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Beyond N-Alkylation: Synthesis, Structure, and Function of N-Amino Peptides

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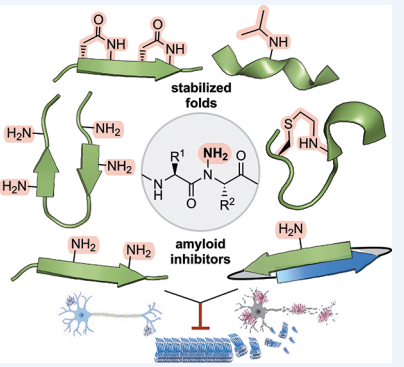
CONSPECTUS: The growing list of physiologically important protein−protein interactions (PPIs) has amplified the need for compounds to target topologically complex biomolecular surfaces. In contrast to small molecules, peptide and protein mimics can exhibit three-dimensional shape complementarity across a large area and thus have the potential to significantly expand the "druggable" proteome. Strategies to stabilize canonical protein secondary structures without sacrificing side-chain content are particularly useful in the design of peptide-based chemical probes and therapeutics.



Substitution of the backbone amide in peptides represents a subtle chemical modification with profound effects on conformation and stability. Studies focused on N-alkylation have already led to broad-ranging applications in peptidomimetic design. Inspired by nonribosomal peptide natural products harboring amide N-oxidations, we envisioned that main-chain hydrazide and hydroxamate bonds would impose distinct conformational preferences and offer unique opportunities for backbone diversification. This Account describes our exploration of peptide N-amination as a strategy for stabilizing canonical protein folds and for the structure-based design of soluble amyloid mimics.

We developed a general synthetic protocol to access N-amino peptides (NAPs) on solid support. In an effort to stabilize β-strand conformation, we designed stitched peptidomimetics featuring covalent tethering of the backbone N-amino substituent to the preceding residue side chain. Using a combination of NMR, X-ray crystallography, and molecular dynamics simulations, we discovered that backbone N-amination alone could significantly stabilize β-hairpin conformation in multiple models of folding. Our studies revealed that the amide NH_2 substituent in NAPs participates in cooperative noncovalent interactions that promote β-sheet secondary structure. In contrast to Cα-substituted α-hydrazino acids, we found that N-aminoglycine and its N′-alkylated derivatives instead stabilize polyproline II (PPII) conformation. The reactivity of hydrazides also allows for late-stage peptide macrocyclization, affording novel covalent surrogates of side-chain−backbone H-bonds.

The pronounced β-sheet propensity of Cα-substituted α-hydrazino acids prompted us to target amyloidogenic proteins using NAP-based β-strand mimics. Backbone N-amination was found to render aggregation-prone lead sequences soluble and resistant to proteolysis. Inhibitors of Aβ and tau identified through N-amino scanning blocked protein aggregation and the formation of mature fibrils *in vitro*. We further identified NAP-based single-strand and cross-β tau mimics capable of inhibiting the prion-like cellular seeding activity of recombinant and patient-derived tau fibrils.

Our studies establish backbone N-amination as a valuable addition to the peptido- and proteomimetic tool kit. α-Hydrazino acids show particular promise as minimalist β-strand mimics that retain side-chain information. Late-stage derivatization of hydrazides also provides facile entry into libraries of backbone-edited peptides. We anticipate that NAPs will thus find applications in the development of optimally constrained folds and modulators of PPIs.

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novel covalent surrogate of side-chain−backbone H-bonds that are prevalent in folded proteins. Tethering of a backbone hydrazide to the cysteine side chain afforded macrocyclic N-amino peptides of various sizes.

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- Rajewski, B. H.; Makwana, K. M.; Angera, I. J.; Geremia, D. K.; Zepeda, A. R.; Hallinan, G. I.; Vidal, R.; Ghetti, B.; Serrano, A. L.; Del Valle, J. R. β-Bracelets: Macrocyclic Cross-β Epitope Mimics Based on a Tau Conformational Strain. *J. Am. Chem. Soc.* **2023**, *145*, 23131−23142.[4] *This manuscript describes the design and synthesis of macrocyclic β-arch peptides that mimic the Alzheimer's disease fold of tau. N-Amination of a selected aggregation-prone macrocycle afforded potent inhibitors of tau aggregation and cellular propagation induced by patient-derived seeds.*

INTRODUCTION

Peptide-based chemical probes are well-suited to engage expansive biomolecular surfaces that are difficult to target with small molecules.[5,6] However, conformational flexibility imposes an entropic penalty on protein binding and underlies the poor proteolytic stability and cell impermeability often observed in linear peptides. Chemical modifications that stabilize peptide secondary structure in a predictable fashion have found broad utility in the design of PPI modulators. Side-chain constraint, macrocyclization, and incorporation of noncanonical residues are among the most widely used approaches for optimizing the physicochemical and pharmacological properties of parent sequences. Substitution of the backbone amide, alone or in combination with these approaches, is especially attractive since it maintains side chains important for biomolecular recognition.

Amide N-alkylation has dominated the landscape of backbone amide substitution as a peptidomimetic strategy.[7,8] Notable examples include peptoids,[9] peptide tertiary amides,[10] membrane-permeable and orally available N-methylated macrocycles,[11,12] and N-methylated proteomimetics.[13] Less attention has been devoted to backbone N-heteroatom substituents that differ significantly from carbon in electronegativity, size, and reactivity (Figure 1). Seminal contributions from the groups of Marraud,[14] Marshall,[15] Aleman,[16] and others[17−19] explored the conformation of short peptides featuring NH_2 or OH substituents on the backbone amide. Given the paucity of Cα-substituted N-hydroxy peptides (NHPs) and N-amino peptides (NAPs) reported in the literature, we set out to expand the repertoire of backbone modifications that are accessible by solid-phase synthesis to study their folding propensities.

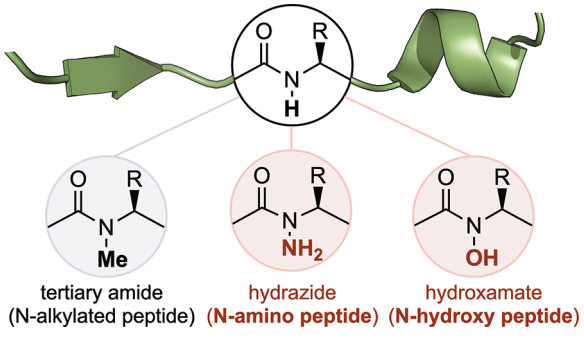

Figure 1. Backbone amide substitution in peptides and proteins.

This Account details our efforts toward the synthesis, conformational analysis, and biological application of N-amino peptides. Our inspiration was nonribosomal peptide natural products that feature main-chain hydrazide or hydroxamate bonds and exhibit unique structural properties.[20] Transferring these motifs into designed peptide mimics represents an exercise in diverted structure and function, whereby conformational lessons from nature are leveraged in new and varied biological applications. Peptidomimetic art often imitates life in this way, with notable examples including the use of α-quaternary amino acids (from peptaibols[21]) to enhance α-helicity and resistance to proteolysis;[22,23] γ-substituted prolines (from collagen[24]) to tune the conformation of proteins and drugs;[25,26] and the aforementioned N-methyl amino acids (from cyclosporin A[21]) to impart cell permeability and modulate receptor subtype specificity.[7,11] In a similar vein, we sought to utilize backbone N-oxidations found in nature for the design of stabilized secondary structures. From these studies, N-amination has emerged as a particularly versatile backbone modification to control form and function, thus expanding the tool kit of peptide and protein mimicry.

SYNTHESIS AND PROPERTIES OF N-HETEROATOM-SUBSTITUTED PEPTIDES

The widespread adoption of peptidomimetic strategies remains largely driven by synthetic accessibility. Advances in the biosynthesis of noncanonical peptides and solid-phase peptide synthesis (SPPS) methods have led to an ever-expanding list of well-characterized peptide modifications. Our first objective was to develop a convenient method to incorporate hydrazide and hydroxamate bonds into peptides on solid support. Earlier reports had described the solution-phase synthesis of some short N-amino and N-alkoxy peptides,[14,19] as well as submonomer-based assembly of aza- and oxypeptoids.[17,18,27,28] After exploring several methods, we opted for a dipeptide building block approach that would allow for the incorporation of multiple hydrazides or hydroxamates into the peptide backbone using automated Fmoc-based SPPS protocols.

To prepare the orthogonally protected dipeptide building blocks required for NAP synthesis, we employ an electrophilic amination strategy using the oxaziridine reagent (TBDOT) originally reported by Armstrong and co-workers.[29] Treatment of α-amino esters with TBDOT followed by Nα acylation and ester hydrogenolysis provides dipeptide carboxylic acids in good overall yields (Figure 2A).[30] Alternatively, N-hydroxy dipeptides for NHP synthesis can be accessed via mono-N-oxidation, followed by acylation and ester deprotection.[31−33] The resulting dipeptide acids can then be used in conventional SPPS, similar to any standard Fmoc-protected monomer. Relative to Fmoc-protected dipeptides lacking amide substitution, these building blocks exhibit unusual resistance to epimerization upon activation and incorporation onto a solid support. We attribute this to suppressed oxazolonium formation resulting from strong electronic withdrawal by the N-heteroatom substituent.[34] Both N-hydroxy and N-amino

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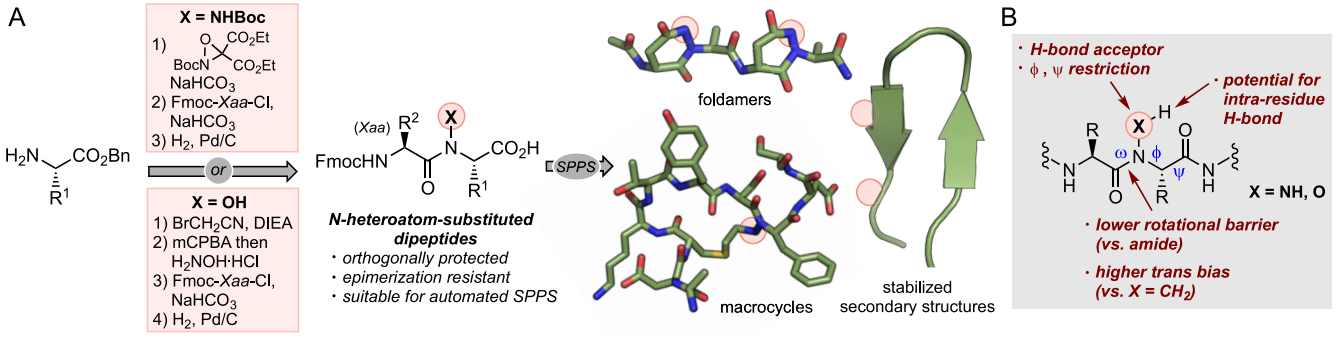

Figure 2. (A) Synthesis of N-amino and N-hydroxy dipeptide building blocks and incorporation into peptide and protein mimics. (B) Effects of peptide N-heteroatom substitution on local conformation.

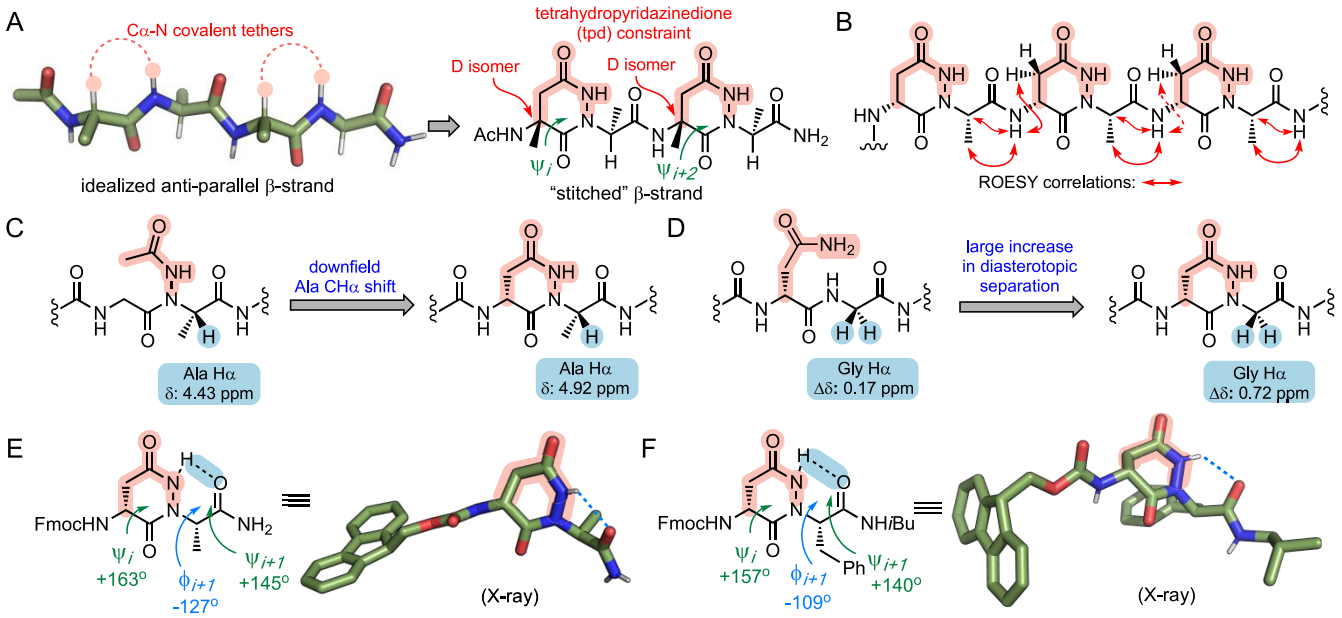

Figure 3. (A) Design of stitched β-strands featuring multiple tetrahydropyridazinedione (tpd) constraints. (B) Representative tritpd β-strand mimic featuring an extended conformation in solution. (C) Downfield Ala Hα NMR shift in a tpd peptidomimetic versus an electronically matched acyclic analogue. (D) High ^{1}H NMR Gly Hα diastereotopic separation in a tpd peptidomimetic versus an acyclic analogue. (E and F) X-ray crystal structures of tpd dipeptides exhibiting C6 hydrogen bonds.

dipeptide derivatives have been used to prepare a diverse array of NHP and NAP foldamers, macrocycles, and stabilized secondary structures on solid support.

The incorporation of hydrazide and hydroxamate bonds into the peptide backbone perturbs the local conformation in several important ways (Figure 2B). As observed in early studies on hydrazide-containing natural products, electrostatic repulsion between the N-heteroatom substituent and the preceding carbonyl O lone pair can strongly destabilize *cis* amide geometry.[35] As a result, acylated piperazic acid residues exhibit enhanced *trans* rotamer bias and lower rotational barriers relative to tertiary amides such as proline. This repulsive effect is maintained at physiological pH since the hydrazide N′ in NAPs is only weakly basic (conjugate acid pK_a ≈ 1−3).[1,36] The steric imposition of an amide substituent also increases ϕ and ψ rotational barriers and favors $A^{1,3}$ strain-minimized conformations. Hydroxamate OH and hydrazide N′H$_2$ groups may engage in H-bonds as donors or acceptors, thus leading to diverse and interchangeable modes of conformational restriction. Finally, the nucleophilicity of hydrazides provides unique opportunities for peptide constraint through covalent tethering. With these features in mind, we began an exploration of secondary structure mimics featuring hydrazide and hydroxamate bonds within the peptide backbone.

β-STRAND/SHEET STABILIZATION THROUGH PEPTIDE N-HETEROATOM SUBSTITUTION

Aberrant PPIs between β-sheet domains are implicated in several diseases, making them attractive targets for pharmacologic intervention.[37,38] While some of these interfaces involve multiple strands from each protein (β-sheet face interactions), many targets engage only a single strand of an interacting partner. This has motivated efforts toward β-strand mimics that do not require an auxiliary strand to stabilize an extended conformation.[39−47] Such designs may have the added benefit of reduced molecular weight and potentially enhanced membrane permeability relative to template-based approaches.

Stitched β-Strand Foldamers

To develop a minimal β-strand constraint, we initially explored tethering of the backbone amide N to the Cα of the preceding

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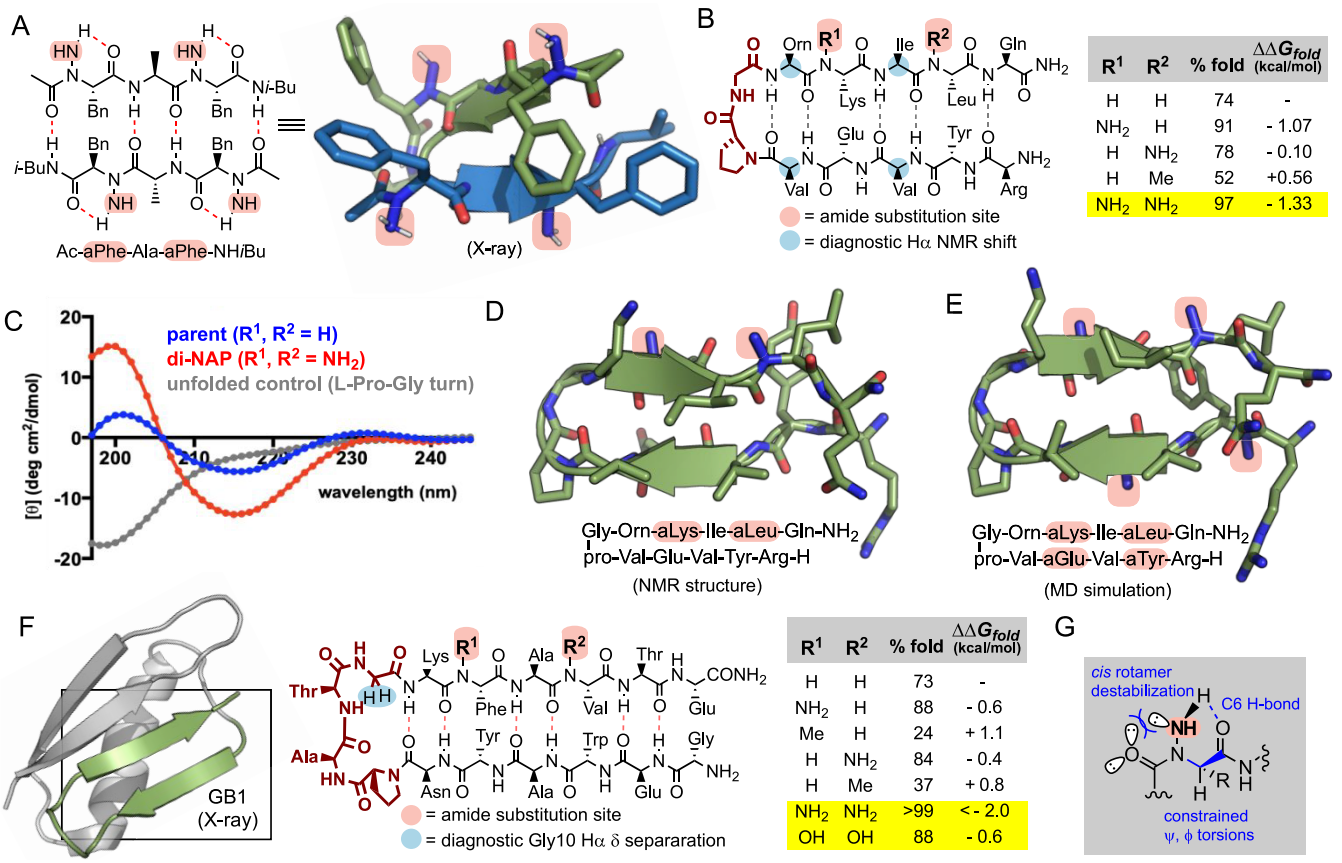

Figure 4. (A) X-ray crystal structure of a di-N-aminated tripeptide that forms an antiparallel β-sheet dimer. (B) Stabilization of a 12-residue β-hairpin model peptide by outer-edge N-amination. (C) Circular dichroic spectra of unfolded, partially folded, and di-N-aminated β-hairpin peptide analogues. (D) Structure of a di-N-aminated β-hairpin peptide determined by NOE distance-restrained MD. (E) Structure of a tetra-N-aminated β-hairpin peptide determined by MD. (F) Stabilization of a 16-residue antiparallel β-hairpin model derived from GB1 by outer-edge N-amination and N-hydroxylation. (G) Cooperative noncovalent interactions contributing to the β-sheet propensity of α-hydrazino acids.

residue. This design was partly inspired by the β-turn-inducing Freidinger lactam constraint, with the key distinction of engaging the side chain of a preceding residue in the D configuration.[48] As shown in Figure 3A, the (D)-tetrahydropyridazine-3,6-dione (tpd) motif effectively restricts the endocyclic ψ dihedral angle to the β-sheet region of Ramachandran space.

Early on, we had noted that the hydrazide N′H_2 in NAPs spontaneously cyclizes during cleavage from the solid support when positioned 5 or 6 atoms away from a side-chain carboxylic acid derivative. Tpd-constrained peptides were thus readily obtained by the cleavage, deprotection, and concomitant cyclization of N-aminated aspartyl dipeptides.[49] A series of "stitched" β-strand mimics featuring multiple tpd subunits exhibited ROESY $H\alpha_i \rightarrow NH_{i+1}$ and $H\beta_i \rightarrow NH_{i+1}$ correlations indicating an extended conformation in solution (Figure 3B).[50] In comparison to electronically matched controls, tetrapeptide mimics containing a single tpd ring exhibited downfield Hα NMR shifts characteristic of residues in a β-strand conformation (Figure 3C). Interestingly, the tpd ring imposes considerable constraint on the subsequent (acyclic) residue. This was supported by quantum mechanical calculations carried out on tpd model dipeptides.[51] In addition, we observed a significant decrease in Gly Hα diastereotopic separation when comparing a tpd-containing tetrapeptide to an acyclic control tetrapeptide (Figure 3D). These data suggested that a C6 H-bond between the hydrazide N′H and carbonyl O restricts the ψ and ϕ torsions of the acyclic residue. X-ray crystal diffraction of two Fmoc-protected tpd dipeptide amides revealed the presence of this H-bond as well as extended backbone dihedral angles similar to those encountered in β-sheets (Figure 3E,F).

β-Sheet Folds Stabilized by Backbone N-Amination and N-Hydroxylation

The apparent persistence of a rigidifying C6 H-bond in our tpd peptides raised an important question: could the same β-strand-stabilizing effect be realized through simple backbone N-amination, without the cyclic constraint? Previous computational studies carried out on N-acetylated α-hydrazino acids revealed an unusually high *trans* ω rotamer bias due to lone pair repulsion.[16] We thus expected that opening of the tpd ring would not incur a significant penalty with respect to the required ω geometry. Indeed, the diaminated tripeptide depicted in Figure 4A crystallized as an antiparallel β-sheet dimer harboring C6 bonds at each of the aPhe residues (Note: α-hydrazino acid residue one- and three-letter codes include an "a" prefix, i.e., Nα-aminophenylalanine = aF or aPhe).[1] These H-bonds also serve to shield the edge of the dimer, precluding further β-sheet-like association with other peptide strands.[1]

Our next step was to quantify the impact of N-amination on β-sheet stability. We introduced α-hydrazino acid residues into the strand regions of an antiparallel β-hairpin model developed by Gellman and co-workers (Figure 4B).[52] N-Amination of outer-edge amides in this system led to an increase in the

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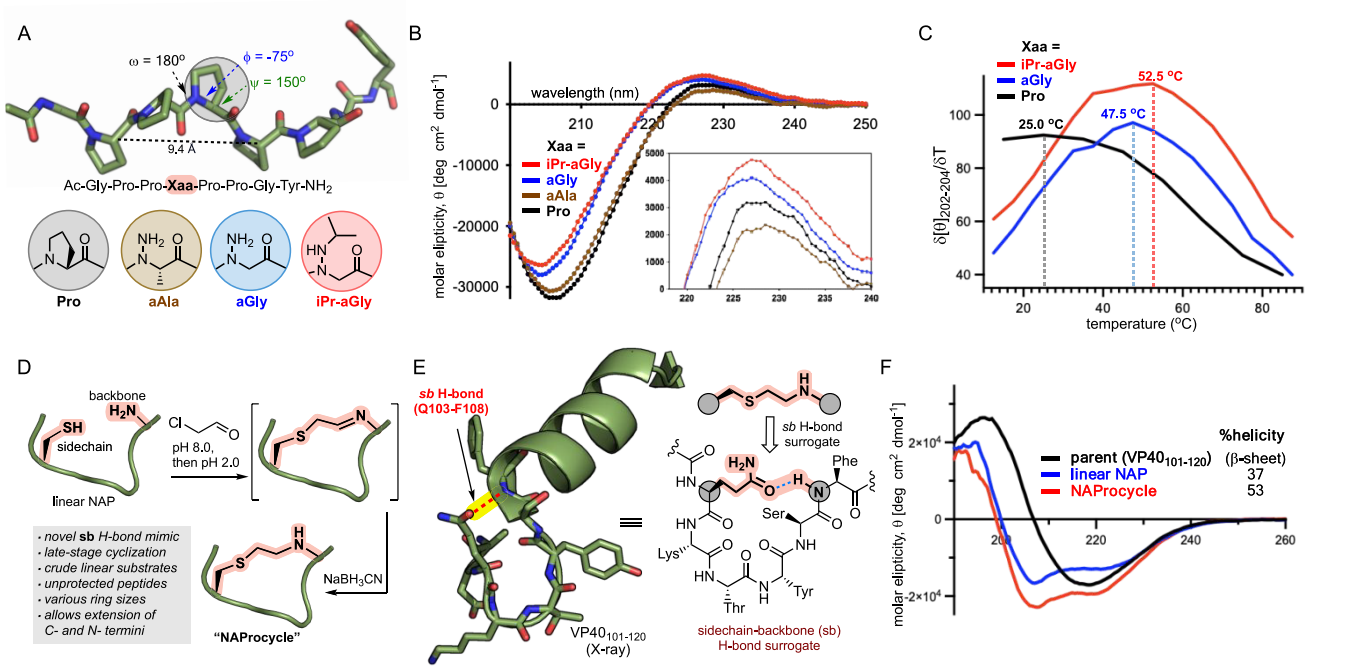

Figure 5. (A) Structural features of the PPII helix and sequences of PPII host–guest peptide analogues. (B) Both aGly and iPr-aGly enhance the PPII helicity of the model peptide relative to Pro, as monitored by CD. (C) iPr-aGly increases the thermal stability of the PPII helix relative to Pro and aGly. (D) Late-stage side-chain–backbone macrocyclization of NAPs. (E) Q103-F108 *sb* H-bond in the X-ray crystal structure of the VP40 dimer and design of a covalent H-bond surrogate. (F) Conformation of an NAProcyclic VP40 mimic, linear parent peptide, and linear N-aminated control, monitored by CD.

folded population relative to the unsubstituted parent peptide, as monitored by changes in diagnostic Hα NMR chemical shifts.[1] In the case of the aLys9/aLeu11 analogue, we observed a remarkable stabilizing effect from di-N-amination on a single strand. Circular dichroism (CD) and interstrand NOEs also indicated a stabilized β-hairpin fold relative to the parent sequence (Figure 4C). Dolenc and co-workers recently performed NOE distance-restrained molecular dynamics (MD) simulations on the same di-NAP β-hairpin as well as an analogue featuring four backbone N-amino groups placed on the outer edge of the peptide (Figure 4D,E).[53] These studies revealed that alternating α-hydrazino acids with canonical residues is particularly effective at stabilizing β-strand conformation, primarily through intraresidue C6 H-bonds.

We utilized a separate β-hairpin model peptide derived from the protein G immunoglobulin-binding domain B1 (GB1)[54] to further investigate the impact of backbone N-amination and N-hydroxylation on conformational stability (Figure 4F). The folded population of this 16-residue peptide can be conveniently quantified by measuring the diastereotopic ^{1}H NMR shift separation of Hα protons at the diagnostic Gly10 residue. Once again, outer-edge backbone N-amination in the strand regions led to a significant increase in folded population relative to unsubstituted or N-methylated control peptides.[51] Interestingly, N-hydroxylation also led to an increase in folded population, although its stabilizing effect was weaker than that of N-amination.[31] In the course of synthesizing the NHPs, we observed consistently lower yields due to base-promoted elimination of the hydroxamate OH. For this reason, we focused our subsequent efforts on the more synthetically accessible and chemically stable NAPs. The β-strand/sheet stabilizing effect of backbone N-amination can be attributed to a series of cooperative noncovalent interactions summarized in Figure 4G.

■ PEPTIDE N-AMINATION IN OTHER FOLDS

Stabilization of PPII Conformation by N-Aminoglycine Derivatives

We more recently turned our attention to the conformational propensity of N-aminoglycine (aGly), which lacks a substituent at Cα. For these studies, we focused on the polyproline II (PPII) helix, which accounts for nearly 10% of all protein structure and is the major ordered component of the collagen triple helix.[55] The PPII helix is defined by ψ and φ dihedral angles near +150° and −75° and is stabilized largely through $n_O \rightarrow \pi^*_{C=O}$ delocalization across sequential residues (Figure 5A).[56,57] While Pro amides exhibit increased *cis* rotamer propensity, PPII helices consist entirely of *trans* amide rotamers. Our previous work demonstrated that *cis* rotamer geometry is destabilized in NAPs due to electron repulsion.[1] Additionally, the electron-withdrawing hydrazide N′H$_2$ group lowers the amide rotational barrier, enhances carbonyl electrophilicity, and therefore lowers the $\pi^*_{C=O}$ LUMO energy.[58] Consistent with these characteristics, we found that the incorporation of aGly into a host–guest model peptide[59] enhanced PPII helicity and thermal stability relative to those of both Gly and Pro, as measured by CD (Figure 5B,C).[3] In contrast, the PPII fold was severely disrupted when the Cα-substituted aAla was incorporated as the guest residue.

We then performed late-stage reductive alkylation on the aGly PPII peptide to study the effect of hydrazide N′ substitution on folding. Out of a small set of derivatives, we identified N′-isopropyl-N-aminoglycine (iPr-aGly) to be a remarkably effective stabilizer of PPII secondary structure relative to both Pro and aGly (Figure 5B,C). Further

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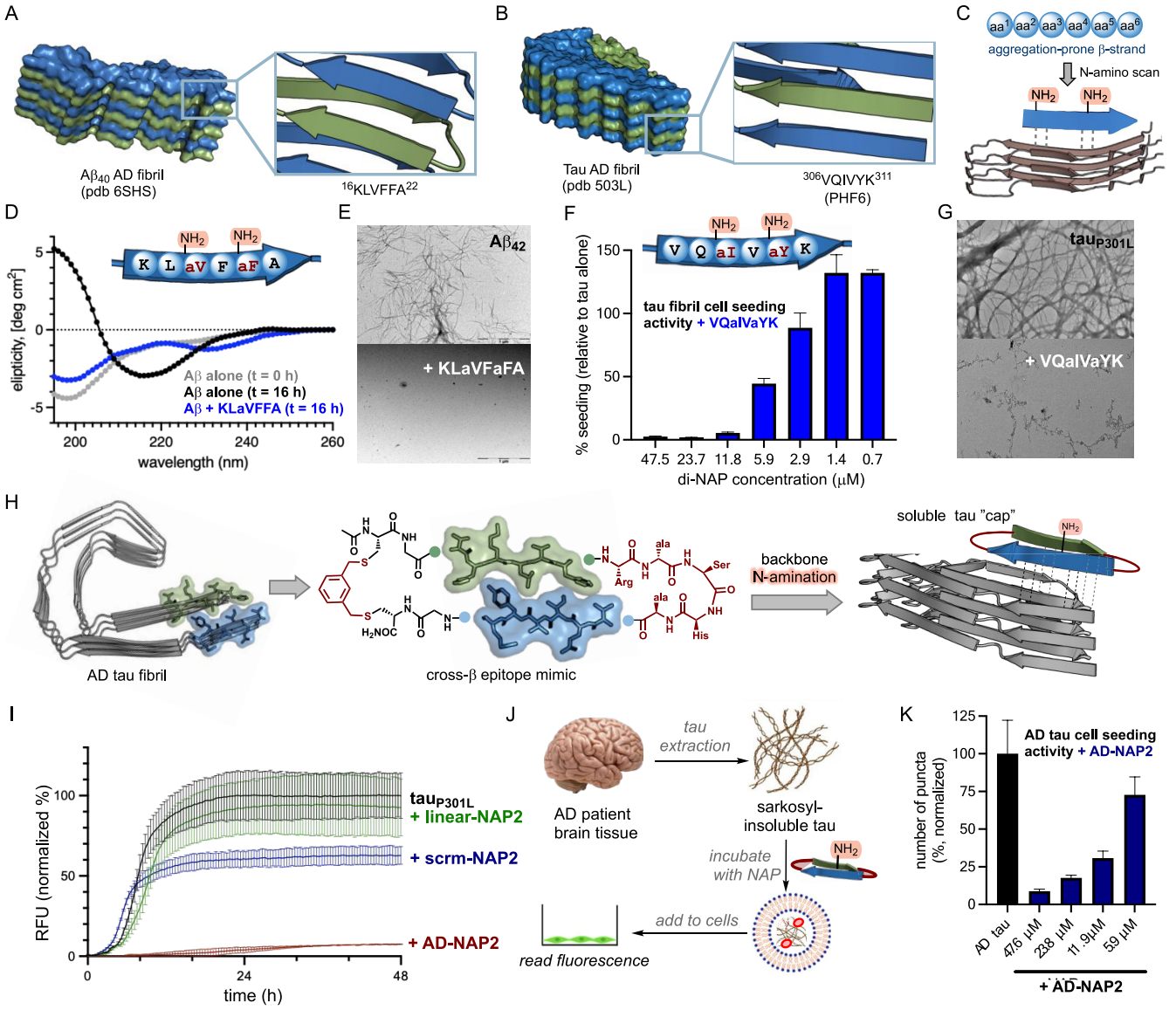

Figure 6. (A) Cryo-EM structure of Aβ_{40} fibrils extracted from AD patient tissue, with the aggregation-prone KLVFFA β-strand module highlighted in the zoomed box. (B) Cryo-EM structure of tau fibrils extracted from AD patient tissue, featuring the aggregation-prone VQIVYK β-strand module highlighted in the zoomed box. (C) Identification of amyloid ligands through N-amino scanning of aggregation-prone strands. (D) KLaVFaFA inhibits Aβ transition to the β-sheet structure, as monitored by CD. (E) KLaVFaFA inhibits the formation of Aβ fibrils by TEM. (F) VQaIVaYK inhibits the seeding activity of recombinant tau in biosensor cells. (G) VQaIVaYK inhibits the formation of mature tau fibrils by TEM. (H) Design of macrocyclic N-aminated cross-β epitope mimics based on the cryo-EM structure of AD tau. (I) An N-aminated cross-β tau mimic, AD-NAP2, potently inhibits tau aggregation in a sequence and macrocycle-dependent manner by ThT. (J) Extraction of AD tau from patient-derived tissue and evaluation of cellular seeding activity in the presence of inhibitors. (K) AD-NAP2 blocks the seeding capacity of patient-derived AD tau fibrils in a biosensor cell assay.

experiments revealed that, unlike aAla, aGly does not exhibit increased *trans* ω rotamer propensity relative to Pro. However, a peptide analogue featuring aGly C-terminal to the polyproline core showed a further increase in PPII helicity. This suggests that the PPII stabilizing effect of N-amination is largely attributable to the enhanced $\pi^*_{C=O}$ acceptor capacity of the hydrazide carbonyl. A similar enhancement of n → π^* delocalization in the collagen fold had been achieved through the incorporation of thioamides into the backbone.[60,61] The impact of N-amination on PPII stability is particularly notable given the low PPII propensity of Gly relative to Pro and several polar residues.[59]

Our work with aGly highlights the role of Cα substitution in destabilizing PPII conformation when used in conjunction with N-amination. This substituent combination within the same residue instead strongly favors β-sheet conformation. Enhancement of PPII stability upon aGly N′ substitution with an isopropyl group mirrors previous trends with bulky peptoid residues that stabilize the collagen triple helix.[62] Since Gly is more commonly encountered in turns and loops, we recently also examined the impact of aGly substitution within the type II′ β-turn of the hairpin model peptide in Figure 4B. We observed significant destabilization of the hairpin fold upon N-amination of the D-Pro-Gly motif, which was exacerbated further by N′-isopropyl substitution. Taken together, these studies underscore the ability of N-amination to dramatically alter or reverse the inherent conformational preferences of proteinogenic residues.

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Macrocyclic NAPs as H-Bond Surrogates

An important feature of NAPs is the potential to engage the pendant hydrazide $N'H_2$ group in chemoselective reactions at a late stage of the synthesis. As demonstrated above, a single aGly-containing substrate could be used to generate derivatives through reductive alkylation of the crude peptide. We leveraged this property to access a new class of macrocyclic peptides that are tethered to the backbone via the N-amino group. While several synthetic approaches have focused on peptide macrocyclizations involving side-chain−side-chain and head/tail−side-chain linkages, there are fewer examples of side-chain−backbone (*sb*) tethering.[63−68] Crude, unprotected Cys-containing NAPs were S-alkylated with chloroacetaldehyde and then subjected to intramolecular reductive alkylation at low pH to afford *sb* macrocycles (Figure 6D).[2] These reactions are tolerant of other reactive side chains within the peptide and can be used to generate diverse ring sizes.

The described mode of cyclization allows for mimicry of *sb* H-bonds, a common motif in folded peptides and proteins.[69] For example, ST and Asx turns feature H-bonds between the amide NH and Ser/Thr or Asp/Asx side-chain donors.[70] These capping motifs are prevalent at the termini of α-helices and often demarcate transitions to loops or other secondary structures within proteins. Larger H-bonded *sb* macrocycles are also widely represented in the Protein Data Bank (PDB).

To demonstrate a novel H-bond surrogate approach, we opted to covalently constrain a loop-helix motif observed in the X-ray crystal structure of the viral matrix protein VP40.[71] The VP40 dimer structure harbors an *sb* H-bond between Q103 (side-chain acceptor) and F108 (amide donor) that caps the α2 helix (Figure 5E). We synthesized a 20-residue peptide corresponding to the loop-helix sequence that featured both a Q103C substitution and N-amination at F108. Macrocyclization between C103 and the N-aminated F108 residue provided a covalently tethered peptide with a ring size matching that of the native *sb* macrocycle. The analysis of secondary structure by CD revealed that the covalent tether leads to increased helicity relative to a linear NAP control as well as the parent peptide (Figure 5F). Interestingly, the 20-residue parent peptide exhibited a β-sheet-like structure despite its α-helical conformation in the protein X-ray structure. N-Amination at F108 was sufficient to restore helical conformation, while *sb* covalent tethering further enhanced folding.

APPLICATIONS IN THE DESIGN OF AMYLOID AGGREGATION INHIBITORS

The β-sheet promoting effects of Cα-substituted α-hydrazino acids prompted us to utilize NAPs to inhibit the aggregation of amyloidogenic proteins. Deposits of fibrils rich in parallel β-sheet structure are correlated with several neurodegenerative diseases, and their assembly is particularly difficult to target selectively with small molecules.[72−74] Pathological amyloid growth is a complex multistep process often nucleated by the exposure of short aggregation-prone β-strand modules within each protomer.[75] The structure-based design of peptidic ligands based on these modules requires that the truncated lead segment be rendered aggregation-resistant without compromising protein affinity. Backbone N-amination was ideally suited to the design of soluble amyloid mimics since both side-chain faces are intact but only one H-bonding edge is preserved from the parent strand.

NAP Scanning of Amyloidogenic β-Strands

The hydrophobic hexapeptide sequences KLVFFA (residues 16−22) and VQIVYK (residues 306−311; PHF6) in β-amyloid (Aβ) and tau, respectively, are accepted as key drivers of aggregation (Figure 6A,B).[76,77] Pioneering work from the laboratories of Doig[78] and Meredith[79] demonstrated the β-breaker characteristics of N-methylated peptides based on KLVFFA in Aβ. A similar approach using Pro-substituted mimics of VQIVYK was employed by Segal and co-workers to target tau aggregation *in vitro*.[80] In a key study, Nowick and co-workers developed a series of peptidomimetic macrocycles that display amyloidogenic recognition strands and are precluded from self-aggregation by a templating strand harboring an unnatural tripeptide surrogate.[81] Inspired by these approaches, we carried out N-amino scans of aggregation-prone hexapeptides from Aβ and tau (Figure 6C). Analogues containing more than one N-amino group were limited to those modified on a single H-bonding edge to allow for a full complement of H-bonds along the protein recognition edge. In the Aβ mimetic series, we identified a di-N-aminated hexapeptide, KLaVFaFA, that blocked the transition of $A\beta_{42}$ from random coil to β-sheet conformation by CD (Figure 5D).[82] In agreement with these results, co-incubation of $A\beta_{42}$ with KLaVFaFA also significantly inhibited the aggregation of Aβ in a thioflavin-T (ThT) fluorescence assay and by transmission electron microscopy (TEM) (Figure 6E).

We then turned our attention to peptidomimetics targeting tau, a protein whose inclusions are more closely correlated to poor prognosis in neurodegenerative disease.[83] Two aggregation-prone β-strand modules within tau, PHF6 and PHF6*, served as lead sequences in a similar N-amino scan, affording 14 NAP-based tau mimics. A di-N-aminated derivative, VQaIVaYK, was found to reduce the aggregation of an isoform of human tau harboring a pathological P301L mutation in a ThT assay.[84] We then employed a HEK293 biosensor cell line that expresses a tau-yellow fluorescent protein fusion (tau-RD[LM]-YFP) to monitor the aggregation status of endogenous tau upon exposure to recombinanat tau_{P301L} fibril seeds. Incubation of VQaIVaYK with monomeric tau_{P301L} prior to cell treatment strongly inhibited the seeding activity of the protein (Figure 6F). We then confirmed that VQaIVaYK blocked the growth of mature tau fibrils by TEM (Figure 6G). Notably, VQaIVaYK could also reduce the cellular seeding activity of mature (preformed) tau_{P301L} fibrils, though higher concentrations of inhibitor were required to suppress endogenous tau aggregation in this case. These results demonstrate that NAP-based tau mimics are capable of blocking the transition of tau from a disordered to a fibrillar state and of inhibiting further templated growth by interacting with mature fibril seeds.

N-Aminated Cross-β Tau Epitope Mimics

Recent high-resolution cryo-EM structures of tau fibrils extracted from patient-derived tissue have revealed that conformationally distinct tau folds correlate with specific pathology.[85] An important consequence of these conformational "strains" is the face-to-face side-chain interactions between established aggregation-prone hexapeptides (PHF6 and PHF6*) and distinct cross-β modules, depending on the disease. We hypothesized that the mimicry of entire cross-β tau epitopes would enable the discovery of more effective aggregation inhibitors and potentially selective caps of pathological tau strains. To test this, we designed a macrocyclic

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mimic of the Alzheimer's disease (AD) tau strain featuring the PHF6 hexapeptide as well as its opposing AD cross-β sequence, THKLTF (Figure 6H). This highly aggregation-prone AD-derived macrocycle was then mono-N-aminated at two different sites within the PHF6 strand. The resulting soluble AD-NAPs were incapable of self-assembly and potently inhibited tau_{P301L} fibrilization in a dose-dependent manner by ThT and TEM. Sequence-scrambled macrocycles and acyclic control NAPs showed little to no inhibitory activity (Figure 6I). AD-NAPs also blocked the cellular seeding activity of recombinant tau_{P301L} at substoichiometric concentrations. Given that these mimics were based on the AD cross-β epitope, we tested whether they could inhibit the seeding activity of tau fibrils extracted from AD patient-derived tissue (Figure 6J). AD-NAPs were able to block seeding by AD tau fibrils in a dose-dependent manner (Figure 6K).

In addition to their antiseeding activity, notable features of our NAP-based tau mimics include excellent solubility, rigidified solution conformations, high proteolytic stability, and an observed lack of cell toxicity.[84,4] Even in analogues devoid of activity against the target protein, we found that a single amide-to-hydrazide replacement was sufficient to render the amyloidogenic parent hexapeptides soluble.[84] In addition, our di-NAP tau strand mimic, VQaIVaYK, showed no effect on the *in vitro* aggregation of $A\beta_{42}$. Beyond the β-strand substantiating effects of N-amination, these features pave the way for the design of ligands that can discriminate between structurally related amyloids or even conformational strains of the same protein.

CONCLUSIONS

Substitution of backbone amides in peptides can dramatically alter conformation while preserving side-chain functionality. N-Alkylation has thus found widespread use in the design of new peptidomimetics, foldamers, and peptide-based therapeutics. Despite the potential for even more pronounced effects on folding and stability, the study of N-aminated and N-hydroxylated peptides lagged far behind peptide tertiary amides. To address this gap, we developed robust protocols for the Fmoc-based SPPS of NAPs and NHPs and embarked on a systematic study of the conformational effects of peptide N-amination.

A predominant characteristic of Cα-substituted α-hydrazino acids is their high propensity for β-sheet conformations. N-Amination along a backbone edge also precludes the formation of insoluble aggregates that typically result from β-strand self-assembly. Amide-to-hydrazide replacement affords soluble β-strands that differ from a parent sequence by only two atoms. These characteristics combine to make N-amination a truly minimalist approach toward β-strand/sheet mimics. Here, we highlight N-amino scanning in the discovery of proteolytically stable inhibitors of amyloid aggregation and propagation. However, one can easily envision a similar strategy to indentify inhibitors of heterotypic β-sheet PPIs.

Hydrazides are remarkably versatile peptide bond surrogates. Their nucleophilicity toward aldehydes and ketones at low pH allows for chemoselective alkylation or macrocyclic tethering of unprotected NAPs. In addition to conformational tuning, late-stage diversification enables the rapid generation of backbone-modified peptide libraries for biological screening. The reduced amidicity of hydrazides also translates into a lowering of the C−N (ω) rotational barrier, suggesting that NAPs may be useful tools for studying and modulating the kinetics of slow-folding peptides and proteins. Finally, the ability of the hydrazide $N'H_2$ to engage as a donor in stable intraresidue or $i \rightarrow i-1$ H-bonds as well as in polar interactions with a protein target further distinguishes it from the N-alkyl substituent in peptide tertiary amides. Chameleonic intramolecular H-bonding in NAPs is of particular interest given the role that desolvation plays in the membrane permeability of peptides. We expect that further investigation along these and related lines will help to realize the considerable potential of NAPs as modulators of biomolecular interactions.

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Notes

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