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A Novel and Rapid Encoding Method Based on Mass Spectrometry for "One-Bead-One-Compound" Small Molecule Combinatorial Libraries

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Abstract: A novel and efficient encoding method based on mass spectrometry for "one-bead-one-compound" small molecule combinatorial libraries has been developed. The topologically segregated bifunctional resin beads with orthogonal protecting groups in the outer and inner regions are first prepared according to our previously published procedure. Prior to library synthesis, the inner core of each bead is derivatized with 3−4 different coding blocks on a cleavable linker. Each functional group on the scaffold is encoded by an individual coding block containing a functional group with the same chemical reactivity. During the library synthesis, the same chemical reactions take place on the scaffold (outer layer of the bead) and coding blocks (inner core of the bead) concurrently. After screening, the coding tags in the positive beads are released, followed by molecular mass determination using matrix-assisted laser desorption ionization Fourier transform mass spectrometry. The chemical structure of library compounds can be readily identified according to the molecular masses of the coding tags. The feasibility and efficiency of this approach were demonstrated by the synthesis and screening of a model small molecule library containing 84 672 member compounds, with a model receptor, streptavidin. Streptavidin binding ligands with structural similarity (17) were identified. The decoding results were clear and unambiguous.

Introduction

Combinatorial chemistry has become an essential component of the drug discovery process during the past decade. In 1991, we first introduced the "one-bead-one-compound" (OBOC) combinatorial library method.[1] Using a "split-mix" synthesis procedure,[1,2] peptide or chemical libraries can be generated such that each bead displays only one compound entity. With an on-bead screening assay, literally millions of compound beads can be screened against specific molecular targets in a few days.[1] Individual positive beads can then be isolated for structure determination. This approach has been successfully applied to the identification of ligands for a large number of biological targets.[3] Peptide beads can easily be microsequenced with Edman degradation using an automatic protein sequencer. In contrast, structure determination of small molecule beads is challenging. To solve this problem, various indirect encoding methods have been developed and the subject has been reviewed.[3,4] In most cases, a coding tag is synthesized on each bead in addition to the library component. This tag defines the chemical history of any particular bead and hence the structure of the compound it supports. It is released from the bead after biological screening and analyzed by a highly sensitive analytical technique. For example, electron capture capillary gas chromatography has been successfully used for the detection of volatile halocarbon tags released from the beads via photolytic[5] or oxidative[6] cleavage; reverse-phase HPLC with fluorescence detection has been used to determine the dansylated secondary amine tags released by mineral acid hydrolysis,[7] and automatic microsequencing has been used to decode the peptide tags.[8,9] Several physical encoding methods have also been described, but they are either not possible with highly diverse libraries (e.g., 250 000 compounds)[10] or too big to be enclosed in a single 80 μm bead.[11]

Mass spectrometry (MS) has been widely used in the analysis of combinatorial libraries due to its intrinsic sensitivity, speed

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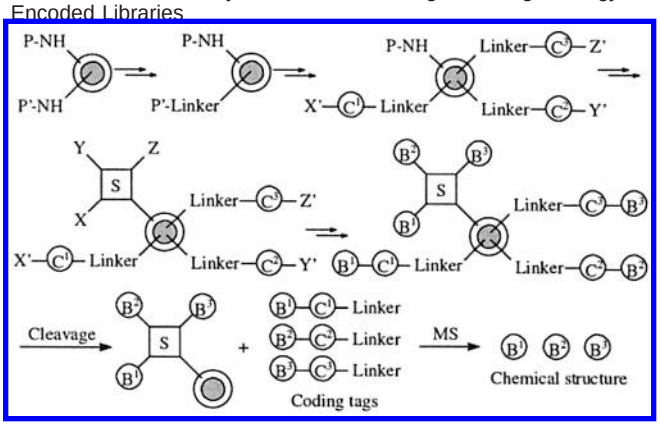
of analysis, specificity of detection, and automation capability. Sequencing of peptides by MS has been developed,[12,13] and databases for MS sequencing of peptide libraries are commercially available. Because simultaneous cleavage and ionization occur under laser irradiation, peptides covalently attached to a single polymeric bead by a photosensitive linker can be directly sequenced by matrix-assisted laser desorption ionization (MALDI) MS.[13] Structure-indicating fragments can be readily obtained by collision-induced dissociation (CID) or tandem MS (MS/MS). The single-bead analysis approach for peptides has also been applied to peptidomimetic and small molecule compound libraries.[14] For small libraries, the component on a single bead can be directly identified with the exact molecular mass and analysis of fragments. However, direct structure identification of a compound on a single bead isolated from a large diverse library of over 100 000 compounds will be very difficult if not impossible. This is because compounds from such combinatorial libraries often have molecular masses differing by less than 200 Da, and some of them are poorly ionizable or nonionizable. Some indirect MS-based encoding/decoding strategies have been described. For example, stable isotopes (^{13}C or deuterium) have been incorporated to the coding tags to generate different isotopic patterns for structure characterization.[15] Another elegant approach, but one limited to libraries of ionizable compounds with repeating subunits, such as peptides, has been developed by Youngquist et al.[16] A small amount of a termination reagent is added at each synthetic step to generate a series of sequence-specific terminated products. Analysis of such products generated from one single bead, with MALDI-MS, enable one to reconstruct the complete peptide sequence.

Current chemical encoding methods have played an important role in the advancement of OBOC combinatorial chemistry. However, those methods often require orthogonal chemistries for tagging and, therefore, additional synthetic steps. For example, in the halocarbon encoding method developed by Still et al.,[5,6] an additional 16−24 h are needed to encode each building block. In addition to the increased time and cost, the tagging molecules themselves potentially could interfere during the screening. To address these problems, our group has developed a new peptide-based encoding method that enables

Scheme 1. General Synthetic and Encoding/Decoding Strategy of Encoded Libraries



us to topologically segregate the testing compounds from the coding tags.[9] The resin beads are first derivatized with orthogonal protecting groups in the outer and inner regions separately. A coding tag precursor consisting of a sequence of α-amino acids, of which the side chains can be derivatized, is then constructed in the interior of the beads. During the library synthesis, building blocks are coupled to the outer scaffold and the side chains of the inner coding peptide simultaneously. In this way, the extra synthetic steps for coding tags are eliminated by combining them with the library synthesis. After biological screening, the structures of active compounds can be easily determined by direct sequencing of the coding peptides with Edman degradation. The utility and reliability of this encoding method has been confirmed. However, like other encoding strategies, this method has its limitations. First, this decoding method is based on Edman degradation and, therefore, is slow and expensive. Second, building blocks have to be carefully chosen to avoid retention time overlap of their amino acid derivatives during sequencing. Third, unless one wants to synthesize trifunctional α-amino acids with unusual side chains, choices for scaffolds are limited to those having the same functional groups as the side chains of commercially available trifunctional amino acids. Most recently, we described an improvement of the previously mentioned peptide encoding method by using mass spectrometry to decode the peptide tag.[17] This new development alleviates the first two limitations outlined previously. However, the analysis of the decoding results could be complicated, and the method has not yet been routinely applied to our library screening efforts.

In an effort to continue to improve the OBOC technology, we have recently developed a new and highly efficient MS-based encoding strategy that is particularly suited to the small organic molecule libraries (Scheme 1). In this encoding system, the topologically segregated bifunctional resin beads are used to segregate the library compounds (outer layer) from coding tags (inner core). Each functional group (X, Y, and Z) on the scaffold (S) is encoded by an individual coding block (C^1, C^2, and C^3) containing a functional group (X′, Y′ and Z′) that has the same chemical reactivity as the one on the scaffold. The chemical reactions for library synthesis occur on the scaffold as well as on inner coding blocks at the same time to form the coding tags. After screening, the coding tags in the positive beads are released by chemical cleavage and characterized by

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MS. The structures of active compounds can be readily identified according to the exact molecular masses of coding tags. To demonstrate the feasibility and efficiency of this encoding method, a model small molecule library containing 84 672 members is synthesized and screened against streptavidin.

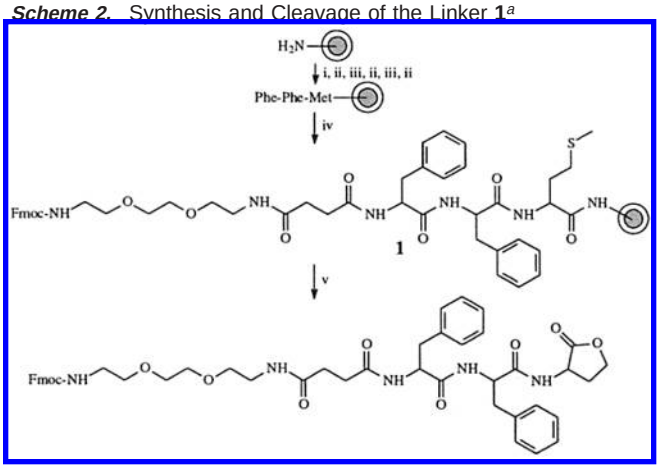
Results and Discussion

General Encoding Strategy. The general synthetic and encoding strategy for our encoded libraries is illustrated in Scheme 1. The resin beads are first derivatized with orthogonal protecting groups in the outer (P, 60%) and inner (P′, 40%) regions separately using our published procedure.[9] A cleavable linker, which can facilitate the mass determination of coding compounds, is then built in the interior of the bead. Coding blocks (C^1, C^2, and C^3) chosen according to the functional groups (X, Y, and Z) on the scaffold (S) are then anchored to the linker. Each coding block contains only one functional group, which has the same chemical reactivity as the one on the scaffold. The scaffold for library synthesis is then coupled to the outer layer of the beads. During the library synthesis, the same building blocks (B^1, B^2, and B^3) are coupled to the scaffold and inner coding blocks concurrently. After screening, the positive beads are isolated and treated with cleavage reagents. Only the inner coding tags are released from the beads. The cleavage solution containing coding tags is then analyzed with high resolution MS. According to the molecular masses of coding tags, the chemical structure of the library compound on the outer layer of the bead can be readily identified.

We use matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) for decoding due to its high resolution, high accuracy, and high sensitivity. Since we need only the molecular masses of coding tags to identify the structure of a library compound, a very small amount (picomole or even femtomole) of coding compounds is enough for MALDI-FTMS detection. Considering a library based on a scaffold with four diversities, if we use 100 different building blocks in each synthetic step, we will get a library containing 100^4 compounds. The total number of coding tags for such a library is only 400. MALDI-FTMS, with an accuracy down to a few ppm, can easily discriminate all the 400 different coding tags as long as they are not isomers, which can be easily avoided by careful selection of building blocks and coding blocks. Because each coding tag constitutes only about a 10% equivalent of the whole bead, and they all reside in the bead interior, interference by the coding tags during biological screening should be minimal.

Linker Design. An ionization linker has been used to enhance ionization of poorly ionizable or nonionizable molecules.[18] The linker also provides a mass shift which overcomes signal overlap with matrix molecules. To effectively decode each bead with mass spectrometry, the linker should meet the following four criteria. First, the linker must be inert to the chemical reactions for library synthesis and stable under the conditions used for various biological screenings. Second, the linker should be highly sensitive to the ionization method so that the final coding tags with different structures can be readily detected. Third, its cleavage must be clean and efficient. Fourth, the linker should have excellent solubility in the extraction solvent. A simple peptide-like linker (Scheme 2, **1**) that meets these four criteria has been designed and synthesized on solid phase using the standard Fmoc chemistry.[19] In principle, any chemically cleavable or photosensitive linkers can be used as the cleavable part as long as they are compatible with the library synthesis and screening. Methionine is chosen in our study because its cleavage by cyanogen bromide (CNBr) is clean and specific and the final homoserine lactone product[20] is chemically stable. This cleavage method has been successfully applied to single-bead analysis of peptides.[16] Two phenylalanines are coupled to the methionine to increase the molecular weight of the linker. Finally, a linear hydrophilic molecule is introduced to the linker to enhance solubility of the coding tag in the extraction solvent (50% acetonitrile/water). The whole linker has excellent chemical stability and is very suitable for MALDI-FTMS detection. The oxygen atoms, the amide bonds, and the side chain of phenylalanines in the linker allow efficient formation of primarily sodiated species and, therefore, provide efficient ionization.

Scheme 2. Synthesis and Cleavage of the Linker **1**[a]



[a] Reagents and conditions: (i) 3 equiv of Fmoc-Met-OH, DIC, and HOBt in DMF, rt, 1 h; (ii) 20% piperidine in DMF, rt, 30 min; (iii) 3 equiv of Fmoc-Phe-OH, DIC, and HOBt in DMF, rt, 1h; (iv) 3 equiv of Fmoc-$NH(CH_2CH_2O)_2(CH_2)_2NHCO(CH_2)_2COOH$, DIC, and HOBt in DMF, rt, 3h; (v) 0.25 M CNBr in 70% formic acid, rt, overnight.

To evaluate the utility of the linker in encoding, we synthesized five different compounds (Scheme 3) in the inner core of topologically segregated bifunctional TentaGel beads (inner core:outer layer = 2:3) via the linker shown in Scheme 2. MS analysis of a single bead from this synthesis revealed the expected five mass signals (Figure 1). The result is clear and unambiguous, indicating that the linker does meet the requirements for the proposed encoding method.

Selection and Relative Reactivity of Coding Blocks. Coding blocks are chosen according to the functional groups on the scaffold for encoded library synthesis. Each of the coding blocks should have an identical or related functional group on the scaffold and a carboxyl group through which the coding block is anchored to the linker. Due to the commercial availability of thousands of organic acids, one can easily find coding blocks for almost any selected scaffold. An ideal coding block should react with the corresponding building block to generate a stable product that is inert to subsequent chemical reactions. For instance, proline could be used to encode reductive alkylation

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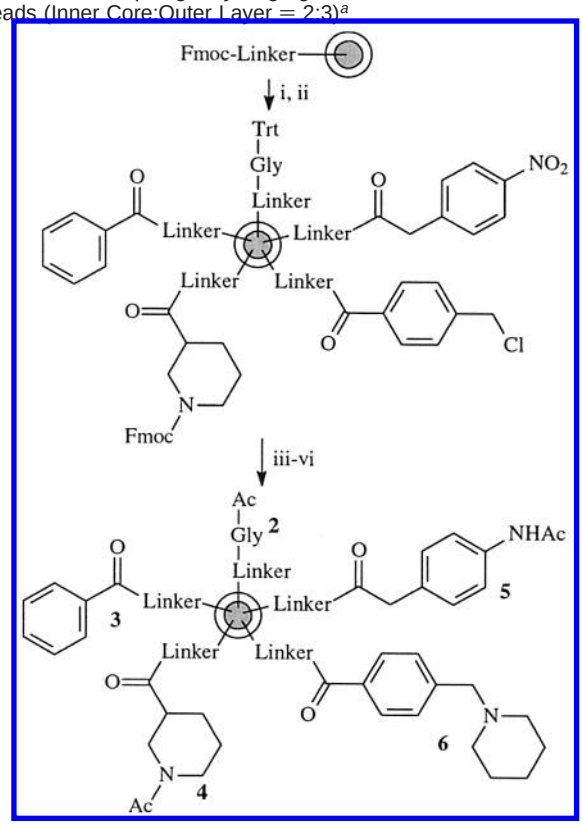
Scheme 3. Synthesis of Five Compounds on the Linker in the Inner Core of Topologically Segregated Bifunctional TentaGel Beads (Inner Core:Outer Layer = 2:3)[a]



[a] Reagents and conditions: (i) 20% piperidine in DMF, rt, 30 min; (ii) 2 equiv of Trt-Gly-OH, benzoic acid, Fmoc-nipecotic acid, 4-nitropheny-lacetic acid, and 4-(chloromethyl)benzoic acid, 10 equiv of DIC and HOBt in DMF, rt, 1 h; (iii) 1% TFA and 5% TIS in DCM, rt, 2 min × 4; (iv) 0.25 M piperidine in 5% DIEA/DMF, rt, overnight; (v) 2 M $SnCl_2 \cdot 2H_2O$ in DMF, rt, 6h; (vi) 20 equiv of Ac_2O, 5 equiv of DIEA in DCM, rt, overnight.

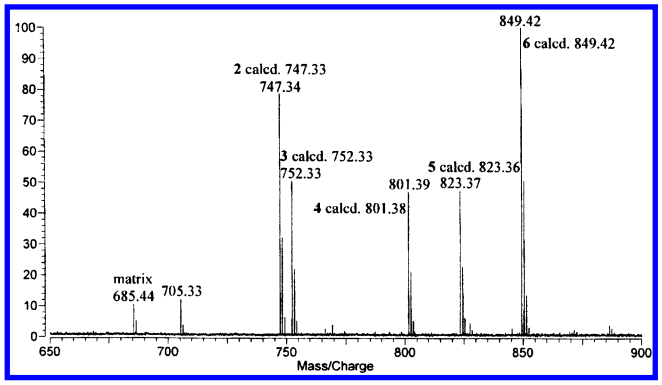
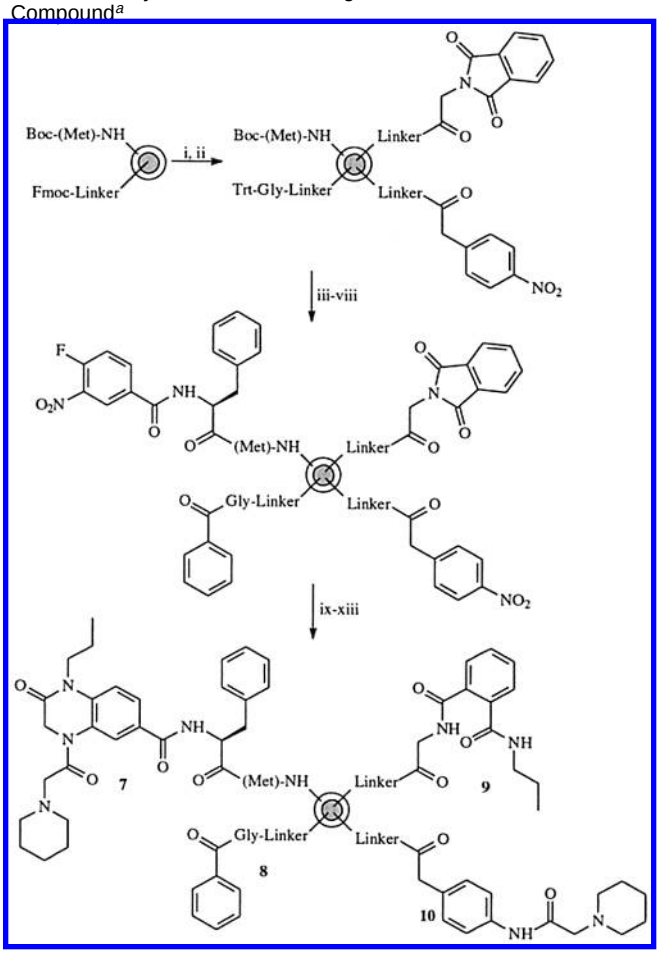


Figure 1. MALDI-FTMS spectrum of five tags in the 40% inner region of a single TentaGel bead.

on the scaffold to form a stable tertiary amine, while glycine is not a good choice unless the reductive alkylation is the last synthetic step. This is because the secondary amine generated is still reactive to acylation or reductive alkylation.

A number of small molecule libraries based on 4-fluoro-3-nitrobenzoic acid scaffold have been reported by several groups.[21,22] The *ortho*-nitro fluoride in this molecule has been shown to undergo facile aromatic nucleophilic substitution with sulfur or nitrogen nucleophiles followed by the reduction of the nitro group. To illustrate the selection of coding blocks in our encoding method, we use 4-fluoro-3-nitrobenzoic acid as a

Scheme 4. Synthetic and Encoding Reactions of a Model Compound[a]



[a] Reagents and conditions: (i) 20% piperidine in DMF, rt, 30 min; (ii) a mixture of Trt-Gly-OH (2.01 equiv), *N*-phthaloylglycine (0.34 equiv), and 4-nitrophenylacetic acid (3.45 equiv), HOBt (6 equiv), and DIC (6 equiv) in DMF, rt, 2 h; (iii) 1% TFA and 5% TIS in DCM, 2 min × 4; (iv) 5 equiv of benzoic acid, HOBt, and DIC in DMF, rt, 2 h; (v) 50% TFA in DCM, rt, 30 min; (vi) 5 equiv of Boc-Phe-OH, HOBt, and DIC in DMF, rt, 2 h; (vii) 50% TFA in DCM, rt, 30 min; (viii) 5 equiv of 4-fluoro-3-nitrobenzoic acid, HOBt, and DIC in DMF, rt, 2 h; (ix) 0.25 M propylamine in 5% DIEA/DMF, rt, overnight; (x) 2 M $SnCl_2 \cdot 2H_2O$ in DMF, 3 h × 2; (xi) 20 equiv of chloroacetic anhydride and 4 equiv of DIEA in DCM, rt, overnight; (xii) 10% DIEA in DMF, rt, 6h; (xiii) 10% piperidine and 10% DIEA in DMF, rt, overnight.

model scaffold (Scheme 4). The carboxyl group of the scaffold is usually connected to the solid support via an amide or an ester bond. If an additional diversity is desired, an amino acid may be introduced prior to the coupling of the scaffold. The

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Table 1. Relative Coupling Efficiency of Some Coding Blocks

coding blocks	relative reactivity	encoded reaction
benzoic acid	1.00	
acrylic acid	1.79	Michael addition
bromoacetic acid	17.76	nucleophilic substitution
4-bromomethylphenylacetic acid	1.39	nucleophilic substitution
4-(chloromethyl)benzoic acid	1.81	nucleophilic substitution
4-maleimidobutyric acid	0.47	nucleophilic substitution
N-phthaloylglycine	5.71	nucleophilic substitution
N-tritylglycine (Trt-Gly-OH)	1.55	amino acid coupling
N-Fmoc-piperidinecarboxylic acid	0.93	reductive alkylation
3-nitrophenylacetic acid	2.75	aromatic reduction and acylation
4-nitrophenylacetic acid	0.70	aromatic reduction and acylation

amino acid can easily be encoded by a precoupled carboxylic acid in the interior of the bead. Either nitrobenzoic acid or nitrophenylactetic acid is a good choice to encode the nitro group. However, we cannot use an *ortho*-nitro compound to encode the *ortho*-nitro fluoride, since the nitro group in the coding block will be reactive to the subsequent reactions. Alternately, we might select bromoacetic acid, 4-bromomethylphenylacetic acid, or 4-(chloromethyl)benzoic acid as the coding block if the building block is going to be a sulfur nucleophile or a secondary amine.[23] In this case, the reaction product will be a stable thioether or tertiary amine. Those acids can be also used to encode Michael addition, since the same nucleophiles are used as the reactant. For primary amine building blocks, which react with the previously mentioned coding blocks to generate a secondary amine, 4-maleimidobutanoic acid or *N*-phthaloylglycine can be chosen because the reaction generates a stable diamide compound (Scheme 4).[24]

The loading of each of the coding blocks should be similar so that comparable signal intensity of each decoding peak can be obtained during MS analysis. This can be achieved by adjusting the concentration of the coding blocks used in the ligation, according to the inverse of their relative coupling efficiency. The relative coupling efficiency of 10 coding blocks were determined using benzoic acid as the standard reference (Table 1). Equal equivalents of a coding block and benzoic acid were coupled to the linker on TentaGel. After cleavage and MS analysis, the relative coupling efficiency of the coding block is expressed as the signal intensity ratio of the coding block to the reference benzoic acid.

A model compound (**7**, Scheme 4) from a published small molecule library using 4-fluoro-3-nitrobenzoic acid scaffold was synthesized and encoded based on our selection of coding blocks.[22] The synthetic and encoding reactions are shown in Scheme 4. The first diversity, that is, the amino acid, was encoded by a precoupled acid on the Trt-Gly-OH (**8**, Scheme 4). *N*-phthaloylglycine and 4-nitrophenylacetic acid were used to encode the *ortho*-nitro fluoride and the nitro group on the scaffold, respectively. The resin beads containing a presynthesized linker in the inner core were divided into two portions. One portion of the resin was treated with TFA to remove an outside Boc protecting group, followed by coupling with Boc-Met-OH. As a result, both the outer and inner layers of these

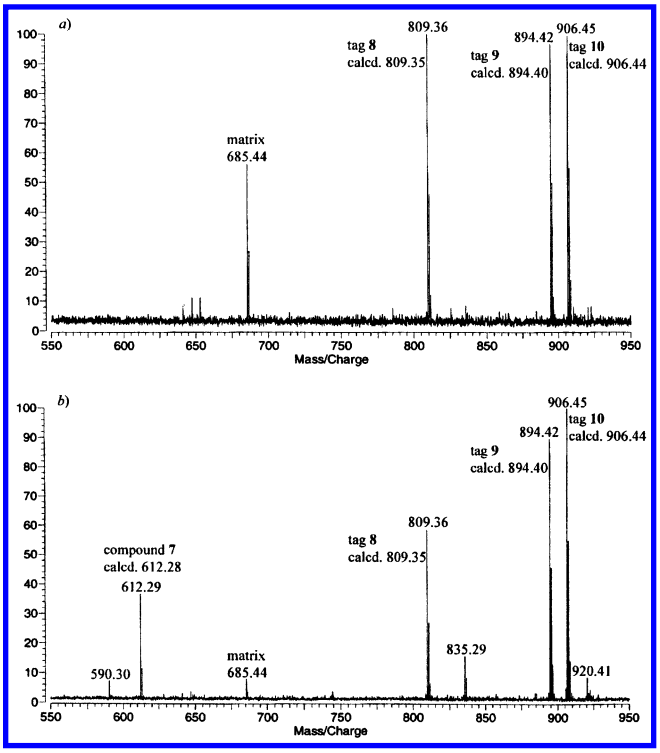


Figure 2. MALDI-FTMS spectra of single-bead analysis for the resin containing library compound **7** and coding tags **8**−**10**: (a) outside-noncleavable bead; (b) outside-cleavable bead.

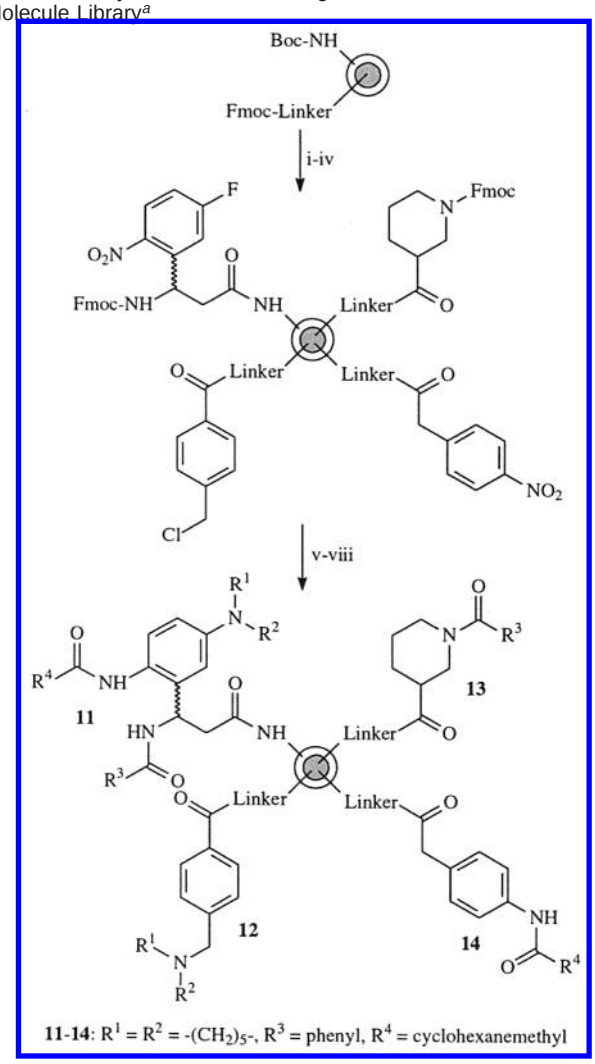
beads contained a cleavable linker. In contrast, the remaining portion of the resin contained a cleavable linker in the inner core only. The inside Fmoc group of both portions of resin was then removed. A mixture of Trt-Gly-OH, *N*-phthaloylglycine, and 4-nitrophenylacetic acid, whose concentrations have been adjusted according to their relative reactivity (Table 1), was coupled to the interior of the resin. The Trt (trityl) protecting group on Trt-Gly-OH was removed using 1% TFA (trifluoroacetic acid), and benzoic acid was coupled to glycine to encode the outside phenylalanine. After Boc deprotection and Boc-Phe-OH coupling, the scaffold was then coupled to outside phenylalanine. Propylamine reacted with inside *N*-phthaloylglycine to form a stable amide bond (**9**) when replacing the fluoride on the scaffold. Both of the outside and inside nitro groups were reduced with tin(II) chloride, followed by acylation with chloroacetic anhydride. The tetrahydroquinoxalin ring was then formed by treating the resin with a base. Finally, substitution of the remaining chloride with piperidine generated library compound **7** and coding tag **10**. A single bead from both portions of resin was then treated with cyanogen bromide and analyzed with MALDI-FTMS (Figure 2).

The results from outside-noncleavable (Figure 2a) and outside-cleavable (Figure 2b) beads are consistent. The signals of three coding tags are clearly seen in both cases, while that of the library compound **7** appears in Figure 2b. The molecular mass 590.30 corresponds to the protonated product of library compound **7**. It should be noted that the signal of the library compound is much weaker than those of coding tags, even though its concentration is much higher. This means the library compound is not as sensitive as the coding tags to the MALDI-FTMS method.

Synthesis and Screening of a Model Encoded Library. To demonstrate the utility and efficiency of our encoding method,

(23) Dankwardt, S. M.; Newman, S. R.; Krstenansky, J. L. *Tetrahedron Lett.* **1995**, *36*, 4923−4926.
(24) Yuan, C.; Williams, R. M. *J. Am. Chem. Soc.* **1997**, *119*, 11777−11784.

Scheme 5. Synthetic and Encoding Reactions of a Model Small Molecule Library[a]



[a] Reagents and conditions: (i) 20% piperidine in DMF, rt, 30 min; (ii) a mixture of 4-(chloromethyl)benzoic acid (1.18 equiv), *N*-Fmoc-3-piperidinecarboxylic acid (2.45 equiv), 4-nitrophenylacetic acid (2.37 equiv), HOBt (6 equiv), and DIC (6 equiv) in DMF, rt, 2 h; (iii) 50% TFA in DCM, rt, 30 min; (iv) 5 equiv of (*R*,*S*)-*N*-Fmoc-β-amino-5-fluoro-2-nitrobenzenepropanoic acid, HOBt and DIC in DMF, rt, 5 h; (v) 1 M secondary amine in 5% DBU/DMF, rt, overnight; (vi) 10 equiv of carboxylic acid, HOBt and DIC in DMF, rt, 4 h; (vii) 2 M $SnCl_2 \cdot 2H_2O$ in DMF, 3 h $\times$ 2; (viii) 30 equiv of carboxylic acid, 30 equiv of DIC, and 6 equiv of DIEA in DCM, rt, 12 h $\times$ 2.

a simple encoded small molecule library was synthesized and screened against streptavidin using (*R*,*S*)-*N*-Fmoc-β-amino-5-fluoro-2-nitrobenzenepropanoic acid as the scaffold (Scheme 5). 4-(Chloromethyl)benzoic acid, *N*-Fmoc-3-piperidinecarboxylic acid, and 4-nitrophenylacetic acid were chosen to encode the *para*-nitro fluoride, Fmoc-protected amino group, and nitro group on the scaffold, respectively. At the beginning of library synthesis, a secondary amine was used as the first building block to replace both the *para*-nitro fluoride on the scaffold and the chloride of the coding block 4-(chloromethyl)benzoic acid. The Fmoc protecting group was removed in this step simultaneously. A carboxylic acid or a Boc-protected amino acid was then coupled to the amino group on the scaffold as well as the coding block 3-piperidinecarboxylic acid, followed by reduction of nitro groups with tin(II) chloride. In the last synthetic step, a carboxylic acid (anhydride, acyl chloride, or sulfonyl chloride)

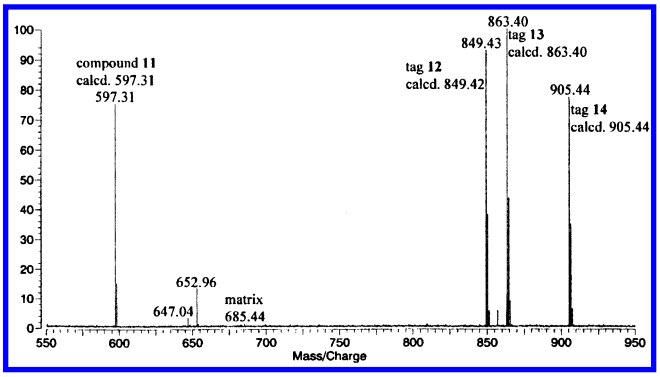


Figure 3. MALDI-FTMS spectrum of single-bead analysis for the resin containing library compound **11** and coding tags **12**−**14**. As in Figure 2b, this model compound was synthesized on beads with a cleavable linker on both the outer layer and inner core.

reacted with the aryl amino groups on both the scaffold and the coding block. The Boc and acid-labile side-chain protecting groups of amino acids were removed by treatment with TFA after library synthesis. A randomly selected model compound (Scheme 5, **11**) from this library was synthesized and encoded prior to the library synthesis. The model compound was linked to the solid support via methionine to make it releasable by CNBr. The decoding result is shown in Figure 3. The obtained molecular masses of library compound **11** and three coding tags are consistent with the calculated values, indicating the structures of library compounds can be reliably decoded.

In the library synthesis, we selected 42 secondary amines, 42 carboxylic acids or Boc-protected amino acids, and 48 carboxylic acids (anhydrides, acyl chlorides, or sulfonyl chlorides) as the first (BB1), second (BB2), and third (BB3) building blocks, respectively. The molecular weights of coding tags were calculated prior to library synthesis to avoid any ambiguity in the final decoding. After the library synthesis was completed, 50 resin beads were randomly picked for single-bead analysis. MALDI-FTMS was used to unambiguously decode 46 of them. No signals were obtained from four samples, probably due to the loss of the beads during sample transfer.

This 84 672-member library (42 $\times$ 42 $\times$ 48) was screened against streptavidin at an extremely dilute streptavidin−alkaline phosphatase conjugate concentration (1:100 000 or 50 pM) using an enzyme-linked colorimetric assay.[9,25] Positive beads (20) were isolated, and 18 of them were successfully analyzed with MALDI-FTMS. Of these 18 beads, 2 yielded identical decoding results (Table 2, entry 1). A typical decoding spectrum is shown in Figure 4. Most of the streptavidin ligands shown in Table 2 share structural similarities. At the position of the first building block, morpholine was found 7 times, and thiomorpholine was found 3 times. The second building block was quite variable, but a neutral amino acid appeared to be preferable. At the position of the third building block, 4-pyridinecarboxylic acid was seen frequently (8 times). 4-Pyridinecarboxylic acid has been found to be important for streptavidin binding in our previous work.[9] Its structural analogues, 3-pyridinepropanoic acid and pyrazinecarboxylic acid, were also observed in some of the ligands. The streptavidin binding affinity of these ligands was confirmed by resynthesis of these compounds on regular TentaGel resin and rescreening against streptavidin using the same method. Two of these compound beads (Scheme 6,

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Table 2. Decoding Results of Positive Beads Obtained from Streptavidin Binding Assay

entry	BB1 (R^1 and R^2)	BB2 (R^3)	BB3 (R^4)
1[a]	morpholine	Ala	4-pyridinecarboxylic acid
2	morpholine	Ala	3-benzoyl-2-pyridinecarboxylic acid
3	morpholine	Gly	pyrazinecarboxylic acid
4	morpholine	Gly	4-oxo-benzenebutanoic acid
5	morpholine	Ser	4-oxo-benzenebutanoic acid
6	morpholine	Gly	3,5-dimethoxybenzoic acid
7	thiomorpholine	acetic acid	4-pyridinecarboxylic acid
8	thiomorpholine	Ala	4-pyridinecarboxylic acid
9	thiomorpholine	Ala	[1,1′-biphenyl]-4-carboxylic acid
10	ethyl 1-piperazinecarboxylate	(*S*)-α-amino-cyclohexaneacetic acid	4-pyridinecarboxylic acid
11	ethyl 1-piperazinecarboxylate	Leu	4-pyridinecarboxylic acid
12	ethyl 1-piperazinecarboxylate	Ser	3-pyridinepropanoic acid
13	1-(2-pyridyl)piperazine	Gly	4-pyridinecarboxylic acid
14	1-(2-cyanophenyl)piperazine	Leu	4-pyridinecarboxylic acid
15	decahydroquinoline	Gly	4-pyridinecarboxylic acid
16	1-(2-furanylcarbonyl)piperazine	acetic acid	4-oxo-benzenebutanoic acid
17	1,4-dioxa-8-azaspiro[4.5]decane	Ser	3-pyridinepropanoic acid

[a] The structure was found twice.

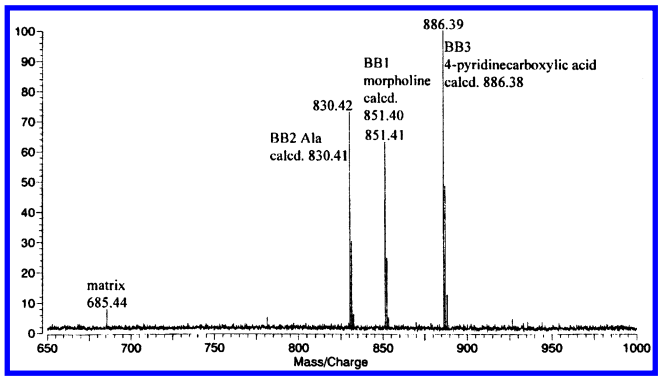
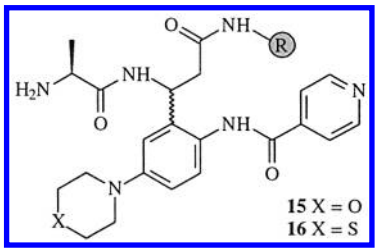


Figure 4. MALDI-FTMS decoding spectrum of a positive bead from the library screening against streptavidin.

Scheme 6. Chemical Structures of Two Streptavidin Binding Ligands



compounds **15** and **16**) stained very dark suggesting higher binding affinities than those of the others.

Conclusion

The encoding method described in this report is simple, fast, and robust and can easily encode small molecule libraries with over a million unique compounds. The extra encoding steps necessary for many other chemical encoding techniques are eliminated in this method, because the library synthesis steps and the encoding steps are combined. One additional but very important advantage of combining compound synthesis and encoding steps is that the decoding information enables us to estimate the quality of library compounds on an individual bead. Since the coupling chemistry on the scaffold and the coding block is either identical or very similar, the quality of a specific coding tag on the decoding spectrum reflects the quality of the corresponding coupling reaction on the scaffold. The topological segregation of coding tags from the library compound avoids interference by the coding tag during biological screening. To save time, the linkers and some of the coding blocks can be coupled to a large batch of resin in advance, and only a portion (e.g., 10 mL of 90 μm settled beads, which is equivalent to 7.5 million beads) is needed for the synthesis of each library. Because the molecular masses of coding tags can be accurately determined in a single analysis step, no combination with other techniques such as HPLC or MS/MS is needed for decoding. We can now manually analyze 20−30 beads in an hour. We envision that, with automation, over 500−1000 beads can be analyzed in a single day. The sensitivity of this encoding method is extremely high. We have been able to decode as small as a 10% fragment of a single 90 μm TentaGel bead with no ambiguity. With this encoding approach, we may decrease the size of the beads to 30 μm and still obtain more than adequate mass signals for accurate decoding. However, further decreases in bead size to less than 30 μm will make the library synthesis and screening technically more difficult to handle. Work is currently underway in our laboratory to fully automate the sample preparation and the laser desorption ionization steps. We are also developing software to facilitate both the selection of building blocks and the decoding process, as well as archiving the massive amounts of data that we expect to obtain.

Experimental Section

Material and General Methods. TentaGel S NH_2 resin (90 μm, 0.26 mmol/g) was purchased from Rapp Polymere GmbH (Tübingen, Germany). All the calculations for synthesis were based on a substitution of 0.26 mmol/g. (*R*,*S*)-*N*-Fmoc-β-amino-5-fluoro-2-nitrobenzenepropanoic acid was purchased from InnovaChem (Tucson, AZ). Alloc-Osu (*N*-(allyloxycarbonyloxy)succinimide) was purchased from Senn Chemicals (Dielsdorf, Switzerland). N^{α}-Fmoc-protected amino acids and HOBt (1-hydroxybenzotriazole) were purchased from GL Biochem (Shanghai, China). N^{α}-Boc-protected amino acids were purchased from Chem-Impex International (Wood Dale, IL) and Advanced ChemTech (Louisville, KY). Streptavidin−alkaline phosphatase conjugate was purchased from Upstate Biotechnology (Lake Placid, NY). All solvents and other chemical reagents were purchased from Aldrich (Milwaukee, WI) and were analytical grade. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX 500 MHz spectrometer at 25 °C. A commercial MALDI-FTMS instrument (IonSpec Corp., Irvine, CA), equipped with an external MALDI source, a 4.7 T superconducting

magnet, and an Nd:YAG laser operating at 355 nm, was used for MALDI-FTMS analysis. All the experiments were carried out at room temperature unless otherwise noted. For Fmoc deprotection, the resin was treated with 20% piperidine/*N*,*N*-dimethylformamide (DMF) for 30 min and then washed thoroughly with DMF, methanol (MeOH), and DMF 3 times each. For Boc deprotection, the resin was incubated with 50% TFA/dichloromethane (DCM) for 30 min and then washed thoroughly with DCM (twice), 2.5% *N*,*N*-diisopropylethylamine (DIEA)/DCM (3 times), DCM (twice), MeOH (3 times), and DMF (3 times).

Synthesis of *N*-(9-Fluorenylmethoxycarbonyl)-4-{2-[2-(2-aminoethoxyl)ethoxy]ethylamino}-4-oxo-butanoic Acid (*N*-Fmoc-2,2′-(ethylenedioxy)bis(ethylamine) Monosuccinamide, Fmoc-Ebes-OH).[26] 2,2′-(ethylenedioxy)bis(ethylamine) (1.46 mL, 10 mmol) was dissolved in 50 mL acetonitrile. A solution of succinic anhydride (1.0 g, 10 mmol) in 25 mL of acetonitrile was added dropwise under vigorous magnetic stirring over 1 h. The stirring was stopped after 3 h. After the waxy product settled, the organic solvent was decanted and discarded. The product was redissolved in 100 mL of 50% acetonitrile/water and chilled in an ice bath for 30 min. A solution of *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu, 4.4 g, 13 mmol) in 25 mL of acetonitrile was added dropwise under vigorous magnetic stirring over 1 h. The pH of the reaction mixture was maintained at 8−9 by adding DIEA throughout the reaction. The reaction was allowed to proceed overnight at room temperature. The solvents were removed in vacuo. The residue was dissolved in 100 mL of 5% aqueous $NaHCO_3$ solution and washed with ethyl acetate. The aqueous phase was then acidified with 1 M HCl to pH 2 and extracted with ethyl acetate (50 mL × 3). The combined organic phase was washed with water and dried over anhydrous $MgSO_4$. The solution was concentrated to a small volume and diluted with hexanes. A white solid was obtained with a yield of 72.6%. Its purity was determined to be 98% by HPLC analysis based on the absorption at 254 nm. Mp 111−113 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ/ppm 7.91 (s, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.69 (d, J = 7.5 Hz, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.33 (m, 2H), 6.28 (s, 1H), 4.29 (d, J = 6.8 Hz, 2H), 4.21 (t, J = 6.8 Hz, 1H), 3.5−3.6 (m, 4H), 3.3−3.4 (m, 4H), 3.1−3.2 (m, 4H), 2.40 (t, J = 7.0 Hz, 2H), 2.30 (t, J = 7.0 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ/ppm 174.6, 171.9, 156.9, 144.6, 141.5, 128.3, 128.0, 125.9, 120.8, 70.2, 69.8, 66.0, 47.4, 40.8, 39.3, 30.8, 30.1; MALDI-FTMS (M + Na^+) m/z calcd, 493.195; found, 493.198.

Solid-Phase Synthesis of the Cleavable Linker 1. The sequence Phe-Phe-Met was synthesized on TentaGel S NH_2 resin using standard Fmoc chemistry with 1,3-diisopropylcarbodiimide (DIC) and HOBt as the activating system.[19] A ninhydrin test was carried out to monitor amino acid coupling and Fmoc deprotection.[27] After Fmoc deprotection of the last Phe, a mixture of Fmoc-Ebes-OH (3 equiv), HOBt (3 equiv), and DIC (3 equiv) in DMF was added to the resin. The reaction mixture was gently shaken overnight until the ninhydrin test was negative. The obtained Fmoc-linker resin was washed with DMF, DCM, MeOH 3 times each and then dried in vacuo.

Preparation of Topologically Segregated Bifunctional TentaGel Resin Beads with 60% Boc Outside and 40% Fmoc-Linker 1 Inside (Outside-Boc-Inside-Fmoc-Linker-Bifunctional Resin). TentaGel S NH_2 resin beads (1.0 g, 0.26 mmol) were swollen in water for 48 h. The water was drained, and a solution of Alloc-OSu (31.1 mg, 0.156 mmol) in a DCM/diethyl ether mixture (50 mL, v/v = 55:45) was added to the resin, followed by addition of DIEA (55 μL, 0.312 mmol). The resulting mixture was shaken vigorously for 1 h. The resin was washed 3 times with DCM and 6 times with DMF. Fmoc-Linker **1** was then built in the inner region of the resin beads using the previously mentioned procedure. The resin was washed 3 times with DCM. In the presence of argon, a solution of $PhSiH_3$ (770 μL, 6.24 mmol) in 4 mL of DCM was added to the resin followed by a solution of Pd(PPh_3)$_4$ (75.1 mg, 0.065 mmol) in 12 mL of DCM.[28] The mixture was shaken in an argon atmosphere for 30 min. This process was repeated once. The resin was washed with DCM, DMF, and DCM 3 times each. A solution of di-*tert*-butyl dicarbonate (1.19 mL, 5.2 mmol) in 10 mL of DCM was added to the resin, followed by the addition of DIEA (226.4 μL, 1.3 mmol). The mixture was shaken until the ninhydrin test was negative. The obtained outside-Boc-inside-Fmoc-linker-bifunctional resin was washed with DCM, DMF, DCM and MeOH 3 times each and then dried in vacuo. The percentage of inner region was determined to be 39% using quantitative UV absorption analysis of the dibenzofulvene−piperidine adduct released by treatment with piperidine.[29]

Determination of the Relative Reactivity of the Coding Blocks. Fmoc-linker resin (20 mg, 0.0052 mmol) was swollen in DMF overnight, followed by Fmoc deprotection. A mixture of the coding block (0.0156 mmol), benzoic acid (1.91 mg, 0.0156 mmol), HOBt (4.22 mg, 0.0312 mmol), DIC (4.9 μL, 0.0312 mmol), and 0.4 mL of DMF was agitated for 30 min and then added to the resin. The reaction mixture was agitated until the ninhydrin test was negative. The resin was washed with DMF, DCM, and MeOH thoroughly. Beads (50) were randomly picked and divided into five groups for cleavage and MALDI-FTMS analysis.

Synthesis of Compound 2−6. Outside-Boc-inside-Fmoc-linker-bifunctional resin (20 mg, 0.0052 mmol) was swollen in DMF overnight, followed by Fmoc deprotection. The resin was incubated with a mixture of Trt-Gly-OH (3.30 mg, 0.0104 mmol), benzoic acid (1.27 mg, 0.0104 mmol), *N*-Fmoc-3-piperidinecarboxylic acid (3.65 mg, 0.0104 mmol), 4-nitrophenylacetic acid (1.88 mg, 0.0104 mmol), 4-(chloromethyl)benzoic acid (1.77 mg, 0.0104 mmol), HOBt (5.62 mg, 0.0416 mmol), and DIC (6.5 μL, 0.0416 mmol) in 0.4 mL of DMF until the ninhydrin test was negative. The resin was washed with DMF (0.5 mL × 3), MeOH (0.5 mL × 3), and DCM (0.5 mL × 3) and then treated with 1% TFA and 5% triisopropylsilane (TIS) in DCM (0.5 mL and 2 min, 4 times). After the resin was washed with DCM (0.5 mL × 3), MeOH (0.5 mL × 3) and 5% DIEA/DMF (0.5 mL × 3), it was agitated with 0.25 M piperidine in 5% DIEA/DMF overnight. To the resin thoroughly washed with DMF, MeOH, and DMF was added 0.5 mL of 2 M $SnCl_2 \cdot 2H_2O$ in DMF. The mixture was shaken for 6 h, followed by washing with DMF (0.5 mL × 3), DCM (0.5 mL × 3), MeOH (0.5 mL × 3), and DCM (0.5 mL × 3). All the amino groups were then blocked by incubation with acetic anhydride (9.8 μL, 0.104 mmol) and DIEA (3.6 μL, 0.0208 mmol) in 0.5 mL of DCM overnight. The resin was washed with DMF, DCM, and MeOH thoroughly.

Synthesis of Model Compound 7 and Tags 8−10. Outside-Boc-inside-Fmoc-linker-bifunctional resin (20 mg, 0.0052 mmol) was swollen in DCM overnight, followed by Boc deprotection. A solution of Boc-Met-OH (3.89 mg, 0.0156 mmol), HOBt (2.11 mg, 0.0156 mmol), and DIC (2.5 μL, 0.016 mmol) in DMF was added to the resin. The mixture was agitated until the ninhydrin test was negative. The resin was washed 5 times with DMF.

Outside-Boc-inside-Fmoc-linker-bifunctional resin (either with or without outside methione, 20 mg, 0.0052 mmol) was swollen in DMF overnight, followed by Fmoc deprotection. A mixture of Trt-Gly-OH (3.32 mg, 0.0105 mmol), *N*-phthaloylglycine (0.36 mg, 0.001 77 mmol), 4-nitrophenylacetic acid (3.25 mg, 0.0179 mmol), HOBt (4.22 mg, 0.0312 mmol), DIC (4.9 μL, 0.0312 mmol), and 0.4 mL DMF was agitated for 30 min and was added to the resin. The reaction mixture was agitated until the ninhydrin test was negative. The resin was washed with DMF (0.5 mL × 3), MeOH (0.5 mL × 3), and DCM (0.5 mL × 3) and then treated with 1% TFA and 5% TIS in DCM (0.5 mL and 2

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(27) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595−598.
(28) Grieco, P.; Gitu, P. M.; Hruby, V. J. *J. Pept. Res.* **2001**, *57*, 250−256.
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min, 4 times). After the resin was washed with DCM (0.5 mL × 2), 2.5% DIEA/DCM (0.5 mL × 2), DCM (0.5 mL × 2), MeOH (0.5 mL × 3), and DMF (0.5 mL × 3), it was agitated with a mixture of benzoic acid (3.18 mg, 0.026 mmol), HOBt (3.52 mg, 0.026 mmol), DIC (4.1 μL, 0.026 mmol), and 0.4 mL of DMF for 2 h, followed by Boc deprotection. A solution of 4-fluoro-3-nitrobenzoic acid (0.026 mmol), HOBt (3.52 mg, 0.026 mmol), and DIC (4.1 μL, 0.026 mmol) in 0.4 mL of DMF was added to the resin. After the ninhydrin test was negative, the resin was washed thoroughly with DMF, MeOH, and DMF 3 times each. A 0.5 mL aliquot of 0.25 M propylamine in 5% DIEA/DMF was added to the resin, and the resulting mixture was agitated overnight. The resin was washed extensively with DMF and then agitated with 0.5 mL of 2 M $SnCl_2 \cdot 2H_2O$ in DMF for 3 h. The reduction was repeated. The resin was washed thoroughly with DMF, MeOH, DMF, and DCM and then agitated with a solution of chloroacetic anhydride (17.8 mg, 0.104 mmol) and DIEA (3.7 μL, 0.021 mmol) in 0.4 mL of DCM overnight. The solution was drained. After the resin was washed with DCM (0.5 mL × 3), MeOH (0.5 mL × 3), and DMF (0.5 mL × 3), it was treated with 10% DIEA/DMF for 6 h. The mixture was filtered, and a solution of 10% piperidine and 10% DIEA in DMF was added to the resin. The resulting mixture was shaken overnight. The resin was washed with DMF, DCM, and MeOH 3 times each.

Synthesis of the Model Encoded Library. Outside-Boc-inside-Fmoc-linker-bifunctional resin (0.5 g, 0.13 mmol) was swollen in DMF overnight, followed by Fmoc deprotection. A mixture of 4-(chloromethyl)benzoic acid (26.17 mg, 0.1534 mmol), *N*-Fmoc-3-piperidinecarboxylic acid (113.92 mg, 0.3185 mmol), 4-nitrophenylacetic acid (55.81 mg, 0.3081 mmol), HOBt (105.46 mg, 0.78 mmol), DIC (122.1 μL, 0.78 mmol), and 10 mL of DMF was agitated for 30 min and then added to the resin. The resulting mixture was agitated until the ninhydrin test was negative. The resin was washed with DMF, MeOH, and DCM 3 times each, followed by Boc deprotection. A solution of (*R,S*)-*N*-Fmoc-β-amino-5-fluoro-2-nitrobenzenepropanoic acid (272.66 mg, 0.65 mmol), HOBt (87.88 mg, 0.65 mmol), and DIC (101.8 μL, 0.65 mmol) in 8 mL of DMF was added to the resin. The reaction mixture was shaken until the ninhydrin test was negative. The resin was filtered and washed with DMF, MeOH, and DMF 3 times each. The resin was then split into 42 aliquots; to each of those a solution of one of 42 secondary amines in 5% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)/DMF was added. The reaction was allowed to proceed overnight. The resin beads were combined and washed with DMF, MeOH, and DMF 3 times each. The resin was split into 42 equal portions again. Each one of the 42 carboxylic acids and N^{α}-Boc-protected amino acids (0.031 mmol) was dissolved in a solution of HOBt (4.19 mg, 0.031 mmol) in 0.5 mL of DMF followed by addition of DIC (4.9 μL, 0.031 mmol). The solutions were added to the 42 portions of resin individually. The reaction mixtures were shaken for 4 h. The resin beads were combined and washed with DMF, MeOH, and DMF 3 times each, followed by incubation with 10 mL of 2 M $SnCl_2 \cdot 2H_2O$ in DMF for 3 h. The reduction was repeated. After washing thoroughly with DMF, DCM, MeOH, and DCM 3 times each, the resin was split into 48 aliquots. To each aliquot of resin, a solution of 1 of 40 carboxylic acids (0.081 mmol) and DIC (12.7 μL, 0.081 mmol) in 0.7 mL of DCM (or a solution of 1 of 8 anhydrides, acyl chlorides, and sulfonyl chlorides (0.041 mmol) in 0.7 mL of DCM) was added followed by addition of DIEA (2.8 μL, 0.016 mmol). The reaction was carried out for 12 h and repeated. Complete acylation was confirmed by a negative chloranil test.[30] The resin beads were combined, washed with DCM, DMF, DCM, and MeOH 3 times each, and then dried in vacuo. The bead-supported library was treated with a cleavage mixture consisting of TFA, TIS, and water (v/v/v = 95:2.5:2.5) for 2 h to remove the Boc and side-chain protecting groups of amino acids. After the bead-supported library was extensively washed with DCM, DMF, DCM, and MeOH, it was stored in MeOH at 4 °C.

Library Screening. A 0.2 mL aliquot of the bead-supported library was transferred into a 10 mL disposable polypropylene column with a polyethylene frit. The beads were washed with water (4 mL × 10) and then agitated with 4 mL of $PBSTGNaN_3$ buffer (8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4, plus 0.1% Tween-20, 0.1% gelatin, and 0.05% sodium azide) for 1 h. To the beads washed with $PBSTGNaN_3$ buffer (4 mL × 5) streptavidin−alkaline phosphatase conjugate solution (2 mL) was added at a dilution of 1:100 000 (original concentration was 1 mg/mL) in $PBSTGNaN_3$ buffer. The mixture was incubated for 1 h. The beads were filtered and washed with TBS buffer (2.5 mM Tris-HCl, 13.7 mM NaCl, 0.27 mM KCl, pH 8.0, 4 mL), followed by washing with BCIP buffer (0.1 M Tris-HCl, 0.1 M NaCl, 2.34 mM $MgCl_2$, pH 8.8, 4 mL × 2). A solution of the alkaline phosphatase substrate 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in BCIP buffer (1.65 mg/mL, 2 mL) was added to the resin to develop color for 1 h. The enzymatic reaction was stopped by washing with $PBSTGNaN_3$ buffer (4 mL × 5) and water (4 mL × 5). The blue-colored beads were retrieved and treated with 6.0 M guanidine−HCl (pH 1.0) solution to strip the protein off the beads and then were washed twice with water. The indigo dye was removed by incubating the beads with acetone for 15 min. After the beads are washed with ethanol twice, they are ready for cleavage.

Cleavage and MALDI-FTMS Analysis of a Single Bead. A single resin bead was transferred to a 200 μL polypropylene microcentrifuge tube in ethanol under a microscope. The solvent was evaporated in vacuo, and 10 μL of 0.25 M CNBr in 70% formic acid was added. The mixture was gently shaken overnight in the dark. The cleavage was stopped by freezing and lyophilizing to dryness. The residue was redissolved in 10 μL of 50% acetonitrile/water. The sample was applied on the probe with 2 μL aliquots. Sodium dopant (0.01 M NaCl in 50% ethanol/water, 1 μL) was added to the probe tip followed by matrix solution (0.4 M of 2,5-dihydroxybenzoic acid in ethanol, 1 μL). Hot air was used to quickly crystallize the sample on the probe.

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Supporting Information Available: Structures of building blocks used in library synthesis and molecular weights of the corresponding coding tags. This material is available free of charge via the Internet at http://pubs.acs.org.

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