Structure-Property Relationship Study of *N*-(Hydroxy)Peptides for the Design of Self-Assembled Parallel β -Sheets.

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Supporting Information Placeholder

ABSTRACT: The design of novel and functional biomimetic foldamers remains a major challenge in creating mimics of native protein structures. Herein, we report the stabilization of a short β -sheet by incorporating *N*-(hydroxy)glycine (Hyg) residues into the backbone of peptides. These short peptide-peptoid hybrids form unique parallel β -sheet structures by self-assembly. Spectroscopic and crystallographic data collected suggest that the local conformational perturbations induced by *N*-(hydroxy)amides are outweighed by a network of strong interstrand hydrogen-bonds.

In recent years, much interest has been devoted to the development of peptoids (*N*-substituted glycines)¹ due to their proteolytic stability and the ease to introduce various side chains without incorporating the asymmetric α -stereocenters typically found in peptides.² A major drawback in controlling these peptoid secondary structures is the lack of hydrogen bonding motifs and the high backbone flexibility of the central glycine methylene. Interestingly, one subclass of peptoid blocks, namely *N*-hydroxy- α -amino acids can be found in numerous bioactive natural products such as aurantimycins, polyoxypeptins, dentigerumycin and penicisulfuranols A–F,³ and other bacterial siderophores.⁴ Despite an intriguing potential arising from the secondary hydroxamate functional group as hydrogen-bond donor/acceptor and as metal chelator,^{5,6} the notorious lack of stability of this motif (pH-dependent decarboxylation and possible dismutation) has hampered most synthetic studies.⁷ Seemingly, only sparse examples of synthetic and structural studies have been reported on the propensity of *N*-(hydroxy)glycine (Hyg) to induce secondary interactions like β -turn⁸ and sheet-like structures (e.g. **1**, Figure 1).⁹ Given the enhanced acidity of *N*-(hydroxy)amides in comparison to typical amide groups,¹⁰ we became interested in testing their hydrogen-bond donor/acceptor potential into *N*-(hydroxy)peptoid-peptide hybrids to study their abilities to generate β -sheet tertiary structures (e.g. **2**, Figure 1). *N*-(hydroxy)⁹ and *N*-(alkoxy)peptides¹¹ are known to adopt a *trans*-conformation preferentially, which is also appropriate to the design of novel β -sheets. Herein, we report the stabilizing effect of Hyg blocks inside a unique intra/inter-molecular hydrogen-bond network for the self-assembly of particularly short β -sheet backbones (Figure 1). This work provides a foundation to understand and rationalize the role of *N*-(hydroxy)amides as hydrogen-bond donor/acceptor motifs in peptide-peptoid hybrids.

The synthesis of *N*-hydroxy- α -amino acids in both racemic¹² and asymmetric¹³ manners have been previously reported; However the insertion of these peptoid blocks into peptides has been fairly scarce.¹⁴ In order to develop a robust synthesis of *N*-(hydroxy)peptoid-peptide hybrids, a series of *N*-(benzyloxy)glycine derivatives **3–6** was prepared with typical protecting groups for liquid- or solid-phase peptide synthesis (Figure 1). For the initial study of *N*-terminal elongation, dipeptide-peptoids **7a–c** were synthesized starting from *N*-(OBn)-Gly-OEt **6a** which was coupled with three different Fmoc-protected amino acids (Scheme 1). As for *N*-alkyl peptoid blocks, *N*-alkoxy residues are known to have a low reactivity for amide

Figure 1. Proposed novel β -sheet scaffold built by intercalation of Hyg blocks (left). Peptoid blocks synthesized in this study (right)

● Parallel β -sheet peptoids containing Hyg reported by Blackwell in 2011

● This work: Intercalation of Hyg blocks with α -amino acids for a study of H-bond network.

89% yield

4, Pg = Ac (83% yield)

5, Pg = Fmoc (69% yield)

6a, R = Bn (79% yield)

6b, R = H (72% yield)

bond formation, but a cocktail of HATU/HOAt reagents was found suitable to the synthesis of dipeptide-peptoids **7a-c** in 70-87% yields. Compounds **7a-c** were then engaged in a sequence of deprotection/coupling towards the tripeptide-peptoids **9a-c**. Unfortunately, under the large set of conditions tested,¹⁵ *N*-terminal free dipeptide **8** proved difficult to isolate and the coupling towards **9a-c** failed. Instead, large amounts of degradation

Scheme 1. *N*-terminal elongation toward tripeptide-peptoid **9a-c**

Reaction conditions: **a.** Fmoc-AA-OH (4.0 eq.), HATU (4.0 eq.), HOAt (4.4 eq.), DIEA (4.0 eq.) in CH₂Cl₂/DMF (3:1); **b.** Piperidine or NHEt₂; **c.** **3** (4.0 eq.), HATU (4.0 eq.), HOAt (4.4 eq.), DIEA (4.0 eq.) in CH₂Cl₂.

were observed along with the intramolecular cyclization into diketopiperazines (DKPs) **10a-c**. Although Hyg favors *trans* amide bonds thermodynamically, the low transition-state energy barrier previously reported for the *cis-trans* isomerization of this motif (~16 Kcal/mol)^{7b} can explain the ease of forming DKPs. While *N*-(benzyloxy)peptoid oligomers are known to be exclusively in a most stable *trans* amide conformation,¹¹ the fast kinetics of *cis-trans* isomerization do not allow the *cis* rotamer to be observed on the NMR time scale. To obtain a direct evidence of the genuine *cis-trans* isomerization, we thought to force the equilibrium toward the *cis* rotamer by exploit the chelating nature of the Hyg residue. Dipeptide-peptoid **7c** was therefore hydrogenated to the corresponding Fmoc-Ile-Hyg-OEt dipeptide which was used as chelating ligand to form a gallium(III) complex (3:1 ligand/metal ratio).¹⁶ In this complex, the strong NOESY (nuclear Overhauser effect spectroscopy) ¹H-¹H cross-peak signal between the Ile H_α and the Hyg methylene detected by NMR, supports the presence of a *cis* *N*-(hydroxy)amide rotamer. Taken together, this spectroscopic evidence of a *cis* rotamer and the facile DKP formation (**8** → **10**) further support that *N*-(hydroxy) and *N*-(benzyloxy)amides undergo fast *cis-trans* isomerization.

To circumvent the formation of DKP **10**, we turned our attention to a strategy of *C*-terminal elongation (Scheme 2). Dipeptides **11a-c** were synthesized by coupling the acetyl Hyg **4** with three different *t*butyl α-amino esters by activation with EDCi or HATU. When similar conditions were tested to couple Fmoc-Hyg **5** with Ile-O*t*Bu, an unexpected cleavage of the benzyl protecting group occurred. The coupling was therefore optimized with EEDQ to deliver dipeptide **11d** without epimerization.¹⁷ A smooth deprotection with TFA lead quantitatively to all the corresponding free carboxylic acid of dipeptides **11a-d** which were directly coupled with **6a** in presence of HATU/HOAt to deliver tripeptide-peptoids **9a-d**. Unfortunately, **9c** was highly epimerized at the Ile α-stereocenter (2:3 dr). To improve the pivotal amide-bond formation, the coupling reaction was further evaluated with the unprotected block **6b**. In this case, the epimerization of tripeptide-peptoids **9c'** could not be detected by ¹H

NMR, but the racemization was later confirmed in the crystal structure of **12c** (Figure 2). *N*-(hydroxy) and *N*-(benzyloxy)glycine blocks are less reactive than typical *N*-methylated amino acids which are already known to be poor nucleophiles in amide bond formation. Due to the oxygen electronegativity and the steric hindrance carried by the hydroxy- and benzyloxy-side chains during the approach to the activated carbonyl π* orbital, such peptoid ligation is particularly slow and difficult, leading ultimately to potential epimerization. To address this issue, several activating cocktails were evaluated for the difficult coupling of **6a**. To our delight, tripeptide-peptoid **9c** was prepared with ~17% of epimerization by using EEDQ as activating agent. Given the electron-withdrawing nature of the *N*-(benzyloxy) side chain, we hypothesized that the *N*-terminal acetyl of **11c** might be unusually basic and participates into the Ile epimerization (see Figure SI-1). Thus by switching to a Fmoc-protecting group in **11d**, the EEDQ-coupling was achieved successfully to produce **9d** in 21% yield with minimal epimerization (~3%).^{15,17} To circumvent the low yield of this coupling, further optimizations were required. A cocktail of pivaloyl chloride with a hindered base (lutidine) provided tripeptide-peptoid hybrid **11d** in 50% yield while maintaining low epimerization (< 5%).¹⁵ Finally, benzyl groups from all the synthetic tripeptide-peptoids **9a-d**, **9c'** were removed under typical hydrogenation conditions to afford **12a-d** in 79% to 99% yields.

Scheme 2. *C*-terminal elongation toward tripeptide-peptoid **12a-d**

Reaction conditions: **a.** from **4** (1.0 eq.), DIPEA (1.5 eq.), for **11a-b**: EDCi (1.2 eq.), HOBt (1.2 eq.) in CH₂Cl₂, for **11c**: H-AA-O*t*Bu (2.0 eq.), HATU (1.5 eq.), HOAt (1.5 eq.) in DMF; for **11d**: from **5** (1.0 eq.), EEDQ (1.2 eq.) in CH₂Cl₂; **b.** TFA / CH₂Cl₂ (1:1); **c.** for **9a-9c'**: **6a** or **6b** (2.0 eq.), HATU (2.0 eq.), HOAt (2.0 eq.), DIEA (2.0 eq.) in DMF, for **9d**: PivCl (1.2 eq.), lutidine (1.2 eq.) in CH₂Cl₂; **d.** Pd/C 10% mol.

The synthesis of “turnless” β-sheets inspired by short protein sequences or by peptidomimetics remains a major challenge.¹⁸ Indeed, motifs capable of enhancing non-bonded interstrand interactions to stabilize artificial short β-sheets are rare.¹⁹ To assess the presence of secondary and potential tertiary structures in the synthetic peptide-peptoid hybrids **9a-d**, **9c'** and **12a-d**, a careful solution NMR spectral analysis at 18 °C was performed (Figure 2a-c).^{15,20} A combination of ¹H, ¹H-¹H NOESY and ¹H-¹³C heteronuclear multiple-quantum correlation (HMBC) NMRs in solvents of various polarity (CDCl₃, CD₃CN, DMSO-*d*₆) facilitated the conformational and structural analysis of these peptoid-peptide hybrids.¹⁵ The *cis-trans* equilibrium constants for both *N*-(benzyloxy) and *N*-(hydroxy)glycine residues in peptides **7a**, **11a** and **9a** were obtained with *K*_{trans/cis} values >20 in each case, meaning that independently of the solvent, these amides have an exclusive thermodynamic preference for the *trans* geometry.^{15,21} Interestingly, in all constructs **9**, **11-12**,

Figure 2. NOESY data in the region of the N-OH proton of **12c** in a) CDCl_3 , b) CD_3CN and c) $\text{DMSO}-d_6$. d) X-ray structure of **12c** with ellipsoids shown at the 50% probability level: solid-state conformation of **11c** with interstrand hydrogen bonds represented by dashed lines.

the methylene protons ($\text{H}_\alpha/\text{H}_{\alpha'}$) of *N*-(hydroxy) or *N*-(benzyloxy)glycine blocks are always highly diastereotopic (doublets with geminal coupling constant of ~ 17 Hz) with a $\Delta\delta(\text{H}_\alpha/\text{H}_{\alpha'})$ of ~ 0.5 ppm, which typically characterize well-folded structures. Each hydroxyl proton OH^1 and OH^2 from Hyg(1) and Hyg(2) residues were easily assigned and differentiated based on distinct NOESY correlations as well as a clear HMBC correlation between OH^2 and the vicinal Ile carbonyl. Given the downfield chemical shifts of both OH^1 (δ 8.36-8.76 & 9.73-9.91 ppm) and OH^2 (δ 8.85-9.44 & 10.19-10.25 ppm) in CDCl_3 and $\text{DMSO}-d_6$ respectively, tripeptide-peptoid **9c'** and **12a-d**, hydroxyl hydrogens were determined to participate in inter- or intramolecular hydrogen bonds. The difference of ~ 0.5 ppm between δOH^1 and OH^2 suggests that the OH^2 hydroxyl is more strongly hydrogen bonded. As shown in Figure 2C, all NOESY cross-peaks obtained for **12c** in $\text{DMSO}-d_6$ can be attributed to interstrand interactions suggesting that no intermolecular assembly occurs in this solvent. In contrast, in CD_3CN and more markedly in CDCl_3 (Figure 2A-B), a stronger NOESY correlation between OH^1 on one strand and the Ile H_α on the second strand supports the formation of a β -sheet self-assembly. Indeed, in a pair of facing residues i,j on opposite strands from a parallel β -sheet (e.g. Ile/(allo-)Ile in **12c,d**) residue i is H-bonded to both residues $j-1$ and $j+1$, which in this case both are Hyg residues.²² As established by Abraham, the difference in chemical shifts of exchangeable protons, or chemical shift deviation (CSD) due to solvent exposure $\Delta\delta = \delta(\text{DMSO}-d_6) - \delta(\text{CDCl}_3)$ can be used to assess the strength of intra- and intermolecular hydrogen bonds for OH and NH groups.²³ The

$\Delta\delta(\text{H}_\text{N})$ of 0.53 and 0.57 ppm in constructs **12c,d** suggest the presence of relatively strong hydrogen bonds likely arising from an intermolecular interaction between β -strands (see Table SI-8). Similarly, $\Delta\delta(\text{OH}^2)$ of 0.80 and 0.77 ppm in **12c** and **12d** respectively, supports the existence of hydrogen bonds from the Hyg(2) hydroxyls, while the larger $\Delta\delta(\text{OH}^1)$ of 1.13 and 1.11 ppm firmly indicate that OH^1 hydroxyls are more solvent exposed and not engaged in hydrogen bonding.¹⁵ Taken together, the downfield shifts observed for the exchangeable protons ($\text{OH}/\text{H}_\text{N}$) as well as the small CSD values for both Ile H_N and Hyg(2) OH in constructs **12c,d** suggest that such hydrogens participate in strong interstrand hydrogen bonding. These results also suggest that upon hydrogenation of the *N*-(benzyloxy)glycine residues, Hyg blocks play an important role in the self-assembly of constructs **12a-d** into short β -sheets.

These spectroscopic data were further confirmed by the structural features provided in the crystal structure of construct **12c** by X-ray analysis (Figure 2D). In this crystal, the parallel β -sheets are packed in a head-to-head manner forming columns through a single and relatively weak hydrogen bond $\text{OH}^1 \cdots \text{O}=\text{C}$ (*N*-terminal acetyl). Certainly, the low-lying conformation observed in the crystal lattice reflects closely the major conformer of **12c** in solution (CDCl_3). Both H_N and OH^2 groups are shown to participate in intermolecular hydrogen bonding to stabilize a parallel β -sheet structure. Both Φ and Ψ dihedral angles of Hyg(1/2) and Ile residues collected from the crystal structure are in agreement with an extended β -sheet arrangement (see Ramachandran plot, Figure SI-12). A notable feature of the paral-

lel β -sheet is the nitrogen pyramidalization in each *N*-(hydroxy)amide ($\omega(\text{OH}^1)$ 171.5°, and $\omega(\text{OH}^2)$ 170.9°). A similar pyramidalization was previously reported by Kirshenbaum and others for *N*-alkoxy peptoids.²⁴ This phenomenon is likely responsible for decreasing the nitrogen lone pair resonance in the *N*-(hydroxy)amide which in turn accentuates the basicity of the vicinal carbonyl. This stereoelectronic effect likely explains the unique hydrogen-bond acceptor nature of the *N*-(hydroxy)amide carbonyl responsible for the unusual parallel β -sheet alignment. In the original report from Blackwell, the central α -amino acid was instead a *N*-aryl peptoid block (*i.e.* construct **1** vs **2**, Figure 1). In such constructs, the central carbonyls were also good hydrogen-bond acceptors, but these peptoid blocks could not participate in intermolecular H-bond, which could justify the preference for an anti-parallel β -sheet direction. Our results on peptoid-peptide hybrids **12a-d** complement well Blackwell's study by demonstrating that *N*-(hydroxyl)amide can force intermolecular hydrogen bonds with Hyg presented inward and outward of strands for stabilizing a different parallel β -sheet directionality.²⁵

Figure 3. CD spectra comparison between **9d** and **12d**

CD spectra of **9d** in acetonitrile (90 mM) and hexafluoroisopropanol (103 mM), and **12d** in hexafluoroisopropanol (116 mM) at 0 °C. Samples concentrations were determined accurately by UV absorption from the fluorene chromophore (Fmoc).

Secondary and tertiary peptidyl structures have well-established signatures in far-UV circular dichroism (CD), but the assignment of bands in peptoids is more challenging due to their important flexibility. *N*-alkoxy peptoid oligomers have been proposed by Kirshenbaum to adopt a polyproline II type (PPII) secondary structure that exhibit two maxima at 197 and 215 ± 5 nm.²⁶ The CD spectra obtained for tripeptide-peptoid hybrid **9d** present similar bands at 195/216 nm in CH₃CN and 200/218 nm in HFIP, suggesting that a similar PPII secondary structure formed (Figure 3). As shown by the superimposition of CD spectra in Figure 3, the hydrogenation of **9d** into **12d** triggers a dramatic change in the tripeptide-peptoid 3-dimensional structure. Typically, anti-parallel β -sheets are characterized by two bands, a positive maxima at 193-195 nm and a negative exciton at 213-218 nm, profoundly resembling the two bands for parallel β -sheets at 200-203 and 214-221 nm.²⁷ Therefore, the combination of a positive exciton at 201 nm and a large CD band

overlay at 213-219 nm obtained for construct **12d** conclusively supports the formation of a parallel β -sheet. Furthermore, the positive band at 298 nm, characteristic of a π - π^* transition was also observed in the CD spectrum of **12d** (Figure SI-13).²⁸ This band arising from an interaction between the proximate π -systems of both *N*-terminal Fmoc-groups strongly support a parallel arrangement as it is a feature found exclusively in parallel β -sheets. Overall, the structural elucidation by CD from the Fmoc interaction at 298 nm and the exciton minima at 213/219 nm distinctively confirm a parallel β -sheet self-assembly of **12d** in solution.

Figure 4. Temperature dependence of H_N and OH chemical shifts as a function of the temperature in CDCl₃ vs DMSO-*d*₆

A.	<i>T</i> _C CDCl ₃ Δδ/Δ <i>T</i> (ppb/K)			<i>T</i> _C DMSO- <i>d</i> ₆ Δδ/Δ <i>T</i> (ppb/K)		
	NH	OH ¹	OH ²	NH	OH ¹	OH ²
9c'	-4.2	N/A	-13.6	-5.9	N/A	-4.6
<i>trans</i> - 12a	N/A	-14.2	-17.0	N/A	-6.1	-4.2
<i>cis</i> - 12a	N/A	-5.8	-15.8	N/A	-5.5	-4.5
12b	Insoluble			-5.3	-4.5	-4.2
12c	-8.2	-12.2	-12.7	-5.6	-4.0	-4.6
12d	-8.7	-15.6	-12.6	-5.5	-3.7	-4.7

To test the stability of these novel parallel β -sheets, variable temperature NMR spectra (VT-NMR) were recorded between -60 °C and 110 °C (Figure 4). As expected, spectra of tripeptide-peptoids **12c-d** in DMSO-*d*₆ revealed no evidence of tertiary structure as shown by the small temperature dependence coefficients (*T*_C, Δδ/*T* < -4.6 ppb/K) for H_N while both *N*-(hydroxyl)amide hydrogens (OH¹/OH²) might exist in relatively strong intramolecular hydrogen bond (-4.6 < *T*_C < -4.0 ppb/K).^{29,30} The *T*_C representing the slopes of the best-fitted linear regression of OH¹ and OH² chemical shift drifts in function of the temperature were invariably low for all the constructs studied in DMSO-*d*₆ (Figure 4A and Table SI-8). It can be concluded that a strong intramolecular 6-membered ring hydrogen bond stabilizes each Hyg residue in DMSO-*d*₆ in single-stranded molecules (Figures SI-6/7).¹⁵ In contrast, the significantly larger *T*_C(OH¹) and *T*_C(OH²) values (-17.0 < *T*_C < -12.2 ppb/K) in CDCl₃ (Figure 4A) indicate that hydrogen bonds are

weaker in apolar solvents. These counterintuitive results could be explained by the competition between a strong interstrand H-bond and the innate intramolecular H-bond of Hyg residues (as shown in DMSO-*d*₆). Competing intra- and intermolecular H-bonds result in a net weakening of the hydroxyls' hydrogen bond network. The results obtained for β -sheets **12c,d** are in line with the interactions reported earlier by Blackwell for a peptoid tetramer β -sheet (e.g. **1**, Figure 1). While examining the temperature dependency on the *N*-(hydroxy)amide chemical shifts in CDCl₃, it was found that both hydroxyl signals shifted in a non-linear fashion (Figure 4B). The sigmoidal dependency of chemical shifts of *N*-(hydroxy)amide protons (OH¹/OH²) as a function of temperature is distinctive of structural transition.³¹ The melting curves observed for β -sheets **12c,d** indicate a rupture of folding at elevated temperatures. Thus melting temperatures (*T*_m) corresponding to the hydrogen-bond network cleavage within the parallel β -sheets of **12c,d** were estimated from the best-fitted curves of thermal transition to the Boltzmann equation.¹⁵ *T*_m(OH¹) of 23 and 14 ± 5 °C, and *T*_m(OH²) of 46 and 29 ± 5 °C were calculated for β -sheets of **12c** and **12d** respectively (Figure 4B, Table SI-6). In both molecules, the significantly higher melting temperatures (> 15 °C) associated with the H-bond rupture of hydroxyl OH² is a direct evidence that the interstrand hydrogen bond from Hyg(2) stabilizes the entire structure.

In conclusion, an epimerization-free coupling of the readily available *N*-(hydroxy)glycine peptoid blocks (Hyg) has been developed for C- and N-terminal insertion into peptide-peptoid hybrids. The synthetic constructs self-assembled in solution to form short parallel β -sheets which were characterized by a combination of NMR and CD experiments and confirmed by the crystal structure of **12c**. A detailed examination of the major differences in NOESY cross-strand correlations, CSDs, *T*_C and *T*_m in various solvents clearly highlight the role of *N*-(hydroxy)amides from Hyg residues in the self-assembly and the stabilization of these tertiary structures. Our results demonstrated that *N*-(hydroxy)amides not only favor exclusively *trans* amide conformers *via* intramolecular H-bonds (*K*_{*trans/cis*} > 20), but are also amenable to strong interstrand (intermolecular) H-bonds in solvents of low polarity. Recent studies from Del Valle have shown that the insertion of *N*-(amino)³² and *N*-(hydroxy) α -amino acids^{14d} at specific non-hydrogen bonded sites (outward positions) can stabilize β -sheets within hairpins. In contrast, the present study establishes that a combination of *N*-(hydroxy)amides at hydrogen-bonded (inward) and non-hydrogen-bonded sites (outward) can be favorable due to strong interstrand H-bonds and an enhanced carbonyl basicity. Given the typical difficulty to create stable β -sheets within short peptide constructs,^{18,19} the ability of Hyg to improve hydrogen-bond donor/acceptor properties of backbone amides will certainly find numerous applications in foldamers. Ongoing studies in our laboratory will leverage the unique stereoelectronic properties of Hyg and other *N*-hydroxy α -amino acids to study the assembly of parallel and anti-parallel β -hairpins in water.³³

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge

Tables of selected results from the C- and N-terminal coupling optimization and epimerization is reported. Complete experimental procedures and characterization data including ¹H, ¹³C, NOESY and HMBC NMR spectra, as well as HPLC chromatograms for *d.r.* determination are available online.

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Author Contributions

This project was conceived by S.P.R. and the manuscript was written through contributions of S.P.R. and A.D.R.. All authors have given approval to the final version of the manuscript.

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