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Late-Stage Sidechain-to-Backbone Macrocyclization of *N*-Amino Peptides

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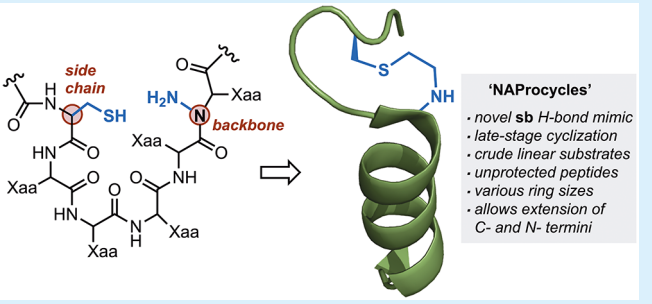
ABSTRACT: Cysteine-containing *N*-amino peptides undergo chemoselective reactions with haloaldehydes to afford ethylene-bridged cyclic peptides. This bis-alkylation strategy provides macrocycles harboring a novel covalent H-bond surrogate. Mimicry of a native sidechain-to-backbone (*sb*) H-bond is demonstrated in the context of a model loop-helix peptide. The described method is amenable to the synthesis of diverse ring sizes from crude unprotected linear substrates under aqueous conditions.



Macrocyclization is now a mainstay of peptidomimetic drug design owing to the conformational restriction, improved proteolytic stability, and often superior pharmacokinetic properties of cyclic peptides relative to their linear counterparts.[1] Peptide macrocycles can be designed to mimic the biologically active conformations of lead sequences, resulting in enhanced target binding and selectivity. Diversity-oriented cyclization strategies, where a common linear substrate gives rise to a library of peptide macrocycles, have also been widely employed in screening campaigns. Such compounds continue to find widespread utility as ligands for expansive and shallow protein surfaces that are difficult to target with small molecules.[2] New synthetic methods to introduce covalent tethers into polypeptides remain highly desirable.

While several synthetic approaches toward peptide macrocycles have been reported, the majority result in head-to-tail, sidechain-to-head or tail (lariat-type), or sidechain-to-sidechain modes of cyclization (Figure 1A).[3] Less attention has been paid to sidechain-to-backbone (*sb*) tethering, presumably due to challenges associated with installing reactive handles on internal amides within a chain.[3d,4] Our group was particularly interested in addressing this gap since *sb* H-bonds are commonly encountered in folded peptides and proteins.[5] These motifs feature the interaction between a backbone amide donor and a sidechain acceptor, typically from Asx, Glx, Ser, or Thr. We envisioned that replacing this H-bond with a covalent surrogate would result in a novel class of constrained peptide macrocycles (Figure 1B). Here, we report a versatile method to tether the Cys sidechain and the backbone amide substituent within *N*-amino peptides (NAPs). These macrocycles (termed "NAProcycles") can be formed from crude unprotected linear substrates and incorporate an internal *sb* peptide constraint, thus allowing the extension of both the C- and N-termini.

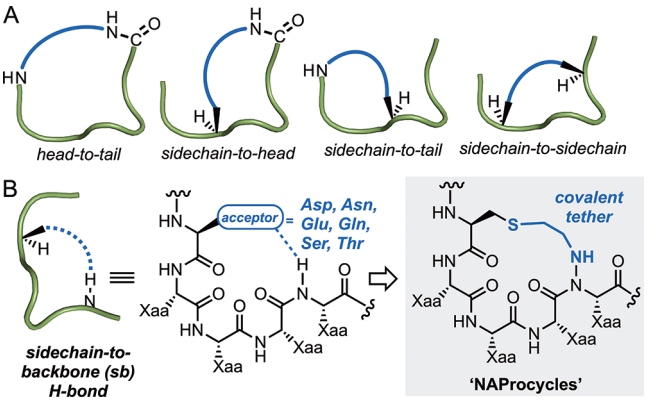


Figure 1. (A) Common modes of peptide macrocyclization. (B) Sidechain-to-backbone (*sb*) H-bonds within peptides and proteins and the structure of *N*-amino peptide macrocycles (NAProcycles).

We previously showed that backbone *N*-aminated peptides exhibit conformational characteristics distinct from their alkylated (peptide tertiary amide) counterparts. In contrast to *N*-methyl peptides, the hydrazide bonds in NAPs retain an almost native-like preference for the *trans*-amide geometry and can engage in intra-residue H-bonds that stabilize extended

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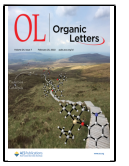

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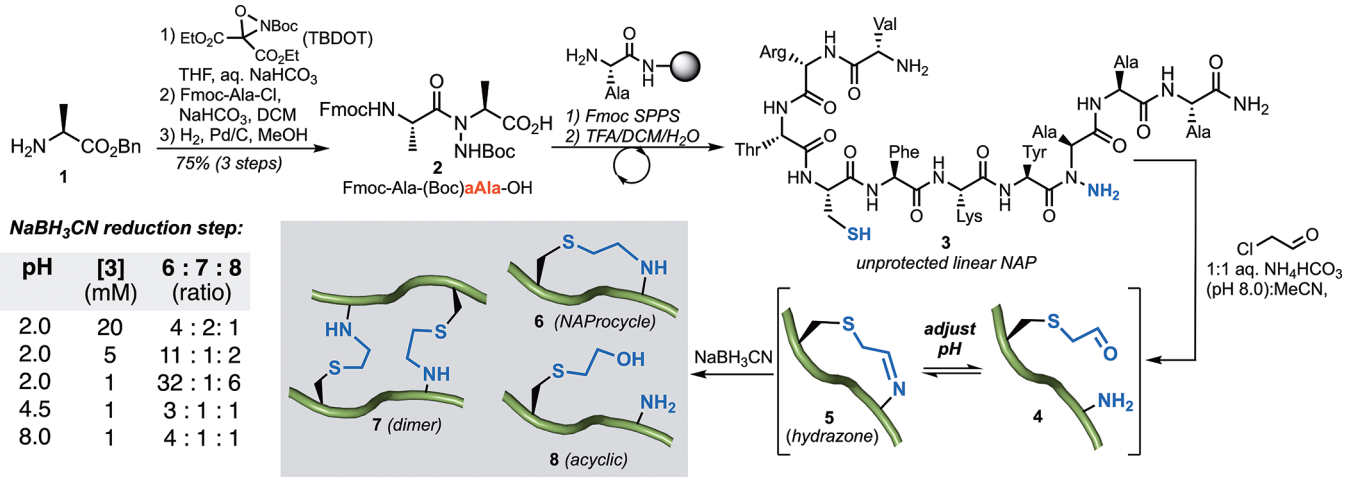
Scheme 1. Synthesis of Linear NAP 3 and Screening of Macrocyclization Conditions



NaBH$_3$CN reduction step:

pH	[3] (mM)	6 : 7 : 8 (ratio)
2.0	20	4 : 2: 1
2.0	5	11 : 1: 2
2.0	1	32 : 1: 6
4.5	1	3 : 1 : 1
8.0	1	4 : 1 : 1

conformations.[6] Moreover, *N*-amino dipeptide building blocks are readily incorporated into peptides by solid-phase peptide synthesis (SPPS) and are highly resistant to the racemization commonly observed during the condensation of *N*-alkylated peptide fragments.[7] Hydrazides have long been used as reactive handles in aqueous environments, enabling the chemoselective modification of peptides and proteins.[8] We thus sought to employ the reductive alkylation of the *N*-amino substituent in NAPs in conjunction with Cys sidechain alkylation[9] to provide a novel *sb* H-bond surrogate.

To screen for optimal macrocyclization conditions, we prepared a linear test substrate where the reactive Cys and *N*-aminoalanine (aAla) handles were spaced four residues apart. We also included nucleophilic sidechains in our sequence (Arg, Lys, and Tyr) in addition to a free N-terminus to establish functional group tolerance in the alkylation and reduction steps. The required N-aminated building block for peptide synthesis was prepared by reacting **1** with *t*-butyl-diethyl-oxaziridinetricarboxylate (TBDOT), followed by condensation with Fmoc-Ala-Cl and hydrogenolysis of the benzyl ester (Scheme 1).[10] Dipeptide derivative **2** was incorporated into the linear peptide by conventional Fmoc SPPS on Rink amide MHBA resin (HATU/NMM condensations). TFA-mediated cleavage and deprotection provided NAP **3**, which was purified by preparative RP-HPLC and aliquoted to screen the cyclization conditions.

Treating **3** with 2.5 equiv of chloroacetaldehyde in aq. NH_4HCO_3/MeCN (pH 8) resulted in its full conversion to the Cys-alkylated intermediate **4** within 1 h, as judged by LCMS. We then adjusted the reaction mixture to pH 2 using TFA, which resulted in the formation of cyclic hydrazone **5** (Figure 2). Excess solid $NaBH_3CN$ was added to the mixture to provide hydrazide **6** as the major product. When the cyclization was carried out at a 20 mM substrate concentration, we observed a significant amount of dimeric species **7** in addition to a minor amount of the acyclic alcohol **8**. Decreasing the peptide concentration to 1 mM provided the highest proportion of NAProcycle **6** relative to the byproducts. Attempts to carry out the reduction step at a higher pH (4.5 or 8.0) resulted in poor conversion and less favorable product ratios. No cyclization was observed when the linear substrate lacked an *N*-amino substituent (compound **S1**, Supporting Information), thus confirming that amine groups are not competent nucleophiles under the reaction conditions.

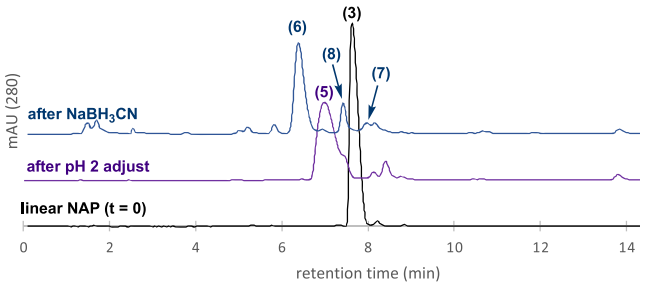


Figure 2. RP-HPLC spectra after hydrazone formation and reduction.

To evaluate the scope of macrocyclization with respect to ring size, we varied the position of the reactive Cys and aAla residues. We also carried out cyclizations using crude linear substrates to improve the overall efficiency. Figure 3 depicts NAProcycles prepared from crude peptides directly following cleavage from the resin. In addition to the 20-membered ring in compound **6**, we synthesized 17- and 14-membered NAProcycles (**9** and **10**, respectively) in good overall yields based on the initial resin loading. In contrast, 11-membered NAProcycle **11** was obtained in only trace amounts following repeated RP-HPLC purifications. In this case, we observed poor conversion to the cyclic hydrazone intermediate by LCMS following the adjustment to pH 2 (prior to reduction). The *S*-alkylated linear byproduct and macrocyclic dimer were major products, presumably due to the ring strain associated with the high content of sp^2-hybridized atoms within the 11-membered transition state. Peptide **12** was designed to mimic a *sb* H-bond in which the amide donor is N-terminal to the acceptor. This 16-membered NAProcycle was formed in a 19% overall yield based on resin loading. Finally, we reacted **3** with *p*-chloromethybenzaldehyde in place of chloroacetaldehyde to afford macrocycle **13**. Although the overall isolated yield was lower relative to that of **6**, this result demonstrates the ability to introduce linker diversity through variation of the bis-electrophile reagent.

A key feature of NAProcycles is the presence of a backbone H-bond surrogate within a peptide that extends further in both the C and N directions. This may be particularly useful for mimicking H-bonds that bridge secondary structures. In folded proteins, α-helix N-caps are prevalent motifs that often feature a sidechain H-bond acceptor situated N-terminal to the helix.[11]

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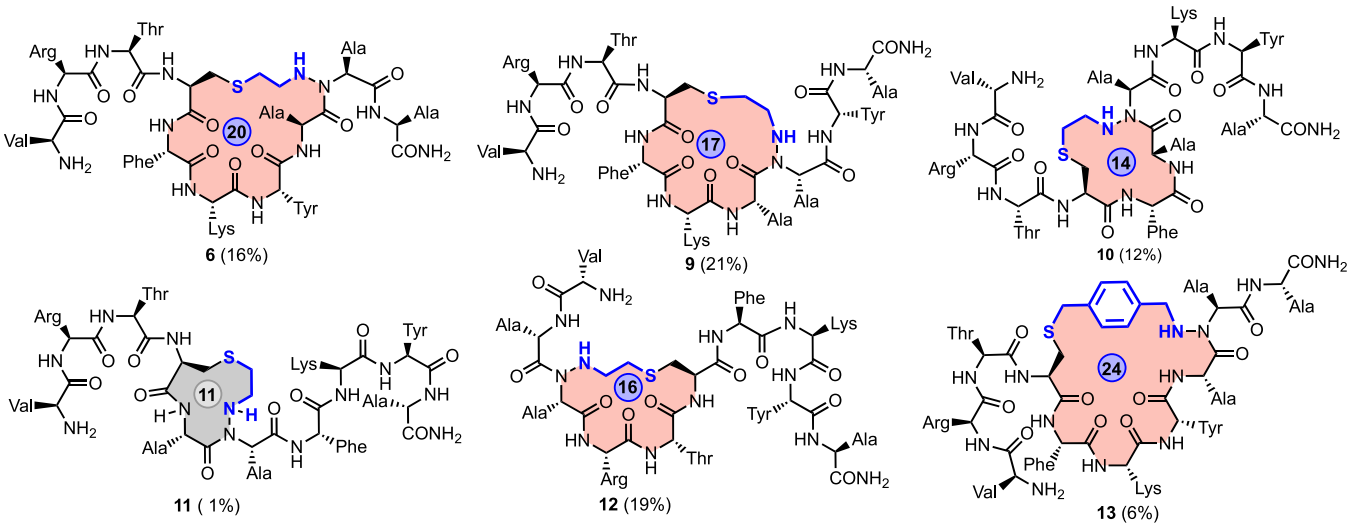


Figure 3. NAProcycles synthesized from crude linear substrates (with isolated overall yields following RP-HPLC purification).

This acceptor can interact with the otherwise "unsatisfied" amide donors at the start of the helix while also imposing a conformational constraint amenable to helix formation. Though most helix N-caps involve smaller H-bonded cyclic motifs (i.e., Asx or ST turns),[12] a cursory inspection of high-resolution protein structures in the Protein Data Bank (PDB) reveals several larger loop-helix *sb* macrocycles. We identified, for example, a 20-membered *sb* H-bond in the X-ray crystal structure of VP40, a viral matrix protein whose assembly plays a critical role in the budding of mature virions.[13] Here, a Gln sidechain engages the first amide NH of the α2 helix (Figure 4). We sought to demonstrate the utility of our approach by

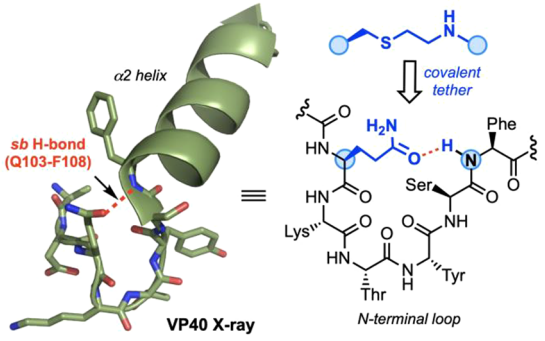
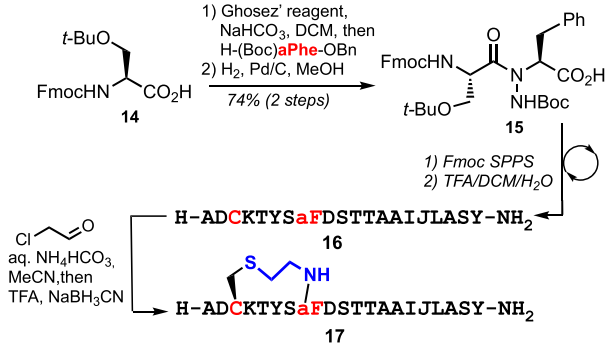


Figure 4. C20 *sb* H-bond in the α2 helix of VP40 (PDB 4LDB).

replacing the native Q103-F108 *sb* H-bond with a covalent tether. We thus designed a model peptide that includes five residues from the N-terminal loop of VP40 in addition to the α2 helix residues.

The Ser−aPhe (aPhe = *N*-aminophenylalanine) dipeptide building block (**15**) required for NAP synthesis was prepared by a route analogous to that used for **2** (Scheme 2). Fmoc-SPPS, cleavage, and deprotection provided linear aPhe-containing peptide **16**, which featured the additional Gln → Cys mutation required for cyclization. We also replaced a Met residue within the wt VP40 helix with norleucine to avoid the potential alkylation of the thioether sidechain. Crude NAP **16** was subjected to cyclization with chloroacetaldehyde under our optimized conditions to provide **17** following preparative RP-HPLC purification. We also prepared a series of control

Scheme 2. Synthesis of VP40-Based NAProcycle 17 and linear control sequences[a]



Control sequences	
18	H-ADQKTYSFDSTTAAIJLASY-NH_2
19	H-ADCKTYSFDSTTAAIJLASY-NH_2
20	H-ADQKTYSaFDSTTAAIJLASY-NH_2

[a]J = norleucine and aF = *N*-aminophenylalanine.

sequences for comparison. Peptide **18** represents the unmutated parent sequence, while peptides **19** and **20** feature a single Gln → Cys and Phe → aPhe mutation, respectively.

We used circular dichroism (CD) to assess the predominant secondary structure of **16**−**20** as well as the extent of folding at 75 μM in 1:1 H_2O/MeCN (peptides **18** and **19** gave turbid mixtures in pure water and required the addition of MeCN to dissolve) (Figure 5). Interestingly, parent peptide **18** exhibited a maximum molar ellipticity at 195 nm and a minimum at 217 nm, clearly indicating a β-sheet conformation. Although $VP40_{108-121}$ is helical in the crystal structure of the dimer, the addition of the N-terminal loop residues in our truncated model peptide appears to promote an alternate sheet-like structure. Peptide **19** also exhibited a β-sheet CD signature, albeit with a less pronounced minima, indicating that a single Gln → Cys mutation does not significantly impact the preferred fold. We observed a clear shift to an α-helix conformation in the case of NAP **20**. This dramatic effect suggests that the unsatisfied Phe amide donor in **18** is important for promoting a β-sheet-like fold. We have previously shown that N-amination disrupts canonical H-

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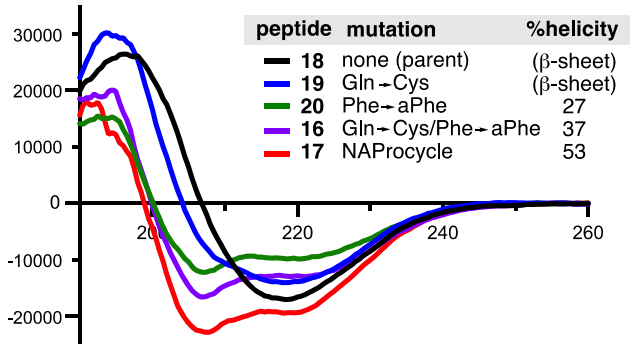

Figure 5. Circular dichroism spectra of **16**–**20** in 50% MeCN/H_2O.

bonding on the substituted edge,[6] likely precluding **20** from adopting such a conformation. We used the mean residue molar ellipticity at 222 nm[14] to determine that **20** was 27% helical in 1:1 H_2O/MeCN. The helicity increased to 37% in the case of linear NAP **16**, which is the precursor to cyclization and harbors both the Gln → Cys and Phe → aPhe mutations. NAProcycle **17** exhibited the strongest helicity (53%) among all the peptides examined. While *N*-amination was sufficient to induce a conformational switch from β-sheet to α-helix, we were gratified to find that NAProcycle tethering resulted in significant secondary structure stabilization relative to the linear analogues.

In summary, we have described a novel peptide macrocyclization approach based on covalent sidechain-to-backbone tethering. This ethylene bridging strategy leverages the reactivity of hydrazino acid and Cys residues within crude unprotected peptides to provide macrocycles of various ring sizes. Our initial studies suggest that additional macrocycle diversity can be realized through varying the haloaldehyde bis-electrophile. The ease of synthesis of linear NAP substrates renders this a particularly useful method for tethering to the peptide backbone. Studies employing NAProcycles as stabilized mimics of protein folds and bioactive peptides are currently underway in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.2c00204.

Detailed experimental procedures; characterization data for novel compounds; and copies of RP-HPLC, HRMS, and NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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