Peptide-Bond Modification for Metal Coordination: Peptides Containing Two Hydroxamate Groups

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Abstract: Peptide-bond modification via N-hydroxylation has been explored as a strategy for metal coordination to induce conformational rigidity and orient side chains for specific molecular recognition. N-Hydroxyamides were prepared by reacting N-benzyloxyamino acid esters or amides with Fmoc–AA–Cl/AgCN (Fmoc: 9-fluorenylmethoxycarbonyl; AA: amino acid) in toluene or Fmoc–AA/HATU/DIEA in DMF (HATU: O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIEA: N,N-diisopropylethylamine; DMF: N,N-dimethylformamide), followed by deblocking of benzyl protecting groups. Novel linear and cyclic N,N-dihydroxy peptides were efficiently assembled using Fmoc chemistry in solution and/or on a solid support. As screened by electrospray ionization–mass spectroscopy (ESI-MS), high iron-binding selectivity and affinity were attainable. Compounds having a spacer of two α-amino acids between the amino acids bearing the two hydroxamates, i.e., a spacer of 8 atoms, generated 1:1 iron complex species in the gas phase. Moreover, high performance liquid chromatography (HPLC), uv/vis, and ¹H-NMR analyses provided direct evidence for complex formations in solution. Significantly, the representative compound cyclo(Leu–[CON(OH)]–Phe–Ala–Pro)₂ (P₈) may serve as a robust metal-binding scaffold in construction of a metal-binding library for versatile metal-mediated molecular recognition.

Keywords: peptide-bond modification; metal coordination; conformational rigidity; molecular recognition; N-hydroxyamide; hydroxamate

INTRODUCTION

Metals play essential roles in biological systems both structural and functional. Iron exists at the active center of proteins responsible for oxygen and electron transport. Functional roles of metals are found in such diverse metalloenzymes as oxidases, hydrogenases, reductases, dehydrogenases, deoxygenases, and dehy-
Zinc organizes the conformation required for molecular recognition in zinc-finger proteins, originally isolated as transcription factors, but now recognized as having diverse functions in biology. Metal-binding compounds have received great attentions for their potentials in conformational control, biomimetic design, elucidation of metal-mediated catalytic mechanism, and chelation therapy, as novel enzyme inhibitors, and in the development of metallo-drugs, etc. We and others have utilized metal coordination as a strategy for preorganizing peptide structures for molecular recognition by enzymes and receptors. Others have used metal coordination as a means of stabilizing secondary structures and quaternary structures such as coiled coils and helix bundles as well as controlling the allosteric states of receptors. Coordination of different metals allows subtle variation of the conformation of ligands with the same molecular template that should assist elucidating the receptor-bound conformation and optimizing the complementarity between peptide ligand and enzyme/receptor (Figure 1).

Most reports of metal-binding peptides and peptidomimetics focus on metal–peptide complexes utilizing peptide side chains, the amino, imidazole, carboxylate, sulfhydryl, indole, phenol groups, etc., as metal-binding sites. As side chains of peptides often play very important roles in molecular recognition at biological receptors, we have focused on modifying peptide bonds to build metal-binding sites within the backbone. This strategy leaves the side chains free to interact with receptors while the conformation of the backbone is preorganized via metal coordination. As combinatorial approaches have proved valuable for the discovery of molecules with novel structures having interesting biological activities, we sought to develop metal-binding scaffolds that would be useful in efficient construction of a metal-binding library. Metal coordination is expected to not only preorganize the complex’s conformation for molecular recognition, but the use of different metals will also enhance structural diversity by subtle conformational effects on the template that orients the side chains.

We envision that an ideal metal-binding scaffold should be endowed with the following features: (1) exhibits high metal-binding affinity and selectivity generating a limited number of stable structures; (2) coordinates a single metal ion specifically within the ligand architecture; (3) allows for the generation of high structural diversity and similarity via an efficient combinatorial approach; and (4) possesses significantly diverse bioactivities where the structure–function relationships can be explored by metal coordination. As peptides are common chemical messengers in biological systems, we sought minimal structural perturbation that would confer metal-binding properties on the peptide backbone. It is well known that a hydroxamate group is a very powerful bidentate ligand for metal coordination. For example, the naturally occurring trihydroxamate-containing siderophores such as desferrioxamine (DFO) bind and transport ferric ions essential for growth and proliferation of microorganisms. The conformation, metal-binding properties, and interesting biological activities of N-hydroxy peptides have received attention. Peptidyl hydroxamic acids have been developed as potent inhibitors of several metalloproteases including matrix metalloproteinases. Reported that N-hydroxylation of a single amide bond in the T-cell epitope SIINFEKL by replacement of Asn with N-hydroxyglycine renders a T-cell receptor agonist into an antagonist and enhances enzymatic stability. Recently, hydroxamate-containing peptidomimetics show significant inhibition of HIV protease in vitro. The metal-binding properties and potential biological activities of peptides containing multiple N-hydroxamides led to exploration of N-hydroxyamide-containing peptides as metal-binding scaffolds.

Earlier efforts to design and synthesize tri-N-hydroxyamide-containing peptides by mimicking the siderophore, DFO, were reported by the Akiyama group. More recent studies by Hara et al. have focused on iron complexation by oligomers of Ala–Ala–Ψ[CO–NOH]–β-Ala. In contrast, we initially focused on N,N'-dihydroxy peptides. Dihydroxamic acids, such as the siderophore rhodotorulic acid and its analogs have been reported to form 1:1 complexes with iron(III) at low pH. While a cyclic N,N'-dihydroxy dipeptide has been reported, the best of our knowledge, metal-binding properties of N,N'-dihydroxy peptides have not been characterized. Compared to hexadentate N,N',N'-trihydroxy peptides, tetradentate N,N'-dihydroxy peptides possess only two bidentate metal-binding motifs. Nevertheless, in order to efficiently construct metal-binding libraries containing multiple N-hydroxamide groups, a close scrutiny of N,N'-dihydroxy peptides and their metal-binding properties was the initial goal. First, it would provide understanding of the structure vs metal-binding properties; second, it should help in the ra-
tional design of improved \(N,N',N''\)-trihydroxypeptides; third, a robust and compact metal-binding scaffold based on \(N,N''\)-dihydroxypeptides would allow the rational incorporation of additional versatile metal-binding sites (in side chains, for example) for further molecular diversity.

The aims of this study were as follows: (a) to develop efficient syntheses of \(N,N''\)-dihydroxypeptides; (b) to establish rapid methods for screening their metal-binding properties; (c) to elucidate the structure vs metal-binding relationship for further molecular design and other potential applications; and finally, to provide a metal-binding scaffold for construction of a two-dimensional combinatorial library where ligand structure would comprise one dimension, and different bound metals would compromise the second dimension.

SYNTHESIS

Initially, novel dihydroxamate-containing pseudo-oligopeptides that featured one hydroxamate group at the C-terminus and the other close to the N-terminus using an oligopeptide sequence as linker were targeted. The synthetic route to such \(N,N''\)-dihydroxy-oligopeptides should allow facile synthesis in solution phase and/or on a solid support\(^{49,50}\) while the internal hydroxamic acid [—CON(OH)—] could be synthesized by acylation of protected \(N\)-hydroxyamino acid derivatives prepared by a variety of procedures.\(^{51–54}\) Three \(O\)-benzyl protecting \(N\)-hydroxyamino acids derivatives were used as building blocks, i.e., \(N\)-benzoxoxy-glycine tert-butyl ester 1, \(N\)-benzoxoxy-alanine tert-butyl ester 2, and \(N\)-benzoxoxy-phenylalanine 3 as building blocks. THF: tetrahydrofurane; DIEA: \(N,N\)-diisopropylethylamine.

Peptide-Bond Modification

[SCHEME 1] Synthesis of \(N\)-benzoxo-glycine tert-butyl ester (1), \(N\)-benzoxo-alanine tert-butyl ester (2), and \(N\)-benzoxo-phenylalanine (3) as building blocks. THF: tetrahydrofurane; DIEA: \(N,N\)-diisopropylethylamine.
Compared to the amino groups of usual amino acid building blocks, the amino groups of N-benzyloxy amino acid derivatives have poor basicity and nucleophilicity due to the steric and electronic effects of the N-benzyloxy group. Powerful acylation methods, such as acid chloride, mixed anhydride, and O-(7-azabenzotriazol-lyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), were required for efficient syntheses of N-hydroxypeptides. Compared to the amino acid anhydride and HATU methods, the acid chloride method was attractive due to its facile preparation from the corresponding Fmoc-amino acid and oxalyl chloride in DCM. Importantly, acylation with Fmoc–AA chlorides (AA: amino acid) reportedly occurs without significant racemization. In addition, for solution synthesis, product purification was facilitated without the side products derived from other coupling agents. Perlow et al. successfully used Fmoc–l-isoleucine acid chloride in the acylation of a sterically hindered N-Cbz-piperazic acid N-terminus, and the acylation efficiency was further improved by the combination with AgCN in toluene. This powerful acylation method, therefore, was applied to the incorporation of N-benzyloxyamino acids into peptides in solution. Wang and Phanstiel have also successfully acylated N-(benzyloxy)amino acid derivatives with Fmoc-amino acid chlorides without racemization. Braslau et al. have utilized a rearrangement of O-acyl hydroxamic substrates prepared by coupling Fmoc-amino acid chlorides to N-protected hydroxylamine esters to generate di- and tripeptide hydroxamic acids.

As summarized in Scheme 2, the reaction of N-benzyloxyglycine tert-butyl with Fmoc–Val–Cl in the presence of AgCN in toluene afforded dipeptide 6 (85%). Cleavage of the tert-butyl group of 4 with TFA provided the corresponding acid that was coupled with alanine tert-butyl ester to give tripeptide 7. A tetrapeptide 8 was obtained from 7 similarly via cleavage with TFA and then coupling with leucine tert-butyl ester. Compound 8 was further coupled with O-benzylhydroxylamine to give 9. Following removal of the Fmoc group with 20% piperidine in DMF, the two O-Bz protecting groups were removed successfully with ammonium formate in the presence of Pd/C (5%) in CH3OH to afford the desired pseudo-peptide P1 with one hydroxamate close to the N-terminus and the other one located at the C-terminus. Based on the above Fmoc/tert-butyl chemistry in solution, P2 was prepared from 2 similarly.

In contrast, the low nucleophilicity of the N-benzyloxyamino group allowed us to link the carboxylate of N-benzyloxy-phenylalanine with the free amino group of a normal amino acid or peptide building block using conventional peptide coupling methods without further protecting the N-benzyloxyamino group.

As summarized in Scheme 3, 3 was coupled with alanine tert-butyl ester smoothly using EDCI and HOBT in DCM to afford dipeptide 10 in high yield. Similarly, 10 was also reacted with Fmoc–Leu–Cl/AgCN in toluene to afford the tripeptide Fmoc–Leu–Ψ[CON(OBz)]–Phe–Ala–OBut (11) that was further elongated to give the tetrapeptide, Fmoc–Leu–Ψ[CON(OBz)]–Phe–Ala–Pro–OBut (12).

The pseudo-peptide 12 was deblocked with 20% piperidine/DMF or 50% TFA/DCM (DCM: dichloromethane) to afford two different intermediates 13.
and 14 with different free termini that were used as modules to assemble the target compounds via fragment condensation reactions, either in solution or on a solid support. For example, 13 was coupled with 14 in solution to form the dimer 15. After further deprotecting 15 with piperidine/DMF and TFA/DCM, the resulting linear octapeptide 16 was cyclized in the presence of (benzotriazol-1-ylxy)-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) and DIEA in DMF to afford 17. Finally, two target compounds, the linear P7 and its cyclic analog P8, were obtained by deprotecting 16 and 17, respectively. (See Scheme 4.)

The modular approach described above has provided convenient access to a small library of mono-, di-, and tri-hydroxamate-containing pseudo-oligopeptides, making use of N-benzyloxyamino acids as building blocks in solution syntheses. Meanwhile, solution methods were adapted to solid phase because of the advantages, i.e., facile workup by filtration, and nearly complete coupling reactions driven via excessive reactants and repeated couplings. As summarized in Scheme 5, solid-phase synthesis started with commercially available N-Fmoc-hydroxyamine 2-chlorotrityl resin to introduce the N-hydroxamate at the C-terminus. The peptide chain was elongated using
conventional Fmoc chemistry after removal of the Fmoc group and attachment of the first amino acid using HATU/DIEA in DMF. As described in Scheme 5, the N-benzyloxyamino acids were similarly incorporated on the solid support using DIC/HOBT without further protecting their N-benzyloxyamino groups. Completion of this coupling was conveniently monitored by the ninhydrin test as the benzyloxyamino group was negative to this test. By cleaving an aliquot of the resin and detecting the product by electrospray ionization–mass spectroscopy (ESI-MS), no attachment of additional N-benzyloxyamino acid to the desired products were found. In order to form the N-benzyloxyamide efficiently on solid phase, HATU that has proven a powerful activating agent in many difficult coupling reactions was used. After cleavage with TFA, the isolated products proved that the coupling mediated by HATU were successful.

As showed in Table I, an array of N,N′-dihydroxamate-containing peptides with structural diversity and similarity can be efficiently constructed by varying the amino acids, the number and positions of N-hydroxylation, and the linker between the two hydroxamates in solution or on a solid support. Obviously, the key steps are the couplings with benzyloxyamino groups.

Our results show that a library of hydroxamate-containing peptides with structural diversity and similarity can be efficiently constructed by varying the amino acids, the number and positions of N-hydroxylation, and the linker between the two hydroxamates in solution or on a solid support. Obviously, the key steps are the couplings with benzyloxyamino groups.

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**Table I The Synthesized N,N′-Dihydroxypeptides**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
<td>H–Val–Ψ[CON(OH)]–Gly–Ala–Leu–NHOH</td>
</tr>
<tr>
<td>P2</td>
<td>H–Val–Ψ[CON(OH)]–Ala–Ala–Leu–NHOH</td>
</tr>
<tr>
<td>P3</td>
<td>H–Val–Ψ[CON(OH)]–Phe–Ala–Leu–NHOH</td>
</tr>
<tr>
<td>P4</td>
<td>H–Val–Ψ[CON(OH)]–Phe–Ala–Pro–Leu–NHOH</td>
</tr>
<tr>
<td>P5</td>
<td>H–[Leu–Ψ[CON(OH)]–Phe–Ala]₂–OH</td>
</tr>
<tr>
<td>P6</td>
<td>Cyclo–[Leu–Ψ[CON(OH)]–Phe–Ala]₂</td>
</tr>
<tr>
<td>P7</td>
<td>H–[Leu–Ψ[CON(OH)]–Phe–Ala–Pro]₂–OH</td>
</tr>
<tr>
<td>P8</td>
<td>Cyclo–[Leu–Ψ[CON(OH)]–Phe–Ala–Pro]₂</td>
</tr>
<tr>
<td>P9</td>
<td>H–Leu–Ψ[CON(OH)]–Phe–Ala–Pro–Leu–NHOH</td>
</tr>
<tr>
<td>P10</td>
<td>H–Leu–Ψ[CON(OH)]–Ala–Pro–Leu–NHOH</td>
</tr>
<tr>
<td>P11</td>
<td>H–Leu–Ψ[CON(OH)]–Phe–Ala–Pro–Ala–Leu–NHOH</td>
</tr>
<tr>
<td>P12</td>
<td>H–Leu–Ψ[CON(OH)]–Phe–Ala–NHOH</td>
</tr>
</tbody>
</table>

*P1, P2, P3, P4, P9, and P12 were synthesized in solution phase as depicted in Scheme 2; P5, P6, P7, and P8 were synthesized as summarized in Schemes 3 and 4; P10 and P11 were assembled on a solid support as summarized in Scheme 5.*
to form $N$-benzyloxyamides, where powerful acylation methods are necessary. Significant side products due to overreaction or racemization were not observed during purification of the target compounds by HPLC. In addition, two different coupling methods, Fmoc–AA chloride and HATU, were used to synthesize $P_9$ and both products were identical on HPLC. Nevertheless, further experiments to elucidate possible racemization during synthesis of hydroxamate-containing peptides are required as the proposed mechanism for Fmoc–AA chloride/AgCN involves an oxazolone intermediate usually thought to mediate racemization. Compared with Fmoc–amino acid chlorides, Fmoc–amino acid fluorides possess some advantages due to their stability, their lack of conversion to oxazolones in the presence of tertiary amines, and their ability to affect acylation in the total absence of base. Fmoc–AA fluorides may be an alternative method of choice for both efficient acylation of benzylamino groups on a solid support and minimization of racemization, and should be explored. Note-worthy, after deblocking the $O$-benzyl protecting groups, the crude products also contained the species $[M-16/H]^+$ in their ES-MS spectra, presumably from overreduction of the desired products to their corresponding amide analogs, especially noted in the synthesis of the $N$-benzyloxy-glycine-based $P_1$ in Table I. This side reaction, which both decreases yields and complicates purification, may be overcome using alternative synthetic strategies with different protection of the $N$-hydroxyl groups.

METAL-BINDING PROPERTIES

It is well known that hexadentate trishydroxamate compounds are good ligands for iron coordination based on the discovery and characterization of siderophores, natural products used to facilitate ferric ion uptake by microorganisms, such as DFO. Thus, it was logical to screen the $N,N'$-dihydroxy peptides for their iron-binding properties and metal-binding selectivities. The soft nature of electrospray ionization makes ESI-MS a powerful tool for studying noncovalent interactions including ligand–metal coordination. ESI-MS was used as a fast screening method to evaluate metal-binding of $N$-hydroxamide peptides, while other characterization techniques including HPLC, uv-vis, and NMR were used in addition to gain a more comprehensive knowledge of their properties as metal ligands.

**Fe(III) BINDING PROPERTIES**

**ESI-MS Analysis**

Typically, solutions (200 µL) of a peptide ligand (100.0 µM) and different equivalents of Fe(NO₃)₃ in methanol were subjected to ESI-MS analysis. All positive-ion ESI-MS were collected under the same conditions (see the experimental section) established to produce singly charged species $[LH]^+$ or $[LNa]^+$ of all free peptide ligands with high abundances. Figure 2 showed the effects of addition of Fe(III) on the ESI-MS of compounds $P_7$–$P_9$. No significant abundances of free ligand peaks remained in the spectra upon addition of Fe(III) (1.2 equiv) and the spectra were especially clean. The respective base peaks at $m/z$ 644.30, 960.73, and 942.73 were readily interpreted as the singly charged ions resulting from their coordination with Fe(III), i.e., $[L + Fe-2]^+$. These results indicated that Fe(III) complexes of $P_7$–$P_9$ could be readily detected in the gas phase by ESI-MS.

Titration with Fe(III) further revealed the binding stoichiometries of compounds $P_7$–$P_9$. The ESI-MS obtained usually contained multiple peaks that arose from ionizations of free ligands, metal complexes, and related molecular fragments. It is difficult to calculate the relative concentrations due to possible differences in ionization, and thus the binding constants $K_c$ were only estimated. As shown in Figure 3, the relative abundance in each ESI-MS as a factor instead of $K_c$ served to evaluate the relative metal-binding affinity of each compound. Relative abundance was calculated based on the percentage of the resulting singly charged species $[LM]^+$ to the total observed free ligand-related peaks including $[LH]^+$, $[LNa]^+$, and $[LH_2]^{2+}$ by the following equation:

$$K_c = \frac{[LM^{n+}]}{[L][M^{n+}]}$$

Relative abundance = $\frac{[LM^{n+}]}{[LM^{n+}] + [LH]^+ + [LNa]^+ + [LH_2]^{2+}}$ (%)
Figure 3 clearly showed the dependence of the relative abundances of complex upon added Fe(III) concentration. As the concentration of Fe(III) increased, the relative abundances of the singly charged species \([L@Fe]^+\) increased, and the relative abundances of the singly charged species \([LH]^+\) of the three compounds decreased correspondingly. As for both P7 and P8, complexation with Fe(III) was nearly complete upon addition of 1.2 equiv Fe(III).

In contrast, compounds P3, P5, and P6 generated complicated spectra upon the addition of Fe(III). Figure 4 shows the spectra of P3, P5, P6, and their coordination with 1.2 equiv of Fe(III). The lowered abundances of species \([LH]^+\) clearly indicated that these compounds participated in coordination with Fe(III). As for P3 and P5, species from intramolecular coordination and intermolecular coordination showed similarly significant abundances. As for P6, a species of bimolecular coordination was observed, but no significant species of intramolecular coordination was apparent. The type of coordination correlated with Fe(III) concentration. Compared to the desired intramolecular coordination, bimolecular coordination prevailed at 0.4 equiv of Fe(III). As the Fe(III) concentration was further increased, the spectra became more complicated, and both intramolecular and bimolecular coordination were apparent. Compared to Fe(III) coordination of P7–P9, results with P3, P5, and P6 clearly showed the impact of ligand structure on the metal-binding property as revealed by ESI-MS.

To further compare the relative Fe(III) binding affinity of ligands, competition experiments were performed. All ESI-MS were recorded for solutions of two different peptide ligands (100 μM of each ligand) in CH3OH pretreated with 1 equiv Fe(NO3)3. By comparing the relative abundances of the resulting intramolecular Fe(III) complex species to their corresponding free ligand in their ESI-MS (Figure 5), the relative binding affinities of compounds in the following order—P7 > P9, P5 > P6, P4 > P3, P8 > P7, P8 > P9, P8 > P6, and P7 > P5—were estimated.
HPLC Analysis

ESI-MS only reflects the relative compositions of the ion species for ligand–metal coordination in the gas phase. To ascertain coordination of $N,N'$-dihydroxypeptides with Fe(III) in solution, all solutions prepared for ESI-MS were also subjected to HPLC analysis. Figure 6 shows HPLC profiles of three representative compounds P7–P9. The addition of Fe(III) to compound P9 only produced a minor new peak at 13.2 min. Compared to P9, the addition of Fe(III) to compound P7 (peak at 14.4 min) produced a new peak of higher abundance at 16.4 min. Nevertheless, instead of the free-ligand peak of P8 at 18.1 min, a new broad peak was eluted at 23.2 min upon addition of Fe(III). After isolation by semipreparative HPLC and analysis by ESI-MS these new peaks were related with the desired Fe(III) complexes as showed in Figure 2. The high Fe(III)-binding affinity of P7–P9 allowed the resulting complexes to be separated from free ligands by HPLC. The relative area of new peaks to free ligand peaks clearly reflected the relative stabilities of the resulting Fe(III) complexes in solution phase in the following order—P8 > P7 > P9—consistent with the observation from ESI-MS. Obviously, HPLC is a convenient method to evaluate the relative ligand properties of $N,N'$-dihydroxypeptides in solution.
UV-Vis Spectroscopy

UV-vis spectroscopy has been widely used to study complexation of hydroxamates with Fe(III). A typical 1:3 complex such as ferrioxamine B shows maximum absorption ($\lambda_{\text{max}}$) at 420 nm while a 1:1 complex of a monohydroxamic acid with iron (III) at low pH has a $\lambda_{\text{max}}$ at 520 nm. A 1:2 complex formation was identified with $\lambda_{\text{max}}$ at 466 nm observed during transformation of a 1:3 complex to a 1:1 complex.\textsuperscript{7,13,26} Similarly, coordination of $N,N'$-dihydroxyamide pep-

FIGURE 4 Positive-ion ESI-MS for the solutions of $N,N'$-dihydroxyamide ligands P3, P5, and P6 (100 $\mu$M) with different mole ratios of Fe(NO$_3$)$_3$ in CH$_3$OH, showing the spectral complexity upon the addition of Fe(III).

FIGURE 5 Positive-ion ESI-MS for the competition solutions of different dihydroxyamide ligands P3 and P6–P9 (100 $\mu$M) with 1 equiv of Fe(NO$_3$)$_3$ in CH$_3$OH.
tides with Fe(III) increased absorption at wavelengths ranging from 420–520 nm. For peptides P7–P9, the \( \lambda_{\text{max}} \) were 474, 446, and 458 nm, respectively (Figure 7) within the reported range for 1:2 complexes.

Titration with Fe(III) showed that the absorption intensities were dependent upon the Fe(III) concentration, and reached equilibrium near 1 equiv for P7 and P8, but requiring approximately 3 equiv for P9. These characteristic peaks could be applied to both qualitative and quantitative identification of complex formation. It is noteworthy that the addition of Fe(III) also significantly enhanced the absorption in the uv region significantly and was accompanied by a shift of \( \lambda_{\text{max}} \) to longer wavelength (226 nm).

**1H-NMR Spectroscopy**

1H-NMR experiments were undertaken to further characterize the metal-binding properties of representative compound P8 with higher affinity to reduce presence of free ligand, especially the effect of metal coordination on the structure (conformation) of P8. Ga(III) possesses similar coordination properties as Fe(III), and has been used as a surrogate of Fe(III) in 1H-NMR experiments. Studies via ESI-MS analysis confirmed that coordination of 8 with Ga(III) was very similar to Fe(III). Therefore, the Ga(III) complex of P8 was prepared by mixing 2.0 equiv of Ga2(SO4)3 with compound P8 in CH3CN/H2O(3:2) and stirring overnight. The resulting complex was lyophilized, confirmed by HPLC and ESI-MS (m/z 955.47), and dissolved in DMSO-d6 for 1H-NMR experiments. Proton chemical shifts of P8 were assigned by analysis of correlated spectroscopy (COSY), total COSY (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY) spectra. Only Ala and Leu have spin propagation from the amide NH through the \( \text{H}^\alpha \), \( \text{H}^\beta \), and \( \text{H}^\gamma \) protons [Figure 8(b)]. Correlated proton resonances from 1.3 to 5.2 ppm in Figure 8(a) were unambiguously assigned to \( \beta \), \( \gamma \), and \( \delta \) protons of (NOH)Phe and Pro residues. Observed sequential \( \alpha(i)\text{-NH}(i + 1) \) NOE, such as Phe(NOH)–Ala and Al

**FIGURE 6** The HPLC profiles for solutions of P7–P9 containing 3 equiv Fe(III), showing the existences and relative stabilities of their Fe(III) complexes in HPLC mobile phase. The HPLC analysis is performed on a Vydac C-18 column using a linear gradient of 0.1% TFA in H2O and 0.1% TFA in CH3CN and from 90:10 to 10:90 over a period of 30 min, flow rate 1.0 mL/min, detection at 220 nm. Complexes were confirmed by ESI-MS of HPLC peaks.

**FIGURE 7** UV-visible spectra for coordination of P7–P9 with 1, 3, and 5 equiv of Fe(III) in CH3CN/H2O (7:3).
FIGURE 8  Expansion of (a) Hα-aliphatic and (b) NH-aliphatic region of 600 MHz TOCSY spectra of P8 cyclo(Leu-$\psi$(CON(OH))Phe-Ala-Pro)$_2$ in DMSO-$d_6$. 
Pro–Leu cross peaks in Figure 9(b), further confirmed the (NOH)Phe–Ala and Pro–Leu connectivities, respectively. The presence of proline in P8 resulted in cis/trans-isomerization of the Ala–Pro amide linkage. This was reflected by two observed sets of chemical shifts for each individual proton resonance in one-dimensional (1D) (Figure 10) and two-dimensional (2D)-TOCSY spectra (Figure 8). Correlated Pro(α–β–γ–δ) spin propagation was used to further differentiate the cis/trans-isomers, thus two groups of the correlated-cross peaks, from 4.61 to 1.65 ppm and from 4.22 to 1.43 ppm, shown in Figure 8(a) were related to different isomers. Furthermore, a strong NOE cross peak between Ala-α (4.47 ppm) and Pro-δ (3.36 and 3.54 ppm) ['ααααP8 in Figure 9(a)] indicated a trans-Ala–Pro amide linkage while an observed Ala-α (4.46ppm)–Pro-α (4.22ppm) cross peak ['ααααP8 in Figure 9(a)] suggested a cis-amide conformation. P8 revealed a symmetrical spectra pattern and the observed Phe(NO)(NO)-Leu α NOE cross peak [Figure 9(c)] confirmed the presence of the hydroxamate group in P8. All sequential connections between adjacent residues were observed and the continuous path shown in Figure 9 indicated a cyclic [Leu–Phe(NO)–Ala–Pro] segment in P8. Complete assignments of proton resonance for each residue are available in Table II. The population of cis/tran-conformers estimated from 1D spectrum was approximately 20/80%.

The coordination geometry of P8 was investigated by TOCSY and NOESY spectra of the Ga(III)–P8 complex. The disappearance of the N–OH resonance (such as peaks at 9.08 and 10.24 ppm) tA/H9251 revealed a symmetrical spectra pattern and the observed Phe(NO)(NO)-Leu α NOE cross peak [Figure 9(c)] indicated complex formation between Ga(III) and the ligand in different complexes. The relative abundances vs different metals plots were showed in Figure 12.

Based on the relative abundance, it was found that compounds P7–P9 exhibited high preference for intramolecular coordination with Fe(III). These compounds were further screened for their binding properties towards other metals of medical interest. Initially, solutions of P3 and P7–P9 (100 μM) in methanol were pretreated with 1.2 equiv amount of different metals including Fe(III), Ga(III), Fe(II), Cu(II), Co(II), Mn(II), Ni(II), and Zn(II). The relative abundances vs different metals plots were showed in Figure 12.

As identified by ESI-MS, compounds P7–P9 exhibited high affinity for intramolecular coordination with Fe(III). These compounds were further screened for their binding properties towards other metals of medical interest. Initially, solutions of P3 and P7–P9 (100 μM) in methanol were pretreated with 1.2 equiv amount of different metals including Fe(III), Ga(III), Fe(II), Cu(II), Co(II), Mn(II), Ni(II), and Zn(II). The relative abundances vs different metals plots were showed in Figure 12.
FIGURE 9  Expansion of (a) Hα-aliphatic, (b) NH-aliphatic, and (c) NOH-aliphatic region of 600 MHz NOESY spectra of P8 cyclo(Leu–Ψ[CON(OH)]Phe–Ala–Pro)₂ in DMSO-d₆. Cross peaks of the continuous sequential path for residues between Leu and Pro are indicated.
FIGURE 10 The 600 MHz proton spectrum of P8 cyclo(Leu-Ψ[CON(OH)]Phe-Ala-Pro)₂ in DMSO-d₆.
RELATION BETWEEN STRUCTURE AND METAL BINDING

As described above, the metal-binding properties of $N,N'$-dihydroxypeptides have been evaluated by ESI-MS, HPLC, uv-vis, and $^1$H-NMR. Even though the results are qualitative, they can assist in understanding the relationship between structure and metal binding.

The Metal-Binding Selectivity of $N,N'$-Dihydroxypeptides

As exemplified by P8, $N,N'$-dihydroxypeptides showed high binding selectivities for Fe(III) over Cu(II), Mn(II), Zn(II), Ni(II), and Cd(II). The high selectivities can be ascribed to the two hydroxamate groups that provide hard donors of negatively charged oxygen for the hard acidic Fe(III). The affinity of $N,N'$-dihydroxypeptides for Fe(III) complex formation is in accordance with the Hard and Soft Acid and Base (HSAB) principles of Pearson.77,78

The Effects of Linker Length on Fe(III) Coordination Mode

ES-MS analysis indicated clearly that compounds P7–P9 had higher tendencies for forming 1:1 intramolecular complexes with Fe(III) in the gas phase than compounds P3, P5, P6, and P12. These tendencies reflected the importance of linker length in determining Fe(III) coordination mode and binding affinity. As we have seen with compounds P7–P9, a two $\alpha$-amino acid spacer between the amino acids bearing the N-hydroxyl groups, i.e., a spacer of 10 $\alpha$-amino acid atoms between the two hydroxamates, or even longer linker, provides enough conformational flexibility and suitable orientations for both hydroxamate groups to match both geometrical and electronic requirements of Fe(III) coordination. Adversely, a shorter linker such single amino acid spacer as seen in compounds P3, P5, P6, and P12 led to unfavorable intramolecular coordination with Fe(III) and intermolecular coordination became competitive (Figure 14). There were a variety of other peaks present in the spectra of compounds P3, P5, P6, and P12 that could not be assigned completely, suggesting that iron coordination may generate additional higher coordination species, or may promote fragmentation of ligands during the process of mass spectrometry.

It is significant to identify suitable linkers connecting the two hydroxamates that may be the structural determinants for forming thermodynamically stable Fe(III) complexes. Caudle et al.45 used ES-MS to show conclusively that for dihydroxmate ligands with a methylene-bridge linker; the $n \times 8$ species forms monomer whereas the $n \times 2$ species forms a dimer. The naturally occurring nonpeptidic Tris-hydroxamate siderophore, desferrioxamine, possesses a high Fe(III) binding constant and the linker between the two hydroxamates is 9 atoms. The Akiyama group reported the design and synthesis of DFO-mimicking trihydroxamate-containing peptides with a ten-atom spacing between two hydroxamates consistent with our observation on dihydroxamate-containing peptides. We have constructed Fe(III)-binding trihydroxypeptides with high affinity based on the above results.4

The Effects of Backbone Cyclization on Metal-Binding Properties

ESI-MS-based competition experiment and HPLC analysis confirmed that the relative Fe(III) binding affinities of P8 is higher than P7. As shown in Figure 12, compound P8 actually exhibited higher binding affinities than P7 to all metals tested. These results suggest that the advantages of a macrocyclic effect including preorganization and favorable entropic contributions may prevail against possible cyclization-induced disadvantages including losses of poten-

<table>
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<td>(NOH)Phe</td>
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<td>(4.55)</td>
<td>(1.83, 1.70)</td>
<td>($\gamma$ 1.60; $\delta$ 0.90, 0.79)</td>
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<td>(4.46)</td>
<td>(3.20, 3.07)</td>
<td>(NOH 9.08)</td>
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<td>Pro (4.22)</td>
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<td>$\gamma$ 1.82, 1.65; $\delta$ 3.54, 3.36</td>
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<td>(2.23, 2.01)</td>
<td>( $\gamma$ 1.79, 1.43; $\delta$ 3.36, 3.27)</td>
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* Numbers in parentheses are the chemical shifts of the $cis$-isomer.
FIGURE 11 Expansion of (a) Hα-aliphatic region of 600 MHz TOCSY, (b) Hα-aliphatic, and (c) N(OH)Phe, aromatic–aliphatic region of 600 MHz NOESY spectrum of Ga(III)–P8 Ga(III)–cyclo(Leu–[CON(OH)]Phe–Ala–Pro)2 complex in DMSO-d6.
tial metal-binding sites (N-terminal amino group and C-terminal carboxylic acid), decreased conformational flexibility, and increased steric strain.

Cyclization has widely been used as a strategy for studying the bioactive conformation and in the discovery of peptidomimetics. Improvements in biological potency, receptor selectivity, metabolic stability, and bioavailability have also been reported. As exemplified by P8, cyclization can be used as a strategy for preorganizing linear N,N'-dihydroxypeptides for improving binding affinity in metal coordination. In contrast, cyclization of P5 led to the decreased relative binding affinity as seen in P6, indicating that cyclization of two hydroxamates connecting with unsuitable length may enhance the unfavorable enthalpic contributions to intramolecular complex formation.

The Cooperation of Two Hydroxamates with Other Metal-Binding Groups

Typically, a Fe(III) complex features an octahedral geometry with 6 ligand atoms. Siderophores of high Fe(III) binding affinities such as DFO contain three bidentate hydroxamate groups. It is suggested that two hydroxamate groups in N,N'-dihydroxypeptides cooperate with other groups in complexation such as peptide amide bonds, N- and C-terminal groups, and/or side chains for forming stable Fe(III) complex. This is evidenced by peptide P7 and P9. P7 contains a C-terminal carboxylate that is also good ligand for Fe(III) coordination due to its negatively charged oxygen donors, and shows higher Fe(III) binding affinity than peptide P9.

The Effects of Neighboring Groups on Fe(III) Binding Affinity

The effects of neighboring groups of hydroxamates on the Fe(III) binding affinity was explored by comparing peptides P1–P3, P9, and P11. Competition experiments showed the following order: P1 > P2, suggesting that neighboring groups play a significant role in affinity.

The Effects of Metal Coordination on the Structure

NMR spectroscopy showed that coordination of peptide P8 with Ga(III) caused disappearance of the protons in the N-hydroxamate groups, clearly indi-
cating that metal-binding sites are located at the two \( N \)-hydroxyamides. It also revealed that Ga(III) coordination further defined the structure of the cyclic compound, but that several isomers existed. Leong and Raymond\(^76\) reported that at least \( P7 \) isomers existed in Fe(III) trihydroxamate complexes due to different wrapping of the linear chain that positions the hydroxymate group as well as alternative orientations of the hydroxamates as bidentate ligands. As pointed out early,\(^3\) multiple isomers of metal–ligand complexes may lead to ambiguous interpretations regarding the conformation of the complex responsible for observed activity. Nevertheless, multiple conformers from metal coordination also generate molecular diversity with enhanced potential for sampling biologically relevant conformations in the context of a combinatorial library.

Comparing the different methods of analysis used, ESI-MS showed advantages in characterizing a series of ligands for metal coordination. Among them, direct observation of complex speciation and ready comparison of relative ligand affinity by ESI-MS were significant, supporting the use of ESI-MS for screening metal-binding libraries. It is widely recognized that gas-phase information obtained from ESI-MS accurately reflects noncovalent reactions in solution, but it is noteworthy that different analytical methods may lead to different observations due to different conditions for analysis. For example, compared to high concentrations used in NMR studies, the low concentration of ligands in the gas phase for ESI-MS might minimize intermolecular reactions. Moreover, solubility is an important factor in ESI-MS analysis that may preclude identification of some metal complexes insoluble in aqueous methanol or acetonitrile solutions. For a comprehensive understanding of ligand behavior in metal coordination, a combination of different analytic approaches remains essential.

**CONCLUSIONS**

Peptide amide bonds were successfully modified to bear \( N \)-hydroxyl groups for metal coordination. An array of model \( N,N' \)-dihydroxypeptides were designed and synthesized for probing the relation between structure and metal binding. The strategies for synthesis both in solution and solid support presented here are amenable to the construction of metal-binding peptides libraries. As demonstrated by ESI-MS, high iron-binding affinity and selectivities were attainable with \( N,N' \)-dihydroxypeptides. Compounds \( P8 \) and \( P9 \) are two representatives of \( N,N' \)-dihydroxypeptides capable of forming complexes with Fe(III) with high affinity and selectivity due to coordination of the bidentate hydroxamate groups with Fe(III) consistent with ligand architecture. Cyclic compound \( P8 \) is especially attractive as a robust and compact metal-binding scaffold for combinatorial chemistry. By variation of the amino acid residues, a metal-binding library could be constructed utilizing this scaffold. Moreover, the structure-metal binding relationship revealed here should facilitate further molecular design and fine-tuning of novel metal-binding ligands with altered or enhanced function.

**EXPERIMENTAL**

**General**

\(^1\)H-NMR spectra were recorded on Varian HG-300 (300 MHz) or Varian Inova-600 (Varian Inc., Palo Alto, CA) spectrometer (Table III). Routine positive ESI-MS experiments were performed on a Waters ZQ mass spectrometer at cone voltage 60 kV. For all measurements, the test solutions were injected at a flow rate of 10 \( \mu \)L/min for 2 min. UV/vis measurements utilized a Cary model 3E spectrophotometer. Chromatographic purifications were performed by

![FIGURE 14](image-url) Schematic representation of three possible complexes of \( N,N' \)-dihydroxypeptide ligands with Fe(III). As detected by ESI-MS, the size of the linker between the two hydroxamate groups dominates the coordination mode and a two amino acid linker favors intramolecular coordination.
Table III  Some $^1$H-NMR Data for the Key Intermediates and Final Products

**P3: H–Val–Ψ[CON(OH)]–Phe–Ala–Leu–NHOH**

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<tr>
<td>Phe</td>
<td>5.09</td>
<td>3.18, 3.09</td>
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<tr>
<td>Ala</td>
<td>8</td>
<td>4.31</td>
<td>1.18</td>
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<tr>
<td>Leu</td>
<td>7.99</td>
<td>4.19</td>
<td>1.51</td>
<td>1.4</td>
<td>0.87, 0.83</td>
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**P1: H–Val–Ψ[CON(OH)]–Gly–Ala–Leu–NHOH**

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<td>8.14</td>
<td>4.34</td>
<td>1.16</td>
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<td>Leu</td>
<td>8</td>
<td>4.18</td>
<td>1.51</td>
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**P4: H–Val–Ψ[CON(OH)]–Phe–Ala–Pro–Leu–NHOH**

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**P5: H–Leu–Ψ[CON(OH)]–Phe–Ala–Leu–Ψ[CON(OH)]–Phe–Ala–OH**

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**P6: cyclo[Leu–Ψ[CON(OH)]–Phe–Ala–]$_2$**

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N-Benzyloxy-Glycine tert-Butyl Ester (1)

To a solution of BzONH₂ (2.71 g, 22.0 mmol) and DIEA (1.55 g, 12.0 mmol) in THF (20 mL) was added dropwise tert-butyl bromoacetate (2.15 g, 11.0 mmol) in THF (15 mL). The resulting solution was refluxed under nitrogen atmosphere for 8 h. After the solvent was removed under reduced pressure, the residual oil was dissolved in CH₂Cl₂ (20 mL); successively washed with H₂O, 5% aqueous citric acid, 5% aqueous NaHCO₃, and brine; dried over Na₂SO₄; and filtered. Evaporation of the solvent under reduced pressure gave a crude product, which was then purified by flash chromatography on silica gel using hexane/CH₂Cl₂ (100:0, 100:20, 100:40, 60:40) as eluent to afford 2.22 g (65% yield) of 1 as an oil.

N-Benzyloxy-Alanine tert-Butyl Ester (2)

To a stirred and cooled (~15°C) solution of tert-butyl (R)-(−) lactate (2.0 g, 13.68 mmol) in CH₂Cl₂ (15 mL), was added dropwise a solution of trifluoromethanesulfonic anhydride (4.63 g, 16.41 mmol) in CH₂Cl₂ (10 mL), following by adding 2,6-lutidine (1.80 g, 16.80 mmol). After 30 min, O-benzyl-hydroxyamine (2.5 g, 20.3 mmol) was added and the reaction mixture was stirred at 0°C for 30 min and room temperature for 2 h. The solvent was removed, and the residue was dissolved in CH₂Cl₂; washed with H₂O, 5% aqueous citric acid, 5% aqueous NaHCO₃, and brine; dried over Na₂SO₄; and filtered. Evaporation of the solvent under reduced pressure gave a crude product, which was then purified by flash chromatography on silica gel using hexane/CH₂Cl₂ (100:0, 100:20, 80:20) as the eluent to afford the title compound 2 as an oil (1.8 g, 53%).

d-3-Phenyllactic Acid Allyl Ester (4)

To a stirred solution of d-3-phenyllactic acid (3.50 g, 21.06 mmol) in 5% NaHCO₃ (36 mL) was added allyl bromide (2.80 g, 23.14 mmol) and Aliquat 336 (8.51 g, 21.06 mmol) in CH₂Cl₂ (40 mL). The reaction mixture was stirred vigorously for 120 h. The organic layer was separated and the aqueous layer was extracted with CH₂Cl₂ (2 × 10 mL). The combined CH₂Cl₂ was dried over anhydrous K₂CO₃, concentrated, and purified by flash column chromatography (CH₂Cl₂/hexane, 100:0, 100:20, 100:40, 50:50) to afford the title compound 4 as a colorless oil (3.9 g, 89% yield).
**N-Benzylxoy-Phenylalanine Allyl Ester (5)**

The title compound was prepared from 4 (3.0 g, 14.55 mmol) under the same conditions described for 2. The crude product was purified by flash column chromatography on silica gel using CH2Cl2/hexane (100:0, 100:40, 100:60, 40:60) as the eluent to afford 5 (2.5 g, 55% yield).

**N-Benzylxoy-Phenylalanine (3)**

To a mixture of 5 (2.0 g, 6.42 mmol) and tetrakis(triphenylphosphine) palladium(0) (0.74 g, 0.64 mmol) in THF (10 mL) under a nitrogen atmosphere was added morpholine (6.6 g, 75.75 mmol). The reaction mixture was stirred at room temperature for 8 h. The solvent was removed under reduced pressure and the residue was dissolved in 5% NaHCO3 and filtered; the filtrate was acidified with 1N HCl. The precipitate was collected by filtration and dried to afford 6a (1.75 g, 75% yield). This crude product was used without further purification. Fifty milligrams of the crude product was further purified using HPLC to give 38.2 mg of pure 3. ES-MS: Observed for [MH]+: m/z 272.15.

**Fmoc-Val-Ψ[CON(OBz)]-Gly-OBut† (6a)**

To a stirred solution of 1 (1.0 g, 4.21 mmol) and Fmoc-Val-Cl (1.80 g, 5.03 mmol) in 15 mL of toluene was added AgCN (0.70 g, 5.23 mmol). The insoluble material was filtered off, and the solvent was removed under reduced pressure. The residue was taken up in CH2Cl2 and purified by flash column chromatography on silica gel using CH2Cl2/CH3OH (100:0, 100:0.5, 100:1) to afford 6a (1.75 g, 75%). ES-MS: Observed for [MH]+: m/z 559.31.

**Fmoc-Val-Ψ[CON(OBz)]-Ala-OBut† (6b)**

Using the same procedure as described for 6a, the title compound was prepared from 2 (1.0 g, 3.98 mmol) and Fmoc-Val-Cl (1.44 g, 4.02 mmol) and AgCN (0.66 g, 4.94 mmol). Purification by flash column chromatography using CH2Cl2/CH3OH (100:0, 100:0.5, 100:1) as the eluent afforded 1.64 g (72%) of 6b as a white solid. ES-MS: Observed for [MH]+: m/z 573.27.

**Ψ(OBz)-Phe-Ala-OBut† (10)**

To a stirred and cooled solution of 3 (1.0 g, 3.69 mmol), HOBT (0.50 g, 3.69 mmol), H-Ala-OBut†-HCl (0.81 g, 4.46 mmol), and N-ethylmorpholine (0.51 g, 4.43 mmol) in DMF (15 mL) was added EDCI (0.88 g, 4.59 mmol). The reaction mixture was stirred at room temperature for overnight. After the solvent was removed under reduced pressure, the residue was dissolved in CH2Cl2 (25 mL), successively washed with H2O, 5% aqueous citric acid, and 5% aqueous NaHCO3, and brine, dried over Na2SO4, and filtered. Evaporation of the solvent under reduced pressure gave a crude product that was purified by flash chromatography on silica gel using CH2Cl2/CH3OH (100:0, 100:0.5, 100:0.5) as the eluent to afford 1.21 g (82%) of 10 as a white solid.

**Fmoc-Leu-Ψ[CON(OBz)]-Phe-Ala-OBut† (11)**

The title compound was prepared from 10 (2.0 g, 5.02 mmol), Fmoc-Leu-Cl (2.24 g, 6.02 mmol), and AgCN (0.82 g, 6.12 mmol) using the same procedure described for 6. Purification by flash column chromatography using CH2Cl2/CH3OH (100:0, 100:0.5, 100:1) as the eluent afforded 3.1g (84%) of 11 as a white solid. ES-MS: Observed for [MH]+: m/z 734.39.

**Fmoc-Leu-Ψ[CON(OBz)]-Phe-Ala-Pro-OBut† (12)**

Compound 11 (2.0 g, 2.73 mmol) was dissolved in TFA (15 mL), stirred for 20 min, concentrated, and dried under vacuum overnight. The obtained acid was dissolved in DMF (15 mL) together with Pro-OBut† (0.56 g, 3.29 mmol) and HOBT (0.44 g, 3.25 mmol). To this stirred and cooled (0°C) solution, EDCI (0.63 g, 3.29 mmol) was added. The reaction mixture was stirred at room temperature, concentrated under vacuum and dissolovd in CH2Cl2 (30 mL). The solution was washed successively with H2O, 5% aqueous citric acid, 5% aqueous NaHCO3, and brine; dried over Na2SO4; and filtered. Evaporation of the solvent gave a crude product that was purified by flash chromatography on silica gel using CH2Cl2/CH3OH (100:0, 100:0.5, 100:1:1) as the eluent afforded 3.1g (84%) of 12 as a white solid. ES-MS: Observed for [MH]+: m/z 831.45.

**Fmoc-Leu-Ψ[CON(OBz)]-Phe-Ala-Pro2-OBut† (15)**

Compound 12 (0.5 g, 0.60 mmol) was dissolved in TFA (8 mL), stirred for 20 min, concentrated, and dried under vacuum to afford the acid intermediate 13. Compound 12 (0.5 g, 0.60 mmol) was dissolved in 20% piperidine/DMF (10 mL) solution, stirred for 15
min, concentrated under vacuum, treated with hexane (3 × 5 mL). The unsoluble residue was dried under vacuum to afford the amine intermediate 14. To a stirred solution of 13 and 14, HOBT (0.10 g, 0.74 mmol), and PyBOP (0.38 g, 0.73 mmol) in DMF (10 mL) at room temperature was added DIEA (0.19 g, 1.47 mmol). The reaction mixture was stirred for 8 h and concentrated under vacuum. The residue was dissolved in CH2Cl2; successively washed with H2O, 5% aqueous citric acid, 5% aqueous NaHCO3; and brine, dried over Na2SO4; and filtered. The solvent was removed under reduced pressure to give a crude product that was purified by flash chromatography on silica gel using CH2Cl2/CH3OH (100:0, 100:0.5, 100:1, 100:1.5, 100:2, 100:2.5) as the eluent to afford 0.45 g (55%) of 15 as a white solid. ES-MS: Observed for [MH]+: m/z 1365.63.

Cyclo{Leu–Ψ[CON(OBz)]–Phe–Ala–Pro}2 (17)

Compound 15 (0.20 g, 0.15 mmol) was dissolved in TFA (5 mL), stirred for 20 min at room temperature, concentrated, and dried under vacuum. This obtained acid intermediate was dissolved in 20% piperidine/DMF (5 mL), stirred for 15 min, concentrated, and treated with hexane (3 × 5 mL). The unsoluble residue was dissolved in DMF (800 mL). To this stirred solution were added DIEA (38.8 mg, 0.30 mmol), HOBT (20.3 mg, 0.15 mmol), and PyBOP (106.1 mg, 0.20 mmol). The reaction mixture was stirred at room temperature for 72 h, and concentrated. The residue was dissolved in CH2Cl2 (20 mL); successively washed with H2O, 5% aqueous citric acid, 5% aqueous NaHCO3; and brine; dried over Na2SO4; and filtered. Evaporation of the solvent under reduced pressure gave a crude product that was purified by flash chromatography on silica gel using CH2Cl2/CH3OH (100:0, 100:0.5, 100:1, 100:1.5, 100:2, 100:2.5) as the eluent to afford 96.2 mg (60%) of 17 as a white solid. ES-MS: Observed for [MH]+: m/z 1069.00.

Cyclo{Leu–Ψ[CON(OH)]–Phe–Ala–Pro}2–OH• TFA (P7)

Compound 15 (100 mg, 0.073 mmol) was similarly deblocked first with TFA and then 20% piperidine as described for 17. The obtained crude material was dissolved in cooled CH3OH (10 mL). To this stirred solution was added 5% Pd/C (35 mg), followed by ammonium formate (100 mg, 1.59 mmol). The mixture was stirred at room temperature for 4 h, filtered, concentrated, and purified by preparative HPLC (CH3CN/H2O gradient, 5–80%; 30 min) to afford P7 (23.1 mg, 31%) as a white solid. ES-MS: Observed for [MH]+: m/z 907.71.

Cyclo{Leu–Ψ[CON(OH)]–Phe–Ala}2 (19)

The title compound was prepared from 18 (0.20 g, 0.17 mmol) using the same procedure described for 17. Purification by flash chromatography on silica gel using CH2Cl2/CH3OH (100:0, 100:0.5, 100:1, 100:1.5, 100:2) as the eluent to afford 70.0 mg (47%) of 19 as a white solid. ES-MS: Observed for [MH]+: m/z 875.76.

H–{Leu–Ψ[CON(OH)]–Phe–Ala–Pro}2–OH (P5)

Using the same procedure as described for P7, the title compound (18.3 mg, 26%) was prepared from 18 (100.0 mg, 0.085 mmol). ES-MS: Observed for [MH]+: m/z 713.56.

Cyclo{Leu–Ψ[CON(OH)]–Phe–Ala}2 (P6)

The title compound was prepared from 19 (70.0 mg, 0.08 mmol) using the same procedure as described for P8 (15.0 mg, 27%). ES-MS: Observed for [MH]+: m/z 695.43.
**Fmoc–Val–Ψ[CON(OBz)]–Phe–Ala–OBut**

The title compound was prepared from 10 (1.0 g, 2.51 mmol), Fmoc–Val–Cl (1.08 g, 3.02 mmol), and AgCN (0.41 g, 3.06 mmol) using the same procedure as described for 6a. Purification by flash column chromatography on silica gel using CH$_2$Cl$_2$/CH$_3$OH (100:0, 100:0.5, 100:1) as the eluent afforded 1.43 g (79%) of 20 as a white solid.

**Fmoc–Val–Ψ[CON(OBz)]–Phe–Ala–Leu–NHOBz**

Compound 20 (1.0 g, 1.39 mmol) was dissolved in TFA (10 mL), stirred for 20 min, concentrated, and dried under vacuum. The obtained acid intermediate was dissolved in DMF (15 mL) together with H–Leu–NHOBz (0.394 g, 1.67 mmol) and HOBT (0.23 g, 1.70 mmol), and dissolved. To this stirred solution at 0°C was added EDCI (0.33 g, 1.72 mmol). The reaction mixture was stirred for 4 h. The solvent was removed under vacuum, and the residue was dissolved in CH$_2$Cl$_2$ (20 mL); successively washed with H$_2$O, 5% aqueous citric acid, 5% aqueous NaHCO$_3$, and brine; dried over Na$_2$SO$_4$; and filtered. Evaporation of the solvent under reduced pressure gave a crude product that was then purified by flash chromatography on silica gel using CH$_2$Cl$_2$/CH$_3$OH (100:0, 100:0.5, 100:1) as the eluent to afford 0.74 g (60%) of 10 as a white solid. ES-MS: Observed for [MH]$^+$: m/z 883.48.

**H–Val–Ψ[CON(OH)]–Phe–Ala–Leu–NHOH**

Compound 21 (0.5 g, 0.57 mmol) was dissolved in 20% piperidine/DMF (10 mL), stirred for 15 min, concentrated, and treated with hexane (3 × 10 mL). The insoluble residue was dissolved in CH$_3$OH (20 mL). To this stirred solution was added 5% Pd/C (100 mg), followed by ammonium formate (300 mg, 4.76 mmol). The mixture was stirred at room temperature for 4 h, filtered, concentrated, and purified by preparative HPLC (CH$_3$CN/H$_2$O gradient, 5–60%, 30 min). The pure fractions were identified by ESI-MS, combined, and lyophilized to afford P3 (0.14 g, 41%). ES-MS: Observed for [MH]$^+$: m/z 480.64.

**H–Leu–Ψ[CON(OH)]–Phe–Ala–Pro–Leu–NHOH**

The title compound was prepared from 12 (500 mg, 0.60 mmol) via Fmoc–Leu–Ψ[CON(OBz)]–Phe–Ala–Pro–Leu–NHOBz using the similar procedure as described for P3. Purification by HPLC afforded 127.0 mg in an overall yield of 30%. ES-MS: Observed for [MH]$^+$: m/z 591.47.

**Synthesis of H–Leu–Ψ[CON(OH)]–Ala–Ala–Pro–Leu–NHOH**

The peptide was prepared manually on N-Fmoc-hydroxylamine 2-chlorotrityl resin (0.7 mmol/g, 1 g). The resin was preswollen in DCM for 1 h and then deblocked with 20% piperidine in DCM for 20 min. After washing with DCM (5 × 2 mL) and DMF (5 mL × 3), the first residue Fmoc–Leu was attached by reacting the resin with a solution of Fmoc–Leu (5 equiv), HATU (4 equiv); and DIPEA (4 equiv) in DMF for 18 h. The chain elongation was realized using a conventional Fmoc–AA/HBTU/HOBT/DIEA coupling protocol. Ψ[N(OH)]–Ala was incorporated into resin-bound peptide by using a mixture of Ψ[N(OH)]–Ala (3 equiv)/DIC (3 equiv)/HOBT (3 equiv) for 16 h. After it was filtered and washed with DMF (5 mL × 3), the resulting resin-bound peptide was reacted with a solution of Fmoc–Leu (3 equiv)/HATU (2 equiv)/DIEA (2 equiv) in DMF for another 16 h. It was filtered, washed with DMF, and deblocked with 20% piperidine/DMF for 20 min. The resin was treated with TFA:water:trisopropylsilane (95:2.5:2.5) (0.5 h × 3) to provide the crude product containing 5 mg of metal (130 mg), that was deblocked similarly using ammonium formate in the presence of Pd/C (5%) in methanol as described for compound P10. The crude peptide hydroxylamide was purified by semipreparative HPLC to afford the desired compound (57 mg, 13%). ES-MS: Observed for [MH]$^+$: m/z 515.33.

**Preparation of Test Solutions of Metal Complex for ESI-MS, HPLC, and UV-Vis Analysis**

Stock solutions (1 mM) of the peptide ligands and some metal salts including CuSO$_4$ · 5H$_2$O, MnCl$_2$, ZnSO$_4$ · 7H$_2$O, NiCl$_2$ · 6H$_2$O, Co(NO$_3$)$_2$ · 6H$_2$O, FeSO$_4$ · 7H$_2$O, CdCl$_2$, and Fe(NO$_3$)$_3$ · 9H$_2$O, Ga$_2$(SO$_4$)$_3$ in methanol or water were first prepared. Typically, test solutions (300 μL each) containing 100 μM of ligands were then prepared by mixing 30 μL of a ligand stock solution and 30 μL of a metal stock solution in 240 μL of methanol. Different aliquots of a metal solution were added to afford test solutions with different metal/ligand ratios for titration studies while different metal solutions were added into one solution for competition experiments. All the test solutions were shaken for 8 h to insure equilibrium before subjecting to ESI-MS analysis.
Preparation of 20@Ga$^{3+}$ Complex for 2D $^1$H-NMR Studies

The amount of 3.8 mg of Ga$_2$(SO$_4$)$_3$ and 4.0 mg of 20 (i.e., 8 in Table I) were first dissolved in 7 mL of CH$_3$CN/H$_2$O (7:3). The resulting solution was stirred at room temperature overnight and lyophilized to afford a dry white solid that was dissolved in d$_6$-DMSO and filtered for $^1$H-NMR experiments.

$^1$H-NMR Experiments

NMR spectra were recorded with a Varian Inova-600 (Varian Inc., Palo Alto, CA) spectrometer and the data were processed with VNMR software. The TOCSY spectra were recorded using a MELV-17 mixing sequence of 120 ms flanked by two 2 ms trim pulses. Phase-sensitive 2D, spectra were obtained by employing the hypercomplex method. A total of 256 $\times$ 2048 data matrix with 16 scans per t1 increment were collected. Gaussian and sine-bell apodization functions were used in weighting the t2 and t1 dimensions, respectively. After two-dimensional Fourier transformation, the 2048 $\times$ 2048 frequency domain representation was phase and baseline corrected in both dimensions. The NOESY spectrum resulted in a 256 $\times$ 2049 data matrix with 32 scans per t1 increment. Spectra were recorded with 250 and 420 ms mixing time. The use of a 420 ms mixing time (at 298 K) afforded a better result. The hypercomplex method was used to yield phase-sensitive spectra. The time domain data were zero filled to yield a 2048 $\times$ 2048 data matrix and were processed in a similar way as the 2D TOCSY spectrum described above.

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