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Fluorogenic tagging of peptides via Cys residues using thiol-specific vinyl sulfone affinity tags

Gergely B. Cserép [a], Zsuzsa Baranyai [b], Dávid Komáromy [a], Kata Horváti [b], Szilvia Bősze [b], Péter Kele [a,*]

[a] "Lendület" Chemical Biology Research Group, Hungarian Academy of Sciences, Research Centre for Natural Sciences, Institute of Organic Chemistry, Magyar tudósok körútja 2, Budapest H-1117, Hungary
[b] MTA-ELTE Research Group of Peptide Chemistry, Pázmány Péter sétány 1a, Budapest H-1117, Hungary

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ABSTRACT

Fluorescent tagging of Cys-containing peptides is presented herein. The procedure follows a two-step sequential reaction scheme using thiol specific bifunctional chemical reporters and fluorogenic labels. Vinyl-sulfone bearing chemical reporters have been synthesized and demonstrated to selectively modify cysteine under physiological conditions in the presence of other nucleophilic amino acids. Bifunctional chemical reporters decorated with a terminal alkyne moiety, suitable for modification with azide containing fluorogenic labels were also synthesized. Such fluorogenic (*turn on*) labels in combination with these new vinyl-sulfone tags can be used generally in fluorescent modulation schemes of thiol-bearing biomolecules.

1. Introduction

Tagging of biomolecules with fluorescent probes by means of bioorthogonal methods has drawn much interest lately.[1] Static or dynamic, in vitro or in vivo real-time fluorescence imaging of biological structures requires chemical transformations using biologically inert, non-toxic, and non-reactive functional groups.[2] At the same time these bioorthogonal groups need strong affinities towards their counterpart function in a high yielding reaction at rates comparable to that of biological processes.[2] To date only a limited set of reactions is available that satisfy the above mentioned criteria. Among these, the Staudinger ligation[3] of azides and phosphanes, the inverse electron demand Diels–Alder reaction between tetrazines and strained alkenes,[4] the photoactivatable dipolar cycloaddition of tetrazoles and alkenes,[5] and the 1,3-dipolar cycloaddition of azides and alkynes (CuAAC or SPAAC)[6,7] are probably the most widely utilized. Bioorthogonal, fluorescent modulation schemes most often follow a two-step process. The first step involves the implementation of a chemical reporter that can subsequently be labeled with a specific tag carrying a bioorthogonal function.[8] Installation of such handles can be achieved, e.g., by taking advantage of the metabolic machinery of living systems using bioorthogonalized building blocks of biopolymers.[9] However, these modified building blocks are not always accepted by the metabolic pathways of live systems. Another option for site specific introduction of chemical reporters is offered by genetically encoded unnatural amino acids bearing a bioorthogonal function.[10] This method, however, is limited solely to proteins that are directly encoded in the genome and cannot be applied to other biomolecules or to follow, e.g., post-translational modifications. In such cases, introduction of bioorthogonalized handles through selective chemical reactions is the method of choice. There are only a few naturally occurring moieties with distinct reactivity that enable selective manipulation of target structures. For example, N-terminal modification of proteins becomes possible by making use of the relatively lowered pK_a of N-termini.[11] Single-site modification of proteins, however, still remains a challenging task. The thiol function of cysteine residues as being the second least abundant amino acid can offer a functionality that can be selectively modified with chemical reporters.[12] To target thiol groups under physiological conditions chemical biologists can use native chemical ligation techniques with synthetic surrogates, disulfide exchange[13] or direct alkylation schemes with iodoacetamides or maleimides.[11] Other techniques involve the treatment of Cys containing sequences with *O*-mesitylenesulfonylhydroxylamine.[14] Lately, thiol-ene/thiol-yne

* Corresponding author. Tel.: +36 1 382 6986; e-mail address: kele.peter@ttk.mta.hu (P. Kele).

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chemistry has also become an emerging tool of chemical biology research.[15] The reaction between thiols and alkenes/alkynes can be triggered either by radical initiators (e.g., UV-light) or nucleophilic catalysts (e.g., PPh_3). Another group of Michael-acceptors is represented by reagents bearing vinyl-sulfone motif. Earlier works by Masri et al.[16a] and Hemelaar et al.[16b] demonstrated that the excellent reactivity of electron deficient vinyl sulfones towards thiols allows fast and high yielding modulation of sulfhydryls without the need for any further auxiliaries.[16] A report by Santoyo-Gonzales et al. provides a recent example for a vinyl-sulfone warhead with multiple functionality.[16c,d] Contrary to its superior activity only few examples were reported on the use of vinyl sulfones in bioorthogonal ligation schemes. Besides their reactivity, vinyl-sulfones are mostly water soluble, highly stable compounds, and their Michael-addition reaction with thiols proceeds almost quantitatively and basically without any by-products.[16c,d] The Michael product of vinyl-sulfones is also much more stable compared to other thiol specific reagents (e.g., maleimides).[16] Herein we report on the synthesis of hydrophilic vinyl-sulfone and alkyne containing linkers that can site-selectively target thiols. These bifunctional linkers can serve as chemical reporters enabling further functionalization by means of alkyne-azide 1,3-dipolar cycloaddition, e.g., with fluorogenic labels. Recently, we have reported on the synthesis of new fluorogenic systems that offered low-background fluorescent tagging schemes with genetically encoded cyclooctyne bearing non-canonical amino acids.[17] Combination of the specificity of water soluble vinyl-sulfone based bifunctional chemical reporters with the power of fluorogenic probes provides us with a generally applicable technique for site specific and selective fluorescent modulation of peptides and proteins. Furthermore the use of fluorogenic probes is also advantageous, for example, in the emerging field of super-resolution microscopy techniques.

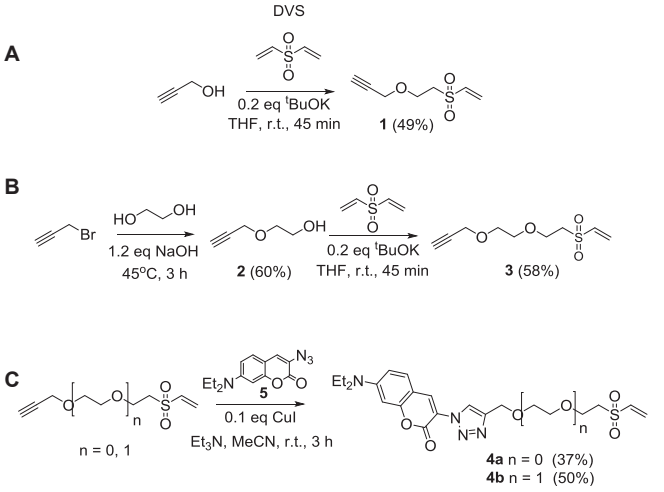
2. Results and discussion

2.1. Synthesis of bifunctional linkers

In the design of these hydrophilic bifunctional chemical reporters we have considered bioorthogonal functions that offer concise synthesis, provide high yielding reactions within reasonable timeframes, and most importantly that can be targeted by fluorogenic dyes. More particularly, we aimed at constructing thiol-specific chemical reporters that can be further modified with fluorogenic azides.[17] Therefore we have designed chemical reporters with ethylene glycol cores decorated with vinyl-sulfone and terminal alkyne moieties as thiol and alkyne specific moieties, respectively. The synthesis of **1** was achieved by reacting propargyl alcohol with divinyl-sulfone (DVS, Scheme 1A). The reaction was found to give the best yield (50%) when carried out in THF in the presence of 4 equiv of DVS and catalytic amount of ^{t}BuOK. To construct a more hydrophilic chemical reporter, ethylene glycol was reacted with propargylbromide to provide **2** (Scheme 1B). Subsequent reaction of **2** with DVS under similar conditions used for the synthesis of **1** provided **3** in 60% yield. A portion of **3** and **1** was further subjected to cycloaddition reaction with 3-azido-7-diethylaminocoumarin, **5** to furnish **4a** and **4b** (Scheme 1C).

2.2. Reactivity and selectivity studies

In order to elaborate the reactivity and selectivity of bifunctional vinyl-sulfones we have chosen a heptapeptide sequence (DTHFPIC) containing a single Cys residue. Unlike other Cys-containing peptides, this sequence has the unique feature of existing in monomeric form for longer time periods without the formation of disulfides.[18,19] Former HPLC and MS studies have shown that no dimerization was observed following a 24 h incubation time.[18]



Scheme 1. Syntheses of bifunctional chemical reporters and fluorescently labeled thiol active reagents.

When an excess amount of **4a** was incubated with DTHFPIC in a buffered medium (50 mM HEPES, pH 8), the peptide could still be detected after 15 min, however, it was completely consumed after 1 h, with simultaneous appearance of a new peak in the HPLC chromatogram (ESI). Mass spectrometry confirmed that the new peak corresponds to a DTHFPIC-**4a** adduct. Considering that the heptapeptide contains further side chains with nucleophilic character, e.g., His, Thr, we compared the MS/MS spectra of the free peptide and the labeled adduct. To our delight, fragmentation patterns confirmed that the peptide forms a covalent bond uniquely with Cys (ESI).

Next, we studied the pH range of reactions, especially in the presence of other nucleophilic residues, e.g., Lys side chain. For this, we have synthesized another peptide sequence that contained an N-terminal Lys-Gly elongation (KGDTHFPIC). First, we explored whether the altered sequence retained stability.[19] Gratifyingly, minimal disulfide-formation was observed after 24 h incubation (ESI). At first, we examined the reaction between **1** and KGDTHFPIC nonapeptide at pH 8, using 1.5 equiv vinyl sulfone reagent. The reaction conducted this way was almost instantaneous as HPLC chromatogram showed nearly complete consumption of the peptide within 1 min (ESI). Then we lowered the pH to 7.4 and used 1.5 equiv of **1** to label the nonapeptide. As expected, at lower pH the reaction required longer time and complete consumption of the peptide could be observed only after 90 min (ESI). Using equimolar amounts of the reagents at the same pH required 120 min for complete conversion of the starting materials. MS, and MS/MS analysis of the products have confirmed that the peptide was labeled with **1** exclusively through the Cys side chain (ESI).

2.3. Sequential labeling of peptide

Our next aim was to demonstrate the possibility of fluorescence tagging of Cys-residues via sequential labeling schemes. For this, we have used the KGDTHFPIC nonapeptide sequence. We anticipated that a two-step tagging scheme of the peptide through its thiol function by appropriately functionalized vinyl sulfone chemical reporter and fluorogenic labels would provide us with reaction mixtures that contain a sole fluorescent entity, i.e., the fluorescently labeled peptide (Scheme 2). First, we have prepared and purified two derivatives of the peptide: a) KGDTHFPIC-**3** and b) KGDTHFPIC-**4b**.

Secondly, KGDTHFPIC-**3** was mixed with 2 equiv of fluorogenic dye **5** in the absence and in the presence of a copper catalyst. In

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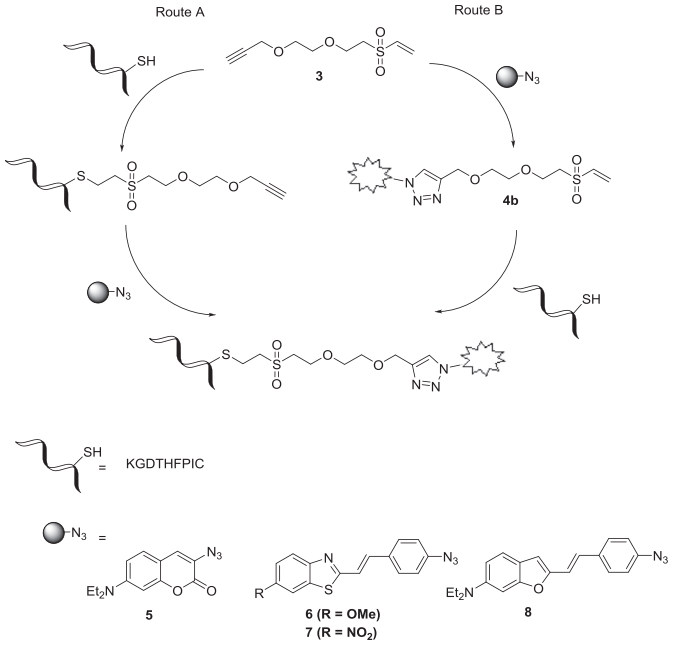

Scheme 2. Sequential (Route A) and direct (Route B) labeling strategies of Cys containing peptides.

a parallel experiment KGDTHFPIC was labeled with excessive amount (2 equiv) of **4b**. All reaction mixtures were loaded directly onto SDS-gel. Following gel electrophoresis the gel was irradiated with UV light to trigger fluorescence of the products. As expected, the purified sample of KGDTHFPIC-**4b** contains a single fluorescent product (Fig. 1A, line 3) indicating the position of the product of KGDTHFPIC-**3**+**5** reaction in the presence of copper catalyst (Fig. 1A, line 5).

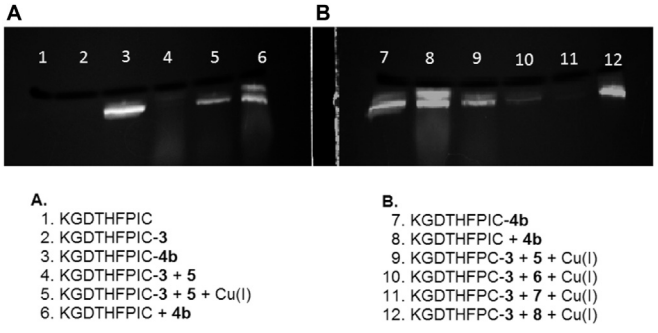

Fig. 1. Fluorescence images of gels. (A) Comparison between sequential (lane 5) and direct labeling routes (lane 6). (B) Sequential labeling with further fluorogenic labels (lanes 9–12) in comparison with direct labeling (lane 8).

The fact that line 5 of Fig. 1A shows only a single fluorescing band, implies that the excess amount of fluorogenic label **5** does not contribute to the fluorescent signal. The image also shows that the same reaction in the absence of copper does not result in fluorescent species (Fig. 1A, line 4). On the other hand, when the peptide was reacted directly with 2 equiv of fluorescent chemical reporter **4b** a second fluorescent band appears just above the product line indicating the presence of unreacted **4b** (Fig. 1A, line 6). These findings support the superiority of sequential modification routines (Route A) over direct fluorescent labeling schemes.

In a separate experiment we have tested the performance of other fluorogenic labels **6**–**8** that have recently been developed in our laboratory.[17] Fig. 1B shows that all fluorogenic labels gave similar results, with label **8** giving the best performance. The inefficiency (very low fluorescence) of label **7** is probably due to poor compatibility of excitation and emission channels with the fluorescent product. Differences in fluorescent quantum yields could also be accounted for the lower intensities (for full and color images see ESI, color of compound **6** is more intense).

3. Conclusions

We have presented a fluorescent tagging scheme using clickable fluorogenic labels and hydrophilic, clickable, chemical reporters with thiol-specific vinyl-sulfone warhead. Systematic studies have demonstrated that these water soluble vinyl-sulfones have strong and chemoselective affinity towards thiol groups under physiological conditions. In peptide tagging experiments these bifunctional linkers have been shown to be suitable for further modification, e.g., with azide containing fluorogenic labels. The use of these fluorogenic (*turn on*) labels may offer reduction of background fluorescence resulting in higher signal-to-noise ratios, a feature highly demanded, e.g., in the field of super-resolution microscopy. These results suggest a generalizable chemical modulation scheme of cysteine containing peptides and proteins that allow further manipulation, e.g., with fluorogenic labels in a sequential manner.

4. Experimental

4.1. General

Unless otherwise indicated, all starting materials were obtained from commercial suppliers (Sigma–Aldrich, Alfa Aesar, Merck) and used without further purification. Analytical thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} precoated glass TLC plates with 0.25 mm silica. Column chromatography was carried out with flash silica gel (0.040–0.063 mm) from Merck. Semipreparative RP-HPLC purification was carried out using a Knauer HPLC system under the following conditions: gradient elution: 5 min equilibration at 0% B, 0–90% B 90 min (A eluent: 0.1% TFA/water v/v; B eluent: 0.1% TFA/acetonitrile:water/80:20 v/v), flow rate: 4 ml/min, detection: 220 nm, column: Phenomenex Jupiter C18, 10 mm ID×250 mm, 10 μm, 300 Å. The NMR spectra were recorded on a Bruker DRX-250 spectrometer. Chemical shifts (δ) are given in parts per million (ppm) using solvent signals as the reference. Coupling constants (J) are reported in Hertz (Hz). Splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quadruplet), quint (quintuplet), m (multiplet), dd (doublet of a doublet), and td (triplet of doublets). Mass spectrometry of the peptide derivatives was carried out on a Bruker Daltonics Esquire 3000+ ion trap mass spectrometer while high resolution mass spectrometry (HRMS) measurements were carried out on an Agilent 6230 MS-TOF system. All melting points were measured on Büchi 501 apparatus and are uncorrected. IR spectra were obtained on Bruker IFS55 spectrometer on a single-reflection diamond ATR unit.

For peptide synthesis, *N*,*N*′-diisopropylcarbodiimide (DIC), triisopropylsilane (TIS) were purchased from Fluka (Buchs, Switzerland). The amino acid derivatives were obtained from Reanal (Budapest, Hungary) and IRIS Biotech (Marktredwitz, Germany). 1-Hydroxybenzotriazole (HOBt) and 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) were purchased from IRIS Biotech. Fmoc-Rink Amide MBHA resin was purchased from NovaBiochem (Läufelfingen, Switzerland). Acetonitrile, trifluoroacetic acid (TFA), *N*-methylpyrrolidone (NMP), and dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). Ninhydrin was bought from Sigma–Aldrich (St. Louis, MO, USA). *N*,*N*-Dimethylformamide (DMF) was purchased from Reanal (Budapest, Hungary).

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4.2. Synthesis

*4.2.1. 3-(2-(Vinylsulfonyl)ethoxy)prop-1-yne (**1**).* To a solution of propargyl alcohol (120 μL, 2.05 mmol) and DVS (820 μL, 8.20 mmol) in anhydrous THF (15 mL) under argon atmosphere was added dropwise a suspension of ^{t}BuOK (46 mg, 0.41 mmol) in anhydrous THF (20 mL). The reaction mixture was stirred at room temperature for 45 min when TLC (DCM:MeOH/200:1) showed total conversion of the alcohol. After evaporation of the solvent the product was extracted with EtOAc (5×10 mL) and purified on silica (DCM:MeOH/500:1) to give 174 mg (49%) of the desired product as a colorless oil. R_f=0.28 (DCM:MeOH/500:1). IR: ν(neat)=1095; 1126; 1311; 1358; 1386; 1445; 1612; 2116; 2877; 3060; 3269 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz): δ=2.47 (1H, d, J=2.3 Hz), 3.26 (2H, t, J=5.6 Hz), 3.91 (2H, t, J=5.6 Hz), 4.14 (2H, d, J=2.3 Hz), 6.09 (1H, d, J=9.9 Hz), 6.38 (1H, d, J=16.6 Hz), 6.70 (1H, dd, J_1=16.6 Hz, J_2=9.9 Hz). ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ=54.7, 58.4, 63.2, 75.4, 78.4, 129.2, 137.4. HRMS (ESI): $[M+H]^+$ calcd for $C_7H_{11}O_3S^+$: 175.0429, found: 175.0431.

*4.2.2. 2-(Prop-2-ynyloxy)ethanol (**2**).* A solution of propargylbromide (2.5 mL, 80% in toluene, 22.5 mmol) and ethylene glycol (2.5 mL) was flushed with argon and cooled to 0 °C in an ice bath. Powdered NaOH (1.08 g, 45.0 mmol) was added and the reaction mixture was stirred at 45 °C for 3 h. The precipitate was filtered, washed with DCM (2×3 mL), and the filtrate was extracted with DCM (3×10 mL). After evaporation of the solvent the crude product was purified using silica gel column chromatography (Hexane:EtOAc/2:1) to yield 1.34 g (60%) of **2** as a pale yellow oil. R_f=0.25 (Hexane:EtOAc/2:1). IR: ν(neat)=1106; 1246; 1729; 2115; 2868; 2935; 3287; 3400 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz): δ=2.42 (1H, t, J=2.2 Hz), 2.99 (1H, s), 3.55 (2H, t, J=4.4 Hz), 3.66 (2H, t, J=4.4 Hz), 4.12 (2H, d, J=2.2 Hz). ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ=58.1, 61.2, 71.0, 74.6, 79.3. HRMS (ESI): $[M+H]^+$ calcd for $C_5H_9O_2^+$: 101.0603, found: 101.0602.

*4.2.3. 3-(2-(2-(Vinylsulfonyl)ethoxy)ethoxy)prop-1-yne (**3**).* To a solution of **2** (320 mg, 3.2 mmol) and DVS (480 μL, 4.8 mmol) in anhydrous THF (15 mL) under argon atmosphere was added dropwise a suspension of ^{t}BuOK (72 mg, 0.71 mmol) in anhydrous THF (30 mL). The reaction mixture was stirred at room temperature for 45 min when TLC (Hexane:EtOAc/5:3) showed total conversion of the alcohol. After evaporation of the solvent the product was extracted by EtOAc (5×10 mL) and purified on silica (Hexane:EtOAc/2:1) to give 402 mg (58%) pale yellow oil. R_f=0.27 (Hexane:EtOAc 5:3). IR: ν(neat)=1122; 1353; 1385; 1461; 2115; 2872; 3266 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz): δ=2.43 (1H, d, J=2.4 Hz), 3.24 (2H, t, J=5.7 Hz), 3.64 (4H, m), 3.87 (2H, t, J=5.7 Hz), 4.16 (d, 2H, J=2.4 Hz), 6.06 (1H, d, J=9.9 Hz), 6.36 (1H, d, J=16.6 Hz), 6.78 (1H, dd, J_1=16.6 Hz, J_2=9.9 Hz). ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ=54.9, 58.2, 64.6, 68.6, 70.2, 74.7, 79.3, 128.7, 137.8. HRMS (ESI): $[M+H]^+$ calcd for $C_9H_{15}O_4S^+$ $[M+H]^+$: 219.0691, found: 219.0689.

*4.2.4. 7-(Diethylamino)-3-(4-((2-(vinylsulfonyl)ethoxy)methyl)-1H-1,2,3-triazol-1-yl)-coumarin (**4a**).* 7-Diethylamino-3-azido-coumarin (100 mg, 0.39 mmol) and 3-(2-(vinylsulfonyl)ethoxy)-prop-1-yne (**1**) (67 mg, 0.39 mmol) were dissolved in MeCN (10 mL) and triethylamine (50 μL) and CuI (7.4 mg, 0.039 mmol) were added. The mixture was stirred at room temperature for 1 h when TLC (Hexane:EtOAc/1:1) showed completion of the reaction. After evaporation of the solvent the product was extracted by EtOAc (3×5 mL) purified on silica (Hexane:EtOAc/1:1→1:3 gradient) to yield 62 mg (37%) yellowish powder. R_f=0.14 (Hexane:EtOAc 1:1). Mp=152–154 °C. IR: ν(neat)=1249; 1291; 1432; 1465; 1484; 1526; 1596; 1621; 1708; 2875; 2925; 3069; 3179 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz): δ=1.24 (6H, t, J=7.1 Hz), 3.28 (2H, t, J=5.6 Hz), 3.46 (4H, q, J=7.1 Hz), 3.97 (2H, t, J=5.6 Hz), 4.72 (2H, s), 6.10 (1H, d, J=9.9 Hz), 6.40 (1H, d, J=16.6 Hz), 6.55 (1H, d, J=2.4 Hz), 6.68 (1H, dd, J_1=8.9 Hz, J_2=2.4 Hz), 6.76 (1H, dd, J_1=16.6 Hz, J_2=9.9 Hz), 7.41 (1H, d, J=8.9 Hz), 8.39 (1H, s), 8.5 (1H, s). ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ=12.4, 45.0, 55.0, 63.9, 64.4, 97.0, 107.0, 110.1, 116.6, 123.7, 129.1, 130.0, 134.8, 137.7, 143.7, 151.7, 155.8, 156.9. HRMS (ESI): $[M+H]^+$ calcd for $C_{20}H_{25}N_4O_5S^+$: 433.1546, found: 433.1544.

*4.2.5. 7-(Diethylamino)-3-(4-((2-(2-(vinylsulfonyl)ethoxy)ethoxy)-methyl)-1H-1,2,3-triazol-1-yl)-coumarin (**4b**).* 7-Diethylamino-3-azido-coumarin (70 mg, 0.28 mmol) and 3-(2-(vinylsulfonyl)-ethoxy)-prop-1-yne (**1**) (63 mg, 0.28 mmol) were dissolved in MeCN (10 mL) and triethylamine (50 μL) and CuI (53 mg, 0.28 mmol) were added. The mixture was stirred at room temperature for 1 h when TLC (Hexane:EtOAc/1:1) showed completion of the reaction. After evaporation of the solvent the product was extracted by EtOAc (3×5 mL) from the brown residue and was purified on silica (Hexane:EtOAc/1:1→1:3 gradient) to yield 67 mg (50%) product as a yellowish powder. R_f=0.12 (Hexane:EtOAc/1:2). Mp=101–103 °C. IR: ν(neat)=1188, 1243, 1265, 1291, 1346, 1381, 1472, 1596, 1624, 1695, 1714, 2870, 2904, 2976, 3060, 3160 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz): δ=1.22 (6H, t, J=7.0 Hz), 3.25 (2H, t, J=5.6 Hz), 3.44 (4H, q, J=7.0 Hz), 3.67 (4H, m), 3.88 (2H, t, J=5.6 Hz), 4.72 (2H, s), 6.07 (1H, dd, J=9.9 Hz), 6.36 (1H, d, J=16.6 Hz) 6.53 (1H, s), 6.66 (1H, d, J=8.9 Hz), 6.79 (1H, dd, J_1=16.6 Hz, J_2=9.9 Hz), 7.39 (1H, d, J=8.9 Hz), 8.35 (1H, s), 8.53 (1H, s). ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ=12.3, 44.9, 54.9, 64.3, 64.6, 69.2, 70.4, 96.9, 106.9, 110.0, 116.7, 123.6, 128.8, 130.0, 134.8, 137.8, 144.4, 151.5, 155.7, 156.9. HRMS (ESI): $[M+H]^+$ calcd for $C_{22}H_{29}N_4O_6S^+$: 477.1808, found: 477.1810.

4.3. Peptide synthesis, tagging reactions

4.3.1. Peptide synthesis. The peptides (DTHFPIC, KGDTHFPIC) were synthesized manually by Fmoc/^{t}Bu solid phase strategy on Fmoc-Rink Amide MBHA resin. The protocol of the synthesis was the following: (i) Fmoc deprotection with 2% piperidine/2% DBU/DMF (v/v), 2+2+5+10 min; (ii) washing with DMF (5×1 min); (iii) coupling with 3 equiv of Fmoc-amino acid derivative–DIC–HOBt dissolved in NMP, 60 min; and (iv) washing with DMF (5×1 min). Peptides were cleaved from the resin with the TFA/H_2O/TIS (9.5:2.5:2.5 v/v) mixture (2 h, RT). After filtration, compounds were precipitated in cold diethyl ether, centrifuged (4000 rpm, 5 min), and freeze-dried in water. Crude products were purified by semi-preparative RP-HPLC. Purified peptides were analyzed by analytical RP-HPLC, ESI MS, and amino acid analysis (ESI).

*4.3.2. Synthesis of KGDTHFPIC-**3**.* 7.6 mg (7.5 μmol) KGDHTFPIC peptide was dissolved in 1000 μL 50 mM HEPES buffer (pH 8.0) and 2.4 mg (11.2 μmol, 1.5 equiv) **3** in 100 μL DMF was added to the peptide. The reaction was stirred at room temperature and monitored by analytical RP-HPLC. The reaction was totally completed after 1 h. The product was purified by semi-preparative RP-HPLC and analyzed by analytical RP-HPLC, ESI MS, MS/MS (see ESI).

*4.3.3. Synthesis of KGDTHFPIC-**4b**. Route A*: 1.0 mg (0.8 μmol) KGDHTFPIC-**3** was dissolved in 287 μL 50 mM HEPES buffer (pH 8.0). 203 μL 10 mM sodium L-ascorbate (2.5 equiv) solution in 50 mM HEPES buffer (pH 8.0) and 203 μL 10 mM $CuSO_4$–TBTA (2.5 equiv) solution (55% DMSO, 45% distilled water) was prepared and combined. To this mixture 418 μg (1.6 μmol, 2 equiv) **5** in 287 μL DMSO and the 287 μL solution of KGDTHFPIC-**3** was added finally. The reaction was stirred at room temperature and monitored by analytical RP-HPLC. The reaction was completed after 24 h. The product was purified by semipreparative RP-HPLC and analyzed by analytical RP-HPLC, ESI MS, MS/MS (ESI).

Route B: 2.5 mg (2.5 μmol) KGDHTFPIC peptide was dissolved in 400 μL 50 mM HEPES buffer (pH 8.0) and 2.3 mg (4.9 μmol, 2 equiv) **4b** in 400 μL DMF was added to the peptide. The reaction was

stirred at room temperature and monitored by analytical RP-HPLC. The reaction was totally completed after 7 h. The product was purified by semi-preparative RP-HPLC and analyzed by analytical RP-HPLC, ESI MS, MS/MS (ESI).

4.4. Gel chromatography

SDS-PAGE gel electrophoresis was carried out using PhastGel® high density gels and PhastGel SDS buffer strips on PhastSystem electrophoresis system (Pharmacia, Uppsala, Sweden). The following samples were prepared:

1. 1 μg/μL KGDTHFPIC in distilled water.
2. 1 μg/μL KGDTHFPIC-**3** in distilled water.
3. and 7. 1 μg/μL KGDTHFPIC-**4b** in distilled water.
4. 1 μg/μL KGDTHFPIC-**3** and 2 equiv **5** in distilled water/DMSO.
5. and 9. 1 μg/μL KGDTHFPIC-**3** in HEPES buffer, 2 equiv **5** in DMSO, 2.5 equiv 10 mM sodium L-ascorbate solution, 2.5 equiv 10 mM $CuSO_4$–TBTA solution, the sample was mixed for 20 h at room temperature before the gel electrophoresis.
6. and 8. 1 μg/μL KGDTHFPIC in HEPES buffer, 2 equiv **4b** in DMSO, the sample was stirred for 20 h at room temperature before the gel electrophoresis.
10. 1 μg/μL KGDTHFPIC-**3** in HEPES buffer, 2 equiv **6** in DMSO, 2.5 equiv 10 mM sodium L-ascorbate solution, 2.5 equiv 10 mM $CuSO_4$–TBTA solution, the sample was mixed for 20 h at room temperature before the gel electrophoresis.
11. 1 μg/μL KGDTHFPIC-**3** in HEPES buffer, 2 equiv **7** in DMSO, 2.5 equiv 10 mM sodium L-ascorbate solution, 2.5 equiv 10 mM $CuSO_4$–TBTA solution, the sample was mixed for 20 h at room temperature before the gel electrophoresis.
12. 1 μg/μL KGDTHFPIC-**3** in HEPES buffer, 2 equiv **8** in DMSO, 2.5 equiv 10 mM sodium L-ascorbate solution, 2.5 equiv 10 mM $CuSO_4$–TBTA solution, the sample was mixed for 20 h at room temperature before the gel electrophoresis.

40–40 μL 2× running buffer (10 mM Tris/HCl, 1 mM EDTA, 2.5% SDS, 0.01% Bromophenol Blue, pH 8.0) was added to 40 μL of each samples. The samples were heated at 100 °C for 5 min. 4–4 μL of the samples were applied for the gel. The separation was carried out according to the PhastSystem, Separation Technique File No. 112.[20] The gel was irradiated with UV light and photographed. The images were taken using Genesnap software (Syngene, Frederick, USA).

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2014.05.103.

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