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N-Hydroxy peptides: Solid-phase synthesis and β-sheet propensity

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ABSTRACT

Peptide backbone amide substitution can dramatically alter the conformational and physiochemical properties of native sequences. Although uncommon relative to N-alkyl substituents, peptides harboring main-chain N-hydroxy groups exhibit unique conformational preferences and biological activities. Here, we describe a versatile method to prepare N-hydroxypeptides on solid support and evaluate the impact of backbone N-hydroxylation on secondary structure stability. Based on previous work demonstrating the β-sheet-stabilizing effect of α-hydrazino acids, we carried out an analogous study with N-hydroxy-α-amino acids using a model β-hairpin fold. In contrast to N-methyl substituents, backbone N-hydroxy groups are accommodated in the β-strand region of the hairpin without energetic penalty. An enhancement in β-hairpin stability was observed for a di-N-hydroxylated variant. Our results facilitate access to this class of peptide derivatives and inform the use of backbone N-hydroxylation as a tool in the design of constrained peptidomimetics.

INTRODUCTION

N-Hydroxy-α-amino acid residues are encountered in several natural metabolites and are biosynthetic precursors to structurally complex non-ribosomal peptides.[1] Several N-hydroxy peptide (NHP) natural products exhibit potent antibacterial properties, cancer cell cytotoxicity, or activity against endogenous

hormone receptors.[2] The introduction of a hydroxamate group into the peptide backbone also serves to enhance metal chelating properties,[3] stability toward some proteases,[4] and the potential for enhanced hydrogen-bonding interactions with target proteins. For these reasons, several designed peptidomimetics harboring N-hydroxy-α-amino acid residues have found utility as biologically active analogues of native peptides.[4-5]

Studies on the conformational impact of peptide N-hydroxylation or N-alkoxylation have been mostly limited to glycine derivatives.[6] Although these residues lack side chain substituents, their N-acyl derivatives were found to exhibit a strong preference for the *trans* amide conformation. The hydroxamate group can also engage in both intra- and intermolecular hydrogen bonding interactions to stabilize extended or turn geometries. Despite these initial reports, the effect of backbone amide hydroxylation on peptide secondary structure remains largely unexplored.[7] Conformational studies on NHPs featuring Cα-substituted N-hydroxy residues could provide design principles relevant to the stabilization of folded peptides. Although solution-phase syntheses of several Cα-substituted NHPs have been described,[3-4,5b,6b,7-8] access to wider array of diastereopure derivatives via solid-phase methods would enable further investigation.

We previously reported the solid-phase synthesis and conformational analysis of several N-amino peptides (NAPs) as novel β-strand mimics.[9] Amide N-amination restricts backbone dihedral angles through cooperative non-covalent interactions as shown in Figure 1. A key feature of NAPs is their ability to engage in an intraresidue (C6) H-bond analogous to that observed in conformationally extended peptides and β-sheets (C5).[10] Due to lone pair repulsion across the hydrazide bond, NAPs also avoid the significant *cis* amide rotamer propensity observed with N-alkylated peptides (peptoids, prolyl-containing peptides, and N-methyl peptides).[11] These factors result in stabilization of β-sheet-like conformation as determined

by mutational studies using β-hairpin model systems.[9] We envisioned that N-hydroxy-α-amino acid residues would exhibit similar or even enhanced β-sheet propensities given the greater electron density and hydrogen bond donor capacity of the OH group relative to NH_2. Here, we describe the solid-phase synthesis of diastereopure NHPs and examine the impact of N-hydroxy-α-amino acids on β-hairpin folding. Our results provide insights into the structure and stability of this underexplored class of N-heteroatom-substituted peptides.

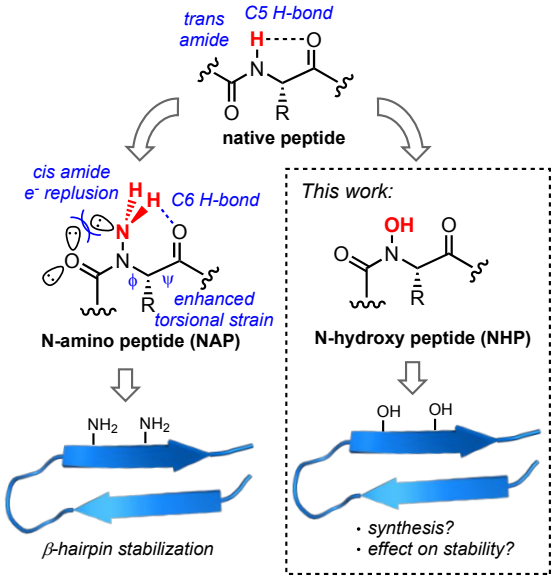
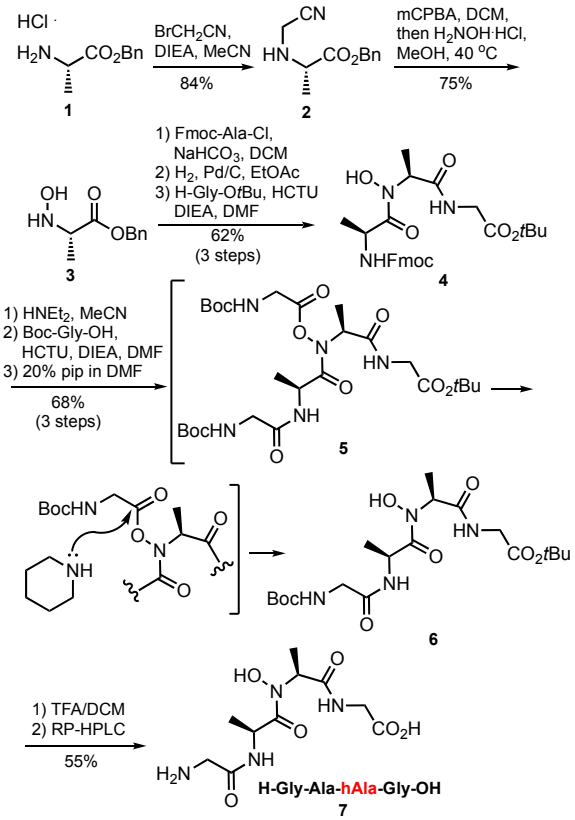


Figure 1. Structures and properties of N-heteroatom-substituted peptides.

RESULTS AND DISCUSSION

As shown in Figure 1, N-heteroatom substitution introduces electron repulsion that may partially counteract the *cis* amide rotamer propensity of peptide tertiary amides. To quantify the impact of backbone N-hydroxylation on *trans/cis* amide bias we employed a Gly-Ala-Xaa-Gly tetrapeptide model system originally developed by Raleigh and co-workers[12]. This peptide has previously been used to determine the rotamer preferences of proline, pipecolic acid, ε-oxapipecolic acid, and other cyclic residues in place of Xaa.[12-13] We adapted this sequence in order to directly compare a series of acyclic residues in the Xaa position, including those with N-heteroatom substituents. Following Fukuyama's

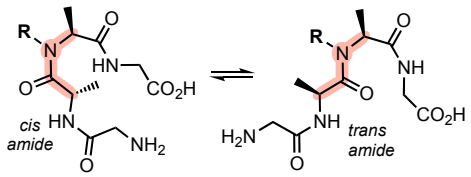
method,[14] we prepared the N-hydroxyalanine (hAla) variant in solution starting from *O*-benzyl-protected L-alanine **1** as shown in Scheme 1. N-alkylation was effected with bromoacetonitrile to give secondary amine **2**. Oxidation with *m*CPBA then provided the nitrone intermediate, which was subjected to aminolysis to give hAla benzyl ester **3**. Selective N-acylation was achieved in the presence of pre-formed Fmoc alanine acid chloride and $NaHCO_3$.[15] Elaboration of the dipeptide using standard protocols then provided tetrapeptide **7**. Notably, we observed diacylated derivative **5** as the major product during condensation with Boc-Gly-OH. However, deacylation of the backbone N-OH occurred upon treatment with a 20% solution of piperidine in DMF, providing tetrapeptide **6** in good yield.



Scheme 1. Solution-phase synthesis of model NHP **8**.

With NHP **7** in hand, we synthesized alanine, N-methylalanine, and N-aminoalanine variants for comparison by ^{1}H NMR (Figure 2). Analysis of the spectra taken in D_2O revealed that N-heteroatom

substituted analogues **7** and **8** exhibit significantly higher *trans* amide populations relative to N-methyl peptide **9**. While NAP **8** did exhibit a small but detectable population of *cis* rotamer (~6%), NHP **7** exclusively adopts a *trans* amide conformation in solution. These results are consistent with lone pair repulsion in the *cis* conformation and the greater electron density of O relative to N. As expected, control peptide **10** exhibits a *trans*-only conformation in D_2O.



peptide	R	$K_{trans/cis}$
7	OH	>99
8	NH_2	16
9	CH_3	2.8
10	H	>99

Figure 2. Amide *trans/cis* equilibrium constants for **7**-**10** determined by NMR (rt in D_2O).

In order to evaluate the β-strand propensity of N-hydroxy-α-amino acid residues, we utilized a β-hairpin model developed by Horne and co-workers for mutational analysis.[16] This 16-residue sequence is derived from the B1 domain of *Streptococcal* protein G (GB1, Figure 3) and exhibits moderate folding in aqueous media. Its folded population can be quantified by ^{1}H NMR on the basis of diastereotopic separation of the Gly_{10} Hα signals in comparison to cyclic (fully folded) and truncated (random coil) controls. We have also employed this model system to assess the impact of backbone N-amination on β-sheet stability.[9b] In that study, elongation of N-amino peptides was achieved by on-resin acylation of α-hydrazino acid residues using Fmoc-protected acid chlorides. Here, we chose to simplify the SPPS protocol by pre-forming the hydoxamate bond in solution and incorporating modified building blocks as dipeptide fragments. This approach allows us to avoid the use of labile acid chlorides and the generation

of insoluble salts during the automated phase of peptide synthesis. Because diacylated derivatives exemplified by **5** exhibit poor stability,[8e] we chose to circumvent over-acylation during SPPS through the use of *O*-protected hydroxamates. Our target building blocks were thus the protected N-hydroxy dipeptides shown in Figure 3, corresponding to two outer edge amide mutations within the model hairpin sequence.

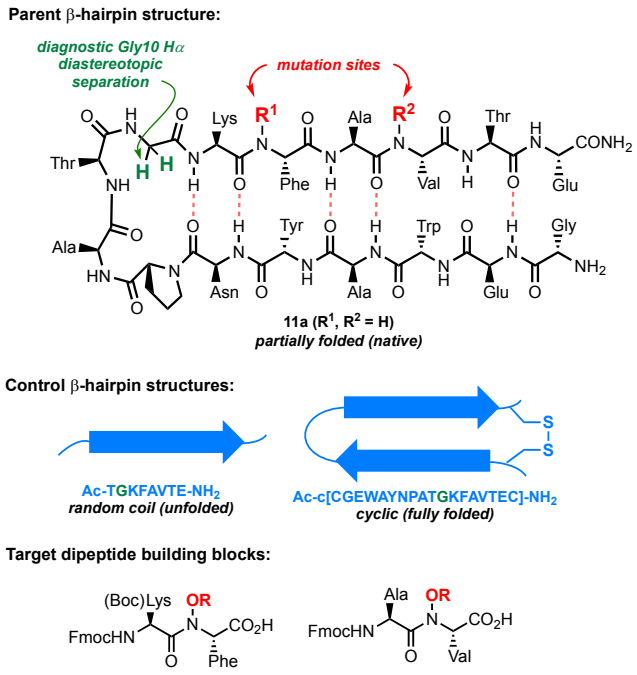
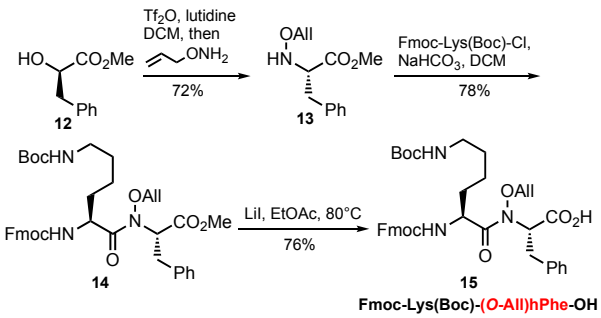


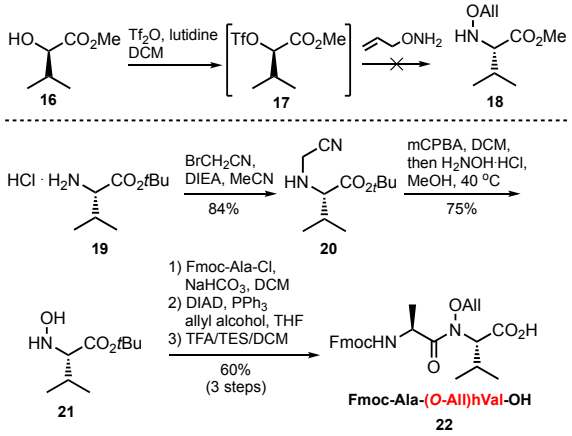
Figure 3. β-Hairpin model system and dipeptide building blocks for Fmoc-SPPS.

The required Lys-hPhe dipeptide derivative was prepared as shown in Scheme 2, starting from methyl D-phenyllactate. Activation and displacement of the triflate intermediate with allyloxamine proceeded smoothly to give **13**. Dipeptide bond formation was then achieved through reaction with Fmoc-Lys(Boc)-Cl, which was generated *in situ* using Ghosez' reagent. Lithium iodide-promoted deesterification then provided building block **15** in 76% yield.



Scheme 2. Synthesis of Lys-hPhe building block **15**.

Efforts to employ a similar S_N2 displacement strategy for the synthesis of the analogous Ala-hVal dipeptide were unsuccessful due to the additional steric bulk of the valine side chain (Scheme 3). As an alternative, we prepared N-hydroxyvaline *t*-butyl ester using the nitrone methodology described above (see Scheme 1). Condensation of **21** with Fmoc-Ala-Cl was followed by Mitsunobu reaction with allyl alcohol and final TFA deprotection to give **22** in 60% yield over three steps.



Scheme 3. Synthesis of Ala-hVal building block **22**.

Condensation of dipeptides or larger fragments can often lead to significant C-terminal epimerization. With access to N-allyloxy dipeptide acids **15** and **22**, we sought to ensure that stereochemical integrity would not be compromised under standard coupling conditions during SPPS. Compound **15** was thus

converted to (*S*)-α-methylbenzamide derivative **23** with HCTU and NMM at elevated temperature. A diastereomeric amide derivative differing in configuration at the (*O*-All)hPhe residue was synthesized for comparison by HPLC. Crude analytical traces revealed a > 99:1 d.r. for **23** (Figure 4). This level of diastereopurity demonstrates a lack of epimerization during activation of the dipeptide building block with HCTU/NMM. We attribute this configurational stability to suppressed oxazolonium formation in the case of N-alkoxy peptide fragments. Figure 5 depicts the mechanism of peptide fragment epimerization upon activation of the carboxyl group.[17] The N-allyloxy group is strongly electron withdrawing and presumably destabilizes the transition state leading to the labile oxazolonium intermediate.

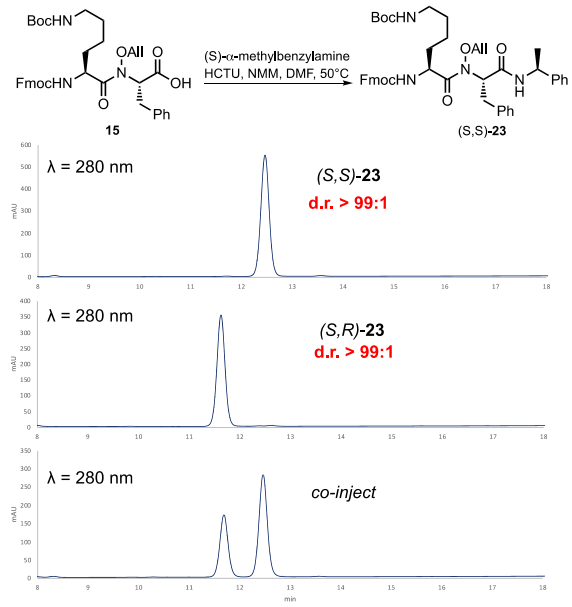


Figure 4. Determination of fragment epimerization during C-terminal condensation by RP-HPLC.

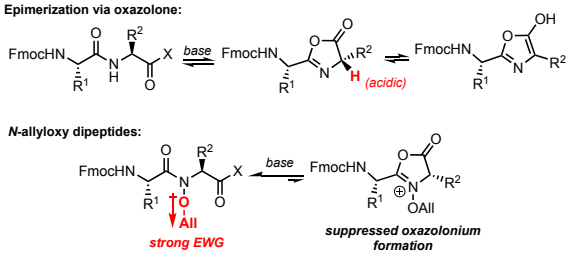
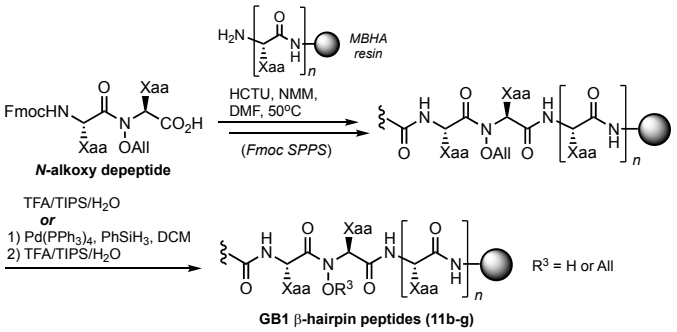


Figure 5. Mechanism and suppression of oxazolone/oxazolonium-mediated epimerization.

Assembly of peptides on solid support employed standard Fmoc chemistry using HCTU/NMM activation (Figure 6). Where appropriate, allyl deprotection with $Pd(Ph_3)_4$ was performed prior to global deprotection with TFA. Figure 6 lists the peptides synthesized for the current study in addition to previously prepared peptides for comparative analysis. All amide substitutions are positioned on the outer edge of the putative hairpin so as not to disrupt interstrand H-bonding interactions. Peptides **11b-c** are mono-N-hydroxylated peptides (mono-NHPs) substituted at either the Phe or Val residue mutations sites. Peptides **11d-e** retain the allyl protecting group to provide comparison between N-hydroxy and N-alkoxy substitutions. To assess the impact of multiple backbone substitutions, peptides **11f-g** were synthesized. Entries **11h-j** from our previous work[9b] are included here for comparison between N-hydroxy and N-amino substitution. Lastly, entries **11k-l** serve as a point of comparison between N-heteroatom and N-methyl substitutions.



peptide	R^1	R^2	sequence
11a	H	H	H-GEWAYNPATGKFAVTE-NH_2
11b	OH	H	H-GEWAYNPATGK-**hPhe**-AVTE-NH_2
11c	H	OH	H-GEWAYNPATGKFA-**hVal**-TE-NH_2
11d	OAll	H	H-GEWAYNPATGK-(***O*-All)hPhe**-AVTE-NH_2
11e	H	OAll	H-GEWAYNPATGKFA-(***O*-All)hVal**-TE-NH_2
11f	OH	OH	H-GEWAYNPATGK-**hPhe**-A-**hVal**-TE-NH_2
11g	OAll	OAll	H-GEWAYNPATGK-(***O*-All)hPhe**-A-(***O*-All)hVal**-TE-NH_2
11h	NH_2	H	H-GEWAYNPATGK-**aPhe**-AVTE-NH_2
11i	H	NH_2	H-GEWAYNPATGKFA-**aVal**-TE-NH_2
11j	NH_2	NH_2	H-GEWAYNPATGK-**aPhe**-A-**aVal**-TE-NH_2
11k	Me	H	H-GEWAYNPATGK-(***N*-Me)Phe**-AVTE-NH_2
11l	H	Me	H-GEWAYNPATGKFA-(***N*-Me)Val**-TE-NH_2

Figure 6. Solid-phase assembly of β-hairpin peptides and list of synthesized sequences.

Detailed NMR studies with **11b-11l** were carried in D_2O at 4 °C (1 mM peptide in 50 mM aq. $NaHPO_4$, pH 6.8). Full ^1H NMR assignments were made on the basis of TOCSY and ROESY spectra. Several inter-strand NOE correlations observed for N-hydroxy and N-alkoxy peptides **11b-11g** were in agreement with those observed in the fully folded cyclic peptide, indicating the presence of β-hairpin structures.[18] Quantitative assessment of folding was accomplished by calculating the Gly_{10} Hα chemical shift separation, which was then converted to fraction folded and ΔΔG relative to **11a** (Table 1). The folded fraction of hPhe derivative **11b** was found to be 86%, corresponding to a stabilization of -0.5 kcal/mol relative to **11a**. This increase in stability is comparable to NAPs **11h** and **11i**. N-hydroxylation at Val **11c** did not enhance hairpin stability, but was still tolerated without energetic penalty. Entry **11f** shows that multiple N-hydroxylations are well supported within the hairpin, but did not provide the same gain in stability as diamination (**11j**). This may be due in part to the negligible effect of Val N-hydroxylation relative to N-amination (**11c** vs. **11i**). The folded fraction of N-alkoxy derivatives **11d-e**, lacking the C6 H-bonding capability of the N-hydroxy mutants, were found to be only 67%, corresponding to a slight destabilization. This trend is also seen in the N-alkoxy double mutant **11g**. While backbone N-methylation in **11k** resulted in significant destabilization (+1.1 kcal/mol), a less pronounced effect was observed in the case of (*N*-Me)Val mutant **11l**. The Val_{14} residue appeared to be less sensitive to mutation in the case of the N-hydroxy, N-alkoxy, and N-methyl variants. Taken together, these results show that backbone N-hydroxylation is well tolerated in the strand region of a β-hairpin, and in some cases, provides enhancement of stability. Given that *O*-allyl variant **11g** exhibited only a single amide rotational isomer by NMR, it is likely that the loss of intraresidue H-bonds contributes to its lower folded population relative to di-NHP **11f**. As with N-amino peptides, a significant increase in folded population was observed for NHPs relative to their N-methylated analogues.

Table 1. Folding thermodynamics of NHPs and analogous peptides determined by NMR (D_2O, pH 6.8, 4 °C).

peptide	mutation	$\Delta\delta Gly_{10}$ (ppm)	% folded	ΔG_{fold} (kcal/mol)	$\Delta\Delta G_{fold}$ (kcal/mol)
11a	none	0.224	73	-0.5	
11b	$hPhe_{12}$	0.255	86	-1.0	-0.5
11c	$hVal_{14}$	0.221	72	-0.5	0.0
11d	(*O*-All)$hPhe_{12}$	0.208	67	-0.4	+0.1
11e	(*O*-All)$hVal_{14}$	0.209	67	-0.4	+0.1
11f	$hPhe_{12}$,$hVal_{14}$	0.260	88	-1.1	-0.6
11g	(*O*-All)$hPhe_{12}$,(*O*-All)$hVal_{14}$	0.203	65	-0.3	+0.2
11h	$aPhe_{12}$	0.261	88	-1.1	-0.6
11i	$aVal_{14}$	0.252	84	-0.9	-0.4
11j	$aPhe_{12}$,$aVal_{14}$	0.301	99[a]	-2.5[a]	-2.0
11k	(*N*-Me)Phe_{12}		24	+0.6	+1.1
	trans	0.096	37	+0.3	+0.8
	cis	0.000	0	0	
11l	(*N*-Me)Val_{14}		56	-0.1	+0.4
	trans	0.213	69	-0.4	+0.1
	cis	0.000	0	0	

[a]A value of 99% was used for calculations as the derived $\%_{fold}$ from $\Delta\delta Gly_{10}$ exceeded 100%.

Finally, it is worth noting that we observed reduced levels of crude purity for NHPs relative to their NAP counterparts. During mid-sequence LCMS analysis, we identified a mass fragment corresponding to loss of allyl alcohol. This prompted us to investigate the stability of N-allyloxy peptides towards basic conditions. Peptide **11d** was thus dissolved in a 20% piperidine/MeOH solution at 5 mM concentration and analyzed by HPLC (Figure 7). After 30 min exposure to base, a small amount of deallylated peptide was detected by LCMS. At 2 h, a prominent peak corresponding to the N-terminal fragment of imine hydrolysis was identified. Interestingly, we observed a series of peaks that featured the mass of the

imine species. We reasoned that these could represent the intact N-acyl imine as well as dehydrophenylalanine tautomers. These results suggest that N-alkoxy peptides are prone to base-promoted elimination across the Nα-Cα bond and that buffered piperidine solutions or lower reaction temperatures may improve the crude purity of NHPs prepared on solid support.

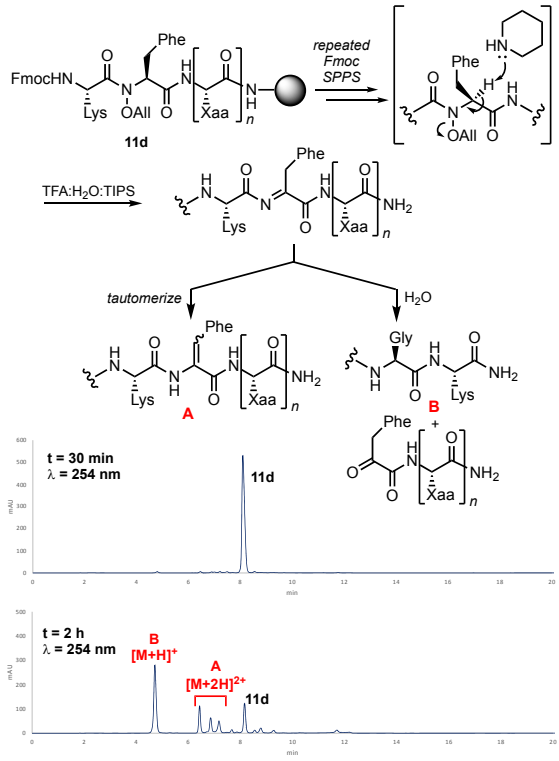


Figure 7. Proposed mechanism of base-mediated decomposition of **11d**.

CONCLUSIONS

In summary, we describe the synthesis of N-hydroxy dipeptide building blocks, their incorporation into NHPs, and initial assessment of β-sheet propensity by NMR. Furthermore, a tetrapeptide model was synthesized in solution and used to demonstrate that NHPs exhibit an unusually high *trans* amide propensity relative to N-amino- and N-methyl peptides. N-Hydroxy-α-amino acid residues were accommodated into the strand region of a β-hairpin fold without energetic penalty. In the case of **11b** and **11f**, mono- and di-N-hydroxylation was found to enhance β-hairpin stability. Capping of the OH

with an alkyl group compromised stability, suggesting that an intraresidue H-bond involving the hydroxamate may play an important role in structural preorganization. In addition to the conformational effects explored here, the current work provides a useful method to access various Cα-substituted NHPs by SPPS. We anticipate that these results will enable further structural and biological studies on this intriguing class of N-heteroatom-substituted peptides.

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CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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[18] See the Electronic Supplementary Information for details.

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