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α -Silylated Diazoalkynes: New Tools for Bioconjugation of Proteins

Tuan Zhao,^[a] Emmanuelle Sachon,^[b] Laurent Micouin,^{*,[a]} and Riccardo Piccardi^{*,[a]}

α -Silylated diazoalkynes are stabilized diazo compounds that can selectively react with carboxylic residues in buffered aqueous media. *In-situ* fluoride induced desilylation increases this reactivity, leading to a very fast reaction. Application to the

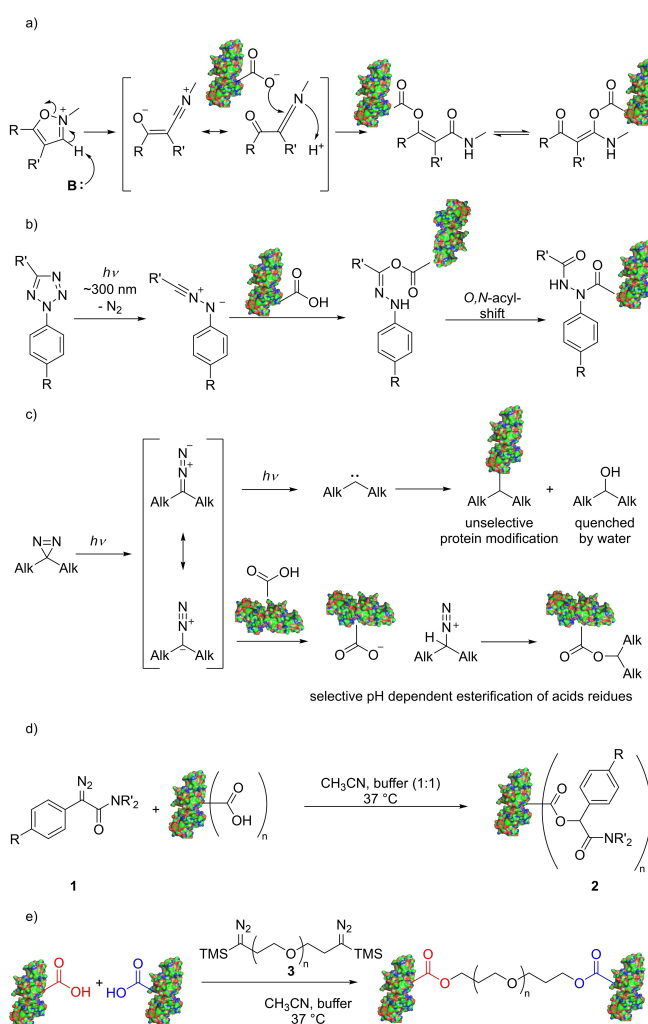
selective functionalization of RNase A, followed by post-functionalization using click chemistry, is described. These new reagents expand the toolbox for native protein modification at carboxylic residues.

Introduction

Direct native protein modification is of extreme interest, with many applications such as fluorophores or photoswitches introduction for biological investigations, PEGylation to improve solubility and biocompatibilities, or antibody-drugs conjugates preparation for targeted drugs delivery.^[1]

Chemical protein bioconjugation relies mainly in targeting nucleophilic reactive amino acids side chains,^[2] in particular the ones of cysteine (Cys)^[3] or lysine (Lys).^[4] Besides these classical transformations, selective modifications based on nucleophilic or redox properties have also been developed to target other residues such as tyrosine (Tyr).^[5] Examples of transformations based on carbon-centered radical species are growing, thanks to the recent progress in the field of (photo)-redox catalysis and electrochemistry.^[6]

Selective modification of acidic residues has drawn attention in the last decade. Small heterocycles (like isoxazolium salts or tetrazoles) have been used to generate reactive intermediate to trap a carboxylate and form a stable enol-ester or an amide (Schemes 1a and 1b).^[7,8] Diazirines have also been largely employed in photoaffinity labeling.^[9] Interestingly, their reactivity has been recently revisited by the group of Woo, who showed that their photolysis can lead to different intermediates, depending on their substitution pattern.^[10] Upon irradiation, aryl diazirines react through a carbene intermediate whereas alkyl diazirines preferentially rearrange into a diazo



Scheme 1. Methods of functionalization of the acidic residues of proteins.

intermediate. Therefore, the two different reaction pathways produce different selectivity. Aryldiazirines are more prone to react with weak acids like Tyr or Cys while alkyl diazirines react preferentially with Glutamic (Glu) and Aspartic acid (Asp) residues in a pH-dependent manner (Scheme 1c).

This esterification of carboxylic acids side chains with diazo compounds was already reported in 1960s.^[11] Diazomethane

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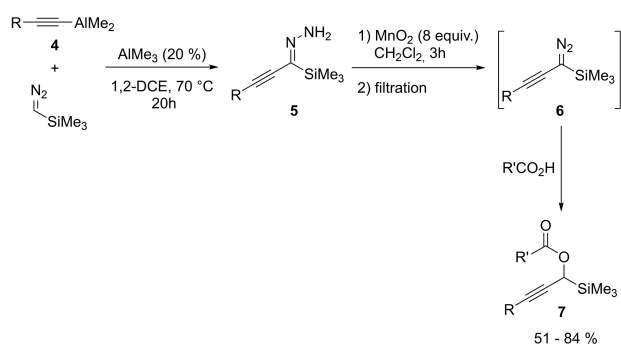
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was the first compound used in esterification of proteins in non-aqueous medium.^[12] Later on, protein functionalization in aqueous medium was described using more stable diazo compounds, such as diphenyldiazomethane^[13] diazoacetoglycinamide^[14] and diazoacetamide.^[15] However, in all these examples, large excess of diazo compounds ($> 10^3$ -fold) was used to compensate their low water stability, leading to hydrolysis.

A major breakthrough in this field was the use of more stable α -aryl- α -diazoacetamide derivatives (Scheme 1d) developed by the group of Raines in their studies on the cytosolic delivery of proteins by esterification of proteins and their intracellular decaying by esterases^[16] or small molecules.^[17] One key feature of this class of reagents is the basicity of these diazo compounds that can be tuned by their aromatic substitution pattern to preferentially abstract a proton from a carboxylic acid but not water in a buffered reaction medium, leading to an improved esterification efficiency.^[18] Interestingly, a substitution by a silyl group is known to affect the basicity and the stability of diazo compounds. Surprisingly, the use of α -silyl diazo derivatives as tools for protein functionalization has only been scarcely reported. However, derivative **3** was indeed reported to esterify and cross-link proteins in aqueous medium at pH 7.4 (Scheme 1e).^[19]

We have recently reported a general access to α -silyl diazoalkynes **6** that we have shown to readily react in dichloromethane with carboxylic acids to afford propargyl esters **7** (Scheme 2).^[20] This synthesis allows to access in a simple manner to a variety of compounds possessing different substituents on the triple bond.

During this study, we could notice that these reagents were also stable in water. This observation prompted us to investigate if compounds such as **6a** (Figure 1) could act as bifunc-



Scheme 2. Synthesis of α -acyloxy- α -alkynyltrimethylsilanes.

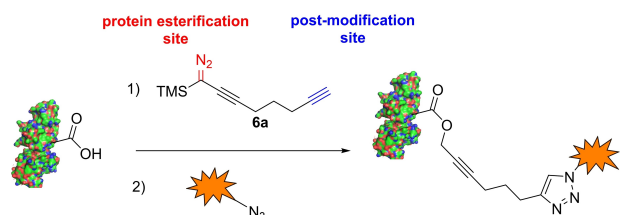


Figure 1. α -silyl diazoalkyne **6a** as versatile conjugation tool for proteins.

ditional conjugation agent by first reacting with acidic residues of a protein followed by a second functionalization of the terminal alkyne.

Results and Discussion

The first part of this work was devoted to adapting the two-step one pot procedure used in the synthesis of propargyl esters **7** to the esterification of amino acids in an aqueous medium.

We investigated the outcome of the reaction using **6b** as model compound with 2 equivalents of *N*-Boc-Gly at different pH. Reactions were conducted in a 2:1 mixture of THF and buffer in order to work in a homogeneous reaction medium. The use of DMF, DMSO or acetonitrile led to the degradation of the starting material during the oxidation step.

Two compounds **7ba** and **8ba** were obtained at pH 5.5 or 6 with a comparable overall combined yield (entry 1 and 2, Table 1). The formation of **8ba** can be explained by the desilylation of diazo **6b** as described by the group of Lloyd-Jones on the esterification of carboxylic acids with trimethylsilyl diazomethane.^[21] This result is also in agreement with the work reported by the group of Lei with compound **3**.^[19]

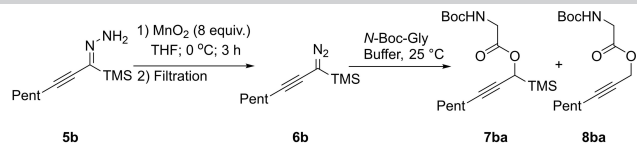
The reaction was then carried out using a buffer at pH 6.5 and 7. In these cases only compound **7ba** was obtained in comparable yields (entry 3 and 4, Table 1).

We investigated the influence of the ratio between the diazo and the amino acid at pH 5.5 and 7.^[16a,18a,19]

When the reaction was conducted at pH 5.5 using equimolar ratio of the two reagents, **7ba** was obtained in 34% yield (entry 2 Table 2), while no further improvement was observed using 2 equivalents of **6b**, probably because higher concentration of diazo favors the hydrolysis reaction (entry 3). On the other hand, when the reaction was carried out at pH 7, using 2 or 1 equivalents of amino acids did not affect the final yield of the reaction (entry 4 and 5) while the use of 2 equivalents of

Table 1. Study of the influence of the pH.				
Entry ^[a]	Buffer ^[b] (pH)	Time (h)	Yield (%) ^[c]	
			7ba	8ba
1	MES (5.5)	0.5	16	13
2	MES (6)	2	11	13
3	MES (6.5)	0.5	24	–
4	HEPES (7)	0.5	21	–

[a] Conditions: oxidation was performed treating a solution of **5b** (0.03 M in THF) with 8 equivalents of MnO_2 . Esterification was performed adding a solution of **6b** (10 mL, 0.03 M in THF) to a solution of *N*-Boc-Gly (5 mL, 0.12 M) in the desired buffer at 25 °C. [b] Concentration of the buffer 0.3 M. [c] Isolated yields calculated from **5b**.

Table 2. Study of the influence of the ratio between **6b** and *N*-Boc–Gly.


Entry ^[a]	5b : <i>N</i> -Boc–Gly	Buffer ^[b] (pH)	Time (h)	Yield (%) ^[c]	7ba	8ba
1 ^[e]	1:2	MES (5.5)	0.5	16 ^[d]	13 ^[d]	–
2 ^[f]	1:1	MES (5.5)	0.5	34	–	–
3 ^[g]	2:1	MES (5.5)	0.5	32	–	–
4 ^[e]	1:2	HEPES (7)	0.5	21 ^[d]	–	–
5 ^[f]	1:1	HEPES (7)	0.5	19	–	–
6 ^[g]	2:1	HEPES (7)	0.5	traces	–	–
7 ^[h]	1:1	MES (5.5)	0.5	23	–	–
8 ^[i]	1:1	MES (5.5)	0.5	18	–	–
9 ^[j]	2:1	MES (5.5)	1	53	–	–

[a] Oxidation was performed treating a solution of **5b** (0.03 M in THF) with 8 equivalents of MnO₂. [b] Concentration of the buffer was 0.3 M; [c] Isolated yields calculated from *N*-Boc–Gly. [d] Isolated yields calculated from **5b** [e] **6b** (10 mL, 0.03 M) was added to *N*-Boc–Gly (5 mL, 0.12 M) in the desired buffer at 25 °C. [f] **6b** (10 mL, 0.03 M) was added to *N*-Boc–Gly (5 mL, 0.06 M) in the desired buffer at 25 °C. [g] **6b** (10 mL, 0.06 M) was added *N*-Boc–Gly (5 mL, 0.06 M) in the desired buffer at 25 °C. [h] Esterification conducted at 40 °C. [i] Esterification conducted at 0 °C. [j] **6b** (10 mL, 0.03 M in THF) was added to *N*-Boc–Gly (5 mL, 0.06 M) in MES buffer at 25 °C, followed by another addition after 30 min of **6b** (2 mL, 0.15 M in THF).

diazo had even a negative effect on the outcome of the reaction (entry 6). The esterification reaction was then conducted at different temperatures (entries 7 and 8) with no improvement compared to the reaction conducted at 25 °C (entry 2).

Finally, we decided to work with successive additions of diazo (Table S1). This approach proved to be beneficial since when 2 equivalents of diazo were added in two portions at 30 min interval, compound **7ba** was isolated in 53% yield (Table 2 entry 9). However, no further improvement was obtained using 3.5 equivalents (Table S1, entry 3), adding the diazo dropwise or adding a more concentrated solution of diazo in solution of amino acid in buffer and THF 1:1 (Table S1, entry 4 and 5).

Conditions used in table 2, entry 9 were selected for labeling studies. Hydrazone **5a**, prepared from the corresponding alkynylaluminum reagent **4a** in 37% isolated yield was oxidized into the corresponding diazo compound and reacted with *N*-Boc–Gly to deliver **7aa** together with **8aa** in respectively 29% and 24% yield. Similar reaction with *N*-Boc–Ser led to the corresponding esters **7ab** and **8ab** in 20 and 18% yield, respectively. In this case, the best results were obtained using three successive additions of the diazoalkyne. As expected, no reaction of the alcohol moiety was observed.^[22] The reaction conducted with *N*-Boc–Glu gave a complex mixture, while with *N*-Boc–Asp–NH₂ **7ac** and **8ac** were obtained in 15 and 13%

yield respectively (scheme 3), showing that carboxylate side chains can also react with diazo **6a**.

The reaction with tripeptides **9** and **10** was then investigated.

8ad could be obtained in 29% yield from **9**. Esterification of **10** led to **8ae** in 32% isolated yield (Scheme 4).

Having established the ability of silylated diazoalkynes to selectively react with short peptides carboxylic residues in buffered medium, we then investigated their ability to label the free carboxylic residues on the bovine pancreatic ribonuclease A (RNase A).^[23]

First attempts using our optimized protocol with **10** to 50 equivalents of diazo to take into account the presence of 11 carboxylic acid residues in the protein led to inconsistent adducts as depicted by MALDI-TOF analyses.

We suspect that these artifacts could be due to the use of THF, reported to be incompatible with the polypropylene that constitutes the Eppendorf® vials used for the experiments.^[24] We therefore modified our protocol by first performing the oxidation step in CH₂Cl₂, solvent removing under vacuum,^[25] and conducting the esterification step in an acetonitrile-buffer mixture. Under these conditions, using *N*-Boc–Gly as a model substrate, silylated esterified amino acid **7aa** was systematically accompanied by desilylated adduct **8aa** (Table 3, entries 1–3). As this mixture could lead to complex mass analyses with proteins bearing multiple carboxylic acid residues, we inves-

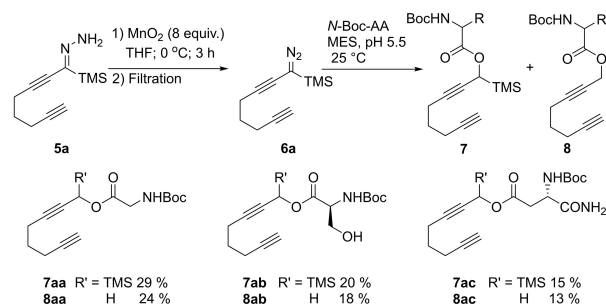
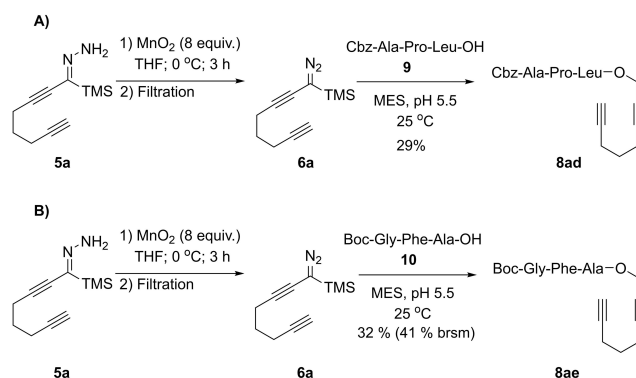
**Scheme 3.** Reaction of **6a** with *N*-Boc–Gly, *N*-Boc–Ser and *N*-Boc–Asp–NH₂**Scheme 4.** Functionalization of tripeptides. Conditions: **6a** (5 mL, 0.03 M in THF) was added to a solution of tripeptide (2.5 mL, 0.06 M) in MES buffer at 25 °C. Equation A: second addition of **6a** (1 mL, 0.15 M in THF) after 30 min. Equation B: second addition of **6a** (1 mL, 0.15 M in THF) after 30 min., followed by third addition of **6a** (1 mL, 0.15 M in THF) after one hour. (see experimental part).

Table 3. Protocol modification.

Entry ^[a]	CsF (equiv.)	Number of additions	Time total (h)	Yield (%) ^[b]	
				7aa	8aa
1 ^[c]	–	1	0.5	31	5
2 ^[d]	–	2	1	32	6
3 ^[e]	–	1	1	38	5
4 ^[f]	2	1	0.5	–	24
5 ^[g]	2	2	1	–	28
6 ^[h]	2	1	1	–	25

[a] Oxidation was performed treating a solution of **5a** in CH₂Cl₂ with 8 equivalents of MnO₂. The solvent was then evaporated and **6a** was dissolved in the desired amount of CH₃CN; [b] Isolated yields based on *N*-Boc-Gly; [c] **6a** (0.6 mmol in 5 mL of CH₃CN) was added to *N*-Boc-Gly (0.3 mmol in 5 mL of MES buffer). [d] **6a** (0.3 mmol in 5 mL of CH₃CN) was added to *N*-Boc-Gly (0.3 mmol in 5 mL of MES buffer). after 30 min **6a** (0.3 mmol in 1 mL of CH₃CN) was added to the reaction mixture which was stirred for another 30 min; [e] as entry 1 but reaction stirred for 1 h; [f] **6a** (0.6 mmol in 5 mL of CH₃CN) was added to a mixture of *N*-Boc-Gly (0.3 mmol in 5 mL of MES buffer) and CsF (0.6 mmol) and stirred for 1 h. [g] **6a** (0.3 mmol in 5 mL of CH₃CN) was added to a mixture of *N*-Boc-Gly (0.3 mmol in 5 mL of MES buffer) and CsF (0.6 mmol). After 30 min, **6a** (0.3 mmol in 1 mL of CH₃CN) was added to the reaction mixture which was stirred for another 30 min. [h] As entry 4 but stirred for 1 h.

tigated the addition of cesium fluoride in the reaction mixture to favor the formation of desilylated adducts (Table 3, entries 4–6). As expected, compound **8aa** was exclusively obtained in 24 to 28% yield depending on the reaction conditions.

Additional experiments were performed to better understand the role of CsF in the desilylation reaction. First, the stability of **6a** at pH 5.5 was evaluated using IR spectroscopy. When the solution of **6a** was dissolved in buffer, we observed a diazo stretching band at 2055 cm⁻¹ whose intensity diminished over 60 minutes (Figure 2A and 2B). The same experiment conducted in the presence of CsF led to the observation of a new band at 2074 cm⁻¹ with a much faster decay within 4 min (Figure 2C and 2D).

Similar experiments were then performed in the presence of *N*-Boc-Gly. Completion of the reaction was observed after 30 minutes in MES buffer at pH 5.5 (Figure 3A and 3B) whereas it took only 100 seconds to see the disappearance of compound **6a** in the presence of CsF (Figure 4A and 4B).

These results clearly outline the stabilizing effect of the silyl group on compound **6a**. CsF promoted desilylation generates a new diazo reagent with improved reactivity towards water and carboxylic residues, leading to desilylated adducts at a faster reaction rate.

Reaction with RNase A was then investigated using 30 equivalents of **6a** solubilized in acetonitrile. We tested different reaction temperatures (37 or 25 °C) and reaction times. Under these conditions, multiple residues were esterified. As

expected, MALDI-TOF analysis revealed that silylated (*M* + 176u) or desilylated (*M* + 104u) esters were present at the same time on protein **11** (Scheme 5A). On the other hand, conducting the same experiment in the presence of CsF (30 equiv.) protein **12** was obtained showing a much cleaner MALDI-TOF (Scheme 5B) with no trace of silylated adducts. The reaction was also conducted using a lower RNase A concentration (100 μM) and a large excess (300 equiv.) of diazo and CsF. A similar conjugation pattern was confirmed by MALDI-TOF analysis (see Supporting Information).

Trypsin digestion of the modified RNase A was analyzed using liquid chromatography coupled to mass spectrometry (MS) to characterize the sites of modification. MS/MS fragmentation allowed to unambiguously characterize Asp14, Glu49, Asp53 and Val124 (C-term) as the dominant modified residues. However, we cannot rule out other residues being modified in low amount (below fmol), which would not be detectable by MS. These results are in line with the ones reported by Raines and coworkers who described that diazoacetamide compounds were able to react with Asp14, Glu49, Glu111 and Val124 (C-term) positions.^[16a] Attempts to decrease the quantity of **6a** to only 10 equiv. led to identical MALDI-TOF spectra. However, MS/MS analysis after trypsin digestion could only clearly confirm esterification of Glu49.

Finally, the modular functionalization of the terminal alkyne was investigated first with the introduction of the coumarin fluorogenic probe **13** using copper catalyzed alkyne azide cycloaddition (CuAAC) (Scheme 6 and Figure 5).

Experimental conditions described by Finn, using Tris(3-hydroxypropyltriazolyl-methyl)amine (THPA) and aminoguanidine^[26] led to the introduction of the coumarin moiety, as confirmed by both MALDI-TOF and by UV and fluorescence spectroscopy. Biotin functionality could be installed using the same procedure with compound **14** (Scheme 6 and Figure 5).

Conclusions

In summary, we have reported that silylated diazo alkynes, easily prepared in a two-step process from trimethylsilyldiazo-methane, can react with carboxylic residues in buffered aqueous solution. We have also shown that these compounds can be further activated by a CsF induced *in-situ* desilylation reaction, delivering new species that can react with proteins in a fast manner. Finally, we have shown that this strategy enables the installation of a terminal triple-bond onto a protein, allowing facile diversification via click chemistry.

Experimental Section

General Remarks

All reactions were carried out under argon atmosphere in flame-dried glassware using dry solvents, unless otherwise specified. Dry solvents (1,2-dichloroethane, CH₃CN, CH₂Cl₂, THF) and all commercially available compounds were purchased from Merck, Acros

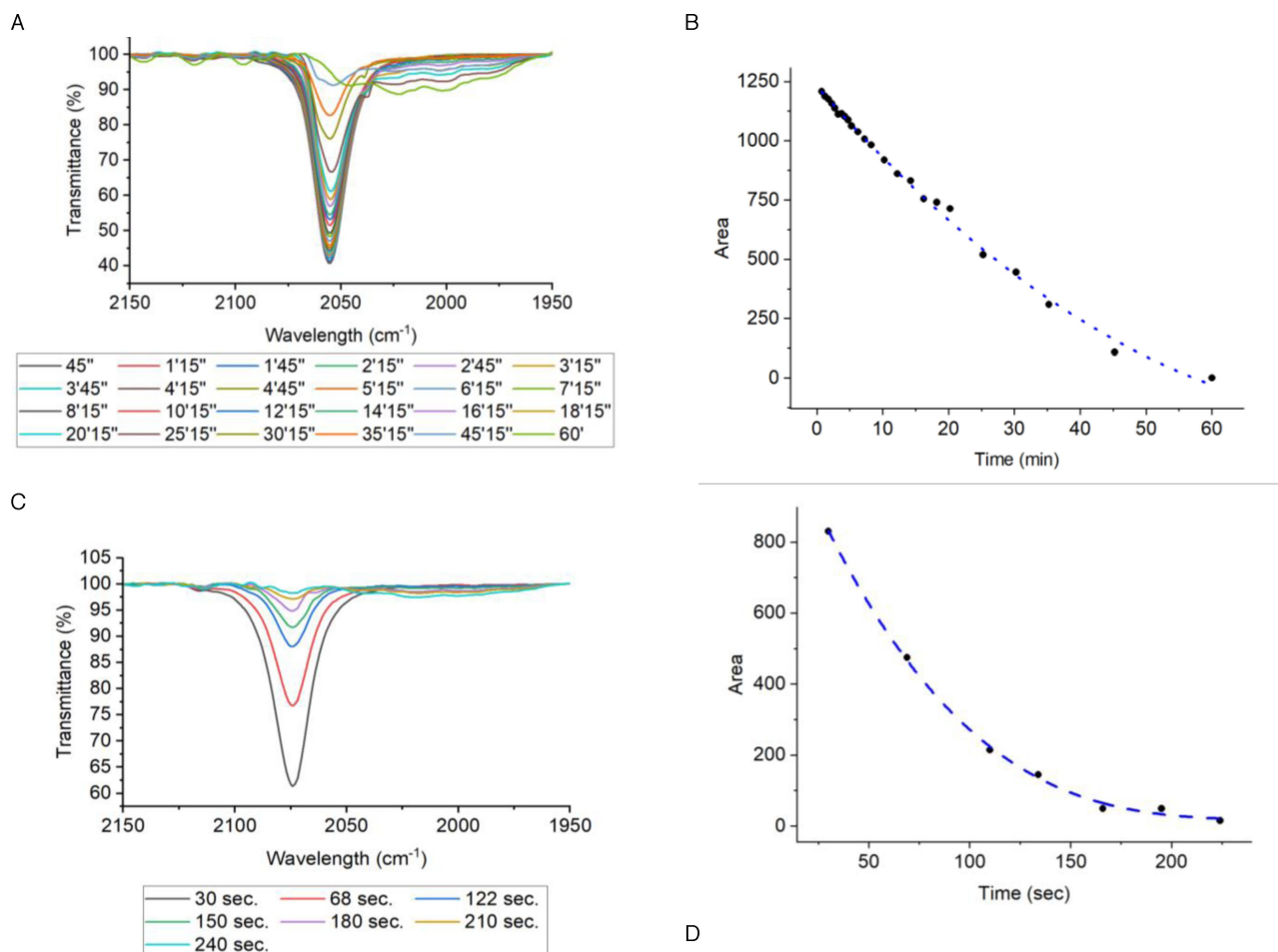


Figure 2. IR stability studies of **6a** in MES buffer 0.6 M pH 5.5. **A)** time-dependent transmittance variation at 2055 cm⁻¹; **B)** time-dependent variation of band area at 2055 cm⁻¹; **C)** time-dependent transmittance variation at 2074 cm⁻¹ in presence of CsF (2 equiv.); **D)** time-dependent variation of band area at 2074 cm⁻¹ in presence of CsF (2 equiv.).

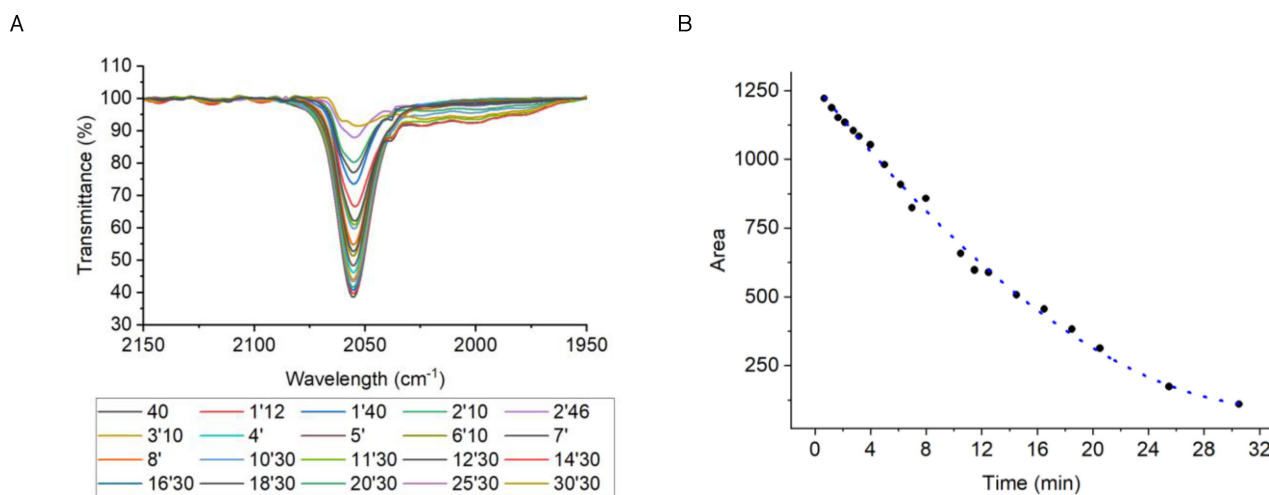


Figure 3. IR reactivity studies of **6a** with *N*-Boc-Gly in MES buffer 0.6 M pH 5.5. **A)** time-dependent transmittance variation at 2055 cm⁻¹ **B)** time-dependent variation of band area at 2055 cm⁻¹.

Organics or Alfa Aesar and used as received. Peptides were purchased from Bachem AG.. MES buffer (0.3 M, pH=5.5) used in the reactions with amino acids and tripeptides was prepared by

adding a 5 M aqueous solution of NaOH to a 0.3 M aqueous solution of MES till pH 5.5. MES buffer (0.15 M, pH 5.5) used in the reaction with RNase A was obtained in a similar manner starting

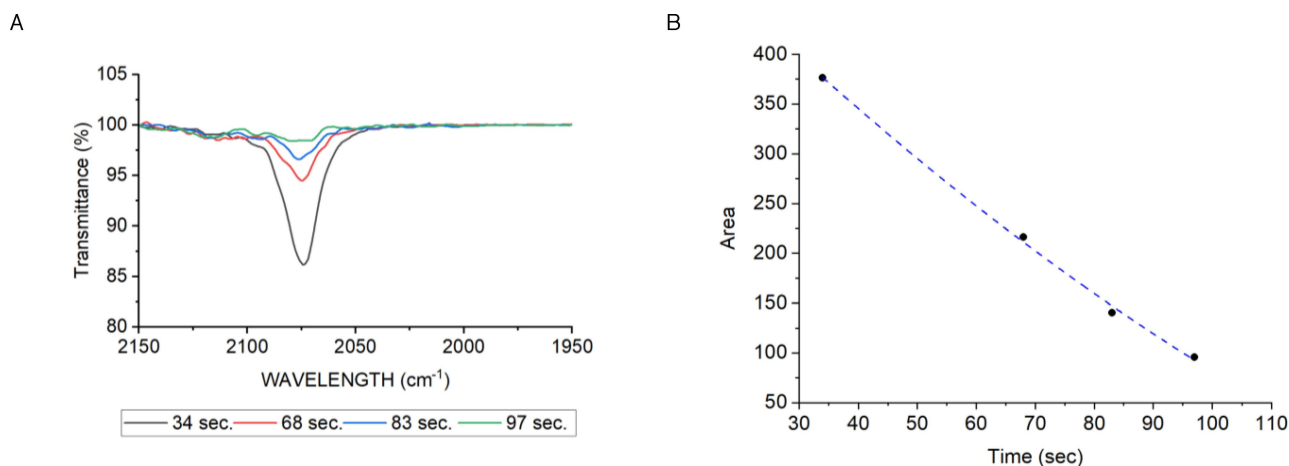
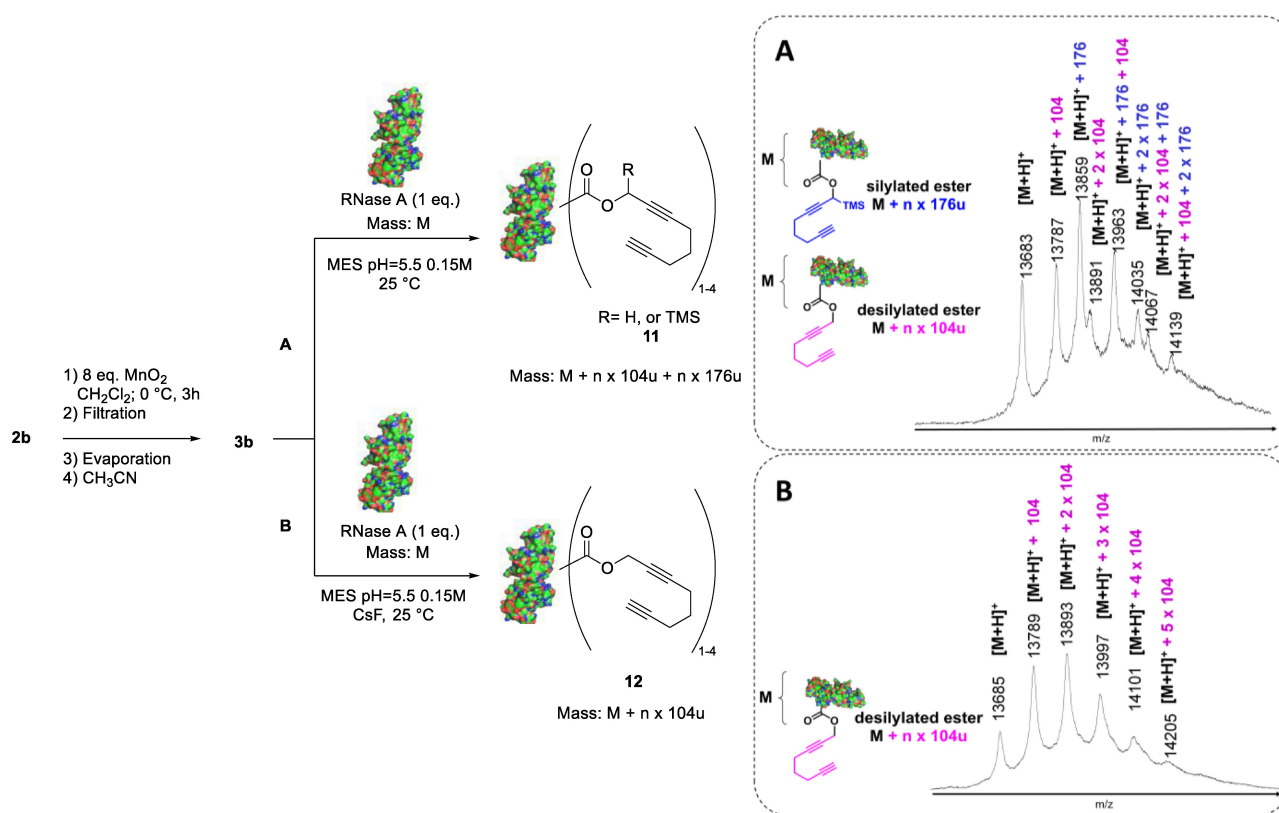


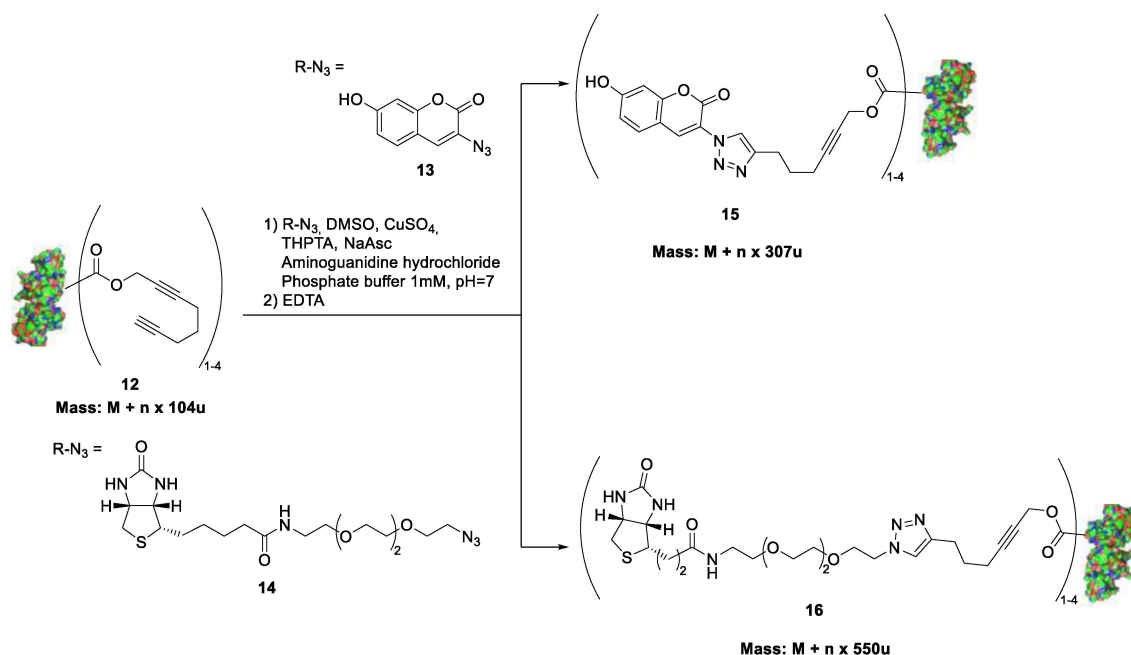
Figure 4. IR reactivity studies of **6a** with *N*-Boc-Gly in MES buffer 0.6 M pH 5.5. A) time-dependent transmittance variation at 2074 cm⁻¹ in the presence of CsF (2 equiv.); B) time-dependent variation of band area at 2074 cm⁻¹ in the presence of CsF (2 equiv.).



Scheme 5. Reaction between diazo **3b** and RNase A without A) or with CsF B) and corresponding MALDI-TOF, linear positive ions with SA.

with a solution of MES 0.15 M. Phosphate buffer (0.1 M, pH 7.0) was obtained by adding a solution of K₂HPO₄ trihydrate (900 mg, 4.0 mmol) in H₂O (50 mL) to a solution of KH₂PO₄ monohydrate (840 mg, 6.0 mmol) in H₂O (50 mL). The pH of the resulting solution was adjusted to pH 7 by adding an aqueous solution of NaOH (2 M). The pH of the buffer solutions was measured by a pH-meter FiveEasy from METTLER TOLEDO. Isolation of proteins was performed by Ultra-centrifugal filtration: the solution of the protein was placed in an AMICON® Ultra-4 centrifugal device with a membrane of regenerated cellulose with a cutoff of 3 kDa, and centrifugated for 35 min at 4000 rpm. Yields refer to chromatographically

and spectroscopically (¹H NMR) homogeneous materials, unless otherwise stated. Reactions were monitored by TLC carried out on 0.2 mm E. Merck silica gel plates (60 F₂₅₄) using UV lamp as visualizing agent and KMnO₄ solution as developing agent. Flash column chromatography was performed on silica gel (particle size: 60–230 mesh). ¹H (500 MHz), ¹³C (125 MHz) NMR spectra were recorded on a Bruker Avancell 500 in CDCl₃ or DMSO-*d*₆ and calibrated using residual undeuterated solvent as an internal reference. Chemical shifts (δ) are given in ppm relative to tetramethylsilane (0 ppm). Multiplicity is indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), p (pentuplet), sext



Scheme 6. Click reaction of 12 and azides 13 and 14.

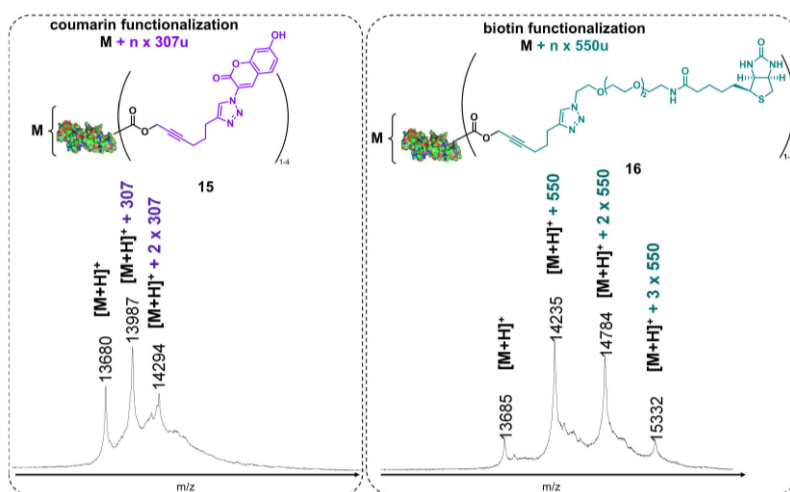


Figure 5. MALDI-TOF, linear positive ions, with SA of compounds 15 and 16.

(sextet), m (multiplet), dd (doublet of doublet), td (triplet of doublet), brs (broad singlet), brd (broad doublet). Coupling constants J are reported in Hz. All NMR spectra were obtained at 300 K unless otherwise specified. High Resolution mass spectra were recorded on a ThermoFischer Exactive Orbitrap spectrometer.

IR spectra were obtained using a spectrometer Perkin Elmer Spectrum ONE using an ATR MIRACLE (PIKE) or a CaF₂ liquid cell. Absorption and fluorescence spectra were recorded on UV-2700 spectrophotometer (Shimadzu) and F-7000 fluorescence spectrometer (Hitachi), respectively. MALDI-TOF analyses were performed by the mass spectrometry MS³U platform at Sorbonne University. Spectra were acquired using a Bruker Autoflex IV in a linear positive-ion mode and using sinapinic acids as the matrix. Trypsin digestion and MS/MS experiments to identify modified protein residues were performed at PROTEOM'IC Facility of Cochin Institute.

Mixed dimethylaluminum reagents^[27] and **5b**^[20a] were prepared following literature procedures.

Preparation of 5a: a 2 M solution of AlMe₃ in toluene (0.6 mL, 1.2 mmol) was added to a 1.56 M solution of (hepta-1,6-diy-1-yl)dimethylaluminum in toluene (3.8 mL, 5.6 mmol) and DCE (28 mL), followed by a 2 M solution of trimethylsilyldiazomethane in Et₂O (4.2 mL, 8.4 mmol). The flask was sealed, and the reaction mixture was stirred at 70 °C for 20 h. The reaction mixture was cooled down and then poured in a mixture of Rochelle's salts and ice and the resulting mixture was stirred for 1 h. The two phases were separated, and the aqueous layer was extracted 3 times with EtOAc; the combined organic layers were washed with brine, dried over MgSO₄, filtered and the solvent was evaporated affording an orange oil that was purified by flash chromatography (pentane/Et₂O 85:15 then 80/20). **5a** was obtained as a light orange oil, 426 mg (2.06 mmol, 37% yield).

1-(trimethylsilylocta-2,7-diyn-1-ylidene)hydrazine (**5a**): $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 6.51 (brs, 2H), 2.61 (t, $J=7.0$ Hz, 2H), 2.35 (td, $J=7.0$, 2.6 Hz, 2H), 1.98 (t, $J=2.6$ Hz, 1H), 1.81 (p, $J=7.0$ Hz, 2H), 0.18 (s, 9H). $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 138.6, 104.8, 83.2, 74.2, 69.3, 27.5, 18.8, 17.6, -2.3 . HRMS (ESI) m/z : calcd for $\text{C}_{11}\text{H}_{19}\text{N}_2\text{Si}^+$ ($[\text{M}+\text{H}]^+$) 207.1312, found 207.1314.

General procedure A for the preparation of diazo derivatives in THF: MnO_2 (2.4 mmol, 8 eq.) was added to a solution of hydrazone **5** (0.3 mmol, 1 eq.) in THF (10 mL) at rt. The suspension was stirred for 4 hours and then the solids were filtered off using a syringe filter. The resulting solution was used in the following step without any further purification.

General procedure B for the preparation of solution of diazo compounds in CH_3CN : Hydrazone (0.6 mmol, 1 eq.) was added at 0°C to a suspension of MnO_2 (4.8 mmol, 8 eq.) in CH_2Cl_2 (10 mL). After 1 h, the ice bath was removed, and the mixture was stirred at rt for 2 hours. The mixture was filtered through a syringe filter to separate the solid precipitate. The filtrate was concentrated under reduced pressure in a cold bath (**CAUTION**, pure diazo compounds can be explosive!). The pure diazo compound was finally dissolved in CH_3CN (5 mL).

General procedure C for the esterification of amino acids in a THF: MES mixture 2:1: A solution of **6** (10 mL, 0.03 M in THF), obtained following general procedure **A**, was added to a solution of amino acid (5 mL, 0.06 M in MES buffer). The mixture was stirred at 25°C for 30 min, then a solution of **6** (2 mL of a 0.15 M in THF) was added. After 30 min, Et_2O was added, and the two phases were separated. The aqueous phase was extracted twice with Et_2O , then the combined organic layers were washed with brine, dried over MgSO_4 and concentrated under vacuum.

Reaction of **6b with *N*-Boc-Gly in a THF: MES mixture 2:1:** The reaction was conducted following general procedure **C** starting from 53 mg (0.3 mmol, 1 equiv.) of *N*-Boc-Gly and 126 mg (0.6 mmol, 2 equiv.) of **5b**.^[20a] The crude product was purified by column chromatography (cyclohexane/ Et_2O 9:1 to 8:2) to give **7ba** as a yellow oil (56 mg, 53%).

1-(Trimethylsilyloct-2-yn-1-yl (tert-butoxycarbonyl)glycinate (**7ba**).^[20b] $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 5.21 (t, $J=2.4$ Hz, 1H), 5.02 (bs, 1H), 4.00–3.75 (m, 2H), 2.21 (td, $J=7.1$, 2.2 Hz, 2H), 1.48 (sext, $J=7.2$ Hz, 2H), 1.44 (s, 9H), 1.39–1.27 (m, 4H), 0.88 (t, $J=7.1$ Hz, 3H), 0.12 (s, 9H). $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 170.3, 155.7, 89.4, 80.0, 76.2, 60.4, 42.7, 31.1, 28.5, 28.4, 22.3, 19.1, 14.1, -3.8 . HRMS (ESI) m/z : calcd for $\text{C}_{18}\text{H}_{33}\text{NNaO}_4\text{Si}^+$ ($[\text{M}+\text{Na}]^+$) 378.2071, found 378.2065.

Oct-2-yn-1-yl (tert-butoxycarbonyl)glycinate (**8ba**). Compound **8ba** was isolated during the optimization of the reaction conditions. $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 5.01 (s, 1H), 4.73 (t, $J=2.2$ Hz, 2H), 4.10–3.76 (m, 2H), 2.19 (tt, $J=7.2$, 2.2 Hz, 2H), 1.50 (pent, $J=7.2$; 2H), 1.44 (s, 9H), 1.38–1.25 (m, 4H), 0.88 (t, $J=7.1$ Hz, 3H). $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 170.0, 155.8, 88.5, 80.2, 73.5, 53.8, 42.5, 31.1, 28.4, 28.2, 22.3, 18.8, 14.0. HRMS (ESI) m/z : calcd for $\text{C}_{15}\text{H}_{25}\text{NO}_4\text{Na}^+$ ($[\text{M}+\text{Na}]^+$) 306.1676, found 306.1682.

Reaction of **6a with *N*-Boc-Gly:** The reaction was conducted following general procedure **B** starting from 53 mg (0.3 mmol, 1 equiv.) of *N*-Boc-Gly and 126 mg (0.6 mmol, 2 equiv.) of **5a**. The crude product was purified by column chromatography (Heptane/ Et_2O 85:15 then 75:25) to give **7aa** (31 mg, 0.089 mmol, 29%) and **8aa** (20 mg, 0.07 mmol, 24%) as light orange oils.

Reaction of **6a with *N*-Boc-Gly in a mixture CH_3CN : MES 1:1:** A solution of **6a** (4.6 mL, 0.12 M in CH_3CN , 115 mg, 0.56 mmol), obtained following general procedure **C**, was added to a solution of *N*-Boc-Gly (4.6 mL, 0.06 M, in MES buffer, 49 mg, 0.28 mmol). The

mixture was stirred at 25°C for 1 hour, then Et_2O was added, and the two phases were separated. The aqueous phase was extracted twice with Et_2O , then the combined organic layers were washed with brine, dried over MgSO_4 and concentrated under vacuum. The crude product was purified by column chromatography (Heptane/ Et_2O 85:15 then 75:25) to give **7aa** (38 mg, 0.107 mmol, 38%) and **8aa** (4.0 mg, 0.014 mmol, 5%) as light orange oils.

Reaction of **6a with *N*-Boc-Gly with CsF:** A solution of **6a** (5.0 mL, 0.12 M in CH_3CN , 124 mg, 0.6 mmol) in CH_3CN , obtained following general procedure **C**, was added to a solution of *N*-Boc-Gly (5 mL, 0.06 M in MES buffer, 53 mg, 0.30 mmol) containing CsF (91 mg, 0.6 mmol). The mixture was stirred at 25°C for 1 hour, then Et_2O was added, and the two phases were separated. The aqueous phase was extracted twice with Et_2O , then the combined organic layers were washed with brine, dried over MgSO_4 and concentrated under vacuum. The crude product was purified by column chromatography (Heptane/ Et_2O 85:15 then 75:25) to give **8aa** as a light orange oil (21 mg, 0.074 mmol, 25%).

1-(Trimethylsilylocta-2,7-diyn-1-yl (tert-butoxycarbonyl)glycinate (**7aa**). $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 5.20 (t, $J=2.3$ Hz, 1H), 5.01 (brs, 1H), 4.00–3.80 (m, 2H), 2.37 (td, $J=7.0$, 2.4 Hz, 2H), 2.28 (td, $J=7.0$, 2.6 Hz, 2H), 1.95 (t, $J=2.6$ Hz, 1H), 1.71 (p, $J=7.0$ Hz, 2H), 1.44 (s, 9H), 0.13 (s, 9H). $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 170.3, 155.7, 87.9, 83.5, 80.1, 77.0, 69.0, 60.2, 42.6, 28.4, 27.7, 18.2, 17.6, -3.8 . HRMS (ESI) m/z : calcd for $\text{C}_{18}\text{H}_{30}\text{NO}_4\text{Si}^+$ ($[\text{M}-\text{H}]^+$) 352.1939, found 352.1938.

Octa-2,7-diyn-1-yl (tert-butoxycarbonyl)glycinate (**8aa**). $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 4.99 (s, 1H), 4.74 (t, $J=2.1$ Hz, 2H), 4.05–3.82 (m, 2H), 2.36 (tt, $J=2.1$, 7.0 Hz, 2H), 2.30 (td, $J=2.6$, 7.0 Hz, 2H), 1.96 (t, $J=2.6$ Hz, 2H), 1.73 (p, $J=7.0$ Hz, 2H), 1.45 (s, 9H). $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 169.9, 155.8, 87.1, 83.4, 80.2, 74.3, 69.1, 53.6, 42.5, 28.4, 27.3, 17.9, 17.7. HRMS (ESI) m/z : calcd for $\text{C}_{15}\text{H}_{20}\text{NO}_4^-$ ($[\text{M}-\text{H}]^-$) 278.1398, found 278.1404.

Reaction of **6a with *N*-Boc-Ser-OH:** A solution of **6a** (10 mL, 0.03 M in THF, 62.0 mg, 0.3 mmol), obtained following general procedure **A**, was added a solution of *N*-Boc-Ser-OH (5 mL, 0.06 M in MES buffer, 61.6 mg, 0.3 mmol). The mixture was stirred at 25°C for 30 min, then a solution of **6a** (2 mL, 0.15 M, 62.0 mg, 0.3 mmol) was added. After 30 min, a solution of **6a** (1 mL, 0.15 M, 31.0 mg, 0.15 mmol) was added. Finally, after 30 min, Et_2O was added, and the two phases were separated. The aqueous phase was extracted twice with Et_2O , then the combined organic layers were washed with brine, dried over MgSO_4 and concentrated under vacuum. Flash chromatography (pentane/ EtOAc 8:2) afforded **7ab** (mixture of isomers, 23 mg, 0.06 mmol, 20%) and **8ab** (18 mg, 0.05 mmol, 17%).

1-(Trimethylsilylocta-2,7-diyn-1-yl(tert-butoxycarbonyl)-L-serinate as a mixture of diastereoisomers (**7ab**). $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 5.54–5.48 (m, 1H, one isomer), 5.48–5.41 (m, 1H, one isomer), 5.27–5.21 (m, 1H, one isomer), 5.20–5.15 (m, 1H, one isomer), 4.45–4.39 (br, 1H, one isomer), 4.39–4.33 (br, 1H, one isomer), 4.00–3.84 (m, 2H, both isomers), 2.40–2.31 (m, 2H, both isomers), 2.31–2.23 (m, 2H, both isomers), 1.97–1.93 (m, 1H, both isomers), 1.70 (sext, $J=7.0$ Hz, 2H, both isomers), 1.44 (s, 9H, both isomers), 0.13 (s, 9H both isomers).

$^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 170.9, 170.6, 155.9, 88.0, 87.9, 83.5, 80.4, 77.0, 69.0, 64.0, 63.9, 60.8, 60.7, 56.1, 55.9, 28.4, 27.6, 27.6, 18.2, 18.1, 17.6, 17.5, -3.9 . HRMS (ESI) m/z : calcd for $\text{C}_{19}\text{H}_{32}\text{NO}_5\text{Si}^+$ ($[\text{M}+\text{H}]^+$) 382.2044, found 382.2040, $\text{C}_{19}\text{H}_{31}\text{NNaO}_5\text{Si}^+$ ($[\text{M}+\text{Na}]^+$) 404.1864, found 404.1857.

Octa-2,7-diyn-1-yl (tert-butoxycarbonyl)serinate (**8ab**). $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 5.46 (br s, 1H), 4.83–4.68 (m, 2H), 4.41 (br s, 1H), 4.04–3.95 (m, 1H), 3.95–3.85 (m, 1H), 2.40–2.32 (m, 2H), 2.30 (td, $J=$

7.0, 2.6 Hz, 2H), 2.26–2.06 (br, 1 H) 1.99–1.90 (m, 1H), 1.72 (p, $J=7.0$ Hz, 2H), 1.45 (s, 9H). ^{13}C NMR (125 MHz, CDCl_3) δ 170.4, 155.8, 87.2, 83.4, 80.5, 74.2, 69.2, 63.7, 55.9, 54.0, 28.4, 27.2, 17.9, 17.6. HRMS (ESI) m/z : calcd for $\text{C}_{16}\text{H}_{22}\text{NO}_5^+$ ($[\text{M}+\text{H}]^+$) 310.1649, found 310.16543, $\text{C}_{16}\text{H}_{23}\text{NNaO}_5^+$ ($[\text{M}+\text{Na}]^+$) 332.1468, found 332.14735.

Reaction of 6a with N-Boc-Asp-NH₂: A solution of **6a** (10 mL, 0.03 M in THF, 62.0 mg, 0.3 mmol), obtained following general procedure A, was added a solution of N-Boc-Asp-NH₂ (5 mL, 0.06 M in MES buffer, 69.7 mg, 0.3 mmol). The mixture was stirred at 25 °C for 30 min, then a solution of **6a** (2 mL, 0.15 M, 62.0 mg, 0.3 mmol) was added. After 30 min, Et₂O was added, and the two phases were separated. The aqueous phase was extracted twice with Et₂O, then the combined organic layers were washed with brine, dried over MgSO₄ and concentrated under vacuum. Flash chromatography (*n*-heptane/EtOAc 5:5 to 4:6) afforded **7ac** (mixture of isomers, 18 mg, 0.044 mmol, 15%) and **8ac** (15 mg, 0.04 mmol, 13%).

1-(trimethylsilyloct-2,7-diyn-1-yl (3S)-4-amino-3-((tert-butoxycarbonyl) amino)-4-oxobutanoate (**7ac**). ^1H NMR (500 MHz, CDCl_3) δ 6.48 (brs, 1H, NH, 2 isomers), 5.81 (brd, 1 H, NH Boc, 1 isomer), 5.57 (brd, NHBoc, 1 isomer), 5.59, (brs, NH, 1 isomer), 5.18 (brs, 1 H, 2 isomers), 4.53, (brs, 1 H, 2 isomers), 3.12–2.96 (m, 1 H, 2 isomers), 2.79–2.61 (m, 1H, 2 isomers), 2.37 (td, $J=7.0$, 2.3 Hz, 2 H, 2 isomers), 2.29 (tm, $J=7.0$ Hz, 2H, 2 isomers), 1.96 (t $J=2.6$ Hz, 1 H, 2 isomers), 1.72 (p, $J=7.0$ Hz, 2H, 2 isomers) 1.53–1.39 (m, 9 H, 2 isomers), 0.14 (9 H, 1 isomer); 0.12 (9H, 1 isomer) ^{13}C NMR (125 MHz, CDCl_3) δ 173.2, 173.0, 172.2, 171.4, 155.7, 155.5, 87.9, 87.7, 83.6, 83.6, 80.7, 77.0, 69.1, 69.0, 60.1, 59.9, 50.4, 50.4, 36.4, 35.9, 28.4, 27.7, 27.7, 18.2, 17.6, -3.8, -3.8. HRMS (ESI) m/z : calcd for $\text{C}_{20}\text{H}_{32}\text{N}_2\text{NaO}_5\text{Si}$ ($[\text{M}+\text{Na}]^+$) 431.19727, found 431.19781.

octa-2,7-diyn-1-yl (S)-4-amino-3-((tert-butoxycarbonyl)amino)-4-oxobutanoate (**8ac**). ^1H NMR (500 MHz, CDCl_3) δ 6.47 (brs, NH, 1 H), 5.69 (d, $J=7.5$ Hz, NHBoc, 1H), 5.51 (brs, NH, 1H), 4.73, (dt, $J=15.2$, 2.1 Hz, 1H), 4.67 (dt, $J=15.2$, 2.1 Hz, 1H), 4.55 (brs, 1H), 3.06, (dt, $J=7.3$, 4.2 Hz, 1H), 2.71, (dt, $J=7.3$, 6.1 Hz, 1H), 2.36, (tt, $J=7.0$, 2.1 Hz, 2H), 2.30, (td, $J=7.0$, 2.7 Hz, 2H), 1.97, (t, $J=2.6$ Hz, 1H), 2.71, (dt, $J=7.3$, 6.1 Hz, 1H), 1.73, (p, $J=7.0$ Hz, 2H), 1.45, (s, 9H). ^{13}C NMR (125 MHz, CDCl_3) δ 173.0, 171.6, 155.6, 87.0, 83.5, 80.8, 77.4, 74.3, 69.2, 53.5, 50.3, 35.9, 28.4, 27.3, 17.9, 17.6. HRMS (ESI) m/z : calcd for $\text{C}_{17}\text{H}_{25}\text{N}_2\text{O}_5^+$ ($[\text{M}+\text{H}]^+$) 337.1758, found 337.1751.

Reaction of 6a with N-Cbz-Ala-Pro-Leu-OH (9): A solution of **6a** (5 mL, 0.03 M in THF, 31 mg, 0.15 mmol), obtained following general procedure A, was added to a solution of **9** (2.5 mL, 0.06 M, 65 mg, 0.15 mmol) in MES buffer (0.3 M, pH=5.5). The mixture was stirred at 25 °C for 0.5 hour, then a solution of **6a** (1 mL, 0.15 M, 31 mg, 0.15 mmol) was added. After 30 min Et₂O was added, and the two phases were separated. The aqueous phase was extracted twice with Et₂O, then the combined organic layers were washed with brine, dried over MgSO₄ and concentrated under vacuum. Flash chromatography (pentane/EtOAc 1:1) afforded **8ac** (23 mg, 0.043 mmol, 29%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.19 (d, $J=7.4$ Hz, 1H), 7.46 (d, $J=7.3$ Hz, 1H), 7.40–7.24 (m, 5H), 5.07–4.93 (m, 2H), 4.76–4.58 (m, 2H), 4.40–4.14 (m, 3H), 3.65–3.47 (m, 2H), 2.79 (t, $J=2.5$ Hz, 1H), 2.34–2.26 (m, 2H), 2.36–2.18 (m, 2H), 2.06–1.64 (m, 6H), 1.60 (p, $J=7.0$ Hz, 2H), 1.57–1.43 (m, 2H), 1.17 (d, $J=6.9$ Hz, 3H), 0.90 (d, $J=6.6$ Hz, 3H), 0.84 (d, $J=6.5$ Hz, 3H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 171.8, 171.7, 170.7, 155.6, 137.0, 128.3, 127.7, 127.6, 86.2, 83.4, 75.1, 71.7, 65.3, 58.8, 52.6, 50.3, 47.9, 46.5, 39.1, 28.8, 27.0, 24.4, 24.1, 22.6, 21.4, 17.0, 16.8, 16.7. HRMS (ESI) m/z : calcd for $\text{C}_{30}\text{H}_{40}\text{N}_3\text{O}_6^+$ ($[\text{M}+\text{H}]^+$) 538.2912, found 538.29067.

N-Boc-Gly-Phe-Ala-OH (10): Boc₂O (149 mg, 0.68 mmol, 2.7 eq.) was added to a stirred solution of N-Gly-Phe-Ala-OH (100 mg, 0.25 mmol, 1 eq.) in a mixture of THF (2 mL), H₂O (1.7 mL) and an

aqueous solution of NaOH (0.3 mL, 1 M). The reaction mixture was stirred at room temperature and the evolution of the reaction was followed by TLC. Upon completion, HCl (1 M in H₂O) was added to the mixture to adjust the pH to 5. Then the reaction mixture was stirred for one hour and filtered. The solid was dissolved in THF (10 mL) and filtered using a syringe filter. The solution was then transferred in a flask and the solvent was evaporated to give **10** as a white amorphous solid (121 mg, 0.31 mmol, 90% yield). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 12.53 (s, 1H), 8.34 (d, $J=7.3$ Hz, 1H), 7.86 (d, $J=8.4$ Hz, 1H), 7.27–7.21 (m, 4H), 7.20–7.14 (m, 1H), 6.90–6.83 (m, 1H), 4.58–4.50 (m, 1H), 4.21 (p $J=7.2$ Hz, 1H), 3.53 (dd, $J=16.7$, 6.2 Hz, 1H), 3.40 (dd, $J=16.7$, 6.0 Hz, 1H), 3.01 (dd, $J=14.0$, 4.2 Hz, 1H), 2.76 (dd, $J=13.8$, 9.4 Hz, 1H), 1.36 (s, 9H), 1.29 (d, $J=7.5$ Hz, 3H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 173.8, 170.7, 169.0, 155.7, 137.6, 129.2, 127.9, 126.2, 78.0, 53.3, 47.4, 43.0, 37.6, 28.1, 17.0. HRMS (ESI) m/z : calcd for $\text{C}_{19}\text{H}_{28}\text{N}_3\text{O}_6^+$ ($[\text{M}+\text{H}]^+$) 394.1973, found 394.1965, $\text{C}_{19}\text{H}_{26}\text{N}_3\text{O}_6^-$ ($[\text{M}-\text{H}]^-$) 392.1827, found 392.1827.

Reaction of 6a with N-Boc-Gly-Phe-Ala-OH (10): A solution of **6a** (5 mL, 0.03 M in THF, 31 mg, 0.15 mmol), obtained following general procedure A, was added to a solution of **10** (2.5 mL, 0.06 M in MES buffer, 59 mg, 0.15 mmol). The mixture was stirred at 25 °C for 30 min, then a solution of **6a** (1 mL, 0.15 M in THF, 31 mg, 0.15 mmol) was added. After 30 min, solution of **6a** (1 mL, 0.15 M in THF, 31.0 mg, 0.15 mmol) was added. After another 30 min, Et₂O was added, and the resulting two phases were separated. The aqueous phase was extracted twice with Et₂O, then the combined organic layers were washed with brine, dried over MgSO₄ and concentrated under vacuum. Flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 98:2) afforded **8ad** (24 mg, 0.048 mmol, 32%) and **10** (11 mg, 0.028 mmol, 19%).

^1H NMR (500 MHz, CDCl_3) δ 7.33–7.17 (m, 5H), 6.79 (d, $J=7.7$ Hz, 1H), 6.41 (brs, 1H), 5.10 (brs, 1H), 4.73–4.63 (m, 3H), 4.49 (quint., $J=7.0$ Hz, 1H), 3.84–3.70 (m, 2H), 3.16–2.99 (m, 2H), 2.40–2.33 (m, 2H), 2.33–2.24 (m, 2H), 1.98–1.93 (m, 1H), 1.73 (p, $J=7.0$ Hz, 2H), 1.43 (s, 9H), 1.36 (d, $J=7.2$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 171.6, 170.4, 169.9, 156.2, 136.1, 129.5, 128.9, 127.4, 87.2, 83.4, 80.8, 74.2, 69.2, 54.4, 53.9, 48.5, 44.5, 38.3, 28.4, 27.3, 18.1, 17.9, 17.6. HRMS (ESI) m/z : calcd for $\text{C}_{27}\text{H}_{36}\text{N}_3\text{O}_6^+$ ($[\text{M}+\text{H}]^+$) 498.2599, found 498.2607.

Reaction of 6a (30 equivalents) with RNase A with CsF: Following general procedure B we prepared a stock solution 22.0 mM of **6a** in CH_3CN . Then we prepared a stock solution 22.0 mM of CsF in MES buffer (pH 5.5, 0.15 M). RNase A (2.5 mg, 0.18 μmol) were placed in an Eppendorf® and dissolved in 250 μL of the stock solution of CsF in MES buffer. Then 250 μL of the stock solution of diazo were added and the resulting mixture was placed in an orbital shaker at 25 °C with a speed of 800 rpm. After 1 h, the mixture was diluted to 4 mL. Protein **12** was isolated by Ultra-centrifugal filtration. Lyophilization afforded the modified protein that was analyzed by MALDI-TOF and subjected to trypsin digestion coupled to MS/MS spectrometry to identify the modified residues.

Reaction of 6a (10 equivalents) with RNase A with CsF: Following general procedure B we prepared a stock solution 7.14 mM of **6a** in CH_3CN . Then we prepared a stock solution 7.14 mM of CsF in MES buffer (pH 5.5, 0.15 M). RNase A (7.0 mg, 0.51 μmol) were placed in an Eppendorf® and dissolved in 700 μL of the stock solution of CsF in MES buffer. Then 700 μL of the stock solution of diazo were added and the resulting mixture was placed in an orbital shaker at 25 °C with a speed of 800 rpm. After 1 h it was diluted to 4 mL and the protein **12** was isolated by Ultra-centrifugal filtration. Lyophilization afforded the modified protein that was analyzed by MALDI-TOF, and then subjected to trypsin digestion coupled to MS/MS spectrometry to identify the modified residues. This protein was also further functionalized.

Reaction of 6a (300 equivalents) with RNase A with CsF: Following general procedure **B** we prepared a stock solution 30 mM of **6a** in CH₃CN. Then we prepared a stock solution 30 mM of CsF in MES buffer (pH 5.5, 0.15 M). RNase A (2.7 mg, 0.2 μmol) was dissolved in 2.0 mL of the solution of CsF in MES buffer. 900 μL of this solution were placed in an Eppendorf® and then 900 μL of the stock solution of diazo were added. The resulting mixture was placed in an orbital shaker at 25 °C with a speed of 800 rpm. After 1 h it was diluted to 4 mL and the protein **11** was isolated by Ultra-centrifugal filtration. Lyophilization afforded the modified protein that was analyzed by MALDI-TOF.

Synthesis of 15 by post-functionalization of 12 (prepared with 10 equivalents of 6a) with coumarin 13. We prepared different stock solutions: A solution of **13** (13.1 mg, 64.5 μmol) in DMSO (1.0 mL) – stock solution **A**. A solution of CuSO₄ (70 mg, 280 μmol) in H₂O (5.0 mL) – stock solution **B**. A solution of THPTA (Tris(3-hydroxypropyltriazolylmethyl) amine, 15.6 mg, 35.8 μmol) in H₂O (125 μL) – stock solution **C**. A solution of aminoguanidine hydrochloride (AMG) (30.9 mg, 279.5 μmol) in H₂O (500 μL) – stock solution **D**. A solution of NaAsc (sodium ascorbate) (55.5 mg, 280.1 μmol) in H₂O (500 μL) – stock solution **E**. A solution of EDTA disodium salt (1.0 g, 2.97 mmol) in H₂O (10 mL) – stock solution **F**.

Stock solution **A** (40 μL) was added to **12** (2 mg, 0.14 μmol) dissolved in phosphate buffer (500 μL). A pre-mix of stock solution **B** (25 μL) and stock solution **C** (25 μL) were added to the reaction mixture. Stock solution **D** (125 μL) and stock solution **E** (125 μL) were then added sequentially. The tube containing the reaction mixture was placed in a rotary mixer and rotated slowly for 1 h. Stock solution **F** (1 mL) was finally added to quench the reaction. The resulting mixture was diluted to 4 mL and the protein was isolated by Ultra-centrifugal filtration (this step was conducted twice). Lyophilization of the concentrated solution afforded **15** that was analyzed by MALDI-TOF showed the presence of the desired product. UV-Vis and fluorescence spectra were recorded.

Synthesis of 16 by post-functionalization of 12 (prepared with 10 equivalents of 6a) with Biotin derivative 14. We prepared different stock solutions: A solution of **14** (8.8 mg, 19.8 μmol) in DMSO (250 μL) – stock solution **A**. A solution of CuSO₄ (70 mg, 280 μmol) in H₂O (5.0 mL) – stock solution **B**. A solution of THPTA (Tris(3-hydroxypropyltriazolylmethyl) amine, 15.6 mg, 35.8 μmol) in H₂O (125 μL) – stock solution **C**. A solution of aminoguanidine hydrochloride (AMG) (30.9 mg, 279.5 μmol) in H₂O (500 μL) – stock solution **D**. A solution of NaAsc (sodium ascorbate) (55.5 mg, 280.1 μmol) in H₂O (500 μL) – stock solution **E**. A solution of EDTA disodium salt (1.0 g, 2.97 mmol) in H₂O (10 mL) – stock solution **F**.

Stock solution **A** (20 μL) was added to **12** (1 mg, 0.072 μmol) dissolved in phosphate buffer (250 μL). A pre-mix of stock solution **B** (25 μL) and stock solution **C** (25 μL) were added to the reaction mixture. Stock solution **D** (70 μL) and stock solution **E** (70 μL) were then added sequentially. The tube containing the reaction mixture was placed in a rotary mixer and rotated slowly for 1 h. Stock solution **F** (1 mL) was finally added to quench the reaction. The resulting solution was then diluted to 4 mL and the protein was isolated by Ultra-centrifugal filtration (this step was conducted twice). Lyophilization of the concentrated solution afforded **16** that was analyzed by MALDI-TOF showed the presence of the desired product.

Supporting Information

All NMR spectra, IR experiments, UV and fluorescence spectra of **15**, MALDI-TOF analysis, data of trypsin-digestion and MS/MS

experiments are included in the Supporting Information. Additional references cited within the Supporting Information.^[28]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: click chemistry · protein bioconjugation · RNase A · silylated diazo alkyne

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