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A Highly Divergent Approach for Synthesis of Metal-Binding Peptide Libraries

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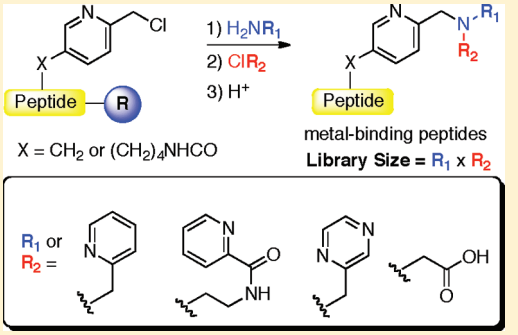
Supporting Information

ABSTRACT: A highly divergent approach for the synthesis of metal-binding peptides is described. This approach builds pyridine-based chelators in a stepwise fashion on resin and provides rapid access to a diverse array of metal-binding peptides in minimal synthetic steps.



Metal-binding peptides have important applications in medicine, imaging, metal-ion detection, and catalysis.[1−7] Their remarkable scope in chemistry and biology today is due in part to properties of the synthetic ligand, whose strong affinities ensure that peptides remain bound to metals in biological settings. Within this area of research, ligands have been attached to several positions of peptides, including the *N*- and *C*- termini[8] and side chains.[9]

Synthesis of metal-binding peptide libraries is important, because it allows rapid identification of the best peptide that exhibits strong and stable binding to the desired metal while maintaining biological activity. Even though much effort has been directed toward the synthesis of metal-binding peptides, we recognized that it was difficult to create libraries where the structure of the ligand could be varied readily. This was because in peptide−ligand conjugates methods often relied on attaching chelators to single amino acids prior to elaboration of the peptide chain.[2,10] To address this issue, a divergent approach for the synthesis of peptide−ligand conjugates was created that could generate multiple metal-binding peptides from a single peptide on resin (Figure 1, Divergent Strategy).[11] The approach used unnatural pyridine-based amino acids as anchor points to attach secondary amines (HNR_1R_2) to the peptide. This late-stage transformation fully elaborated the metal-binding unit, thereby providing a straightforward way to access diverse ligand structures that could be used to tune properties such as metal-binding affinity and reactivity of the metal center. With this approach, the only limit to how many metal-binding peptides could be prepared from a single peptide on resin was the number of available secondary amines. For instance, if one wanted to create 100 metal-binding peptides, each with a different ligand, 100 independent secondary amines would be needed to complete the

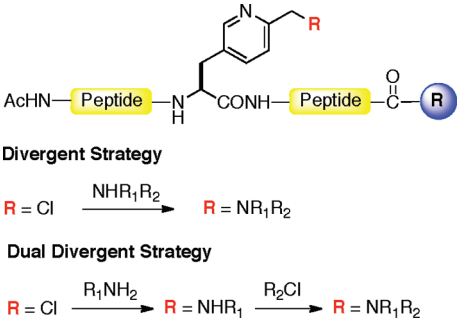


Figure 1. Comparison of divergent and dual divergent strategies.

library. To advance this strategy, we recognized that if ligands were built in a stepwise fashion on resin, where the two groups on the amine (R_1 and R_2) were attached one by one to the peptide, rather than in a single step, this would expedite the synthesis of larger libraries. This strategy, named the dual divergent approach, would reduce the number of starting materials needed to 20 for creating a library of 100 compounds (10 R_1NH_2 and 10 R_2Cl), would not require the tedious solution-phase synthesis of secondary amines and would allow one to take advantage of the split-pool synthesis method to create large libraries of metal-binding peptides with different ligand structures.[12]

The known dipeptide substrate **1** bound to Rink amide resin was examined as a model in order to develop optimal conditions for the dual divergent strategy (Scheme 1). Deprotection of the TBS group in **1** with buffered TBAF, followed by chlorination of

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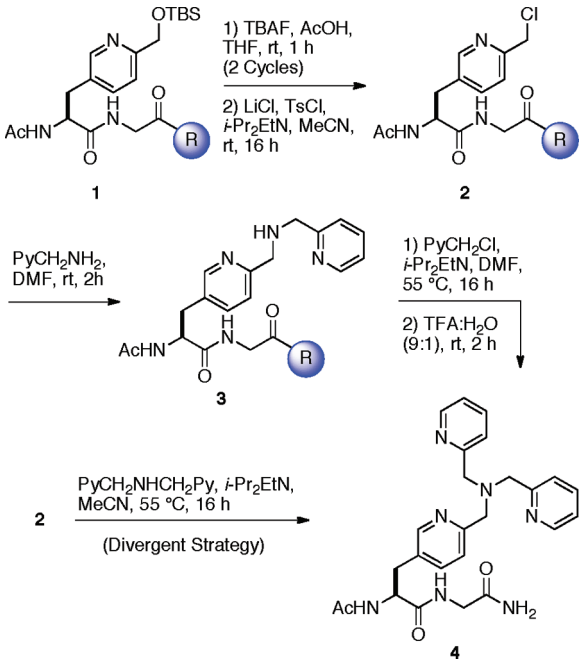
Scheme 1. Optimization of the Dual Divergent Strategy on Model Dipeptide 1



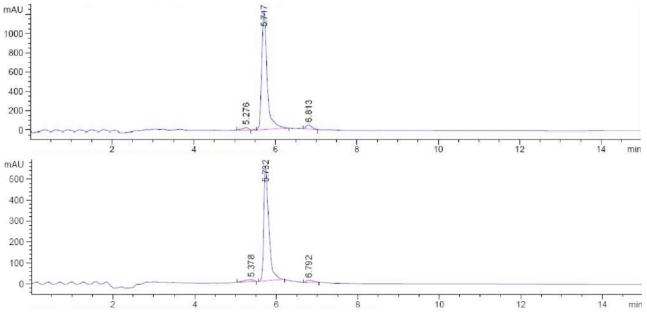
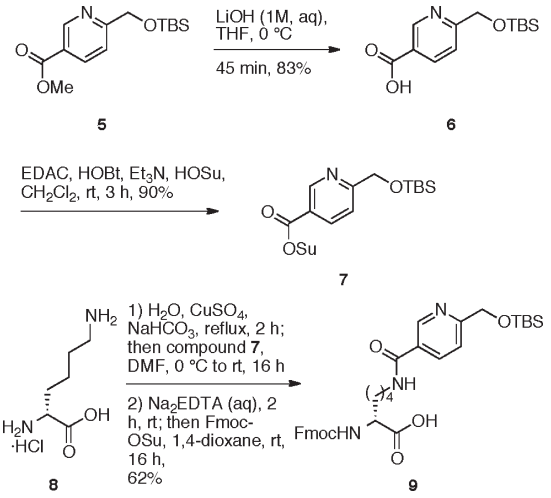


Figure 2. HPLC chromatograms of crude **4** prepared by the divergent (top) and dual divergent (bottom) strategies.

the primary alcohol using TsCl and LiCl furnished resin-bound chloride **2**. Amination of **2** with 2-picolylamine under conditions developed for divergent strategy (MeCN, 55 °C, 20 h) did not give the desired secondary amine **3** in high purity. Instead, a complex mixture of secondary and tertiary amines was observed by ^{1}H NMR spectroscopy and ESMS after cleavage from the resin, including a structure that was the apparent product of the reaction between peptides **2** and **3**. Similar results were found when CH_2Cl_2 was used as the solvent. Gratifyingly, amination of **2** with 2-picolylamine in DMF for 2 h at rt gave the desired secondary amine **3** in high purity. Likewise, the second alkylation with 2-(chloromethyl)pyridine proceeded cleanly at 55 °C in DMF and afforded **4** in excellent purity after cleavage from the resin with TFA/H_2O. In fact, HPLC chromatograms of **4** (Figure 2) prepared from the divergent (**2** to **4**) and dual divergent (**2** to **3** to **4**) approach were indistinguishable.

In order to determine the scope of the dual divergent approach, a more complex peptide scaffold was sought. The decapeptide luteinizing-hormone-releasing hormone (LHRH)

Scheme 2. Synthesis of Fmoc-D-HPL(OTBS)-OH (9)



was chosen due to its diverse array of functional groups (lactam, imidazole, indole, ether, guanidinium), as well as its biological significance. LHRH peptides selectively deliver cytotoxic agents to cancer cells in vivo by taking advantage of the fact that many cancer cell lines express the LHRH receptor, whereas most normal cells do not.[13,14] Analogues such as AN-152, a doxorubicin (DOX) conjugate now in phase II clinical trials for ovarian cancer, contains the residue D-Lys in place of Gly at position 6 of the peptide.[15] Besides providing a point of attachment for the cytotoxic agent DOX, the residue D-Lys helps to maximize serum stability[16] while maintaining binding affinity of the peptide for the LHRH receptor.[17] To approach the synthesis of metal-binding LHRH analogues, a new pyridine-containing amino acid derived from D-Lys (**9**, Scheme 2) was developed for the dual divergent approach. This amino acid, named HPL for hydroxymethylpyridyllysine, would be used to attach chelators to the end of the D-Lys side chain.

Synthesis of amino acid **9** began from ester **5** (Scheme 2), which is available in two steps and 92% overall yield from the commercially available compound dimethyl pyridine-2,5-dicarboxylate.[11,18] Saponification of ester **5** with LiOH followed by formation of the activated ester with HOSu, EDAC, and HOBt furnished *N*-hydroxysuccinimide ester **7** in two steps and 75% overall yield from **5**. Amino acid **9** was prepared in 62% overall yield from **7** and D-Lys·HCl (**8**) by a method adapted from the literature.[19,20] This sequence involved in situ protection of the α-amino group of D-Lys through a Cu^{II} chelate, followed by amidation of the ε-amino group with **7**. Isolation of the Cu^{II} chelate, followed by removal of the Cu^{II} with Na_2EDTA and in situ Fmoc protection of the α-amino group with Fmoc-OSu, gave amino acid **9**.

With amino acid Fmoc-HPL(OTBS)-OH (**9**) in hand, the LHRH analogue **10** was prepared by Fmoc solid-phase synthesis (Scheme 3). A peptide synthesizer was used for all steps, except for incorporation of amino acid **9**, which was done manually. *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) was employed as the coupling reagent. After construction of the decapeptide, selective deprotection of the TBS group in **10** with buffered TBAF followed by chlorination of the primary alcohol **11** with TsCl and LiCl furnished resin-bound peptide **12**.

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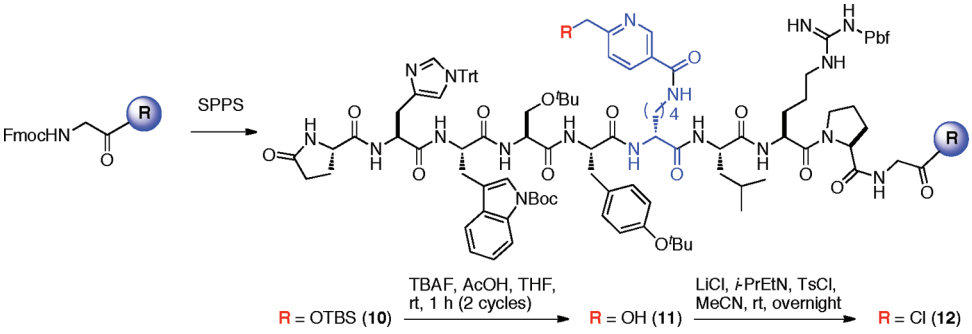
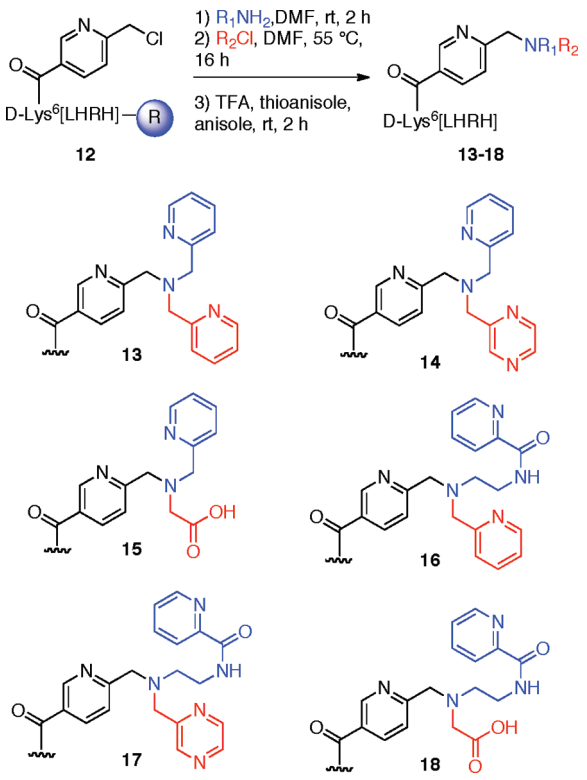
Scheme 3. Synthesis of Decapeptide 12, an LHRH Analogue Containing D-HPL at Position 6



Scheme 4. Synthesis of LHRH Analogues 13−18 Containing Metal-Binding Ligands



A library of six LHRH peptides containing metal chelators was prepared from chloride **12** by the dual divergent approach. Two amine (R_1NH_2) and three chloride (R_2Cl) starting materials were chosen to provide a diverse array of metal-binding groups in the final products. Examples chosen were a carboxylate O-donor and several N-donors, including pyridine, pyrazine, amide, and basic amines. The first step of the synthesis involved alkylation of resin-bound chloride **12** with either 2-picoylamine (for **13**−**15**) or *N*-(2-aminoethyl)picolinamide (for **16**−**18**). After the first alkylation, each product was split into three and treated with 2-(chloromethyl)pyridine, 2-(chloromethyl)pyrazine, or *tert*-butyl 2-chloroacetate. After cleavage from resin, analyses by ^{1}H NMR spectroscopy and HPLC indicated all six peptides were obtained in >80% purity. The final six peptides (**13**−**18**) were purified by reversed-phase HPLC and characterized by ^{1}H NMR, HPLC, and MALDI-TOF. The resultant chelators are analogues of the known ligands TPA and $PaPy_3$, which demonstrate attractive properties for biological applications. These properties include strong affinities for biologically relevant metal ions such as Mn^{II}, Fe^{II}, Cu^{II}, and Zn^{II},[21] catalysis in small molecule oxidation,[22,23] O_2 activation,[24] and selective delivery of NO.[25] In fact, metal-binding experiments with compounds **13** (TPA derivative) and **16** ($PaPy_3$ derivative) confirmed that the peptide−ligand conjugates bind Zn^{II} and Fe^{III}, respectively, in a fashion similar to that of the parent ligands. In the case of **13**, Zn^{II} binding in D_2O was confirmed by ^{1}H NMR spectroscopy (Figure S1, Supporting Information), where similar shifts were observed for the pyridyl protons between Zn^{II}-bound and unbound forms of TPA and **13**. Likewise, titration of **16** with $Fe^{III}(ClO_4)_3$ in H_2O produced a red species whose UV−vis spectrum (Figure S2, Supporting Information, λ_{max} = 505 nm, ε = 1520 M^{-1} cm^{-1}) agreed well with the literature and experimental data for the Fe^{III} complex of $PaPy_3$ (λ_{max} = 500 nm, ε = 1830 M^{-1} cm^{-1}).[26]

In conclusion, a highly divergent approach was developed for the synthesis of metal-binding peptides. The stepwise manner that chelators can now be synthesized in will provide ready access to large libraries of metal-binding peptides from commercially available materials with minimal reaction steps. Furthermore, the ability to assemble multidentate ligands by a solid-phase approach, rather than relying on solution-phase methods that can involve longer sequences and the use of protecting groups, represents a significant advancement in the construction of such chelators. We anticipate that the ability to build analogues of multidentate ligands in a rapid and straightforward fashion will expedite the discovery of catalysts and biologically active molecules. Such efforts are now underway in our laboratory.

EXPERIMENTAL SECTION

Materials. All reagents were purchased from commercial suppliers and used as received. Rink amide MBHA resin (200−400 mesh, 0.75 mmol/g, 1% DVB) was purchased from Chem-Impex International, Inc. Compounds bis(pyridin-2-ylmethyl)amine,[27] 2-(chloromethyl)pyrazine,[28] *N*-(2-aminoethyl)picolinamide,[26] **2**,[11] and **5**[11] used in this report were synthesized according to literature procedures.

Peptide Syntheses. All coupling reactions were performed on a 0.15−0.4 mmol scale by a solid-phase peptide synthesis method using Nα-9-fluorenylmethyloxycarbonyl (Fmoc) amino acid with HBTU-activated esters in a peptide synthesizer and/or peptide synthesis vessel (manual synthesis). Typical protocols for coupling steps involved 1−4 h coupling cycles with 2−4 equiv of Fmoc amino acid, except for coupling with **9**, where only 2 equiv was used. During coupling reactions,

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Fmoc amino acids were preactivated with HBTU, *i*-Pr_2EtN, and DMF for 1−2 min. Fmoc deprotection was performed using 20% piperidine/DMF with two cycles, 20−30 min each. During manual synthesis, the resin was washed after each step with DMF (2 × 2 mL), *i*-PrOH (2 × 2 mL), and CH_2Cl_2 (2 × 2 mL). The Kaiser test was used to monitor the coupling reactions and deprotection of the Fmoc group. The general synthetic route to obtain LHRH peptide−ligand conjugate is depicted in Schemes S1−S2, Supporting Information. Steps are summarized in Table S1, Supporting Information.

TBS Cleavage and Chlorination. A mixture of the peptide (0.3 mmol scale), 1 M TBAF (1.2 mmol), acetic acid (0.3 mmol), and THF (2 mL) was shaken in a peptide synthesis vessel for 1 h at rt. Two cycles were performed in order to ensure complete cleavage of TBS group. The resin was successively washed with DMF (2 × 2 mL), *i*-PrOH (2 × 2 mL), and CH_2Cl_2 (2 × 2 mL), dried under vacuum, and stored at −30 °C.

A mixture of peptide (75 μmol), lithium chloride (3.75 mmol), *i*-Pr_2EtN (1.13 mmol), TsCl (0.75 mmol), and MeCN (1.7 mL) was shaken in peptide synthesis vessel for 20−24 h at rt. The resin was successively washed with DMF (2 × 1 mL), *i*-PrOH (2 × 1 mL), and CH_2Cl_2 (2 × 1 mL), dried under vacuum, and stored at −30 °C.

Alkylation. A mixture of resin-bound chloride (60 μmol), primary amine (6 mmol), and DMF (1.6 mL) was shaken in a peptide synthesis vessel for 2 h at rt. The resin was successively washed with DMF (2 × 1 mL), *i*-PrOH (2 × 1 mL), and CH_2Cl_2 (2 × 1 mL), dried under vacuum, split into three portions (20 μmol each), and transferred into vials. This resin was mixed with *i*-Pr_2EtN (0.45 mmol), chloride (0.2 mmol), and DMF (1.1 mL) and stirred slowly (<200 rpm) at 55 °C for 18−20 h. The resin was successively washed with DMF (2 × 1 mL), *i*-PrOH (2 × 1 mL), and CH_2Cl_2 (2 × 1 mL) and dried under vacuum.

Cleavage of the Peptides from Resin. The peptide−ligand conjugates were cleaved from resin by stirring the resin (25−30 mg) with a 27:1:2 mixture of TFA/anisole/thioanisole (2 mL) for 2 h at rt. The resin was removed by filtration, and the filtrate was mixed with cold Et_2O (10 mL). The resulting precipitate was isolated by centrifugation. The residue was washed with cold ether (2 × 4 mL), dried under vacuum, and used for purification/analysis.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures for the preparation of **6**, **7**, **9**, and **13**−**18** including characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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