Peptide Research

Synthesis of Combinatorial Libraries with Only One Representation of Each Structure

Magda Stanková, Shelly Wade, Kit S. Lam[1] and Michal Lebl
Selectide Corporation, Tucson, AZ, USA; [1]Arizona Cancer Center and College of Medicine, Tucson, AZ, USA

ABSTRACT

A technique of generating libraries in which every compound is displayed only once and all possible combinations are prepared with absolute certainty is presented. The method is based on the stepwise division of a continuous carrier in each step of randomization. Polypropylene membrane and cotton thread were evaluated as potential supports for this approach to library construction. Cotton thread accommodates 400 nmol of test compound per cm, but its mechanical properties limit the dimension of libraries. An alternative combinatorial library of "restructurable toothbrushes" is suggested.

INTRODUCTION

Techniques in combinatorial chemistry are gaining wide acceptance among modern methods for the generation of new pharmaceutical leads (20,23). The combinatorial approach used in our laboratories is based on a strategy involving the synthesis of libraries containing a different structure on each particle of the solid phase support, interaction of the library with a soluble receptor, identification of the "bead" which interacts with the macromolecular target and determination of the structure carried by the identified "bead" (34). An alternative to this approach is the sequential release of defined aliquots of the compounds from the solid support, with subsequent determination of activity in solution, identification of the particle from which the active compound was released and elucidation of its structure by direct sequencing (44), or by reading its code (28,40,41).

The synthesis of libraries with a unique compound on each solid-phase particle employs a simple principle for the generation of equimolar mixtures of peptides in solution that was first described by Furka (17–19). This principle was later applied to the construction of soluble libraries for iterative screening (26) and to bead-based libraries screened with solid-phase-binding protocols (34). Representation of all possible structures can be achieved with near certainty only in cases in which the number of particles used for the synthesis is at least an order of magnitude higher than the number of synthesized structures (5), or the number of possible structural permutations is much greater than the number of particles used. In the latter case, however, only a fraction of the library is actually synthesized.

In the previously mentioned methods for library construction, it is desirable to perform the synthesis on a defined number of solid-phase particles exactly matching the number of compounds resulting from the randomization of a given set of building blocks. With this ideal situation, we would be able to eliminate the statistical uncertainty of library generation by the split and mix procedure. An assay of the library might then be performed by distributing the "beads" into microplate wells and releasing the compounds with the subsequent determination of their biological activity, without the danger of missing an active compound or detecting the identical structure several times. This argumentation is relevant especially in the cases of small libraries (the range of tens of thousands structures), where it is not unusual to find only a single active compound (see, e.g., Reference 45). Large libraries of millions of structures may contain numerous analogs and the "motif" can be found even with a noncomplete library (36).

The standard polymeric bead, 100 μm in diameter, accommodates, depending on polymer substitution, anywhere from 100 to 500 pmol of test compound. Released and dissolved in 100 μL of solvent, this amount affords a 1 to 5 μM solution. Thus, the amount of compound available on a given particle clearly defines the lower limit of detectable activity of an active structure. A higher yield of released test compound from an individual solid-phase particle (from bigger or higher substituted particles) would allow for the utilization of higher concentrations and/or simultaneous screening for ligands of several targets.

MATERIALS AND METHODS

Instrumentation

UV/VIS absorption spectra were recorded on a Hewlett-Packard HP

8452A Diode-Array spectrophotometer (Palo Alto, CA, USA) using a 1-cm quartz cuvette. Sequencing by Edman degradation was performed on an ABI 4778 protein sequencer (Applied Biosystems, Foster City, CA, USA) and a Porton PI 3010 instrument (Porton Instruments, Tarzana, CA, USA).

Materials

Commercial-grade solvents were used without further purification. Protected amino acids were obtained from Bachem (Torrance, CA, USA), Advanced ChemTech (Louisville, KY, USA) or Propeptide (Vert-le-Petit, France). Monoclonal anti-β-endorphin antibodies (clone 3-E7) were purchased from Boehringer Mannheim, Mannheim, FRG. Streptavidin conjugated to alkaline phosphatase was obtained from Pierce Chemical (Rockford, IL, USA), and the goat-anti-mouse alkaline phosphatase (AP) complex was obtained from Bio-Rad (Richmond, CA, USA). d-Biotin was purchased from Sigma Chemical (St. Louis, MO, USA).

General Procedures

Solid-phase synthesis was performed manually in polypropylene syringes (30). Fmoc protecting groups were cleaved with 50% piperidine/DMF for 1 × 10 min. DIC in DMF was used for the activation of Nα-Fmoc amino acids. The completeness of each condensation reaction was monitored by the bromophenol blue method (31). The coupling protocol included washing with DMF (6–8 times) between coupling and deprotection, and between deprotection and coupling. Final deprotection, was done by mixture K (29).

Synthesis of the Directed Library on Cotton String

Cotton string (5 m, 6 m/g) was treated for 1 h in 25% trifluoroacetic acid (TFA) in dichloromethane (DCM). It was washed by DCM (3×), neutralized by 5% diisopropylethylamine in DCM (5 min) and washed by DCM and DMF (3×). Fmoc-β-Ala (2 mmol) was coupled overnight by DIC (2 mmol) and HOBt (2 mmol) activation with the addition of *N*-methylimidazole (3.5 mmol). Cotton was washed by DMF and substitution was determined by photometrical determination of the cleaved Fmoc group—0.41 mmol/g (683 nmol/cm). Five pieces of the cotton string (25 cm each) were placed into five polypropylene syringes, and Fmoc-protected amino acids (Phe, Tyr(But), Ala, Leu, Gly) were coupled by DIC/HOBt protocol. After completion of the coupling (monitored by the bromophenol blue method), the strings were washed with DMF, placed into one syringe, and the Fmoc group was cleaved. After washing with DMF and a 2% solution of HOBt in DMF, the mixture of 19-L (cysteine excluded) of Fmoc amino acids, with the molar ratio (determined in pilot experiments) adjusted for different reactivities (12,22,42), was coupled to all cotton pieces. After completion of this coupling, the cotton was washed, the Fmoc group was cleaved and the washed cotton string was divided and placed into five syringes in the following way: From each string, 5 cm of the cotton was cut and placed into the different syringes. In this way all syringes had only one 5-cm piece of cotton cut from each 25-cm string and none had more than one piece. Coupling of the five amino acid derivatives (same as above) was performed, the cotton was washed, the Fmoc group was removed and the cotton was subdivided again. In this case, 1-cm pieces were cut from all 5-cm pieces and placed into five syringes. Coupling of the same five amino acids was performed, the Fmoc group was cleaved and the side-chain protecting group was cleaved by a mixture of TFA, DCM and anisole (50:45:5) for 2 h. The cotton pieces were washed with DCM, MeOH and dried. Quantitative amino acid analysis of a sample of one string revealed the substitution of 400 nmol/cm.

Synthesis of the Directed Library on Functionalized Cross-linked Teflon® Membrane

Hydrophilic aminopropyl functionalized membrane (UV cross-linked aminopropyl methacrylamide, *N,N*-dimethylacrylamide and methylene-bisacrylamide on Teflon membrane, 16 × 16 cm [Perseptive Biosystems, Bedford, MA, USA]) with approximately 35 nmol/cm^2 substitution was placed into 50-mL Falcon tubes (Becton Dickinson, Lincoln Park, NJ, USA) and acylated by Fmoc-β-Ala using the DIC/HOBt procedure in DMF. Fmoc-Gly, Fmoc-β-Ala and Fmoc-Gly were coupled consecutively. After deprotection, the membrane was divided into two parts, and Fmoc-Phe and Fmoc-Leu, respectively, were coupled to them. After completion of the coupling (bromophenol blue monitoring) and deprotection, the pieces were divided again into two halves and recombined for the coupling of Fmoc-Gln and Fmoc-Phe. For the next coupling, the membrane was divided again into two pieces and recombined for the coupling of Fmoc-Pro and Fmoc-Gly. The pieces resulting from these couplings (8 × 4 cm) were then divided into 19 strips (8 × 0.21 cm), and the strips were placed into 19 small polypropylene tubes. Nineteen natural amino acids (excluding Cys) were used for coupling in this stage. After the coupling was completed and Fmoc deprotection, the strips were cut into 19 pieces (4 × 2.1 mm) and again divided into 19 vessels. The same set of 19 Fmoc amino acids was used for the last coupling. All pieces were combined, the Fmoc group was removed and the side-chain protecting groups were cleaved by mixture K (29). Membrane pieces were washed by TFA (2×), DCM (5×), MeOH (3×) and water (5×). Substitution, based on measurement of the absorbance of the last Fmoc release, was 43.3 nmol/cm^2.

Screening of the Directed Library on Cross-Linked Teflon Membrane

The peptide library was screened according to published protocol (33). The peptide squares were first washed with double-distilled water. After thorough washing, the squares were washed and coated with 0.05% gelatin (wt/vol) to block nonspecific binding. Washing with PBS/Tween (137 mM NaCl, 2.7 mM KCl, 4.3 Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2, with 0.1% Tween-20) and 2× PBS/Tween/gelatin (2× PBS, 0.1% Tween-20 [vol/vol] and 0.05% gelatin [wt/vol]) was followed by an incubation with a 20 nM streptavidin-alkaline phosphatase in 2× PBS/Tween/gelatin. Again the library was washed with PBS/Tween, 2× PBS/Tween/gelatin and with TBS (137 mM NaCl, 2.7 mM KCl, 25 mM Tris base, pH 7.4). The standard substrate 5-bromo-4-chloro-3-indolyl phosphate was then added. The library and substrate were then transferred to petri dishes for color development. Fifty-five color squares were collected, washed with 6 M

guanidine hydrochloride, pH 1.0, decolorized with DMF, gelatin coated and competed with 100 nM d-Biotin with 20 nM steptavidin-AP in 2× PBS/Tween/gelatin. Incubation with substrate yielded 17 colorless, competed squares. Reincubation with 20 nM streptavidin-AP produced 17 positively reacting squares following treatment with substrate.

The remaining library was then recycled with 6 M guanidine hydrochloride, pH 1.0, DMF, and gelatin-coated. After washing with PBS/Tween and 2× PBS/Tween/gelatin, 250 ng/mL biotinylated anti-β-endorphin antibody in 2× PBS/Tween/gelatin were added. Following thorough washing, streptavidin-alkaline phosphatase was added. The library was then washed, substrate was added, and color development proceeded as described above. Twenty-one squares developed color, and subsequent recycling prepared them for specificity determination. Using 2× PBS/Tween/gelatin as the buffer, 200 ng/mL anti-β-endorphin antibody were added. A thorough washing was followed with 1.5 nM goat-anti-mouse AP. The squares were then washed with PBS/Tween, 2× PBS/Tween/gelatin and TBS, and substrate was added. As a result, 3 squares developed the color that indicates binding.

Screening of the Directed Library on Cotton

Cotton pieces were cut in half and placed into matching positions of the two matching microplates (one equipped with the filter bottom and a regular plate—"structure evaluation plate"). The filter plates were placed into a dessicator, which was repeatedly evacuated and filled with ammonia gas. After 20 h exposure to ammonia, the dessicator was evacuated and ammonia removed. The release buffer PBS/Tween was added to each well (100 μL) and the plates were shaken overnight at room temperature. The solution was filtered to the test plates.

For the inhibition ELISA, 96-well Immulon plates (Dynatech Labs, Bethesda, MD, USA) were used. Each well was coated initially with 50 μL of streptavidin (20 μg/mL) in bicarbonate buffer (pH 9.4) (Pierce Chemical) overnight, followed by three washes with PBS. Wells were treated with 200 μL of bovine serum albumin (BSA, 3 mg/

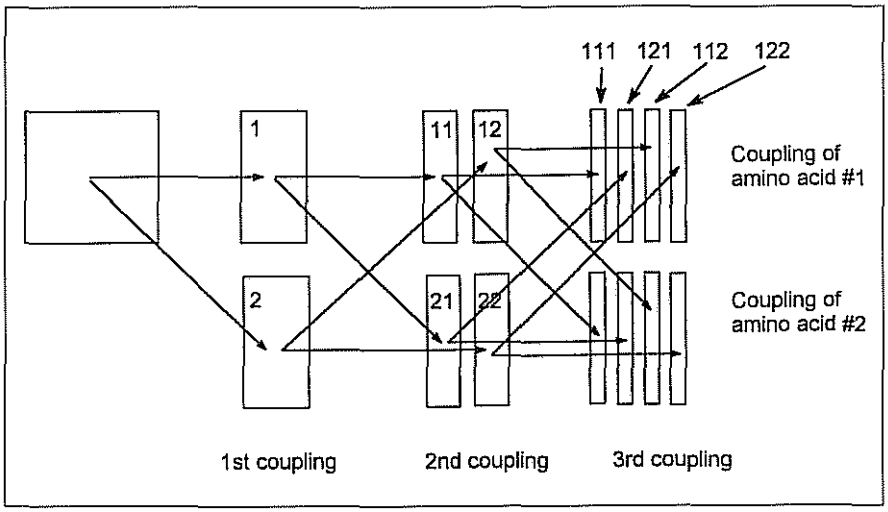

Figure 1. General scheme for the synthesis of directed libraries. Example of the library with three positions randomized by two amino acids in each step, generating the library of eight tripeptides.

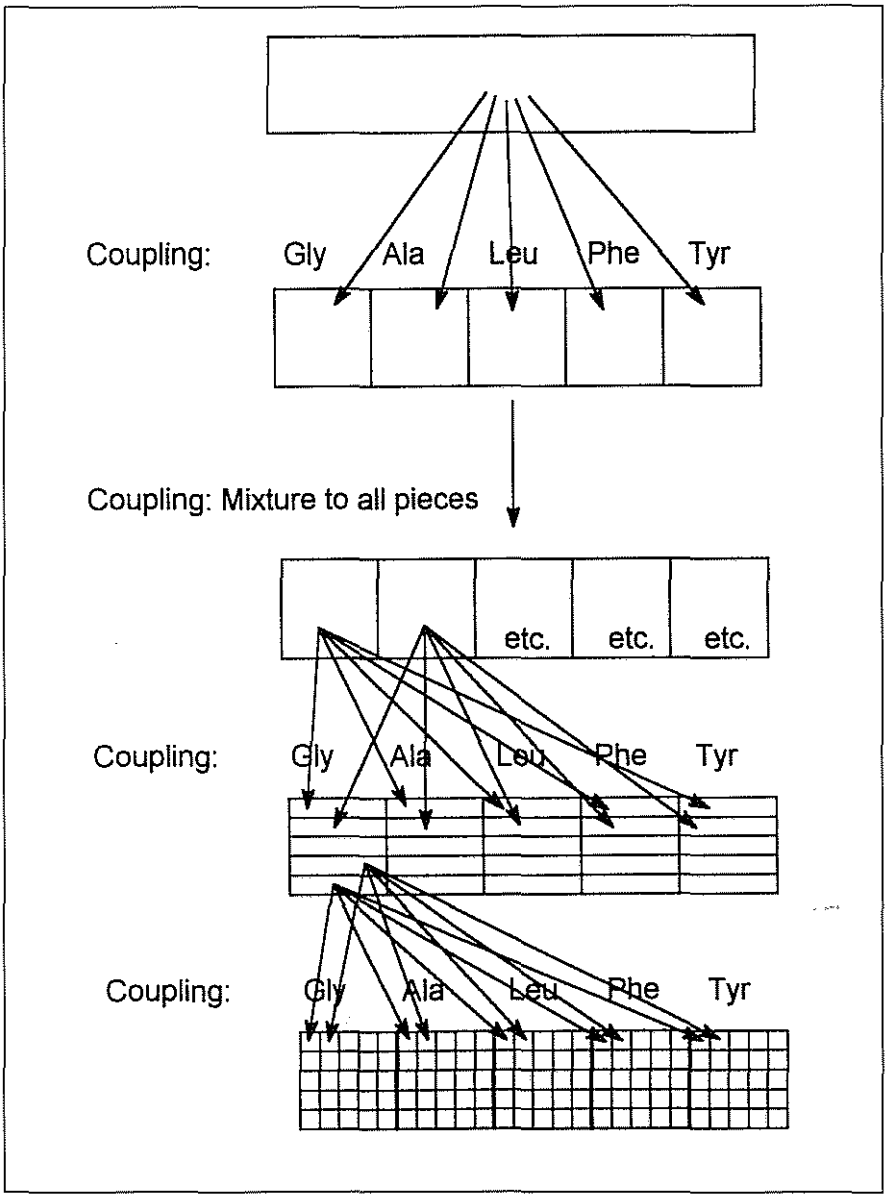

Figure 2. Scheme of the synthesis of model library of 125 tetrapeptide mixtures on cotton string.

mL) in PBS to prevent nonspecific adsorption and washed three times with PBS/Tween. Then, 50 μL biotinylated Tyr-Gly-Gly-Phe-Leu (44) (10 ng/mL) were added. After 1 h the plates were washed with PBS/Tween, and 50 μL of the solution that was released from the cotton pieces (or Tyr-Gly-Gly-Phe-Leu, positive control) were added, followed by 50 μL of anti-β-endorphin (40 ng/mL). After 1 h of incubation at room temperature, the plates were washed with PBS/Tween, and 50 μL of a 1:1000 dilution of goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad) were added. One hour later, the plates were washed, and 100 μL of a solution of 30 μL of 30% H_2O_2 in 10 mL of 2,2′-azidobis(3-ethylbenzthiazolinesulfonic acid) substrate in citrate buffer (pH 4.2) were added. Fifteen minutes later, the ELISA plates were read at 405 nm. Only one showed significant inhibition. A sample of the cotton piece (0.3 mm) from the matching well in the "structure evaluation plate" was sequenced, giving the sequence Tyr-Gly-Mix-Phe.

RESULTS AND DISCUSSION

Solid-phase synthesis of peptides is normally performed on a beaded polymer (39); however, alternative forms of solid support can be employed. The solid supports include plastic pins (21), membrane (8), glass (13), sheets of paper (14,16) or cotton (12,35). For a review of the multiple synthesis techniques, see References 15 and 27. Prior experience with cotton (11) led to the selection of cotton threads as a solid support for the synthesis of "directed" or "nonrandom" libraries. The principle of such libraries is simple and is illustrated in Figure 1. The continuous support is divided into as many parts as the number of building blocks utilized in the first randomization step, and the pieces are individually reacted with the appropriate residue. After completion of the reaction, each fragment of the carrier is then divided into as many parts as the number of building blocks in the second randomization step, with the appropriate pieces from each fragment pooled for coupling. In this manner, the synthesis can continue until peptides of desired length are completed, or until the mechanical limit of handling the support is reached. The mechanical limit of cotton thread is achieved at a dimension of several millimeters, which defines the practical capacity of a library of 10 to 50 000 compounds. However, even libraries of these proportions are of substantial value, especially in the case of nonpeptidic libraries, in which no more than three or four randomizations are usually performed. While the structure of the compound on the individual solid-support fraction (particle) is not known when utilizing this approach, the uniqueness of each compound and completeness of the library can be ascertained.

As an alternative carrier to cotton threads, functionalized polypropylene membranes were tested. The lowest manageable area was determined to be 1 mm^2, and since the membrane is only 10 μm thick, a library of several million compounds can be feasibly constructed, although automation of

this process is strongly recommended. A reasonable arrangement for the described technology might be "restructurable toothbrush," constructed from bunches of threads of functionalized material with better mechanical properties than cotton. The toothbrush can be disassembled into bunches and reassembled again for the next randomization step. Individual bunches of threads can be disassembled after two or three rounds of randomization and recombined for the next step. For the last randomization step, the threads can be cut into pieces.

To prove the idea of "nonrandom" libraries, we prepared two model peptide libraries. The first library was prepared on cotton thread and contained only 125 peptide mixtures (see Figure 2). Cotton thread (125 cm) was modified by beta alanine (11) and glycine was attached to it. The thread was then divided into five pieces, and Gly, Ala, Leu, Phe and Tyr, respectively, were coupled to each piece. After complete coupling and Fmoc deprotection, a mixture of L-amino acids was coupled to all parts. In the two successive steps, the thread fragments were again sectioned and acylated with the same set of five amino acids. Ultimately, all fragments were combined, deprotected, neutralized, washed and dried. This resulted in the generation of a library of 125 tetrapeptide motifs (mixtures of 19 peptides on each particle having positions 1, 2 and 4 defined). Each piece was then cut in half and both halves were placed into two matching microplates, one of them equipped with the filter. The filter plate was then exposed to ammonia gas in a dessicator to release the peptides from the cotton. Gas ammonolytical cleavage, described earlier (2–4), was chosen as a cleavage method because it allows convenient handling of the carrier, even after the performed release (released peptide is physically attached to the dry support particle). Also, peptide can be extracted directly to the testing buffer without the need for pH adjustment. After extraction of the peptide with buffer, the peptide was transferred to another plate and the anti-β-endorphin antibody binding assay was performed. One well was observed to react positively, and sequencing of the corresponding cotton fraction identified the sequence Tyr-Gly-Mix-Phe ("Mix" is a mixture of all amino acids), corresponding to the known motif for anti-β-endorphin antibodies. Further analysis revealed that one centimeter of cotton thread can yield 400 nmol of released peptide, allowing for the preparation of 40 mL of a 10-μM solution of test compound, more than enough for multiple assays.

Table 1. Sequences Found on Positive Squares Selected for Binding to Streptavidin and Anti-β-Endorphin

Streptavidin	Anti-β-Endorphin
Gly-His-Pro-Gln-Phe	Tyr-Gly-Gly-Phe-Leu
Met-His-Pro-Gln-Phe	Tyr-Gly-Gly-Phe-Phe
Lys-His-Pro-Gln-Phe	Tyr-Gly-Pro-Phe-Leu

The effectiveness of our approach is further demonstrated with the 2888-peptide library, in which a 16 × 16-cm sheet of functionalized Teflon membrane was used for synthesis. The membrane was acylated by protected β-alanine, and a linker composed of a repeated sequence of β-alanine and glycine was constructed. The scheme of the synthesis, given in Figure 3, shows the generation of a library with the structure Xxx-Xxx-Pro/Gly-Gln/Phe-Phe/Leu (Xxx is one of 19 L- amino acids used for the randomization). This structure contains one copy of the sequence Leu-His-Pro-Gln-Phe (38 copies of Xxx-His-Pro-Gln-Xxx), with His-Pro-Gln being the known motif for streptavidin, and one copy of Tyr-Gly-Gly-Phe-Leu, containing the sequence Tyr-Gly-Xxx-Phe, the known motif for anti-β-endorphin binding (1,7,9,25, 32,34,38,43). The library was then screened with two model targets, streptavidin and anti-β-endorphin, using the solid-phase-binding protocol.

Screening with streptavidin yielded 55 positive squares. The specificity of the peptides was determined by addition of the known competitor biotin. Seventeen of the squares contained peptides that did not react with streptavidin in the presence of biotin; however, all 17 of the squares again stained positively in the absence of biotin with approximately equal intensity. The peptides on three squares were sequenced individually (results given in Table 1). Small pieces of all the squares were cut and sequenced at the same time. This multiple sequencing experiment (37) revealed the requirements of individual positions of the peptide chain. In position 1, Asp, Asn, Thr, Ser, Gly, Glu, Ala, His, Tyr, Pro, Met, Val, Trp, Phe, Ile, Lys and Leu were found. Position 2 contained His; position 3, Pro; position 4, Gln; and position 5, Phe. We can conclude that almost anything can be placed in position 1 and that phenylalanine is preferred over leucine in position 5. The sequence -His-Pro-Gln- was found in all seventeen squares.

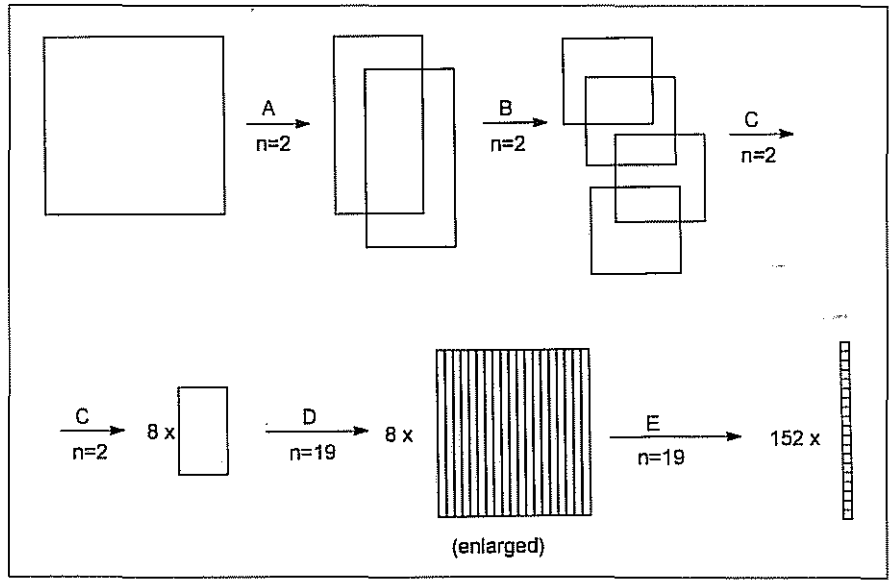

Figure 3. Scheme of the synthesis of membrane library of 2888 pentapeptides. n = Number of amino acids randomized in the particular position.

Two squares, identified as nonspecific binders, were sequenced to find out the major cause of the false-positive binding. The sequences were Arg-Arg-Gly-Gln-Phe and Arg-Arg-Gly-Pro-Phe. It seems that clusters of positively charged residues might be responsible for the observed nonspecific binding.

Incubation with anti-β-endorphin provided 21 stained squares. These positively interacting particles were destained and reincubated with the anti-β-endorphin antibody using an alternative detection scheme. In the first staining, the antibody was labeled with biotin, and detection of bound target was achieved by incubation with the streptavidin-alkaline phosphatase complex. The second round of detection was performed by incubation with the anti-mouse antibody-alkaline phosphatase complex. In this way, the nonspecific interactions of the first detection scheme were eliminated, and, since the second detection was done only with selected particles, the probability of detecting the nonspecific interactions (specific interactions of the detection system) was significantly decreased. The second incubation detected only three positive squares. They were sequenced and the results found are given in Table 1. Predicted sequences with the motif Tyr-Gly-__-Phe were found.

CONCLUSION

The experiments described demonstrate the effectiveness of "nonrandom" or "directed" libraries as an alternative to random libraries in both solid-phase binding and solution assays. By this technique, the amounts of individual compounds in the library are limited by the size of the carrier used for their synthesis, and depend on the choice of the experimenter. Alternatives to continuous, dividable carriers for the synthesis of large amounts of library compounds are the application of "tea bags" (24), paper disks (16), cotton pieces (11) or pins (21). In these cases the synthesis of nonrandom libraries can be performed in such a way that the structure of the compound (in each bag, on each piece of paper or cotton, or on each pin) is known, since every component of the library can be labeled. However, the synthesis of millions, or even ten of thousands, of structures is unrealistic. The strategy of "directed" libraries allows for the incorporation of simple coding schemes, which permit simple (e.g., visual) identification of important structural features in the library, based on applied physical characteristics of the support, such as the color (6) or shape of the material (length in the case of thread). The use of coding by shape was applied to our model library on functionalized membrane, in which control peptides on triangular particles were added to the library prior to screening. In principle, the positionally defined multiple peptide synthesis techniques (13,14) can be classified as directed library techniques, but they require individual synthesis of all components (see, e.g., References 10 and 14) or sophisticated instrumentation (13).

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Address correspondence to:

Michal Lebl
Selectide Corporation
1580 East Hanley Boulevard
Tucson, AZ 85737, USA