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ARTICLE

Labelling of Endogenous Target Protein via N–S Acyl Transfer-Mediated Activation of *N*-Sulfanylethylanilide

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The ligand-dependent incorporation of a reporter molecule (e.g., fluorescence dye or biotin) onto a endogenous target protein has emerged as an important strategy for elucidating protein function using various affinity-based labelling reagents consisting of reporter, ligand and reactive units. Conventional labelling reagents generally use a weakly activated reactive unit, which can result in the non-specific labelling of proteins in a ligand-independent manner. In this context, the activation of a labelling reagent through a targeted protein-ligand interaction could potentially overcome the problems associated with conventional affinity-based labelling reagents. We hypothesized that this type of protein-ligand-interaction-mediated activation could be accomplished using *N*-sulfanylethylanilide (SEAlide) as the reactive unit in the labelling reagent. Electrophilically unreactive amide-type SEAlide can be activated by its conversion to the corresponding active thioester in the presence of a phosphate salt, which can act as an acid-base catalyst. It has been suggested that protein surfaces consisting of hydrophilic residues such as amino, carboxyl and imidazole groups could function as acid-base catalysts. We therefore envisioned that a SEAlide-based labelling reagent (SEAL) bearing SEAlide as a reactive unit could be activated through the binding of the SEAL with a target protein. Several SEALs were readily prepared in this study using standard 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase protocols. These SEAL systems were subsequently applied to the ligand-dependent labelling of human carbonic anhydrase (hCA) and cyclooxygenase 1. Although we have not yet obtained any direct evidence for the target protein-mediated activation of the SEAlide unit, our results for the reaction of these SEALs with hCA1 or butylamine indirectly support our hypothesis. The SEALs reported in this study represent valuable new entries to the field of affinity-based labelling reagents and are expected to show great utility in protein labelling.

Introduction

Affinity-based labelling has recently attracted considerable attention as an efficient strategy for the introduction of reporter units (e.g., fluorescent dyes) onto the target proteins of bioactive compounds, with the resulting reporter-labelled proteins being used to analyze protein functions.[1-4] The affinity-based labelling reagents generally used in this method are composed of three functional units, including (1) a ligand unit for the selective labelling of a target protein; (2) a reporter unit for the visualization of the target protein; and (3) a reactive unit for the covalent attachment of the reporter unit to the target protein. For example, Hamachi et al. recently developed several labelling protocols based on ligand-directed tosyl chemistry.[5] However, the reactive units of these tosyl chemistry-based reagents are only weakly activating, which can sometimes lead to the labelling of non-target proteins in a ligand-independent manner. The reactivities of certain groups can be carefully tuned against non-target proteins to highly stable labelling reagents under physiological conditions.[6,7] The labelling of non-target proteins with an activated reagent has been reported to be largely insignificant. However, we envisaged that the development of an unprecedented labelling reagent that is efficiently activated by binding to its target, but remains otherwise unreactive would be an ideal tool for protein labelling. As part of our ongoing efforts towards the chemical synthesis of proteins using *N*-sulfanylethylanilide (SEAlide) peptide as a crypto-thioester,[8] we envisioned that application of the SEAlide unit to the labelling of proteins could result in the development of unprecedented labelling reagents as mentioned above. The amide-type SEAlide remains intact in the absence of phosphate salts and does not undergo acylation reactions with any of the nucleophilic functional groups found on peptides/proteins. However, the addition of a phosphate salt induces the N–S-acyl-transfer-mediated conversion of the stable amide species to the corresponding reactive thioester, which can acylate a variety of different nucleophiles (Scheme 1A).[9, 10] In this case, the phosphate salt acts as an acid-base catalyst, facilitating the N–S acyl transfer under neutral conditions. Protein surfaces are composed of numerous hydrophilic groups including carboxyl,

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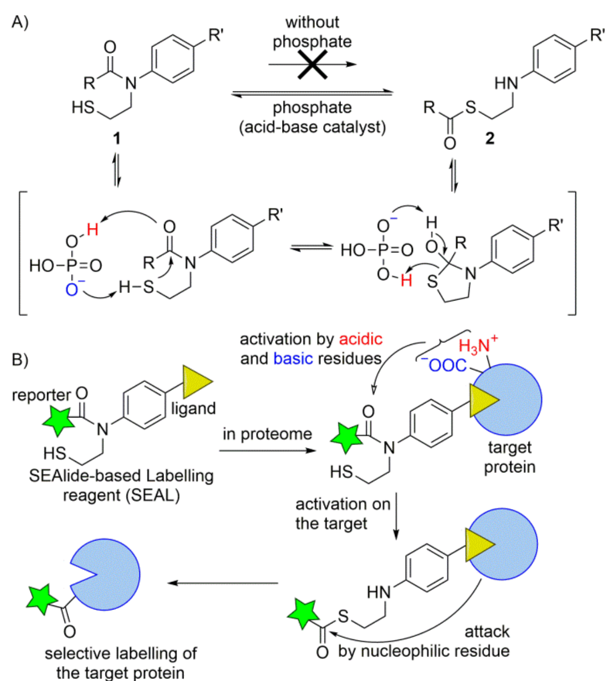
imidazole, amino and guanidine groups, which can function cooperatively as acid-base catalysts for the N–S acyl transfer. With this in mind, we hypothesized that the unactivated amide-type SEAlide moiety could be immobilized on the surface of a protein, where it could be converted to an active thioester-type SEAlide through a side chain-driven N–S acyl transfer. The resulting thioester could then show considerable promise as a reactive unit for the development of affinity-based labelling reagents (Scheme 1B). Based on our hypothesis, we designed a SEAlide-based labelling reagent (SEAL) bearing a SEAlide unit to allow for the formation of a connection the ligand and reporter units as shown in Scheme 1. By not interacting with the target, the SEAL would remain in its amide form. This would therefore prevent the SEAL from acting as an acylating agent because it would not be efficiently concentrated on the protein surface, which would be required as an acid-base catalyst. In contrast, the binding of the SEAL to the target protein via the formation of specific interactions between the ligands and targets would allow for the labelling reagent to be concentrated for a certain period on the target protein, allowing it to work as an acid-base catalyst. This would therefore result in the N–S-acyl-transfer acylation of the nucleophilic residues in a ligand-dependent manner.



Scheme 1 Design of the SEAL. A) Phosphate catalyzed activation of the SEAlide unit via N–S acyl transfer. A plausible mechanism for this transformation is shown in parentheses; B) Concept for the affinity labelling of a target protein using SEAL.

Based on this hypothesis, we prepared unreactive amide-type SEALs and successfully applied this labelling reagent to the selective affinity-based labelling of two model proteins, including human carbonic anhydrase (hCA) and cyclooxygenase 1 (COX-1).

Results and discussion

Molecular design of the SEAL

The cytosolic protein hCA was selected as a model target protein for a proof of concept study. Benzenesulfonamide (SA) was used as a specific ligand for hCA.[11-13]

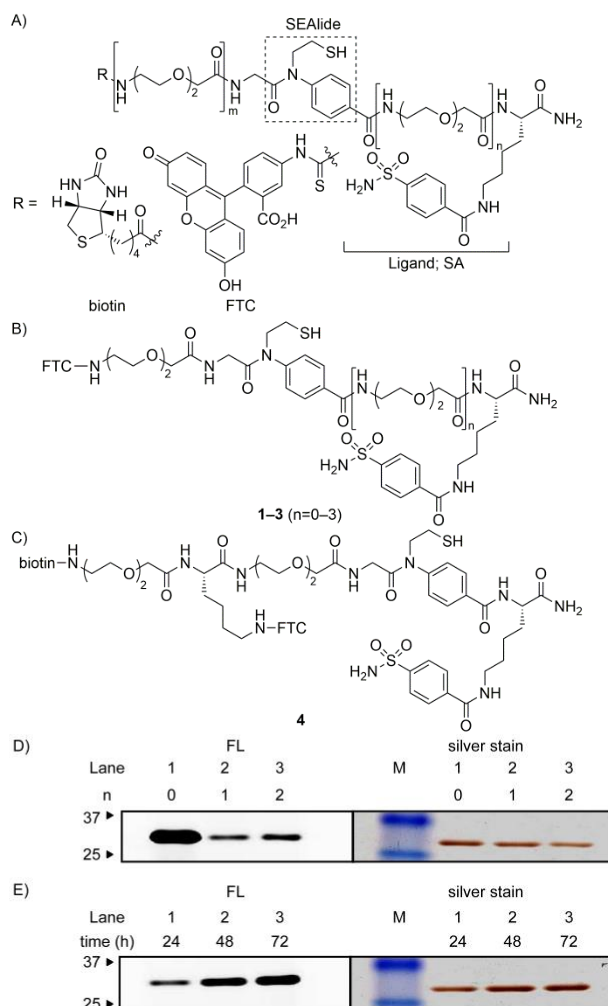


Fig. 1 Selective protein labelling using SEAL. A) Chemical structures of the SEALs containing the SA ligand for hCA and two different reporters (i.e., biotin and FTC). B and C) Chemical structures of SEALs **1**–**4**. D) SDS-PAGE analysis for the covalent labelling of purified hCA1 with **1**, **2** and **3** in a test tube. Reaction conditions: hCA1 (10 μM) and the SEAL (100 μM) were incubated in 50 mM HEPES buffer (pH 7.2) at 37 °C for 72 h. E) Time course experiment for the labelling of hCA1 with **1**. Reaction conditions: hCA1 (10 μM) and SEAL **1** (100 μM) were incubated in 50 mM HEPES buffer (pH 7.2) at 37 °C for the specified time.

A fluorescein-thiocarbamoyl (FTC) group or biotin was used as a reporter unit. The SA ligand and reporter units were connected by SEAlide to give the SEALs shown in Fig. 1A. Flexible ethylene glycol (mini-PEG) linkers of various lengths were also embedded into the SEALs. The incorporation of an S-protected SEAlide unit as a stable synthetic intermediate allowed for the desired SEALs

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to be readily prepared using standard 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase protocols, representing a considerable advantage over other techniques generally used for the synthesis of labelling reagents.

Optimization of the structure of the SEAL

We initially evaluated a variety of mini-PEG linkers of different lengths to determine which one would allow for most efficient labelling of hCA. FTC-modified SEALs **1-3** were prepared bearing varying numbers of mini-PEG repeats and incubated with purified hCA1 (10 μM) at 37 °C in 50 mM HEPES buffer (pH 7.2). Aliquots of the different reaction mixtures containing FTC-labelled hCA1 were collected every 24 h and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorescence gel imaging (FL). The results revealed that the incubation experiment involving **1** (n=0 for the chain length) afforded maximal fluorescence intensity (Fig. 1D, Lane 1). This effect was found to be specific for **1** highlighting the selective nature of this SEAL for hCA1 based on the specificity of the ligand for the ligand-binding cavities of the target protein, which is crucial for efficient labelling. Notably, the labelling of hCA1 with **1** reached a plateau within 48 h. (Fig. 1E).

We also examined whether SEALs worked through site-specific labelling. In this case, the use of the dual modified SEAL **4** containing biotin and a FTC group allowed for the labelled and unlabelled hCA1 proteins to be individually detected by SDS-PAGE. Purified hCA1 (10 μM) was incubated with **4** (100 μM) at 37 °C in 50 mM HEPES buffer (pH 7.2). The labelled hCA1 was isolated by SDS-PAGE and subjected to proteolytic digestion using trypsin in the gel, and the resulting digested peptide fragments were extracted from the gel and analysed by nanoLC-MS/MS. The resulting of this analysis revealed that Lys137, which is located in close proximity to the ligand-binding site, was labelled with the reporter (Fig. S1).

Selective labelling of target proteins

The labelling selectivity of SEAL **1** was subsequently examined using a protein mixture containing hCA1, enolase, ovalbumin (OVA) and glutathione *S*-transferase (GST). The protein mixture was incubated with **1** for 48 h at 37 °C in 50 mM HEPES buffer (pH 7.2). The resulting mixture was analyzed by SDS-PAGE, followed by fluorescence gel imaging, which revealed the present of a strong fluorescence band corresponding to the FTC-labelled hCA1 (Fig. 2). These results therefore demonstrated that we could achieve the selective labelling of a target protein using the SEAL system in a mixture of different proteins, whilst leaving the none-target protein unchanged.

To verify the versatility of these SEAL system for the selective labelling of target proteins, we attempted to label cyclooxygenase 1 (COX-1) using a SEAL containing indomethacin (IM), which is well-known as a specific ligand for COX enzyms.[14] SEAL **5** was therefore synthesized containing biotin as a reporter (Fig. 3A).

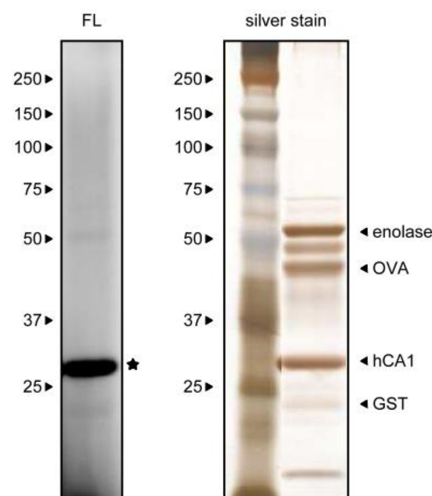

Fig. 2 SDS-PAGE analysis of the selective labelling of hCA1 with **1** in a protein mixture consisting of four different proteins in a test tube. Reaction conditions: enolase, GST, hCA1 and OVA (10 μM each) were incubated with **1** (100 μM) in 50 mM HEPES buffer (pH 7.2) at 37 °C for 48 h.

A mixture of Tris·HCl buffer (pH 8.0) containing bovine serum albumin (BSA), COX-1 and enolase was incubated with **5** for 48 h at 4 °C. Notably, this experiment was conducted at 4 °C because COX-1 was found to be unstable at 37 °C in aqueous buffer in the absence of a stabilizer.

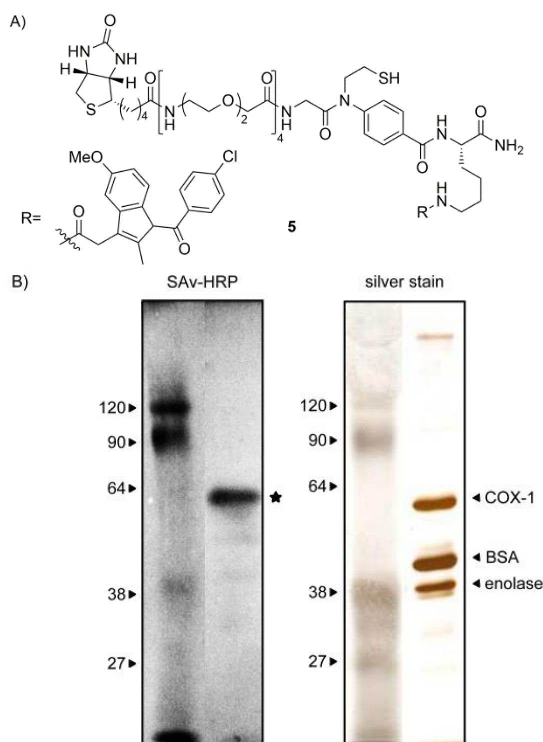

Fig. 3 SDS-PAGE analysis for the selective labelling of COX-1 in a protein mixture. A) Chemical structure of **5**, bearing indomethacin (IM) as a ligand for COX-1 labelling. B) SDS-PAGE analysis for the labelling of COX-1 with **5** in a mixture of three different proteins. Reaction conditions: BSA, COX-1 and enolase (1.4 μM each) were incubated with **5** (14 μM) in 80 mM Tris·HCl buffer (pH 8.0) at 4 °C for 48 h.

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The incubation mixture was subsequently analyzed by SDS-PAGE, which revealed the presence of a single biotin-labelled protein using a streptavidin-horseradish peroxidase conjugate (SAv-HRP). Fig. 3B shows that a single band corresponding biotinylated-COX-1 was detected at approximately 64 kDa.

Labelling of intracellular hCA

We also investigated the application of this strategy to intracellular protein labelling by attempted the selective labelling of endogenous hCA using a SEAL in human red blood cells (RBCs).[15] Low molecular weight hydrophobic compounds can generally permeate through cell membranes much more effectively than high molecule hydrophilic compounds.[16] With this in mind, we designed and synthesized SEAL **6** to provide improved membrane permeability and enhanced labelling efficiency towards intracellular proteins compared with SEAL **1**. Notably, SEAL **6** had a low molecular weight than **1** and lacked the hydrophilic mini-PEG linker (Fig. 4A). RBCs were incubated in HEPES-buffered saline (HBS) containing **6** (200 μM) for 48 h at 37 °C, before being washed three times with fresh HBS.

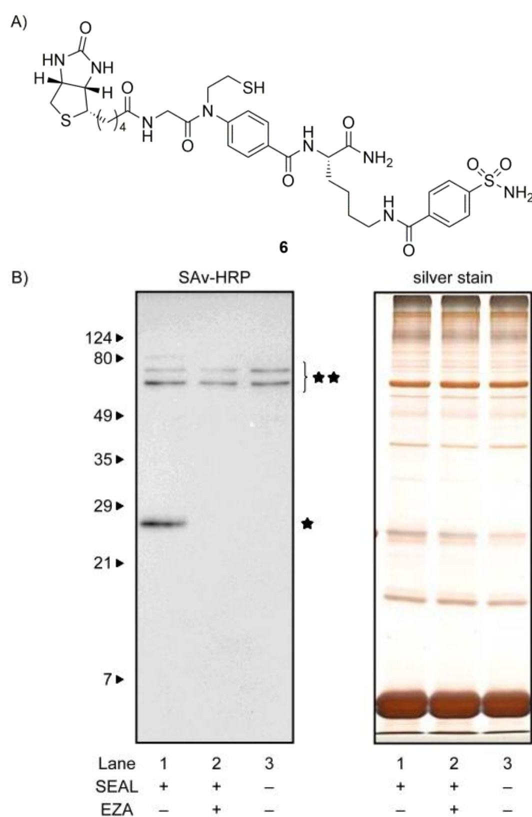

Fig. 4 Intracellular hCA labelling with **6** in RBCs. A) Chemical structure of **6**. B) SDS-PAGE analysis for the labelling of the endogenous hCA in RBCs. The bands were detected with streptavidin-HRP. The bands with a single asterisk (*) correspond to hCA, whereas those with the double asterisk (**) corresponds to non-specific detected with streptavidin-HRP band. Reaction conditions: RBCs were incubated with **6** (100 μM) in HEPES-buffered saline (HBS, pH 7.2) at 37 °C for 48 h.

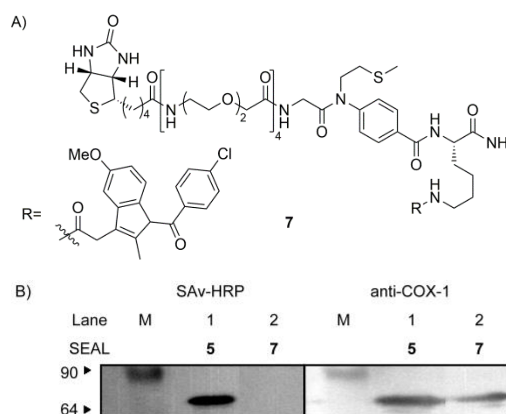

Fig. 5 Elucidation of the mechanism for the SEAL-mediated labelling of the proteins. A) Chemical structure of **7**. B) SDS-PAGE analysis to determine the mechanism of this SEAL-mediated labelling process. Reaction conditions: Purified COX-1 (1.4 μM) was incubated with **5** or **7** (14 μM) in 80 mM Tris·HCl buffer (pH 8.0) at 4 °C for 48 h.

The RBCs were then lysed and the resulting lysate was analyzed by SDS-PAGE, followed by detection with SAv-HRP, which revealed a single band corresponding to biotinylated-hCA at approximately 37 kDa (Fig. 4B). Notably, we did not observe the SA-dependent labelling of the target protein in the presence of ethoxyzolamide (EZA), which is a strong binder against the SA-binding pocket of hCA.[17] These results therefore demonstrated that the use of hydrophobic and low molecular weight SEAL bearing a SA ligand could be used as an efficiently strategy for the intracellular labelling of hCA in a ligand-dependent fashion.

Elucidation of the mechanism of this SEAL-mediated labelling process

The potential involvement of the N–S acyl transfer of the SEAlide moiety during this protein labelling process was examined the using S-protected SEAL derivative **7** (Fig. 5A). COX-1 was incubated with **7** in 80 mM HEPES buffer (pH 8.0) for 48 h at 4 °C. However, the subsequent analysis of this mixture by SDS-PAGE revealed that none of the COX-1 has been labelled, and thereby indicated that the N–S acyl transfer reaction was a critical step in the protein labelling process (Fig. 5B).

Finally, we attempted to address the issue of whether the surface of the protein acts as an acid-base cluster catalyst to enhance the N–S acyl transfer reaction. Although we have not yet collected any direct evidence to suggest that the N–S acyl transfer reaction is accelerated by acid-base residues on protein surfaces, we have collected the following anecdotal evidence: (1) SEAL **6** remained intact in the presence of a large amount of amine or cysteine methyl ester in HEPES buffer (Fig. S2). (2) SEALs immobilized on protein surfaces in a ligand-dependent manner could modify Lys-ε-amino group which would present themselves in a stoichiometric ratio relative to the SEAL under the labelling conditions.

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Conclusions

We have developed a new type of protein-labelling reagent "SEAL" that features a SEAlide moiety with tunable electrophilic reactivity. These SEAL systems were readily obtained using standard Fmoc-based solid-phase protocols. Electrophilically unreactive amide-type SEAlides can be converted to the corresponding electrophilically reactive thioester-type SEAlides in the presence of a phosphate salt, which can act as an acid-base catalyst. With this in mind, we hypothesized that the acidic and basic residues found on protein surfaces could function as acid-base catalysts for the N–S acyl transfer reaction of a SEAL system. It was therefore envisioned that the binding of a SEAL to the surface of a target protein in a ligand-dependent manner could be followed by the activation of the SEAL to a reactive thioester to electrophilic labelling of the target protein. hCA and COX-1 were used as model target proteins with SEAL **1** (hCA-1 ligand) and **5** (COX-1 ligand), respectively, which selectively labelled the target proteins in a mixture of proteins. We also developed a hydrophobic low molecular weight SEAL without a mini-PEG linker that we used to label intracellular hCA1 in RBCs. Taken together, these results highlight the potential of SEALs as protein-labelling reagent. However, one issue remains unresolved, and that is whether the significant difference observed between our newly developed SEALs and conventional labelling reagents can be attributed to the activation of the labelling reagents by the protein surface. Although we collected indirect evidence for the activation of the SEAL by the protein surface, based on the fact that it remained unreactive in the presence of a large excess of amine, the acquisition of direct evidence remains challenging. Research towards obtained direct evidence for the surface-mediated activation of these SEAL system, as well as extending their utility to other ligand-protein pairs including target naïve ligands, is currently underway in our lab. These results will be reported in due course.

Experimental

General procedures

All reactions of small molecules were carried out under a positive presser of argon. For column chromatography, silica Gel 60 N (sperical, neutral, Kanto Chemical Co. Inc.) was employed. Thin layer chromatography was performed on precoated plates (0.25 nm, silica gel Merck Kiesegel 60F245). Mass spectra were recorded on a Waters MICROMASS® LCT PRIME™ (ESI-TOF) or a Bruker Esquire2000T (ESI-Ion trap). NMR spectra were recoded using a Bruker AV400N at 400 MHz frequency for ^{1}H and a JEOL JNM-AL300 at 75 MHz frequency for ^{13}C in methanol-d_4. Chemical shifts are calibrated to the solvent signal. For HPLC separations, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1 mL/min) or a 5C_{18}-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% (v/v) TFA aqueous solution (solvent A) and 0.1% (v/v) TFA in MeCN (solvent B) was used for HPLC elution. Fluorescence and chemiluminescent signals were detected with LAS-4000mini (Fujifilm).

Synthetic of SEALs

SEALs were prepared by Fmoc-solid phase protocols using Fmoc-mini-PEG™, Fmoc-Gly-SEAlide-OH[8d], Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH (for hCA1 labelling) and Fmoc-L-Lys(ivDde)-OH (for COX-1 labelling).

Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH

To a stirred solution of Fmoc-L-Lys-OH (346 mg, 0.940 mmol) in DMF (2.5 mL) were added 4-sulfamoylbenzoic acid N-hydroxysuccinimide ester (365 mg, 1.22 mmol) and DIPEA (486 μL, 2.82 mmol), and the reaction mixture was stirred for 3.5 h. The mixture was concentrated *in vacuo*, and the obtained residue was dissolved in EtOAc. The organic layer was washed with 5% (*w/v*) aqueous citric acid, H_2O and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by column chromatography ($CHCl_3$/MeOH = 9:1) and 427 mg of Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH (0.754 mmol, 80%) was obtained as a white powder: $[\alpha]^{19}_{D}$ 2.79 (c 0.64, CH_3OH); ^{1}H NMR (400 MHz, CD_3OD) δ= 1.45-1.58 (m, 2H), 1.58-1.81 (m. 3H), 1.91 (m, 1H), 3.42 (t, *J* = 7.0 Hz, 2H), 4.18 (dd, *J* = 4.4, 9.2 Hz, 1H), 4.24 (t, *J* = 6.8 Hz, 1H), 4.34 (dd, *J* = 6.8, 10.7 Hz, 1H), 4.39 (dd, *J* = 6.8, 10.7 Hz, 1H), 7.31 (t, *J* = 7.6 Hz, 1H), 7.40 (t, *J* = 7.0 Hz, 2H), 7.68 (d, *J* = 7.6 Hz, 2H), 7.70 (d, *J* = 7.6 Hz, 1H), 7.81 (d, *J* = 7.6 Hz, 1H), 7.95 (d, *J* = 8.8 Hz, 2H), 7.98 (d, *J* = 8.8 Hz, 2H), ^{13}C NMR (75 MHz, CD_3OD): δ=24.4, 29.9, 32.3, 40.9, 55.2, 67.9, 120.9, 126.2, 127.3, 128.1, 128.8, 129.0, 142.6, 145.2, 145.3, 147.6, 158.7, 168.8, 175.9; HRMS (ESI-TOF) *m/z* calcd for $C_{28}H_{29}N_3O_7NaS$ ($[M+Na]^+$) 574.1624, found 574.1625)

Synthesis of SEAL 1

Synthesis of SEAL **1** was performed by using Fmoc-based solid-phase protocols on NovaSyn® TGR resin (loading: 0.22 mmol/g, 45 mg, 0.010 mmol). Coupling of Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH (17 mg, 0.030 mmol) with the aid of DIC (4.6 μL, 0.030 mmol)/HOBt·H_2O (4.6 mg, 0.030 mmol) in DMF was performed, and treatment with 20% (*v/v*) piperidine in DMF was used for the removal of Fmoc protection to give a resin. Fmoc-Gly-SEAlide-OH (1.4 mg, 0.020 mmol) was coupled on the resulting resin by HATU (7.2 mg, 0.019 mmol) and DIPEA (3.3 μL, 0.019 mmol) in DMF at room temperature in DMF for 2 h. After deprotection of Fmoc group with 20% (*v/v*) piperidine in DMF, coupling of Fmoc-mini-PEG™ (12 mg, 0.030 mmol) in DMF were conducted by Fmoc-based solid-phase protocols. To obtained resin were added fluorescein-5-isothiocyanate (FITC, 9.7 mg, 0.025 mmol) and DIPEA (43 μL, 0.025 mmol) in DMF, and the reaction was performed for 2 h. The resulting completed resin was treated with TFA-TES-H_2O (95:2.5:2.5, 3.3 mL) for 2h. After filtration of the resin, the filtrate was concentrated to provide the crude product. The obtained crude product was purified by preparative HPLC to yield SEAL **1** (1.51 mg, 14%, an yellow lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 20.9 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 30 to 40% over 30 min. MS (ESI-TOF) *m/z* calcd for $C_{51}H_{54}N_8O_{14}S_3$ ($[M+H]^+$): 1099.3, found 1099.6.

Synthesis of SEAL 2

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SEAL **2** was synthesized in a manner similar to that employed in the synthesis of SEAL **1**. NovaSyn® TGR resin (loading: 0.22 mmol/g, 45.0 mg, 0.01 mmol) was used to yield SEAL **2** (2.85 mg, 23%, an yellow lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 20.7 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 30 to 40% over 30 min. MS (ESI-TOF) *m/z* calcd for $C_{57}H_{65}N_9O_{17}S_3$ ($[M+H]^+$): 1244.4, found 1244.8.

Synthesis of SEAL 3

SEAL **3** was synthesized in a manner similar to that employed in the synthesis of SEAL **1**. NovaSyn® TGR resin (loading: 0.22 mmol/g, 45.0 mg, 0.01 mmol) was used to yield SEAL **3** (2.90 mg, 21%, an yellow lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 20.4 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 29 to 39% over 30 min. MS (ESI-TOF) *m/z* calcd for $C_{63}H_{76}N_{10}O_{20}S_3$ ($[M+H]^+$): 1389.5, found 1389.9.

Synthesis of SEAL 4

SEAL **4** was synthesized in a manner similar to that employed in the synthesis of SEAL **1** on NovaSyn® TGR resin (loading: 0.22 mmol/g, 227 mg, 0.050 mmol). Coupling of Fmoc-L-Lys(4-sulfamoyl-benzoyl)-OH (85 mg, 0.15 mmol) with the aid of DIC (23 μL, 0.15 mmol)/HOBt·H_2O (20 mg, 0.15 mmol) in DMF was performed, and treatment with 20% (*v/v*) piperidine in DMF was used for the removal of Fmoc protection to give a resin. Fmoc-Gly-SEAlide-OH (72 mg, 0.10 mmol) was coupled on the resulting resin by using HATU (36 mg, 0.095 mmol) and DIPEA (17 μL, 0.095 mmol) in DMF at room temperature for 2 h. After deprotection of Fmoc group of the resulting resin with 20% (*v/v*) piperidine in DMF, stepwise coupling of Fmoc-mini-PEG™ (58 mg, 0.15 mmol) in DMF, Fmoc-Lys(ivDde)-OH (86 mg, 0.15 mmol) in DMF and biotin (61 mg, 0.25 mmol) in DMF-DMSO (3:1 (*v/v*)) were conducted by Fmoc-based solid-phase protocols. The ivDde group on the Lys residue was removed by treatment with 2% (v/v) hydrazine monohydrate in DMF for 17 h, and the generated amine was treated with FITC (49 mg, 0.13 mmol) in DMF and DIPEA (22 μL, 0.13 mmol) for 5 h. The resulting completed resin was treated with TFA-TES-H_2O (95:2.5:2.5, 16 mL) for 2h. After filtration of the resin, the filtrate was concentrated to provide a crude product. The obtained crude product was purified by preparative HPLC to yield SEAL **4** (22.0 mg, 28%, an yellow lyophilized powder).Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 19.5 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 28 to 38% over 30 min. MS (ESI-TOF) *m/z* calcd for $C_{79}H_{102}N_{14}O_{23}S_3$ ($[M+2H]^{2+}$): 799.6, found 799.5.

Synthesis of SEAL 5

SEAL **5** was synthesized in a manner similar to that employed in the synthesis of SEAL **4**. NovaSyn® TGR resin (loading: 0.25 mmol/g, 160 mg, 0.040 mmol) was used to yield SEAL **5** (0.38 mg, 0.6%, a white lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 25.7 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 38 to 48% over 30 min. MS (ESI-TOF) *m/z* calcd for $C_{70}H_{99}ClN_{12}O_{20}S_2$ ($[M+H]^+$): 1527.6, found: 1527.9.

Synthesis of SEAL 6

SEAL **6** was synthesized in a manner similar to that employed in the synthesis of SEAL **1**. NovaSyn® TGR resin (loading: 0.24 mmol/g, 208 mg, 0.050 mmol) was used to yield SEAL **6** (13 mg, 33%, a white lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 16.8 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 23 to 33% over 30 min. MS (ESI-TOF) *m/z* calcd for $C_{34}H_{46}N_8O_8S_3$ ($[M+H]^+$):791.3, found: 791.1.

Synthesis of SEAL 7

To a solution of SEAL **5** (10.6 mg, 6.87 μmol) in DMF was added MeI (69.0 μL, 1.11 mmol) and DIPEA (69.0 μL, 6.87 μmol) with stirring at room temperature for 1 h. The reaction mixture was purified by preparative HPLC to yield SEAL **7** (4.43 mg, 42%, a white lyophilized powder). Analytical HPLC conditions: gradient of solvent B in solvent A, 10-60% over 30 min. Retention time = 25.9 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 35 to 49% over 30 min. MS (ESI) *m/z* calcd for $C_{71}H_{101}ClN_{12}O_{20}S_2$ ($[M+H]^+$): 1541.7, found 1541.7.

Optimization of structure of SEALs

Purified human carbonic anhydrase 1 (hCA1) was purchased from SIGMA-Aldrich and used without further purification. The hCA1 (10 μM) with **1-3** (100 μM) was incubated at 37 °C in 50 mM HEPES buffer (pH 7.2). At every 24 h, the reaction aliquots were subjected to ultrafiltration (14,000 g, 15 min, himac CT 15RE (Hitachi Koki Co., Ltd.)) using Amicon® Ultra-0.5 Centrifugal Filter Devices (Merck Millipore) and the concentrate was mixed with the same volume of 2×SDS sample loading buffer followed by heating at 100 °C for 5 min. The heated reaction mixtures were analyzed using SDS-PAGE in 14% in polyacrylamide gels. For the chemiluminescence imaging of the biotinylated proteins, the proteins were transferred to Amersham Hybond-P PVDF Membrane (GE Healthcare) and detected with a streptavidin-horseradish peroxidase conjugate (SAv-HRP, GE Healthcare) and ECL plus Western Blotting Detection System (GE Healthcare). For silver staining of all proteins, proteins in a gel were stained with Silver Stain KANTO Ⅲ (KANTO CHEMICAL CO., INC.).

To determaine that the labelling of hCA1 was site-specifically occurred, purified hCA1 (10 μM) was labelled using **4** (100 μM) according to the method described above. The labelled hCA1 was subjected to SDS-PAGE in 14% in polyacrylamide gel and stained with CBB Stain One (Nacalai tesqe). The gel containing of the labelled hCA1 with trypsin was incubated at 37 °C in Tris·HCl buffer (pH 8.0) for 20 h. The digested labelled-hCA1 was analyzed on nanoLC-MS/MS.

LC: UltiMate 3000 RSLCnano system (Thermo Fisher Scientific Inc.)

MS: Orbital Elite (Thermo Fisher Scientific Inc.)

Column: Acclaim PepMap RSLC Nano Column (75 μm×15 cm, Thermo Fisher Scientific Inc.)

Solvent A: 0.1% formic acid aq.; solvent B 0.08% formic acid/80% MeCN aq.

Selective labelling of hCA1 in protein mixture

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A protein mixture containing of enolase, GST, hCA1 and OVA (10 μM each, purchased from SIGMA) with **1** (100 μM) was incubated in 50 mM HEPES buffer (pH 7.2) at 37 °C for 48 h. The reaction mixture was subjected to ultrafiltration and the concentrated mixture was blended with the same volume of 2 ×SDS sample loading buffer followed by heating at 100 °C for 5 min. The heated samples were applied to 14% SDS-PAGE, and analyzed by *in-gel* fluorescence imaging and silver-staining.

Selective labelling of COX-1 in protein mixture

As a protein mixture, BSA purchased from SIGMA, cyclooxygenase 1 (COX-1) purchased from Cayman Chemical and enolase (1.4 μM each) was used.

The mixture of proteins with **6** was incubated in 80 mM Tris·HCl buffer (pH 8.0) at 4 °C for 48 h. The reaction mixture was analyzed using SDS-PAGE in 10% polyacrylamide gels followed by chemiluminescence imaging according to the methods described above. For silver staining of all proteins, proteins in a gel were stained with Silver Stain KANTOⅢ.

Intracellular protein labelling

Human red blood cells (hRBCs) were purchased from BizCom Japan, Inc., and purified by the use of a centrifugation (3,000 g, 15 min). Purified hRBCs were resuspended in HEPES-buffered saline (HBS) and incubated with **5** (200 μM) in the absence or presence of ethoxyzolamide (EZA, 2 mM) at 37 °C for 48 h. The incubated hRBCs were washed three times with HBS and lysed by sonication. The resulting lysate was centrifuged (12,000 g, 30 min) and the resulting supernatant was collected and separated with **5** by the use of ultrafiltration. The resulting mixture was analyzed using SDS-PAGE in 10% polyacrylamide gels followed by chemiluminescence imaging according to the methods described above. For silver staining of all proteins, proteins in a gel were stained with Silver Stain KANTOⅢ.

Elucidation of a mechanism of the labelling using a SEAL

Purified COX-1 (1.4 μM) was incubated with **6** or **7** (14 μM) in 80 mM Tris·HCl buffer at 4 °C for 48 h. The reaction mixtures were subjected to ultrafiltration followed by SDS-PAGE analysis. The biotinylated COX-1 was detected with SAv-HRP using ECL plus Western Blotting Detection System.

The immunodetection of COX-1 was accomplished with COX-1 monoclonal antibody and anti-mouse IgG-HRP conjugate (both Santa Cruz Biotechnology).

To demonstrate of the stability of **5**, butylamine (1 M) and **5** (10 μM) were incubated in 50 mM HEPES buffer (pH 7.2) at 37 °C. At every 24 h, reaction aliquots containing **5** were analyzed by HPLC.

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