Design, Synthesis, and Metal Binding of Novel *Pseudo*-Oligopeptides Containing Two Phosphinic Acid Groups

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ABSTRACT:

Phosphinic compounds have potential as amide-bond mimetics in the development of novel peptidomimetics, enzyme inhibitors, and metal-binding ligands. Novel pseudo*-oligopeptides with two phosphinic acid groups embedded in the peptide backbone serving as amide-bond surrogates, Ψ[P(O,OH)—CH_2], were targeted. A series of linear and cyclic* pseudo*-oligopeptides with two phosphinic acid groups arrayed at different positions in the peptide sequence were designed, including Ac—Phe—{(R,S)—AlaΨ[P(O,OH)—CH_2]Gly}$_2$—NH_2 (P2), Ac—NH—(R,S)—AlaΨ[P(O,OH)—CH_2]Gly—Phe—(R,S)—AlaΨ[P(O,OH)—CH_2]Gly—NH_2 (P3), Ac—NH—(R,S)—AlaΨ[P(O,OH)—CH_2]Gly—Phe—Phe—(R,S)—AlaΨ[P(O,OH)—CH_2]Gly—NH_2 (P4), cyclo{NH—(R,S)—AlaΨ[P(O,OH)—CH_2]Gly—Phe}$_2$ (P5), and cyclo[NH—(R,S)—AlaΨ[P(O,OH)—CH_2]Gly—Phe—Phe]$_2$ (P6). They were synthesized via conventional Fmoc chemistry on solid support utilizing Fmoc-protected phosphinic acid-containing* pseudo*-dipeptide fragment, i.e. Fmoc—(R,S)—AlaΨ[P(O,OCH_3)—CH_2]Gly—OH. The* pseudo*-peptides containing two phosphinic acid groups exhibited the highest binding affinity and selectivity for Fe(III) among the 10-metal ions screened by ESI-MS analysis—Cu(II), Zn(II), Co(II), Ni(II), Mn(II), Fe(II), Fe(III), Al(III), Ga(III), and Gd(III). P4 and P6 with 11-atom linkages between the two phosphinic acids preferred intramolecular metal binding to form 1:1 ligand/metal complexes. As revealed by competition experiments, P4 showed the highest relative binding affinity among the six compounds tested. Noteworthy, P4 also showed higher relative binding affinity than similar dihydroxamate-containing* pseudo*-peptides reported previously. The novel structural prototype and facile synthesis along with selective and potent Fe(III) binding strongly suggest that* pseudo*-peptides containing the two or more phosphinic groups as amide-bond surrogates deserve further exploration in medicinal chemistry.*

Keywords: pseudopeptide bond; phosphinic acid; metal binding

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INTRODUCTION

Phosphinic acid functionalities (phosphinates, R_1—P(O,OH)—R_2, R_1, R_2 alkyl or aryl) derived from phosphinic acid $H_2P(O,OH)$ have been widely used in design of bioactive phosphinic *pseudo*-peptides and peptidomimetics as they can serve as isosteres or surrogates of peptide bonds. More especially, they can mimic the transition state of enzyme-catalyzed peptide-bond hydrolysis. Phosphinic acid compounds have shown advantages in both chemical and enzymatic stabilities over other phosphorus analogs, such as phosphonates [—P(O,OH)—O—] and phosphonamides [—P(O,OH)—NH—]. The incorporation of the phosphinic moiety has provided an efficient approach for discovering and developing novel inhibitors targeting such diverse enzymes including thermolysin,[1,2] matrix metalloproteinases,[3–11] *N*-acetylated-α-linked acidic dipeptidase (NAALADase),[12–15] angiotensin converting enzyme,[16,17] HIV-1 protease,[18] renin,[19,20] thrombin,[21] farnesyltransferase,[22] glutamine synthetase,[23] folylpoly-gamma-glutamate synthetase,[24,25] etc. In our efforts to discover and develop novel peptidomimetics that selectively chelate metals, we became interested in pseudopeptides containing two phosphinic acid moieties.[26] To the best of our knowledge, the phosphinic-containing compounds reported in the literature to date contain only one phosphinic acid group, and peptides containing two phosphinic compounds have not yet been reported by others. Thus, our work has explored the construction and properties of such di-substituted compounds.

Metals play very important roles in living systems as the three-dimensional structures and enzymatic activities of many proteins are dependent on bound metals. A compound might exhibit significant pharmacological activity by interacting with the metal centers of proteins to influence both their structures and functions. Detailed knowledge of the metal-binding selectivity of a compound is essential for understanding of their mechanism of action. The rational design of potent, selective, and specific enzyme inhibitors with low toxicity also requires such knowledge. Phosphorus compounds exhibited significant metal-binding properties[27] and their uses in metal chelators as MRI contrast agents and others have also been described.[28] This research investigates possible synergistic effects of two phosphinic groups in a peptide framework on their metal-binding properties.

A series of such linear and cyclic *pseudo*-oligopeptides were synthesized based on a phosphinic amino-acid or *pseudo*-dipeptide module. Their metal-binding properties were screened by electrospray ionization mass spectrometry (ESI-MS) analysis. The results clearly demonstrated the high Fe(III)-binding affinity and selectivity of such di-phosphinic acid-containing pseudo-peptides with 11-atom linkage between the two phosphinic acids in the backbone.

RESULTS AND DISCUSSION

Molecular Design

As shown in Figure 1, we have focused on a divalent system where the two phosphinic acid groups were embedded in the peptide backbone with different linkages. Because of the key roles of the phosphinic acid functionality, such a divalent system should provide a platform to study possible synergism of the two phosphinic acid groups as a function of structural modification.

α-Functionalized phosphinic acid derivatives such as α-hydroxyl- and α-amino-phosphinic analogs have received considerable attention in medicinal chemistry and related fields.[29–31] We have used phosphinic acid-containing α-amino acids as building blocks for the construction of the di-phosphinic acid-containing pseudopeptides because of their facile synthesis and ready incorporation into peptides. In the present work, a phosphinic amino acid analog, NH_2—(*R,S*)—$CH(CH_3)$—P(O,OH)—CH_2—CH_2—COOH or *pseudo*-dipeptide H—(*R,S*)—AlaΨ[P(O,OH)CH_2]Gly—OH, was used.

As shown in Table I, several dimeric *pseudo*-peptides including linear and cyclic analogs were designed with the two phosphinic groups embedded in the backbone and their intergroup distance varied by different peptide linkages including one or two phenylalanine residues. The design provided a convenient approach for the initial construction of complex molecules based on a single phosphinic acid building block. To highlight the role of phosphinic acid group in metal binding, the linear *pseudo*-peptides were amidated at both N- and C-termini to minimize the influences of charged C- and N-termini on metal-binding effects. In addition, such amidation may improve related functions such as cellular permeability, enzymatic stability, and biological activity as often reported for many other biologically active peptides.

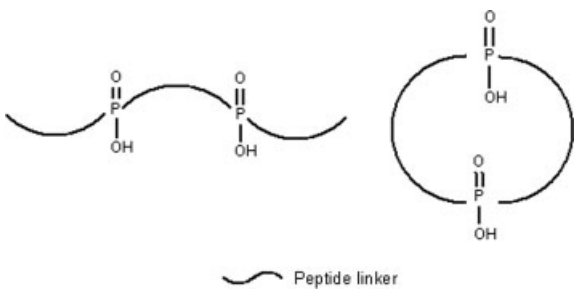


FIGURE 1 Schematic linear and cyclic *pseudo*-peptides containing two phosphinic acids in the backbones.

Table I Designed Di-Phosphinic Acid-Containing Pseudopeptides

Entry No.	Structures
P1	Ac—Phe—(*R*,*S*)—AlaΨ[P(O,OH)CH_2]Gly—NH_2
P2	Ac—Phe—[(*R*,*S*)—AlaΨ[P(O,OH)CH_2]Gly]$_2$—NH_2
P3	Ac—(*R*,*S*)—AlaΨ[P(O,OH)CH_2]Gly—Phe—(*R*,*S*)—AlaΨ[P(O,OH)CH_2]Gly—NH_2
P4	Ac—(*R*,*S*)—AlaΨ[P(O,OH)CH_2]Gly—Phe—Phe—(*R*,*S*)—AlaΨ[P(O,OH)CH_2]Gly—NH_2
P5	Cyclo[(*R*,*S*)—AlaΨ[P(O,OH)CH_2]Gly—Phe]$_2$
P6	Cyclo[(*R*,*S*)—AlaΨ[P(O,OH)CH_2]Gly—Phe—Phe]$_2$

Cyclization has been widely used to restrict conformational freedom and gain substantial entropy of binding in molecular recognition by preorganization. Other potential effects of cyclization including reverse-turn mimicry, metabolic stability, enhanced binding affinity, selectivity, and specificity. Cyclization of *pseudo*-peptides with two phosphinic groups embedded in the backbone is a first step in discovering and developing novel, diverse, and optimal enzyme inhibitors as potential therapeutics and/or imaging agents.[30–32]

Synthesis

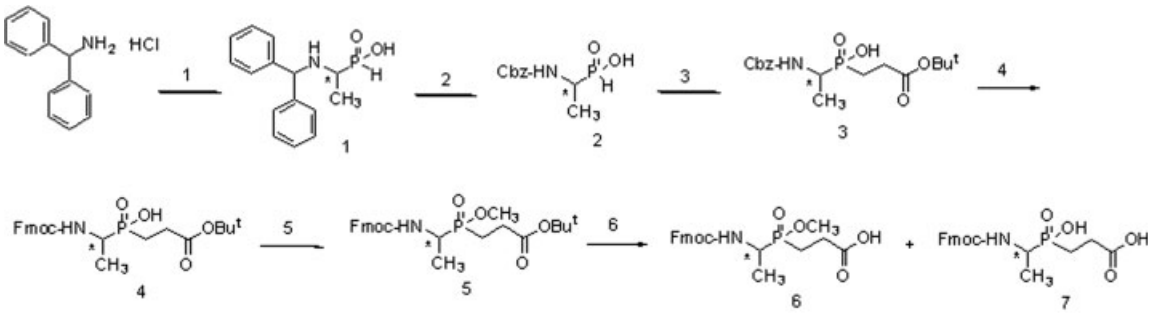
In the synthesis of phosphinic compounds, a P—C bond can be formed via a Michael-type addition of a substituted bis (trimethylsilyl)phosphonite to an acrylate derivative. Different strategies for synthesis of phosphinic *pseudo*-peptides have been developed. For example, α-amino-phosphinic acid pseudo-dipeptides can first be prepared as building blocks in solution for incorporation into peptides as reported by Meldal and coworkers.[8,33] Meldal's group also explored direct P—C formation on a solid support during peptide elongation.[34] Even though this method showed some advantages, the relatively harsh conditions for P—C bond formation might generate some undesired side reactions with certain side chains of the peptides and certain peptide-resin linkages. In contrast, solution syntheses can provide a large quantity of α-amino-phosphinic acid pseudo-dipeptides as building blocks conveniently. Another consideration was protection of the phosphinic acid due to its potential reactivity during the coupling reactions. Different ester groups including methyl,[24,26] ethyl, isopropyl,[15,35] benzyl,[13] 1-adamantyl,[33] *tert*-butyl,[13] and TMS[24] have been used to protect a phosphinic acid group. They are usually removed by hydrolysis as one of the final synthetic steps.

As summarized in Figure 1, the Fmoc-protecting α-amino-(*R*,*S*)-phosphinic acid methyl ester analog, i.e. Fmoc—NH—(*R*,*S*)—$CH(CH_3)$—P(O)(OCH_3)—CH_2—CH_2—COOH (6) was initially selected as a building block for incorporation into peptides. Therefore, acetaldehyde was first reacted with both aminodiphenylmethane and 50% aqueous hypophosphorous acid at reflux to form the benzhydryl-protected intermediate (1). Compound 1 was refluxed with concentrated hydrochloric acid to expose the amino group which was then protected with Cbz-Cl in the presence of sodium carbonate in aqueous dioxane to give 2. The Michael addition of 2 to *tert*-butyl acrylate was realized in the presence of bis(trimethylsilyl)acetamide (BSA) to afford 3. The Cbz protecting group of 3 was removed with ammonium formate in the presence of 10% Pd/C in methanol, followed by reaction with Fmoc-Cl in aqueous dioxane solution containing 5% Na_2CO_3 to form 4. Finally, Compound 4 was converted to the corresponding methyl ester (5) with EDCI in methanol.

When deblocking the *tert*-butyl group of 5 with TFA, we found that the methyl ester was also deprotected to give a mixture of Fmoc—NH—(*R*,*S*)—$CH(CH_3)$—P(O)(OCH_3)—CH_2—CH_2—COOH (6) and its acid analog Fmoc—NH—(*R*,*S*)—$CH(CH_3)$—P(O)(OH)—CH_2—CH_2—COOH (7), with a low yield of the desired methyl ester product 6. TFA in dichloromethane (20%) was also tried instead of TFA, but removal of the methyl ester group was similarly observed. In both cases, a longer time-exposure of TFA led to almost complete hydrolysis with 7 as the major product.

The hydrolysis of phosphinate and phosphonate esters can be mediated by both acids and bases in aqueous solutions; the mechanism of TFA-mediated hydrolysis was reported by Reiter and Jones.[36] The hydrolysis of phosphinic acid esters containing a beta-carboxamido group is conveniently and quantitatively effected by treating the ester with 10:90 H_2O:TFA. The rate of hydrolysis is dependent on the electron density of the amide, suggesting the intermediacy of a cyclic imidate structure. In our case, the mechanism of the methyl ester hydrolysis remains unclear as we only used TFA or its diluted solution in DCM without water. The stability of phosphinic acid methyl ester along with its two adjacent amide and acid groups may play certain roles in this promoted hydrolysis.

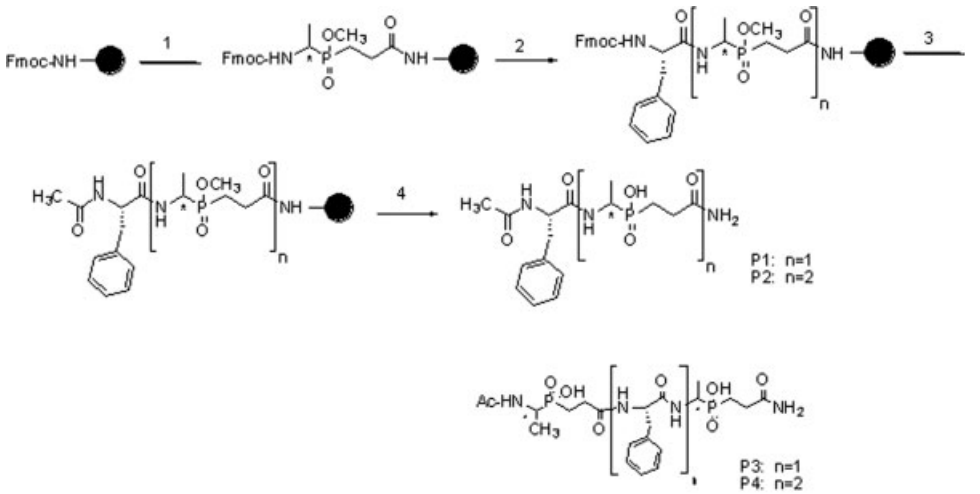
The Fmoc-protected phosphinic acid methyl ester analog 6 was first purified by column chromatograpy and used as a building block for incorporation into peptides on solid support.[26] Compound 6 was attached to a Rink amide resin in the presence of HBTU/HOBT/DIEA in DMF (Scheme 2). The chain was further elongated with Fmoc-Phe via conventional Fmoc chemistry. Compound 6 was also used for an additional coupling to introduce the second phosphinic acid group on the Rink amide MBHA resin. Finally, the resin-bound peptide were acetylated at the N-termini with Ac_2O in the presence of DIEA in DCM. Aqueous TFA (95%) was



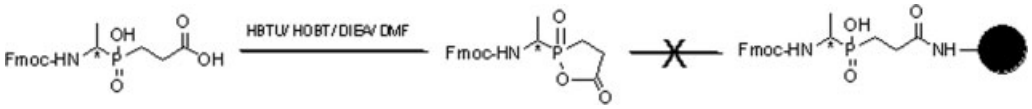
SCHEME 1 Synthesis of Fmoc—(*R*,*S*)—AlaΨ [P(O,OCH$_3$)CH$_2$]Gly—OH analogs.

used to cleave the peptide from the resin. ESI-MS analysis found that the desired products including P1, P2, P3, and P4 along with their methyl ester analogs were obtained with $[MH]^+$ peaks at 370.8, 533.8, 534.0, and 680.8 daltons, respectively. Noteworthy, the TFA cleavage caused the hydrolysis of one or two methyl ester groups. Nevertheless, some of the corresponding mono-methyl esters were also found, so the crude cleaved products were further hydrolyzed using LiOH in aqueous methanol to afford the desired target products.

The low yield and difficult purification of 6 led us to use the deprotected mixture of 6 and 7 directly after TFA deprotection without further purification where similar results were obtained. On the basis of the successful synthesis using a mixture of 6 and 7 described earlier, we tried to elucidate the role of 7 in synthesis of such phosphinic pseudopeptides. Therefore, 7 was further purified by flash column chromatograpy to get rid of any 6 completely, and then used as a building block in the presence of HBTU/HOBT/DIEA in DMF for attachment onto Rink amide resin. Unfortunately, no significant attachment was found by both Kaiser test and the TFA cleavage followed by ES-MS analysis. After detecting the reaction filtrate by ES-MS, we found a peak of high abundance at 387.5 instead of compound 7 in the filtrate. As showed in Scheme 3, we deduced that this peak must be the inner anhydride analog of 7 from its dehydration effected by the coupling agents. The high tendency of dehydration should be ascribed to the high stability of the resulting five-membered inner anhydride ring that finally prevented the carboxylic acid and active ester from attacking the amino group of the resin to form an amide bond. As 7 and its inner anhydride analog can be prepared in high yields conveniently, their potentials in construction of novel phosphinic pseudopeptides and related bioactive molecules deserve further exploration in our future studies.



SCHEME 2 Synthesis of pseudo-peptides containing one or two phosphinic acid groups.

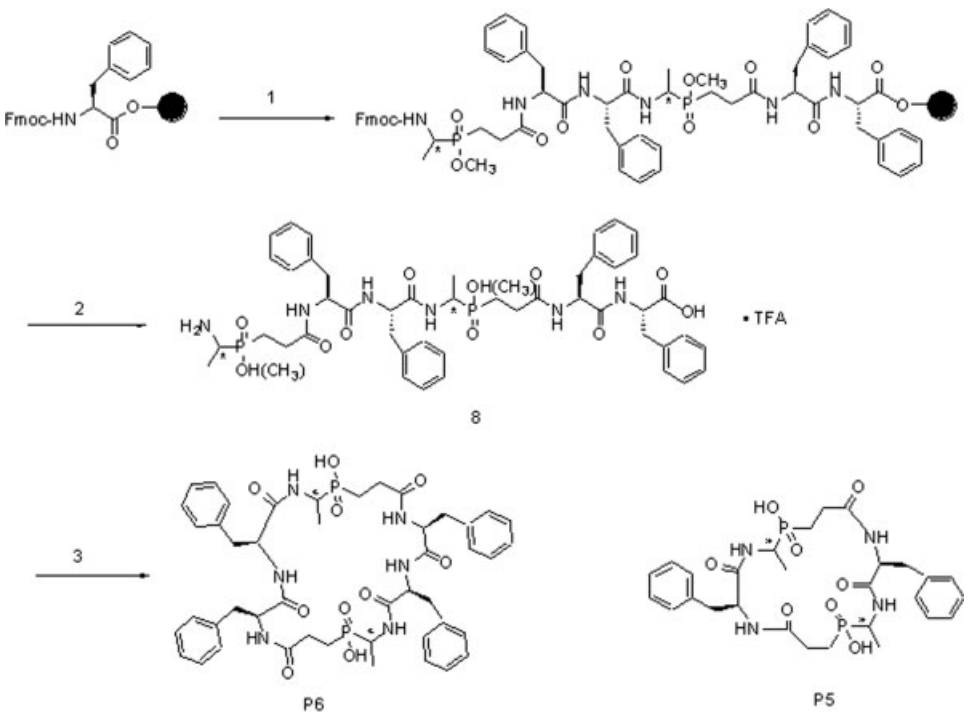


SCHEME 3 The transformation of 7 into its inner anhydride analog by the coupling agents.

Scheme 4 summarizes the synthesis of the two cyclic compounds P5 and P6. The linear di-phosphinate precursor i.e. H—[NH—(*R,S*)—CH(CH_3)—P(O)(OCH_3)—CH_2—CH_2—CO—Phe—Phe]$_2$—OH (8) was similarly assembled from Fmoc-Phe Wang resin and cleaved with TFA from the resin. Similarly, ES-MS analysis showed the cleaved linear products were mixtures of the di-phosphinic acids and their mono and dimethyl ester analogs. TFA cleavage might lead to complete hydrolysis of one phosphinic acid methyl ester especially at an elevated temperature with an extended cleavage time.

The head-to-tail cyclization of 8 was realized in the presence of PyBOP/HOBT/DIEA in a diluted solution of DMF to afford the cyclic product as detected by ES-MS. The crude cyclic product was further hydrolyzed with LiOH in aqueous methanol to give P6 (ES-MS: $[MH]^+$ 914.8). Similarly, P5 (ES-MS: $[MH]^+$ 620.7) was synthesized via its linear precursor mixture of H—[NH—(*R,S*)—CH(CH_3)—P(O)(OH)—CH_2—CH_2—CO—Phe]$_2$—OH and its methyl ester assembled from Fmoc-Phe Wang resin.

Our results justify the direct use of the crude linear precursors for the cyclization. It was suggested that the free phosphinic acid group in the peptide backbone might not interfere the head-to-tail cyclization between C-terminal carboxylic acid and N-terminal amino group under the conditions we used. Previously, there are also some reports on synthesis of phosphinic-peptides without protecting the phosphinic acid groups.[34,37] Compared to a carboxylic acid group, a phosphinic acid in the pseudopeptides synthesized above might be weaker and slower in coupling with an amino group due to its electrophilicity and steric hindrance. This would favor the head-to-tail cyclization while minimizing any amino-to-phosphinic acid reaction. Of course, it is essential to use 2-chlorotrityl resin instead of Wang resin to minimize the hydrolysis of phosphinic acid ester during TFA-mediated cleavage and preparing such fully protected



SCHEME 4 Synthesis of cyclic pseudo-peptides containing two phosphinic-acid groups.

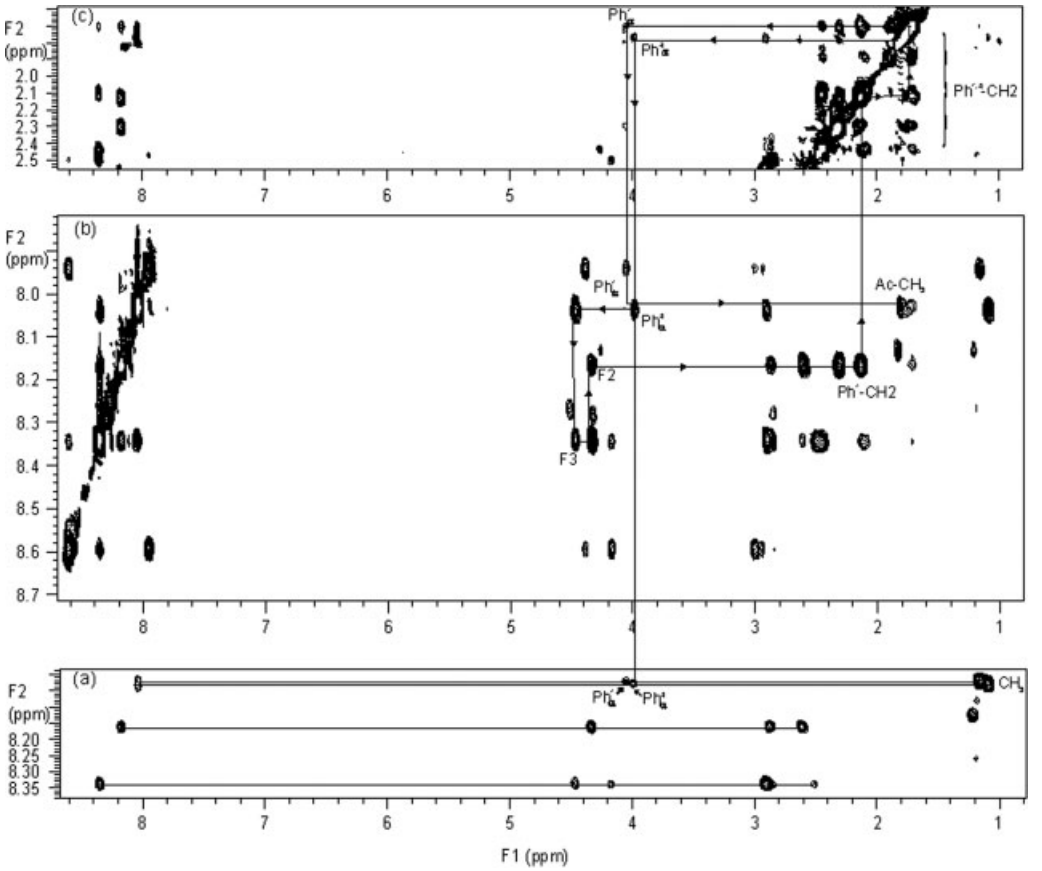


FIGURE 2 Expansion of 600 MHz (a) TOCSY NH-αH, NOESY (b) NH—αH, and (c) Ph1,4—CH_2 region of P4 in DMSO-d_6.

linear phosphinic pseudo-peptides for cyclization and other structural modifications in future.

All the crude products were purified by semi-preparative HPLC using a gradient increase of aqueous acetonitrile eluant containing 0.1% TFA from 10 to 80% over 30 min. Multiple fractions thus collected were found to contain the desired product as identified by ESI-MS. These fractions were further analyzed by analytical HPLC using a gradient increase of aqueous acetonitrile eluant containing 0.1% TFA from 10 to 90% over 10 min. Some fractions showing close retention time were combined for lyophilization. All the final products thus obtained are supposed to exist in acidic forms as they do not contain any free amino groups to form some salts with TFA or phosphinic acid groups.

The stereochemistry of such diphosphinic pseudopeptides should be complex as α-aminomethyl-phosphinic acid obtained in Scheme 1 contains a diastereomeric center at its α-carbon. The phosphorus atom itself can structurally be an asymmetric center, but the prototopic transfer of the acidic proton between the two phosphoryl (P═O) and acidic (P—OH) groups would make rapid interchange of any diastereomeric forms in solution.[38] So the products obtained above might be a mixture of several stereoisomers. They were directly used in the following ^{1}HNMR and metal-binding studies without further clarification of their stereochemistry. It would be significant to separate and identify the stereoisomers,[39] or incorporate optically pure phosphinic acid dipeptide building blocks into the peptides,[40,41] to explore the impact of chirality on the related structures and functions of such diphosphinic *pseudo*-peptides.

^{1}HNMR Spectroscopy

Proton chemical shifts of P4 and P6 were assigned by analysis of TOCSY and NOESY spectra. Both P4 and P6 were expressed in the following simplified forms, i.e. (Ac—Ph1—F2—F3—Ph4—NH_2) (P4) and (cyclo[Ph1—F2—F3—Ph4—F5—F6]) (P6), where F and Ph represent phenylalanine and —NH—(*R,S*)—$CH(CH_3)$—P(O,OH)—CH_2—CH_2—CO— residues, respectively. The Ph1 and Ph4 residues were identified first by spin propagation from amide NH through α and β—CH_3 protons (Figure 2a). The methyl-

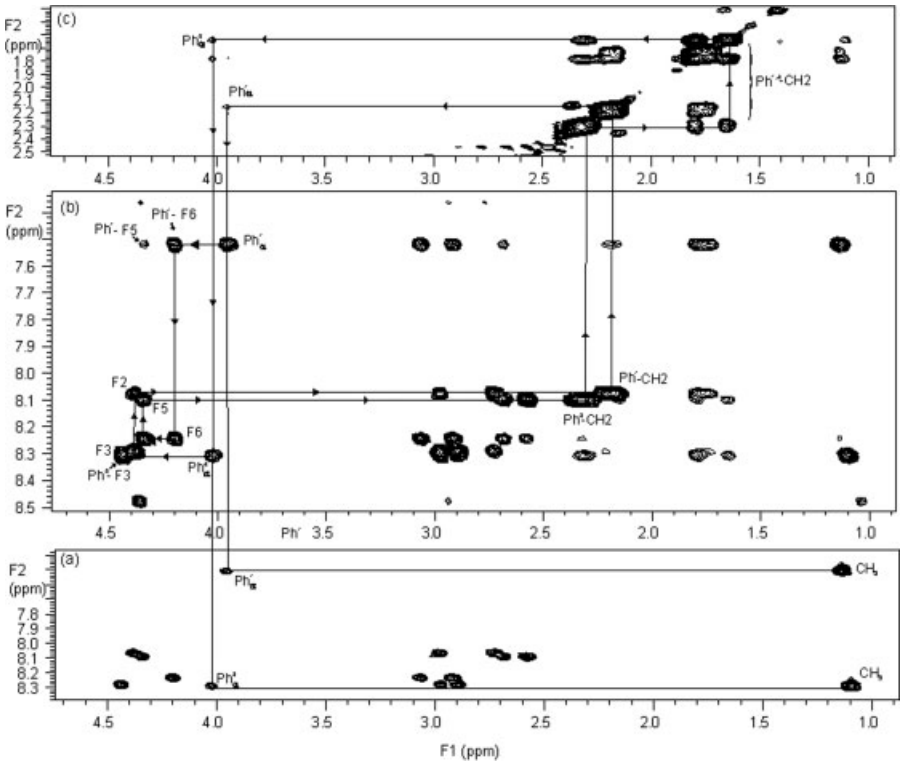


FIGURE 3 Expansion of 600 MHz (a) TOCSY NH—αH, NOESY (b) NH—αH, and (c) Ph1, 4—CH_2 region of P6 in DMSO-d_6.

ene protons show unique resonances between 1.6 and 2.4 ppm (Figure 2c). Sequential assignments were obtained by using the NH—αH fingerprint region of the NOESY spectra. The F—Ph sequential connections were identified by the F(NH)—Ph(CH2) NOE cross peaks (Figure 2b). The structure of Ph residues was confirmed by the observed cross peaks between CH_2 and α protons in CHα—P(O,OH)—CH_2 fragment of Ph1 and Ph4. All the NH(i + 1)—αH(i) and F—NH(i + 1)—Ph—CH_2 ((i) connections between adjacent residues were observed and a continuous path indicates the segment from Ac—Ph1 to Ph4—NH_2 in Figure 1).

Cyclization through the amide bond in sample P6 was evidenced by the observed Ph1 (NH)—F6 (αH) and Ph4(NH)—F3(αH) NOE's (Figure 3b). Complete sequential assignment in the cyclic compound was obtained by the observed F—NH(i + 1)—Ph—CH_2((i)—Ph—αH(i) NOE's in addition to the NH(i + 1)—αH(i) cross peaks in the Ph—F moiety. Chemical shift assignments for each residue are summarized in Table II. As shown in Figure 3, strong NH (i + 1)—αH (i) cross peaks were observed, indicating P6 adopted a *trans*-amide peptide conformation in the 24-membered ring. Furthermore, the presence of Ph1(NH)—F5(αH), (NH(i + 2)—αH(i)), cross peak (Figure 3b) revealed that a turn conformation appeared at the Ph1—F6 linkage. The observed down field shift of Ph4—NH resonance indicates the aromatic ring-current from nearby F5, F3 and F2 residues are effective due to the turn of the peptide near the F6 residue.

Metal-Binding Analysis by ES-MS

ES-MS has been demonstrated to be an efficient, sensitive, and powerful method for screening the relative metal-bind-

Table II Proton Chemical Shifts (ppm) of P4 and P6 in DMSO-d_6

Compounds	NH	αH	βH	CH_2
P4				
Ph1	8.02	4.06	1.17	2.23, 1.72
F2	8.16	4.33	2.88, 2.62	
F3	8.34	4.47	2.91, 2.51	
Ph4	8.08	3.99	1.10	2.32, 1.77
P6				
Ph1	7.51	3.95	1.13	2.19, 1.77
F2	8.06	4.38	2.98, 2.73	
F3	8.28	4.43	2.98, 2.90	
Ph4	8.29	4.02	1.10	2.32, 1.80
F5	8.09	4.34	2.69, 2.58	
F6	8.23	4.20	3.06, 2.92	
7	7.06	3.73		2.38, 1.77

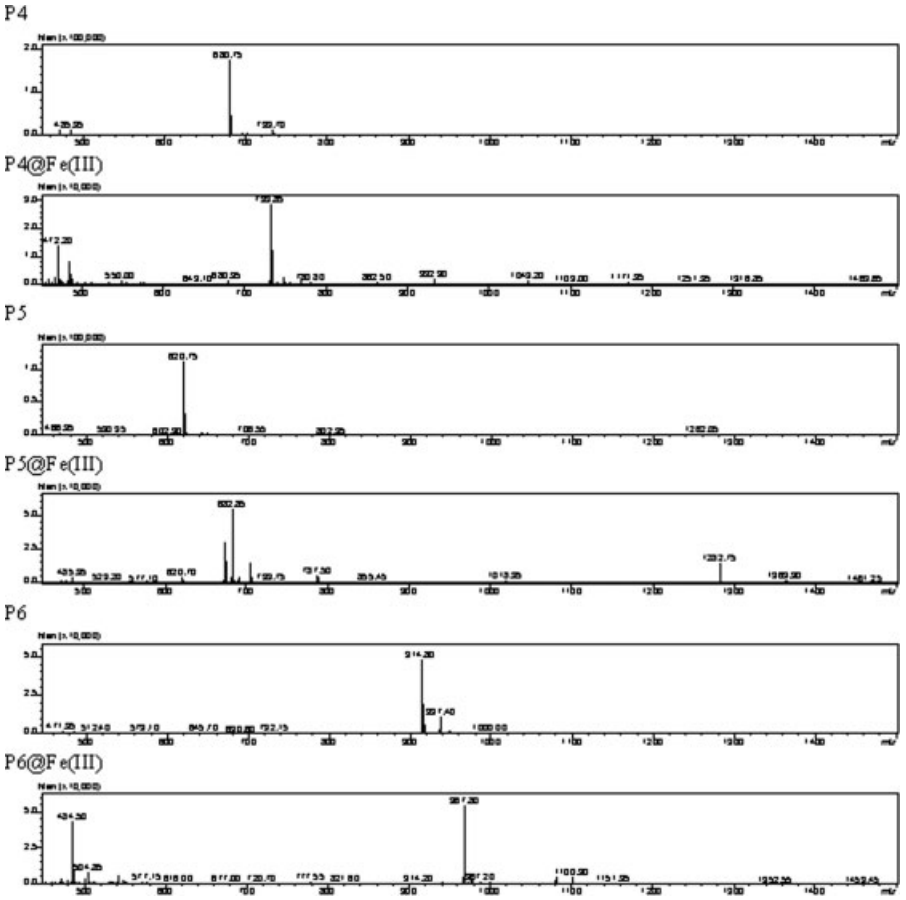


FIGURE 4 ES-MS spectra of P4, P5, and P6 at 100.0 μM with 2 equiv Fe(III) in aqueous acetonitrile.

ing properties of ligands.[42–53] It is especially good for compounds in limited amounts due to enhanced sensitivity. Previously, we have studied the metal-binding properties of dihydroxamate- and trihydroxamate-containing peptides using ES-MS.[54,55] We have extended the ES-MS-based metal-binding assays to study the diphosphinic acid-containing pseudo-peptides. The three compounds, i.e. P4, P5, and P6, were initially chosen for screening with 10 different metal ions, Cu(II), Zn(II), Co(II), Ni(II), Mn(II), Fe(II), Fe(III), Al(III), Ga(III), and Gd(III). Therefore, 10.0 μl (1.0 m*M*) of a ligand solution was mixed with 10.0 μl (2.0 m*M*) of a freshly prepared metal-ion solution. The resulting solution was then diluted to 100.0 μl to make a test solution with the ligand at 100.0 μM and kept at room temperature for 48 h before being subjected to ESI-MS analysis.

Metal-Binding Affinity and Selectivity

The three di-phosphinic acid-containing pseudo-peptides exhibited high binding affinity and selectivity for both Fe(III) and Al(III) compared to the other eight metals tested, leading to the almost complete disappearance of the free ligands in the ES-MS spectra. Figure 4 showed the spectra of P4, P5, P6, and their corresponding Fe(III) complexes. The desired singly charged peaks $[LH]^+$ and $[(L—2H)Fe(III)]^+$ were found to be highly abundant at 680.75 (P4), 733.85 (P4@Fe), 620.75 (P5), 673.60 (P5@Fe), 914.80 (P6), and 967.80 (P6@Fe). Noteworthy, the Fe(III) binding of P5 showed some spectral characteristics different from P4 and P6. Except the desired peak at 673.60 $[(P5—2H)@Fe]^+$, 682.85 was the highest peak observed which might result from hydration of its Fe(III) complex, i.e. $[(P5—2)@Fe@0.5H_2O]^+$.

Previously, we have determined the relative metal-binding affinity of a ligand by calculating the percentage of the binding ligand peaks with the total ligand-related peaks in a positive ES-MS spectrum. Compared to other compounds including P1, P2, P3, and P5, both P4 and P6 showed relatively simple, neat ES-MS spectra. In addition to the singly charged peaks, the corresponding doubly charged peaks $[(L—2H)FeH]^{2+}$ were also observed, but their abundances were low. Some peaks related to a ligand-sodium complex

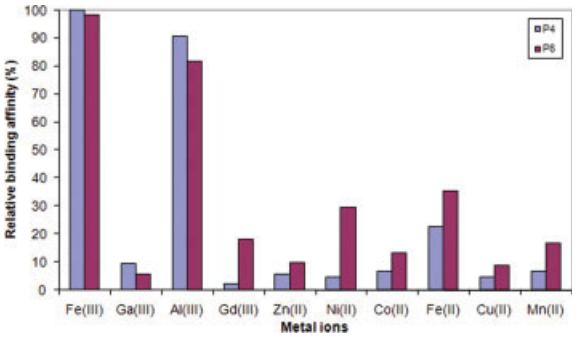


FIGURE 5 Relative binding affinities of P4 and P6 with 1.2 equiv of different metal ions by ES-MS.

were also observed. Therefore, we calculated the relative binding affinity based on the following equation:

$$[LFe_1 + LFe_2]/[LFe_1 + LFe_2 + L_2 + L_1 + LNa] \times 100\,(\%)$$

where L_1 and LFe_1 were the singly charged peak abundance of a ligand and its complex while LFe_2 and L_2 were the doubly charged peak abundance of a ligand and its complex.

As shown in Figure 5, the relative binding affinities of P4 and P6 (1 equiv) with different metal ions (1.2 equiv) obtained under the conditions described above were compared. Their corresponding singly charged peaks are at 733.8 (P4@Fe), 748.7 (P4@Ga), 744.7 (P4@Zn), 736.5 (P4@Ni), 737.7 (P4@Co), 736.6 (P4@Fe(II)), 741.6 (P4@Cu), 704.7 (P4@Al), 732.8 (P4@Mn), 835.7 (P4@Gd), 914.8 (P6@Ga), 977.6 (P6@Zn), 972.7 (P6@Ni), 971.75 (P6@Co), 968.8 (P6@Fe(II)), 976.2 (P6@Cu), 938.8 (P6@Al), 967.5 (P6@Mn), 1068.5 (P6@Gd), respectively. They were all identical with the calculated singly charged peaks based on $[(L—2H)MH]^+$ where L and M represent the molecular weight of a ligand and the atomic weight of a metal, respectively. Clearly, both P4 and P6 showed high binding affinities with Fe(III) and Al(III) compared to the other eight metal ions, Co(II), Ni(II), Fe(II), Zn(II), Mn(II), Cu(II), Ga(III), and Gd(III). But Fe(II) appeared a little stronger than the other seven metal ions.

As screened above, the diphosphinic peptides showed highest binding affinity to Fe(III). In addition, some phosphinic acid compounds have been reported to exhibit inhibitory activity with Zn-containing metalloproteinases.[14,56,57] Therefore, we further studied the bindings of P6 with two different metal ions, i.e. Fe(III) and Zn(II) at different equivalents. Titrations showed that the metal binding had a clear dependence on the metal ion types and their concentrations. As showed in Figure 6, Fe(III) bindings were nearly completed at a 1.2 equiv Fe(III). But even a high molar ratio of Zn(II) to P6 (5:1) could not drive complete Zn(II) binding. Clearly, the titration experiments further confirmed the difference of Fe(III) and Zn(II) in relative binding affinity and selectivity with P4 and P6 as previously determined.

The Relation of Structure with Metal Binding

Just like P4, P5, and P6, the other two diphosphinic acid compounds P2 and P3 also showed high binding affinity and selectivity for Fe(III). The two oxygen of a phosphinic acid group were supposed to be the ligands for Fe(III) binding. The relative binding affinity should be generally consistent with the hard-soft and acid-base principle for metal coordination (HSAB Principle, **h**ard cations prefer hard anions; soft cations prefer soft anions).[58] Competition experiments revealed relative binding affinities and selectivities in the following orders: Fe(III) > Al(III) > Fe(II) > Ga(III). It indicated that other factors such as ion size and ionization could also be determinants for metal binding with the di-phosphinic *pseudo*-peptides.

To further understand the structure and binding relationships, all the compounds listed in Table I were compared for their metal-binding properties, especially with Fe(III). Competition experiments revealed that the relative binding affinity in the following orders: P4 > P3, P2 > P1, P4 > P2, P6 > P5, P4 > P6, and P3 > P5. These results indicated the importance of two phosphinic acid groups as well as effects of linkages and backbone on Fe(III) binding. Compared to the monomeric P1, the higher binding affinity of diphosphinic acid P2 clearly confirmed the expectation that the synergism of two phosphinic acid groups would enhance the binding affinity.

Comparison of P2, P3, and P4 clearly showed that the length between the two phosphinic acid groups played a key role in their relative binding affinities. It seems that the distance of 11 atom between the two phosphinic acid in P4 were more favorable for Fe(III) binding. This was consistent with

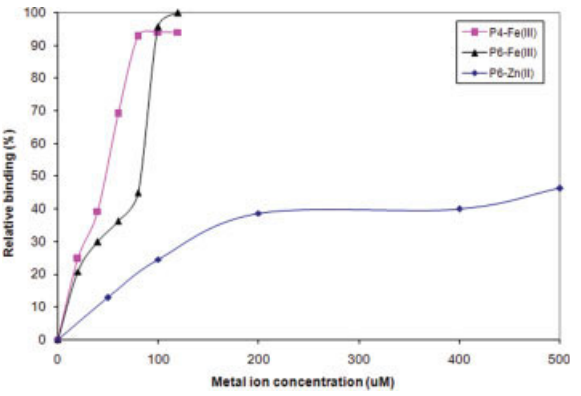


FIGURE 6 Titrations of P4 and P6 at 100.0 μM in 50% aqueous acetonitrile with Fe(III) and Zn(II).

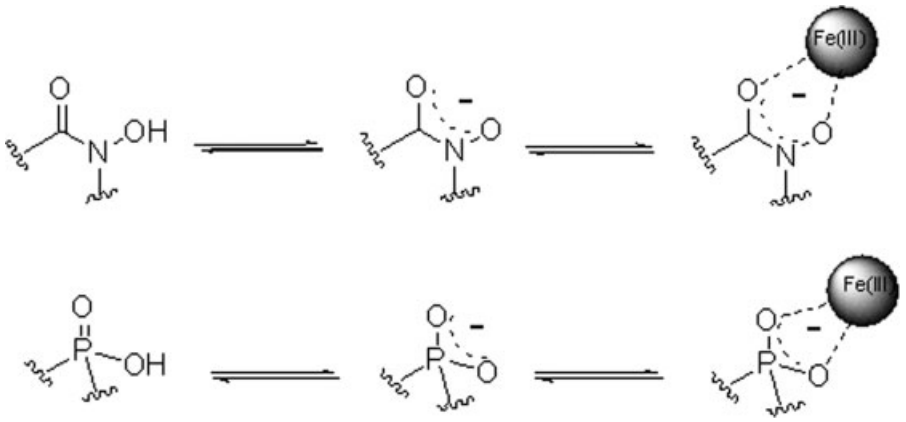


FIGURE 7 The structural similarity between hydroxamic and phosphinic acids for Fe(III) binding.

the observation we have reported previously with di- and trihydroxamate peptide systems where the two hydroxamates distanced at 10 atoms favored 1:1 intra-ligand coordination with higher relative Fe(III) binding affinity.[54,55] This distance might also determine metal-binding modes of such di-phosphinic *pseudo*-peptides. As showed in Figure 2, the Fe(III) binding of both P4 and P6 showed neat spectra with highly abundant singly charged peak compared to P2 and P3, so the 1:1 intraligand Fe(III) binding of P6 and P4 should predominate to form the corresponding M@L complexes. Contrastly, two phosphinic groups of P3 and P5 at the shorter lengths were complicated with both inter and intraligand binding ($M@L + M@L_2$).

Contrary to previous results with hydroxymate-containing peptides,[54,55] the relative binding affinity order P4 > P6 and P3 > P5 indicated that cyclization decreased the relative binding affinity. In this case, cyclization may change the relative orientation of the two phosphinic acid groups by decreasing backbone flexibility and increasing steric hindrance, not favoring the coordination geometry of Fe(III) and thus decreasing the relative binding affinity. As described earlier, the highly abundant hydration peak of P5@Fe(III) might also evidence the impact of cyclization on metal binding of P5 as hydration appeared to stabilize the resulting 1:1 intra-ligand metal complex.

It is very known that hydroxamate-based compounds are good ligands for Fe(III) binding. Previously, we found a series of *pseudo*-peptides containing two dihydroxamates arrayed at a 10 atom distance, i.e H—Val—Ψ(CONOH)—Phe—Ala—Pro—Leu—NHOH, H—[Leu—Ψ(CONOH)—Phe—Ala—Pro]$_2$—OH, and cyclo[Leu—Ψ(CONOH)—Phe—Ala—Pro]$_2$, showed high relative Fe(III) binding affinity as identified by ES-MS.[54,55] Therefore, we chose to compare P4 with such three dihydroxamate-containing peptides by competition experiments. So an aqueous solution of Fe(III) (2 mM, 10 μl) was added into a mixture of P4 (1 m*M*, 20 μl) and the dihydroxamate-containing peptides (1 m*M*, 20 μl) in 150 μl of 50% acetonitirle. As detected by ES-MS, P4 showed higher relative binding affinity than the three compounds. These results further confirmed that the di-phosphinic *pseudo*-peptides are good ligands for Fe(III) binding. This also inspired us to compare the structures of hydroxamic and phosphinic acid groups as Fe(III) binding ligands. Both hydroxamic and phosphinic acid groups, i.e. —(C=O)—(NOH)— and —(P=O)—(OH), contain two oxygen atoms and show certain structural similarity (Figure 7). Just like hydroxamic acid, we deduced that the orientation of two bidentate oxygen atoms on phophorus might fit the geometry of Fe(III) coordination. Compared with abundant literatures of hydroxamate-based ligands, phosphinic acid-based ligands have seldom been reported. Our results suggested further studies on their coordination chemistry should be essential.

As reported above, we initially used ES-MS to screen the metal binding selectivity and affinity of the diphosphinic *pseudo*-peptides under the same conditions. Compared to TFA (p*K*a 0.23) and HCl acid, the phosphinic acids are usually weak in acidity. Koval et al. reported the p*K*a values of the phosphinic acid group were typically 1.5–2.25 which changed with some substituted groups such as C-terminal carboxylic groups (2.94–3.50), imidazolyl moiety of histidine (6.55–8.32), and N-terminal amino groups (7.65–8.28).[39] So the pH of the tested solutions could greatly impact the ionization of such phosphinic *pseudo*-peptides and the related metal binding properties. The addition of different metal salts could also lead to pH variation of the test solutions. In addition, the anions from different metal salts might also exert their influences on the metal binding. Therefore, it is important to consider all these factors in further metal binding studies.

CONCLUSIONS AND PERSPECTIVES

A series of novel diphosphinic acid-containing pseudopeptides including linear and cyclic analogs were designed and synthesized. They were assembled utilizing a Fmoc-α-amino-phosphinic acid analog, i.e. Fmoc—(*R,S*)—AlaΨ[P(O,OCH_3)—CH_2]Gly—OH, as a building block via conventional Fmoc chemistry on solid support. The successful synthesis demonstrated that it should be feasible to synthesize other phosphinic acid-containing pseudopeptides using a similar strategy. Especially, the synthetic strategies developed in the present work should allow for incorporating diverse α-amino phosphinic acid analogs, including their optically pure isomers, into peptides and related structures. Thus, the di-phosphinic acid-containing *pseudo*-peptides provide additional points of diversity–for the library construction of *pseudo*-peptides for screening in the design, discovery, and development of novel peptidomimetics, enzyme inhibitors, selective cytotoxins, and metal chelators.

As screened by ES-MS, such *pseudo*-peptides bound Fe(III) with high affinity and selectivity. The established structure-metal binding relations provides a basis for discovery and further development of novel metal chelators via multiple approaches such as fine-tuning the linkages of two phosphinic acid groups, backbone cyclization, and side chains in future. Compared with hydroxamate groupd, phosphinic acids show certain similarities in structure and metal binding, so diphosphinic pseudo-peptides should be further studied for their coordination chemistry. As Fe(III) plays very important roles in living systems, their Fe(III) binding suggests further exploration of their potential in biological systems. For example, the use of such compounds as potential therapeutics for the treatment of iron-overload diseases[59,60] such as thalassemia and hemochromatosis as well as cancers[61] needs further research.

EXPERIMENTAL SECTION

General

^{1}H NMR spectra were recorded on Varian HG-300 (300 MHz) or Varian Inova-600 (Varian, Palo Alto, CA) spectrometer. Routine positive electrospray ionization-mass spectrometry (ESI-MS) analysis was performed on a Waters ZQ mass spectrometer. UV/Vis measurements utilized a Cary model 3E spectrophotometer. Flash column chromatographic purifications were performed on silica gel eluted with dichloromethane and methanol. The semi-preparative HPLC and analytical HPLC analyses were performed on Vydac C-18 columns. All the solvents including *N,N*-dimethylformamide, acetonitrile, dichloromethane, methanol, hexane, toluene, and ethyl acetate were obtained from Fisher Scientific. HBTU, DIEA, trifluoroacetic acid, piperidine, and all Fmoc-amino acids were purchased from Advanced ChemTech (Louisville, KY). All the metal salts and other chemicals were purchased from Sigma-Aldrich.

N-Diphenylmethyl-(*R,S*)-AlaΨ[PH(O, OH) (1)

To a stirred refluxing mixture of aminodiphenylmethane hydrochloride (33 g, 150.0 mmol) and 50% aqueous hypophosphorous acid (30 g, 230.0 mmol) in water (100 ml) was added dropwise acetaldehyde (8.8 g, 200 mmol) in water (60 ml). The mixture was heated for 2 h and cooled. The precipitated solid was collected by filtration, washed with water, and dried. Flash column chromatographic purification afforded 32 g (70%) of the title compound. ES-MS: $[MH]^+$ 275.9.

Cbz-(*R,S*)-AlaΨ[PH(O, OH)] (2)

1 was refluxed in 100 ml of 18% concentrated HCl for 2 h. After cooled to room temperature and extracted with diethyl ether, the aqueous layer was concentrated to afford the title compound (18.5 g, 84%). To a stirred and cooled (0°C) solution of **1** (18.0 g, 123.0 mmol) in dioxane/H_2O (1:1, 600 ml) and 10% Na_2CO_3 (460 ml) was added dropwise a solution of Cbz-Cl (31.5 g, 185.0 mmol) in dioxane (100 ml). The mixture was stirred for another 2 h at room temperature, acidified with diluted HCl solution to pH 3–4, and concentrated. The resulting mixture was treated with methanol and filtered. The filtrate was concentrated to afford the crude product which was then triturated with ethyl acetate/hexane (30:70). The resulting white solid was collected by filtration, washed with ethyl acetate/hexane (30:70), and dried under vacuum to afford the title compound (23 g, 80%). ES-MS: $[MH]^+$ 243.9.

Cbz—(*R,S*)—AlaΨ[P(O, OH)CH_2]—Gly—OBu^t (3)

To a solution of **2** (15.0 g, 62.0 mmol) and *tert*-butyl acrylate (23.8 g, 186.0 mmol) in anhydrous acetonitrile (150 ml) under nitrogen atmosphere was added bis(trimethylsilyl)acetamide (BSA) (31.5 g, 155.0 mmol). The mixture was stirred at room temperature overnight and cooled in ice-water, followed by adding 20 ml of methanol to decompose the excessive BSA. The resulting mixture was concentrated under reduced pressure. The residue was dissolved in ethyl acetate, and the solution was successively washed with water, and saturated $NaHCO_3$, dried over $MgSO_4$, and concentrated. Flash column chromatographic purification gave 8 g (35%) of the title compound. ES-MS: $[MH]^+$ 371.9.

Fmoc—(*R,S*)—AlaΨ[P(O, OH)CH_2]—Gly—OBu^t (4)

3 (5 g, 13.0 mmol) was N-deprotected by stirring with 10% Pd/C (0.4 g) in methanol (300 ml) in the presence of excessive ammonium formate overnight at room temperature. The mixture was filtered and the filtrate was concentrated. The residue was dissolved in dioxane/H_2O (1:1) (200 ml) and a solution of Fmoc—Cl (5.0 g, 19.0 mmol) in dioxane (100 ml) was added slowly. The mixture was stirred for another 4 h at room temperature, acidified with diluted HCl solution to pH 3–4, and concentrated. The resulting mixture was treated with methanol and filtered. The filtrate was concentrated, triturated with ethyl acetate/hexane (30:70). The crude product which was further purified using flash column chromatography to afford **4** (4.8 g, 80%). ES-MS: $[MH]^+$ 460.0.

Fmoc—(*R*,*S*)—AlaΨ[P(O, OCH_3)CH_2]—Gly—OBu^t (5)

4 (2.0 g, 4.3 mmol) was dissolved in methanol (100 ml) and cooled in ice, followed by adding EDCI (6.2 g, 32.0 mmol). The mixture was stirred overnight, concentrated, and washed with water. The title compound was obtained in a quantitative yield. ES-MS: $[MH]^+$ 473.9.

Fmoc—(*R*,*S*)—AlaΨ [P(O, OCH_3)CH_2]—Gly—OH (6)

4 (2.0 g, 4.3 mmol) was dissolved in 20% TFA in DCM (50 ml) and the solution was stirred at room temperature for 8 h, concentrated, dried under vacuum overnight to afford title compound with its acid analog 7. 20 mg of **7** was further purified by HPLC using aqueous acetonitrile eluant containing 0.1% TFA in a gradient increase from 10 to 40% over 30 min for ^1H NMR analysis. ES-MS: $[MH]^+$ 403.8.

Synthesis of P1, P2, P3, and P4

General Procedure. The synthesis was based on the conventional Fmoc chemistry. Rink amide MBHA resin (0.5 g, 0.61 mmol/g) was swelled in DMF, deprotected with 20% piperidine in DMF (10 min × 2), and washed with methanol and DMF. A solution of Fmoc-protected amino acid including Fmoc-Phe and 6 (0.9 mmol), HOBT (0.12 g, 0.9 mmol), and PyBOP (0.47 g, 0.9 mmol) in DMF (4 ml) was added into the reaction vessel, following by adding DIEA(0.23 g, 1.8 mmol). The mixture was shaken for 2 h. About 1–2 mg resin was checked by Kaiser test. If the coupling was complete, another deprotection/coupling cycle was repeated to obtain the desired sequence without N-terminal protection. The resin obtained was acetylated using Ac_2O (6 equiv) in the presence of DIEA (3 equiv) in DCM (6 ml) in the synthesis of P1, P2, P3, and P4. The resin was cleaved with TFA/H_2O (95:5 v/v, 5 ml) for 2 h (5 times). The TFA filtrate was concentrated, dissolved in aqueous methanol (~20 ml) containing LiOH (50 mg, 2.0 mmol), and stirred overnight. The mixture was concentrated, redissolved in TFA, and concentrated. The crude product was purified by HPLC using aqueous acetonitrile eluant containing 0.1% TFA in a gradient increase from 10 to 40% over 30 min. The fractions were identified by ESI-MS and lyophilized to afford the desired products (20–30% yield). Similarly, the products were further purified using aqueous acetonitrile eluant at isocratic 10% (P1), 20% (P2 and P3), and 30% (P4). ES-MS: $[MH]^+$ 370.8(P1), 533.8 (P2), 534.0 (P3), and 680.8 (P4).

Synthesis of P5 and P6

The linear di-phosphinate precursor **8** for **P6** was assembled starting from Fmoc-Phe Wang resin (0.5 g, 0.65 mmol/g) using the conventional Fmoc chemistry as described above. The resin without N-terminal Fmoc protection was cleaved with TFA/H_2O (95:5 v/v, 5 ml) for 2 h (3 times). The TFA filtrate was concentrated to afford 8 (ES-MS: $[MH]^+$ 932.7). A solution of 8 in 10 ml of DMF was added dropwise into a solution of PyBOP (0.30 g, 0.57 mmol), HOBT (0.10 g, 0.74 mmol), and DIEA (0.25 g, 2.0 mmol) in DCM (450 ml) and DMF (50 ml). The mixture was stirred overnight, concentrated, and washed with water. The solid was dissolved in aqueous methanol (~20 ml) containing LiOH (100 mg, 4.0 mmol) and stirred overnight. The mixture was concentrated, redissolved in TFA, and concentrated. The crude product was purified by HPLC using aqueous acetonitrile eluant containing 0.1% TFA in a gradient increase from 10 to 70% over 30 min. The fractions were identified by ESI-MS and lyophilized to afford the desired product (90 mg, ~30% yield). Part of the product was further purified by HPLC for NMR and metal binding studies. ES-MS: $[MH]^+$ 914.8.

Similarly, the linear di-phosphinate precursor (ES-MS: $[MH]^+$ 638.8) for **P5** was prepared from Fmoc-Phe Wang resin (0.5 g, 0.65 mmol/g) as described above. It was cyclized in the presence of PyBOP(0.41 g, 0.78 mmol), HOBT(0.11 g, 0.78 mmol), and DIEA(0.40 g, 3.12 mmol) in a diluted solution (500 ml) of DCM and DMF. Semi-preparative HPLC purification using aqueous acetonitrile eluant containing 0.1% TFA in a gradient increase from 10 to 70% over 30 min gave P5 (50 mg, ~25% yield). Part of the product was further purified by HPLC for NMR and metal binding studies. ES-MS: $[MH]^+$ 620.7.

Preparation of Metal Binding Solutions for ESI-MS Analysis

Stock solutions (2 m*M*) of the peptide ligands in methanol and some metal salts including nickel(II) chloride hexahydrate (99.999%), zinc sulfate heptahydrate (99.999%), iron(III) nitrate nonahydrate (99.99+%), copper(II) sulfate pentahydrate (99.999%), iron(II) sulfate hepahydrate (99.99%), cobalt(II) nitrate hexahydrate (99.998%), manganese(II) chloride, gallium(III) sulfate (99.999%), aluminum sulfate hydrate (99.998%), and gadolinium (III) chloride (99.99%) in distilled water were first prepared. Typically, a test solution (100 μl each) containing 100 μM of ligands was prepared by mixing 10 μl of a ligand stock solution (1.0 m*M*) and 10 μl of a metal ion stock solution (2.0 m*M*) in 80 μl of 50% aqueous acetonitrile. 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 also added into one solution for competition experiments. All the test solutions were swirled for at least 24 h to insure equilibrium before subjecting to ESI-MS and UV-vis analysis. The relative metal-binding affinity of a ligand was calculated as the percentage of the relative abundance of the binding ligand peaks with the total ligand-related peaks in a positive ES-MS spectrum according to the following equation:

$$[LFe_1 + LFe_2]/[LFe_1 + LFe_2 + L_2 + L_1 + LNa] \times 100\,(\%)$$

where L_1 and LFe_1 were the singly charged peak abundance of a ligand and its complex while LFe_2 and L_2 were the doubly charged peak abundance of a ligand and its complex.

Competition Experiments

10.0 μl of ligand 1 (1.0 m*M*) and 10.0 μl of ligand 2 (1.0 m*M*) were mixed in 75.0 μl of 50% aqueous acetonitrile, followed by adding a solution of $Fe(NO_3)_3$ in water (2.0 m*M*, 5.0 μl). The mixture (200 μl) was swirled at room temperature for at least 24 h. Different ligand pairs such as P1/P2, P2/P3, P3/P4, P3/P5, P4/P6, and P5/P6 were used and the resulting solutions were subjected for ESI-MS analysis.

^{1}H NMR Experiments

The NMR spectra were recorded with a Varian Inova-600 (Varian, Palo Alto, CA) spectrometer and the data were processed with VNMR software. The total correlation (TOCSY) spectra were recorded using an 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 2 × 256 × 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 × 2048 frequency domain representation was phase and baseline corrected in both dimensions. The NOESY spectrum resulted 2 × 256 × 2049 data matrix with 32 scans per t1 increment. Spectra were recorded with 250 ms 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 × 2048 data matrix and were processed in a similar way as the 2D TOCSY spectrum described above.

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