1,6-Thiol Addition of Trifunctional *N*-Alkylpyridinium for Site-selective Dual Functionalization of Proteins

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Abstract:

We report *N*-alkylpyridinium reagents for site-selective dual modification of proteins *via* a regioselective 1,6-thiol addition reaction. The *N*-alkylpyridinium derivatives can be synthesized in two reaction steps and demonstrate high labelling efficiency and chemoselectivity towards cysteine residues. This reaction is combined with strain-promoted azide-alkyne click and inverse-electron-demand Diels−Alder reactions to achieve dual functionalization of proteins in a sequential one-pot reaction. A Rho-inhibiting enzyme is functionalized with a cancer cell-targeting peptide and a fluorescent dye for successful *in vitro* uptake imaging and concomitant inhibition of specific Rho-mediated cellular pathways. The ease of synthesis, fast kinetics, high solubility and regioselectivity make *N*-alkylpyridinium reagents unique for protein/peptide modification to increase their functional diversities for medical applications.

Introduction

Protein-based therapeutics have led to new paradigms in the treatment of diseases with high targeting efficiency and specificity.[1] In 2023, the global protein therapeutics market size raised to over $400 billion and could further expand to about $700 billion in 2033, with many drug candidates currently undergoing clinical trials.[2-5] Nowadays, site-selective protein modification has already become an indispensable tool to customize protein-based therapeutics beyond their evolutionary roles.[6-12] Rationally designed bioconjugation reagents that exploit the reactivity of selected amino acid residues have allowed the precise conjugation of new functionalities such as cytotoxic drugs, cell targeting groups or imaging agents to native peptides or proteins.[13-17] In this way, a protein can be repurposed to acquire new features, such as a new bioactivity profile, cellular internalization or fluorescence

for bioimaging, or disease treatment, among others.[18-21]

However, designing therapeutics against serious diseases such as cancer often requires the preparation of structurally complex (macro)molecules that combine different functionalities to enable stratified treatments. For example, there is a growing interest in integrating drugs and imaging agents into the therapeutic protein to facilitate real-time monitoring and assessment of treatment efficacy. In addition, protein therapeutics, such as traditional antibody drug conjugates (ADCs) loaded with a single type of anticancer drug, may exhibit suboptimal therapeutic efficacy, drug resistance and unfavourable pharmacokinetics.[22] Therefore, ADCs with two different drugs are crucial when combination therapy is needed to overcome drug resistance and address tumor heterogeneity.[23-25] However, the preparation of these multimodal protein conjugates has predominantly relied on statistical modifications on the protein surface,[26-29] potentially leading to heterogeneous products with different pharmacokinetic profiles batch-to-batch variability and compromised protein activity, thus posing substantial obstacles for further *in vivo* applications. In this regard, site-selective dual modification of proteins has emerged as a promising approach to allow for the simultaneous introduction of two different functionalities at a specific site, offering well-defined multimodal protein conjugates with tailored properties.[30]

Due to the variety of reactive groups at the protein surface, it is rather challenging to select two different amino acid residues for dual modification that provide orthogonal reactivities and sufficient surface abundance.[31-35] Thus, one promising approach relies on the design of a trifunctional bioconjugation reagent that already carries two payloads (such as two drugs or fluorescent dyes) to modify a specific residue on a protein surface.[36, 37] But such reagents often require arduous multi-step synthesis involving tedious protection/deprotection processes, in addition to potential poor water solubility or steric hindrance, leading to low modification efficiency and heterogeneous product mixtures.[38, 39] Therefore, structurally simple trifunctional bioconjugation reagents that readily react with a single, surface-exposed amino acid residue, while providing two additional bioorthogonal groups for the straightforward *in situ* dual functionalization of native proteins are urgently needed. Among all the amino acids, cysteine residues occur in low abundance on the protein surface and exhibit high nucleophilicity, which is important for high site selectivity and reactivity. However, until now, trifunctional bioconjugation reagents targeting cysteine residues[36, 40-44] are still much less explored compared to the vast library of reagents for single cysteine modification. In addition, some of the trifunctional bioconjugation reagents also suffer from complicated synthesis, low modification efficiency or limited chemoselectivity, which significantly restrict their broader usage.[34] Thus, this field will greatly benefit from the development of novel structurally simple trifunctional bioconjugation reagents targeting cysteine residues with easy synthesis, excellent chemoselectivity and high labelling efficiency.

Herein, we introduce *N*-alkylpyridinium as a new and versatile bioconjugation reagent for site-selective dual functionalization of peptides and proteins at cysteine residues. Assisted by the Density Functional Theory (DFT) calculations, the non-reactive (*E*)-3-(pyridin-4-yl)acrylic acid is converted to the *N*-alkylpyridinium reagents that can react with thiol groups with excellent chemoselectivity and fast kinetics *via* a 1,6-thiol addition reaction. Various *N*-alkylpyridinium derivatives containing alkyne, azide and tetrazine groups can be obtained by a simple and straightforward two-step synthesis with commercially available starting materials. In addition, the 1,6-thiol addition reported here is compatible with SPAAC and iEDDA reactions thus allowing the *in situ* one-pot dual functionalization of native proteins. As a proof of concept, *Clostridium Botulinum* C3, a model enzyme that catalyzed ADP-ribosylation of Rho proteins[45, 46] and only naturally uptaken into macrophages and monocytes,[47, 48] was dual-modified with a fluorescent dye (Cy5) and a cell-targeting peptide *via* two bioorthogonal reactions. In this way, C3 enzyme was able to reach Rho proteins as intracellular drug targets, altered the morphology of cancer cells, expanding the evolutionary optimized features of C3 toxins for potential biomedical applications.

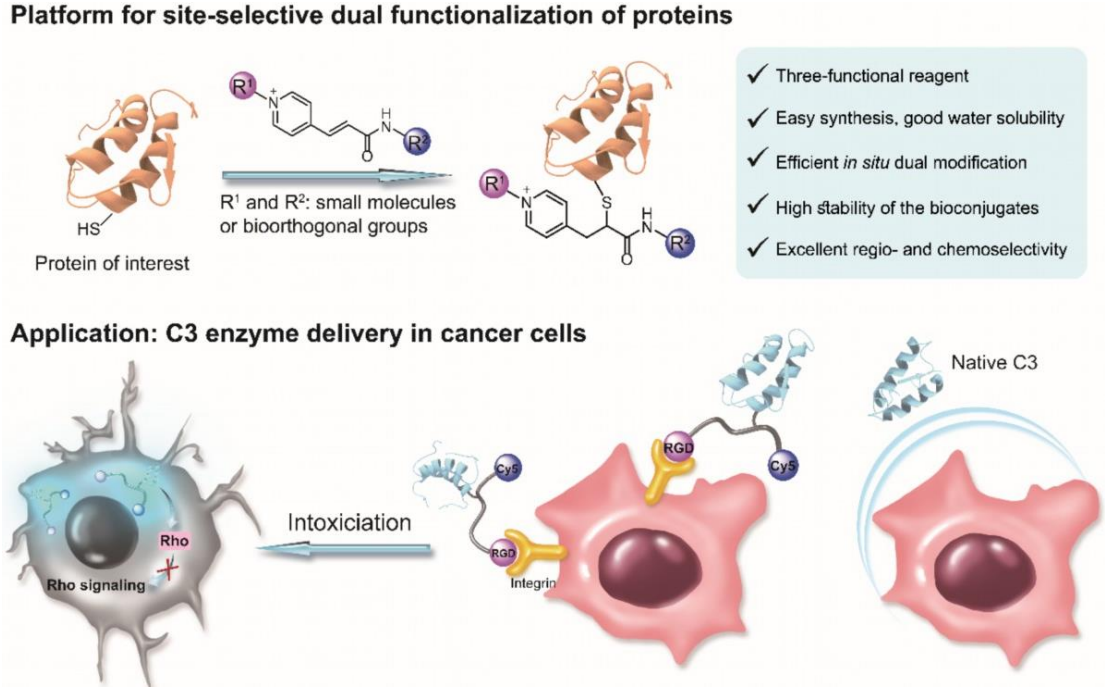


Fig. 1 Overview of *N*-alkylpyridinium reagents to achieve dual modification of functional proteins and proof-of-concept application with a toxin enzyme.

Results

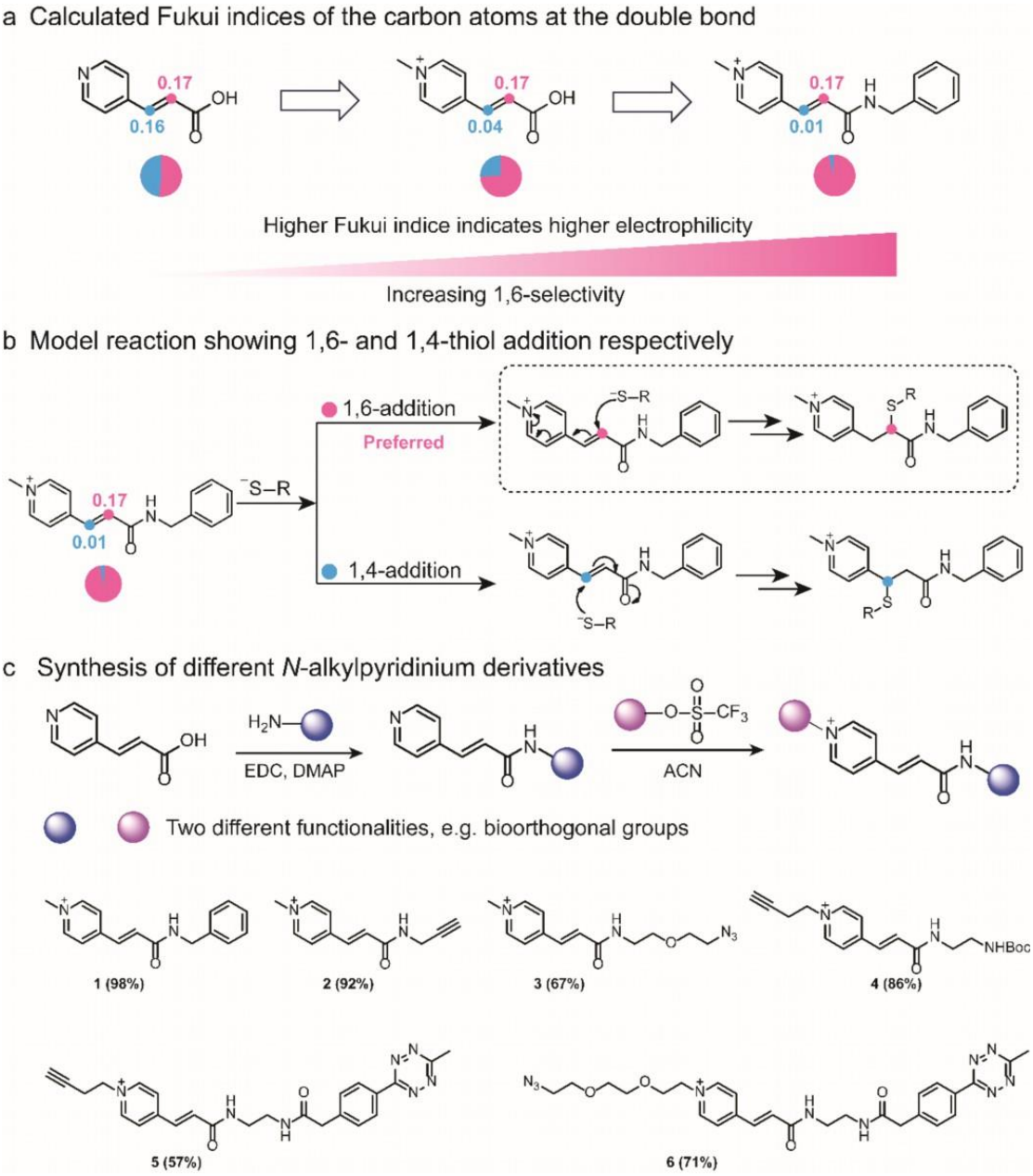
Chemical design of *N*-alkylpyridinium reagents

Capitalizing on the trifunctional structure of the commercially available (*E*)-3-(pyridin-4-yl)acrylic acid, bearing a conjugated double bond, along with a carboxylic acid and a pyridine group, we envision a new class of trifunctional reagents for the fast and efficient *in situ*, site-selective dual modification of native peptides and proteins in aqueous media. The

nitrogen quaternization of pyridine increases the electrophilicity of the conjugated double bond in the *para* position through electron density delocalization. In this way, *N*-alkylpyridinium derivatives can react with cysteine residues through 1,6-addition reaction (Fig. 1), which has rarely been explored for bioconjugation.[49] In addition, according to the hard-soft acid-base (HSAB) theory by Pearson, we hypothesize that the *N*-alkylpyridinium reagents that have a more extended conjugated system and diffused electron density are considered soft electrophiles, which prefer to react with soft nucleophiles, such as thiolates rather than amines (hard nucleophiles).[50] Therefore, we envision that 1,6-thiol addition between *N*-alkylpyridinium reagents towards thiol groups will demonstrate better chemoselectivity over the commonly reported 1,4-thiol addition in the literature.[51-53]

In order to design the reagent, the impact of *N*-quaternization and different substituents at the carbonyl group on the selectivity of the addition reaction at the double bond was first estimated based on the Fukui indices (Scheme 1a, SI Fig. S7.1). The Fukui index is calculated by DFT to determine the electrophilicity or nucleophilicity of the different atoms in a molecule. Higher Fukui index f+ indicates higher electrophilicity of a specific atom.[54] For the carbon atoms at the double bond of (*E*)-3-(pyridin-4-yl)acrylic acid, the calculated Fukui indices of 0.16:0.17 (C_{blue}:C_{pink}, Scheme 1) indicate that the reagent would undergo non-regioselective addition reaction. Quaternization of the nitrogen (0.04:0.17), as well as amidation of the carboxylic acid (0.01:0.17) favors the 1,6-addition reaction, whereas other substituents such as ester, ketone or nitrile groups have no positive effect on the Fukui indices (SI Fig. S7.1). Moreover, due to the positive charge of the *N*-alkylpyridinium group, the corresponding bioconjugation reagents reveal low partition coefficients (*n*-octanol to water, cLog $P_{o/w}$) (SI Fig. S3.1), thus providing water solubility to suppress potential protein aggregation and denaturation during the bioconjugation reactions.[55, 56]

Accordingly, we employed amide functionalization of the carboxylic acid group, followed by *N*-methylation on the commercially available (*E*)-3-(pyridin-4-yl)acrylic acid to prepare compound **1** (Scheme 1b). To explore the versatility of *N*-alkylpyridinium reagents for site-selective dual modification of proteins, different reactive handles such as azido, alkyne, or tetrazine groups were also incorporated to offer *N*-alkylpyridinium derivatives **2**–**6** in good to high yields (57–92%). Compared to other reported dual modification reagents, such as allyl sulfone reagents that require five-step synthesis,[43] the *N*-alkylpyridinium derivatives **1**–**6** can be readily obtained in only two reaction steps. In this way, complicated protection and deprotection processes can be avoided that are often required for the synthesis of other dual functionalization reagents, which otherwise might lead to the reduction of the overall yield and tedious purification steps.



Scheme 1. a) Fukui indices for (*E*)-3-(pyridin-4-yl)acrylic acid, after quaternization of the Nitrogen and amide formation reveals increasing selectivity for 1,6-addition (pink) over the more common 1,4-addition (blue); (b) Model reaction showing the 1,6 and 1,4 addition reaction respectively; c) Synthetic route of the various *N*-alkylpyridinium derivatives (**1**–**6,** yields from 57%-98%).

Reaction profile of *N*-alkylpyridinium with nucleophiles

The chemoselectivity of *N*-alkylpyridinium towards different nucleophiles such as thiol or amino groups was first investigated with compound **1** due to its structural simplicity for analysis and characterization. Compound **1** (1 equiv, 4 mM) was incubated with thiol-containing compound **7** (1.5 equiv) and amine-containing compound **8** (1.5 equiv) in ACN:PB (pH 7) (*v/v* = 2:3) and the reaction progress was monitored using high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). Only the thioether derivative **9** was formed with quantitative conversion (Fig. 2b and SI Fig. S4.1).

Besides the observed reaction with thiol groups, compound **1** did not show any cross-reactivity with other amino acid residues that contain nucleophilic side chains, such as tyrosine, methionine, arginine and others under the same reaction conditions (Fig. 2c, SI Fig. S4.2). Next, we investigated the influence of pH on the thiol addition reaction. Quantitative conversion to product **9** was observed after 4 h at pH 6–9 (Fig. 2d), showing the efficiency and the robustness of the reaction. There was a decrease in conversion to around 80% at pH 5, probably due to the lower amount of thiolate anions under more acidic conditions (Fig. 2d, SI Fig. S4.3). Next, the reaction conversion was investigated over time with the same model reaction at pH 7. Compounds **1** and **7** (2 equiv) were incubated in ACN:PB (pH 7) at room temperature and the reaction was monitored by HPLC at different time intervals. The data showed that quantitative conversion was already achieved within the first hour of the reaction (Fig. 2e, SI Fig. S4.5).

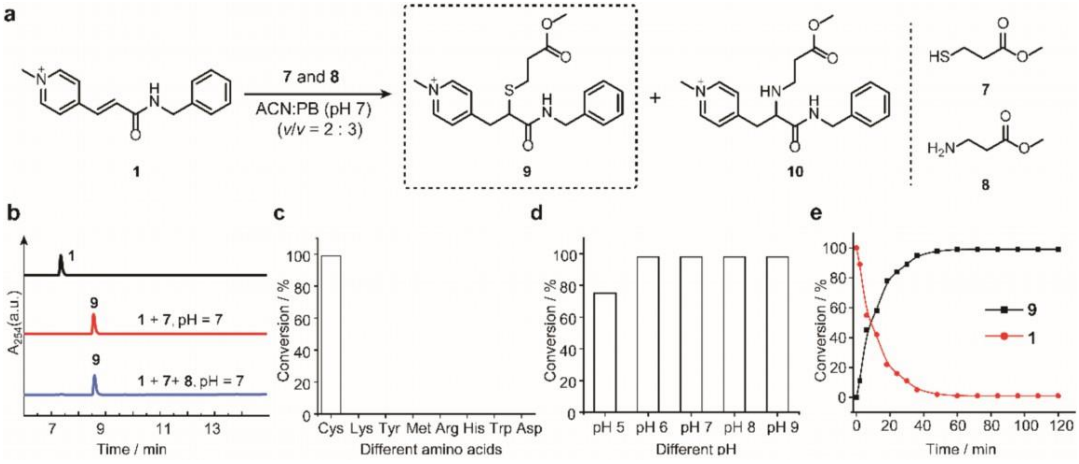


Fig. 2 (a) Model reactions of compound **1** with **7** and **8**. (b) HPLC traces of model reaction between compound **1** and **7** (and **8**) at 4 mM, pH 7, after 4 h. (c) Evaluation of chemoselectivity towards different nucleophilic amino acids. (d) Evaluation of the conversions of the reactions between compound **1** and **7** at different pH. (e) Reaction profile of compound **1** and formation of compound **9** determined as percentage with reference to the internal standard (Fmoc-Phe-OH) by the integration of the HPLC peak.

Besides reactivity, sufficient stability of *N*-alkylpyridinium reagents in solution is also an important feature for the subsequent bioconjugation reaction. Therefore, the stability of compound **1** was evaluated at three different pH (pH 6, 7 and 8) for 24 h. According to the HPLC data, no decomposition of compound **1** was observed for up to 24 h related to the internal standard Fmoc-Phe-OH (SI Fig. S5.1-S5.3). In contrast, maleimide reagents can easily and quickly hydrolyze to unreactive maleamic amides, especially at basic pH ($t_{1/2}$ < 2 h).[57]

Regioselectivity of the *N*-alkylpyridinium derivatives with NMR studies and DFT calculations

The thiol-addition of **7** to **1** can potentially afford two products, **9** and **9'** (Fig. 3a), which are indistinguishable by MS analysis. Thus, the regioselectivity was investigated with nuclear magnetic resonance (NMR) spectroscopy using compound **9**. A comparison of the proton

NMR of **1** and **9** indicated that the peaks at 8.64 ppm and 8.13 ppm assigned to the vinylic protons of **1** are absent in the product, demonstrating that the reaction took place at the double bond. The details and assignment of the proton NMR spectrum of the isolated product **9** are given in Fig. S6.2 in SI, which confirms the formation of a single product, rather than a mixture of different regioisomers. The heteronuclear single quantum coherence (HSQC), Heteronuclear Multiple Bond Correlation (HMBC) and H-H correlated spectroscopy (COSY) spectra of compound **9** were acquired to further confirm the site of the thiol-addition reaction (C_5 or C_6) (Fig. 3a, Fig.S6.3-6.6). HMBC data showed two cross peaks between C_3 and H_5 (Fig. 3b), indicating the thiol group was added to the vinylic C_6 in a 1,6-thiol addition reaction. No single cross peak was observed between C_3 and H_5 in the HMBC spectrum, confirming that the thiol-addition did not occur at C_5. Furthermore, both C_4 and C_7 showed a single cross peak with H_6 but two cross peaks with H_5 in the HMBC spectrum, which further confirmed the 1,6-regioselectivity (SI Fig. S6.5). The pronounced reactivity preference for the 1,6-addition is in agreement with the relative electrophilicity of each carbon (C_5 and C_6) in compound **1** based on the calculated Fukui indices (Fig. 3c).

Further DFT calculations were employed to gain a deeper understanding of the energy profile of the 1,6-addition with thiol derivatives (Fig. 3d). The Gibbs free energy profiles for the thiol-addition at C_5 and C_6 were determined to be 14.1 kcal/mol and 7.6 kcal/mol, respectively, using methylthiolate as a model reagent. The DFT calculation further corroborated the experimental results that the thiol-addition at C_6 is selectively favored and only product **9** was obtained. The calculated activation energy for maleimide conjugation with the same model thiol was determined to be 10.8 kcal/mol, which is comparable to the new modification strategy reported here. Calculation of proton affinity (PA) of the 1,6-addition adduct intermediate indicates a preference for protonation at the carbon atom of the former double bond in the final reaction step (Fig. 3d). Furthermore, the analysis of the computed frontier molecular orbitals of compound **1** reveals that the lowest unoccupied molecular orbital (LUMO), the acceptor orbital, is mainly located at the conjugated pyridinium, while the more energetic LUMO+2 is located at the conjugated amide (SI Fig. S7.2). This observation indicates that the carbon atom leading to the 1,6-addition product is more electrophilic, which is consistent with the calculated Fukui indices (Fig. 3c).

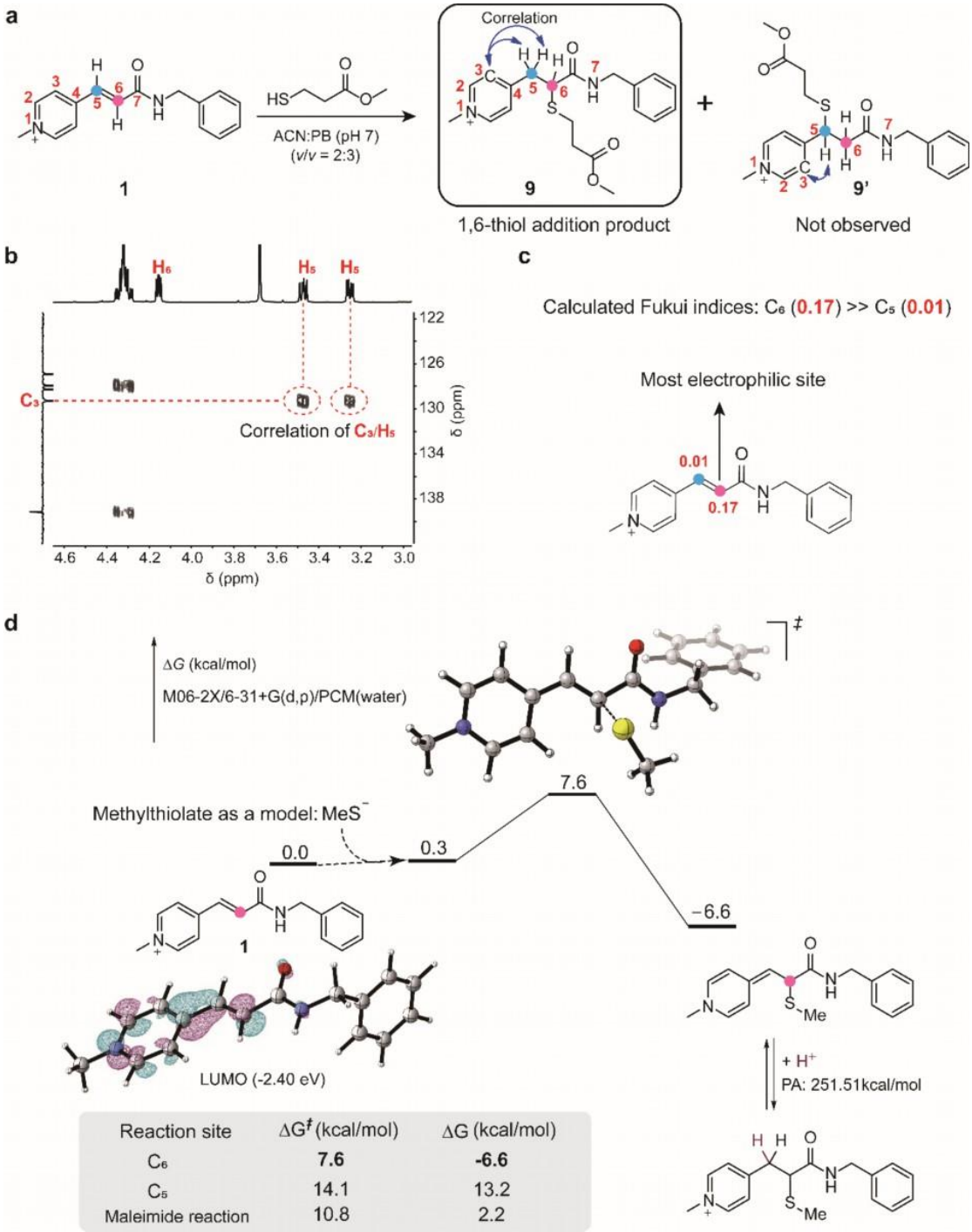
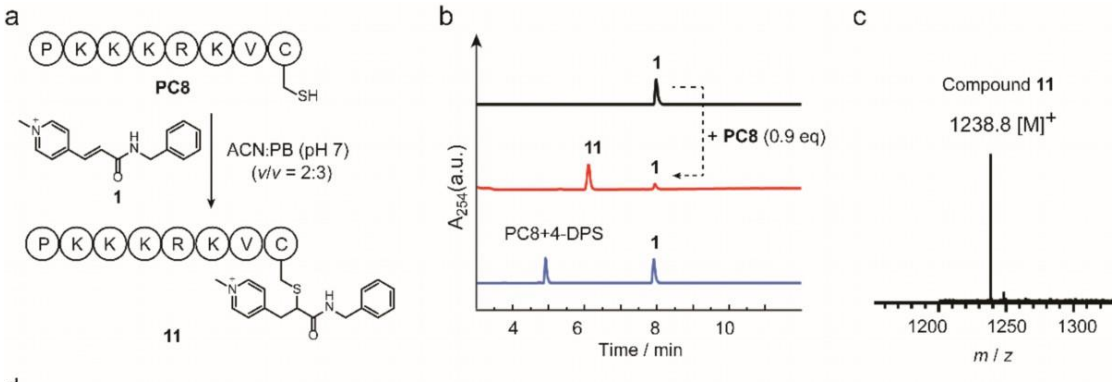


Fig. 3 (a) Model reaction between **1** and **7** to form two different regioisomers: **9** and **9'** (b) HMBC spectrum of the obtained thiol-addition product. (c) Calculated Fukui indices of atom 5 (C_5) and 6 (C_6). (d) Gibbs free energy profiles of the reaction between **1** and methylthiolate at site C_6 *via* 1,6-thiol addition reaction and the proton affinity of the 1,6-adduct intermediate to offer the final product. LUMO of compound **1** was localized at the conjugated pyridinium.

Site-selective modification of peptides

The model reaction between **1** and **7** used for characterization shows high chemoselectivity and regioselectivity. Next, we have used the *N*-alkylpyridinium **1** to modify cysteine residues in more complex structures such as peptides and proteins. PC8, a nuclear targeting peptide containing an accessible cysteine and four lysines in its sequence, was selected

to demonstrate the chemoselectivity and regioselectivity of *N*-alkylpyridinium reagents towards thiol *versus* amino groups (Fig. 4a). HPLC analysis of the crude reaction mixture of reagent **1** (1 equiv) with PC8 (0.9 equiv) in ACN:PB (50 mM, pH 7, *v/v* = 1:10) shows complete conversion to the corresponding 1,6-conjugate **11** after 4 h along with the remaining excess of starting material **1** (Fig. 4b). As a control, thiol-reactive reagent 4,4'-dithiodipyridine (4-DPS) was used to mask the cysteine residue in PC8 peptide *via* a thiol-disulfide exchange reaction. No reaction occurred between reagent **1** and the 4-DPS modified PC8 peptide, even in the presence of four lysine residues (SI section 8.2). These data clearly show the excellent chemoselectivity and high efficiency of *N*-alkylpyridinium reagents for reacting with cysteine residues in peptides. Other peptides such as integrin receptor binding peptide RGDC, CEIE peptide, chemokine receptor 4 antagonist WSC02 (sequence in Fig. 4), antimicrobial peptide Tet (sequence in Fig. 4) and pan-coronavirus (CoV) fusion inhibitor EK1C (sequence in Fig. 4) with different lengths, sequences and cysteine positions were also successfully modified with **1** under mild reaction conditions in moderate to high isolated yields (57–95%, Figure 4d, SI section 8.1), demonstrating the general applicability of *N*-alkylpyridinium reagents for site-selective modification of peptides at the cysteine residue. Thereafter, we used PC8 conjugate **11** as a model to investigate the stability of these new 1,6-conjugates under various conditions, including different pH buffers and in presence of glutathione (GSH), which is found in the cytosol of cells.[58] Under these physiologically relevant conditions, bioconjugate **11** showed high stability and only minor degradation (<16% in 24h). At basic reaction conditions, the 1,6-elimination reaction occurs (SI section 8.3) as side reaction and about 29% degradation product was observed after incubating **11** at pH 8 for 24h. Similarly to other addition reactions, the resulting thioethers such as thiosuccinimides can undergo thiol exchange reactions.[59] However, **11** remains stable even in the presence of 1 mM GSH at pH 7.4, where only little degradation was observed (about 18%) after 45 h incubation (SI section 8.3). Altogether, these results demonstrate high chemoselectivity and modification efficiency of *N*-alkylpyridinium reagents for site-selective modification of peptides at cysteine residues to afford fairly stable conjugates under physiologically relevant conditions and in the presence of GSH.



d

Peptide	Sequence	Isolated yields	Calc. (*m/z*)	Obs. (*m/z*)	
CEIE	NH_2-CEIE-COOH	95%	373.2	**373.2**	$[M+H]^{2+}$
RGDC	NH_2-RGDC-COOH	57%	702.3	**702.4**	$[M]^+$
PC8	NH_2-PKKKRKVC-COOH	65%	1238.9	**1238.8**	$[M]^+$
WSC02	NH_2-IVRWSKKVPCVS-COOH	86%	827.5	**827.9**	$[M+H]^{2+}$
Tet	NH_2-HLNILSTLWKYRC-COOH	94%	1036.0	**1036.2**	$[M+H]^{2+}$
EK1C	NH_2-SLDQINVTFLDLEYEMKKL-EEAIKKLEESYIDLKELC-COOH	92%	1563.1	**1563.6**	$[M+2H]^{3+}$

Fig. 4 (a) PC8 peptide modification with compound **1** in PB. (b) HPLC trace of **1**, the reaction mixture of PC8 and **1** (1.1 equiv), and the reaction mixture of DPS-modified PC8 peptide and compound **1** (from top to down). (c) ESI-MS of **11** (calculated: 1238.9 $[M]^+$, found: 1238.9 $[M]^+$). (d) Site-selective modification of different peptides with compound **1**.

As protein modification often involves the use of reagents in large excess to enhance conversion, specificity for thiol over amino groups could be compromised, as in the case of maleimides.[60, 61] Therefore, we compared the thiol selectivity of *N*-alkylpyridinium and maleimide reagents when used in increasing equivalents with Tet peptide. The Tet peptide was selected as it contains a cysteine and a lysine residue in its sequence. Notably, when up to 10 equiv of the respective bioconjugation reagent (compound **1** and maleimide shown in Table S1) were applied, only the *N*-alkylpyridinium derivative **1** afforded the single-modified Tet conjugate, whereas the Michael addition reaction of maleimide and Tet resulted in a mixture of single and double-modified Tet (Table S1). These results highlight the higher thiol selectivity of *N*-alkylpyridinium over maleimide reagents, even when a large excess of reagent is required, such as for efficient protein bioconjugation.

Site-selective modification of proteins

Next, the functionalization of proteins containing accessible cysteine residues with *N*-alkylpyridinium reagents was accomplished. Ubiquitin, a small regulatory protein that plays an important role in protein degradation and that contains a cysteine residue at its K63 position was selected for site-selective modification using the bioconjugation reagents **1**, **5** or **6**. Ubiquitin K63C (1 equiv) was incubated with compounds **1**, **5** (8 equiv) or **6** (30 equiv) in PB (50 mM, pH 7) overnight to ensure efficient modification. Electrospray ionization-high

resolution MS (ESI-HRMS) analysis showed successful modification of ubiquitin and different functionalities have been introduced successfully (Fig. 5a, SI section 9.1). To validate the site selectivity towards the cysteine residue on the ubiquitin surface, the solvent accessible thiol group (K63C) was masked with 4-DPS, which prevented any further modification, even when compound **1** was used in 30 equiv excess (SI Fig. S9.8).

To further demonstrate the general applicability of our functionalization method, the anti-MMR nanobody targeting the macrophage mannose receptor in tumor-associated macrophages,[62-65] was selected for modification with compound **6** under similar conditions as previously used for ubiquitin. The successful modification was confirmed by the correlation of the observed and calculated molecular mass (found: 15617 Da, calculated: 15619 Da) as shown in Fig. 5b. In addition, the C3bot1 toxin (**C3**) from *Clostridium botulinum* was also selected for dual modification using compound **6** to incorporate the bioorthogonal pairs for late-stage functionalization. The C3 enzyme specifically mono-ADP-ribosylates the GTPases Rho A, -B, and -C and thereby inhibits Rho-mediated signal transduction and associated cellular processes.[45, 46, 48] ESI-HRMS data in Fig. 5c confirmed the successful site-selective modification to afford **C3-*N_3-Tz*** conjugate (found 25305 Da, calculated: 25038 Da). Noteworthy, CD spectra of the various modified proteins ubiquitin (K63C), anti-MMR nanobody and C3 enzyme indicate that the bioconjugation reaction did not disturb the native secondary structure of these proteins (SI Fig. S9.9, S9.13 and S9.17).

Then, having demonstrated that two bioorthogonal groups can be successfully introduced into different protein substrates, we investigated in the following steps the possibility of late-stage re-engineering of a native protein to confer additional properties beyond its natural function and mode of action. The C3 enzyme of *Clostridium Botulinum* selectively ADP-ribosylates the small GTP-binding proteins RhoA/B/C and inhibits their downstream signalling pathways, which blocks cell migration and has been applied for cancer applications.[66-70] However, the application of C3 is limited as it is poorly internalized into the cytosol of epithelial cells as compared to monocytic cells including monocytes, macrophages, osteoclasts and dendritic cells.[47, 48, 71, 72] This greatly restricts the usage of native C3 protein for pharmacological inhibition of Rho-signalling in most cancer cells.[73] Thus, as a proof-of-concept, we used the *N*-alkylpyridinium reagent **6** for late-stage dual functionalization of Cys-containing C3 to enhance its delivery into A549 lung cancer cells and to track its uptake and fate within these cells. In this way, orthogonal reactions can be achieved in a sequential one-pot fashion to incorporate two payloads. Specifically, a combination of two different payloads was attached to the purified **C3-*N_3-Tz*** *via* two orthogonal click reactions as shown in Fig 5e. The cell-penetrating RGD peptide was selected to redirect and mediate the uptake of C3 into cancer cells expressing integrin αvβ3 receptor, while the Cy5 served as a fluorophore for simultaneous tracking of the uptake of the resultant conjugate through confocal microscopy. The peptide (RGDC-DBCO) and the fluorophore (Cy5-TCO) were

both attached to *N*-alkylpyridinium at Cys-13 of C3 enzyme, in order to achieve dual functionalization *via* two biorthogonal reactions, i.e., the SPAAC and iEDDA reaction respectively, affording **C3-*RGD-Cy5*** (found: 27110 Da, calculated: 27112 Da) in a one-pot reaction. The molecular mass increased (2075 Da) over the three reaction steps, which is in agreement with the calculated values (Fig. 5g). As a negative control, a FRET pair (Cy3 and Cy5 dyes) was also introduced to C3 sequentially *via* two orthogonal reactions (Cy3-DBCO *via* SPAAC and Cy5-TCO *via* iEDDA reaction) to afford the dual-functionalized protein conjugate **C3-*Cy3-Cy5*** (full chemical structure in SI) with the molecular weight of 26885 Da (Fig 5f).

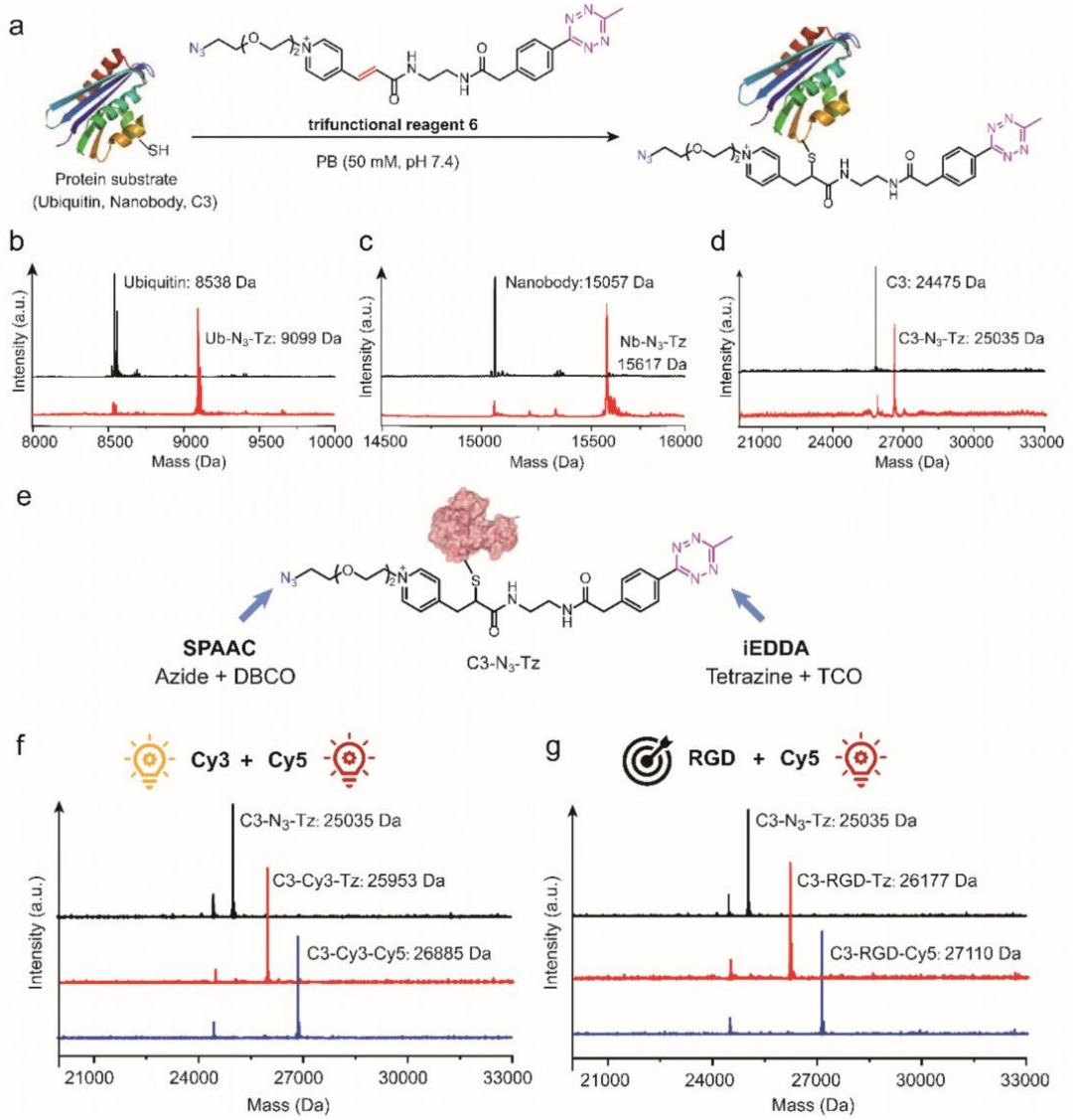


Fig. 5 (a) General scheme of site-selective modification of proteins with compound **6** and late-stage dual modification with SPAAC and iEDDA reactions. (b) Deconvoluted ESI-MS of **Ub-*N_3-Tz*** (calculated: 9101 Da, found: 9099 Da). (c) Deconvoluted ESI-MS of **Nb-*N_3-Tz*** (calculated: 15619 Da, found: 15617 Da). (d) Deconvoluted ESI-MS of **C3-*N_3-Tz*** (calculated: 25038 Da, found: 25035 Da). (e) Dual functionalization of **C3-*N_3-Tz*** conjugate with pairs of two different functionalities, such as RGD peptide, Cy3 and Cy5. (f) Deconvoluted ESI-MS of **C3-*Cy3-Tz*** (calculated: 25956 Da, found: 25953 Da), and **C3-*Cy3-Cy5*** (calculated: 26888 Da, found: 26885 Da). (g) Deconvoluted ESI-MS of C3-*RGD-Tz* (calculated: 26180 Da, found: 26178 Da), and **C3-*RGD-Cy5*** (calculated: 27112 Da, found: 27110 Da).

Thereafter, we applied both dual-modified C3 proteins to A549 lung cells, which overexpress the integrin αvβ3 receptor[74] and investigated the uptake of the C3 proteins into the cells via confocal microscopy. As shown in Fig. 6a, the functionalization of C3 with RGD now promoted efficient cell internalization of **C3-*RGD-Cy5*** in comparison to the control **C3-*Cy3-Cy5*** (Fig. 6a). Based on this observation, the effect of the different chemical modifications of C3 *in vitro* was investigated in more detail. It is well-established that C3 internalization into cellular cytosol can lead to intoxication, showing highly characteristic morphological cells changes with shrunken/rounded cell bodies and the formation of protrusions.[75] Thus, the cell morphology was investigated after incubation of A549 cells with various C3 substrates using phase contrast microscopy (Fig.6b). After 5 h of treatment with two concentrations of the C3 substrates, **C3-*RGD-Cy5*** showed the most efficient uptake and intoxication (Fig. 6b-c) compared to the negative controls (**C3** and **C3-*Cy3-Cy5***). The difference was most pronounced at 300 nM indicating a concentration-dependent effect (Fig. 6c). The efficient internalization of **C3-*RGD-Cy5*** into the cytosol of the cells was further confirmed by the biochemical analysis of the ADP-ribosylation status of Rho from these cells (Fig. 6d), using Western blot analysis of cell lysates after incubation with C3 substrates (see Fig. 6d and SI for detailed description of analysis). A strong band indicates that Rho was not or only slightly modified in the intact cells during toxin treatment, while a weak band indicates ADP-ribosylation by C3. As shown in Fig. 6d, strong bands were observed in the Western blot of untreated (Ctrl), native **C3** and **C3-*Cy3-Cy5*** treated cells. In contrast, the cells intoxicated by **C3-*RGD-Cy5*** showed a weaker signal for Biotin-ADP-ribosylated Rho in the same Western blot. **C3-*RGD-Cy5*** was more efficiently internalized into the cytosol of the cells and the dual-functionalized C3 retained its catalytic function to exhibit ADP-ribosyltransferase activity in the cytosol, which is in agreement with earlier results.[69, 73, 75] The reaction with the trifunctional *N*-alkylpyridinium reagent preserved the catalytic activity of C3 and significantly enhanced cytosolic uptake into cancer cells, resulting in intracellular ADP-ribosylation, thus successfully transferring the catalytic properties of C3 into cancer cells.

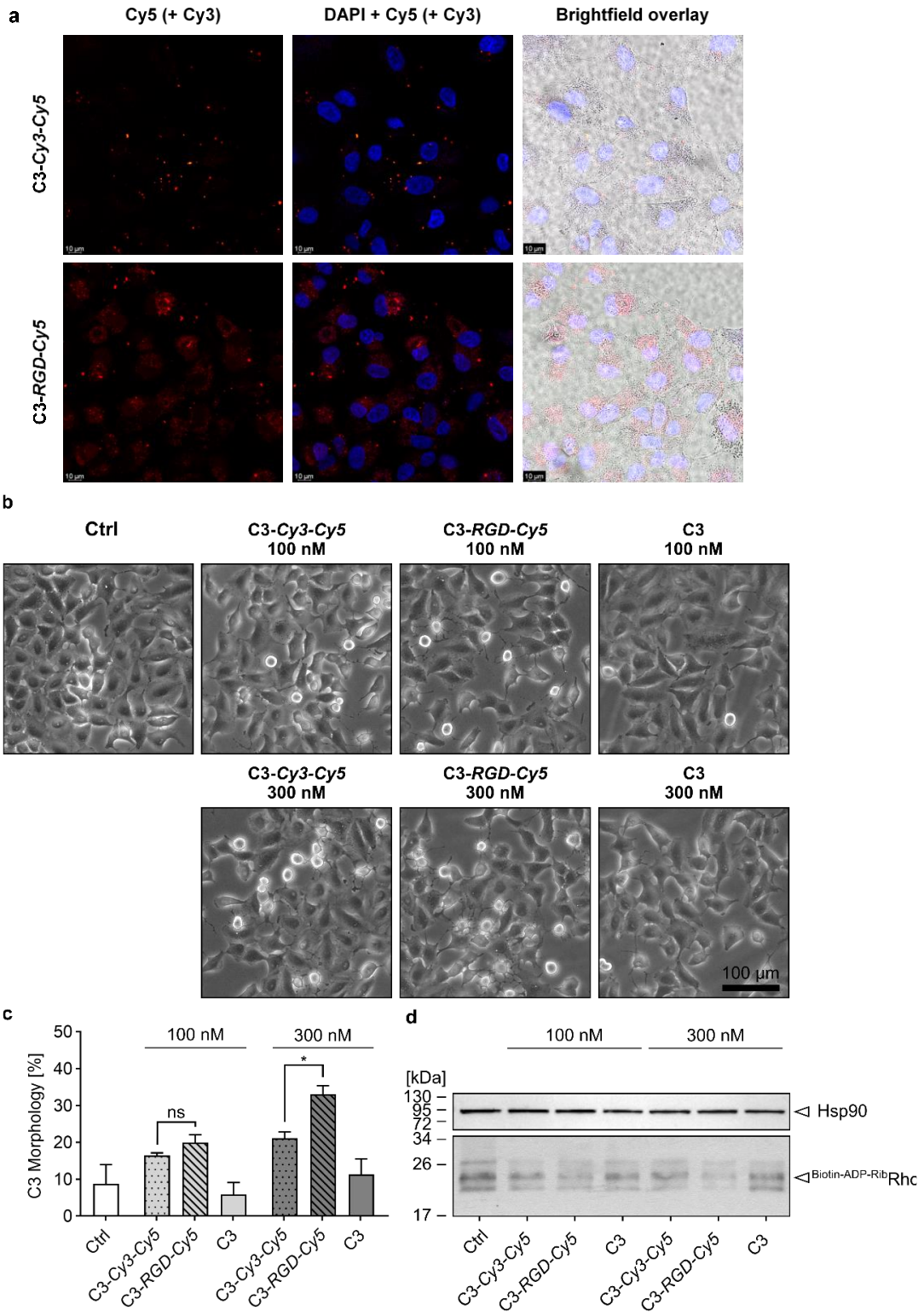


Fig. 6. (a) Fluorescence confocal microscopy showing uptake of C3 protein conjugates, **C3-*Cy3-Cy5*** and **C3-*RGD-Cy5*** into A549 cells treated with 300 nM of the respective C3 variants for 24 h and co-stained with NucBlue. DAPI channel at 405 nm, Cy5 channel at 649 nm and Cy3 channel at 554 nm. Scale bar: 10 µm. (b–d) A549 cells were treated with 100 nM or 300 nM of **C3**, **C3-*Cy3-Cy5*** or **C3-*RGD-Cy5*** at 37 °C for 5 h or left untreated (Ctrl). (b) Phase contrast microscopic images of intoxicated A549 cells. Scale bar: 100 µm. (c) Quantification of cells showing C3-morphology as percentage of total cell number. Values are given as mean ± SD and a t-test with Welch's correction was performed (n = 2, ns $p > 0.05$, * $p < 0.05$). (d) Intracellular Rho ADP-ribosylation status of cells after treatment for 8 h. Non-modified Rho in each cell lysate was biotin-labelled in the sequential ADP-ribosylation reaction by C3. Biotin-ADP-ribosylated Rho was detected in a Western Blot by streptavidin-peroxidase conjugate, where a weak signal

correlates with a high ADP-ribosyltransferase activity of C3 conjugates in living cells. Hsp90 served as loading control (n = 4). Detailed experimental information is provided in SI.

Discussion

We introduce *N*-alkylpyridinium reagents for site-selective dual functionalization of peptides and protein for the preparation of well-defined protein conjugates. The facile synthesis of the trifunctional *N*-alkylpyridinium derivatives with two reactive bioorthogonal handles were accomplished in good yields using a commercially available starting material. These reagents reveal excellent chemoselectivity, regioselectivity and high modification efficiency towards thiols, even in the presence of other nucleophiles. A broad range of cysteine-containing peptides and proteins can be site-selectively modified by *N*-alkylpyridinium derivatives through 1,6-addition reaction under mild, aqueous reaction conditions with excellent chemoselectivity, outperforming the broadly applied maleimides. As an alternative to arduous direct dual derivatisation using conventional bioconjugation reagents or genetic encoding of noncanonical amino acids,[76] we have shown that various dual functionalized bioconjugates can be easily obtained *in situ* by bioorthogonal reactions with high efficiency. Due to their ease in synthesis, these bioconjugation reactions can also be carried out by non-chemists. As a proof-of-concept, we demonstrated the functionalization of bacterial C3 enzyme with *N*-alkylpyridinium carrying two bioorthogonal handles for the conjugation of different payloads in a simple one-pot reaction under physiological conditions. In this way, additional functionalities were conferred to the C3 enzyme to transform beyond its natural function, improving its uptake into the cytosol of cancer cells and thereby increasing its activity in vitro. Our approach enables the *in situ* customisation of native peptides and proteins with different functions to tailor protein therapeutics to specific needs. We envisage that the bioconjugation strategy presented herein can be used by chemists, biologists and medical doctors to overcome the evolutionary limitations imposed by nature and expand the arsenal of protein therapeutics to better address the rapidly evolving diseases such as cancer or infections in the future.

Methods

General procedure for the synthesis of *N*-alkylpyridinium derivatives

Commercially available compound (*E*)-3-(pyridin-4-yl)acrylic acid (1 equiv) was dissolved in DMF (10 mg/mL). After that, EDC (1.2 equiv) and DMAP (0.12 equiv) were added and the resultant mixture was stirred at rt for 30 min. Then, the amine-containing substrate (1.5 equiv) was added and the reaction mixture was stirred overnight at rt. After that, the solvent was evaporated under reduced pressure and the crude product was purified by flash column chromatography to get compound that contains the first functionality. Thereafter, the alcohol-containing substrate (1 equiv) was dissolved in anhydrous DCM, followed by the addition of 2,6-dimethyl pyridine (1.5 equiv). After that, trifluoromethane sulfonic anhydride (1.5 equiv) was added at 0°C and the resultant mixture was stirred for 30 min. Without

further purification, the resultant triflyl-derivative was added to the aforementioned compound and the mixture was stirred overnight at rt. Thereafter, the reaction mixture was purified by HPLC to get the respective *N*-alkylpyridinium derivatives which were characterized by ^{1}H NMR, ^{13}C NMR ESI-LRMS and HRMS.

Reaction profile of *N*-alkylpyridinium with nucleophiles

Compound **1** was chosen as a model compound to react with a thiol-containing substrate **7** and an amine-containing substrate **8** to investigate the reactivity of *N*-alkylpyridinium derivatives towards thiol and amino groups. Compound **1** (50 µg, 0.20 µmol, 1 equiv) was also incubated with compound **7** (35.6 µg, 0.30 µmol, 1.5 equiv) and **8** (41.4 µg, 0.30 µmol, 1.5 equiv) in 50 µL ACN:PB (50 mM, pH 7) (*v/v* = 2:3) mixture. The resultant mixture was incubated at rt for 4h. Then the mixture was injected to the HPLC to monitor the reaction. In addition, compound **1** (50 µg, 0.20 µmol, 1 equiv) was also incubated with different amino acids, such as tyrosine (71.6 µg, 0.40 µmol, 2 equiv), methionine (59 µg, 0.40 µmol, 2 equiv), arginine (69 µg, 0.40 µmol, 2 equiv), histidine (61.3 µg, 0.40 µmol, 2 equiv), tryptophan (80.7 µg, 0.40 µmol, 2 equiv) and aspartic acid (53 µg, 0.40 µmol, 2 equiv), separately. All the reaction mixtures were incubated at rt for 4h. Thereafter, 10 µL of mixture was injected to HPLC to check if compound **1** reacted with these amino acids or not.

General procedure for site-selective modification of peptides

To a solution of peptide (1-2 mM) in 50 mM PB, pH 7 was added 1.2 equiv of compound **1** and mixed at rt. After 4-5 h, the conjugates were isolated by semi-preparative HPLC from the crude reaction mixtures with using a Zorbax Eclipse XDB-C18 HPLC column (80 Å, 9.4 × 250 mm, 5 µm) at a flow rate of 4 mL/min with acetonitrile (solvent A, containing 0.1% *v/v* TFA) and Milli Q water (solvent B, containing 0.1% *v/v* TFA). The modified peptide conjugates were characterized by ESI-LRMS and ESI-HRMS in positive mode.

General procedure for site-selective modification of proteins

Procedure for site-selective modification of proteins

A solution of the protein of interest (1 mg/mL, 1 equiv) was prepared in 50 mM PB buffer, pH 7. Next, the corresponding *N*-alkylpyridinium reagent (8-40 equiv depending on reagents and the protein itself) was added and the resultant mixture was incubated overnight at room temperature. After that, the reaction mixture was purified by using ultrafiltration tube (GE healthcare) to remove the excess bioconjugation reagents and organic solvent with using water as exchange solvent. The obtained protein conjugate was characterized by ESI-HRMS.

Procedure for dual functionalization of C3 enzyme

To the Cys-C3bot1 solution (0.372 mg/mL in 50 mM PB, pH 7.4) (300 µL, 0.0045 µmol), compound **6** (25.0 mg/mL in DMF) (20 equiv, 2.00 µL, 0.090 µmol) was added and the

resultant mixture was incubated for 5 h at 20°C. The reaction mixture was transferred to a ultrafiltration tube (0.5 mL, 10 kDa, PES filter) to remove the excess compound **6** and organic solvent using 50 mM PB, pH 7.4 as exchange solvent (3x, 12000G, 10 min) to deliver **C3-*N_3*-*Tz*** conjugate. The obtained **C3-*N_3*-*Tz*** was characterized by ESI-HRMS to confirm the successful modification.

A solution of RGDC-DBCO (20 mM in 50 mM PB, pH 7.4) (10 equiv, 0.75 µL, 0.015 µmol) was added, to the previously purified **C3-*N_3*-*Tz*** conjugate solution (100 µL) and mixed for 4h under same conditions. Then, Cy5-TCO.TEA (5 mg/mL in H_2O/1% DMSO) (10 equiv, 3.18 µL, 0.015 µmol) was added and mixed for 1 h. After confirming the successful C3 dual modification (*m/z* 27110) by ESI-HRMS, the reaction mixture was purified by UF in a 6 mL tube (6x, 12000G, 5 min) using 50 mM PB pH 7.4 as exchange solvent to afford the **C3-*RGD-Cy5*** conjugate.

Confocal Microscopy

A549 cells were seeded at a density of $2*10^5$ cells per well in DMEM (10% FBS, 1 % penicillin/streptomycin) within an IBIDI8-well confocal slide. After adhering for 24h, cells were treated with 300 nM of **C3-*RGD-Cy5*** and **C3-*Cy3-Cy5*** for 24h at 37°C, 5 %CO_2. After incubation, cells were washed thrice with PBS, co-stained with NucBlue and fixed with 4% PFA. Cells were imaged with a Leica Stellaris 8 microscope HC PL APO CS2 40x/1.25GLYC objective, 405 nm at 1.0% laser power for NucBlue excitation, 554 nm at 0.6% for Cy3 and 649 nm at 2.6% for Cy5.

Intoxication Studies

A549 cells were seeded in a 24-well microtiter plate with a cell number of $5*10^5$ cells per ml 48 h prior treatment and incubated at 37°C, 5 % CO_2. They were then treated with 100 nM or 300 nM of Cys-C3bot1 (**C3**), **C3-*Cy3-Cy5***, **C3-*RGD-Cy5***, respectively or left untreated as control. Phase contrast microscopy was used to monitor changes in cell morphology after intoxication.

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Author Contributions

L.X.: Initiating the project and conceptualization of the chemical design. Conducting synthetic and experimental work, performing 1,6-thiol additions to the peptide and protein substrates, data analysis and processing, preparing figures and drafting the manuscript.

M.J.S.A.S: Performing dual post-functionalization of C3-toxin enzyme, repeating 1,6-thiol additions to peptides and proteins to finalize analysis, planning and designing confocal uptake studies for C3-toxin enzyme. Data analysis and processing, drafting the manuscript.

J.A.S.C.: Calculation of Fukui indices, DFT calculations and interpretation, drafting discussion of DFT results.

J. B. and N. S.: Conducting experimental work, data analysis and processing for biochemical/biological evaluation of activity of C3-toxin enzyme, drafting the discussion for biological evaluation of activity of C3-toxin enzyme.

H. B.: Involved in the design and analysis of the biochemical/biological evaluation for C3-toxin enzymes, correcting the biological discussion, supervision of J.B. and N.S.

S.L.K.: Discussion of the design, concept and results, correcting the manuscript, initiated collaboration for the project.

T. W.: Involved in the inception and design concept, discussion of the concept and results, correcting the manuscript, acquired funding for the project, supervision of L.X. and M.J.S.A.S.

Competing interests

The authors declare no competing interests.