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Macrocyclization of Unprotected Peptide Isocyanates

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Supporting Information

ABSTRACT: A chemistry for the facile two-component macrocyclization of unprotected peptide isocyanates is described. Starting from peptides containing two glutamic acid γ-hydrazide residues, isocyanates can be readily accessed and cyclized with hydrazides of dicarboxylic acids. The choice of a nucleophilic linker allows for the facile modulation of biochemical properties of a macrocyclic peptide. Four cyclic NYAD-1 analogues were synthesized using the described method and displayed a range of biological activities.

Peptide macrocyclization is a powerful tool for enhancing and modulating bioactivity, cell permeability, proteolytic stability, and other important properties of peptides. The pharmaceutical promise of macrocyclized or "stapled" peptides has drawn serious attention from the scientific community, resulting in a number of peptide macrocyclization strategies. The best known approach involves ring-closing metathesis of α,α-disubstituted olefin-bearing amino acid residues and results in all-hydrocarbon bridge tethering of two residues on the same helical face.[1,2] Successful applications of this approach include the development of p53/MDM2 interaction inhibitors,[3−5] BCL-2 family proteins activators and repressors,[6−9] β-catenin binders,[10,11] and others.[12,13] Among other techniques now available for macrocyclization are cysteine modification reactions, including arylation,[14,15] alkylation,[16−18] and disulfide bond formation;[19] side-chain to side-chain reactions, such as lactam[20,21] and oxime formation;[22] and cycloaddition reactions.[23] Combined together, these impressive advances in the field of peptide macrocyclization enable access to a wide range of structurally and chemically different substrates.

In this study we investigated the possibility of using isocyanates as reactive handles to achieve facile two-component cyclization with bifunctional nucleophiles, such as hydrazides of dicarboxylic acids. This approach enables the formation of cyclic systems of variable size and topology in a modular fashion. Additionally, the cyclization proceeds selectively over unprotected amino acid side chains and results in unique, hydrophilic bridges of variable rigidity, which modulate the biochemical properties of peptides.

The idea behind our approach is illustrated in Figure 1. Facile oxidation of an unprotected peptide containing two glutamic acid γ-hydrazides with sodium nitrite in water at pH 3−4 yields the peptide containing two side-chain acyl azides.[24] Without intermediate isolation, the peptide is then introduced to a bifunctional nucleophile to allow for a Curtius rearrangement followed by a cyclization between generated isocyanates and the nucleophile. If dihydrazides are used as the nucleophile, a double N′-substituted semicarbazide bridge tethering two diaminobutyric acid side chains is constructed. Starting peptide hydrazides can be prepared on-resin during peptide synthesis from γ-allyl-protected glutamic acid in two straightforward steps. First, the fully protected peptide is treated with $Pd(PPh_3)_4/SiPhH_3$ to remove allyl ester protection from the glutamic acid side chain. Then, free acid is activated with HBTU/DIEA and introduced to a solution of hydrazine in DMF, which cleanly yields glutamic acid γ-hydrazides (Supporting Information (SI) sections 2.2 and 3.1).

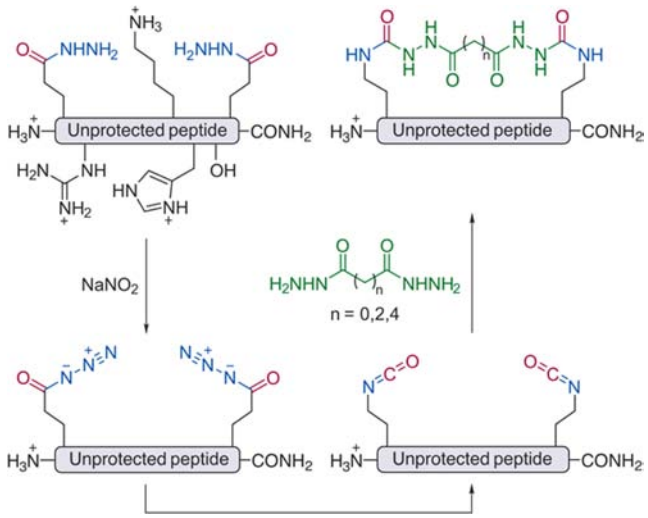

Figure 1. Concept of the study. Two-component macrocyclization of unprotected peptide isocyanates with dicarboxylic acid hydrazides.

To investigate the feasibility of our approach, we prepared[25,26] a model peptide, NH_2-GALPYXVKSXFG-

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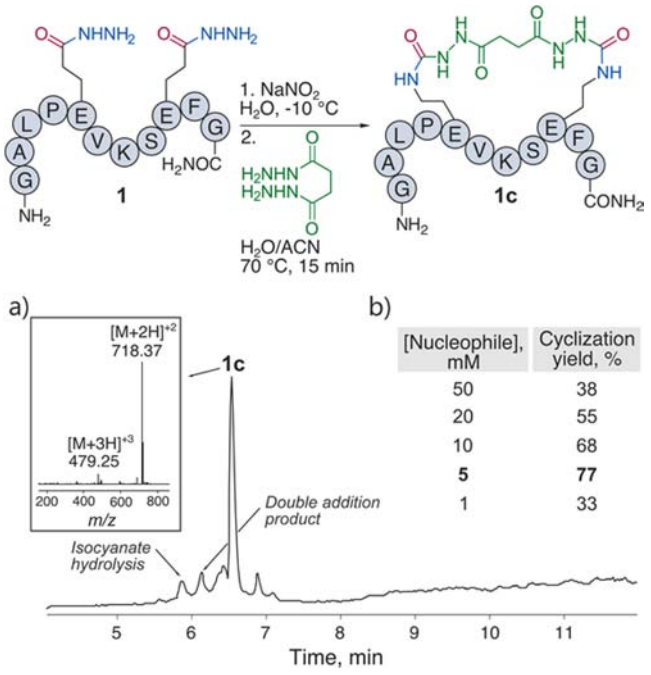

Figure 2. Development of isocyanate macrocyclization reaction. (a) HPLC-MS (TIC) chromatogram of the crude macrocyclization reaction between peptide **1** and succinic dihydrazide with MS inset for the expected cyclized product. The peptide was oxidized with 20 mM $NaNO_2$ at −10 °C for 10 min, diluted into a 5 mM solution of the nucleophile in water/acetonitrile (1:3, v/v) mixture and incubated at 70 °C for 10 min. (b) Yield of **1c** as a function of nucleophile concentration.

$CONH_2$, where X is glutamic acid γ-hydrazide (**1**) and studied its cyclization with succinic dihydrazide (Figure 2a). In our previous study (see the accompanying manuscript: Vinogradov, A. A.; Simon, M. D.; Pentelute, B L. *Org. Lett.* **2016**, DOI: 10.1021/acs.orglett.6b03625), we established that nucleophile concentration is key to successful isocyanate conjugation in aqueous solvents. Thus, we began by screening various concentrations of succinic dihydrazide. In a typical reaction, 3.1 mM peptide in 200 mM sodium phosphate aqueous buffer at pH 3.2 was oxidized with 20 mM sodium nitrite at −10 °C for 10 min. An excess of succinic dihydrazide in water/acetonitrile (1:3, v/v) was then added to the peptide, and the mixture was thermostated at 70 °C for 10 min. The reaction outcome was analyzed by HPLC-MS. As summarized in Figure 2b, we investigated five different nucleophile concentrations and found that the reaction proceeded most efficiently at 5 mM succinic dihydrazide, yielding 77% macrocyclized product. Lower nucleophile concentrations resulted in significant hydrolysis of at least one isocyanate functionality, which abolished the cyclization, while more concentrated nucleophile promoted double addition over the cyclization.[27]

Encouraged by the results of this model study, we decided to further explore the substrate scope, the nucleophile influence, and the effects of the cycle size on the outcome of the reaction. To this end, we prepared two more peptides: the first, NH_2-GA**X**PYLVKS**X**FG-$CONH_2$ (**2**), had two hydrazides separated by six amino acids (*i*, *i* +7 cycle), and the second, NH_2-GALPYX**X**VKSEFV-$CONHNH_2$ (**3**), was a peptide containing a single glutamic acid γ-hydrazide and a valine C-terminal hydrazide separated by five amino acids to study the possibility of a side chain to C-terminus cyclization. As linkers for the cyclization we chose the commercially available dihydrazides of variable reactivity, size, and rigidity. Carbohydrazide (**a**) and oxalyldihydrazide (**b**) represented short, rigid, and generally less reactive linkers, while succinic dihydrazide (**c**) and adipic dihydrazide (**d**) were longer staples with several sp^3 carbon centers to allow for greater flexibility of resulting constructs.

As summarized in Figure 3, we performed a total of 12 reactions to study the reaction. The peptides were cyclized with 5 mM nucleophiles essentially following the protocol outlined above, and the reaction mixtures were analyzed by HPLC/MS. Additionally, peptides **1** and **1a**−**d** were isolated by RP-HPLC and characterized by ^1H and ^{13}C NMR to confirm the product identity and purity (SI section 3.3). In all cases, we observed

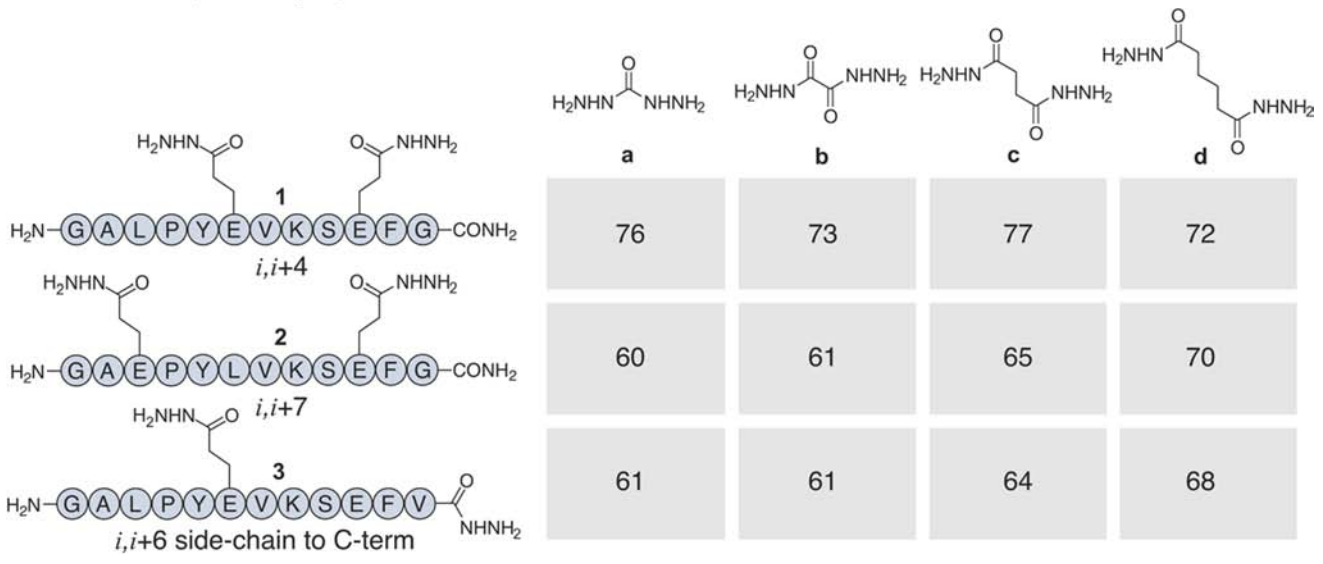

Figure 3. Substrate and linker scope of the isocyanate macrocyclization. Peptides were oxidized with 20 mM $NaNO_2$ at −10 °C for 10 min, diluted into a 5 mM solution of the nucleophile in water/acetonitrile (1:3, v/v) mixture and incubated at 70 °C for 10 min. Numbers in each cell represent HPLC/MS yields of the expected macrocyclized product formed from a corresponding peptide and a linker. TIC chromatograms were manually integrated and yields were determined from relative peak areas.

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smooth cyclization with similar reaction profiles, although reaction yields varied for different constructs. Peptide **1**, which had isocyanates spaced in the (*i*, *i* + 4) manner, generally cyclized more efficiently than peptides **2** and **3**, indicating, not surprisingly, that the cyclization is more robust for smaller ring sizes. Interestingly, we also found that the side-chain to C-terminus cyclization of **3** proceeded nearly as efficiently as the side-chain to side-chain cyclization of **2** for all studied linkers which suggests that the cycle size is the primary factor influencing the reaction yield. From the linker standpoint of view, we found no major discrepancy in reactivity between four studied dihydrazides, although linker **c** generally reacted better than the others, possibly due to a more optimal size and substantial flexibility of the resulting ring.

Having established the scope of the reaction, we turned to investigating how biological properties of these unusually cyclized peptides with double N′-acylated semicarbazide tethers are affected. We first studied whether stapled peptides **1a**−**d** are protected against proteolytic degradation by digestive proteases, a property which at least some stapled peptides were reported to possess.[28] To this end, we incubated cyclized peptides as well as the unstapled control **1** with two common proteases, trypsin and proteinase K, and analyzed the digestion outcomes by HPLC/MS. We found that cyclized peptides were significantly more stable toward both proteases than the unstapled control (SI section 3.6). For example, after 1 h of incubation with trypsin only 13% of unstapled peptide **1** remained undigested, which is in stark contrast with 94% remaining for **1d**. Generally, we found that longer linkers provided better protection against digestion: peptides stapled with adipic dihydrazide linker were most stable toward proteolysis. Separately, we also investigated the chemical stability of macrocyclized peptides under different conditions. We found that peptides **1a**−**d** were completely stable to both acidic and basic conditions as well as to the action of reducing (TCEP, DTT) and oxidizing ($NaNO_2$) agents (SI section 3.5) after 24 h of incubation. Taken together, these data suggest that the tethering with unnatural linkers does not compromise the chemical stability of a peptide and, moreover, renders it more resistant to proteolysis.

To study the effects of macrocyclization on biological activity and conformational behavior of a peptide, we prepared peptide NH_2-ITF**X**DLL**X**YYGKKKK(Biotin)-$CONH_2$ (an NYAD-1 analogue, **4**), a known binder of the C-terminal domain of HIV-1 capsid assembly polyprotein (C-CA).[29] Earlier, this peptide was successfully stapled using olefin methathesis and cysteine perfluoroarylation approaches.[14,30,31] In both studies, researchers found that the peptide cyclization promoted α-helical conformation and improved binding to C-CA. As illustrated in Figure 4, we cyclized peptide **4** with linkers **a**−**d** under the standard conditions to obtain a total of four stapled NYAD-1 analogues, **4a**−**d**. To probe the influence of the stapling on the conformation of the peptides, we turned to far-UV CD spectroscopy (Figure 4a and SI section 2.8). Although all tested NYAD variants displayed at least some degree of α-helicity as evident by characteristic minima at 208 and 222 nm, the nature of the linker influenced the peptide conformation. Stapling with succinic dihydrazide increased the α-helical character of the peptide, from 11% for unstapled peptide **4** to 26% for **4c**. NYAD-1 analogue **4d** stapled with the longer and more flexible adipic dihydrazide linker also led to an increase in helicity of the peptide, albeit to a lesser extent: the α-helicity of **4d** was estimated at 19%. In stark contrast, cyclization with

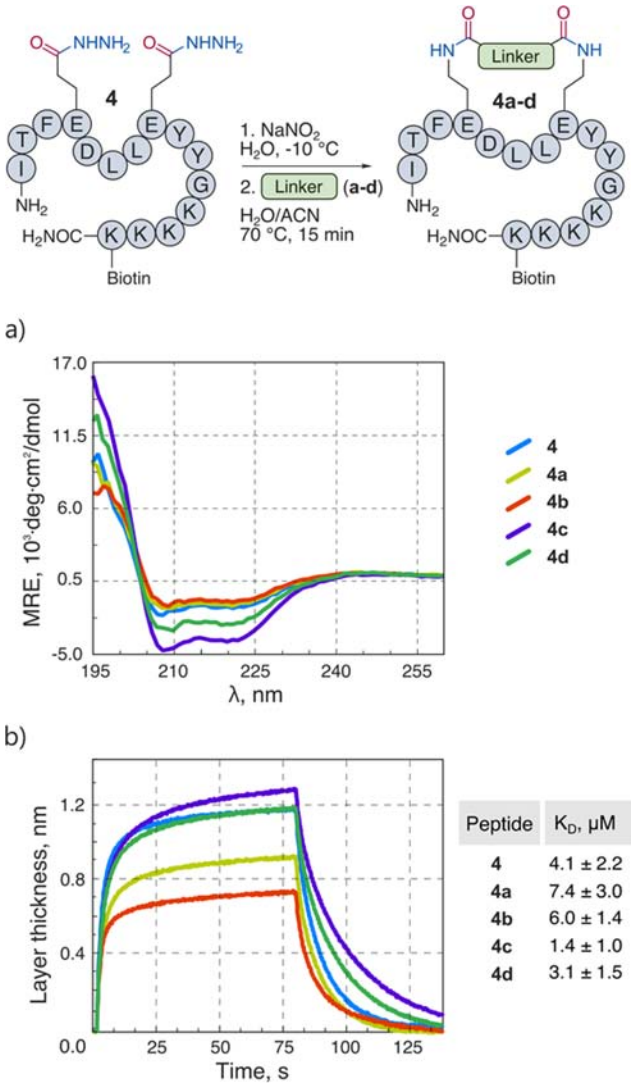

Figure 4. Effects of the isocyanate macrocyclization on the biological properties of NYAD-1 and its macrocyclized analogues. The peptide was oxidized with 20 mM $NaNO_2$ at −10 °C for 10 min, diluted into a 5 mM solution of the nucleophile in a water/acetonitrile (1:3, v/v) mixture, and incubated at 70 °C for 10 min to prepare the cyclic constructs **4a**−**d**. (a) CD spectra of peptides **4**, **4a**−**d**. (b) Bilayer interferometry binding sensograms of immobilized **4**, **4a**−**d** with 10 μM C-CA. K_D values in μM ± one standard deviation are displayed.

short and rigid linkers **a** and **b** resulted in practically no change in the CD spectrum as compared to peptide **4**. Consistent with previous studies,[20] these observations indicate that both linker length and rigidity are important considerations for increasing the α-helical content of a cyclized peptide.

Finally, we evaluated how isocyanate macrocyclization affects the binding affinity of peptide **4** toward C-CA by measuring binding kinetics using bilayer interferometry on an Octet RED96 system (SI section 2.9 and 3.7). Consistent with previous reports,[14,30] the binding affinity of NYAD-1 analogues directly correlated with the α-helicity of the peptides. As indicated in Figure 4b, we observed an approximately 3-fold improvement in the binding constant for the most helical analogue **4c** over the unstapled control **4** (K_D (**4c**) = 1.4 μM versus K_D (**4**) = 4.1 μM). At the same time, the least helical stapled peptides **4a** and **4b** bound C-CA less tightly than the unstapled peptide with K_D values of 7.4 and 6.0 μM

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respectively. Thus, the five NYAD-1 analogues demonstrated a range of biological properties, such as α-helicity and binding affinity based on the nature of the dihydrazide linker. These results indicate that it is possible to fine-tune biochemical properties of a peptide by modulating the nature of a dihydrazide linker used for cyclization.

In conclusion, we developed a unique approach to peptide macrocyclization, which relies on conjugating peptide isocyanates with commercially available dicarboxylic acid hydrazides. Fully unprotected peptide isocyanates are readily available via a Curtius rearrangement of corresponding acyl azides and react with bifunctional nucleophiles in aqueous solvents in a chemo- and regioselective fashion. Our method complements existing tools by providing a two-component macrocyclization platform that does not rely on cysteine modification or expensive customized amino acids, and provides an access to abiotic, hydrophilic semicarbazide-bearing constructs unobtainable by existing approaches. This methodology allows the construction of rings of variable size, rigidity, and topology, including side-chain to side-chain and side-chain to C-terminus cyclizations. Resulting stapled peptides proved to be chemically stable and showed improved proteolytic stability compared to their linear analogues. We also demonstrated that the biological activity of a peptide can be enhanced and fine-tuned using our approach. We believe that this macrocyclization platform will expand the design scope for developing novel biologically active macrocyclized peptides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b03626.

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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