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Synthesis, Screening, and Sequencing of Cysteine-Rich One-Bead One-Compound Peptide Libraries

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Cysteine-rich peptides are valued as tags for biarsenical fluorophores and as environmentally important reagents for binding toxic heavy metals. Due to the inherent difficulties created by cysteine, the power of one-bead one-compound (OBOC) libraries has never been applied to the discovery of short cysteine-rich peptides. We have developed the first method for the synthesis, screening, and sequencing of cysteine-rich OBOC peptide libraries. First, we synthesized a heavily biased cysteine-rich OBOC library, incorporating 50% cysteine at each position (Ac-X8-KM-TentaGel). Then, we developed conditions for cysteine alkylation, cyanogen bromide cleavage, and direct MS/MS sequencing of that library at the single bead level. The sequencing efficiency of this library was comparable to a traditional cysteine-free library. To validate screening of cysteine-rich OBOC libraries, we reacted a library with the biarsenical FlAsH and identified beads bearing the known biarsenical-binding motif (CCXXCC). These results enable OBOC libraries to be used in high-throughput discovery of cysteine-rich peptides for protein tagging, environmental remediation of metal contaminants, or cysteine-rich pharmaceuticals.

Introduction

Cysteine-rich peptides are of significant and growing interest due to the increasing use of tetracysteine tags for fluorescence imaging of living cells and as tools for bioremediation and phytoremediation of toxic heavy metal contaminants such as cadmium and mercury. A library of 205 designed tetracysteine peptides immobilized on cellulose has been successfully screened against biarsenical probes. But, the drawback is that these spatially addressed libraries tend to be orders of magnitude smaller than biological or bead-based libraries. Cell-based cysteine-rich libraries have also been successfully used to identify optimized peptide tags for biarsenicals. However, cytotoxicity is an intrinsic liability in the use of cell-based libraries to identify peptides that can sequester heavy metals. In contrast, phage libraries can be screened under abiotic conditions and are less susceptible to cytotoxic effects. They have been used to identify peptides that bind to immobilized metals, but immobilized metals present few open coordination sites for tight peptide binding of phage-displayed peptides. A complementary method is needed for identification of cysteine-rich peptides that tightly bind to free metals and cytotoxic ligands.

One-bead one-compound (OBOC) peptide libraries are powerful tools to simultaneously screen millions of peptides for exceptional reactivity and selectivity. They can incorporate both ribosomal and nonribosomal amino acids and can be screened under conditions toxic to cells. However, due to the problems caused by cysteine during synthesis, screening, and sequencing, cysteine is generally excluded from OBOC libraries.

Standard conditions (1:1 DMF/CH2Cl2)7 for the synthesis of polycysteine peptides using tritylcysteine are inefficient due to the bulk and hydrophobicity of the S-trityl group, leading to aggregation and ultimately to racemization. Alternative protecting groups such as S-acetamidomethyl and S-r-butylthiocrysteine can be used to deter aggregation, but at the cost of reagent toxicity, additional side reactions, and additional deprotection steps. Thiols will form disulfides under ambient conditions, so screening and sequencing must be performed under reducing conditions to prevent disulfide formation. Free cysteine thiols can undergo side reactions during Edman degradation and during cyanogen bromide cleavage—chemistry used in the two traditional methods of direct sequencing of OBOC-derived peptides. In addition, cysteine can be difficult to distinguish from arginine by Edman degradation.

The first cysteine-rich OBOC library has recently appeared in the literature. While this library was neither screened nor sequenced, randomly chosen metal-soaked beads were extracted and analyzed to reveal interesting variations in metal affinities. Clearly, a robust method for sequencing cysteine-rich OBOC libraries would be invaluable. A wide range of methods are available to identify the sequences of peptides on single beads. After screening an OBOC library and isolating bead hits, there are either indirect or direct approaches to identify the sequences of these library hits. The indirect approaches like recursive deconvolution are laborious while the use of encoding tags introduces various complications.

The direct sequencing of individual beads from OBOC peptide libraries traditionally uses Edman degradation which is restricted to α-amino acids11 and requires access to a free

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N-terminus. Surprisingly, after two decades of research using OBOC peptide libraries, free cysteines have been included in variable positions and identified by Edman sequencing in only a handful of instances. Continuing advances in the efficiency and economy of mass spectrometry have led to powerful new methods of peptide sequencing based on chemical formation of mass ladders. Tandem mass spectrometry (MS/MS) is still the most direct approach to peptide sequence determination and does not share the structural limitations imposed by Edman chemistry. Therefore it is surprising that there are so few examples of MS/MS sequencing applied to peptides obtained from single beads. In no case have single-bead MS/MS analyses been applied to oligomers containing cysteine. Despite the lack of precedence, we chose to use MS/MS as our method to sequence thiol-rich compounds from a highly diverse OBOC library in order to reduce post-screening chemical manipulations and facilitate compatibility with capped N-termini, beta peptides, and peptoids.

A wide range of major and minor problems conspire to make the synthesis, screening, and sequencing of cysteine-rich OBOC peptide libraries, at best, impractical and, at worst, impossible, using any combination of existing methods. Herein we describe the first synthesis, screening, and direct sequencing of a cysteine-rich OBOC library and validate this methodology by screening for and discovering peptides that form highly fluorescent complexes with the biarsenal FLAsH·EDT2.

Results and Discussion

Cysteine leads to problems during synthesis, screening, and sequencing of OBOC libraries. Therefore, we first developed robust conditions for the synthesis of a cysteine-rich peptide library and optimized the cleavage and sequencing of peptides from individual beads from a cysteine-rich combinatorial library. We then screened a cysteine-rich library in the presence of thiol and phosphine reducing agents to identify optimal ligands for the biarsenal FLAsH·EDT2.

Cysteine-Rich Peptides are Efficiently Synthesized using Tritylcysteine in N-Methylpyrrolidinone (NMP)

Cysteine is often incorporated into peptides with an S-acetamidomethyl protecting group that can be removed using mercuric acetate. However, mercury is difficult to remove from cysteine-rich peptides and can lead to beta elimination, generating dehydroalanine intermediates. Indeed, removal of acetamidomethyl groups from polycysteines like Ac-CCPKWCCCKM-NH2 using mild conditions (60 mM Hg(OAc)2 in 1:1 AcOH/DMF) led to low yields due to formation of dehydroalanines which were apparent in the mass spectra of the products. The problems of mercury deprotection have been overcome in the synthesis of Ac-(γ-Glu-Cys)2Gly-NH2 using S-t-butyllithiocysteine, but it required an additional deprotection step. Tritylcysteine is a good alternative for incorporation of cysteine since the S-trityl protecting group is removed under standard acidic deprotection conditions. However, the standard conditions (1:1 DMF/CH2Cl2) for the synthesis of polycysteine peptides using tritylcysteine is inefficient due to the bulk and hydrophobicity of the S-trityl group, leading to aggregation and ultimately to racemization. Through experimentation, we found that using NMP as the solvent for all couplings during synthesis of polycysteines alleviates the problems with tritylcysteine.

Cleavage of Beads for Sequence Analysis. As a prelude to construction and screening of a cysteine-rich library it was essential to demonstrate that we could cleave cysteine-rich sequences from TentaGel using cyanogen bromide. We synthesized a biarsenical-binding tetracysteine peptide (Ac-WDCCPGCCKM) on TentaGel in order to optimize conditions for cyanogen bromide cleavage. The first problem we encountered was centered on the reactivity of the cysteine thiols. While there is precedent for chemo-selective cyanogen bromide cleavage of full-length proteins containing cysteine, we found that cyanogen bromide cleavage of the cysteine-rich peptide led to a wide range of side products (Figure 1), presumably due to thiocyanate and disulfide formation. To overcome these problems with cyanogen bromide cleavage, we first treated the beads with dithiothreitol (DTT) and then alkylated the cysteine thiols with iodoacetamide in a sodium carbonate buffer. These steps led to a dramatic improvement in the purity of the cleavage products (Figure 2).

The alkylation/cleavage chemistry was readily applied to individual beads in Millipore Ultrafree-MC Centrifugal Filter Units adapted for microcentrifuge tubes. Unfortunately, single beads are poorly solvated in the alkylation step and often float at the aqueous–air interface, leading to incomplete reduction and alkylation. To ensure reproducibility, we included methanol as a cosolvent in the alkylation procedure. Under these conditions the beads are well-solvated and sink to the bottom of the tube, leading to clean, reproducible alkylation.

Synthesis and Sequencing of a Cysteine-Rich OBOC Library. A cysteine-rich decapeptide library (Ac-X8-KM-TentaGel) with a potential diversity of 188 (all amino acids excluding methionine and isoleucine) was synthesized on 5 g of 130 µm TentaGel (approximately 6.5 million beads). Ile was excluded because Leu and Ile are isobaric (have exactly the same mass). Lysine was included near the C-terminus to favor the formation of y-ions in the MS/MS spectrum of the doubly charged peptide. The library was biased toward cysteine by coupling half of the resin to Fmoc-tritylcysteine.
during each round of coupling. Fourteen beads were selected at random from the library and subjected to the cysteine alkylation and cyanogen bromide cleavage conditions. The samples were then subjected to nanoflow LC-MS/MS for sequencing of the doubly charged molecular ions. For these cleaved peptides, which are different from traditional tryptic peptides, manual interpretation of the MS/MS spectra proved to be more reliable than automated interpretation. The MS/MS for the peptide cleaved from bead Cys01 (Figure 3), and to be more reliable than automated interpretation. The MS/MS for the peptide from Cys01 (Figure 3), and to be more reliable than automated interpretation. The MS/MS for the peptide from Cys01 (Figure 3), and to be more reliable than automated interpretation. The MS/MS for the peptide from Cys01 (Figure 3), and to be more reliable than automated interpretation. The MS/MS for the peptide from Cys01 (Figure 3), and to be more reliable than automated interpretation.

Table 1. Sequences from a Cysteine-Rich Peptide Library

| bead   | sequence          |
|--------|-------------------|
| Cys01  | Ac-CVCFCRCCKM     |
| Cys02  | Ac-CVCCCYGKKM     |
| Cys03  | Ac-CVWKTCCKMK     |
| Cys04  | Ac-CFCMcCCKKKM    |
| Cys05  | Ac-CmCCACCKMK     |
| Cys06  | Ac-DYWCRCCKM      |
| Cys07  | Ac-CCKKCGFGKMK    |
| Cys08  | Ac-FLAKHCCCKM     |
| Cys09  | Ac-CLNFPPAKXM     |
| Cys10  | Ac-XXDQRCLAXM     |

Subsequently, a mammalian cell library and a cellulose array were used to identify optimized CCXXCC tetracysteine peptide tags for FlAsH. Two types of optimized tetracysteine motifs appear to be superior to all others: CCGGCC and CC(K/R)XCC. The proven superiority of these FlAsH tags provided us with the opportunity to validate our protocols for screening and sequencing of our cysteine-rich library.

Inclusion of a Bona Fide FlAsH Target Complements Limited Library Coverage. Our library was heavily biased toward cysteine by coupling 50% of the resin to cysteine at each stage of the library synthesis. This kind of user-control over amino acid composition is difficult to achieve with biological libraries synthesized on a DNA synthesizer, but is easy to achieve with OBOC peptide libraries. While the potential diversity of our library (Ac-X8KM; X ≠ Met, Ile) is around 10 billion (188), the actual diversity of our library was limited by the amount of resin used (about 6.5 million beads) and the fact that 50% cysteine was incorporated at each and every position. We found it convenient to screen 1/3 g resin, about 430,000 beads. This amount of resin does not fully cover the potential sequence space, but the cysteine bias optimizes the coverage toward known tetracysteine sequences. In order to ensure the presence of an optimized FlAsH sequence (WDCCPGCCCK), twenty-three beads with the known biarsenical tag Ac-WDCCPGCCCKM were added to the library.

Library Screened with FlAsH·EDT2). The beads (0.33 g resin) were suspended in 50 mL of a low sodium/high potassium pH 7.2 phosphate buffer that approximates the ion content of mammalian cytoplasm.28 Several reducing agents, commonly used in cell staining with FlAsH, were included in the screening buffer: beta-mercaptooethanol (1 mM), the bis-thiol BAL29 (0.5 mM), and tris-carboxyethylphosphine (2 mM). Triton X-100 (0.02% v/v) was also added to reduce nonspecific binding. The library, containing about 130 µmol total peptide, was exposed to 0.005 µmol FlAsH·EDT2 over 6 h. The beads were then washed with phosphate buffer containing Triton X-100. To increase the stringency of the assay, the beads were exposed to screening buffer containing 1 mM BAL, but devoid of FlAsH·EDT2 for 10 h. Secondary thiol washes are a common method to improve contrast when staining mammalian cells with biarsenicals. The beads were then rinsed with and visualized in bicarbonate buffer. The suspended beads were screened under a long wave UV lamp, which has sufficient output within the FlAsH excitation range to readily identify hits. Six beads exhibited strong to weak green fluorescence and were removed for sequencing (Figure 4). Each bead was then subjected to reduction, alkylation, and cleavage, and the cleaved peptide was analyzed by tandem mass spectrometry.

Three Tetracysteine Sequences Obtained from Six Beads. Three of the six cleaved peptides, FB1, FB2, and FB6 gave unambiguous sequences (Table 2). The other three samples contained very little peptide, insufficient for MS/MS analysis, possibly due to interference with residual FlAsH. To our satisfaction, one of the three sequences matched the sequence of the three beads doped into the library. Surprisingly, this bead exhibited the weakest green fluorescence of the six beads selected for sequencing. Since the fluorescence of bead FB6 was on the borderline of our
with 5% dichlorodimethylsilane in dichloromethane, rinsed,
Gel. All glassware used in the library synthesis was silylated
chelating species,9 and cysteine-rich pharmaceuticals.30
(Xxx)8LysMet (Xxx
libraries. A diverse, heavily biased cysteine-rich library Ac-
screening, and sequencing of cysteine-rich OBOC peptide
identified: CCPCCC.
contains a potential FlAsH tag that has not previously been
been passed over. It is possible, but less likely, that the other
two doped beads corresponded to the unsequencable samples
FB3–FB5. All three of the FlAsH-binding sequences contain a
CCXXXXC tetracysteine motif and suggest that it is superior
to other short tetracysteine patterns: e.g., CXCXCCXC,
CXXXCCXXC, etc. FlAsH-binding peptide FB1 contains one of the two known efficient sequences (CCKXCC and
CCPGCC); peptide FB6 contains the other. Peptide FB2
contains a potential FlAsH tag that has not previously been
identified: CCPCCC.

| name | rel. fluor. items | sequence |
|------|------------------|----------|
| FB1  | + + +            | Ac-CCPCCCRM |
| FB2  | + + +            | Ac-CCPCCTRNM |
| FB3  | + +              | -        |
| FB4  | + +              | -        |
| FB5  | + +              | -        |
| FB6  | +                | Ac-CCPCCCRM |

* Consensus tetracysteine motif in yellow.

criteria for selection, the other two doped beads may have
been passed over. It is possible, but less likely, that the other
two doped beads corresponded to the unsequencable samples
FB3–FB5. All three of the FlAsH-binding sequences contain a
CCXXXXC tetracysteine motif and suggest that it is superior

to other short tetracysteine patterns: e.g., CXCXCCXC,
CXXXCCXXC, etc. FlAsH-binding peptide FB1 contains one of the two known efficient sequences (CCKXCC and
CCPGCC); peptide FB6 contains the other. Peptide FB2
contains a potential FlAsH tag that has not previously been
identified: CCPCCC.

Conclusion

We have developed the first method for the synthesis,
screening, and sequencing of cysteine-rich OBOC peptide
libraries. A diverse, heavily biased cysteine-rich library Ac-
(Xxx)3LysMet (Xxx = 50% Cys ≠ Met, Ile) was synthesized
on Tentagel resin with Fmoc-tritylcysteine, using NMP to
avoid tritylcytstine aggregation. Conditions were developed for iodoacetamide alkylation and cyanogen bromide cleavage of single beads, and the samples were analyzed by tandem
mass spectrometry. Over half of the peptide samples cleaved
from beads gave unambiguous sequences. Cysteine-free
peptides yielded slightly more unambiguous sequences than
the cysteine-rich sequences. The method was validated by
synthesizing and screening a cysteine-rich library against the
profluorescent reagent FlAsH. Using this method, we suc-
cessfully identified sequences containing two of the known
short biarsenical tag motifs: CCPGCC and CCKXCC. Our
synthesis and sequencing methodology expands the power
of OBOC libraries to include short, cysteine-rich peptides
for applications such as biarsenical binding tags,27 metal-
chelating species,9 and cysteine-rich pharmaceuticals.30

Experimental Section

Cysteine-Biased Library Synthesis: Ac-X8-KM-Tenta-
Gel. All glassware used in the library synthesis was silylated
with 5% dichlorodimethylsilane in dichloromethane, rinsed,
and oven-dried. Solutions of 0.5 M Fmoc-amino acids and
0.5 M HBTU/HOBt in NMP were prepared for the coupling
reactions. Beads were transferred between vessels with a
silylated 10 mL electric pipettor as suspensions in NMP. The
couplings were performed with 5 g of 130 μm Rapp Polymer
TentaGel HL NH2 resin (0.45 mmol/g, batch no. 124.782)
with 3 equiv of each amino acid, 3 equiv HBTU, 3 equiv
HOBt, and 6 equiv DIPEA in NMP.

The first two residues (Met and Lys(Boc)) were sequential-
tially coupled to the entire batch of TentaGel in a 500 mL
solid phase synthesis vessel. To ensure 50% incorporation
of cysteine at each randomized position, one-half of the resin
was coupled to Fmoc-Cys(Trt)-OH in the 500 mL solid phase
synthesis vessel; the remaining resin was equally divided
between 17 20 mL scintillation vials. The vials were gently
agitated on a shaker during the coupling reaction. After
coupling for 90 min, the resin was pooled in the SPPS vessel,
rinsed with DMF (3 × 50 mL), and deprotected for 30 min
with 50 mL of 20% piperidine in DMF. The resin was again
rinsed with DMF (3 × 50 mL). After the final amino acid
was coupled, the N-terminus of each peptide in the library
was acetylated in the SPPS vessel; the remaining resin was acetylated in the SPPS vessel with 5 mL acetic anhydride
and 24 mL DIPEA in NMP. The library was washed with
DMF (3 × 50 mL), methanol (3 × 50 mL), and dichloro-
methane (3 × 50 mL). The protecting groups were
removed by treatment of the resin with 94% TFA, 2.5%
ethanedithiol, 2.5% H2O, and 1% trisopropylsilane for 3 h.
Following cleavage, the beads were washed with methanol
(3 × 50 mL) and pH 7.2 buffer (3 × 50 mL).

Single Bead Isolation and Preparation. The bead library
was suspended in 50 mL pH 7.2 aqueous solution. To select
random beads from the library, 200 μL of suspended beads
were transferred to a 90 mm Petri dish using a P200 Pipetman.
Individual beads were taken up with the P200 Pipetman under a 50×
dissecting microscope and transferred to a prewashed (2 × 250 μL, methanol) Millipore Ultratfile-
MC spin tube with a 0.22 μm Durapore PVDF membrane.
The beads were separated from solvent by centrifugal filtration
by spinning at 12 000 rpm in a benchtop microcentrifuge.
To avoid loss of the single bead, it is essential to confirm
by microscope that the bead is at the bottom of the spin tube
between washes. Beads were subsequently washed with 30%
aqueous methanol (2 × 100 μL). Small wash volumes and
gentle agitation were used to confine the bead to the bottom
of the spin filter.

Bead Sample Alkylation. The cysteine sidechains of
beads isolated from cysteine-containing libraries were re-
duced and alkylated to prevent side reactions during cyano-
gen bromide cleavage: to each tube was added 100 μL of

Figure 4. Photographs of green fluorescent beads FB1–FB6 (left to right). Bead diameters are about 130 μm.
50 mM DTT buffered with 0.4 M NaHCO₃ in 30% aqueous methanol, and the tube was shaken for 1 h. Next, 40 µL of 0.5 M iodoacetamide in 30% aqueous methanol was added to each tube. After shaking for 30 min, 150 µL of 50 mM DTT buffered with 0.4 M NaHCO₃ in 30% aqueous methanol was added and the tube was shaken for 15 min. Finally, the alkylation mixture was spun through the spin tube and the tube progresses to the rinsing phase.

Still inside of the spin tube, the bead was rinsed with 3:7 methanol/0.1% aqueous formic acid (2 × 100 µL) and 1:1 CH₂Cl₂/MeOH (2 × 100 µL). The bead was then transferred as a suspension in 50 µL MeOH to a PCR tube. The MeOH was evaporated under vacuum, leaving the bead in bottom of the tube. The presence and location of the bead was verified under the microscope. To each PCR tube was added 30 µL of a solution of 20 mg/mL cyanogen bromide in 0.1 M HCl. The cleavage reactions were run for 8 h at room temperature in the dark. Each tube was covered from light and was then frozen at −78 °C, and the solvent was removed by lyophilization. A solution of aqueous formic acid in acetonitrile (30 µL of 1:1 (V/V) 0.1% formic acid/MeCN) was added to the PCR tubes, that were then vortexed for one minute to dissolve the residue. The solution was then transferred to a vial for LC-QTOF analysis.

**Bead Sample Sequencing.** Samples were analyzed on a Waters QTOF2 mass spectrometer in positive ion mode equipped with a Waters nanoAcquity HPLC (column: Waters Symmetry 100 μ × 100 mm C18 1.7 µm particle size). Doubly charged ions were selected and isolated for sequencing by survey scanning during chromatographic analyses. The flow rate was 350 nL/min ramped from 5% B to 80% B over 40 min. (A = 94.9% H₂O 5% acetonitrile 0.1% formic acid. B = acetonitrile + 0.1% formic acid.) The electrospray voltage was 3 kV and the cone voltage 30 V. To assist nebulization, nitrogen gas was used at approximately 5 psi. Argon was used as the collision gas at 4 × 10⁻⁵ Torr on the analyzer’s Penning gauge.

The MS/MS spectra were interpreted manually, primarily by assigning the peaks corresponding to the C-terminal y-ions. The y-ion peaks were abundant since every cleaved peptide had LysHsl (Hsl = homoserine lactone) at the C-terminus. The interpretation of the spectra was simplified because the formation of b-ions was suppressed as a result of N-terminal acetylation.

**Screening a Cysteine-Biased Library with FIAsh-EDT₂.** A cysteine-rich OBOC library (0.33 g; Ac-(Xxx)s-LysMet; Xxx = 50% Cys; ≠ Met, Ile) was first prescreened to remove fluorescent beads. The beads were transferred to a 50 mL solid phase synthesis vessel. Once inside the 50 mL vessel, the library was washed with 70:30 100 mM potassium bicarbonate/MeOH (2 × 30 µL). The beads were transferred to, and suspended in, a 90 mm Petri dish using 30 mL of 100 mM potassium bicarbonate and observed in the dark under an 8 W long wave UV lamp (Spectrolite ENF-280C) with a stereo microscope (15×−45× magnification). Seven weakly fluorescent beads were removed from the library.

The library was then transferred back to the solid phase synthesis vessel for screening using 100 mM potassium bicarbonate. At this point three beads bearing the sequence Ac-WDCCPGCCKM, suspended in 10 mL deionized water, were added to the vessel using a 200 µL Pipetman. The library was then washed (3 × 50 mL) with a potassium-rich phosphate buffer (15 mM NaCl, 0.5 mM MgCl₂, 100 mM potassium phosphate, pH 7.2) containing 0.01% (v/v) Triton X-100. To the washed library in the solid phase synthesis vessel was added 50 µL of the potassium-rich phosphate buffer containing 0.03% (v/v) Triton X-100, 1 mM β-mercaptoethanol, 0.5 mM BAL, and 2.6 mM tris-carboxyethylphosphine·HCl. FlAsH-EDT₂ (5 µL) was added as a 1 mM stock solution in DMSO. The vessel was then agitated on a shaker for 6 h.

After 6 h, the screening solution was drained and the resin was extensively washed. The library was first washed with potassium-rich phosphate buffer containing 0.01% Triton X-100 (v/v) (3 × 30 µL). Then, a phosphate buffer containing 0.03% (v/v) Triton X-100, 1 mM β-mercaptoethanol, 1 mM BAL, and 2.5 mM tris-carboxyethylphosphine·HCl was added to the vessel, and the vessel was shaken for 10 h. After 10 h, the solution was drained and the resin was washed extensively with 100 mM potassium bicarbonate buffer (6 × 30 µL) until the odor of thiols from the resin was no longer diminished. Finally, the library was suspended in 200 mL of 100 mM potassium bicarbonate buffer and divided equally into four 90 mm Petri dishes for visualization/bead selection.

**Visualization and Isolation of Fluorescent Beads.** Each of the four library fractions 90 mm Petri dish was observed under an 8 W long wave UV lamp, held 10 cm from the beads, using a stereo microscope to identify fluorescent beads. Fluorescent beads were photographed with a Nikon Coolpix E4500 digital camera, top-mounted on the microscope (exposure times between 1 and 8 s were used). A small amount of MeOH was added by pipet to sink floating beads into the focal plane. After being photographed, the fluorescent beads were isolated from the surrounding nonfluorescent beads using the tip of a 200 µL Pipetman. Once the surrounding beads were cleared, the fluorescent bead was drawn into the tip of the 200 µL Pipetman with about 10 µL of the screening solution. In each case, the presence of a single fluorescent bead was confirmed by observing the pipet tip under the long wave UV lamp. The bead was transferred to a Millipore Ultrafree-MC spin tube by placing the bead-loaded tip of the Pipetman near (but not resting on) the bottom of the tube before dispensing the contents. The presence of the fluorescent bead in the spin tube was confirmed under a UV lamp using a stereo microscope.

**Preparation and Alkylation of Fluorescent Bead Hists.**

These procedures were performed in the manner discussed above, with three exceptions. One, the prewashing of the Millipore Ultrafree-MC spin tube consisted of a water wash (2 × 250 µL), followed by a methanol wash (2 × 250 µL), and finished with a dichloromethane wash (2 × 250 µL). Two, just before adding the CNBr solution, the beads are placed in a drying oven for 5 min, then removed and allowed to cool for an additional 5 min. Third, the CNBr cleavage was shortened to 6.5 h.
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Supporting Information Available. MS/MS spectra for the peptides cleaved from beads Cys02—Cys10 and a Microsoft Excel spreadsheet for prediction of y-ion and b-ion masses. This material is available free of charge via the Internet at http://pubs.acs.org.

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