)-NH_2 (**9**)). The reactions of modifiers **8** and **9** (1.5 mM) with model substrate **2a** using 30 mM $MgCl_2$-0.1% TFA in 2% BMPy·OTf/AcOH (Condition B for **8**) or in AcOH (Condition A for **9**) at 37 °C or 25 °C, respectively, for 6 h also gave the desired modified materials **10** and **11** in high conversion yields (Figure 5 (inside the blue frame) and Figures S16-S19)).

Considering the susceptibility of proteins to facile denaturing, we next evaluated the applicability of the $MgCl_2$-mediated sulfenylation to the Trp-selective labeling of proteins using an ionic liquid as

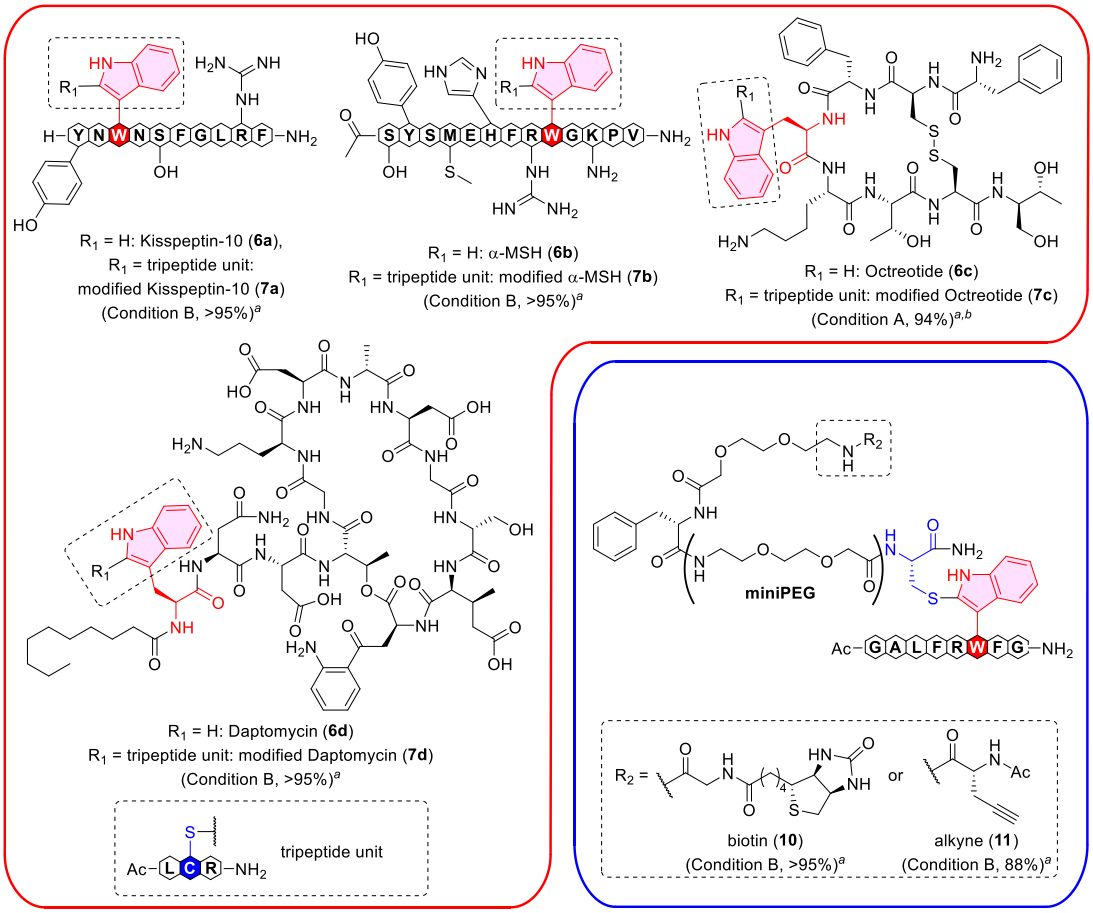


Figure 5. Results of modification of biologically active peptides **6** using Cys(Acm)(O)-containing modifiers **1**, **10**, and **11**. Unless otherwise mentioned, the reactions were conducted under condition A or B: (Condition A): The mixture consisting of a peptide (1.0 mM) and modifier (1.5 mM) was treated in 0.1% TFA/AcOH in the presence of 30 mM $MgCl_2$ at 25 °C for 6 h; (Condition B): The mixture consisting of a peptide (1.0 mM) and modifier (1.5 mM) was treated in 2% BMPy·OTf/AcOH in the presence of both 0.1% TFA and 30 mM $MgCl_2$ at 37 °C from 25 °C for 6 h. [a]Conversion yield calculated using the equation: conversion = 100 [(integ. **7**)/(integ. **6** + **7** + byproducts)], where integ. = integrated peak area of UV absorption. [b]9 h.

solvent. We subjected the six Trp-containing 129-residue lysozyme protein **12** to the reaction. The conjugation of protein **12** (0.1 mM) and the Cys(Acm)(O) peptide **1** (1.5 mM) in 5% H_2O/BMPy·OTf in the presence of 30 mM $MgCl_2$, and 0.1% TFA at 37 °C for 24 h gave a mixture of nonlabelled parent lysozyme **12** and labelled materials **13**, which could not be separated by HPLC (Figures 6a and S20). The MALDI-TOF MS analysis of the mixture indicated that the attempted reaction afforded mono-labelled material as a main component (Figures 6b and S21). In addition, the peptide label was confirmed by the MS/MS analysis to predominantly occur at the Trp108 residue (Figure S22).

The promising results for lysozyme protein prompted us to attempt the reaction incorporating a biotinylated peptide (Ac-Cys(Acm)(O)-miniPEG$_2$-Lys(Biotin)-NH_2 (**14**)) into trastuzumab **15**, a

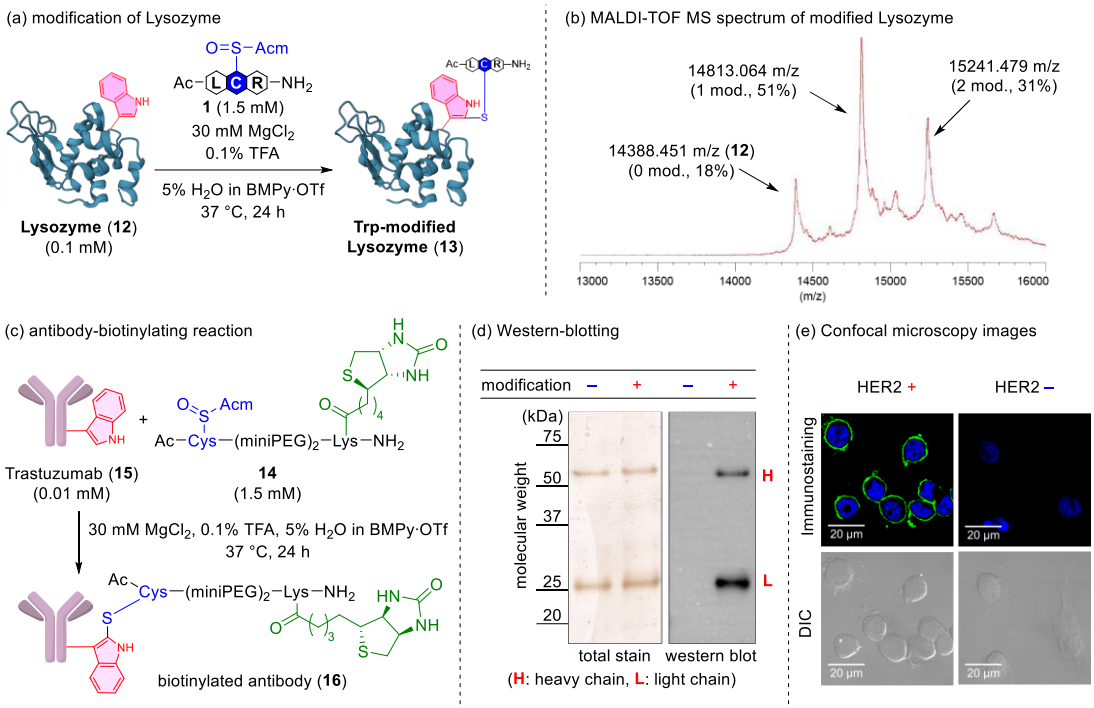
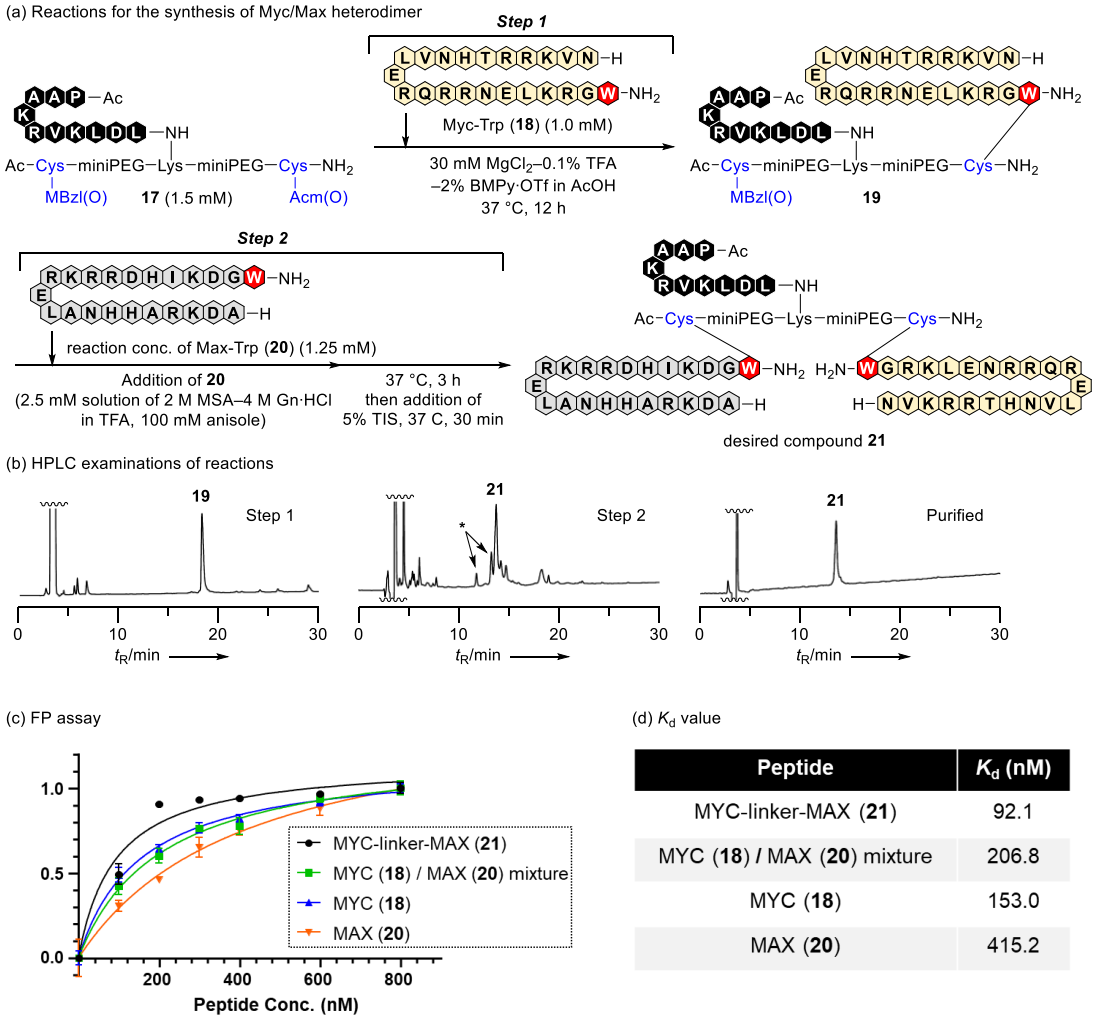


Figure 6. Modification of lysozyme **12** and trastuzumab **15**. (a) Scheme for the modification of lysozyme. (b) The result of MALDI-TOF MS analysis of modified lysozyme 13. (c) Scheme for the modification of trastuzumab **15** with Cys(Acm)(O) and biotin-containing modifier **14**. (d) Western-blotting analysis, using streptavidin−HRP conjugate, of non-modified trastuzumab **15** and biotinylated trastuzumab **16**. (e) Confocal microscopy images of (left side) HER2-overexpressing SK-BR-3 cells or (right side) HER2-unexpressing MDA-MB231 cells stained with biotinylated trastuzumab **16**, visualized by anti-human secondary antibody−fluorophore (DyLight) conjugate (green). Blue: nuclear stain with DAPI. DIC: differential interference contrast.

monoclonal antibody against HER2 antigen as a proof-of-concept trial (Figure 6c).[23] The $MgCl_2$ (30 mM)-mediated reaction of trastuzumab **15** (0.01 mM) with **14** (1.5 mM) in 5% H_2O/BMPy·OTf containing 0.1% TFA for 24 h at 37 °C gave the corresponding labelled proteins **16**. The SDS-PAGE analysis of the obtained protein sample followed by western-blotting with anti-biotin antibody-HRP conjugate showed that peptide label was incorporated on the heavy and light chains (Figures 6d and S26). LC-MS/MS analyses of the protein showed that labeling was predominantly at the Trp110 residue in the heavy chain (Figure S27).

The HER2 antigen-recognition ability of the resulting trastuzumab-biotin conjugate **16** was confirmed by confocal microscopy using SK-BR-3 breast cancer cells (HER2 (+)) and MDA-MB231 cells (HER2 (-)). An immunofluorescence signal by an anti-human IgG−fluorescein dye conjugate was



Peptide	K_d (nM)
MYC-linker-MAX (**21**)	92.1
MYC (**18**) / MAX (**20**) mixture	206.8
MYC (**18**)	153.0
MAX (**20**)	415.2

Figure 7. Heterodimerization of DNA-binding domain-extracted MYC and MAX sequences (**18** and **20**) with the NLS-containing linker **17**. (a) Reaction overview of peptide heterodimerization in a one-pot manner. (b) HPLC charts after each step. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 15% to 30% over 30 min. UV detection at 220 nm. (c) Evaluation of DNA-binding affinity of obtained MYC-linker-MAX conjugate **21** by FP assay compared with MYC and MAX monomers. (d) K_d values of MYC/MAX-derived peptides calculated from the result of FP assay.

observed following treatment of HER2 (+)-cells with the trastuzumab-biotin conjugate **16**, similar to what is observed for unconjugated trastuzumab (Figures 6e and S28). A signal by anti-biotin antibody was also detected (Figure S28).

Additionally, we conducted FACS analysis of the trastuzumab-biotin conjugate **16** using an anti-human-IgG antibody, which indicated that the affinity of the modified **16** remained unchanged. (Figure S29). The ability to perform this reaction and obtain products that retain protein function highlights the potential utility for protein labeling of Trp-sulfenylation enabled by $MgCl_2$-mediated activation of the *S*-Acm oxide unit.

As described above, the *S*-chlorocysteine requisite for Trp-sulfenylation forms from Cys(Acm)(O) by the action of $MgCl_2$ under mildly acidic conditions. However, the generation of chlorocysteine from Cys(MBzl)(O) requires stronger acidic conditions than TFA, due to acid-induced liberation of MBzl cation. Accordingly, we anticipated that Cys(MBzl)(O) would be stable under the mildly acidic Cys(Acm)(O)-converting conditions. Anticipating this selectivity, we prepared a Cys(Acm)(O)- and Cys(MBzl)(O)-containing linker for the one-pot/heterodimerization[24] of Trp-containing peptides. We examined sulfenylation of peptide **2a** with Cys(MBzl)(O)-containing peptide ((Ac-**L**-Cys(MBzl)(O)-**R**-NH_2 (**S10**)) (Figure S30). We found when reacting **2a** (1.0 mM) with **S10** (1.5 mM) in 0.1% TFA/AcOH in the presence of 30 mM $MgCl_2$, and the Cys(MBzl)(O) that no sulfenylation occurred and peptide **S10** remained intact. After adding methanesulfonic acid (MSA), DA·HCl, and anisole (final concentration: MSA (1 M), DA·HCl (2 M); anisole (50 mM)) to the reaction mixture, the sulfenylation began to afford the Trp-sulfenylated peptide **3a**. This provided a preliminary example of heterodimerization using a model system (Figure S31). Next, we examined the more challenging one-pot/stepwise heterodimerization of Trp-tagged MYC and MAX peptides[25] using a nuclear localization sequence (NLS)-embedded Cys(Acm)(O)/Cys(MBzl)(O)-containing linker (Ac-Cys(Acm)(O)-miniPEG-Lys(NLS)-miniPEG-Cys(MBzl)(O)-NH_2 (**17**)) (Figure 7a). The first sulfenylation reaction of the C-terminally Trp-tagged MYC peptide **18** (1.0 mM) with linker **17** (1.5 mM) was achieved in 2% BMPy·OTf/AcOH in the presence of 30 mM $MgCl_2$ and 0.1% TFA at 37 °C for 12 h to afford the desired Cys(MBzl)(O)-containing linker–MYC peptide conjugate **19** quantitatively (Figure 7b). Next, addition to the reaction mixture of an equal volume of MAX peptide **20** solution (2.5 mM in 2 M MSA–4 M Gn·HCl in TFA, 100 mM anisole) initiated the second sulfenylation reaction between the Cys(MBzl)(O) in linker and Trp of **20**. After reacting at 37 °C for 3 h, *S*-sulfenylation on the sulfides resulting from the excess use of sulfenylating reagent (linker) was reversed by treatment with triisopropyl silane (TIS). This one-pot/sequence of reactions for peptide heterodimerization proceeded efficiently to afford heterodimer **21** in 36% isolated yield (Figure 7b, Figures S36 and S37). Finally, the DNA-binding affinity of the resulting heterodimer **21** was examined in fluorescence anisotropy experiments by comparing with the monomer peptides and with a mixture. As shown in Figure 7, the heterodimer **21** exhibited higher binding affinity (92 nM) than the monomers (MYC 153 nM; MAX 415 nM) and the mixture (207 nM) (Figures 7c and 7d).

In summary, $MgCl_2$ plays an important role as a Lewis acid in the conversion of Cys(Acm)(O) to *S*-chlorocysteine. By combining the replacement of a strong acid along with Gn·HCl (or DA·HCl), we were able to develop a peptide- and protein-friendly protocol that allows the generation of *S*-chlorocysteine under mildly acid conditions and subsequent Trp-selective sulfenylation. This methodology permits the Trp-selective modification of a variety of peptides and proteins, including the antibody protein trastuzumab while not affecting protein function. The difference in the chemical behaviors of Cys(Acm)(O) and Cys(MBzl)(O) during their conversion to *S*-chlorocysteine upon the

Acm or MBzl cation liberation allowed us to develop a linker for peptide heterodimerization. The Cys(Acm)(O) and Cys(MBzl)(O) linker enabled the efficient heterodimerization of Trp-tagged peptides by one-pot/stepwise procedures, and successful application to the preparation of a DNA-binding MYC/MAX dimer.

ASSOCIATED CONTENT

The Supporting Information is available.

Materials and methods, synthetic procedures and characterization of compounds, detailed experimental procedures, HPLC data, Data of HPLC and MS analyses, spectroscopic data (PDF)

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