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Rapid Identification of Substrates for Novel Proteases Using a Combinatorial Peptide Library

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Fluorogenic substrates for assaying novel proteolytic enzymes could be rapidly identified using an easy, solid-phase combinatorial assay technology. The methodology was validated with leader peptidase of *Escherichia coli* using a subset of an intramolecularly quenched fluorogenic peptide library. The technique was extended toward the discovery of substrates for a new aspartic protease of pharmaceutical relevance (human napsin A). We demonstrated for the first time known to us that potent fluorogenic substrates can be discovered using extracts of cells expressing recombinant enzyme to screen the peptide library. The straightforward and rapid optimization of protease substrates greatly facilitates the drug discovery process by speeding up the development of high throughput screening assays and thus helps more effective exploitation of the enormous body of information and chemical structures emerging from genomics and combinatorial chemistry technologies.

Introduction

The study of genes and their functions is bringing about a revolution in our understanding of the molecular mechanisms of diseases. A large number of potential targets for therapeutic intervention of both human and microbial origin is expected to become available through genomics. One of the major challenges is now to prioritize these targets. Homology of known genes, as well as information about localization and temporal expression of the genes will be used in the selection process. Genes that are preferentially expressed in diseased tissue, compared to healthy tissues, are likely to be interesting, potential targets.

Proteolytic enzymes represent one major class of targets, since they are involved in a wide variety of disorders including infection diseases and other pathologies. There is a great need for optimal substrates to study the enzymology of the selected proteases, for validation as relevant medicinal targets and to screen for inhibitors. Sensitive fluorescence resonance energy transfer (FRET) experiments are well-suited and extensively used in biomedical research for this purpose. We propose to use a variation of the combinatorial chemistry approach developed by Meldal and co-workers[1] for the rapid, straightforward identification of fluorogenic substrates. These substrates allow the rapid development of high throughput screening assays (HTS) for the fast and reliable testing of ever expanding compound libraries against the increasing number of potential targets.

The technology consists of a solid-phase enzyme assay system using polymer-bound peptide libraries[2−5] and is based on the principle of FRET. Variations of the procedure have been used to optimize substrates for subtilisin carlsberg,[1] cruzipain,[6] cathepsin,[7] and papain.[8] In this report we extend the work of Meldal and co-workers[1] to the *Escherichia coli* (*E. coli)* leader peptidase, with a view of validating the technology employing *N*-(2-aminoethyl)-4-amino-3,6-disulfo-1,8-naphthalimide[9] (Lucifer yellow) and 4-dimethylamino-azobenzene-4′-sulfonyl[10,11] (Dabsyl) as donor/acceptor pair for FRET. Subsequently, we improved the original solid-phase assay technology by using cell extracts transfected with napsin A for the screening of the peptide library instead of using pure protease. One of the substrates found was synthesized in large scale and used for HTS for napsin A inhibitors.

Results and Discussion

Solid-Phase Assay System Based on FRET. The principle components of the assay system are beads of a biocompatible polymer to which is attached a suitable fluorophore as the donor molecule (Figure 1). A peptide library is then built by split synthesis,[2−4] and an appropriate acceptor of the donor fluorescence is coupled to the *N*-terminus of each peptide. We used Lucifer yellow, which was attached to the C-terminal carboxyl group of the Glu, as the fluorescent donor and Dabsyl, which was coupled to the ϵ-amino group of the *N*-terminal Lys, as fluorescence quenching acceptor (Figure 2). This leaves the α-amino group free to allow Edman sequencing, if necessary. The

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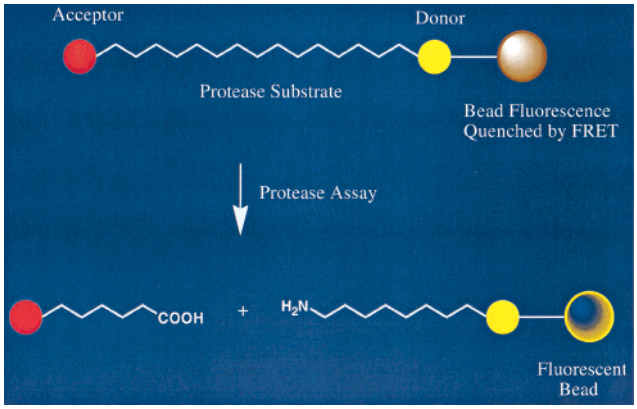

Figure 1. Principle of a solid-phase assay taking advantage of FRET between a fluorescent donor and a quenching acceptor. One bead is represented, and the zigzag line symbolizes a peptide chain. Because the intact peptide bead contains both donor and acceptor, FRET occurs and the bead is nonfluorescent. After digestion of the peptide bead with a protease, cleavage is indicated by an increase in fluorescence.

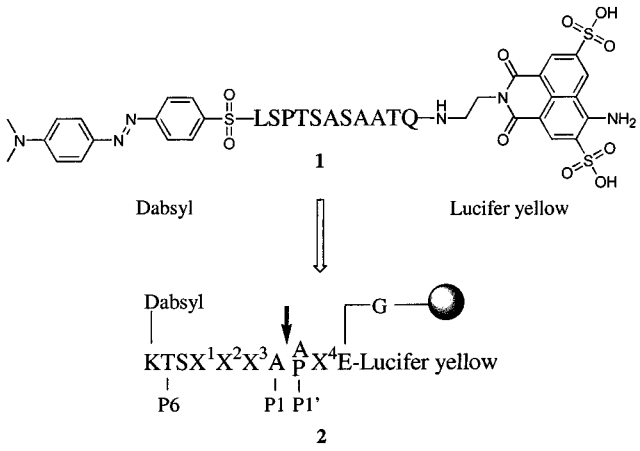

Figure 2. General structure of the peptide library **2** designed from leader peptidase substrate **1**. Black arrow indicates the directed site of cleavage. X represents all possible combinations of the 19 natural L-amino acids. Cys was not incorporated.

Lucifer yellow/Dabsyl pair for FRET screening showed several advantages. The high charge of Lucifer yellow helped solubilize hydrophobic peptides, and its emission maximum (520 nm) is well above the spectral region of most small organic compounds. The broad absorption spectrum (λ_{max} = 466 nm) of the Dabsyl moiety overlaps efficiently with the emission spectrum of Lucifer yellow. The Lucifer yellow/Dabsyl pair is ideally suited for solid-phase assays. When suspended in a slightly acidic buffer and inspected through a red filter ($\lambda_{50\%Transmission}$ = 605 nm), fluorescent beads can even be observed by the naked eye. These features of the Lucifer yellow/Dabsyl pair greatly facilitate the detection and subsequent manipulation of the fluorescent beads.

The choice of an appropriate solid support is critical for the success of library synthesis and enzymatic screening. We used amino $PEGA_{1900}$ resin as the support for our library since the open, hydrophilic structure of this resin in its fully swollen state allows permeation by large biomolecules such as enzymes.[1,12] $PEGA_{1900}$ is a copolymer of poly(ethyleneglycol) and acrylamide. The batch we used in this work was made from a PEG chain with a molecular weight of 1900 (PEG_{1900}). Split synthesis was used to assemble the peptide library. The method of split-mix synthesis results in each bead containing only one species of peptide. The number of beads of any given sequence follows a Poisson distribution. Consequently, sufficient resin was used to make three times the complexity of the library to ensure that it would be statistically representative.[13]

The acceptor we have chosen is an effective quencher of the donor fluorescence, and therefore the beads of the completed one bead-one compound peptide library were nonfluorescent. When the beads are incubated with a protease, the enzyme cleaves its preferred substrate sequences and liberates the quencher from those peptides into the solution. The beads carrying such a cleaved substrate become fluorescent. They can be easily detected under a fluorescence microscope among the quenched and therefore dark beads. The most fluorescent beads are collected and sequenced by Edman degradation. When only a fraction of the substrate moieties have been cleaved, sequence analysis can give the exact position of the cleavage site (from the cleaved peptides) as well as the intact substrate sequence (from the uncleaved peptides). A semiquantitative estimation of the substrate potency can also be made from the relative amounts of cleaved and intact peptide sequences obtained after Edman degradation.

Library Design for *E. coli* Leader Peptidase. Leader peptidases are integral membrane proteins that catalyze the removal of the signal peptides of proteins exported to the periplasm.[14] They are essential for the growth of bacteria and, thus, represent an attractive target for therapeutic intervention in infectious diseases. Extensive statistical evaluation of natural leader peptidase sequences and biochemical studies[15,16] have shown that leader peptidase has a strong preference for small amino acid residues, especially Ala at positions P1 and P3.[14,17] To test the principle of the FRET assay, we synthesized a leader peptidase substrate **1** taking these preferences into account. This formed the basis for the design of our peptide library **2** (Figure 2) generally described as K(Dabsyl)TSX¹X²X³AA/PX⁴E(G-$PEGA_{1900}$-resin)-Lucifer yellow.[18] Nineteen natural L-amino acids were chosen for positions X^1, X^2, X^3, and X^4 in the library. Cys was not included because of possible oxidation and disulfide bond formation. Position P1 was occupied exclusively by Ala, while position P1′ included either Ala or Pro. Our intention was to direct the site of cleavage between Ala-Ala for one-half of the library and to generate noncleavable substrates by fixing the Ala-Pro motif for the other half of the library, since the leader peptidase is inhibited by peptides that include Pro at P1′.[19] Ser and Thr were inserted at position P5 and P6, respectively. The theoretical complexity of the library was therefore 19 × 19 × 19 × 2 × 19 = 260 642 individual peptides. A Gly spacer was inserted between the polymer and the COOH side chain of Glu in order to facilitate library synthesis.

The synthetic strategy made use of standard fluorenylmethoxycarbonyl (Fmoc) chemistry[20] and *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5,*b*]pyridin-1-yl-methylmethaminium hexa-fluoro-phosphate *N*-oxide (HATU)[21] as condensing agent. Boc-Lys(Dabsyl)-OH (where Boc is *tert*-butoxycarbonyl) and Fmoc-Glu-Lucifer yellow were used to incorpo-

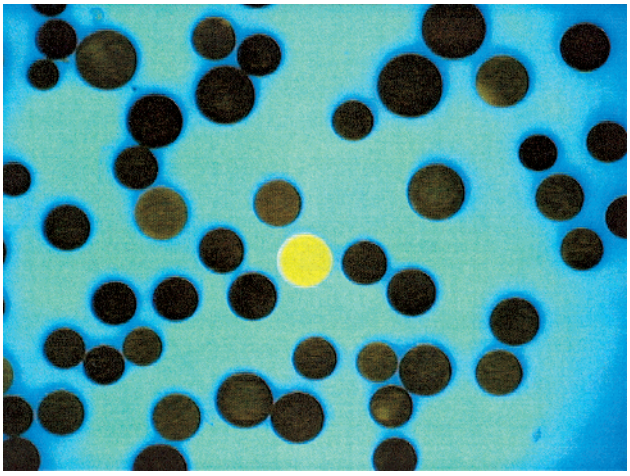

Figure 3. Photomicrograph of beads of the peptide library **2** after incubation with *E. coli* leader peptidase. Small portions of the beads were placed on a glass plate and examined under a fluorescence microscope (Leica MZ12 equipped with a Leica 2 videosystem) using the appropriate violet filter set. The fluorescence and the color contrast of the beads under the microscope were optimal in slightly acidic solutions. Dark beads contained both Lucifer yellow and Dabsyl. Bright yellow fluorescent beads, such as the one in the center, signal peptide cleavage by the enzyme. They were isolated with the help of a glass capillary and submitted for Edman sequencing.

rate the donor/acceptor pair for FRET. Boc-Lys(Dabsyl)-OH was prepared by the reaction of Boc-Lys-OH with dabsyl chloride. Fmoc-Glu-Lucifer yellow was obtained by condensation of the *N*-hydroxysuccinimid ester of Fmoc-Glu-(O*t*Bu)-OH (where *t*Bu is *tert*-butyl) with Lucifer yellow and subsequent treatment with trifluoroacetic acid (TFA).

Validation of the Method Using *E. coli* Leader Peptidase. A subset of the peptide library **2** representing approximately 25 000 beads was incubated with the *E. coli* leader peptidase.[22] A total of 45 bright fluorescent beads was discovered and collected (Figure 3). Edman sequencing analysis showed that all of the isolated $PEGA_{1900}$-bound peptides were cleaved between Ala-Ala. Thirty-nine of them were cleaved at the directed Ala-Ala site (Table 1), while six peptides with Ala for X^3 were digested between X^3-Ala. From the distribution of the amino acids at the different positions of the sequence, the well-known Ala-X-Ala motif defined by von Heijne[16] can be easily deduced. The recently published crystal structure of *E. coli* leader peptidase clearly shows that only Ala fits perfectly at positions P1 and P3 of the active site of the enzyme.[23] For the six peptides cleaved between X^3 and Ala, a highly conserved motif, AXAAPI, was found.

Selected peptides representing the range of on-bead enzymatic activity were synthesized and submitted to enzyme kinetic analysis in solution using leader peptidase (Table 2). HPLC and ESIMS of the products of this reaction showed that the sites of digestion were identical to these found for the solid-phase bound substrates. When compared to the original substrate **1**, the K_m values are rather similar. Since the concentration of active enzyme in our preparation was not known,[22] V_{max} values (per microgram of protein) were listed. Relative catalytic efficiencies, V_{max}/K_m, versus V_{max}/K_m of the original substrate **1**, were calculated. V_{max}/K_m of

Table 1. Observed Amino Acid Distribution for *E. coli* Leader Peptidase[a]

amino acid	P4	P3	P2	P1	P1′	P2′
A	2	25		39	39	3
D						
E	4		3			
F	4		1			2
G						
H			5			
I	7		3			
K	1	1	2			
L	1	1	3			
M	2		4			6
N	1		3			
P	4		1			8
Q	4		5			
R			1			
S			1			4
T	1	1				10
V	3	11	1			6
W	2		2			
Y	3		4			

[a] At different positions relative to the cleavage sites. The numbers were calculated based on the sequences of the 39 bead-bound FRET peptides processed at the directed Ala-Ala cleavage site. P1 and P1′ were Ala as intended by the design of our peptide library **2**. In subsite P3, Ala followed by Val are the most prominent amino acids in the bead-bound substrates. P2 and P4 tolerate a broad variety of residues, while P2′ allows the occurrence of a less broad spectrum of possible amino acids.

substrate **1** was set to 1. Without purified enzyme it is difficult to compare our data with data given in the literature.[24−27] Substrate **3** showed the highest apparent affinity (0.43 μM) and also the highest relative turnover. Comparison of the relative catalytic efficiencies demonstrated that the two best substrates contain the AXAAPI motif.

It would have been possible to test the complete peptide library, but it would have been difficult and we wanted to have more of it available for future screening. On the other hand, it is not necessary to screen the complete library to find useful motifs, and the number of bright fluorescent beads that can be expected in a particular library subset can be theoretically estimated.[5,28] Usually in a proteolytic sequence, only a small number of the amino acids in the sequence are contact residues necessary for specific interaction. For example, leader peptidase has at least two sites with small pockets that accept small residues at P1 and P3 of the substrate, but the other positions in the substrate tolerate a broad range of amino acid residues.[14−16] If one accepts that only a small number of amino acids are critical for binding, than the size of a sample of the library complexity should be adequate to find such a discrete motif. Several peptide binding motifs have been found by screening a subset of large peptide libraries.[5,8,29] In the case of gpIIbIIIa, the motif was observed after testing ca. 50 000 beads of a complexity of 2.47 millions of beads.[29] The substrate specificity of cysteine proteases has been successfully mapped using a subset of ca. 50 000 beads from a library containing 270 000 different peptides.[8] Our work shows that the screening of a subset of the intramolecularly quenched fluorogenic peptide library **2** provides a number of good proteolytic substrates. All together, these results show that the elaborated technology can be useful for the rapid, systematic discovery of peptide substrates.

Table 2. Selected Data for *E. coli* Leader Peptidase Substrates[a]

entry	peptide	cleavage [%][b]	K_m [10^{-6} M][c]	V_{max} [10^{-10} M s^{-1} μg^{-1}][c]	relative catalytic efficiency[d]
1	Dabsyl-LPTSSASA↓ATQ-Lucifer yellow	50	2.24	0.055	1.0
3	K(Dabsyl)TSAFA↓APIQ-Lucifer yellow	75	0.43	0.195	19
4	K(Dabsyl)TSAHA↓APIQ-Lucifer yellow	60	0.65	0.173	11
5	K(Dabsyl)TSKAIA↓APQ-Lucifer yellow	70	1.98	0.123	2.5
6	K(Dabsyl)TSIANA↓AMQ-Lucifer yellow	70	2.20	0.073	1.3
7	K(Dabsyl)TSVAEA↓ATQ-Lucifer yellow	50	3.57	0.039	0.43
8	K(Dabsyl)TSEVMA↓AVQ-Lucifer yellow	60	2.55	0.021	0.34

[a] For peptide **1** and six of the 16 substrates synthesized for kinetic measurements in solution, the arrows indicate the sites of cleavage as detected first on solid phase and as confirmed later with the soluble peptides. Peptides **5**−**8** were cleaved at the intended Ala-Ala sites, while **3**−**4** were digested between X^3-Ala. [b] The degree of the enzymatic cleavage on the beads was calculated as the portion of cleaved substrate with regard to the total amount of recovered substrate during Edman sequencing analysis. On average, 60% cleavage was detected. In the best case, 80% cleavage was found, but two substrates were only cleaved to 10%. [c] Reaction kinetics was followed by measuring the increase in fluorescence at $\lambda_{emission} = 520$ nm periodically during 20 min at six different substrate concentrations (BMG Polarstar). The signals were converted into moles of substrate hydrolyzed per second. Kinetic data were determined from a Lineweaver−Burk plot. [d] For the relative catalytic efficiencies, V_{max}/K_m versus V_{max}/K_m of the original substrate **1** was calculated, whereas V_{max}/K_m of substrate **1** was set to 1.

Identification of Napsin A Substrates. The identification of a potent peptide substrate for the straightforward development of the HTS for napsin A applying the same assay technology was much more challenging. In contrast to the extensive knowledge about leader peptidases, very little is known about napsin. Genes coding for napsin in humans[30,31] and mouse[32] recently has been described. Napsin A is a new member of the aspartic protease family expressed predominantly in kidney and lung. Many aspartic proteases have been extensively studied because of their physiological roles and their involvement in pathological states related to several diseases, including cancer.[33] Therefore, it can be assumed that napsin A, with its specific localization in kidney and lung, may have a unique physiological role and possibly pathological significance.

Although designed specifically for leader peptidases, **2** represents a valuable source of potential substrates for other proteases. We screened a subset of **2** for positive beads carrying putative napsin A substrates. We applied the method to a crude extract from recombinant cells because the purification procedure for napsin A had not been established in our lab at that time.[34] The presence of napsin A in the extract of HEK293 cells was confirmed by immunological studies using an anti-napsin A specific antibody.[35] We developed a two-step procedure for the screening of approximately 25 000 beads of **2** with the extract. The first round was performed to identify and remove fluorescent beads digested by proteases other than napsin A present in the crude cell extract. For this purpose, the 25 000 beads were preincubated with an extract of untransfected HEK293 cells and in the presence of known inhibitors of metallo-, serine, and cysteine proteases, which were added into the reaction mixture to block non-aspartic proteases. The beads were incubated overnight and afterward carefully inspected. Only a very small number of beads turned out to be fluorescent and were removed. The remaining beads were incubated with extract from HEK293 cells transfected with expression vector carrying the human napsin A cDNA.[36] The incubation was performed in the presence of the same inhibitor cocktail and under exactly the same experimental conditions. Twenty-one brightly fluorescent beads were isolated and analyzed by Edman degradation. Twelve beads contained sequences that were cleaved at a single site, while the remaining bright beads carried sequences with several cleavage sites. Since crude cell extracts with multiple proteolytic activities were used, we assume that not necessarily all the discovered substrates can be attributed to the specific cleavage by napsin A. Seven of the peptides showed only one site of enzymatic digestion in the middle of the sequence between X^2 and X^3. These peptides were digested either between Leu-Met, Phe-Phe, or Leu-Phe.

Table 3. Selected Napsin A Substrates[a]

entry	peptide	cleavage (%)[b]
9	K(Dabsyl)TSVL↓MAAPQ-Lucifer yellow	77
10	K(Dabsyl)TSLL↓MAAPQ-Lucifer yellow	66
11	K(Dabsyl)TSEF↓FAPDQ-Lucifer yellow	77

[a] The arrows indicate the sites of cleavage as detected first on solid phase and as confirmed later with the soluble peptides. [b] The degree of the enzymatic cleavage on the beads was calculated as the portion of cleaved substrate with regard to the total amount of recovered substrate during Edman sequencing analysis. Peptide **9** was selected as napsin A substrate for HTS and used to purify and characterize the enzyme.

Peptides **9**, **10**, and **11** were synthesized for further evaluation in solution (Table 3). Using crude as well as purified napsin A,[36] it was shown that all three substrates were cleaved. HPLC and ESIMS of the products of this reaction proved unequivocally that proteolysis occurred specifically at the Leu-Met bond for **9** and **10** and at the Phe-Phe bond of **11**, thus confirming the results of the solid-phase assay. Determination of the initial velocities of enzymatic digestion[35,36] indicated that K(Dabsyl)TSVL-MAAPQ-Lucifer yellow **9** was the best of the three substrates. Thus, we were able to propose a good synthetic substrate for napsin A within a few weeks. This peptide was synthesized in large scale and used for a HTS for napsin A inhibitors as well as for the purification and biochemical characterization of napsin A.

Conclusions

The benefit of this assay technology performed on single beads has been illustrated here for both determining motifs

of peptide substrates and for rapidly finding fluorogenic substrates for novel, poorly characterized proteases. Subsets of one single peptide libray were used for the screening assays with leader peptidase and napsin A. We improved the original assay technology by demonstrating for the first time known to us that even crude extracts containing the enzyme from transfected cells can be used for screening a peptide library. The discovered peptides for napsin A could be used for both HTS purification and biochemical studies of the enzyme. The Lucifer yellow/Dabsyl pair for FRET showed several advantages in screening. Through our work, this combinatorial technology has found a role linking the fast developing fields of genomics, combinatorial chemistry, and HTS, and a wide application of this method is expected.

Experimental Section

Substrate Library Assay Using *E. coli* Leader Peptidase. *E. coli* leader peptidase was cloned and purified using modifications of published procedures.[12] The specific activity of the leader peptidase was not known. A stock solution of *E. coli* leader peptidase in Tris/HCl buffer (20 mM, pH 7.4), $MgCl_2$ (5 mM), Triton X-100 (0.5%), and glycerol (50%) was used. Library **2** (ca. 25 000 beads) was washed with Bis-(Tris)-propane buffer (100 mM, pH 8.5). Bis-(Tris)-propane buffer (100 mM, pH 8.5), $MgSO_4$ (50 mM), and the enzyme preparation (80 μg/mL) were added to the resin in a final volume of 4 mL. After the mixture was shaken for 18 h at room temperature, the resin was filtered off and the beads were intensively washed with Bis-(Tris)-propane buffer (100 mM, pH 8.5) and MES buffer (100 mM, pH 4.8). Fourty-five brightly fluorescent beads were isolated and analyzed by sequencing.

Substrate Library Assay Using Napsin A. Library **2** (ca. 25 000 beads) was washed with sodium acetate (NaOAc) buffer (200 mM, pH 4.0), NaOAc buffer (200 mM, pH 4.0), EDTA (1 mM), protease inhibitors E64 (10 μg/mL, Boehringer Mannheim), PMSF (170 μg/mL), and 500 μL of an extract from untransfected HEK293 cells were added to the resin in a final volume of 2 mL. After the mixture was shaken for 18 h at room temperature, the beads were washed with NaOAc buffer (200 mM, pH 4.0) and MES buffer (100 mM pH 4.8). A few weakly fluorescent beads were removed. The remaining beads were collected and washed with NaOAc buffer (200 mM, pH 4.0). NaOAc buffer (200 mM, pH 4.0), EDTA (1 mM), protease inhibitors E64 (10 μg/mL), PMSF (170 μg/mL), and 500 μL of the extract of napsin A transfected HEK293 cells were added in a final volume of 2 mL. The resin was incubated for 18 h and washed as described above. After washing with MES buffer (100 mM, pH 4.8) the beads were inspected under the microscope. Twenty-one bright beads were isolated and submitted for Edman sequencing.

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Supporting Information Available. Synthesis of peptide libray **2** and synthesis of peptides **3**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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