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C-Terminal Modification of Fully Unprotected Peptide Hydrazides via in Situ Generation of Isocyanates

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Supporting Information

ABSTRACT: A method for chemo- and regioselective conjugation of nucleophiles to fully unprotected peptides and proteins via in situ generation of C-terminal isocyanates is reported. Oxidation of C-terminal peptide hydrazides in aqueous media followed by Curtius rearrangement of acyl azides reliably generates isocyanates, which react with a variety of external nucleophiles, such as hydrazines, hydrazides, aromatic thiols, and hydroxylamines. Multiple peptides and a 53 kDa protein hydrazide were conjugated to different nucleophiles using this reaction.

Chemo- and regioselective conjugation of bioprobes to peptides and proteins is a key challenge in modern bioorganic chemistry. An ideal conjugation reaction should proceed rapidly in aqueous solvents without protecting groups and accept a wide range of probes and substrates. Several outstanding methods embrace these criteria, allowing excellent chemoselectivity in conjugation chemistry.[1] Many of these methods make use of the nucleophilic properties of peptide side chains, in particular cysteine.[2] Less common are methods to conjugate nucleophiles to electrophilic peptides.[3]

Isocyanates are excellent electrophiles, known to react with a broad array of nucleophilic species.[4] Isocyanates are invaluable precursors in the synthesis of peptidomimetics containing urea and carbamate moieties.[5] Synthesis and transformations of peptide isocyanates have been reported for short protected fragments in organic solvents, with constituent amino acids often lacking functional side chains.[6] Thus far, the utilization of isocyanates as reactive handles for conjugation of nucleophiles to complex unprotected peptides in aqueous media is lacking.

Here we fill this gap for the facile generation of C-terminal electrophilic isocyanates. We found that fully unprotected C-terminal acyl azides, obtained by sodium nitrite oxidation of corresponding hydrazides, undergo a Curtius rearrangement to generate reactive isocyanates (Figure 1a). These species cannot be isolated from aqueous solvents, but can rapidly and selectively react with an excess of various external nucleophiles to give conjugation products. To avoid hydrolysis of isocyanates, nucleophiles are added directly to peptide acyl azides, such that any freshly generated isocyanate reacts with the nucleophile rather than water.

We first observed these transformations while preparing peptides for native chemical ligation using the recently reported protocol of Liu and co-workers.[7] We found that isocyanates are generated and then react with 4-mercaptophenylacetic acid (MPAA), to give carbamothioates as side products (Supporting Information (SI) section 5.1). To study this side reaction in more detail we prepared a 10-residue model peptide, H_2N-Ala-Gln-Val-Ile Asn-Thr-Phe-Asp-Gly-Val-$CONHNH_2$. In a typical reaction, 2.80 mM peptide in aqueous phosphate buffer at pH 3.2 was oxidized with 20 mM $NaNO_2$ at −10 °C for 10 min and excess 4-mercaptophenylacetic acid (MPAA) was added to the

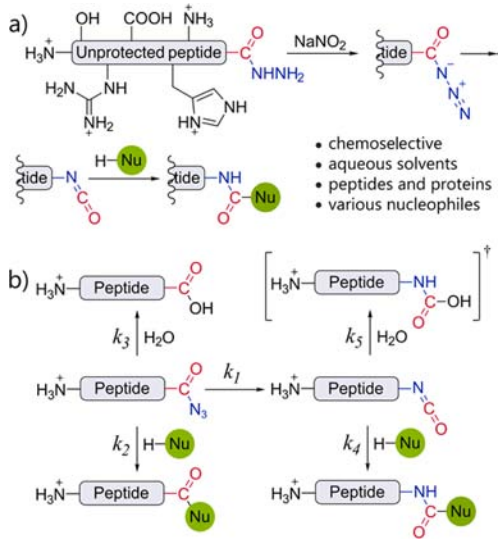

Figure 1. *In situ* generation of peptide isocyanates. (a) One-pot oxidation of C-terminal peptide hydrazides followed by Curtius rearrangement and isocyanate conjugation. (b) A scheme, illustrating formation of the major products observed throughout the work. †This product was not directly observed; rather, it underwent facile decarboxylation during LCMS analysis (SI section 5.1).

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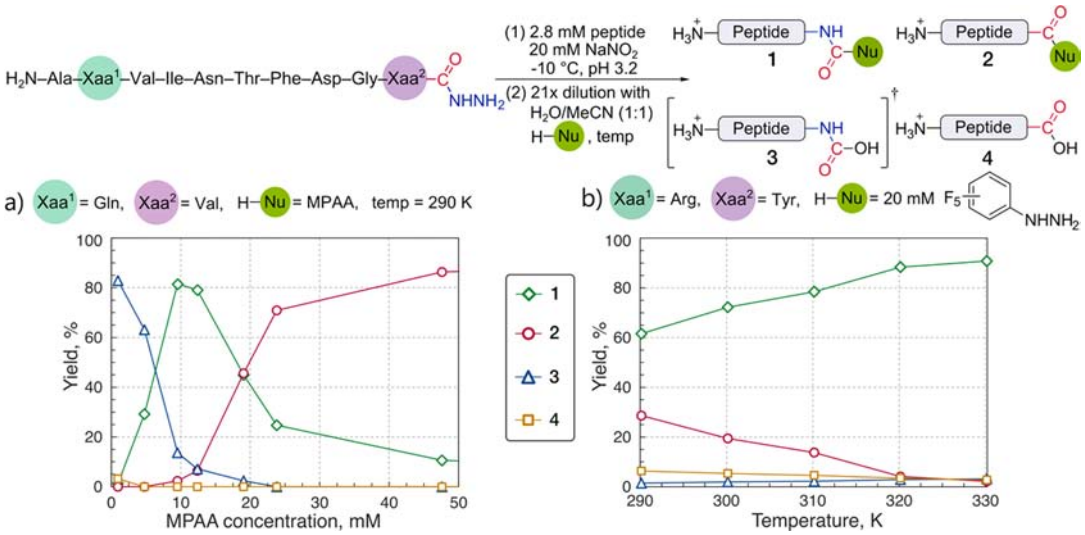


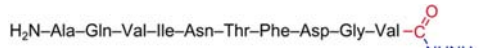
Figure 2. Influence of the parameters on the outcome of the reaction. (a) Distribution of the major reaction products as a function of nucleophile (MPAA) concentration. All data points are in triplicates; ±s.d. is smaller than the size of the marker. (b) Distribution of the major reaction products as a function of temperature. All data points are single measurements. †Hydrolysis was accompanied by formation of several products (SI section 5.1).

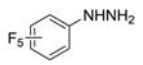
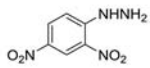
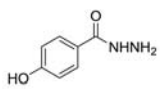
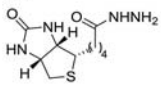
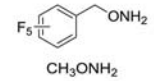
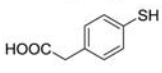
resulting acyl azide. The reaction was then warmed to room temperature (17 °C), and an aliquot of the crude reaction mixture was analyzed by HPLC-MS 2 h later. After investigating different reaction conditions, we found that pH, solvent composition, and the order of the reagent addition had modest effect on the formation of the carbamothioate (SI section 5.2). In contrast, we found that the concentrations of the reagents were crucial for promoting the Curtius rearrangement/isocyanate conjugation. To systematically investigate the effect of nucleophile concentration, we fixed the peptide acyl azide concentration at 133 μM and added varying amounts of MPAA dissolved in water/acetonitrile (1:1, v/v). We found that the carbamothioate formed in 82% HPLC yield when 10 mM MPAA was used (Figure 2a). We also observed that the further dilution of the MPAA nucleophile led to hydrolysis of isocyanate, while a more concentrated nucleophile resulted in thioester formation.

We consistently detected four major products in the reaction mixture: a product of the isocyanate conjugation to nucleophile (Figure 2, compound **1**), a product of nucleophile conjugation to azide (**2**), isocyanate derived hydrolysis products (**3**), and carboxylate (azide hydrolysis product, **4**). Analysis of the kinetic scheme in Figure 1b explains the results summarized in Figure 2a: at high concentration of MPAA and/or peptide, the bimolecular k_2 reaction takes place leading to the corresponding product. At very low concentrations of both reactants, the monomolecular Curtius rearrangement k_1 takes place faster than k_2, and the pseudomonomolecular k_5 takes place faster than bimolecular k_4; this leads to the hydrolysis of the isocyanate observed at low MPAA concentrations. Fortunately, there is a window of concentrations where k_1 is faster than k_2, and k_4 is faster than k_5; this leads to a successful conjugation of nucleophiles to isocyanates. It is challenging to write exact kinetic relationships based on this simplified idea due to the complex nature of interactions between isocyanates and their nucleophiles.[8] Nevertheless, we found that this analysis holds true for a variety of nucleophiles and, moreover, with isocyanate concentration fixed, the optimum nucleophile concentration does not change significantly, when different peptide isocyanates are used (SI section 5.5).

Next, we investigated the nucleophile scope of the reaction with the same model peptide. All reactions were performed under conditions similar to the aforementioned (SI section 5.4) in water/acetonitrile (1:3, v/v). We used strong nucleophiles that remained nucleophilic under acidic conditions and found (Table 1) that electron-deficient monosubstituted hydrazines (entries 1, 2) and hydrazides (entries 3, 4) are excellent nucleophiles and react selectively with isocyanates over a wide range of concentrations to give semicarbazides with high yield

Table 1. Nucleophile Scope of the Reaction



entry	H–Nu	[H–Nu] mM	yield of **1**, %[a]
1		25	96 (82)
2		5.7[b]	73 (48)
3		33	96 (73)
4		33	92 (69)
5		100	72 (53)
6	CH_3ONH_2	100	79 (42)
7		10	82 (60)
8	MeOH	95% (v/v)	89 (66)

[a]HPLC yields are listed. Isolated yields are provided in parentheses. [b]The nucleophile concentration was limited by its solubility.

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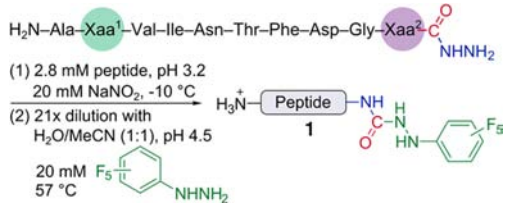
and selectivity. Using a large excess of these nucleophiles ensured that any remaining sodium nitrite is quenched and is inconsequential to the reaction progress. Additionally, we found that hydroxylamine derivatives (entries 5, 6) also conjugate to isocyanates albeit at higher concentrations and with yields lower than in the case of hydrazines. We also demonstrated that the dilution of the acyl azide with primary alcohols affords corresponding carbamates in high yield (entry 8, SI section 5.4.9).Various alkylthiols (β-mercaptoethanesulfonate, β-mercaptoethanol, and *tert*-butylthiol) failed to conjugate to peptide isocyanates under a number of conditions. Notably, we did not observe products of intramolecular cyclizations, or formation of symmetrical ureas, a prominent side reaction in the acyl azide method of peptide synthesis.[9]

The side chain of the C-terminal amino acid is reported to significantly affect the rate of the Curtius rearrangement. More sterically hindered amino acids, such as valine and isoleucine, accelerate the rearrangement, while less sterically hindered glycine and proline significantly decelerate it.[10] Indeed, when we oxidized the model peptide H_2N-Ala-Arg-Val-Ile-Asn-Thr-Phe-Asp-Gly-Tyr-$CONHNH_2$ under standard conditions and used 20 mM perfluorophenylhydrazine in water/acetonitrile (1:1, v/v) as a nucleophile, we observed a modest 62% yield of semicarbazide, with *N*′-perfluorophenyl hydrazide being the side product (29% yield, Figure 2b). To accelerate the Curtius rearrangement and increase the selectivity of the transformation, we performed the reaction at higher temperatures, keeping the temperature of the oxidation step the same. We were delighted to find that the yield of semicarbazide rose almost linearly with increasing temperature, reaching 91% at 57 °C. Additionally, performing the reaction at 57 °C allowed us to reduce the overall reaction time to 30 min (a 10 min oxidation step followed by a 20 min rearrangement). Although the Curtius rearrangement is known to proceed with retention of configuration[11] we confirmed that our conjugation does not lead to the racemization of the C-terminal residue in a separate experiment using two diastereomeric peptides (SI section 5.6).

To further elucidate the effects of the C-terminal residue on the efficiency of the isocyanate conjugation we prepared a small library of peptides H_2N-Ala-Xaa_1-Val-Ile-Asn-Thr-Phe-Asp-Gly-Xaa_2-$CONHNH_2$, where all 20 proteogenic amino acids were scanned in the position Xaa_2.[12] Amino acids in the position Xaa_1 were additionally varied to test the chemoselectivity of the transformations in the presence of potentially reactive peptide side chains. All reactions were carried out under standard conditions (SI section 5.3). With the exception of Xaa_2 = Val, Ile, and Thr, which were reacted at 17 °C, all peptides were incubated with the nucleophile at 57 °C for 20 min.

As summarized in Table 2, we found that the majority of C-terminal amino acids were compatible with the studied chemistry, and in all cases the isocyanate conjugation was compatible with residues in the Xaa_1 position; we did not observe products of the isocyanate cyclization to the Xaa_1. Peptides with C-terminal hydrophobic residues gave corresponding semicarbazides in good yields, with Xaa_2 = Gly (entry 13) as the predictable exception. Interestingly, despite the fact that isocyanates are known to react with aromatic nucleophiles,[4] the C-terminal Tyr and Trp (entries 3 and 4) did not form cyclization products under the studied conditions and instead yielded the desired semicarbazides. Also compatible with the one-pot isocyanate conjugation were C-terminal Arg, Lys, Met, and Cys (entries 5, 6, 8, 11). For Cys the reaction was accompanied by the formation of a product +29 Da heavier than

Table 2. C-Terminal Amino Acid Scope of the Reaction



entry	Xaa^1	Xaa^2	HPLC yield of **1**, %
1	Gln	Val	96[a]
2	Lys	Ile	95[a]
3	Arg	Tyr	91
4	Lys	Trp	89
5	Trp	Arg	85
6	Tyr	Lys	84
7	Asp	Leu	84
8	Gly	Met	83
9	Glu	Ala	72
10	Lys	Pro	62
11	Pro	Cys	57
12	Met	Phe	51
13	Ser	Gly	44
14	Leu	Glu	24
15	Val	His	11
16	Asn	Ser	n. d.
17	His	Thr	n. d.[a]
18	Any	Asp, Asn, Gln	n. a.[b]

[a]Reaction was performed at 17 °C. [b]Fmoc-SPPS failed to produce corresponding hydrazides. n.d. = not determined, n.a. = not applicable.

expected, which we attributed to the nitrothioite (RSNO) formation. Fortunately, nitrothioites can be reduced back to thiols.[13]

Several C-terminal amino acids were incompatible with the reported reaction. The side chains of Thr and Ser both cyclized with the isocyanate, which led to the formation of oxazolidin-2-ones (SI section 5.6, Figure S38).[14] C-terminal His and Glu also led to poor yields of semicarbazides, giving significant amounts of carboxylate. This result may be explained by the cyclization of His and Glu side chains with the acyl azide, followed by the hydrolysis of the cyclic intermediate to release carboxylate.[15] Formation of the cyclic intermediate is supported by the fact that two chromatographically resolved peaks corresponding to peptide thioester were observed when H_2N-Ala-Leu-Val-Ile-Asn-Thr-Phe-Asp-Gly-Glu-$CONHNH_2$ was subject to oxidation/thioesterefication with 200 mM MPAA (SI section 5.6, Figure S39). Consistent with previous reports,[7a] we could not produce C-terminal hydrazides of Asn, Asp, and Gln due to the intramolecular cyclization that spontaneously occurs after the peptide is released from resin (SI section 4.19).

Finally, as a proof-of-concept we demonstrated that the described chemistry can be used to conjugate nucleophiles to longer peptides and proteins. To this end, we successfully conjugated perfluorophenylhydrazine to 26-mer and 37-mer peptides (SI section 5.8). For protein conjugation, we installed a C-terminal hydrazide on a 53 kDa protein (LF_N-DTA-Leu-Pro-Ser-Thr-Gly-Gly-His_5) by means of Sortase A* mediated ligation with Gly_5-Leu-Glu-Ile-$CONHNH_2$ (SI section 5.9).[16] 12 mM $NaNO_2$ in water was added to 90 μM LF_N-DTA-Leu-Pro-Ser-Thr-Gly_5-Leu-Glu-Ile-$CONHNH_2$ in 50 mM acetate buffer at pH 3.8; after 10 min at 4 °C the protein acyl azide was diluted to 22.5 μM with 75 mM perfluorophenylhydrazine in the same

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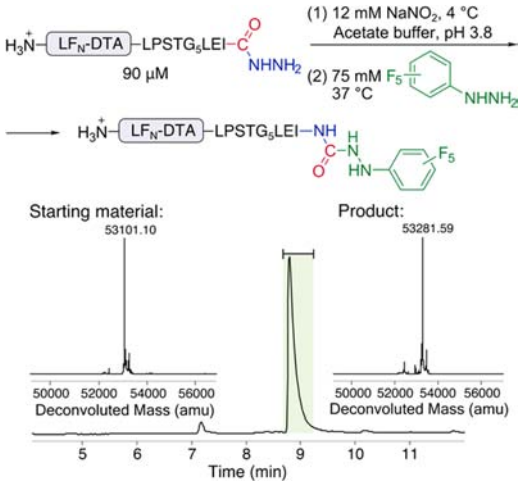

Figure 3. C-terminal labeling of 53 kDa protein with perfluorophenylhydrazine. HPLC-MS (TIC) chromatogram of the crude labeled protein is shown. Highlighted MS peak was deconvoluted (maximum entropy) to obtain the product deconvolution spectrum on the right. The starting material spectrum was obtained the same way (TIC not shown).

buffer (pH 3.8), and the resulting solution was incubated for 1 h at 37 °C. These conditions cleanly afforded perfluorophenyl-labeled C-terminal semicarbazide of LF_N-DTA protein in 92% yield as estimated by the integration of the deconvolution spectrum (Figure 3).

In summary, we have developed a method for the conjugation of nucleophiles to peptides and proteins, making use of C-terminal isocyanates generated in one pot from corresponding hydrazides. The reaction occurs in aqueous solvents; it is chemo- and regioselective, and it is orthogonal to unprotected amino acid side chains. As C-terminal hydrazides are readily available through standard Fmoc-SPPS, there is no need to preinstall electrophilic moieties during SPPS. The conjugation results in formation of peptidomimetic bonds, such as carbamates, semicarbazides, and carbamothioates, and is essentially irreversible. Correct concentrations of reagents are crucial for the success of conjugation (Figure 1b): various nucleophiles in the 5−100 mM range yielded the desired products. Development of practical ligation methods based on this chemistry is underway.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b03625.

Detailed experimental procedures, characterization of synthesized peptides, additional data (PDF)

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Notes

The authors declare no competing financial interest.

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