Synthesis of Peptide−Protein Conjugates Using *N*-Succinimidyl Carbamate Chemistry

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Peptide−protein conjugates are useful tools in different fields of research as, for instance, the development of vaccines and drugs or for studying biological mechanisms, to cite only few applications. *N*-Succinimidyl carbamate (NSC) chemistry has been scarcely used in this area. We show that unprotected peptides, featuring one lysine residue within their sequences, can be converted in good yield into NSC derivatives by reaction with disuccinimidylcarbonate (DSC). No hydrolysis of the NSC group was observed during RP-HPLC purification, lyophilization, or storage. NSC peptides reacted efficiently within minutes with lysozyme used as model protein. To illustrate usefulness of the method consisting of the synthesis of a peptide−protein conjugate of biological interest, a NSC peptide derived from a peptide substrate for tyrosylprotein sulfotransferase (TS) was synthesized and ligated to receptor-binding nontoxic B-subunit of Shiga toxin (STxB). Immunofluorescence studies showed the intracellular delivery of the TS-STxB conjugate and its ability to circulate to the Golgi as the native STxB protein. Moreover, we demonstrate that the TS label could be sulfated by tyrosylprotein sulfotransferases present in the Golgi. Thus, NSC chemistry permitted rapid synthesis of a peptide−protein conjugate worthwhile for studying the transport of proteins from the plasma membrane to the Golgi. The second part of this article describes a more general method for synthesizing peptide−protein conjugates without any limitation of the peptide sequence. The conjugates were assembled by combining NSC chemistry and α-oxo semicarbazone ligation. To this end, a glyoxylyl NSC peptide was synthesized and reacted with lysozyme. The glyoxylyl groups on the protein were then reacted with a semicarbazide peptide to produce the target peptide−protein conjugate. Both reactions, namely, urea bond formation and α-oxo semicarbazone ligation, were carried at pH 8.0 using a one-pot procedure.

INTRODUCTION

Nowadays, various methods are available for the synthesis of peptide−protein conjugates. Site-specific ligation methods, by exploiting a unique functional group present within the protein, lead to well-characterized conjugates. Reaction of a cysteine residue with haloacetyl (*1*) or maleimidyl (*2*) peptides is often used in this field. Alternatively, unnatural functional groups helpful for site-specific ligation such as keto or aldehyde groups can be introduced into protein-specific sites by using chemical methods (*3, 4*), enzymatic techniques (*5*), or living organisms (*6*).

Random peptide−protein conjugates are also useful tools in different fields of research as, for example, the development of vaccines (*7*) or drugs (*8, 9*) or for studying biological mechanisms (*1*). Their preparation is often carried out using *N*-succinimidyl carboxylic ester (NSCE)-based bifunctional reagents (*10*). Usually, the bifunctional reagent is reacted first with the protein leading to formation of amide bonds between surface-exposed ε-amino groups and NSCE moiety. Then, the second functional group is used to attach peptide molecules (*7*). The synthesis of peptide−protein conjugates by direct reaction of NSCE peptides with proteins is less common. For instance, Kida et al. have recently described the synthesis of a Tat-Cys-NSCE peptide by reacting Tat-Cys with 6-maleimidohexanoic acid *N*-succinimidyl ester (*11*). However, the sensitivity of Tat-Cys-NHSE peptide toward hydrolysis precluded its purification by RP-HPLC. Consequently, the crude Tat-Cys-NSCE peptide was stored at −80 °C and directly used in the conjugation experiments. A similar approach was reported by Inglese et al. for the attachment of a protein kinase A substrate to proteins (*12*).

N-Succinimidyl carbamate derivatives (NSC), whose chemistry is related to NSCE, have been scarcely used for the synthesis of conjugates. Morpurgo et al. reported the synthesis of an NSC derivative of biotin for protein labeling (*13*). Later on, Clavé et al. described the synthesis of a fully protected NSC dipeptide scaffold (*14*). This reagent was coupled to the N-terminal amino group of substance P. Pasut et al. described the synthesis of a PEG-β-alanine NSC derivative for protein pegylation (*15*). In these studies, besides the NSC group, no other functional moiety was present on the molecules. To the best of our knowledge, the synthesis of NSC peptides incorporating functional side chains, their purification, and coupling to proteins was not reported in the literature.

We describe here the synthesis of NSC peptides incorporating functional side chains (phenol group of tyrosine, hydroxyl group of serine, imidazole group of histidine, carboxylic acid group of glutamic acid, and/or indole group of tryptophan). These peptide derivatives were successfully purified by RP-HPLC and coupled to proteins. Lysozyme was used as a model protein to optimize preparation of conjugates. Obviously, the partial conversion of NSC group into an isocyanate group during the conjugation experiment is a fact. To illustrate the value of the method consisting of the synthesis of a peptide−protein

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Table 1. Yields for Peptides 1−4a,b and MS Data

peptide	*m/z* calcd	*m/z* found	yield (%)
1a	1601.7[a]	1601.5[a]	73
2a	2183.1[a]	2182.8[a]	34
3a	2339.2[a]	2339.0[a]	42
4a	2495.3[a]	2495.2[a]	47
1b	871.9[b]	872.2[b]	59
2b	1162.6[b]	1163.1[b]	56
3b	1240.6[b]	1241.2[b]	56
4b	1318.7[b]	1319.5[b]	61

[a] $[M+H]^+$ MALDI-TOF analysis in a reflector mode using α-cyano-4-hydroxycinnamic acid as matrix. [b] $[M+2H]^{2+}$ LC-MS analysis (source temperature 120 °C, desolvation temperature 350 °C, cone 30 V, capillary 3000 V).

conjugate of biological interest, a NSC peptide derived from a peptide substrate for tyrosylprotein sulfotransferase (TS) (*10*) was synthesized and ligated to receptor-binding nontoxic B-subunit of Shiga toxin (STxB). STxB binds to glycosphingolipid Gb3 in the plasma membrane of target cells. After binding, STxB is transported to the endoplasmic reticulum, via endosomes and the Golgi. Due to this property, STxB can potentially be used for vectorization of drugs (*16*). The colocalization of TS-STxB conjugate with a Golgi marker was studied by confocal immunofluorescence microscopy. The ability of the TS label within the TS-STxB conjugate to be sulfated by Golgi tyrosylprotein sulfotransferase was also examined.

In the second part of this article, we describe a more general method for synthesizing peptide−protein conjugates, which is not limited by the peptide sequence. The conjugates are assembled using a one-pot procedure combining NSC chemistry and α-oxo semicarbazone ligation. To this end, a glyoxylyl NSC peptide was synthesized and reacted with lysozyme at pH 8.0 to produce glyoxylyl lysozyme. The crude reaction mixture was then reacted with a semicarbazide peptide. Ligation between the glyoxylyl groups on the protein and the semicarbazide group on the peptide resulted in one-pot formation of the target peptide−protein conjugate.

EXPERIMENTAL PROCEDURES

Solid-Phase Peptide Synthesis of Peptides 1−4a. Solid-phase synthesis was performed using the Fmoc/*tert*-butyl strategy on a Novasyn TGR resin (0.1 mmol scale) in a microwave peptide synthesizer (CEM μ WAVES, Saclay, France). Couplings were performed using 5-fold molar excess of each Fmoc L- or D-amino acid, 4.5-fold molar excess of HBTU, and 10-fold molar excess of DIEA. Final deprotection and cleavage from the resin was carried out with TFA/thioanisol/TIS/H_2O 92.5/2.5/2.5/2.5 by vol. The resin beads were filtered, and the filtrate was precipitated in diethyl ether/heptane 1/1 by vol, dissolved in water, and lyophilized. The crude peptide was purified by RP-HPLC on a 100 Å 5 μm C18 Nucleosil column using a linear water/acetonitrile gradient containing 0.05%TFA by vol (6 mL/min, detection 215 nm, 0−40% B in 60 min). Fractions were collected and lyophilized to produce **1−4a** (yields are indicated in Table 1). The purity of peptides **1−4a** was >95% as determined by analytical RP-HPLC and CZE. MS data are in accordance with the proposed structures (see Table 1 and Supporting Information).

Synthesis of NSC Peptides 1−4b. DSC (16.6 mg, 65 μmol) was dissolved in dry DMF (340 μL) at rt under N_2. Peptide **1−4a** (6.5 μmol) and triethylamine (0.45 μL, 3.2 μmol) were solubilized in dry DMF (85 μL) and then added in one portion to DSC solution. The mixture was stirred at rt for 1 h. The crude mixture was diluted with 20 mL of 1% by vol aqueous TFA and purified by RP-HPLC on a 100 Å 5 μm C18 Nucleosil column using a linear water/acetonitrile gradient containing 0.05% TFA by vol (6 mL/min, detection 215 nm, 0−40% B in 60 min). Fractions were collected and lyophilized to produce **1−4b** (yields are indicated in Table 1).

The purity of peptides **1−4b** was >95% as determined by analytical RP-HPLC and CZE. MS data are in accordance with the proposed structures (see Table 1 and Supporting Information).

Synthesis of NSCE Peptide 1c. A solution of suberic acid bis(*N*-succinimidyl ester) DSS (64 mg, 175 μmol) in dry DMF (700 μL) at rt under nitrogen was added to a stirred solution of peptide **1a** (30 mg, 17.5 μmol) and triethylamine (2.4 μL, 17.5 μmol) in dry DMF (340 μL). The mixture was stirred at rt for 1 h. The crude mixture was then diluted with 20 mL of 1% by vol aqueous TFA and purified by RP-HPLC on a 100 Å 5 μm C18 Nucleosil column using a linear water/acetonitrile gradient containing 0.05%TFA by vol (6 mL/min, detection 215 nm, 0−40% B in 60 min). Fractions were collected and lyophilized to produce 15 mg of pure peptide **1c** (46%). The purity of peptide **1c** was >95% as determined by analytical RP-HPLC. MS data are in accordance with the proposed structure (see Supporting Information).

Labeling of Lysozyme, General Procedure. Lysozyme (Appligene ref 130172, 0.41 mg, 0.028 μmol, 0.2 mM final concentration) in 10 mM pH = 7.4 PBS buffer (140 μL) was added to NSC peptide **1−4b** (0.2 μmol). The mixture was stirred at 4 °C for 1 h. Analysis of the reaction mixtures by RP-HPLC and CZE showed that ∼50% of lysozyme molecules were modified. MALDI-TOF analysis of the mixtures revealed the presence of up to 4 peptide chains on lysozyme.

Labeling of STxB. STxB was produced and purified as described elsewhere (*17*). STxB (1.11 mg, 0.14 μmol, 0.32 mM final concentration) in pH = 8.0 sodium phosphate buffer (450 μL) was added to NSC peptide **1b** (2.24 μmol). The mixture was stirred at rt for 1 h. The conversion was ∼70% as determined by LC-MS. MALDI-TOF analysis of the mixture revealed grafting of up to 3 peptide chains on STxB. The conjugate was purified by filtration to produce TS-STxB-a or purified by RP-HPLC to produce TS-STxB-b.

Purification by Filtration, TS-STxB-a. 300 μL of the above reaction mixture was filtered and washed with PBS (3 × 20 mL) on a 3 kD membrane (3000 MWCO PES Vivascience, UK). The total volume after this step was 750 μL. The solution concentration (0.92 μg/μL) was determined using the bicinchoninic BCA assay (*18*).

RP-HPLC Purification, TS-STxB-b. 100 μL of the above reaction mixture was purified by RP-HPLC on a 300 Å 5 μm C18 XBridge column (4.6 × 250 mm) using a linear water/acetonitrile gradient (1 mL/min, eluent A is 0.05% TFA in water, eluent B is 0.05% TFA in CH_3CN/water 4/1 by vol, 0−100% B in 30 min, detection at 215 nm). Fractions were collected and lyophilized to produce TS-STxB-b. The conjugate was dissolved in 100 μL of PBS. The solution concentration (0.5 μg/μL) was determined using the bicinchoninic BCA assay (*18*).

Synthesis of Peptide 5 and Glyoxylyl Peptide 6. Solid-phase synthesis of peptide **5** H-SAAFGGK-NH_2 was performed using the Fmoc/*tert*-butyl strategy on a Novasyn TGR resin (0.1 mmol scale) in a microwave peptide synthesizer (CEM μ WAVES, Saclay, France). Couplings were performed using 5-fold molar excess of each Fmoc L-amino acid, 4.5-fold molar excess of HBTU, and 10-fold molar excess of DIEA. Final deprotection and cleavage from the resin was carried out with TFA/TIS/H_2O 95/2.5/2.5 by vol. The resin beads were filtered and the filtrate was precipitated in diethylether/heptane 1/1 by vol, dissolved in water, and lyophilized to produce 71 mg of peptide **5** (82%). The purity of peptide **5** was >90% as determined by

analytical RP-HPLC and CZE. MS data are in accordance with the proposed structure (see Supporting Information).

A solution of peptide **5** (21.3 mg, 0.024 mmol) in 0.1 M pH 7.0 sodium phosphate buffer (6.4 mL) was reacted for 10 min with sodium periodate (10.5 mg, 0.049 mmol) at rt. The reaction mixture was quenched with ethanolamine (3.5 μL, 0.037 mmol) and purified immediately by RP-HPLC on a 100 Å 5 μm C18 Nucleosil column using a linear water/acetonitrile gradient containing 0.05% TFA by vol (6 mL/min, detection 215 nm, 0−40% B in 60 min). Fractions were collected and lyophilized to produce peptide **6** (11 mg, 64%).

The purity of peptide **6** was 90% by analytical RP-HPLC and 92% by CZE. MS data are in accordance with the proposed structure (see Supporting Information).

Synthesis of Glyoxylyl NSC Peptide 7. A solution of glyoxylyl peptide **6** (11 mg, 0.015 mmol) and triethylamine (1.07 μL, 0.007 mmol) in dry DMF (153 μL) was reacted with DSC (58.7 mg, 0.229 mmol). The mixture was stirred at rt for 1 h. The crude mixture was diluted with 10 mL of 0.3% by vol aqueous TFA and purified immediately by RP-HPLC on a 100 Å 5 μm C18 Nucleosil column using a linear water/acetonitrile gradient containing 0.05% TFA by vol (6 mL/min, detection 215 nm, 0−40% B in 60 min). Fractions were collected and lyophilized to produce glyoxylyl NSC peptide **7** (6 mg, 53%).

The purity of peptide was 87% by analytical RP-HPLC. MS data are in accordance with the proposed structure (see Supporting Information).

Preparation of Peptide−Protein Conjugates. Reaction of Lysozyme with Peptide 7 and then α-Oxo Semicarbazone Ligation with Peptide 8. The synthesis of semicarbazide peptide **8** was described in detail elsewhere (*19*). This peptide sequence is derived from HIV1 POL 476−484 protein.

Labeling with Peptide 7. A solution of lysozyme (0.85 mg, 0.06 μmol, 0.2 mM) in 0.1 M pH 8.0 sodium phosphate buffer (300 μL) was added to peptide **7** (0.30 mg, 0.42 μmol). The mixture was stirred at rt. The reaction was monitored by MALDI-TOF analysis. The reaction was complete after 1 h as visualized by MALDI-TOF mass spectrometry. The MS spectra showed grafting of up to three peptide **7** molecules to lysozyme.

Ligation between Glyoxylyl Lysozyme and Semicarbazide Peptide 8. The above mixture was then added to semicarbazide peptide **8** (1.00 mg, 0.84 μmol). The mixture was stirred at 37 °C for 2 h and then at rt for 72 h. MALDI-TOF analysis showed ligation of up to two semicarbazide peptide **8** molecules to lysozyme.

Retrograde Transport of TS-STxB-a,b Conjugates from the Plasma Membrane to the Trans Golgi Network. *Immunofluorescence Analysis.* 200 000 HeLa cells were placed on coverslip in 24-well test plate (TPP, Switzerland) and incubated with 2 μM TS-STxB-a,b conjugate or STxB for 40 min at 4 °C in PBS++ (PBS containing 0.9 mM $CaCl_2$, 0.52 mM $MgCl_2$ and 0.16 mM $MgSO_4$) and then shifted to 37 °C for 1 h (water-jacketed incubator, Forma Scientific). Cells were fixed with paraformaldehyde (300 μL of a 4% w/v solution in PBS), washed with PBS++, and then permeabilized with saponin solution (300 μL, 0.2% w/v saponin, 2% w/v BSA in PBS buffer). Each coverslip was incubated with 30 μL saponin solution containing 0.2% by vol of 13C4 murine anti-STxB antibody (hybridoma from ATCC #CRL 1794; see ref *20*) and 0.5% by vol of goat antibody against GM130 (C-19, Santa Cruz). Cells were washed with saponin solution and then incubated with 30 μL saponin solution containing 0.5% by vol antimurine Cy3-labeled secondary antibody (Beckman Coulter) and 0.5% by vol antigoat Alexa 488-labeled secondary antibody (Molecular Probes). Coverslips were mounted using 10% aqueous polyvinyl alcohol (Mowiol 4−88 from Fluka), 25% glycerol, and 59% Tris-HCl 0.2 M pH 8.5). Cells were analyzed by confocal microscopy (Leica Microsystems, Mannheim, Germany).

Sulfation Assay. Sulfation assay was performed as described elsewhere (*21*) using STxB, TS-STxB-a (conjugate purified by filtration), TS-STxB-b (conjugate purified by RP-HPLC), and TS-STxB-c. TS-STxB-c is a recombinant STxB protein modified at the C-terminus by two tandem sulfation sites. It is used as a positive control; see ref *22*. Proteins were incubated with 300 000 HeLa cells at 4 °C in sulfate-free Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) for 30 min. Cells were washed with sulfate-free DMEM. 250 μL of radioactive sulfate ($^{35}SO_4^{2-}$, Perkin-Elmer) in sulfate-free DMEM (final activity 480 μCi/mL) were added to cells (120 μCi/300 000 cells). Cells were incubated for 2 h at 37 °C and then lysed in 900 μL of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris pH 8.0) and 9 μL of protease−inhibitor cocktail (1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL antipain, 1 mg/mL pepstatin, 1 M benzamidine, 40 mg/mL phenylmethanesulfonyl fluoride in DMSO). The cell lysate was precipitated with protein G-sepharose (GE Healthcare) loaded with anti-STxB antibody 13C4. Precipitated proteins were analyzed by tris-tricine gel (5 mL gel contains 1.7 mL 30% acrylamide,1.9% bis-acrylamide; 1.7 mL 3 M Tris-HCl with 0.3%SDS pH 8.45; 1.1 mL water; 0.7 mL glycerol; 50 μL 10% ammonium persulfate; 5 μL *N,N,N′,N′*-tetramethylethylenediamine) electrophoresis. Sulfated proteins were detected by autoradiography (STORM860, Molecular Dynamics).

RESULTS AND DISCUSSION

Molecules exposed at the plasma membrane can be circulated to early endosomes for recycling or targeted to lysosomes for degradation. Some molecules are directed to other cell compartments such as the Golgi apparatus or the endoplasmic reticulum (ER). This pathway, termed the retrograde route (*21*), plays a key role in important cellular functions such as antigen presentation (*16*) and cell signaling (*23*).

One strategy used to prove that a protein is transported from the plasma membrane to the Golgi is to modify it with a peptide sequence (TS) which can be enzymatically modified in the Golgi. Peptides presented in Table 1 are derived from the tyrosine sulfation site of rat chromogranin B (*24*). The tyrosine residue within the EEPEYGE motif is sulfated by tyrosylprotein sulfotransferase, an enzyme which is exclusively located in the trans Golgi network. Previous studies have shown TS−protein conjugates utility for studying the retrograde transport of proteins. Sulfation of the TS label was detected by incubating cells with radioactive sulfate followed by immunoprecipitation of the conjugate (*1*).

The work described in the first part of this article was motivated by the need to have an easy access to various TS−protein conjugates. TS peptides were modified at the C-terminus by a Lys(biotin) residue, thereby allowing capture of the TS−protein conjugates using streptavidin-coated beads. TS peptide sequence features four glutamic acid residues, with a negative charge at physiological pH. Thus, the labeling of proteins with TS peptide may significantly lower the isoelectric point (pI) of proteins. The solubility, binding, and biological properties of proteins heavily depend on their pI (*25*−*29*). The interactions of proteins with cell membranes (*28, 30*) are also often dictated by charge effects. Finally, pI control of labeled proteins by varying the label charge has been demonstrated to facilitate purification of labeled proteins by isoelectric focusing techniques (*31*). In this context, synthesis of a series of TS peptides with a range of pI was worth considering. To this end, 2 to 5 arg residues were incorporated at the N-terminus of peptides 1−4. D-Arg residues were chosen to minimize degradation of the peptide label in biological media.

Figure 1. TS-peptides **1**−**4** synthesized in this study. Two to five D-Arg residues were incorporated at the N-terminus of the TS substrate to modulate the pI of peptides.

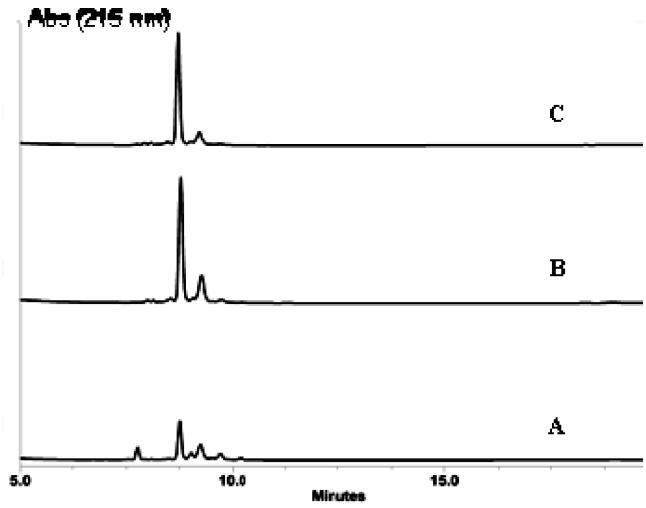


Figure 2. Effect of the DSC mode of addition and stoichiometry on the RP-HPLC yield. Peptide **1a** was reacted at rt in anhydrous DMF with DSC in the presence of triethylamine (2 equiv). (A) Peptide and base were mixed and added over 1 h period to DSC (2 equiv). (B) Peptide and base were mixed and added in one portion to DSC (2 equiv). (C) Same as B using 10 equiv of DSC.

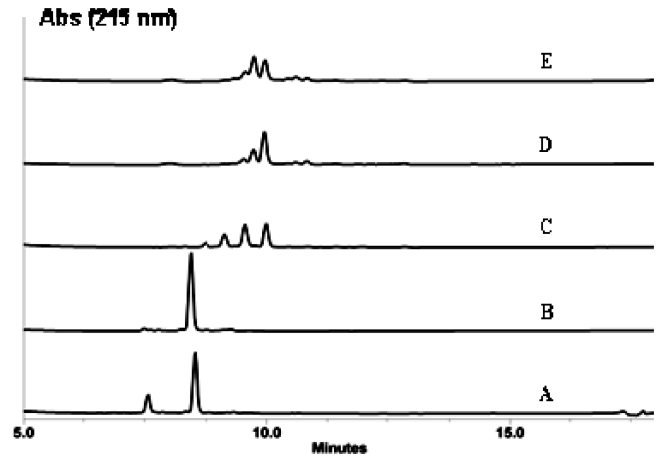


Figure 3. Effect of base stoichiometry on the RP-HPLC yield. Peptide **3a** was reacted at rt in anhydrous DMF with DSC (10 equiv) in the presence of triethylamine. Peptide and base were mixed and added in one portion to DSC. (A) TEA 0.5 equiv, 5 min; (B) TEA 0.5 equiv, 30 min; (C) TEA 10 equiv, 5 min; (D) TEA 10 equiv, 30 min; (E) TEA 10 equiv, 2 h.

NSC Peptide Synthesis and Stability. Peptides **1**−**4b** were obtained by reacting the corresponding precursors **1**−**4a** with DSC in anhydrous DMF. In a first approach, formation of the *N*-succimidylcarbamate moiety was examined using peptide **1a** and 2 equiv of both base and DSC. Slow addition of peptide **1a** to DSC led to a complex mixture (Figure 2A) in which target peptide **1b** represented only 37% of the total detected area. Alternately, addition of peptide **1a** in one portion (Figure 2B) led to a 67% RP-HPLC yield. The use of 10 equiv of DSC slightly improved the yield up to 73% (Figure 2C).

Next, the amount of DSC was fixed to 10 equiv, and we examined the influence of the base amount on the reaction yield using peptide **3a** (Figure 3). The use of an excess of base (Figure 3C−E) produced complex mixtures in which target peptide **3b** was not detected. Alternately, the use of 0.5 equiv of base (Figure 3A,B) permitted the clean conversion of peptide **3a** into NSC peptide **3b**. After 30 min, **3b** was formed nearly quantitatively (Figure 3B). The use of an excess of DSC minimized the formation of urea side-products by reaction of the formed NSC peptide **1**−**4b** with starting peptide **1**−**4a**. The critical role played by base stoichiometry can be explained by the potential conversion of NSC group into the highly reactive isocyanate moiety in basic conditions. Evidence for this degradation is introduced later on. Isocyanate groups react efficiently with amino groups as well as with the phenol group of tyrosine, and thus can be responsible for the multiple side-products observed when two or more equivalents of triethylamine were used. DSC high reactivity allows formation of NSC groups using substoichiometric amounts of base. In these experimental conditions, NSC group is stable and TS-peptides **1**−**4b** could be isolated

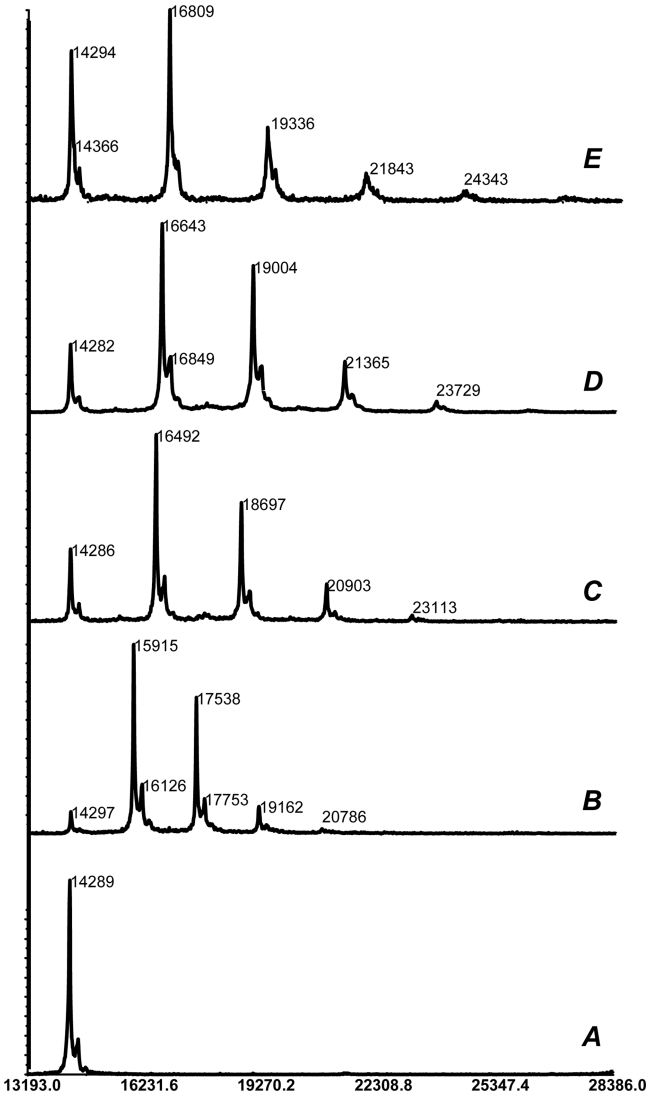


Figure 4. Reaction of lysozyme with peptides **1**−**4b**. MALDI-TOF analysis of the reaction mixtures after 1 h (pH 7.4, 4 °C). (A) without peptide, (B) peptide **1b**, (C) peptide **2b**, (D) peptide **3b**, (E) peptide **4b**.

successfully with a 59−61% yield after RP-HPLC purification. Lyophilized peptides **1**−**4b** were homogeneous by LC-MS and CZE; they displayed the expected molecular ions and were stable for months at −20 °C. ^{1}H and ^{13}C NMR analysis of peptide **1b** confirmed the NSC group presence on the ε-amino group of the C-terminal Lys residue. This procedure was successfully used for converting Ac-YLFSVHWPPLNKA-OH into corresponding NSC derivative Ac-YLFSVHWPPLNK(COOSu)A-OH, showing its compatibility with histidine, serine, tryptophan, and asparagine residues also (see Supporting Information).

For comparison, the same experimental conditions were used to convert peptide **1a** into **1c** in the presence of DSS (Figure 1). The RP-HPLC yield reached only 50% after 90 min of reaction. Peptide **1a** could efficiently be converted into peptide **1c** by using 1 equiv of base (46% isolated yield). Thus, synthesis of NSC peptides requires lower amounts of base compared to NSCE peptides, probably due to the higher reactivity of DSC compared to DSS.

Afterward, we examined the stability of peptides **1b**,**c** in water. Peptides **1b**,**c** were stable enough at room temperature in water containing TFA (pH 2) allowing their purification by RP-HPLC. Typically, hydrolysis of **1b** amounted to 3% after 3 h and only 19% after 18 h. The stability of peptides **1b**,**c** was also studied at pH 6.0. The half-life of NSCE peptide **1c** was 2 h. Peptide **1d** (Figure 1) was the sole species detected by RP-HPLC. The half-life of peptide **1b** was around 10 min. In this case, LC-MS analysis of the mixture revealed, besides peptide **1a**, formation of a novel compound corresponding to isocyanate **1e** (Figure 1). Reaction of this intermediate with benzylamine led to formation of urea **1f**, which displayed the expected fragment ions by matrix-assisted laser desorption ionization-time-of-flight postsource decay analysis.

In situ conversion of NSC peptide **1b** into isocyanate **1e** is probably at the origin of this peptide short half-life. Indeed, carbamates, which cannot be converted into isocyanates such as those derived from secondary amines, are known to be less reactive toward nucleophilic attack than parent carboxylic esters. For example, Gassman et al. have shown that a *p*-nitrophenyl-carbamate derived from a secondary amine is less reactive with nucleophiles than *p*-nitrophenyl acetate (*32*). As demonstrated later on, in situ formation of isocyanate moiety during the conjugation step might also explain the rapid formation of conjugates relative to NSCE derivatives.

Conjugation of Peptides 1−4b to Lysozyme. Lysozyme contains seven Lys residues within its sequence. Thus, 7 equiv of NSC peptide was used in the conjugation experiment. Lysozyme (0.2 mM final concentration) was reacted with NSC peptides **1**−**4b** at pH 7.4 in PBS buffer at 4 °C. A combination of RP-HPLC and CZE analyses showed a conversion of 50% for all tested NSC peptides. Lysozyme labeled with peptide **1b** coeluted by RP-HPLC with the native protein, whereas CZE permitted separation of the labeled protein from the native one due to the low pI of peptide **1b**. Alternately, lysozyme labeled with peptide **4b** could be separated from lysozyme by RP-HPLC, with the labeled material eluting earlier than the unlabeled one, whereas no separation was observed by CZE. The pI of peptide **4b** is close to the pI of lysozyme, explaining why the conjugate between peptide **4b** and lysozyme was not separated from the native protein by CZE. These experiments highlight the interest of varying the NSC peptide pI to match the physicochemical properties.

Figure 4 shows the MALDI-TOF spectra of the reaction mixtures. Up to four peptide chains were linked to lysozyme showing the efficiency of the conjugation reaction. Several studies have proven that NSCE derivatives react with ε-amino groups of proteins and sometimes with hydroxyl-containing amino acids too (*33*). Likewise, reaction of isocyanates with amino, thiol, hydroxyl, and/or imidazole groups within proteins is also possible (*34*). Thus, it cannot be excluded that such reactions might also occur with NSC peptides, which are at least partially converted in situ into isocyanate peptides.

RP-HPLC analysis of the crude reaction mixtures showed also, after 1 h, the absence of NSC peptides **1**−**4b** and two novel peaks in the TS peptide region. The latest eluting peak corresponded to peptides **1**−**4a**, formed by the NSC group hydrolysis. The other peak was attributed to cyclic peptide **1**−**4g** (Figure 5), resulting from the intramolecular reaction between the phenol group of tyrosine and the *N*-succinimidyl carbamate or isocyanate. Indeed, peptide **4g** showed a band at 263.5 nm, typical of an *O*-acetyl tyrosine, whereas the tyrosine residue within peptide **4a** displayed a band at 275.3 nm as expected. Peptide **4g** was isolated by RP-HPLC and treated with 0.1 M NaOH. These experimental conditions led to conversion of **4g** into a compound that coeluted with peptide **4a**. MALDI-TOF and UV spectra of **4a** and of the compound obtained from **4g** were also identical. Overall, these data support the structure proposed for cyclic peptides **1**−**4g**.

Finally, we have compared the reaction rate of peptides **1b**,**c** with lysozyme. LC-MS analysis of the reaction mixtures revealed that reaction of NSC peptide **1b** with lysozyme occurred in less than 5 min (50% conversion). Alternately, the

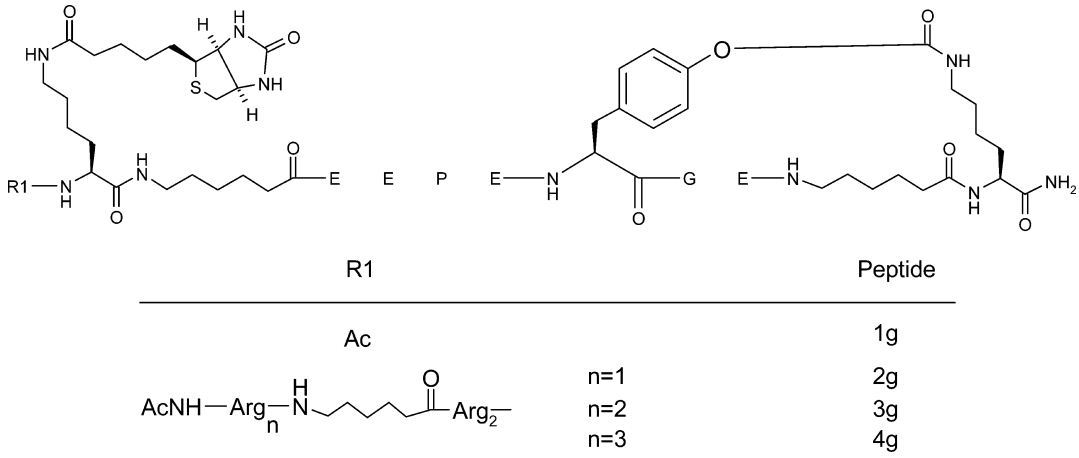


Figure 5. Cyclic peptides **1**−**4c** formed during the labeling reaction by reaction between the phenol group of tyrosine and the NSC group on C-terminal Lys residue.

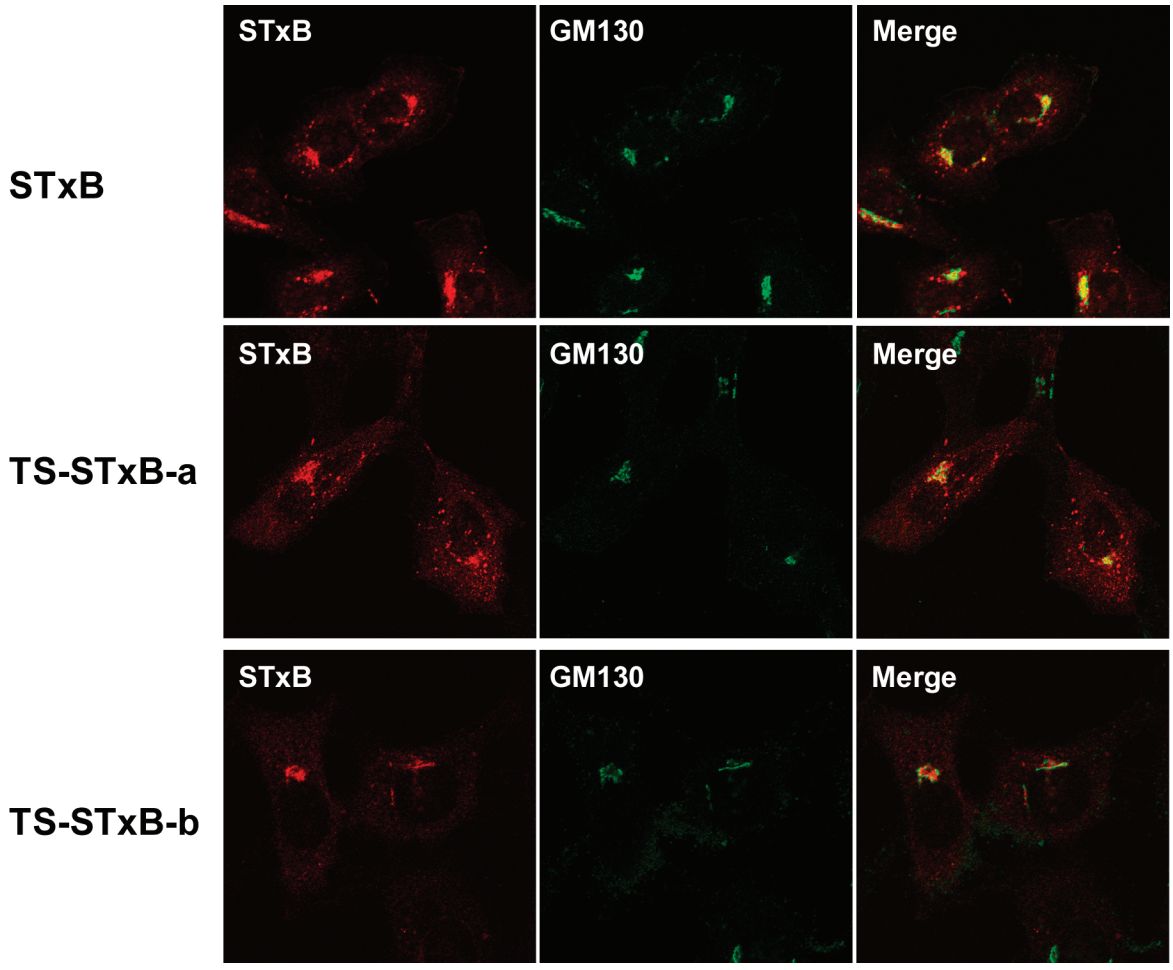


Figure 6. Immunofluorescence study of the retrograde transport of STxB and TS-STxB-a,b conjugates from the plasma membrane to the trans Golgi network. HeLa cells were incubated with 2 μM STxB or TS-STxB-a,b conjugates. Cells were fixed with paraformaldehyde, permeabilized with saponin, and then incubated with 13C4 murine anti-STxB antibody (hybridoma from ATCC #CRL 1794, see ref *20*) and C-19 goat antibody against GM130, a marker of the Golgi. Cells were washed with saponin solution and then incubated with secondary antibodies (Cy3-labeled antimurine, Alexa 488-labeled antigoat). Cells were analyzed by confocal microscopy (Leica Microsystems, Mannheim, Germany).

reaction of lysozyme with peptide **1c** led to only 20% conversion after 5 min and required about 1 h to go to completion (80% conversion). Thus, NSC peptides allow rapid conversion of proteins into peptide−protein conjugates, a property that can be an advantage for the labeling of unstable proteins.

Synthesis of TS-STxB Conjugates and Biological Studies. The retrograde route is used by Shiga toxin (*35*) to exert its toxic effects. This toxin has an AB5 structure. The A domain is responsible for the toxic effects of Shiga toxin. The B-subunit STxB (7.7 kD) forms a pentamer which preferentially binds the eukaryotic receptor globotriaosylceramide (Gb3). The B pentamer has the capacity of targeting the toxin to the retrograde route, allowing transfer of the A-subunit to the cytosol by retrotranslocation from the endoplasmic reticulum. STxB is a

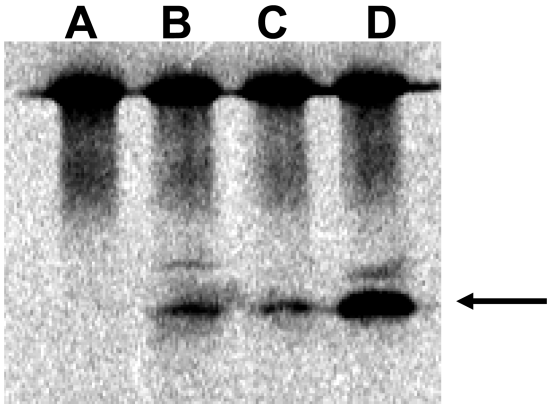


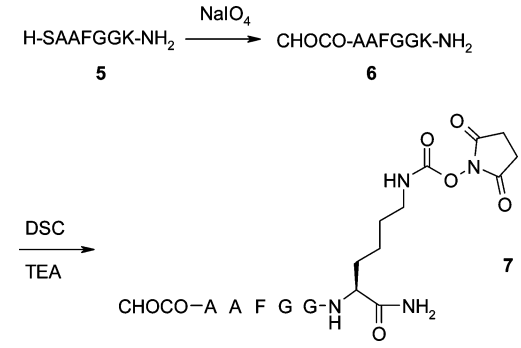
Figure 7. Sulfation assay using STxB (lane A), TS-STxB-a (lane B), TS-STxB-b (lane C), and TS-STxB-c (lane D). Proteins were incubated with HeLa cells at 4 °C in sulfate-free DMEM, and then incubated with $^{35}SO_4^{2-}$ in sulfate free DMEM at 37 °C for 2 h. Cells were lysed and STxB proteins were captured using protein G-sepharose loaded with anti-STxB antibody 13C4. Precipitated proteins were separated by tris-tricine gel electrophoresis, and gels were analyzed by autoradiography. The arrow points to the different STxB proteins.

valuable marker for the retrograde route and was used here to prove the usefulness of TS conjugates prepared using NSC chemistry.

STxB labeling was performed using peptide **1b** as described above. LC-MS analysis of the product revealed that 80% of STxB was modified. MALDI-TOF analysis of the product showed the presence of up to three TS peptides per STxB monomer. The conjugate was purified either by ultrafiltration on a 3 kD membrane (TS-STxB-a) or by RP-HPLC (TS-STxB-b). TS-STxB-a,b were identical by LC-MS. Both conjugates were tested to evaluate the impact of RP-HPLC and lyophilization steps on the TS-STxB-b biological activity.

The retrograde transport of STxB and of TS-STxB-a,b from the plasma membrane to the trans Golgi network was first studied by immunofluorescence confocal microscopy using HeLa cells (Figure 6). The three proteins colocalized with GM130, a protein which is bound to Golgi membranes (*36*). This experiment shows that modification of STxB by NSC

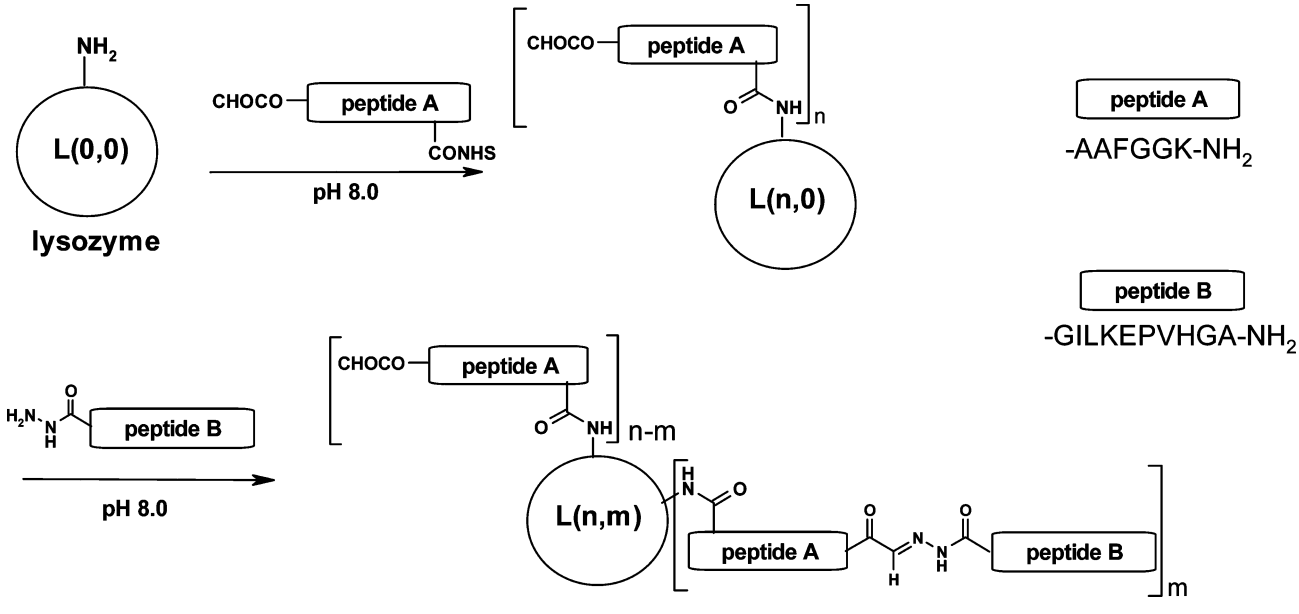
Scheme 2. Synthesis of Glyoxylyl NSC Peptide 7



peptide **1b** did not alter the capacity of the protein to target the retrograde route. We next studied the TS label capacity within the conjugates to be sulfated by tyrosylprotein sulfotransferase, an enzyme which is associated with the Golgi membrane (Figure 7) (*37, 38*). STxB was used as a negative control (lane A, Figure 7), whereas TS-STxB-c, a recombinant STxB protein modified at the C-terminus by two tandem sulfation sites, was used as a positive control (*21*). Cells were first incubated with the different STxB proteins and then in the presence of $^{35}SO_4^{2-}$ in sulfate-free DMEM to allow incorporation of radioactive sulfate within the TS labels. Cells were lysed and STxB proteins were immunoprecipitated using anti-STxB monoclonal antibody. Radioactive bands were detected by autoradiography. The arrow in Figure 7 points to STxB proteins. This experiment shows a radioactive band for the positive control (TS-STxB-c, lane D) but not for the negative control (STxB, lane A). A band is also seen for TS-STxB a or b, showing that the TS label introduced using NSC chemistry is sulfated by tyrosylprotein sulfotransferase present in the Golgi. Overall, these data show that NSC chemistry allows the synthesis of conjugates that preserve the capacity of STxB protein to target the retrograde route and the ability of TS peptide to be sulfated in the cell.

One-Pot Synthesis of Peptide−Protein Conjugates. The method described above can only be used with peptides lacking Lys residues within their sequences such as the TS peptide described above. The two-step strategy depicted in Scheme 1 allows overcoming this limitation. The first step involves reaction of bifunctionnal peptide A with the protein using NSC

Scheme 1. One-Pot Synthesis of Peptide Protein Conjugates Can Be Performed by Combining NSC Chemistry and α-Oxo Semicarbazone Ligation



Scheme 3. Synthesis of Semicarbazide Peptide 8

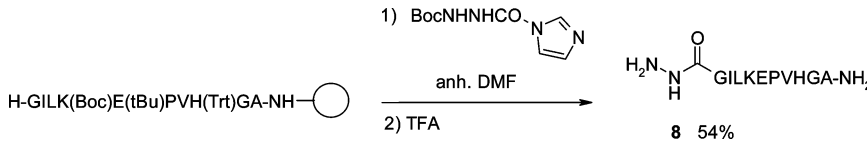


Table 2. MS Data for the One-Pot Reaction of Lysozyme with Glyoxylyl NSC Peptide 7 and Semicarbazide Peptide 8[a]

entries	lysozyme L(*n*,*m*)	calcd	found
1	0,0	14.306	14.306
2	1,0	14.936	14.937
3	2,0	15.567	15.571
4	3,0	16.198	16.198
5	1,1	15.996	15.995
6	2,1	16.626	16.626
7	2,2	17.684	17.684

[a] MALDI MS spectra were acquired in the linear mode by using a PerSeptive Biosystems Voyager DE-STR instrument fitted with a 337 nm nitrogen laser (25 kV, grid 92%, delay time 750 ns). Matrix: 3,5-Dimethoxy-4-hydroxycinnamic acid (20 mg/mL). Entry 1: lysozyme. Entries 2−4: reaction with peptide **7**. Entries 5−7: reaction with peptide **7** and peptide **8**.

chemistry. This allows introducing an α-oxo aldehyde group on the surface of the protein, which can be subsequently ligated chemoselectively with peptide B featuring a semicarbazide group at the N-terminus (Scheme 1). To date, semicarbazone chemistry has been mainly used for linking peptides to surfaces (*39*−*42*). To the best of our knowledge, however, ligation between semicarbazide and glyoxylyl peptides was not reported before. This may be due to the difficulty synthesizing peptides featuring a semicarbazide group at the N-terminus, because this moiety is rapidly hydrolyzed in the acid eluents (pH 2) often used for peptide RP-HPLC analysis or purification. A solution to this problem has recently been described by the use of a nearly neutral buffer instead for the purification step (*19*).

Peptide A synthesized in this work is used as spacer. A Phe residue was inserted into the sequence to facilitate UV detection. However, peptide A can potentially be used for introducing a bioactive sequence or a label depending on the final application.

The strategy described in Scheme 1 requires glyoxylyl NSC peptide **7** and semicarbazide peptide **8** synthesis. Peptide **7** was prepared starting from peptide **5** H-SAAFGGK-NH_2 (Scheme 2). Oxidation of peptide **5** into peptide **6** COCHO-AAFGG-NH_2 by sodium periodate was performed using standard experimental conditions (*4*). Glyoxylyl NSC peptide **7** was then synthesized by treating peptide **6** with 0.5 equiv of triethylamine and an excess of DSC, as described above for NSC TS peptides **1**−**4b**. Attempts to convert peptide **6** into the corresponding NSCE ester with DSS, as described above, failed probably due to imine formation between α-oxo aldehyde and ε-amino moieties in basic conditions.

Model peptide **8** synthesis was performed by coupling Boc-NHNHCO-imidazole to peptidyl resin assembled using Fmoc/*t*-Bu strategy (Scheme 3) (*19*). Semicarbazide peptide **8** was stable during TFA deprotection and cleavage and was purified by RP-HPLC in a pH 6.5 triethylamonium acetate buffer.

Lysozyme was used again as a model protein. Reaction between lysozyme and glyoxylyl NSC peptide **7** was performed in a pH 8.0 sodium phosphate buffer. MALDI-TOF analysis of the reaction mixture revealed grafting of up to three glyoxylyl NSC peptide **7** molecules (L1−3,0 see entries 2−4, Table 2; see also Figure 8). After 1 h, the reaction mixture was reacted with semicarbazide peptide **8** at same pH. Up to two semicarbazide peptide **8** molecules were linked to lysozyme (entries

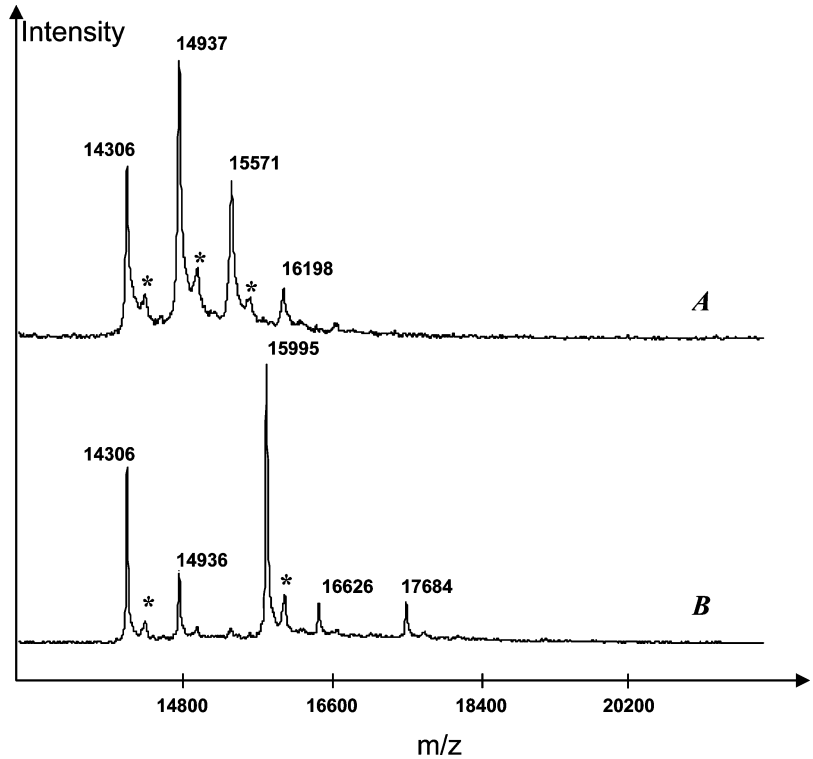


Figure 8. MALDI-TOF analysis of the one-pot conversion of lysozyme L into L(*n*,*m*) peptide-lysozyme conjugates. (A) after reaction with glyoxylyl NSC peptide **7**, (B) after reaction with peptide **7** and semicarbazide peptide **8**. See Table 2 for experimental conditions. The asterisk corresponds to protein−matrix adducts.

5−7, Table 2). Usually, Schiff-base formation between glyoxylyl peptides and peptide hydrazides is carried out at pH 5.5. In this study, successful formation of the α-oxo semicarbazone bond at pH 8.0 permitted the one-pot synthesis of peptide−protein conjugates in a straightforward manner.

In conclusion, we have shown that NSC peptides can easily be synthesized in high yield by reacting unprotected peptide precursors with DSC. The method requires the presence of only one reactive ε-amino group within the peptide sequence. Importantly, NSC peptides were stable during standard RP-HPLC purification at pH 2 and in the lyophilized form for months. Their reaction with ε-amino groups of proteins occurred efficiently and rapidly (<5 min), probably due to in situ partial conversion of NSC group into isocyanate moiety. The method was used for grafting a peptide substrate for tyrosylprotein sulfotransferase to lysozyme, a model protein, or STxB the receptor-binding nontoxic B-subunit of Shiga toxin, a valuable marker for the retrograde route. The biological activity of the STxB conjugate was verified by immunofluorescence confocal microscopy or using a sulfation assay. We have also described a novel method for synthesizing peptide−protein conjugates, which is compatible with all proteinogenic amino acids. The conjugates were assembled by combining NSC chemistry and α-oxo semicarbazone ligation. To this end, a glyoxylyl NSC peptide was synthesized and reacted with lysozyme to produce glyoxylyl lysozyme. The crude reaction mixture was then reacted with a semicarbazide peptide. Ligation between the glyoxylyl groups on the protein and the semicarbazide group on the peptide resulted in formation of the target peptide−protein conjugate. Both reactions were carried out at pH 8.0, thus allowing the one-pot synthesis of the conjugate without the need to adjust pH in between.

Overall, NSC chemistry described here is an interesting tool for preparation of conjugates of biological interest and complements the chemical toolbox of the peptide chemist.

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Supporting Information Available: RP-HPLC, CZE and MS data for all compounds. ^{1}H NMR spectra for peptides **1a**,**b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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