# Fluorescent substrates for covalent protein labeling catalyzed by microbial transglutaminase†

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Novel small substrates with a variety of fluorophores were designed for the covalent labeling of proteins catalyzed by microbial transglutaminase (MTG). The new design is based on the flexibility in the substrate recognition of MTG for the substitution of the N-terminal protecting group of a conventional transglutaminase substrate, benzyloxycarbonyl-L-glutaminylglycine (Z-QG). Here we report for the first time that MTG can accept diverse fluorophores (dansyl, fluorescein, and rhodamine derivatives) in place of the benzyloxycarbonyl moiety when linked *via* a β-alanine or ε-aminocaproic acid linker. The utility of the new fluorescent substrates was demonstrated by site-specific, covalent and quantitative labeling of an MTG-reactive Lys-containing peptide tag fused to the N-terminus of a recombinant bacterial alkaline phosphatase with retention of target protein functionality.

## Introduction

Site-specific labeling of proteins with small molecules has important applications in probing and/or elucidation of protein functions *in vivo* and *in vitro*. The labeling strategy can generally be divided into two categories based on the mode of modification: non-covalent and covalent protein labeling. In particular, site-specific and covalent protein labeling with a variety of small molecules is found to be very useful due to its robustness, especially in diluted cellular environments. Recombinant proteins tagged with a polypeptide, which can be post-translationally modified using a number of methods, are quite useful. In this approach, incorporation of such a tag into the N- and/or C-termini of target proteins is feasibly conducted by standard genetic manipulation.[1] Recent creative research into specific modification of a fused polypeptide tag has evoked a wide variety of new techniques for site-specific and covalent protein labeling.[2–4] Among the approaches for post-translational labeling, enzymatic modification could offer an intriguing choice compared with non-enzymatic approaches in terms of both high site-specificity and mild reaction conditions. For instance, mutant human $O^6$-alkylguanine-DNA alkyltransferase[5] (i.e. SNAP-tag, 182 aa) and mutant haloalkane dehalogenase[6] (i.e. HaloTag, 296 aa) were successfully used as self-labeling polypeptide tags that irreversibly bound functionalized synthetic ligands at the active site both *in vitro* and *in vivo*. Although these methods have been shown to be capable of the site-specific labeling of proteins, the relatively large size of an enzyme tag may influence the expression and intrinsic properties of the target proteins.

Alternatively, a short peptide tag comprising the substrate sequence of transferases and ligases can also be specifically labeled with the corresponding enzymes.[4] Transglutaminase (TGase), an enzyme that functions in the post-translational covalent cross-linking of specific Gln- and Lys-containing peptides and proteins,[7,8] has been recognized as a potent enzyme for post-translational protein labeling. TGase has been employed extensively for *in vitro* labeling of native proteins,[9,10] soluble recombinant proteins[11–13] and a cell surface protein[14] with small molecules. In fact, a useful catalytic property of TGase, in which amine-terminating small probe molecules such as cadaverine derivatives are used as substrates, allows the labeling of a specific Gln residue in native and recombinant proteins. However, little information is available for a Gln-containing small fluorescent probe for labeling a specific Lys residue in target proteins. The pioneering work by Fuchsbauer demonstrated that chemical labeling of the C-terminal carboxylic group of Z-QG with monodansylcadaverine yielded a fluorolabeled Z-QG. The designed fluorescent substrate was efficiently incorporated into reactive Lys residues in αS1-casein by microbial transglutaminase (MTG).[15] In this context, C-terminal fluorolabeled Z-QG and Z-QQPL derivatives were prepared for MTG- and human tissue transglutaminase-mediated labeling of IgG antibodies, respectively.[16] However, all the existing fluorescent Gln-donor substrates of MTG are comprised of the core structure, Z-QG.

Here, we report a set of novel small fluorescent substrates that allow MTG-mediated post-translational modification of a specific Lys residue in the target proteins *in vitro*. We focused on the N-terminus of Z-QG, and it was found that the benzyloxycarbonyl moiety (Z) can be replaced with synthetic dyes if they are joined *via* a linker of a suitable length. Interestingly, MTG accepts a wide variety of fluorophores with the new dipeptide substrate architecture. This new design concept of the substrate peptide

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will expand the utility of MTG to label either a specific Lys residue introduced as a fusion tag to target proteins or naturally occurring Lys-acceptor substrates of this enzyme both *in vitro* and *in vivo.*

## Results and discussion

### Design of new fluorescent substrates for MTG and their validation for protein labeling studies

A model substrate of TGase, Z-QG (Fig. 1), has been widely employed to measure the catalytic activity of TGases from different origins.[17] In particular, Z-QG is one of the most reactive Gln-donor substrates for MTG although GQG is not accepted as a substrate.[18] In addition, the heptapeptides, GLGQGGG and GGLQGGG, are recognized by MTG while GGGQGGG shows little activity.[19] These results suggest that the presence of hydrophobic groups at the N-terminus of the reactive Gln residue is critical for substrate recognition by MTG. This substrate specificity could be explained by the presence of six hydrophobic aromatic residues (W59, Y62, Y69, Y75, Y278, Y291 and Y302) distributed on the protein surface around the active site cleft.[20] Considering these facts, we hypothesized that the replacement of the benzyloxycarbonyl group of Z-QG with a hydrophobic functional moiety might facilitate substrate recognition by MTG.

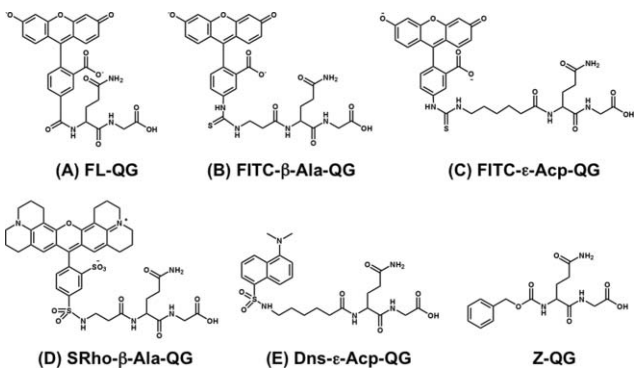


**Fig. 1** Chemical structures of the newly designed substrates and a conventional substrate of MTG. (FL-QG (A), FITC-β-Ala-QG (B), FITC-ε-Acp-QG (C), SRho-β-Ala-QG (D), Dns-ε-Acp-QG (E), and Z-QG.)

Fig. 1 shows the chemical structure of the new fluorescent MTG substrates validated in this study. Firstly, we investigated MTG-mediated protein labeling using FL-QG and FITC-β-Ala-QG, two fluorescein-derivatized substrates with different linker lengths. In FL-QG, the fluorescein moiety on the N-terminus of QG was directly introduced using 5(6)-carboxyfluorescein, to avoid the possible Edman degradation that could occur when fluorescein-4-isothiocyanate is employed. Recombinant *Escherichia coli* alkaline phosphatase (AP) with a N-terminal fused acyl-acceptor substrate peptide tag was employed (abbreviated as NK6-AP[21]) as a model protein.

As shown in Fig. 2A, no reaction was observed with FL-QG. However, insertion of a short linker, β-Ala, between the fluorophore and the N-terminus of QG afforded the MTG-mediated labeling of NK6-AP (lanes 1 and 3 in Fig. 2B). In the

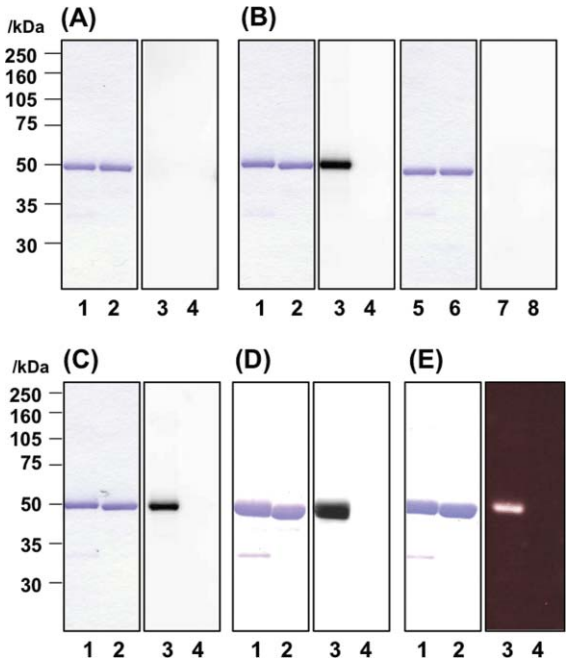


**Fig. 2** MTG-mediated labeling of NK6-AP or wild-type AP with (A) FL-QG, (B) FITC-β-Ala-QG, (C) FITC-ε-Acp-QG, (D) SRho-β-Ala-QG and (E) Dns-ε-Acp-QG. SDS-PAGE analysis of NK6-AP treated with MTG (lane 1) or without MTG (lane 2) and the corresponding fluorescent images of the gel before CBB staining (lanes 3 and 4, respectively). SDS-PAGE analysis of wild-type AP treated with MTG (lane 5) or without MTG (lane 6) and the corresponding fluorescent image of the gel before CBB staining (lanes 7 and 8, respectively).

case of wild-type AP, no labeling was found (lanes 5 and 7 in Fig. 2B), suggesting that the labeling site should be within the fused NK6-tag. Next, we tested FITC-ε-Acp-QG, in which a longer ε-aminocaproic acid linker had been introduced. Fig. 2C clearly showed that FITC-ε-Acp-QG was also enzymatically attached to NK6-AP. No significant difference in reactivity between FITC-β-Ala-QG and FITC-ε-Acp-QG was found at the saturation point (lane 3 in Fig. 2B and C).

In the series of experiments with fluorescein-derivatized substrates (FL-QG, FITC-β-Ala-QG and FITC-ε-Acp-QG in Fig. 2A–2C), it was clearly shown that direct conjugation of the fluorophore to the N-terminus of QG hinders the substrate recognition by MTG. In fact, the active site cleft of MTG is ~16Å deep from the protein surface,[20] so that the γ-carboxyamide side chain of Gln in FL-QG may not have access to the active site of MTG possibly due to the steric hindrance. Accordingly, the existence of a short linker in the designed substrates, FITC-β-Ala-QG and FITC-ε-Acp-QG, made them suitable MTG substrates possibly due to the relaxation of steric constraint at the active site cleft.

To explore the capability of the enzyme to accept a diverse fluorophore, we designed other substrates with relatively large substituents at the N-terminus such as SRho-β-Ala-QG. MTG can also accept a rhodamine-derivatized substrate to label NK6-AP (Fig. 2D), suggesting that the β-Ala linker could be sufficient for substrate recognition. Dns-ε-Acp-QG, derivatized with a relatively small dansyl probe connected *via* a long ε-Acp linker, was also verified to be a substrate of MTG (Fig. 2E).

 

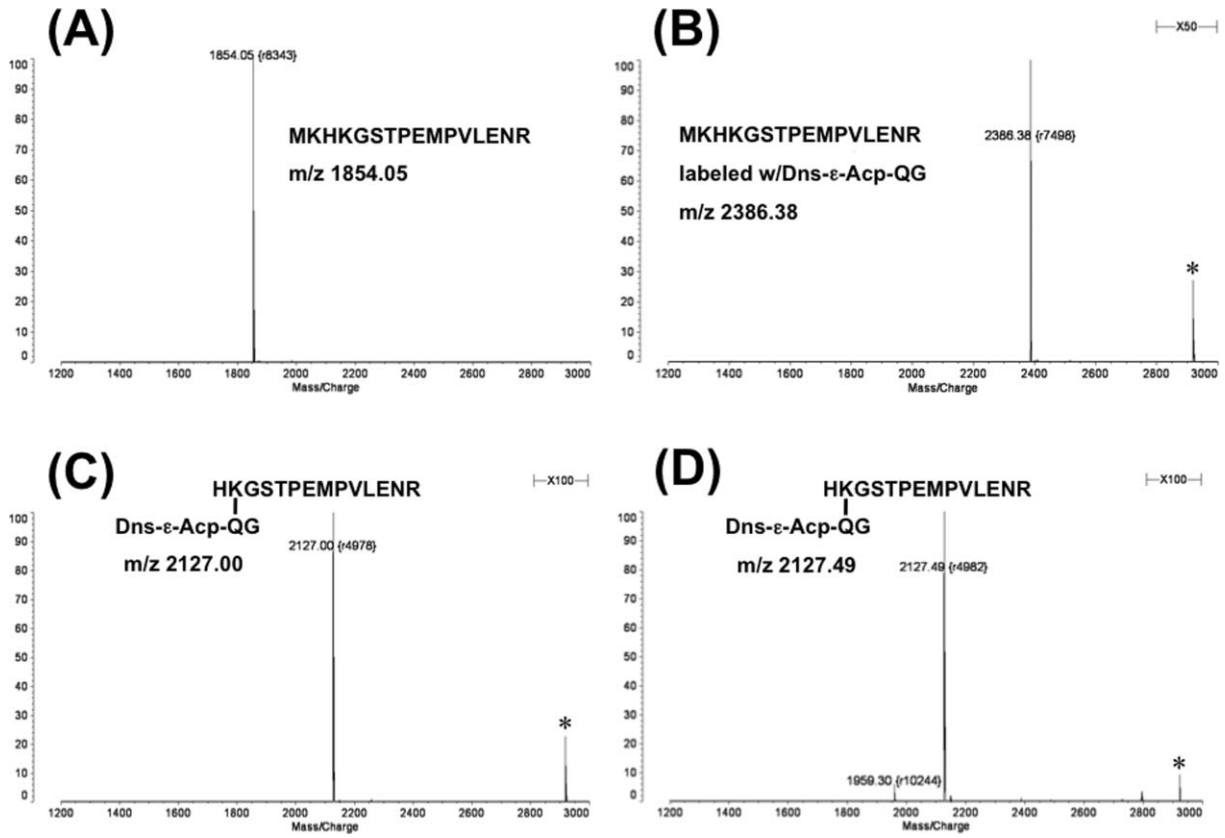


**Fig. 3** Identification of the labeling sites of NK6-AP by mass spectroscopic analysis. Top column: mass chromatograms for the non-treated (A) and Dns-ε-Acp-QG-treated (B) N-terminal synthetic peptide of NK6-AP. Bottom column: mass chromatograms of the tryptic digests of the Dns-ε-Acp-QG-treated synthetic peptide (C) and Dns-ε-Acp-QG-treated NK6-AP (D). Magnifications by a factor of 50 (B) and 100 (C, D) of the doubly labeled peptide ion region were applied.

### Evaluation of the site-specificity of enzymatic protein labeling by MALDI-TOF MS analysis

The practical utility of MTG-mediated *in vitro* protein conjugation has been previously validated.[22–24] Although the SDS-PAGE analysis of labeled wild-type AP may explain the high site-specificity of the protein labeling (Fig. 2B), identification of the cross-linked products will lend further evidence. We first attempted the identification of labeling sites with FITC-β-Ala-QG; however, reactive Lys residues in NK6-AP could not be determined, possibly due to the low stability of the labeled peptide toward laser irradiation. On the other hand, the peptide fragments labeled with Dns-ε-Acp-QG were clearly identified (Fig. 3).

To identify the labeling site of NK6-AP, we first investigated the MTG-mediated labeling of the synthetic N-terminal peptide, MKHKGSTPEMPVLENR, with Dns-ε-Acp-QG (MW 549.23). As shown in Fig. 3A and B, mass chromatograms of the intact synthetic peptide and the Dns-ε-Acp-QG-treated peptide showed a single strong peak, of which m/z values were 1854.05 and 2386.38, respectively. Although a very weak signal that corresponded to a doubly labeled peptide (m/z 2920.00) was also identified at 50-fold magnification of the mass chromatogram (Fig. 3B), the results suggested that single attachment of Dns-ε-Acp-QG to the synthetic peptide had occurred.

There are two possible labeling sites in the N-terminally fused peptide tag (i.e. NK6-tag). To determine the reactive Lys residues labeled, the labeled synthetic peptide was digested with trypsin and subjected to mass spectrometric analysis. Since trypsin exclusively recognizes Lys and Arg residues, MTG-mediated labeling at specific Lys residues should result in different peptide fragmentation patterns. If K2 was labeled, the peptide fragment, GSTPEMPVLENR ($[M + H]^+$ 1329.65), would be detected. However, the corresponding signal was not detected. By contrast, we could clearly identify the singly labeled peptide, HK(Dns-ε-Acp-QG)GSTPEMPVLENR (m/z 2127.00) (Fig. 3C), although the much weaker peak of the doubly labeled N-terminal peptide was also observed. The same trend was observed during the analysis of Dns-ε-Acp-QG-labeled NK6-AP (Fig. 3D). In this case, a trace signal from the doubly labeled peptide was identified. On the basis of these results, we can conclude that primarily K4 was labeled with Dns-ε-Acp-QG by MTG. In addition, the absence of the GSTPEMPVLENR fragment that was observed in the presence of non-labeled NK6-AP indicates that K4 of NK6-AP was quantitatively labeled with Dns-ε-Acp-QG by MTG. Taken together, it was revealed that one of the two Lys residues in the NK6-tag, K4, was preferentially labeled, which demonstrates the substrate specificity of MTG toward an acyl acceptor substrate.

### Efficacy of MTG-mediated protein labeling with new fluorescent substrates

A basic requirement of protein labeling technology is that the protein labeling should be rapid, inert and site-specific. Firstly, it was verified that the fluorescent labeling did not affect the

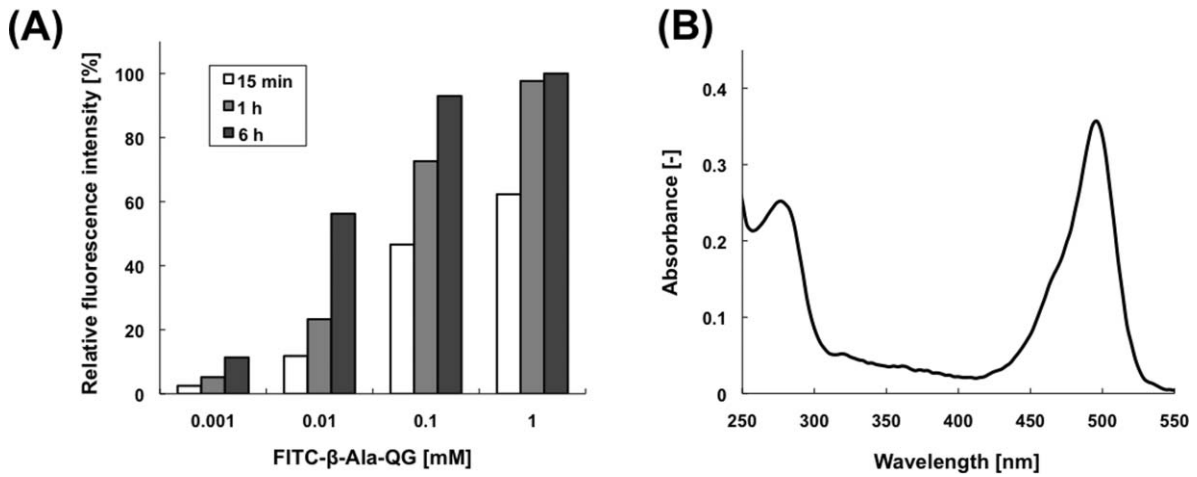


**Fig. 4** (A) Efficacy of MTG-mediated labeling of NK6-AP at different concentrations of FITC-β-Ala-QG. The fluorescence intensity derived from NK6-AP labeled with 1 mM substrate for 6 h was defined as 100%. (B) UV–vis absorption spectrum of NK6-AP labeled with FITC-β-Ala-QG.

enzymatic activity of AP (Fig. S1 in ESI†). As shown in Fig. 4A, MTG-mediated labeling of NK6-AP with FITC-β-Ala-QG was almost completed in approximately 1 h at 4 °C at the substrate concentration of 1 mM. The labeling efficiency decreased concomitantly with the decrease in substrate concentration. Since high substrate concentrations may be impractical for biochemical applications, MTG treatment with 0.1 mM FITC-β-Ala-QG for 6 h at 4 °C could be suitable conditions for attaining a sufficient degree of protein labeling. Preliminary kinetic analyses with 0.1 mM substrates revealed that FITC-β-Ala-QG showed higher reactivity than FITC-ε-Acp-QG (Fig. S2A in ESI†). The protein labeling efficiencies of SRho-β-Ala-QG and Dns-ε-Acp-QG were slightly higher than FITC-β-Ala-QG (Fig. S2B in ESI†). These results imply that both the linker length and the fluorescent moiety structure affect the substrate recognition of MTG.

Fig. 4B shows the UV–visible absorption spectrum of FITC-β-Ala-QG-labeled NK6-AP purified after the enzymatic conjugation. The characteristic absorption bands were observed at 280 nm for NK6-AP and at 493 nm for the FITC moiety of the labeled substrates. On the basis of the molar extinction coefficients, it was calculated that the degree of labeling was 1.06 molecules of FITC-β-Ala-QG per monomeric unit of NK6-AP under the experimental conditions, suggesting quantitative protein labeling.

### Flexibility of MTG in the recognition of unnatural substrate analogues and its utility in covalent protein labeling

In the present design concept, the fluorophore plays a dual role: one is for substrate recognition, and the other is as the intrinsic fluorescent probe. It is noteworthy that MTG accepts variation in both the size of the fluorophore and the linker length in the designed substrates. This flexibility can be attributed to the intrinsic capability of enzymes that act on polypeptide substrates to interact with a variety of potential substrates.[25] Since MTG is known to accept a wider range of substrates and exhibit less substrate specificity than mammalian TGases,[26] it is not surprising that MTG exhibits affinity for the structurally diverse fluorescent substrates. In fact, evaluation of FITC-β-Ala-QG using β-casein as an acyl-acceptor proteinaceous substrate showed that guinea pig liver transglutaminase exhibited much lower activity than MTG (Fig. S3 in ESI†). This exemplifies the trade-off between the flexibility of the enzyme and its substrate specificity, which will be an important consideration in the applications of TGase. A question that still remains is whether the sequence of the Lys-containing substrate can be optimized for the newly designed fluorescent Gln-donor substrates for stoichiometric protein labeling. Recent reports on high throughput screening of TGase substrate sequences using a phage-display system may be useful for identifying preferred substrate sequences.[27,28]

## Conclusions

We designed new small peptidyl substrates with a variety of fluorophores for the covalent labeling of proteins catalyzed by microbial transglutaminase. The protein labeling reaction with new fluorescent substrates was rapid and quantitative under mild conditions, suggesting its practical utility in MTG-mediated site-specific fluorolabeling of proteins. Further studies on the new substrate architecture are currently underway in our group.

## Experimental

### Materials

MTG was provided by Ajinomoto Co. Inc. (Japan) and was purified according to the reported protocol.[18] All the fluorescent substrates used in this study were synthesized by the Peptide Institute, Inc. (Osaka, Japan). The chemical structures and the abbreviations of the designed substrates (Dns-ε-Acp-QG, FITC-β-Ala-QG, FITC-ε-Acp-QG, FL-QG, SRho-β-Ala-QG. Abbreviations: β-Ala, β-alanine; ε-Acp, ε-aminocaproic acid; Dns, dansyl; FITC, fluorescein-4-isothiocyanate; FL, 5(6)-carboxyfluorescein; SRho, sulforhodamine) are shown in Fig. 1. All the substrates were identified using amino acid analysis and ESI-MS and the purity was >96% based on the HPLC analyses. The molar extinction coefficients of the substrates in 100 mM Tris-HCl (pH 8) containing 10 vol% of DMSO were as follows: Dns-ε-Acp-QG: $\lambda_{max}$/nm 330 (ε dm$^3$ mol$^{-1}$ cm$^{-1}$ 2900); FITC-β-Ala-QG: 493 (66600); FITC-ε-Acp-QG, 493 (64300); SRho-β-Ala-QG: 590 (95000). Recombinant *Escherichia coli* alkaline phosphatase

 

(AP) with a N-terminal fused acyl-acceptor substrate peptide tag (NK6-tag; tag sequence: MKHKGS) was prepared according to the previous report and the recombinant AP was abbreviated as NK6-AP.[21] The N-terminal sequence (MKHKG) was verified using N-terminal analysis (TORAY Research Center, Inc., Japan). The synthetic N-terminal peptide of NK6-AP, MKHKGSTPEMPVLENR, was synthesized by Genenet Co., Ltd. (Fukuoka, Japan). The protein concentration was determined using the BCA protein assay kit with a bovine serum albumin standard (Pierce) and based on the concentration, the molar extinction coefficient of the monomeric unit of NK6-AP was as follows: $\lambda_{max}$/nm 280 ($\varepsilon$ dm$^3$ mol$^{-1}$ cm$^{-1}$ 27500). Wild-type *Escherichia coli* alkaline phosphatase was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). All other reagents were of the highest purity commercially available.

### MTG-mediated labeling of NK6-AP with fluorescent substrates

Stock DMSO solutions of the synthetic substrates were prepared (20 mM). The reaction mixture comprised NK6-AP (0.5 mg/mL) and each substrate (1 mM) in 100 mM Tris-HCl buffer (pH 8.0) containing 5 vol% of DMSO. The protein labeling reaction was initiated by the addition of MTG (1 U/mL) at 4 °C. After incubation for 16 h, the reaction products were analyzed by SDS-PAGE. With respect to the fluorescein derivatives, 3 μL of the reaction samples were applied to the gel. In the case of SRho-β-Acp-QG and Dns-ε-Acp-QG, 8 μL of the reaction samples were applied to the gel. Before staining the gel with Coomassie Brilliant Blue (CBB) R-250 (Quick-CBB, Wako Laboratory Chemicals, Osaka, Japan), the fluorescent image of the gels was obtained using a Molecular Imager FX Pro (Bio-Rad Laboratories, Inc.). An excitation wavelength of 488 nm with a 530 (± 15) nm band pass filter for the fluorescein-derivatives and an excitation wavelength of 532 nm with a 640 (± 20) nm band pass filter for SRho-β-Ala-QG were used. For Dns-ε-Acp-QG, the gel image was obtained with a UV illuminator Printgraph (ATTO Co., Japan).

In order to follow the time course of MTG-mediated labeling of NK6-AP with FITC-β-Ala-QG, a small aliquot of the reaction mixture was periodically removed (15 min, 1 and 6 h) and mixed with the standard sample buffer for SDS-PAGE analysis (12 vol% 2-mercaptoethanol, 4 wt% SDS, 20 vol% glycerol in 100 mM Tris-HCl, pH 6.8) to terminate the MTG reaction. The reaction was followed by the increase in the fluorescence of protein bands in the fluorescent image of the SDS-PAGE gel. The MTG concentration was 0.1 U/mL.

For the determination of the degree of protein labeling, FITC-β-Ala-QG-labeled NK6-AP was purified as follows. After the enzymatic conjugation, the labeled NK6-AP was separated from MTG by using a His-tag attached to the C-terminus of NK6-AP.[21] The purification with a Ni-NTA column (HisTrap HP, GE Healthcare) was conducted by following a manufacturer's protocol. The eluted fractions containing the labeled sample were applied to a size-exclusion column (PD10 sepharose column, GE Healthcare) and equilibrated with 100 mM Tris-HCl buffer (pH 8.0) to remove small molecules, including the unreacted FITC-β-Ala-QG. In the calculation of the degree of protein labeling, the molar extinction coefficient of FITC-β-Ala-QG at 280 nm in 100 mM Tris-HCl (pH 8) containing 10 vol% of DMSO ($\varepsilon$ dm$^3$ mol$^{-1}$ cm$^{-1}$ 21100) was used.

### Identification of crosslinking sites of NK6-AP by mass spectroscopy

Dns-ε-Acp-QG was used as an acyl donor to identify the cross-linking sites of Lys residues working as acyl acceptors in NK6-AP. Incorporation of Dns-ε-Acp-QG to NK6-AP (0.5 mg/mL) or the synthetic N-terminal peptide (MKHKGSTPEMPVLENR, 0.1 mM) was conducted by the same labeling protocol described above except for the MTG concentration (0.1 U/mL). After the labeling of NK6-AP, the solution was exchanged with Milli-Q water with Micro Bio-Spin p-30 (Bio-Rad Laboratories, Inc.). After tryptic digestion in 50 mM $NH_4HCO_3$, pH 8.5, containing 3.3 μg/mL trypsin (Promega) at 30 °C overnight, the sample was cleaned and concentrated using μC18 ZipTips (Millipore). Finally, the peptide mixtures were eluted with 1.5 μL of 50% ACN/0.1% TFA onto a MALDI sample plate and mixed with 1 μL of matrices containing 5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) plus 5 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 50% ACN/0.1% TFA prior to MS analysis. MALDI-TOF MS analysis was performed using the AXIMA-CFR plus mass spectrometer (Shimadzu Biotech, Manchester, U.K.). The operating conditions were as follows: nitrogen laser (337 nm) used in reflectron mode with detection of positive ions. The acceleration potential was set to 35 kV using a gridless-type electrode. MALDI-TOF MS spectra were acquired in the auto experiment mode, from m/z 800 to 3000, and internally calibrated with two trypsin autolysis peaks (m/z 842.51 and 2211.10).

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