MOLECULAR DIVERSITY AND LIBRARIES OF STRUCTURES: SYNTHESIS AND SCREENING

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Library, or molecular diversity, approaches are a progressive tool in contemporary investigation of biological interactions and drug discovery. All library approaches include certain degree of rationality and cannot be therefore classified as random or irrational techniques. Reviewed techniques are complementary to so called "rational design"; they can serve either as a tool to generate a lead, or, on the other hand, to optimize a "designed" lead by rapid evaluation of structure–activity relationships. Combinatorial approaches are based on either a random or multiple principle which enables production of large number of diverse molecular structures that can be simultaneously screened and evaluated in a biological assay to find an optimal interacting molecule.
Key words: Combinatorial libraries; Multiple syntheses; Solid phase syntheses; Biological libraries.

1. INTRODUCTION

Traditionally, new medicinal lead structures have been derived from natural products, either on the basis of structure–activity studies of various derivatives, or by means of rational design approaches based on the knowledge of the biological mechanism of action. In addition, many pharmaceutical companies screen vast historical libraries of synthetic compounds. Neither of these methods provides a sufficient number of compounds to support the expectation of discovering the optimal lead structure. Recent trends in drug discovery are based on a quantitatively new philosophy of investigation. The increase in numbers of different structures to be screened was first introduced as a novel synthetic approach – simultaneous multiple syntheses. This and related methodologies enable the simultaneous preparation of up to thousands of unique chemical compounds. Subsequently, random library approaches – both biological and synthetic – started a new age in drug discovery (for the recent reviews see e.g.[1–9]). Typically presented as the antithesis of rational drug design as an approach to drug discovery, nearly all library approaches include rationality and apply useful tricks demanding knowledge both of biological interactions and chemical mechanisms.

In general, libraries can be divided into two categories:

1. Biological libraries (genetically encoded and expressed)

2. Synthetic libraries.

The former is based on screening, testing and evaluation of genetically expressed information (sequences of oligonucleotides or proteins), for which the precursors are randomly generated oligonucleotides. The latter involves various techniques for random or directed syntheses of large numbers of chemical compounds and their screening, testing and optimization.

Each of the library approaches must provide not only for a substantial number of unique structures, but also enable identification and determination of the structures responsible for an observed interaction. This demand is satisfied for biological libraries, where structural information is genetically encoded by DNA, and carried either by phage particles or plasmids or polysomes. Likewise, the structure of peptide molecules generated on a support particle in a random synthetic peptide library can be determined

by Edman degradation. In the case of non-peptidic synthetic libraries, each of the compounds in the library must carry an identification label or code or must be otherwise defined by an iterative process or synthetic algorithm. The biological screening of all types of libraries is based on interaction with a biological target. Synthetic libraries are usually tested either immobilized on a support or free in solution. After biological screening active molecules are analyzed and resynthesized and the tests are repeated and quantified to prove the screening results. Early on, both multiple synthetic methodologies and library approaches were limited to peptides. These molecules, however, do not provide satisfactory lead structures owing to their high bio-degradability. The current emphasis is on the creation of libraries from various "building blocks" other than natural amino acids, use of "scaffolds" and application of diverse chemical reactions providing new molecules. The same trend is apparent in the multiple synthesis field. Nevertheless, peptide libraries and some multiple peptide synthesis approaches have retained an important role in the field of mapping of biological interactions (antibody–antigen, enzyme–substrate/inhibitor, ligand–receptor etc.).

2. BIOLOGICAL LIBRARIES – BRIEF SURVEY

In general, biological libraries are comprised of large numbers of peptides or proteins which are displayed on the surface of filamentous phages, plasmids or polysomes[8,10–12]. These libraries use synthetic or cloned oligonucleotides as an insert to that portion of the virion that enables surface expression of the inserted information. Each of these selection methods has three key steps that are repeated, and each cycle further enriches for ligands with the strongest interaction. The first step is generation of a library, the second is screening against a biological target and selection and the third is amplification of the selected ligands and sequencing of their DNA.

2.1. Phage Libraries

The great advantage of filamentous phage[13–17] is that foreign DNA fragments can usually be inserted into its minor coat protein gene pIII or less often into pVIII (refs[18–21], into the 5′ region) with little effect on phage function. Furthermore, bacteriophages are simple compact structures with only a small number of nonspecific interactions in the selection procedure. The work of Kang et al.[22] deal with connection of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. The fusion peptides that are generated on the phage surface also enable the creation of disulfide bridges between cysteine residues of microproteins displayed on bacteriophage[23].

The pioneering works[24,25] were focused on cloning of antigenic determinants, "epitope libraries", using filamentous phage. Fusion phage bearing specific target determinants can be affinity-purified with antibodies directed against the foreign determinant.

This method has been called "biopanning" (see Fig. 1). It is based, in this case, on the very tight interaction between streptavidin and biotin. The selection is carried out on streptavidin coated petri dishes. The dishes are incubated with biotinylated antibody and then with phages that display clones. The phages bearing antigens are selectively bound to antibodies linked to a dish and nonspecific clones are washed out. The phages that display antigens are then released and expressed in *Escherichia coli*. This process is repeated several times and in such a way that the most specific antigen can be found. The amino acid sequences of the peptides displayed on the phage are then determined by sequencing the corresponding coding region of the viral DNA. The sequencing methodology for identification of ligands obtained from a phage library has been improved by Povinelli and Gibs[26]. Dente et al.[27] have eliminated nonspecific interactions by use of monoclonal antibodies that recognize a filamentous phage particle.

The complexity of a phage library has been checked[28] on a random sample of fifty-two clones from $2 \cdot 10^6$ library. All decapeptide sequences were identified, and biochemical characterization shows that they correspond to structures comprising a wide range and combination of isoelectric, hydropathic, and biochemical properties necessary in drug discovery to access a significant representation of possible peptide structures by affinity or activity screening.

The phage libraries have been screened against various receptors[29]; however, the main field of their use is epitope mapping[17,20,30] and, consequently, screening of biopolymers mimicking natural epitopes[31]. Antibody investigation was focused on either continuous epitopes[19,25,32–35], or conformation dependent epitopes[31,36,37]. A strategy for identification disease-specific phagotopes eliciting antibodies against the original antigen was elaborated by Folgori et al.[38].

Phage display was also used as a tool for generation of monoclonal antibodies[39]. A combinatorial library of the immunoglobulin repertoire in phage was first prepared by

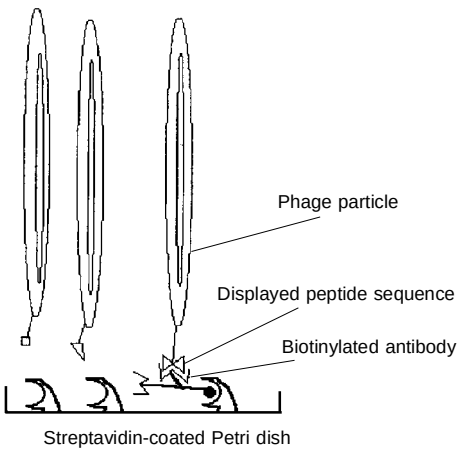


FIG. 1
"Biopanning"

Huse et al.[40]. McCafferty et al.[41] showed that complete antibody V domains can be displayed on the surface of bacteriophage, that the phage binds specifically to antigen and that it can be isolated by affinity chromatography. Barbas et al.[42] and Chang et al.[43] investigated the methods for expression of combinatorial antibody libraries on phage surfaces. Several other genetic approaches for cloning and expression of antibody gene fragments on the surface of filamentous phage have been developed[44–46]. Clackson et al.[47] prepared a random combinatorial library of rearranged randomly linked heavy and light chains which were individually recloned. Finally, Marks et al.[48,49] and Gram et al.[50] have demonstrated that the requirement for immunization can be overcome by the use of phage display. They used a phage-antibody library derived from nonimmunized donors to isolate human antibodies with binding constant 10^4–10^5 M^{-1}. A modified approach, where semisynthetic random oligonucleotides were inserted into phages, was described by Barbas et al.[51]. Combinatorial antibody phage libraries were successfully used in the investigation of antibody response to either HIV-1 (refs[52,53]), or hepatitis B (ref.[54]) or some metals which can coordinate with an antibody protein[55]. Chen et al.[56] and Janda et al.[57] identified diverse catalytic antibodies from combinatorial libraries[58].

Phage libraries enable the identification of peptides binding to the other receptors or proteins, e.g. streptavidin[59], carbohydrate-specific monoclonal antibodies[60], Src SH3 – non-receptor protein kinase[61], calmodulin[62], S-protein[63], concanavalin A (refs[64,65]), chaperonin BiP (ref.[66]), $\alpha_5\beta_1$ Integrin[67] or urokinase receptor[68]. Petithory et al.[69] developed a method for determination of endoproteinase specificity. Some proteinase substrates were also identified using phage libraries[70].

Very important model studies were performed in the field of the streptavidin biotin interaction. These works proved that phage libraries are a good source of specific receptor binding molecules. Devlin et al.[59] found nine different streptavidin-binding peptides in a library. The consensus sequence was HPQ, and binding of these phage clones to streptavidin was inhibited by biotin. Similar results were observed[71] in screening of M13 pIII-fusion phage library. Panning with streptavidin identified the main motifs: HPQ or HPM. Roberts et al.[72] compared the results obtained by both panning with polyclonal antibody against biotin and panning directly with streptavidin. Some similarities were observed, but biotin-sensitive streptavidin binding in ELISA was found only for the motifs (GDWVFI and PWPWLG) identified by direct streptavidin panning. The previously unknown biotin-binding motif, CXWXPPF(K/R)XXC, was selected from a random peptide library expressed on phage by Saggio et al.[73].

In the recent years, the range of applications of phage technology has been extended to the search for peptides binding both antibodies and cell receptors as well as enzymes[74].

2.2. Other Biological Libraries

The peptides-on-plasmids library approach was introduced by Cull et al.[75]. In contrast with phage display, fusion in a plasmid is to the C-terminus of the repressor (peptides are expressed as fusions to the Lac repressor protein), and expression is subsequently intracellular[29]. This type of library can be screened in a manner analogous to the phage system. It was used for construction, selection, and characterization of herpes simplex virus type 1 thymidine kinase mutants[76,77]. Schatz[78] used the peptides-on-plasmids library for identification of a new substrate for protein-biotinylating enzyme, BirA.

Another type of library was created by arrays of polysomes displaying peptides synthesized and isolated in vitro[8]. The polysomes can be affinity purified on immobilized receptors. The advantage of this technique in comparison to other biological libraries is the higher number of clones, about 10^{19} peptides of 10 or more residues long. The library is based on a complex of the nascent peptide displayed on a polysome with its encoding mRNA (refs[79,80]). Screening is similar to those mentioned above.

2.3. Oligonucleotide Libraries

Libraries of oligonucleotides are based on a very convenient method which allows a chemist as well as a biochemist to prepare a mixture of oligonucleotides either synthetically or by genetic means on a very small scale and use a biological mechanism to amplify it by several orders of magnitude. From a classification point of view, oligonucleotide libraries are therefore often on the borderline between biological and synthetic libraries.

This type of libraries was applied to find ligands for a number of targets[81–83]. Sastry et al.[84] introduced method for cloning and amplification of the immunological repertoire (DNA fragments) by polymerase chain reaction (PCR). Tuerk and Gold[85] isolated and amplified high-affinity nucleic acid ligands of a protein (bacteriophage T4 DNA polymerase) from pools of variant sequences. The same procedure (SELEX, for review see ref.[86]) was used for identification of high-affinity RNA ligands (e.g. of HIV-1 proteins[87] or HIV-1 Rev (ref.[88])), ligands to basic fibroblast growth factor[89] or to vascular endothelial growth factor[90]. Ellington and Szostak[91] selected RNA molecules that bind specifically to a variety of organic dyes from a population of random sequence RNA molecules (10^{13} of clones). Griffin et al.[92] discovered and characterized a novel oligonucleotide-based thrombin inhibitor. Bock et al.[93] described so called aptamers (double-stranded DNA or single-stranded RNA molecules that bind specific molecular targets) that bind and inhibit human thrombin. Large randomly generated populations can be enriched in aptamers by in vitro selection and PCR. An oligonucleotide library was also used to determine the binding-site preferences for zinc finger domains[94].

3. SYNTHETIC APPROACHES TO MOLECULAR DIVERSITY

Biological libraries have undeniable importance, but their principal disadvantage is the limitation to natural building blocks. Synthetic approaches overcome this restriction and the diversity generated may be limitless because of the large variety of building blocks and possible chemical reactions. On the other hand, synthetic processes produce a smaller number of individual molecules for biological screening. However, even if a biological method can provide high-affinity lead structures, key molecules for successful contemporary drug discovery require synthetic modification or even fully synthetic analogues.

3.1. Multiple Syntheses

Multiple synthesis, especially multiple peptide synthesis, was initiated about ten years ago. The need at the time was to develop and explore structure–activity relationships, leading to increased demands for synthetic lead compounds. Several sophisticated simultaneous multiple synthetic approaches have been developed and partly or fully automated. The main part of this field concerns solid phase peptide synthesis in a simultaneous multiple mode. Non-peptidic simultaneous multiple synthesis is usually based on the solid-phase methodology as well.

3.1.1. Multiple Peptide Synthesis (MPS)

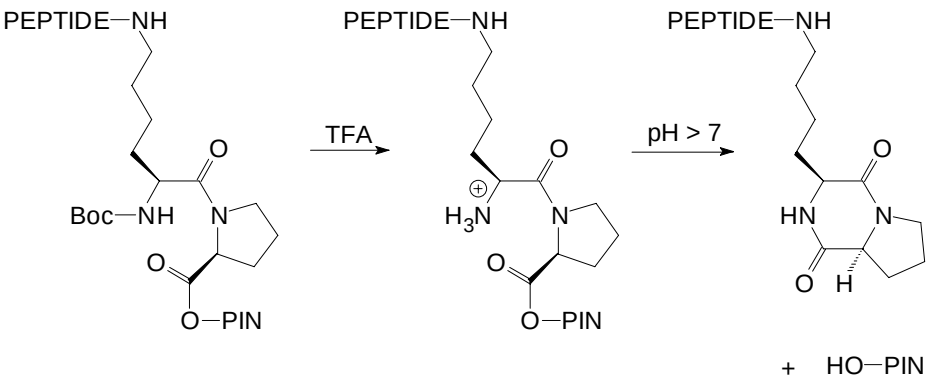
Solid-phase peptide synthesis, introduced by Merrifield[95], opened the way to automation and multiple synthesis, owing to simple handling and a well elaborated protocol. Solid phase peptide synthesis (SPPS) is based on repeated deprotection and coupling steps during the creation of a peptide chain covalently bonded to any support[96,97]. This process eliminates isolation of intermediates and hence simplifies the synthesis which can be carried out simultaneously for different peptides[98]. The MPS approaches can be divided according to solid carrier (resin beads, polypropylene pins, polypropylene membrane, paper sheets, cellulose discs, cotton fabric, glass etc.) utilized for synthesis. The multiple methodologies are designed with a view to prepare peptides either in immobilized form (for immunization, epitope mapping, etc.) or in released form. The rationale for multiple synthesis lies in decreasing synthetic time and in simultaneous biological screening of a great number of various analogues, or investigating the binding site of proteins using overlapping peptide fragments.

The first procedure for rapid concurrent synthesis on solid supports of hundreds of peptides was described by Geysen et al.[99]. This method was carried out on polyacrylic acid-grafted polyethylene rods (pins) fixed in a polyethylene holder in the microtiter plate format (96 rods in one set). Schaaper et al.[100] used polystyrene grafted polyethylene pins. The synthesis was done in teflon microtiter plate-like vessels. The com-

mon Fmoc/*t*-Bu synthetic strategy[96], which can be accomplished in open reactors, is usually used. Each of the rods can covalently bind an average of 50 nmol of a peptide. In this way Geysen et al.[99] prepared 208 overlapping hexapeptide sequences of the 213-amino acid coat protein VP1 of the foot-and-mouth disease virus (FMDV) to identify important binding sites for diverse antisera by an enzyme-linked immunosorbent assay (ELISA).

In several subsequent works this method was improved. In order to carry out a receptor-binding or competitive-binding assay, various linker moieties for the simultaneous release of peptides from pins have been used. A diketopiperazine-forming (DKP) linker[101,102] was used (see Scheme 1). An ester linker based on glycolate and 4-(hydroxymethyl)benzoate was cleaved under mild basic conditions, 0.3% NaOH, and 4% methylamine/water to generate peptides having C-terminal acid, and methylamide, respectively[103,104]. Gas phase ammonolysis of a benzylic ester linkage between a peptide and a functionalized rod[105] was found as another process for multiple parallel cleavage of covalently bound peptides on pins. Subsequently, vapor from the solution of 30% ammonia in tetrahydrofuran was successfully utilized[106]. Finally, the original solid pins were replaced with a detachable crown-shaped polyethylene support with an increased surface area and hence a larger peptide capacity[107,108]. The support was derivatized with trifluoroacetic acid-labile linkers (4-hydroxymethylphenoxyacetic acid or Rink amide linker[109]). Wiesmüler et al.[110] adapted the multipin approach to a fully automated regime. In a similar manner, Gausepohl and Frank[111] designed a synthesizer utilizing pins with detachable rods.

The usefulness of the pin method lies in epitope mapping[112] and other immunoanalytical fields[113,114]. This method was used for study of antibody binding sites of various viral proteins[99,115,116], by overlapping peptide fragments and for mapping of discontinuous epitopes[113,114]. The multipin strategy was used for screening of a T-cell determinant as well[117]. The pin technology was applied for analysis of structure–activity relationships in human tumour necrosis factor α (ref.[118]). The peptide receptor binding



SCHEME 1

studies utilizing the cleavable linkage for releasing of peptides into solution were performed on Substance P (refs[119,120]). The multipin strategy was used for synthesis of analogues of hexapeptide endothelin receptor antagonist by systematic substitution of amino acids at each position[121,122]. Ihlenfeldt et al.[123] compared the pin technology to the biotinylated peptide method for their ability to epitope mapping the hepatitis C virus. The pin method was found faster and more advantageous, especially due to the ability to release linked peptides into solution.

The tea-bag method developed by Houghten[124,125] enables simultaneous preparation of hundreds of peptides (100–200 at a time) at larger scale (up to 80 μmol/bag) in comparison to the multipin strategy. The solid phase resin for synthesis of different peptide sequences is placed in separate solvent-permeable polypropylene packets which are then sealed and labelled. These tea-bags are grouped by the required amino acid to be attached in the particular step of the synthesis and immersed in individual solutions of activated amino acids. Deprotection and washing steps are accomplished by mixing the tea-bags together in a reaction vessel, followed by their subsequent separation for the next coupling. Any standard SPPS procedures can be utilized in this technique, but in most cases Boc/Bzl (ref.[97]) or Fmoc/*t*-Bu chemistry is used. Cleavage of such a great number of peptides from resin can be carried out by hydrogen fluoride in apparatus designed for the multiple cleavage of up to 25 protected bound peptides[126–128]. This method provides fully characterizable peptides which are subsequently screened in solution. A partly automated tea-bag approach has been reported by Beck-Sickinger et al.[129].

The tea-bag (segmental) approach allows to use various synthetic methods and solid phase strategies including application of various carriers. For example, tea-bags with a pellicular carrier and cotton segments were compared in the synthesis of fifty peptides[130]. Activating agents for SPPS, TBTU (benzotriazol-1-yltetramethyluronium tetrafluoroborate)[129] or BOP (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate)[131] were evaluated as well. A great number of peptides synthesized in tea-bags for biological tests provided a good statistical model for HPLC study of prediction of retention times of peptides[132,133].

The main impact of this approach is in epitope mapping[134,135] and in studies of antigen–antibody interactions[136–138]. The tea-bag strategy helped to identify a lymphocyte-activating peptide fragment of the Fc region of human IgG1 (ref.[139]). Overlapping fragments of the large glycoprotein G were analyzed in an ELISA assay against different sera containing virus-specific antibodies to find corresponding binding sites[140]. The tea-bag method has enabled intensive study of immune responses to mycobacterial antigens[141–143]. Ruggeri et al.[144] used synthetic peptides from tea-bags for design of high-affinity antagonists of fibrinogen binding to platelets. Beck-Sickinger et al.[145] identified the binding site for neuropeptide Y by screening of deletion peptide analogues of this hormone obtained by the tea-bag methodology. The tea-bag method was

utilized for synthesis of cyclic disulfide analogues of the complement component C3a (the anaphylatoxic peptide)[146]. The role of single amino acids inserted into a mellitin sequence was observed in relationship to hemolytic activity of these tea-bag method synthesized analogues[147].

The advantage of the tea-bag method is the relatively large number of various unbound peptides in sufficient quantity and quality which can be synthesized during a week or two by one person without any expensive device[128].

Comparable to both above mentioned MPS methods in the number of simultaneously prepared peptides, is spot-synthesis by Frank[148–150]. Spot-synthesis is carried out on cellulose paper sheets adapted to a microtiter plate format represented by 96 positions – spots. In each spot one peptide sequence can be synthesized utilizing conventional Fmoc/*t*-Bu chemistry. The format of the sheet and diameter of the spots (from 3 to 12 mm) can be modified according to the demands of biological tests. The capacity of one spot depends on its diameter and type of paper. Typical loadings range from 0.1 to 1.4 μmol/cm^2. Synthesis is carried out both manually, using a micropipette, or by automatically using a pipetting workstation (Gilson-Abimed)[151].

Evaluation of spotted cellulose sheets representing, for example, overlapping decapeptides derived from the human cytomegalovirus protein (CMV26), the sequences of which are defined by the position on the sheet, can be accomplished using an antibody binding assay for binding to a polyclonal anti-CMV26 antiserum[149]. Antibody binding to a protein or peptide antigen is usually identified by an ELISA assay. In the case of peptides immobilized as spots on a paper cellulose sheet, standard enzyme–conjugate/chromogen combinations which form water insoluble coloured products are used. Coloured spots appear on the paper at the position of the binding sequence. This method was applied for epitope mapping of NS3 and NS4 regions of HCV protein by Flegelová et al.[152] both on a paper sheet and poly(aminopropylmethacrylamide) covered polypropylene membrane. Simmonds et al.[153] analyzed the epitope of the chick link protein using the commercially available SPOTs Epitope Scanning Kit. Both cyclic and unnatural amino acids containing glutathione analogues were prepared on a modified cellulose sheet by Lyttle et al.[154] in order to screen these analogues for binding with antibodies. A computer program that facilitates multiple spot peptide synthesis on cellulose sheets was presented by Molina et al.[155]. Peptides can also be released from the paper support and fully characterized[156]. The method utilizing intramolecular catalysis of an ester bond cleavage in a basic solution by an incorporated basic group[157] was elaborated for direct release of peptides into an aqueous buffer.

A quantitatively new way for parallel preparation of thousands of peptides was developed by Fodor et al.[158–160]. Fodor's light-directed, spatially addressable parallel chemical synthesis is based on solid-phase chemistry and photolabile protecting groups. This approach belongs to the most progressive tools for drug discovery. The synthesis is carried out on the surface of aminopropylsilyl glass microscope slide that is acylated

with Nvoc (*N*-(6-nitroveratryloxycarbonyl))[161] protected amino acid. Photodeprotection is effected by illumination of the support through a lithography mask. A binary masking strategy[158] enables generation of 2^n regions for screening per *n* synthetic steps. The pattern of exposure to light through a mask removes photolabile protecting groups and hence activates regions of the glass slide for chemical coupling. After light deprotection, the first of a set of activated amino acids is exposed to the entire surface, but reaction occurs only in the regions that were addressed by light in the preceding step. When the coupling is finished, the second mask is used for deprotection according to another pattern and this process is repeated and recorded. The sequence of individual peptides is determined by their position on a slide. The number of peptides which can be prepared by this approach depends on photolithographic resolution. For example, the 50 μm checkerboard enables the synthesis of 40 000 peptides on an area of 1 cm^2. Usually, the slide parameters are 1.28×1.28 cm, three identical arrays of peptides are generated per slide, and each synthesis region is 400×400 μm for a total 1 024 sites per array. In addition, each slide is made in duplicate. The synthesis is adapted to an automatic synthesizer and carried out in vacuum flow cells[160]. This multiple synthetic approach can be used in an antibody binding assay[158]. The array of peptides was incubated with fluoresceine-labelled monoclonal antibody. The detection of binding events can be accomplished with a scanning fluorescence microscope with a laser source for excitation[162,163].

One thousand and twenty-four deletion peptide analogues were synthesized by the photolithographic technique to find the shortest active fragment of the C-terminal region of the opioid peptide dynorphin B and screened with a monoclonal antibody against the longer C-terminal region of this peptide. The sequence RQFKVVT was identified[163,164]. This work proceeded with systematic synthesis and screening of various frame shifted sequences (truncated and deletion sequences) and the fragment tightly bound to both the mAb and Fab fragment was found[160]. This approach has also been tried for parallel addressable immobilization of various immunoglobulins on solid support and this "reverse" method should be generally applicable to a wide range of biopolymers with a variety of functional groups for ligand-binding assays[165].

The "photolithographic" method has an exceptional position among other multiple approaches. Rather than a multiple synthesis approach it resembles a library approach by the number of different compounds which can be synthesized and then screened. This approach lies on the boundary between these different methodologies.

There are many other multiple peptide synthetic approaches differing in strategy, supports, level of automation or an experimental arrangement. Simultaneous peptide synthesis using cellulose paper segments as support material were described by Eichler et al.[166]. The synthesis was accomplished either in the shaker or between layers of glass.

The concept of so called "libraries with only one representation of each structure" is based on multiple peptide synthesis on a cotton string or a functionalized Teflon membrane[167]. A support is segmented according to a simple algorithm throughout the synthesis, allowing for separation of coupling steps for different amino acids. Since synthesis produces a full set of possible structures (without any duplication) and the identity of compounds must be defined by analytical techniques, this method of multiple synthesis is appropriately called a library technique.

Two MPS approaches utilizing continuous flow conditions[96] were elaborated. The first method was based on cellulose paper discs arranged into several columns and transferred into a corresponding column[168–170] before each coupling based on a required amino acid to be coupled in a particular peptide sequence. The second method by Krchnak et al.[171,172] utilized the set of the stable-flow columns packed with polystyrene-based resin. The synthesis was performed on a manually operated synthesizer.

Many other MPS methods are carried out in a similar manner in columns or syringes. Albericio et al.[173] as well as Krchnak et al.[171] have used syringes filled with Kel-F-styrene support, or with *p*-methylbenzhydrylamine resin. A manually operated apparatus for parallel multiple column solid-phase peptide synthesis on kieselguhr supported resin was used by Meldal and Holm[174,175].

Gausepohl et al.[176–178] automated both synthesis and cleavage as well as purification of a number of peptides simultaneously, using resin packed columns adjusted in manifold. The device is based on a commercial autosampler GILSON M 222. A robotic workstation for deprotection/cleavage of peptide-resin which enables an automatic final work-up of many peptides was presented by Zuckermann and Banville[179]. The automatic eight channel multiple peptide synthesizer by Nokihara and Yamamoto[180] utilizes polypropylene syringes with a polypropylene filter and beaded solid support. The device also enables simultaneous cleavage of a peptide-resin.

Schnorrenberg et al.[181,182] developed a fully automatic multiple peptide synthesizer (Zinsser SMPS), based on a commercially available modified robotic sample processor. The synthesis is carried out in Eppendorff cups on a polystyrene-based resin. The peptides synthesized by this device were for example used to map an epitope of influenza nucleoprotein[182] or to map endothelin binding sites[181].

A very handy system of manual MPS was presented by Sheppard's group[183,184]. The arrays of independent small columns can be connected into series – multicolumns (for the coupling of the same amino acid), or can react in parallel – independently (for the couplings of several different amino acids).

A cotton planar segmented carrier[185] was used also for MPS in a manual operation[186,187] and also with a centrifuge-based automatic multiple peptide synthesizer[188,189]. This device utilizes cotton sheets as well as resin beads sealed into polypropylene mesh bags for synthesis[189]. The MPS using cotton as a carrier was ap-

plied, for example, to synthesize the complete D-amino acid replacement set of substance P. The histamine-releasing activity of these analogues was then investigated[190].

As a suitable material for multiple peptide synthesis Berg et al.[191,192] tried and recommended long-chain polystyrene-grafted polyethylene film sheets. This support yielded peptides with comparable quality and quantity as beaded support.

3.1.2. Multiple Synthesis of Non-Peptidic Structures

One class of non-peptidic structures which can be prepared by multiple strategy are oligonucleotides and their analogues. A preparation of these molecules can be advantageously accomplished by genetic engineering (see Chapter 2.2.). A general approach for simultaneous multiple chemical synthesis of a large number of oligonucleotides was first described by Frank[193]. His solid phase based method was carried out on cellulose paper discs in glass vials. For each coupling step, the paper discs were sorted according to sequences so that the same protected nucleoside monomer was coupled to several paper discs in a common vial. The synthesis was done in a manually operated device.

A solid phase oligonucleotide synthetic approach that belongs to other powerful approaches of molecular diversity was reported by Fodor et al.[158]. It is based on the above mentioned photolithographic technique of oligonucleotide synthesis (light-directed spatially addressable parallel chemical synthesis), carried out on glass chips using photolabile protecting groups[162]. For protection of nucleosides Holmes and Fodor introduced besides Nvoc also Menpoc[159] (α-methyl-6-nitropiperonyloxycarbonyl) group. The synthesis was accomplished in a similar manner as the synthesis of peptides (Chapter 3.1.1.). Immobilized arrays of oligonucleotides found application in DNA sequence analysis. Sequences which are specific for hybridization of DNA can be easily detected by their localization in the matrix on the planar support[194]. Oligonucleotide arrays could be applied to detect the sequence specificity of RNA and/or DNA, in gene mapping, fingerprinting etc.

The disadvantages of the natural oligonucleotides are similar to peptides. Natural oligonucleotides are rapidly degraded in vivo, primarily via cleavage of the phosphodiester backbone. This fact has stimulated efforts to prepare more resistant oligonucleotides. An incorporation of unnatural building blocks into an oligonucleotide sequence and/or replacement of labile groups with stable ones are possibilities[81].

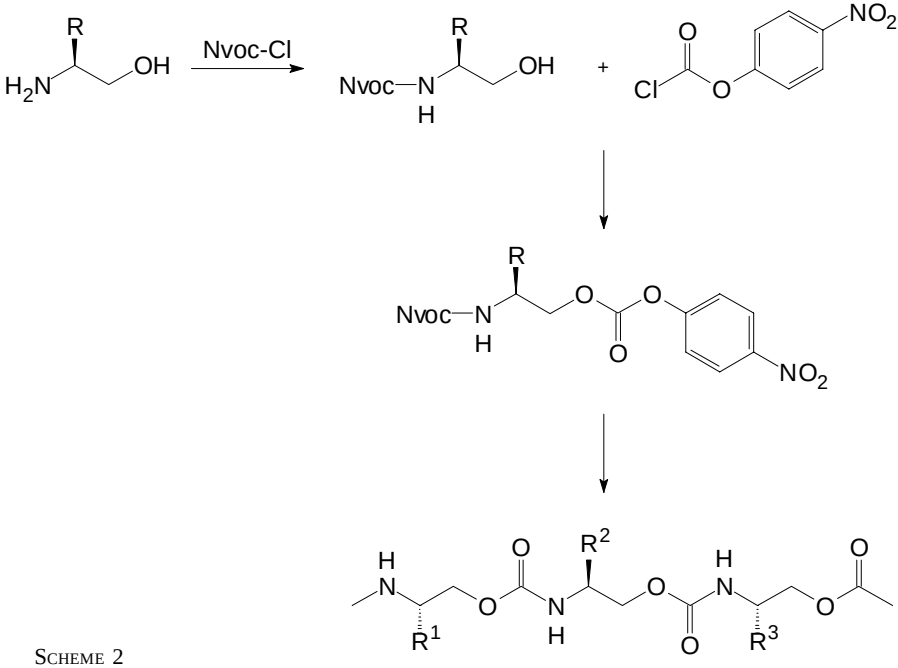
Peptides are extremely important biopolymers with specific cellular functions. Unfortunately, their stability in biological systems is limited. Tremendous progress was made recently both in the field of peptidomimetic synthesis (synthetic modifications of amide bond and utilization of unnatural building blocks) and synthesis of unnatural biopolymers.

Light-directed chemical synthesis was adapted to the chemistry of unnatural biopolymers. Cho et al.[195] introduced a photolithographic solid phase approach for synthesis of oligocarbamates. These molecules rise on solid support by linkage of

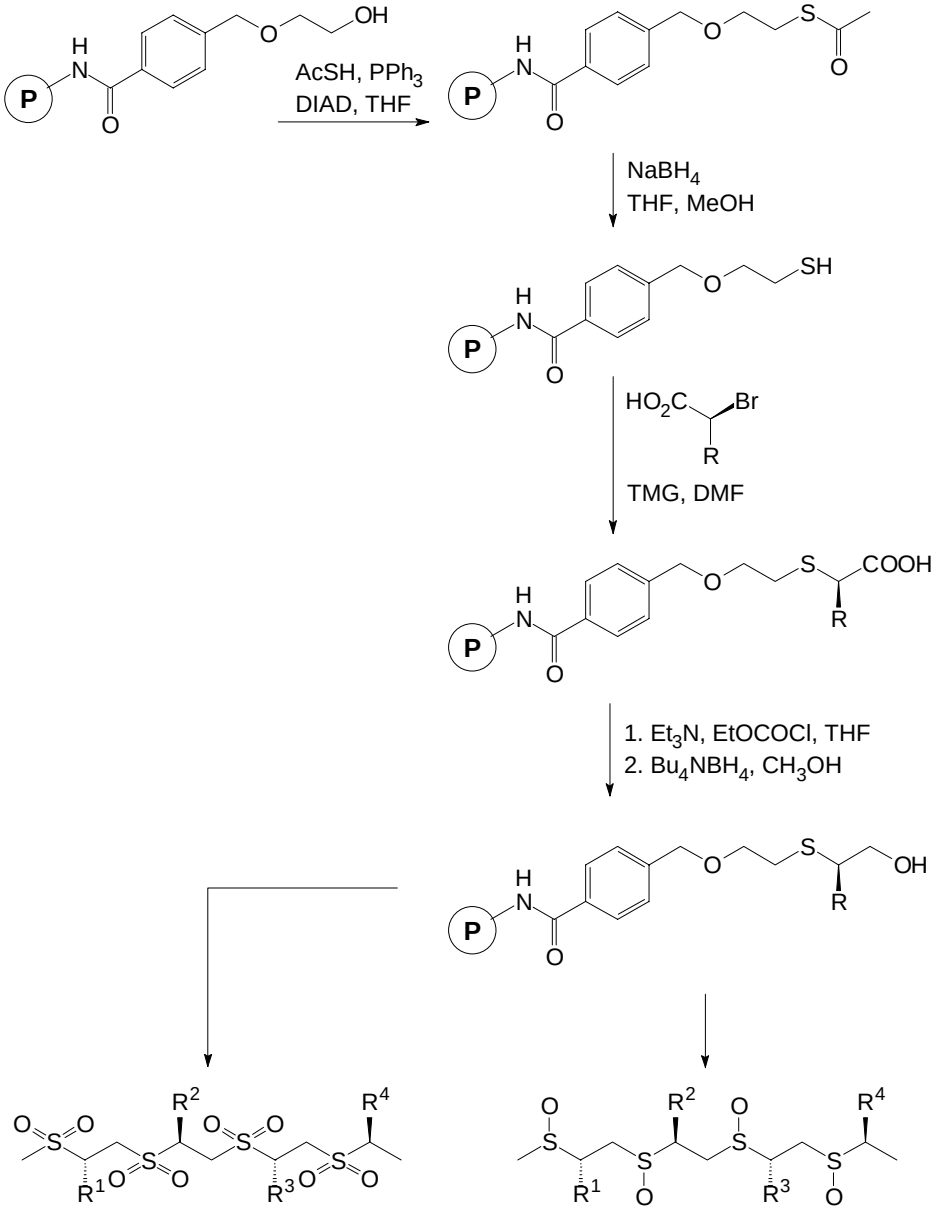
aminocarbonate monomers via a carbamate backbone (Scheme 2). Solid phase synthesis of oligocarbamates involves the sequential base-catalyzed or light-dependent deprotection of the α-amino group of the growing chain followed by coupling to the next protected *p*-nitrophenyl carbonate monomer. The arrays of oligocarbamates bound to a glass slide were screened successfully for their ability to bind a fluorescein labelled monoclonal antibody[163,196]. Moran et al.[196] suggested synthesis of oligothioethers and oligosulfones which can be also accomplished by light-directed chemical synthesis on a glass slide. The synthesis of oligothioethers has five synthetic steps (Scheme 3), nevertheless, all reactions proceed nearly quantitatively. The oligosulfones or oligosulfoxides can be obtained by oxidation of support-bound oligothioethers.

Because of increasing interest in glycoproteins and glycopeptides, new ways for their preparation are under development. They have an important role, for example, in transmembrane proccesses and immunology. Peters et al.[197] described multiple synthesis of *N*-acetylglycopeptide carboxamides in columns packed with poly(dimethylacrylamide) resin (PAL). For synthesis *O*-glycosylated serine and threonine protected building blocks were used. Jansson et al.[198] proposed multiple synthesis of neoglycopeptides by the same method by use of *N*-Fmoc-8-amino-2,6-anhydro-3,8-dideoxy-D-*glycero*-D-*talo*-octonic acid as the building block.

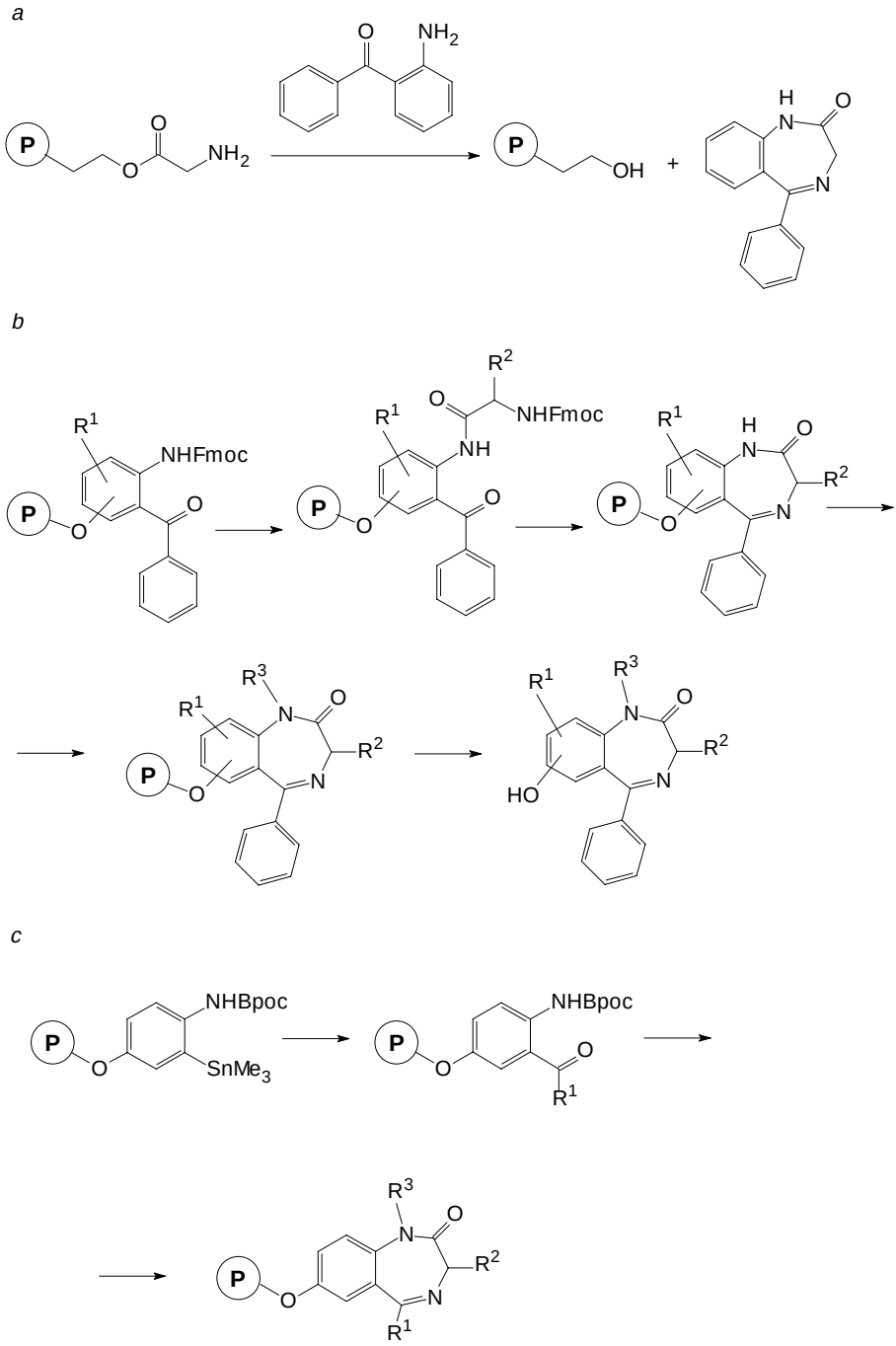
An entirely different approach was used by Bunin and Ellman[199,200]. They did not relate the bioactive structures from natural precursors, their idea is based on the experi-



SCHEME 2


AcSH, PPh3
DIAD, THF
NaBH4
THF, MeOH
HO2C
Br
R
TMG, DMF
1. Et3N, EtOCOCl, THF
2. Bu4NBH4, CH3OH

SCHEME 3


a
P
O
NH2
O
NH2
P
OH
+
H
N
O
N
b
R1
NHFmoc
P
O
O
R2
O
NHFmoc
R1
NH
P
O
O
R1
H
N
O
R2
P
O
N
R3
R1
N
O
R2
P
O
N
R1
R3
N
O
R2
HO
N
c
NHBpoc
P
O
SnMe3
NHBpoc
P
O
O
R1
R3
N
O
R2
P
O
N
R1

SCHEME 4

ence that small synthetic unnatural molecules can affect in biological proccesses instead of biomolecules. The basic structure they chose was 1,4-benzodiazepine[201]. (Interestingly, the first solid phase synthesis of benzodiazepines was described already in 1974 (ref.[202]) – see Scheme 4*a*). Three possible positions in this molecule theoretically suitable for randomization provide a great number of diverse structures (Scheme 4*b*). The first synthetic step is attachment of substituted 2-aminobenzophenone to the acid cleavable linker and subsequently to the support. The second part of the synthesis is carried out on the solid support and consists of (i) coupling of an amino acid, (ii) cyclization into a diazepin ring and (iii) alkylation. The last step of the preparation is cleavage from the support. This scheme is well suited for a simultaneous multiple synthesis. The multipin technology was chosen. A library of 192 structurally diverse 1,4-benzodiazepine derivatives was prepared by means of Geysen's pin apparatus[113] and products of the synthesis were checked by HPLC and MS. The compounds were screened in competitive radioligand binding assay for the CCK A receptor[200]. Later modification of the synthetic scheme addressed the issue of limited availability of 2-aminoaryl ketones – these building blocks were prepared on solid phase using Stille coupling reaction[203] (see Scheme 4*c*).

The preparation of a library of so called "diversomers" (refs[204,205]) is similar. The "diversomers" are in general collections of diverse synthetic compounds. DeWitt et al.[204,205] developed an apparatus and method for the solid phase multiple, simultaneous synthesis of various "diversomers" – benzodiazepines and hydantoins. The device is constructed on the principle of a robotic autosampler. The resin is packed into the glass tubes with a sintered glass filter. The tubes areadjusted in a holder block and the reactions are performed in separate compartments of a supporting block. The synthetic scheme is given in Scheme 5. Forty discrete benzodiazepines and hydantoins were synthesized. The products of syntheses were satisfactorily characterized by TLC, ^{1}H NMR and MS. The benzodiazepines were screened for inhibition of radiolabelled fluoronitrazepam.

3.2. Random Libraries

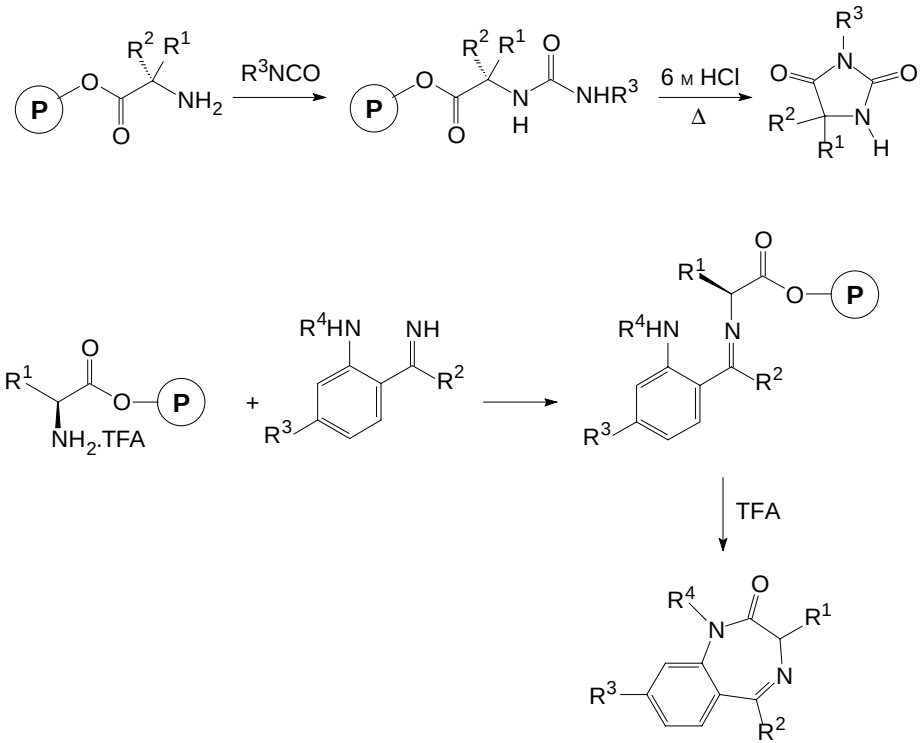
The term library technology means not only the generation of great collections of diverse molecules but also biological screening and evaluation of these compounds. Libraries can be defined by the number of various building blocks used and by a number of synthetic steps carried out during synthesis. The library molecules originate from combinations of these building blocks. The random factor in construction of a large number of diverse molecules is responsible for the complexity and diversity of such a library. Theoretically, a library constructed by combination of N building blocks in n steps (one step maight contain several reactions for incorporation of a building block into molecule) provides N^n various molecules. The probability of existence of all combinations within a library will be discussed in Chapters 3.2.1.2. and 3.2.3.

3.2.1. Peptide Libraries

Peptides, their structure and solid phase synthesis are ideally suited for a library approach. Amino acid building blocks and the amide bond backbone provide simple demands on synthetic arrangement. The first methods for generation of synthetic libraries were based on solid phase peptide synthesis[206–208]. These seminal papers predetermined basic courses of development of peptide library technologies. One course represents the one-bead-one-structure technique[207]. The second uses variously determined and restricted peptide mixtures[208].

3.2.1.1. *One-Bead-One-Structure Libraries*

A method for the synthesis of equimolar multicomponent peptide mixtures was suggested by Furka et al.[206,209]. The multicomponent peptide mixtures were prepared by synthesis on a beaded polymeric support, which was before each coupling step portioned, coupling of different amino acids was carried out in separate vessels and then all the beaded support was mixed. This process (known as portioning-mixing) was repeated several times (according to the desired length of peptides) and finally the pep-



SCHEME 5

tides were cleaved into solution. In this way a mixture of all combinations of chosen amino acids in predefined chains can be prepared. Furka suggested using simple defined peptide mixtures either in biological screening or for study of sequence – HPLC retention time relationships. The authors did not solve the problems relating to the biological screening and isolation and identification of a sequence responsible for an activity.

The concept of a one-bead-one-peptide library was formulated by Lam et al.[207,210]. The method of generating large synthetic peptide libraries is based on Furka's synthetic approach[206] (see Fig. 2). The authors recognized that as a result of the split-and-mix technique each bead should contain only a single peptide species (about 50–200 pmol, according to the loading of the bead). They also developed a rapid technique for screening the library to find beads containing peptides able to bind to a particular acceptor molecule[211]. Acceptor molecules were coupled to a suitable label (e.g. alkaline phosphatase, fluorescein, radiolabel[212], etc.) and added in soluble form to the peptide-bead library. An advantage of such an approach is that beads bearing active sequences can be stained in color-labelled ligand binding assay and are hence visible to the naked eye. A sorting and separation can be carried out using a low-power microscope. The originally described micromanipulator for bead retrieval was later replaced by a technique using micropipettes and pipetting the positive beads from the increasingly diluted slurry of

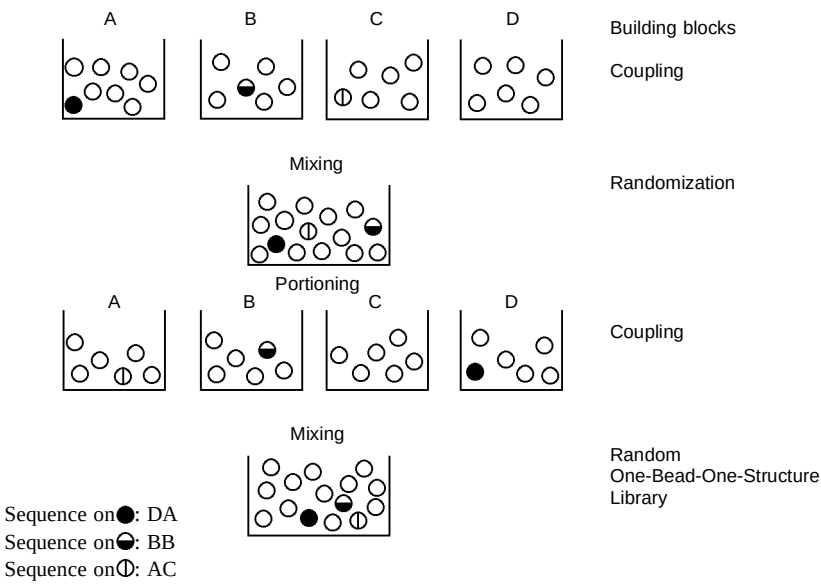


FIG. 2
Methodology for preparation of one-bead-one-structure libraries

beads. Isolated active beads were sequenced by Edman degradation and the structure of the peptide was elucidated. The advantage of this technique over the technique working with mixtures of peptides with iteratively decreased complexity (iterative approach) is that one can obtain several independent lead structures, as well as multiple analogues of the leads. Once a binding motif is identified, a secondary library (based on the motif of the primary screening) can be generated and screened under a more stringent condition to identify leads of higher affinity[213]. The bead-binding screening by one-bead-one-compound technology is described in detail by Lam and Lebl[211]. The bead-binding assay has several attributes: (i) it is extremely rapid, taking only a few hours to screen 10^7–10^8 beads, (ii) the color intensity or fluorescence of the beads is generally proportional to the binding affinity of the ligand, and (iii) the library may be reused several times for different probes. This method does not enable releasing peptides from beads and hence some biological screening methods; e.g., receptor binding assays.

An improved one-bead-one-compound process (Selectide process) was presented[6,214–216]. Multiply cleavable linkers make possible the preparation of an individual peptide sequence in several copies on one bead, so that equimolar portion of a peptide can be released (Scheme 6, ref.[213]). This peptide ester linkage makes possible the screening of a completely random peptide library for activities in solution. The scheme of screening the library of peptides attached to solid support by multiply cleavable linker is illustrated in Fig. 3 (ref.[216]). The random library is distributed into the

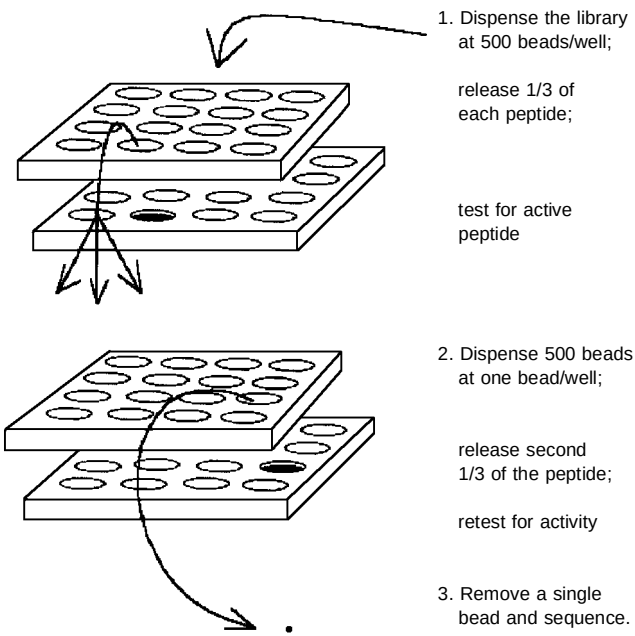
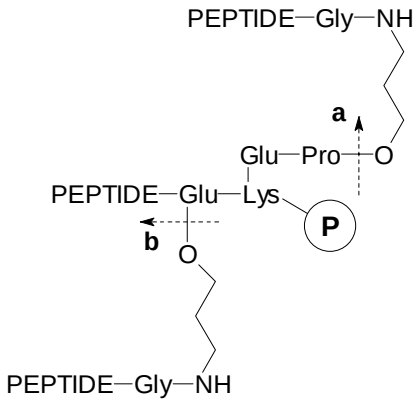


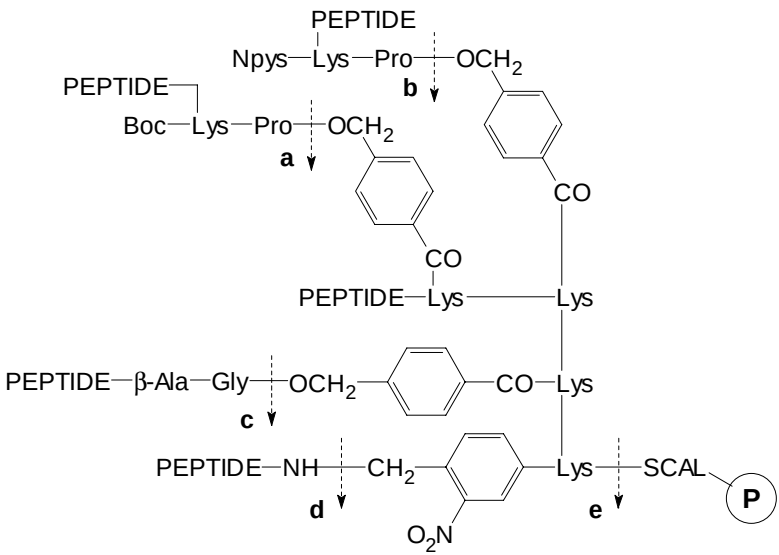
FIG. 3
Scheme of screening library by use of multiply cleavable linker

wells of microtiter filtration plates and the release of the first portion of the compound attached to the solid support is performed under very mild conditions. In this step several hundred to several thousand beads are placed in each well, and therefore a mixture of a substantial number of unrelated compounds is generated in approximately equimolar quan-



a, buffer pH 8.5; **b**, $HO^{\ominus}$, H_2O or NH_3 (g)

SCHEME 6

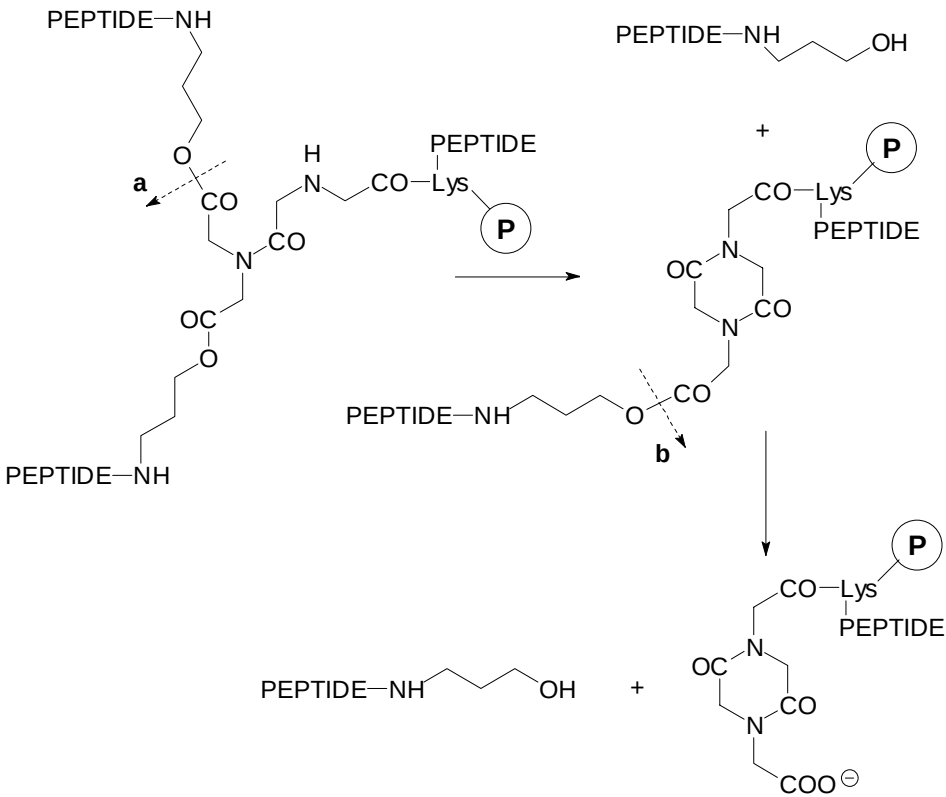


a, TFA; **b**, Ph_3P, TosOH, DMF; **c**, NaOH; **d**, *hν*; **e**, TMSBr, TFA

SCHEME 7

tities. These small aliquots of soluble library are filtered to the testing microtiter plate, and an appropriate biological assay is then performed. Biologically active mixtures are identified and beads from the well responsible for the activity are recovered from the active well of the master filtration plate and redistributed at one to a few beads per well. The second portion of each compound is then released and the biological test is repeated. Finally, the single beads bearing the last active sequence is identified by sequencing.

The structure of the second type of a multiply cleavable linker is given in Scheme 7 (ref.[214]). This linker enables five levels of cleavability. The disadvantage of this multifunctional linker is a different C-terminus of the same peptide sequence cleaved in various ways. The later described doubly cleavable linker (Scheme 8, refs[215,217]) releases the peptide with the same linker residue on the C-terminus. A much simpler



a, pH 8; **b**, pH 13

SCHEME 8

approach was recently described[218] utilizing simultaneous coupling of three different linkers to the solid carrier in the equimolar ratio. Even though this method provides three independent ways of detachment from the solid carrier, it suffers from the fact that in contrast to the more sophisticated linker constructs described above the amount of released peptide is considerably lower.

One-bead-one-compound technology is not limited to linear peptides attached via carboxy terminus. Solid phase methodology makes possible the preparation and testing of all types of cyclized peptides[219–221], or peptides attached via amino terminus or via an amino acid side chain[222–224].

3.2.1.1.1. Applications

The multiple release technique was tested on several model systems. The main model study was performed on a random peptide library from which ligands for anti-β-endorphin were selected. The libraries of peptides of different length both bound to beads and released into solution were screened with anti-β-endorphin monoclonal antibody and consesus sequences were identified[207,210,213,216,225]. D-Amino acid containing ligands of this monoclonal antibody were found by Lam et al.[226]. The ligands recognizing streptavidin and/or avidin were selected from one-bead-one-peptide library too[207,226–228]. The one-bead-one-structure screening was extensively tested on an anti-insulin monoclonal antibody[211,221]. Various motifs identified on the primary screen libraries from tetra- to pentadecapeptides and cyclic peptides were compared and consensus sequences were found.

Releaseable random libraries were used for identification of binding to the gpIIb/IIIa receptor[215,216]. Peptides that compete with fibrinogen for binding to the receptor were found and three sequences were identified as inhibitors of fibrinogen binding to the receptor. Lam et al. demonstrated that through the use of one-bead-one-compound screening process short peptides that interact specifically with a small organic molecule like the indigo carmine can be identified[229]. The Selectide process was applied also for the identification of peptide anchor residues for MHC Class I molecules and for recognition of peptide substrate motifs for cAMP dependent protein kinase, src tyrosine kinase and iodotype-specific peptides for B-cell lymphoma[230]. A pentapeptide library was successfully screened for thrombin ligands and inhibitors[6]. The one-bead-one-peptide technique provided hits in screening for ligands to various targets, for example, factor Xa, HER-2, IL-8, NADPH oxidase, HIV-1 RNase (refs[6,231]). Meldal et al.[232] used the one-bead-one-peptide library for complete subsite mapping of endoprotease specificity. A library of resin-bound potential protease substrates was synthesized and anthranilic acid and 3-nitrotyrosine were used as a donor–acceptor pair for the resonance energy transfer. After treatment with subtilisin Carlsberg, fluorescing beads were collected and sequenced by Edman degradation. The fluorescence is caused by an incomplete quenching due to the cleaved substrate. However, some substrate molecules on a bead

are not accessible for an enzyme and hence the structure of substrate can be determined by sequencing. A statistical distribution of preferred amino acids was obtained for each subsite. The synthesis of library was performed in a manually operated device based on a multi-column system[232-234].

Another approach for characterization of protease function was used by Ator et al.[235]. This technology utilizes random peptide mixtures immobilized on aminopropyl functionalized controlled-pore glass and automatically synthesized in columns. N-Terminus of peptides has a fluorescent label[236]. Protease incubation releases a fluorescence-tagged fragment into the solution. Both released fragments and bound ones are then characterized by Edman sequencing.

Protein kinase substrate specificity was mapped in various ways by Wu et al.[237–239] and Till et al.[240]. The former approach used ^{32}P labelled ATP together with cyclic AMP-dependent protein kinase and a random peptide library. Radiolabelled peptide beads were then isolated for microsequencing and substrates for protein kinase were identified. The latter approach used phosphopeptide-selective mass spectrometry for identification of specific peptide motifs.

3.2.1.1.2. Automation

In consequence with the progress of the one-bead-one-compound technology, a fully automatic synthesizer for preparation of one-bead-one-peptide libraries was designed and constructed[241]. This instrument enables either preparation of a random peptide library from twenty different building blocks or preparation of twenty individual peptides simultaneously. The resin is divided into twenty portions (randomized) by mechanical mixing, blowing nitrogen and subsequent sedimentation. Couplings are carried out separately in individual reaction chambers.

Zuckermann et al. developed an automated peptide synthesizer that is capable of simultaneous synthesis of up to 36 individual peptides and the synthesis of equimolar peptide mixtures based on the split/mix technique[242]. The device consists of an array of reaction vessels and a robotic arm. The resin is portioned by a robotic arm operated syringe using an isopycnic slurry of the beads. The synthesizer was used to synthesize a library of 361 peptides, generated by randomizing two critical binding residues of a 10-mer epitope known to bind an anti-HIV gp120 monoclonal antibody[243]. This group has also identified an anti-gp120 mimotopes from library of 512 component containing unnatural amino acids[244]. They used a combination of recombinant and synthetic peptide library affinity selection methods. A fully automated synthesizer for preparation of peptide libraries was presented by Saneii et al.[245,246]. This commercially available device was used for the synthesis of a library screened for epitope mapping.

3.2.1.2. Tricks and Analytical Techniques for Evaluation of One-Bead-One-Peptide Libraries

The number of structures in peptide libraries synthesized on polymeric beads and screened in one experiment by currently applied techniques ranges from 1 to 10 million. Therefore, one-bead-one-peptide libraries longer than pentapeptides composed of 20 natural amino acids will be incomplete. If it is necessary to test longer peptides, this physical limitation enforces the synthesis of randomly representative yet incomplete libraries. Table I shows relationships between length of a peptide, theoretical number of individual structures and approximate amount of resin (130 μm beads) needed for (hypothetical) synthesis[6].

This tremendous amount of resin theoretically needed for the longer libraries must be decreased by construction of sublibraries with some restrictions. Published structure–activity studies in the field of biologically active peptides have shown that there is generally a limited number (two to four) of "critical" residues in sequence responsible for minimal observable interaction and the rest of the molecule can be replaced by various residues. This obviously may not be true in all cases. This assumption leads to the construction of so-called consecutive one-bead-one-peptide libraries, where only a motif for binding is initially looked for. In following screening of secondary, tertiary and quaternary libraries the most specific sequence is identified (Fig. 4, refs[213,221,222]). Ligands isolated from the primary screen of one-bead-one-peptide libraries often have low to moderate activity. Strategies for optimization use both the consecutive library approach and screening of extension libraries and other analogue libraries[6]. A "homolog library" (ref.[6]) was also used for optimization. In this approach, the residue corre-

TABLE I
Amount of the polymeric support (polystyrene beads 130 μm diameter) needed for the preparation of one copy of the complete library

Length	Number of sequences	Resin amount
3	8 000	8 mg
4	160 000	160 mg
5	3 200 000	3.2 g
6	64 000 000	64 g
7	1 280 000 000	1.28 kg
8	25 600 000 000	25.6 kg
9	512 000 000 000	512 kg
10	10 240 000 000 000	10.2 t

sponding to that in the initially discovered hit is coupled to a fixed percentage (for example 40%) of the solid support, while the remainder of the carrier is randomized. This procedure is repeated in each synthetic step. The screening should confirm the amino acid residue found in the particular position in the primary screening or identify a residue leading to a more avid compound.

The sensitivity of screening of one-bead-one-peptide libraries in solution is limited by concentration of a single peptide in a mixture[215]. The amount of a single peptide is defined by the capacity of a bead. A very important factor is the quality of resin beads and their homogeneity. The concentration of a peptide can also be influenced by varying solubility or affinity to surfaces.

The effect of solid supports on the expected interactions between immobilized ligands and soluble receptors remains unclear. The significant role of a support was observed both in epitope mapping by use of cotton libraries[247] and in screening for substrate specificity of an enzyme using one-bead-one-peptide libraries[232]. In bead-binding screening a support must be resistant to all the organic reagents and solvents used in synthesis and compatible with buffer media in the bioassay. Nonspecific interactions are a potential problem in the screening of bead-bound libraries. This problem is addressed by various blocking agents or multiple rescreening in the presence or absence of a specific ligand as well as by using a dual color substrate system[248] or by using more stringent conditions[6,211].

Since the determination of the structure is critical in the one-bead-one-compound process, a free N-terminus is always required to allow sequencing; however, the C-terminus of peptides in some cases can be essential for biological interactions. A linking moiety for preparation of a library of peptides with a free C-terminus was presented by

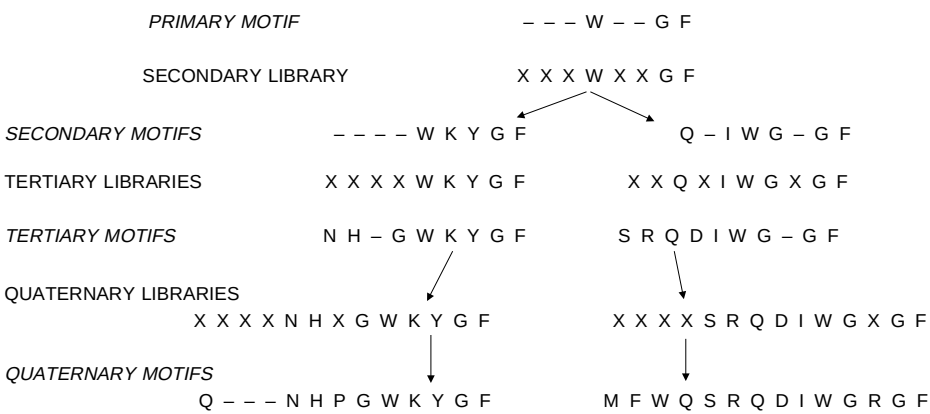


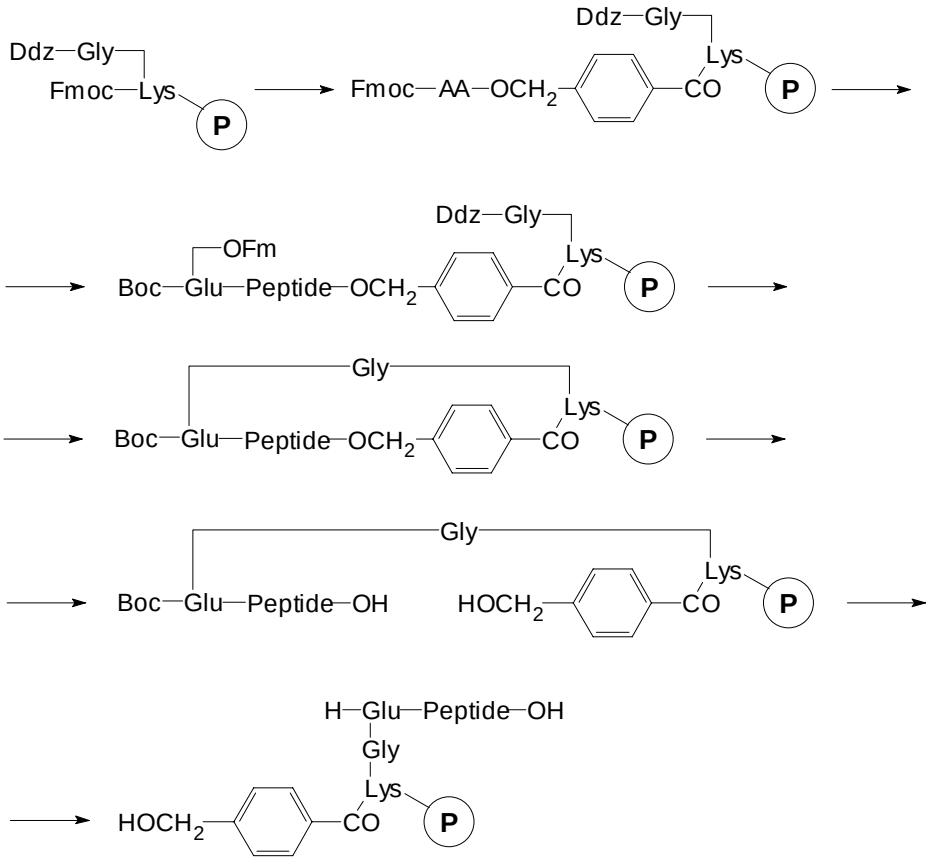
FIG. 4
Construction principle of consecutive libraries

Lebl et al.[222] (Scheme 9) and Holmes and Rybak[223] (Scheme 10). Another strategy for release of C-terminus of immobilized peptides was described by Kania et al.[224].

Some useful tricks were published in the field of bead-based screening. Kassarjian et al. immobilized pentapeptide library beads in thin layer of agarose after incubation with radiolabelled acceptor molecule[212]. Beads that carried specific sequences were identified by autoradiography and subjected to automated gas-phase sequencing. The method was verified in a binding assay with anti-β-endorphin monoclonal antibody.

A screening method based on fluorescence is an alternative for active bead selection. This technique using a cell sorter (FACS) for separation of polymeric beads bound to a fluorescence labelled antibody was developed by two groups[6,249].

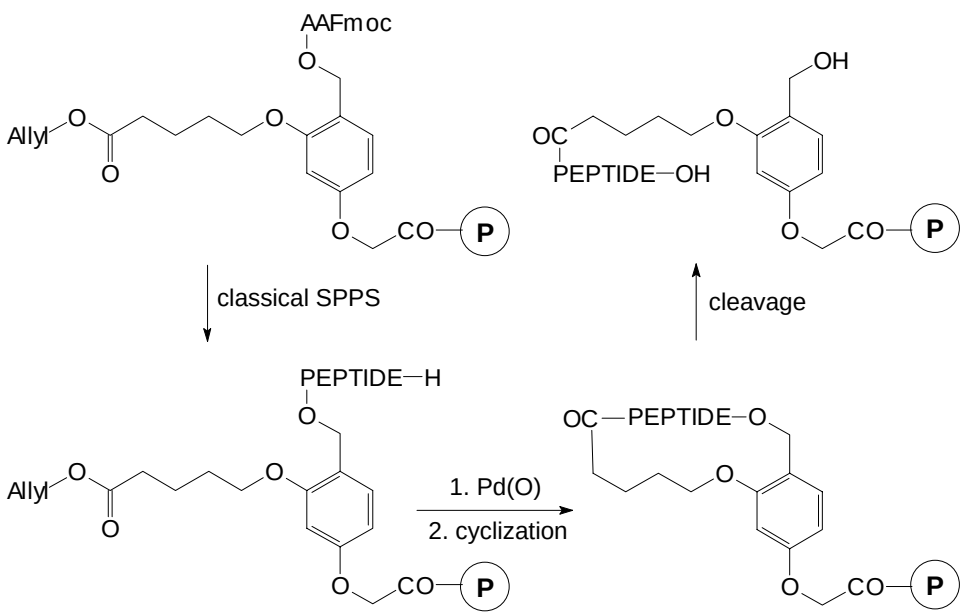
A "multi-use" peptide library was described by Jayawickreme et al.[250]. This type of library was prepared from a one-bead-one-peptide library which was layered on a thin



SCHEME 9

polyethylene sheet fixed between polypropylene rings so that each of the beads is fixed in a defined position. For cleavage of peptides from beads vapors of trifluoroacetic acid (TFA) were used. The resin layer was placed on the agarose bed in which melanophore based G-protein-coupled receptor (GCR) was immobilized. All cleaved peptides diffused on to agarose. The pigment dispersion induced by peptides was indicative of beads containing the active sequences.

Furka's group described several conceptions for library construction and screening[251,252]. They published preparation of partial peptide libraries by so-called binary synthesis which is based on dividing resin before coupling and performing the coupling on only half of resin beads. These libraries contain not only all full length combinations but also all deletion sequences. The number of peptides in a library is substantially increased in this way. This type of library bound to resin was tested for interaction between peptides and cells[251]. The same authors have suggested a so-called "domino strategy" for evaluation of peptide libraries[253–255]. They have designed strategy of "sublibrary kits" so that each of twenty natural amino acids is fixed in a single position of pentapeptide mixtures. In this way one hundred peptide libraries of the first order should be prepared. When two amino acids are fixed in such mixtures the kit of libraries of a second order must comprise four hundred mixtures. The authors supposed that this approach can make easy identification of binding motifs during a biological screening. In addition they introduced pre-colored solid supports which could enable



SCHEME 10

simple sorting of beads according to color. The color is a label for one motif or sequence on a bead[256].

Erb et al.[257] described a "recursive deconvolution" method which can simplify identification and resynthesis of hits identified in biological screening. The method is based on the same principles as typical random divide-couple-recombine (mix/split) approaches with the only difference that in each coupling step part of each pool of a support is stored and labelled. In this way one can finally have all possible pools (catalogue) defined by one building block. By a reversal screening approach, key positions in active sequences can be identified and synthetically optimized. Even though this approach seems attractive and simple, it becomes hopelessly complex when hundreds of libraries with significantly more than twenty building blocks in each randomized positions are used for screening.

3.2.1.2.1. Rapid and/or Multiple Instrumental Analysis

Besides the classical Edman degradation method for characterization of a peptide sequence, more sophisticated and rapid approaches were elaborated. The primary screening of libraries usually gives only preliminary information concerning the importance of some motifs for an observed interaction. The identification of residues with the highest frequence in certain position can be accomplished with advantage by multiple Edman sequencing of positive screened beads[6,211,213,258,259]. Another way for rapid evaluation and determination of short peptide ligands found in library screening is mass spectrometry (MS) sequencing[260]. Multiple peptide sequencing by MS was applied to determination of allele-specific motifs both of a natural peptide mixture from major histocompability complex class I molecules[259,261,262] and synthetic peptide library[259]. Sepetov et al.[263,264] have described the use of hydrogen–deuterium exchange to facilitate sequence analysis by electrospray tandem mass spectrometry which provides an information about the number of labile hydrogens (in –OH, –CONH–, $–NH_2$, $–NHC(NH)NH_2$, –SH and –COOH groups). On-line HPLC-MS (ion-spray) system have been utilized by Metzger et al.[265] for analysis of a synthetic library. The molecular weight of femtomole quantities of small peptides attached to polystyrene beads have been determined with imaging time-of-flight secondary ion MS (ref.[266]). The authors have suggested utilization of this method also to small non-peptidic molecules. Matrix-assisted laser desorption ionization (MALDI) MS was used to quickly read sequence from a series of positively screened peptide-beads[267] or for direct monitoring of SPPS (ref.[268]). The principle of Youngquist's[267,269] and Sepetov's[264] sequencing method is an intentional termination of the synthesis producing all the information needed for the determination of the sequence of the peptide.

Affinity capillary electrophoresis was also used for identification of a peptide ligand from a library that binds most tightly to vancomycin[270]. This method is based on a fact

that complex receptor–peptide (receptor–ligand) has different mobility than inactive (unbound) peptide.

One-bead-one-peptide library approach requires in all cases full determination of positively screened structures. This is a critical point and the biggest disadvantage of random library methods. Other methods (vide infra) utilizing peptide mixtures do not require sophisticated structural elucidation, however they are usually much slower since they demand more synthetic work.

3.2.1.3. Library Approaches Utilizing Peptide Mixtures

Combinatorial libraries in a variety of formats use mixtures of amino acids for incorporating a random factor into peptide chains. These approaches are based on creating a large array of peptides with a conserved sequence motif and randomized remaining parts of the peptide chain. The screening is focused on the identification of the motif responsible for a biological interaction. Even though mixtures of peptides are created, the process is designed in a highly systematic and ordered manner.

Multiple peptide synthesis using a single support as described by Fassina et al.[271] and Tjoeng et al.[272] lies between MPS approaches and library ones. This method utilizes an incorporation of amino acid mixture into one position of peptide chain (magainin 2 and angiotensinogen[272] or oxytocin[271]). The obtained analogues were separated on HPLC or by affinity chromatography and subsequently analyzed. This method enabled preparation of several analogous peptides in one synthesis.

3.2.1.3.1. Iterative Libraries

A true mixture-library approach was developed by Geysen's and Houghten's groups[3,114,208,273]. Houghten's mixture type SPCLs can be divided according to their design and optimization during a biological screeing into three categories:

1. Dual positional synthetic peptide combinatorial libraries (DPSPCLs)

2. Positional scanning synthetic peptide combinatorial libraries (scanning SPCLs)

3. Sizing synthetic peptide combinatorial libraries (sizing SPCLs).

A biological screening of all these libraries uses complex defined peptide mixtures and evaluates an importance of individual motifs characterizing the whole mixture.

DPSPCLs can be generally represented by the formula $O_1O_2XXXX\text{-}NH_2$, where O_1 and O_2 represent positions defining type of a peptide mixture. O_1O_2 equal to all combinations of amino acids used in these two positions. Usually twenty amino acids (natural) are combined. In this way 20^2 individual peptide mixtures defined by two positions O_1O_2 can be created. The remaining positions represented by X are random. Four fully random positions combined from twenty amino acids results in 20^4 peptides in each mixture. Hence $20^2 \times 20^4$ represents the whole number of peptides of this type of library. The individual peptide mixtures were prepared either by a process similar to

one-bead-one-peptide synthesis ("divide-couple-recombine method": equal pools of resin are separately reacted with individual amino acids and then randomly mixed, portioned and separately reacted; the last two positions O_1O_2 are coupled on one separated portion of resin without random mixing)[208] or by using a mixture of amino acids that are incorporated simultaneously during the coupling procedure[274]. The first approach is more laborious and the complexity of a library is limited by the amount of a resin used for synthesis (see Chapter 3.2.1.2.). The latter approach is simple but comprises an inaccuracy in incorporating each amino acid in an equimolar ratio. This problem has been studied by Ostresh et al. regardless of conformational effects which can considerably change competitive coupling rates[274]. The authors have found the relative molar ratios for each amino acid protected derivative necessary for equimolar incorporation when using a 10-fold excess mixture of these derivatives. The optimization of peptide mixture that was identified in biological screening as the most active can be accomplished by an iterative process which is demonstrated in Fig. 5.

Positional scanning[275] is a process based on screening a set of mixtures represented by the formulas: O_1XXXXX, XO_2XXXX, etc., through $XXXXXO_6$. In screening the most active amino acids are searched for each mixture. The selected amino acids defined as the best substituents for each position are then used for synthesis of all possible peptides resulting from their combinations. The total number of peptide mixtures, when twenty natural amino acids are used, is 6×20 and each mixture can contain up to 20^5 individual sequences, so $6 \times 20 \times 20^5$ equals the total number of peptides prepared in one positional scanning library.

Sizing SPCLs[276] were used to determine the optimal peptide length. This type of library is represented by the formulas: OXX, OXXX, etc., through OXXXXXX and OXXXXXXX. When optimal peptide length for individual mixtures (defined by single amino acids) is determined in biological screening the iterative process follows for identification of the most active sequences.

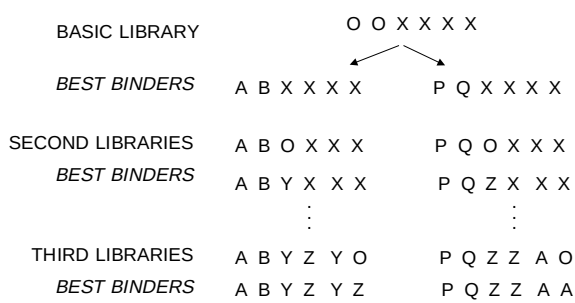


FIG. 5
Library based on iterative process

3.2.1.3.2. Applications

All these soluble peptide combinatorial strategies were evaluated on model systems and can be successfully used in virtually any assay system[3]. A typical O_1O_2XXXX library is usually used in total peptide concentration 1 mg/ml, which yields a theoretical concetration of about 8 ng/ml per peptide. The practical utility of soluble peptide mixtures for epitope mapping was tested by screening the typical Ac-O_1O_2XXXX-NH_2 mixtures by competitve ELISA with a monoclonal antibody against a known epitope (Ac-YPYDVPDYASLRS-NH_2) from influenza virus hemagglutinin and consensus sequences were found by the iterative method[208]. The positional scanning mixtures were screened too[277]. Eichler et al.[247] evaluated scanning SPCLs, using a derivatized cotton carrier for binding to a monoclonal antibody, which had been raised against a longer 36-residue peptide fragment of the hemagglutinin of influenza virus. The cotton libraries were screened either immobilized in a solid phase assay, or in solution. The specificity of the antibody for cotton-bound immobilized peptide mixtures was found to be lower than that found in solution.

The set of mixtures (O_1O_2XXXX) was screened for identification of inhibitors of binding to an antigen peptide[278]; e.g., oncogen-related protein from v-fes and its monoclonal antibody[3,279]. In the same manner an epitope for the surface antigen of hepatitis B virus was identified[280].

The same format of library (O_1O_2XXXX) was screened for identification of the antigenic determinant of anti-β-endorphin monoclonal antibody (3E7, ref.[281]) by competitive ELISA. In the first screening the motif YGXXXX and YAXXXX was found. Finally, the sequence YGGFMT was identified by the iterative process. That is in accord with the sequence of Met-Enkephalin, which is the natural ligand for the opioid receptor. Detailed specificity and dependency on the presence of calcium for binding to a monoclonal antibody against the FLAG sequence revealed potent calcium-independent antigens[282].

Opioid receptor ligands in radio-receptor assays were identified by the above mentioned peptide mixture both using L- (ref.[283]) and D-amino acid libraries[284] as well as positional scanning libraries[275]. Opioid receptor antagonists were determined using the dual positional SPCLs[285]. Both the dual positional[286] and positional scanning[287] SPCLs were applied for identification of inhibitors of the cytolytic peptide mellitin.

Enzyme inhibitors were developed by SPCLs synthesized on cotton carriers[185], cleaved in situ, and then screened[247,288,289]. The hexapeptide cotton library was designed both as dual positional and scanning SPCLs. The random positions were coupled by use of mixtures of amino acids. The synthetic approach was modified so that the deprotected peptides remained attached to the cotton carrier until they were released into solution directly prior to being assayed. The libraries were screened for inhibition of tryptic hydrolysis of N^{α}-benzoyl-D,L-arginine-*p*-nitroanilide. The most in-

hibiting mixtures were found and through an iterative process of synthesis and screening all positions of the sequences were successfully defined.

The SPCLs were screened for new antimicrobial peptides active against *Staphylococcus aureus* and *Streptococcus sanguis* (gram-positive bacteria), *Escherichia coli* and *Pseudomonas aeruginosa* (gram-negative bacteria), and the yeast *Candida albicans* in microdilution assays[3,208].

All types of Houghten's SPCLs comprise one particular feature – their construction is designed with several determining factors as: length, fixed positions, amino acid building blocks, acetylation, etc. All these factors should be kept in mind in the process of evaluation, because they can simplify the identification of single active sequence as well as contribute to discovery of some general principles of an observed interaction. The broad utility of soluble peptide libraries for drug discovery was in general discussed by Houghten[3,290–292].

3.2.1.3.3. Other Approaches Using Peptide Mixtures

Variously formated peptide mixture libraries were used in diverse mapping and screening of biological targets. Soluble mixtures prepared by "divide-couple-recombine method" were screened for optimization of an amino acid in a known sequence responsible for more effective inhibition of platelet adhesion to fibronectin[293]. The iterative approach was used for screening of similarly constructed libraries of tri- and tetrapeptides for identification of HIV protease inhibitors[294].

Pin technology, described in Chapter 3.1.1., was successfully adapted to the library approach and was probably the first application of the library philosophy in screening[114,273]. As much as 400 peptide mixtures (XXO_1O_2XX format, where O_1O_2 are defined positions and X are random) were synthesized on pins and tested with a monoclonal antibody/receptor. Remaining positions were determined iteratively. The importance of each amino acid in each position of the identified hexapeptide sequence was tested by synthesizing all nineteen analogous sequences for each position.

A qualitatively novel approach for construction and screening of peptide libraries was developed on the basis of continuous cellulose membrane supports[295,296]. Authors have used a spot synthesis technique to prepare 20×20 spot-libraries which are defined by their position on a planar support. Each position determines a dipeptide motif of a sublibrary. Libraries of hexapeptides – XXO_1O_2XX – were prepared by spotting on a membrane. Random positions were incorporated using double coupling of 0.8 equivalents of equimolar mixture of amino acids thus compensating for different coupling rates of different amino acids. All the random positions were iteratively identified. The method was verified on beaded support in an antibody binding assay[295]. The cellulose membrane immobilized libraries were tested for the identification of transforming growth factor-α epitope, for binding to a double-stranded DNA as well as for binding of some metals as silver, nickel, technetium. The used libraries were designed as linear all L- or

all D-peptides and cyclized either via a disulfide bridge between two cysteine residues or an amide bond between the α-amino group of the N-terminus and the γ-carboxyl group of a C-terminal glutamic acid[296]. The same authors designed epitope libraries of known HIV-1 p24 epitope, recognized by a monoclonal antibody[297]. The libraries were constructed by replacement of each position by 19 L-amino acids both in soluble form (synthesized on a beaded support and cleaved as a mixture) and immobilized on a cellulose support (synthesized simultaneously on cellulose sheets in spots). The soluble mixture was characterized and the results of screening compared. This laboratory also evaluated a method for screening of peptide–antibody interactions which uses so-called amino acid "clusters" (defined mixtures of variously grouped amino acids)[298]. The continuous cellulose membrane sheet was used again. Each spot was defined by the type of cluster and its position in a peptide sequence. This type of library enables the determination of important motifs or a group of amino acids.

The similar variation of the "spot" technique was used by Frank (Chapter 3.1.1.) for synthesis of a tetrapeptide sublibrary in which each spot contained a mixture of peptides with a certain conserved motif[151]. The peptide synthesizer GILSON 222 was modified to automatize spotting on membrane supports[299]. The synthesized cellulose bound peptide libraries were tested against monoclonal antibodies[299] and for determination of the specificities of cAMP- as well as cGMP- dependent protein kinases[300].

In order to increase the efficiency of biological screening of random mixture peptide libraries, Wallace et al.[301] elaborated a multimeric synthetic peptide combinatorial library method based on the concept of branched multiple antigen peptides by Tam et al.[302] and Houghten's mixture libraries.

Libraries constructed on the basis of sophistically constructed mixtures of amino acids have been published by Blake and Litzi-Davis[303]. The mixtures of amino acids are coupled in order to prepare peptide mixtures in which some amino acids are omitted and others are present in two molar excess. In screening of these libraries activities of defined mixtures are compared and importance of specific amino acids (omitted or redundant) in specific positions is deduced. Zhang et al.[304] synthesized a peptide library of partly random heterodimers randomly linked via sulfide bridges to screen the ability of these derivatives to inhibit angiotensin II binding.

The progress of the past several years in the field of peptide libraries involves various approaches usually using the above mentioned methodologies for synthesis and screening. These variously modified library approaches were used for an antibody binding assay[305–307], substrate specificity mapping[308,309] as well as a receptor binding assay[310].

3.2.1.4. Libraries of Libraries

The concept of a library of libraries[6,311] combines both the one-bead-one-peptide philosophy and the mixture approach. In this type of a library, each individual bead contains single sequence motif in a number of different peptides. The method is based on

the assumption that the individual sequence identified in the peptide library with incomplete representation of all permutations is less valuable than information describing the motif responsible for binding or other biological activity. Figure 6 shows the synthetic scheme for preparing a hexapeptide library of libraries. Random couplings were performed by split synthesis on that portion of the resin equivalent to the number of remaining randomizations divided by the number of remaining synthetic steps. A mixture of amino acids was coupled to the portion of the resin that is equivalent to the number of remaining mixture couplings divided by the number of remaining synthetic steps. Table II illustrates the advantages of the library of libraries approach in the amount of resin needed for the synthesis of a complete library[311].

The advantages of such an approach are obvious: (i) library can contain longer peptide sequences, (ii) the amount of resin does not increase over common synthetic practice, (iii) and not only complexity but also diversity is comparable to one-bead-one-peptide libraries. The library was screened against the anti-β-endorphin monoclonal antibody, streptavidin and thrombin. The results were closely similar to the known peptide ligands of these receptors, however, the number of positive beads with the same motif was several times higher then for a one-bead-one-peptide library.

3.2.2. Libraries of Non-Peptidic Structures

The necessity to modify peptide lead structures in order to obtain more stable and efficient molecules in medicinally important applications stimulated the development

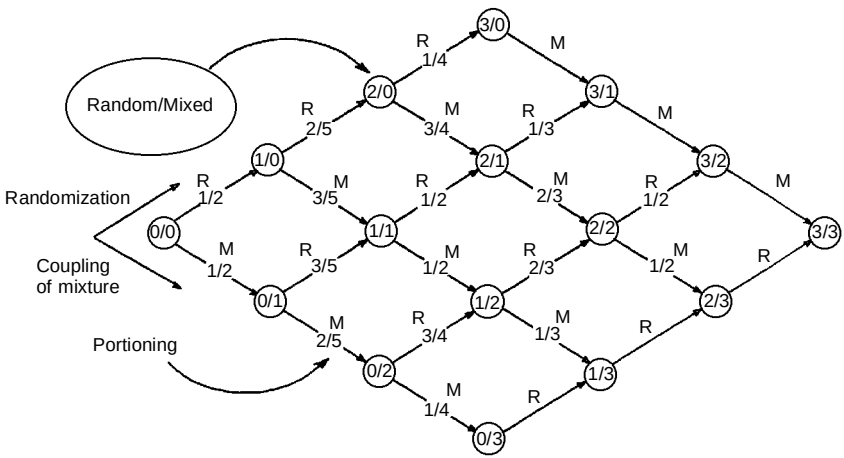


FIG. 6
Synthetic scheme for library of libraries. Example: hexapeptide library with three fixed positions; similar scheme can be applied for library of any length

of non-peptidic libraries. Synthetic random collections of oligonucleotides belong to the category of non-peptidic library too. However, oligonucleotides have the similar disadvantages as peptide libraries and will not be discussed here in detail (see Chapter 2.3., refs[312–314]). Random oligonucleotide libraries containing unnatural building blocks may be mentioned here, for example the library by Latham et al.[315]. This collection of oligonucleotides was prepared with 5-(1-pentynyl)-2′-deoxyuridine in place of thymidine and was tested against human thrombin.

Most pharmaceutically successful compounds are based on structures other than peptides or peptide-like compounds. A library approach can be an important tool for drug research using a great number of randomly prepared compounds based on diverse chemical structures. The demand to prepare a lot of non-sequenceable structures by a library methodology for drug development screening has enforced new methods for characterization and identification.

3.2.2.1. Encoded Libraries

There are three main reasons for the use of coding techniques in the one-bead-one-structure library approach: (i) to enable the structure identification of nonsequenceable

TABLE II
Comparison of the solid carrier amount for the construction of one-bead-one-peptide library and library of libraries

One-bead-one-peptide library			Library of libraries	
Length	Number of individual sequences	Resin amount	Number of motifs	Resin amount
3	8 000	8 mg	8 000	8 mg
4	160 000	160 mg	2 000	32 mg
5	3 200 000	3.2 g	80 000	80 mg
6	64 000 000	64 g	160 000	160 mg
7	1 280 000 000	1.28 kg	280 000	280 mg
8	25 600 000 000	25.6 kg	448 000	448 mg
9	512 000 000 000	512 kg	672 000	672 mg
10	10 240 000 000 000	10.2 t	960 000	960 mg
.	.		.	.
.	.		.	.
15	$32\,770 \cdot 10^{15}$		3 640 000	3.64 g

randomly prepared compounds, (ii) to improve sensitivity of screening, and (iii) to increase the speed and throughput of compound identification[6].

DNA coding, according to the biological pattern, was introduced to solid phase peptide synthesis by Brenner and Lerner[316]. They described synthesis of a peptide in parallel with a coding oligonucleotide on the same support. This technique was developed in parallel in several laboratories[249,317]. A weak point of this coding method is noncompatibility of oligonucleotide synthesis with most of commonly used synthetic methods. The advantage of using nucleic acid coding is the ability to amplify the code by PCR.

Recently, coding by halogenated derivatives of carboxylic acids followed by gas chromatographic determination of cleaved and silylated mixtures of tag molecules was published[318–320]. The "cosynthetic method" uses an electrophoric tag to encode the series of steps and/or reagents used in the synthesis of a library. In this approach the tag molecules are not linked to each other sequentially and this enables the use of a binary code for identification of library compounds.

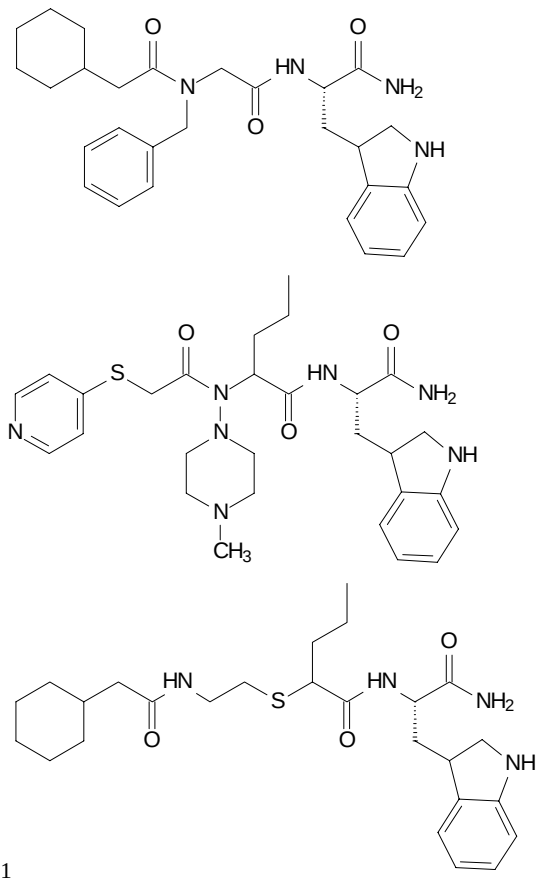
Another type of coding is based on mass spectrometry[264,267]. These methods cap a part of intermediates in each synthetic step by a selective tag. The mixture obtained by cleavage from one positively screened bead is characterized by mass spectrum and by mapping of history of the synthesis the resulting active structure can be deduced. The protein "ladder" sequencing by Chait et al.[321] is carried out in similar manner – in this case the sequence information is generated by partial degradation instead of building a "history ladder".

Two laboratories introduced encoding of nonsequenceable polymeric structures by a peptide sequence[322,323]. Coding peptide is synthesized in parallel to the screening structure using an orthogonal linkage of both chains as well as protecting groups, and different chemistries. Finally, classical Edman degradation of the coding structure is applied for structural analysis. Coding can be carried out in variety of formats. To increase the number of amino acids available for coding, coding utilizing side chain acylation of various diaminocarboxylic acids was introduced[6].

These coding strategies involve the risk that the biological target may specifically interact with the coding sequence. This weakness was overcome by distinguishing the "surface" and "interior" of the polymeric bead[6,324]. The technique is based on orthogonal synthesis of one sequence on the surface of the bead, and a different sequence inside of the bead. Peptides on the surface are well accessible to any receptor. However, the interior of the bead is not accessible to a macromolecular receptors. Providing that one by enzymatic "shaving", or otherwise, specifically discloses the bead surface for synthesis of screened molecules, and interior of the bead is used for building of a coding sequence by orthogonal synthetic scheme, the outside structure can be screened and after identification of positive bead the peptide code inside can be sequenced.

3.2.2.2. Syntheses of Non-Peptidic Libraries and Tools for Their Preparation

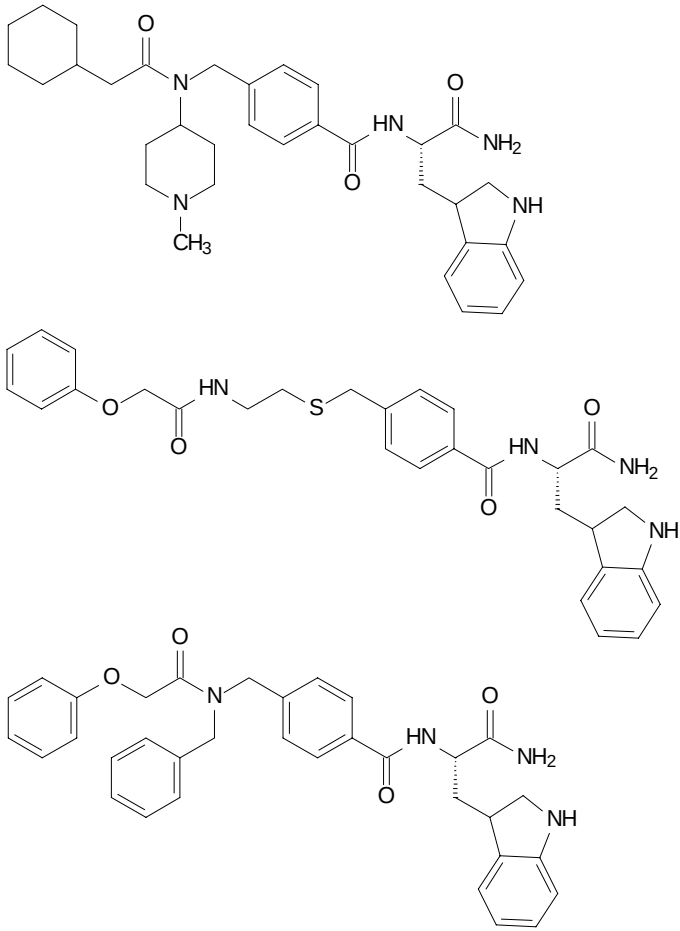
The potential of variously constructed organic libraries combined from a variety of building blocks is enormous. As an example can serve a simple organic library constructed from commercially available amines and carboxylic acids linked via well-developed amide bond formation on a solid support[222,325]. The same authors have used alkylation and acylation reactions for creation of model libraries[323] as well as for libraries screened for pharmaceutically relevant targets (Scheme 11). Unnatural amino acids, aldehydes and carboxylic acids were used for construction of the library by use of reductive alkylation of the primary amino group by various aldehydes and acylation of the resulting secondary amino groups with diverse carboxylic acids[326,327]. A library was screened against streptavidin and the structure of active compounds was determined by mass spectroscopy[326]. A library based on various iminodiacetic acid derivatives was described by Safar et al.[328]. Amide bond formation was the basis for several



SCHEME 11

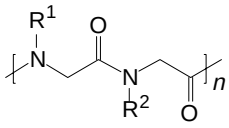
library designs resembling peptide linear and/or branched structure[222,329–331]. However, not only amide bond can form libraries based on repetition of structural units. Examples of the mentioned library types are given in Scheme 12.

The random libraries of *N*-(substituted)glycine dimers and trimers – "peptoids", have been introduced by Zuckermann et al.[335]. The synthesis of these peptidomimetic libraries is performed either by coupling *N*-substituted glycine subunits or by the so called "submonomer" technique in which carboxyl group of bromoacetic acid is coupled to the amino group of preceding amino acid and bromine atom is substituted by an amino group of variety of amines[243,335,340] (Scheme 13). Synthesis of peptoids was adapted to an automatic robotic device[243]. The "peptoid" libraries were screened in

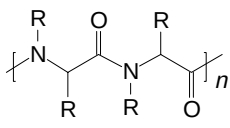


SCHEME 11
(*Continued*)

solution-phase, competitive radioligand-binding assays. Nanomolar ligands for the α1-adrenergic and μ-opiate receptors were identified[341]. Peptoid structures were found to be stable in the presence of proteolytic enzymes[342]. This feature provides a substantial advantage over peptides as potential drug candidates. Modification of "peptoid" side chains by cycloaddition of nitrile oxides with alkenes and alkynes on a solid support



ref.[335]



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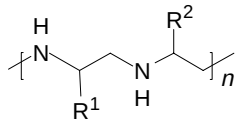

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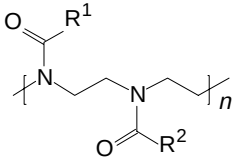
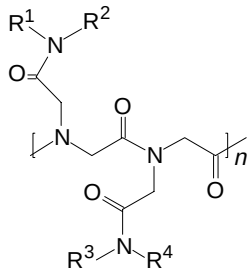


ref.[6]



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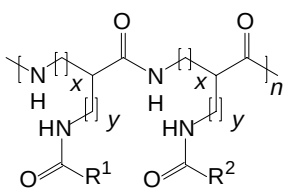

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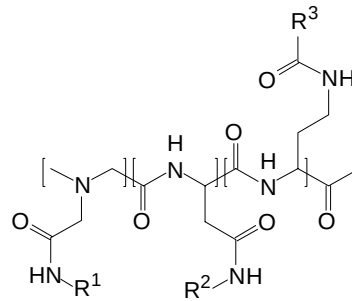

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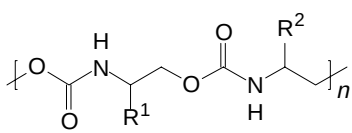

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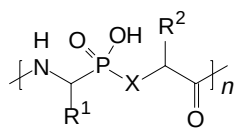
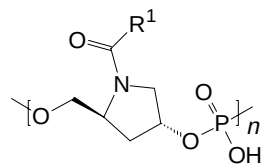


X = NH, O

refs[336,339]



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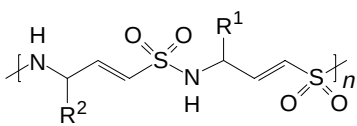

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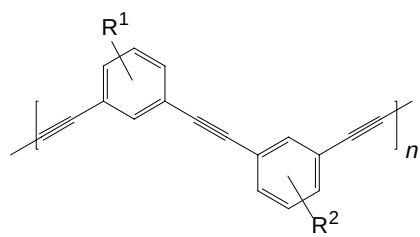


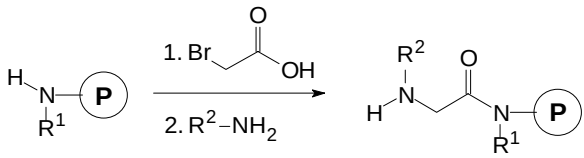
ref.[334]

SCHEME 12

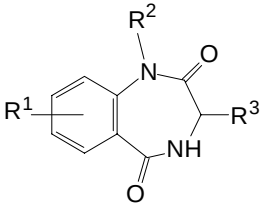
has been described[343]. (Interestingly, cycloaddition of nitrileoxides on solid phase was described[344] already in 1980.) MALDI time-of-flight MS technique was used for determination of structure of peptoids covalently bound to a solid support[345].

A more "organic" library has been synthesized by Chen et al.[346], who used Wittig reaction followed by Michael addition on a solid support. Many well-known reactions of organic chemistry have been successfully accomplished on solid support in order to develop higher molecular diversity for the library technology. It is quite surprising that the significant developments in the solid phase organic chemistry achieved in the past are sometimes overlooked by the researchers excited by the potential of this technique (for reviews see e.g.[347–350]). Let us mention here only the newest additions to the solid phase reaction repertoire. Application of Stille reaction to form carbon–carbon bond[203,351–353] was shown to be perspective for various library constructions. Another way for forming carbon–carbon bonds on a solid support was suggested by Frenette and Friesen[354] as well as by Backes and Ellman[355]. This reaction is based on Suzuki cross-coupling of aryl halides bound to a Merrifield resin with boronic acid derivatives. Both Heck[356,357] and Mitsunobu[358] reactions applied by Krchnak et al.[359,360] to synthesize random molecule collections, belong to noncomplicated approaches useful for creating of variety of compounds. The imagination of organic chemists is limitless – solid phase synthesis of phenylacetylene oligomers[334], oligosaccharides[361], benzodiazepine-2,5-diones[362], isoquinolinones[363], and 3,5-disubstituted-γ-butyrolactones[364] as well as total solid phase synthesis of diverse non-peptidic inhibitors of aspartic proteases[365,366] and various antioxidants[367] – illustrate well this very progressive field of drug discovery. Interesting approach to the generation of molecular diversity is an application of Ugi's four component condensation[368,369]. Structures of libraries resulting from applications of the mentioned reactions are given in Schemes 14–17.

The construction of libraries on a variety of "scaffolds" (ref.[388]) which serve to link diverse functionality belongs among perspective approaches for diversity generation (Schemes 18,19)[6,328,330,331,383,387,389–391]. The scaffolds provide a certain spatial and conformational rigidity and can be rationally designed for a defined biological target. Boyce et al.[392] described a peptidosteroidal random library based on a steroidal scaffold. Patek et al.[383,393] described all-*cis* cyclopentane scaffolding and scaffolding based on a thiazolidine structure. Library based on Kemp's tricarboxylic acid provided potent



SCHEME 13



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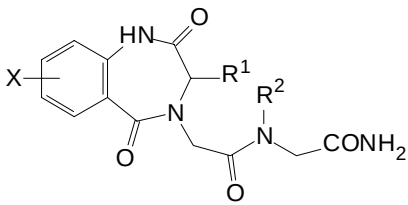

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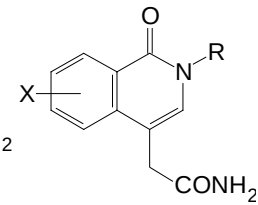

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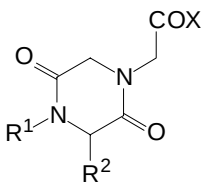

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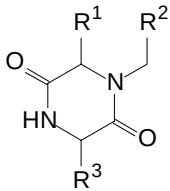

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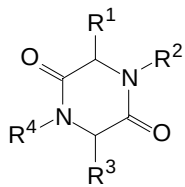

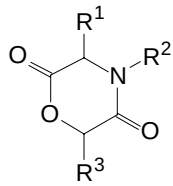

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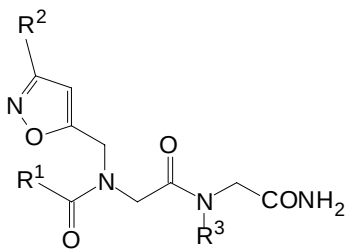

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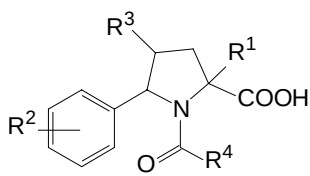

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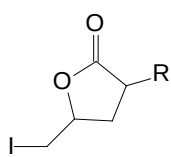

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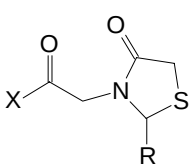

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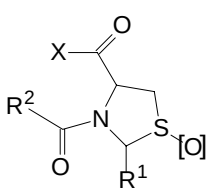

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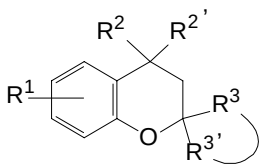


ref.[382]

SCHEME 14

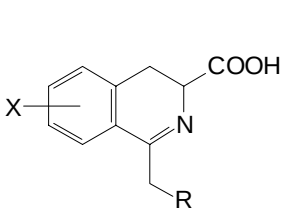
inhibitors of thrombin[6,394]. Carrel et al.[395,396] presented synthesis and screening of libraries prepared on either cubane or xanthene scaffold.

All above mentioned syntheses require either coding techniques or modified analytical methods for determination of structure of solid phase supported synthetic molecules (see Chapter 3.2.1.2.). In addition, magic-angle spinning ^{1}H as well as the solid phase ^{13}C NMR technique were applied[380,397,398] for characterization of bound molecules. FT IR techniques were found indispensable for the analysis of functional group transformations on the solid phase[334,352,399,400].

An iterative approach for identification of active structures from peptide libraries of mixtures (see Chapter 3.2.1.3.), was applied to evaluation of collection of non-peptidic structures. "Libraries from libraries" were created from Houghten's iterative peptide libraries by peralkylation of protected peptides[332,333,401,402]. These *N*-alkylated peptidomimetic polymers were assayed for their ability to inhibit the growth of *Staphylococcus aureus* and *Staphylococcus sanguis*.

Cyclic peptide template libraries based on the cyclic "scaffold" consist of lysines the side chains of which are acylated by variety of carboxylic acids were prepared and assayed[403,404]. Chymotrypsin inhibitors were identified through an iterative approach of the screening.

Oligonucleotide analogue libraries composed of uniform sugar (2′-*O*-methyl) and phosphate modifications with the common nucleotide bases were synthesized and



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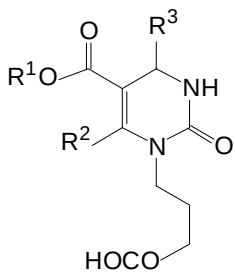

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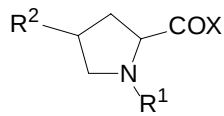

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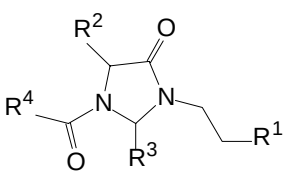

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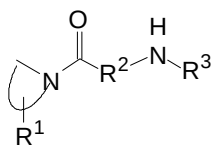

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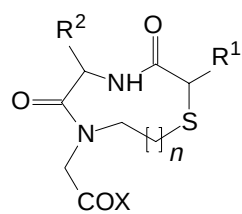

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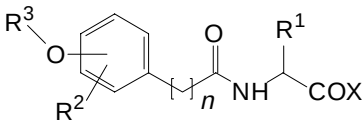
SCHEME 15



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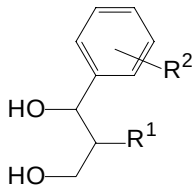

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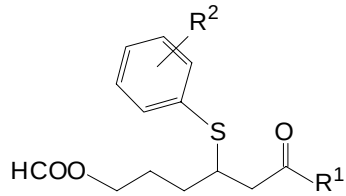

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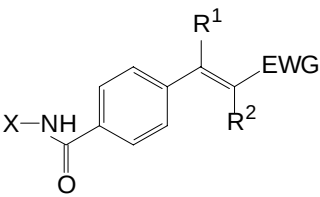

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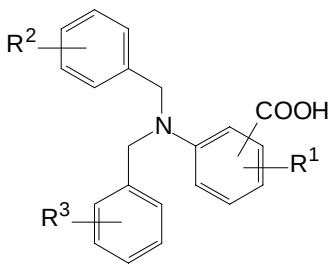

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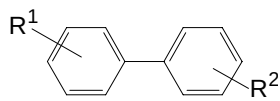
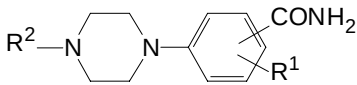


refs[351,353, 354]



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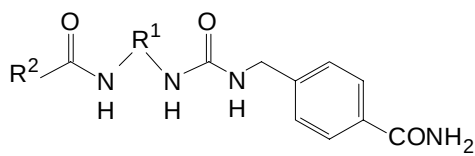

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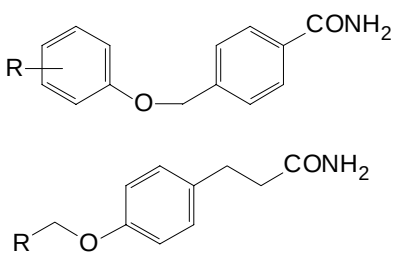

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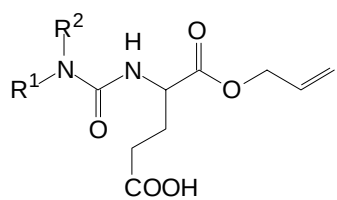

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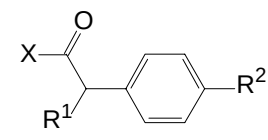
SCHEME 16

screened for inhibition of human herpes simplex virus 1 and binding to the RNA hairpin[405]. These libraries were optimized by the "SURF" (synthetic unrandomization of randomized fragments) method, which is based on an iterative approach[406,407].

3.2.3. Theoretical Analyses of Random Libraries

Very well elaborated random peptide library approaches give well-defined conditions for theoretical analyses and rational design of noncomplete random libraries. It was discussed in Chapter 3.2.1.2. that most commonly only the motif of two to four "critical" amino acid residues in a peptide or protein is responsible for interaction with the bio-



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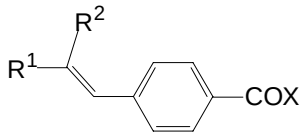
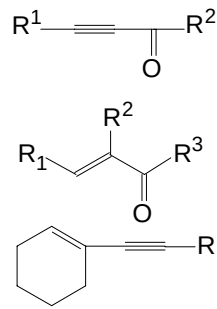


refs[353,356]



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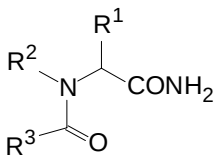
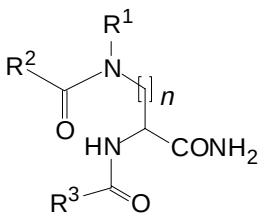


refs[326,379]



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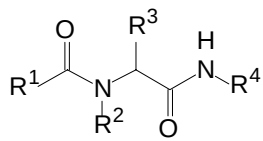

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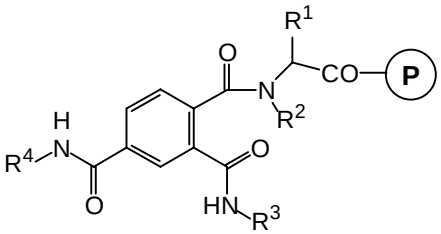
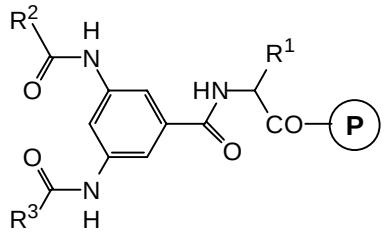
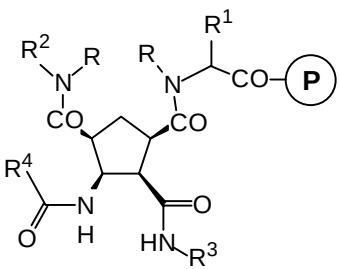
SCHEME 17







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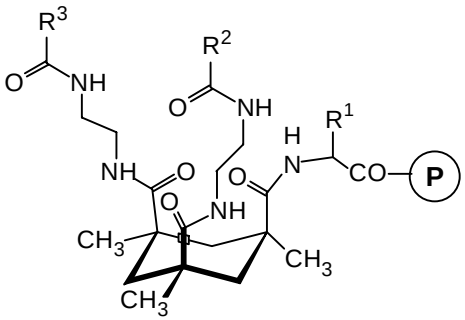

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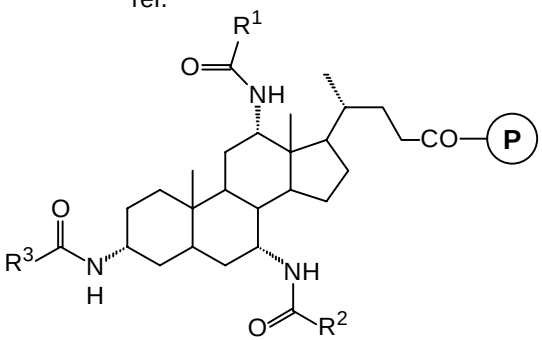
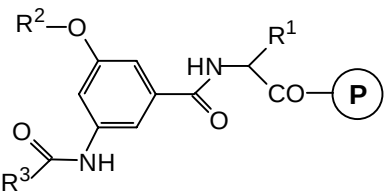
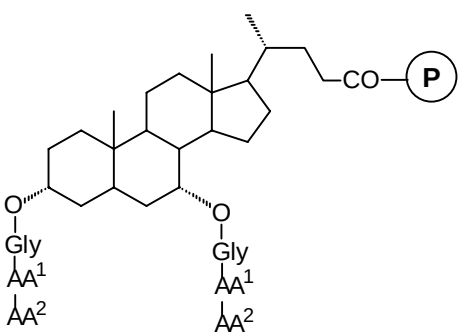




ref.[391]



ref.[392]

SCHEME 18

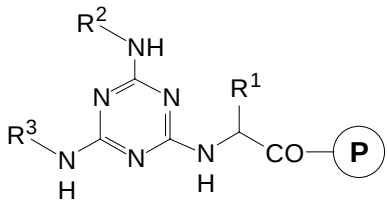
logical target. For any library, the frequency at which a critical sequence motif occurs can be used to calculate a number of critical residues within this motif[222] (Eq. (*1*)).

$$n(\text{crit}) = \frac{\log(sample)}{\log(AA)} - \frac{\log(hits)}{\log(AA)} \quad , \qquad (1)$$

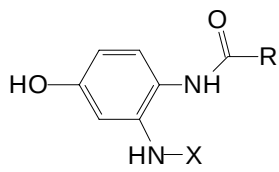
where n(crit) is the number of "critical" residues, *sample* is the number of screened beads from a given library, *hits* is the number of positively identified beads, and *AA* is the number of amino acids used for randomization. As can be seen, the results of this calculation do not depend on the length of the library. On the other hand, the number of expected positive beads can be calculated[6] according to the (Eq. (*2*)).

$$n = (x)(P_f)[S/(A_n)^{n(\text{crit})}] \qquad (2)$$

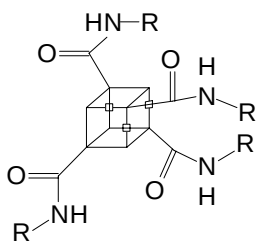
In this equation n is the number of expected positive hits, x is the number of different binding motifs, P_f is the "placement" factor (number of possible placements of each motif in the peptidic chain), S is the number of beads screened, A_n is number of subunits used for randomization, and n(crit) is the number of "critical" residues. In other

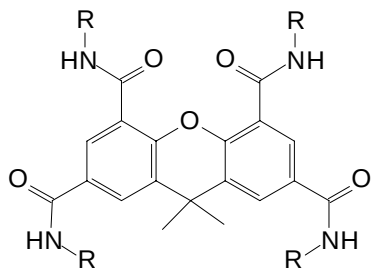


refs[6,330]



ref.[390]





refs[395,396]

SCHEME 19

words the number of positive hits depends on the number of "critical pharmacophores" both peptidic and non-peptidic, as well as on the number of beads tested, but it does not depend on the length of the library. Therefore, screening of even a very incomplete library can provide a reasonable number of positive beads. These formulas were experimentally proven[6] and became a basis for the design of incomplete peptide libraries as well as of the library of libraries[6,311].

The mathematical tools for discussing the probability of binding, catalytic properties as well as synergism of some interacting molecules in a random pool, detection limits etc. were introduced by Kauffman[408–410]. A model system based on oligonucleotide hybridization that addresses these questions using computer simulations has been described by Freier et al.[407]. They have found that iterative deconvolution methods generally find in a large random library either the best binder or one with activity very close to the best, despite experimental and synthetic errors. The presence of many binding molecules in a random pool influenced the profile of subset activities, but did not preclude selection of molecule with near optimal activity. A FORTRAN program simulating typical random library preparation was written by Burgess et al.[411].

Rational approach utilizing knowledge of lipophilicity, shape, branching, chemical functionality and specific binding features was used for computational design of complex libraries[412]. Using structural descriptors and statistical techniques, monomers can be chosen to maximize a library's diversity or to bias a library toward certain features while keeping other features dissimilar.

A variety of theoretical tools prove the rationality of library approaches as well as enabling rational design of great collections of synthetic compounds. They may also show the limitations of such approaches.

4. CONCLUSION

All the above referred tools, both biological and synthetic, for generation of molecular diversity have a great impact on drug discovery methodologies. At present, the majority of these approaches have found commercial application. The number of presented and published works in this field has tremendously increased during the past few years (for the dynamic database of published articles see http://vesta.pd.com). The speed and efficiency of the multiple synthesis and library techniques in the generation of new leads and in the evaluation of structure–activity relationships was most dramatically documented by the successes in the chemistry and biology of peptides. However, due to the outstanding effort dedicated to the development of nonpeptidic molecular diversity, we will not have to wait long for the flood of new leads with nonpeptidic structures.

LIST OF ABBREVIATIONS

Boc	*tert*-butoxycarbonyl
BOP	benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate
Bzl	benzyl
CCK	cholecystokinin
Ddz	2-(3,5-dimethyloxyphenyl)propyloxycarbonyl
DIAD	diisopropyl azodicarboxylate
DKP	diketopiperazine
DMF	dimethylformamide
DPSPCL	dual positional synthetic peptide combinatorial library
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
Fm	fluorenylmethyl
Fmoc	fluorenyl-9-methoxycarbonyl
FT IR	Fourier-transform infrared
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
MALDIMS	matrix assisted laser desorption/ionization mass spectrometry
Menpoc	α-methyl-6-nitropiperonyloxycarbonyl
MHC	major histocompatibility complex
MPS	multiple peptide synthesis
MS	mass spectrometry
NMR	nuclear magnetic resonance
Npys	3-nitro-2-pyridinesulfenyl
Nvoc	6-nitroveratryloxycarbonyl
PCR	polymerase chain reaction
SCAL	safety-catch amide linker (derived from 2-alkoxy-4,4′-bis(methylthio)benzhydrylamine)
SMPS	simultaneous multiple peptide synthesis
SPCL	synthetic peptide combinatorial library
SPPS	solid phase peptide synthesis
TBTU	*O*-benzotriazoyl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate
t-Bu	*tert*-butyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMG	tetramethylguanidine

REFERENCES

1. Madden D., Krchnak V., Lebl M.: Perspect. Drug Disc. Design *2*, 269 (1995).
2. Terrett N. K., Gardner M., Gordon D. W., Kobylecki R. J., Steele J.: Tetrahedron *51*, 8135 (1995).
3. Pinilla C., Appel J., Blondelle S. E., Dooley C. T., Dörner B., Eichler J., Ostresh J., Houghten R. A.: Biopolymers (Peptide Sci.) *37*, 221 (1995).
4. Janda K. D.: Proc. Natl. Acad. Sci. U.S.A. *91*, 10779 (1994).
5. Desai M. C., Zuckermann R. N., Moos W. H.: Drug Dev. Res. *33*, 174 (1994).
6. Lebl M., Krchnak V., Sepetov N. F., Seligmann B., Strop P., Felder S., Lam K. S.: Biopolymers (Peptide Sci.) *37*, 177 (1995).

7. Gordon E. M., Barrett R. W., Dower W. J., Fodor S. P. A., Gallop M. A.: J. Med. Chem. *37*, 1385 (1994).
8. Gallop M. A., Barret R. W., Dower W. J., Fodor S. P. A., Gordon E. M.: J. Med. Chem. *37*, 1233 (1994).
9. Eichler J., Houghten R. A.: Mol. Med. Today *1*, 174 (1995).
10. Scott J. K., Craig L.: Curr. Opin. Biotechnol. *5*, 40 (1994).
11. Schatz P. J.: Curr. Opin. Biotechnol. *5*, 487 (1994).
12. Plückthun A., Ge L.: Angew. Chem., Int. Ed. *30*, 296 (1994).
13. Smith G. P.: Science *228*, 1315 (1985).
14. Smith G. P.: Gene *128*, 1 (1993).
15. Cesareni G.: FEBS Lett. *307*, 66 (1992).
16. Smith G. P., Scott J. K.: Methods Enzymol. *217*, 228 (1993).
17. Cortese R., Felici F., Galfre G., Luzzago A., Monaci P., Nicosia A.: Trends Biotechnol. *12*, 262 (1994).
18. Greenwood J., Willis A. E., Perham R. N.: J. Mol. Biol. *220*, 821 (1991).
19. Felici F., Castagnoli L., Musacchio A., Jappelli R., Cesareni G.: J. Mol. Biol. *222*, 301 (1991).
20. Haaparanta T., Huse W. D.: Mol. Divers. *1*, 39 (1995).
21. Nussey S. S., Bevan D. H., Ang V. T. Y., Jenkins J. S.: Thromb. Haemost. *55*, 34 (1986).
22. Kang A. S., Barbas C. F., Janda K. D., Benkovic S. J., Lerner R. A.: Proc. Natl. Acad. Sci. U.S.A. *88*, 4363 (1991).
23. McLafferty M. A., Kent R. B., Ladner R. C., Markland W.: Gene *128*, 29 (1993).
24. Parmley S. F., Smith G. P.: Gene *73*, 305 (1988).
25. Scott J. K., Smith G. P.: Science *249*, 386 (1990).
26. Povinelli C. M., Gibbs R. A.: Anal. Biochem. *210*, 16 (1993).
27. Dente L., Cesareni G., Micheli G., Felici F., Folgori A., Luzzago A., Monaci P., Nicosia A., Delmastro P.: Gene *148*, 7 (1994).
28. DeGraaf M. E., Miceli R. M., Mott J. E., Fischer H. D.: Gene *128*, 13 (1993).
29. Clackson T., Wells J. A.: Trends Biotech. *12*, 173 (1994).
30. Scott J. K.: Trends Biochem. Sci. *17*, 241 (1992).
31. Felici F., Luzzago A., Folgori A., Cortese R.: Gene *128*, 21 (1993).
32. Cwirla S. E., Peters E. A., Barrett R. W., Dower W. J.: Proc. Natl. Acad. Sci. U. S. A. *87*, 6378 (1990).
33. Dower W. J.: Curr. Biol. *2*, 251 (1992).
34. Stephen C. W., Lane D. P.: J. Mol. Biol. *225*, 577 (1992).
35. Yayon A., Aviezer D., Safran M., Gross J. L., Heldman Y., Cabilly S., Givol D., Katchalski-Katzir E.: Proc. Natl. Acad. Sci. U.S.A. *90*, 10643 (1993).
36. Luzzago A., Felici F., Tramontano A., Pessi A., Cortese R.: Gene *128*, 51 (1993).
37. Balass M., Heldman Y., Cabilly S., Givol D., Katchalski-Katzir E., Fuchs S.: Proc. Natl. Acad. Sci. U.S.A. *90*, 10638 (1993).
38. Folgori A., Tafi R., Meola A., Felici F., Galfre G., Cortese R., Monaci P., Nicosia A.: EMBO J. *13*, 2236 (1994).
39. Chiswell D. J., McCafferty J.: Trends Biotechnol. *10*, 80 (1992).
40. Huse W. D., Sastry L., Iverson S. A., Kang A. S., Alting-Mees M., Burton D. R., Benkovic S. J., Lerner R. A.: Science *246*, 1275 (1989).
41. McCafferty J., Griffiths A. D., Winter G., Chiswell D. J.: Nature *348*, 552 (1990).
42. Barbas C. F., Kang A. S., Lerner R. A., Benkovic S. J.: Proc. Natl. Acad. Sci. U.S.A. *88*, 7978 (1991).
43. Chang C. N., Landolfi N. F., Queen C.: J. Immunol. *147*, 3610 (1991).
44. Posner B., Lee I., Itoh T., Pyati J., Graff R., Thorton G. B., Lapolla R., Benkovic S. J.: Gene *128*, 111 (1993).
45. Willis A. E., Perham R. N., Wraith D.: Gene *128*, 79 (1993).

46. Hogrefe H. H., Mullinax R. L., Lovejoy A. E., Hay B. N., Sorge J. A.: Gene *128*, 119 (1993).
47. Clackson T., Hoogenboom H. R., Griffiths A. D., Winter G.: Nature *352*, 624 (1991).
48. Marks J. D., Hoogenboom H. R., Bonnert T. P., McCafferty J., Griffiths A. D., Winter G.: J. Mol. Biol. *222*, 581 (1991).
49. Marks J. D., Hoogenboom H. R., Griffiths A. D., Winter G.: J. Biol. Chem. *267*, 16007 (1992).
50. Gram H., Marconi L. A., Barbas C. F., III, Collet T. A., Lerner R. A., Kang A. S.: Proc. Natl. Acad. Sci. U.S.A. *89*, 3576 (1992).
51. Barbas C. F., III, Bain J. D., Hoekstra D. M., Lerner R. A.: Proc. Natl. Acad. Sci. U.S.A. *89*, 4457 (1992).
52. Burton D. R., Barbas C. F., III, Persson M. A. A., Koenig S., Chanock R. M., Lerner R. A.: Proc. Natl. Acad. Sci. U.S.A. *88*, 10134 (1991).
53. Barbas C. F., III, Collet T. A., Amberg W., Roben P., Binley J. M., Hoekstra D., Cababa D., Jones T. M., Williamson R. A., Pilkington G. R., Haigwood N. L., Cabezas E., Satterthwait A. C., Sanz I., Burton D. R.: J. Mol. Biol. *230*, 812 (1993).
54. Zebedee S. L., Barbas C. F., III, Hom Y. L., Caothien R. H., Graff R., DeGraw J., Pyati J., LaPolla R., Burton D. R., Lerner R. A., Thornton G. B.: Proc. Natl. Acad. Sci. U.S.A. *89*, 3175 (1992).
55. Barbas C. F., III, Rosenblum J. S., Lerner R. A.: Proc. Natl. Acad. Sci. U.S.A. *90*, 6385 (1993).
56. Chen J. Y. C., Danon T., Sastri L., Mubaraki M., Janda K. D., Lerner R. A.: J. Am. Chem. Soc. *115*, 357 (1993).
57. Janda K. D., Lo C. H. L., Li T., Barbas C. F., III, Wirsching P., Lerner R. A.: Proc. Natl. Acad. Sci. U.S.A. *91*, 2532 (1994).
58. Posner B., Smiley J., Lee I., Benkovic S.: Trends Biochem. Sci. *19*, 145 (1994).
59. Devlin J. J., Panganiban L. C., Devlin P. E.: Science *249*, 404 (1990).
60. Hoess R., Brinkmann U., Handel T., Pastan I.: Gene *128*, 43 (1993).
61. Sparks A. B., Quilliam L. A., Thorn J. M., Der C. J., Kay B. K.: J. Biol. Chem. *269*, 23853 (1994).
62. Dedman J. R., Kaetzel M. A., Chan H. C., Nelson D. J., Jamieson G. A.: J. Biol. Chem. *268*, 23025 (1993).
63. Smith G. P., Schultz D. A., Ladbury J. E.: Gene *128*, 37 (1993).
64. Oldenburg K. R., Loganathan D., Goldstein I. J., Schultz P. G., Gallop M. A.: Proc. Natl. Acad. Sci. U.S.A. *89*, 5393 (1992).
65. Scott J. K., Loganathan D., Easley R. B., Gong X., Goldstein I. J.: Proc. Natl. Acad. Sci. U.S.A. *89*, 5398 (1992).
66. Blondelguindi S., Cwirla S. E., Dower W. J., Lipshutz R. J., Sprang S. R., Sambrook J. F., Gething M. J. H.: Cell *75*, 717 (1993).
67. Koivunen E., Gay D. A., Ruoslahti E.: J. Biol. Chem. *268*, 20205 (1993).
68. Goodson R. J., Doyle M. V., Kaufman S. E., Rosenberg S.: Proc. Natl. Acad. Sci. U.S.A. *91*, 7129 (1994).
69. Petithory J. R., Masiarz F. R., Kirsch J. F., Santi D. V., Malcolm B. A.: Proc. Natl. Acad. Sci. U. S. A. *88*, 11510 (1991).
70. Matthews D. J., Wells J. A.: Science *260*, 1113 (1993).
71. Kay B. K., Adey N. B., He Y. S., Manfredi J. P., Mataragnon A. H., Fowlkes D. M.: Gene *128*, 59 (1993).
72. Roberts D., Guegler K., Winter J.: Gene *128*, 67 (1993).
73. Saggio I., Laufer R.: Biochem. J. *293*, 613 (1993).
74. Cortese R., Monaci P., Nicosia A., Luzzago A., Felici F., Galfre G., Pessi A., Tramontano A., Sollazzo M.: Curr. Opin. Biotechnol. *6*, 73 (1995).

75. Cull M. G., Miller J. F., Schatz P. J.: Proc. Natl. Acad. Sci. U.S.A. *89*, 1865 (1992).
76. Munir K. M., French D. C., Loeb L. A.: Proc. Natl. Acad. Sci. U.S.A. *90*, 4012 (1993).
77. Black M. E., Loeb L. A.: Biochemistry *32*, 11618 (1993).
78. Schatz P. J.: Bio/Technology *11*, 1138 (1993).
79. Kawasaki G.: PCT Int. Appl. WO 91/05058 (1991).
80. Mattheakis L. C., Bhatt R. R., Dower W. J.: Proc. Natl. Acad. Sci. U.S.A. *91*, 9022 (1994).
81. Sherman M. I., Bertelsen A. H., Cook A. F.: Bioorg. Med. Chem. Lett. *3*, 469 (1993).
82. Kenan D. J., Tsai D. E., Keene J. D.: Trends Biochem. Sci. *19*, 57 (1994).
83. Ouellett M. M., Wright W. E.: Curr. Opin. Biotechnol. *6*, 65 (1995).
84. Sastry L., Alting-Mees M., Huse W. D., Short J. M., Sorge J. A., Hay B. N., Janda K. D., Benkovic S. J., Lerner R. A.: Proc. Natl. Acad. Sci. U.S.A. *86*, 5728 (1989).
85. Tuerk C., Gold L.: Science *249*, 505 (1990).
86. Conrad R. C., Baskerville S., Ellington A. D.: Mol. Divers. *1*, 69 (1995).
87. Tuerk C., MacDougal-Waugh S.: Gene *137*, 33 (1993).
88. Giver L., Bartel D. P., Zapp M. L., Green M. R., Ellington A. D.: Gene *137*, 19 (1993).
89. Jellinek D., Lynott C. K., Rifkin D. B., Janjic N.: Proc. Natl. Acad. Sci. U.S.A. *90*, 11227 (1993).
90. Jellinek D., Green L. S., Bell C., Janjic N.: Biochemistry *33*, 10450 (1994).
91. Ellington A. D., Szostak J. W.: Nature *346*, 818 (1990).
92. Griffin L. C., Toole J. J., Leung L. L. K.: Gene *137*, 25 (1993).
93. Bock L. C., Griffin L. C., Latham J. A., Vermaas E. H., Toole J. J.: Nature *355*, 564 (1992).
94. Desjarlais J. R., Berg J. M.: Proc. Natl. Acad. Sci. U.S.A. *91*, 11099 (1994).
95. Merrifield R. B.: J. Am. Chem. Soc. *85*, 2149 (1963).
96. Atherton E., Sheppard R. C.: *Solid Phase Peptide Synthesis*. IRL Press, Oxford 1989.
97. Stewart J. M., Young J. D.: *Solid Phase Peptide Synthesis*. Pierce Chemical Company, Rockford, Illinois 1984.
98. Jung G., Beck-Sickinger A. G.: Angew. Chem., Int. Ed. *31*, 367 (1992).
99. Geysen H. M., Meloen R. H., Barteling S. J.: Proc. Natl. Acad. Sci. U.S.A. *81*, 3998 (1984).
100. Schaaper W. M. M., Beekman N. J. C. M., van Lierop M. J. C., Hensen E. J., Meloen R. H.: *Peptides 1992. Proc. 22. Eur. Pept. Symp., Interlaken 1992* (C. H. Schneider and A. N. Eberle, Eds), p. 312. ESCOM, Leiden 1993.
101. Bray A. M., Maeji N. J., Geysen H. M.: Tetrahedron Lett. *31*, 5811 (1990).
102. Bray A. M., Maeji N. J., Valerio R. M., Campbell R. A., Geysen H. M.: J. Org. Chem. *56*, 6659 (1991).
103. Maeji N. J., Bray A. M., Valerio R. M., Seldon M. A., Wang J. X., Geysen H. M.: Peptide Res. *4*, 142 (1991).
104. Valerio R. M., Benstead M., Bray A. M., Campbell R. A., Maeji N. J.: Anal. Biochem. *197*, 168 (1991).
105. Bray A. M., Maeji N. J., Jhingran A. G., Valerio R. M.: Tetrahedron Lett. *32*, 6163 (1991).
106. Bray A. M., Jhingran A. G., Valerio R. M., Maeji N. J.: J. Org. Chem. *59*, 2197 (1994).
107. Valerio R. M., Bray A. M., Campbell R. A., Dipasquale A. J., Margellis C., Rodda S. J., Geysen H. M., Maeji N. J.: Int. J. Pept. Protein Res. *42*, 1 (1993).
108. Maeji N. J., Bray A. M., Valerio R. M., Wang W.: Peptide Res. *8*, 33 (1995).
109. Valerio R. M., Bray A. M., Maeji N. J.: Int. J. Pept. Protein Res. *44*, 158 (1994).
110. Wiesmüller K. H., Treffer U., Spohn R., Jung G.: Ref.[100], p. 308.
111. Gausepohl H., Frank R. W.: Ref.[100], p. 310.
112. Geysen H. M., Rodda S. J., Mason T. J.: *Peptides, Chemistry and Biology, Proc. 10th Am. Pept. Symp., St. Louis 1987* (G. R. Marshall, Ed.), p. 519. ESCOM, Leiden 1988.

113. Geysen H. M., Rodda S. J., Mason, T. J., Tribbick G., Schoofs P. G.: J. Immunol. Methods *102*, 259 (1987).
114. Geysen H. M., Rodda S. J., Mason T. J.: Mol. Immunol. *23*, 709 (1986).
115. Geysen H. M., Barteling S., Meloen R.: Proc. Natl. Acad. Sci. U.S.A. *82*, 178 (1985).
116. Middeldorp J. M., Meloen R. H.: J. Virol. Methods *21*, 147 (1988).
117. Maeji N. J., Bray A. M., Geysen H. M.: J. Immunol. Methods *134*, 23 (1990).
118. Männel D. N., Ashman K., Stiemer R., Frank R. W.: *Peptides, Chemistry and Biology. Proc. 12th Am. Pept. Symp., Boston 1991* (J. A. Smith and J. E. Rivier, Eds), p. 468. ESCOM, Leiden 1992.
119. Wang J. X., Bray A. M., DiPasquale A. J., Maeji N. J., Geysen H. M.: Bioorg. Med. Chem. Lett. *3*, 447 (1993).
120. Wang J. X., DiPasquale A. J., Bray A. M., Maeji N. J., Geysen H. M.: Bioorg. Med. Chem. Lett. *3*, 451 (1993).
121. Spellmeyer D. C., Brown S., Stauber G. B., Geysen H. M., Valerio R.: Bioorg. Med. Chem. Lett. *3*, 519 (1993).
122. Spellmeyer D. C., Brown, S., Stauber G. B., Geysen H. M., Valerio R.: Bioorg. Med. Chem. Lett. *3*, 1253 (1993).
123. Ihlenfeldt H. G., Kraas W., Seidel C., Wienhus U., Jung G. in: *Innovation and Perspectives in Solid Phase Synthesis, Oxford 1993* (R. Epton, Ed.), p. 555. Mayflower Worldwide Ltd, Birmingham 1994.
124. Houghten R. A.: Proc. Natl. Acad. Sci. U.S.A. *82*, 5131 (1985).
125. Houghten R. A., DeGraw S. T., Bray M. K., Hoffmann S. R., Frizzell N. D.: BioTechniques *4*, 522 (1986).
126. Houghten R. A., Bray M. K., DeGraw S. T., Kirby C. J.: Int. J. Pept. Protein Res. *27*, 673 (1986).
127. Houghten R. A.: Trends Biotechnol. *5*, 322 (1987).
128. Houghten R. A. in: *Macromolecular Sequencing and Synthesis, Selected Methods and Applications* (D. H. Schlessinger, Ed.), p. 185. Alan R. Liss, Inc., New York 1988.
129. Beck-Sickinger A. G., Durr H., Jung G.: Peptide Res. *4*, 88 (1991).
130. Rinnova M., Jezek J., Malon P., Lebl M.: Peptide Res. *6*, 88 (1993).
131. Jezek J., Houghten R. A.: Collect. Czech. Chem. Commun. *59*, 691 (1994).
132. Houghten R. A., Ostresh J. M.: BioChromatography *2*, 80 (1987).
133. Houghten R. A., DeGraw S. T.: J. Chromatogr. A *386*, 223 (1987).
134. Markoff L. J., Bray M., Lai C. J., Chanock R. M., Eckels K., Summers P., Getry M. K., Houghten R. A., Lerner R. A. in: *Vaccines 88, New Chemical and Genetic Approaches to Vaccination* (H. Ginsberg, F. Brown, R. A. Lerner and R. M. Chanock, Eds), p. 161. Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1988.
135. Mathiesen T., Chiodi F., Broliden P. A., Albert J., Houghten R. A., Utte G., Wahren B., Norrby E.: Immunology *67*, 1 (1989).
136. Houghten R. A.: Ref.[134], p. 1.
137. Fieser T. M., Tainer J. A., Geysen H. M., Houghten R. A., Lerner R. A.: Proc. Natl. Acad. Sci. U.S.A. *84*, 8568 (1987).
138. Houghten R. A., Appel J., Pinilla C.: Ref.[134], p. 9.
139. Hobbs M. V., Morgan E. L., Houghten R. A., Thoman M. L., Weigle W. O.: J. Immunol. *138*, 2581 (1987).
140. Norrby E., Mufson M. A., Alexander H., Houghten R. A., Lerner R. A.: Proc. Natl. Acad. Sci. U.S.A. *84*, 6572 (1987).
141. Minden P., Houghten R. A., Spear J. R., Shinnick T. M.: Infect. Immun. *53*, 560 (1986).

142. Minden P., Voegtline M. S., Houghten R. A.: J. Clin. Lab. Anal. *1*, 287 (1987).
143. Voegtline M. S., Houghten R. A., Minden P.: Peptide Res. *2*, 227 (1989).
144. Ruggeri Z. M., Houghten R. A., Russel S. R., Zimmerman T. S.: Proc. Natl. Acad. Sci. U.S.A. *83*, 5708 (1986).
145. Beck-Sickinger A. G., Gaida W., Schnorrenberg G., Lang R., Jung G.: Int. J. Pept. Protein Res. *36*, 522 (1990).
146. Pohl M., Ambrosius D., Grotzinger J., Kretzschmar T., Saunders D., Wollmer A., Brandenburg D., Bittersuermann D., Hocker H.: Int. J. Pept. Protein Res. *41*, 362 (1993).
147. Blondelle S. E., Burcin D. E., Salazar N., Houghten R. A.: Ref.[118], p. 433.
148. Frank R., Güler S., Krause S., Lindenmaier W.: *Peptides 1990. Proc. 21th Eur. Pept. Symp., Platja d'Aro 1990* (E. Giralt and D. Andreu, Ed.), p. 151. ESCOM, Leiden 1991.
149. Frank R.: Tetrahedron *48*, 9217 (1992).
150. Frank R.: Bioorg. Med. Chem. Lett. *3*, 425 (1993).
151. Frank R.: Ref.[123], p. 509.
152. Flegelova Z., Krchnak V., Nemecek V., Pistek T., Suchankova A., Vagner J.: Ref. [123], p. 505.
153. Simmonds R. G., Kingston A. E., Carney S. L.: Ref.[123], p. 681.
154. Lyttle M. H., Berry C. O. A., Villar H. O., Hocker M. H., Kauvar L. M.: *Peptides. Proc. 13th Am. Pept. Symp., Edmonton 1993* (R. S. Hodges and J. A. Smith, Eds), p. 1009. ESCOM, Leiden 1994.
155. Molina F., Pau B., Granier C.: Ref.[123], p. 607. .
156. Frank R.: Ref.[100], p. 59.
157. Hoffmann S., Frank R.: Ref.[123], p. 535.
158. Fodor S. P. A., Leighton R. J., Pirrung M. C., Stryer L., Lu A. T., Solas D.: Science *251*, 767 (1991).
159. Holmes C. P., Fodor S. P. A.: Ref.[123], p. 221.
160. Holmes C. P., Adams C. L., Kochersperger L. M., Mortensen R. B., Aldwin L. A.: Biopolymers (Peptide Sci.) *37*, 199 (1995).
161. Patchornik A., Amit B., Woodward R. B.: J. Am. Chem. Soc. *92*, 6333 (1970).
162. Fodor S. P. A., Rava R. P., Huang X. C., Pease A. C., Holmes C. P., Adams C. L.: Nature *364*, 555 (1993).
163. Jacobs J. W., Fodor S. P. A.: Trends Biotechnol. *12*, 19 (1994).
164. Holmes C. P., Adams C. L., Fodor S. P. A., Yu-Yang P. in: *Perspectives in Medicinal Chemistry* (B. Testa, E. Kyburz, W. Fuher and R. Giger, Eds), p. 489. VHCA, Basel 1992.
165. Rozsynai L. F., Benson D. R., Fodor S. P. A., Schultz P. G.: Angew. Chem., Int. Ed. *31*, 759 (1992).
166. Eichler J., Beyermann M., Bienert M., Lebl M.: *Peptides 1988. Proc. 20th Eur. Pept. Symp., Tübingen 1988* (G. Jung and E. Bayer, Eds), p. 205. Walter de Gruyter, Berlin 1989.
167. Stankova M., Wade S., Lam K. S., Lebl M.: Peptide Res. *7*, 292 (1994).
168. Frank R., Döring R.: Tetrahedron *44*, 6031 (1988).
169. Böldicke T., Maywald F., Wingender E., Collins J., Frank R.: Ref.[166], p. 220.
170. Blankemeyer-Menge B., Frank R.: *Innovations and Perspectives in Solid Phase Synthesis, Oxford 1989* (R. Epton, Ed.), p. 465. SPCC, Birmingham 1990.
171. Krchnak V., Vagner J.: Int. J. Pept. Protein Res. *33*, 209 (1989).
172. Krchnak V., Vagner J.: *Peptides 1990. Proc. 21th Eur. Pept. Symp., Platja d'Aro 1990* (E. Giralt and D. Andreu, Eds), p. 200. ESCOM, Leiden 1991.
173. Albericio F., Ruiz-Gayo M., Pedroso E., Giralt E.: React. Polym. *10*, 259 (1989).
174. Holm A., Meldal M.: Ref.[166], p. 208.

175. Meldal M., Holm C. B., Bojesen G., Jakobsen M. H., Holm A.: Int. J. Pept. Protein Res. *41*, 250 (1993).
176. Gausepohl H., Kraft M., Boulin C., Frank R. W.: Ref.[170], p. 487.
177. Gausepohl H., Kraft M., Boulin C., Frank R. W.: Ref.[172], p. 206.
178. Gausepohl H., Behn C.: Ref.[123], p. 175.
179. Zuckermann R. N., Banville S. C.: Peptide Res. *5*, 169 (1992).
180. Nokihara K., Yamamoto R.: Ref.[118], p. 507.
181. Schnorrenberg G., Gerhardt H.: Tetrahedron *45*, 7759 (1989).
182. Schnorrenberg G., Wiesmüller K. H., Beck-Sickinger A. G., Drechsel H., Jung G.: Ref.[148], p. 202.
183. Hyde C., Johnson T., Sheppard R. C.: Ref.[100], p. 314.
184. Cammish L., Hyde C., Johnson T., Sheppard R. C.: Ref.[123], p. 257.
185. Eichler J., Bienert M., Stierandova A., Lebl M.: Peptide Res. *4*, 296 (1991).
186. Eichler J., Furkert J., Bienert M., Rohde W., Lebl M.: Ref.[172], p. 156.
187. Eichler J., Bienert M., Sepetov N. F., Stolba P., Krchnak V., Smekal O., Gut V., Lebl M.: Ref.[170], p. 337.
188. Lebl M., Stierandova A., Eichler J., Patek M., Pokorny V., Jehnicka J., Mudra P., Zenisek K., Kalousek J.: *Innovation and Perspectives in Solid Phase Peptide Synthesis, Canterbury 1991* (R. Epton, Ed.), p. 251. Intercept Limited, Andover 1992.
189. Pokorny V., Mudra P., Jehnicka J., Zenisek K., Pavlik M., Voburka Z., Rinnova M., Stierandova A., Lucka A. W., Eichler J., Houghten R. A., Lebl M.: Ref.[123], p. 643.
190. Schmidt M., Eichler J., Odarjuk J., Krause E., Beyermann M., Bienert M.: Bioorg. Med. Chem. Lett. *3*, 441 (1993).
191. Berg R. H., Almdal K., Pedersen W. B., Holm A., Tam J. P., Merrifield R. B.: J. Am. Chem. Soc. *111*, 8024 (1989).
192. Berg R. H., Almdal K., Pedersen W. B., Holm A., Tam J. P., Merrifield R. B.: Ref.[148], p. 149.
193. Frank R., Heikens G., Heisterberg-Moutsis G., Blöcker H.: Nucleic Acids Res. *11*, 4365 (1983).
194. Pease A. C., Solas D., Sullivan E. J., Cronan M. T., Holmes C. P., Fodor S. P. A.: Proc. Natl. Acad. Sci. U.S.A. *91*, 5022 (1994).
195. Cho C. Y., Moran E. J., Cherry S. R., Stephans J. C., Fodor S. P. A., Adams C. L., Sundaram A., Jacobs J. W., Schultz P. G.: Science *261*, 1303 (1993).
196. Moran E. J., Wilson T. E., Cho C. Y., Cherry S. R., Schultz P. G.: Biopolymers (Peptide Sci.) *37*, 213 (1995).
197. Peters S., Bielfeldt T., Meldal M., Bock K., Paulsen H.: J. Chem. Soc., Perkin Trans. 1 *1992*, 1163.
198. Jansson A. M., Meldal M., Bock K.: Ref.[123], p. 559.
199. Bunin B. A, Ellman J. A.: J. Am. Chem. Soc. *114*, 10997 (1992).
200. Bunin B. A., Plunkett M. J., Ellman J. A.: Proc. Natl. Acad. Sci. U.S.A. *91*, 4708 (1994).
201. Sternbach L. H.: *The Benzodiazepines*, Raven Press, New York 1973.
202. Camps F., Cartells J., Pi J.: An. Quim. *70*, 848 (1974).
203. Plunkett M. J., Ellman J. A.: J. Am. Chem. Soc. *117*, 3306 (1995).
204. DeWitt S. H., Kiely J. K., Stankovic C. J., Schroeder M. C., Cody D. M. R., Pavia M. R.: Proc. Natl. Acad. Sci. U.S.A. *90*, 6909 (1993).
205. Stankovic C. J., DeWitt S., Schroeder M. C., Kiely J. S., Cody D. M. R., Pavia M. R.: Ref.[123], p. 391.
206. Furka A., Sebestyen F., Asgedom M., Dibo G.: Int. J. Pept. Protein Res. *37*, 487 (1991).
207. Lam K. S., Salmon S. E., Hersh E. M., Hruby V. J., Kazmierski W. M., Knapp, R. J.: Nature *354*, 82 (1991).

208. Houghten R. A., Pinilla C., Blondelle S. E., Appel J. R., Dooley C. T., Cuervo J. H.: Nature *354*, 84 (1991).
209. Furka A., Sebestyen F., Asgedom M., Dibo G.: *14th International Congress of Biochemistry, Prague 1988*. Book of Abstracts, Vol. V, p. 47. Videopress IOJ, Prague 1988.
210. Lam K. S., Salmon S. E., Hersh E. M., Hruby V. J., Al-Obeidi F., Kazmierski W. M., Knapp R. J.: Ref.[118], p. 492.
211. Lam K. S., Lebl M.: Methods: A Companion to Methods in Enzymology *6*, 372 (1994).
212. Kassarjian A., Schellenberger V., Turck C. W.: Peptide Res. *6*, 129 (1993).
213. Lebl M., Lam K. S., Kocis P., Krchnak V., Patek M., Salmon S. E., Hruby V. J.: Ref.[100], p. 67.
214. Lebl M., Patek M., Kocis P., Krchnak V., Hruby V. J., Salmon S. E., Lam K. S.: Int. J. Pept. Protein Res. *41*, 201 (1993).
215. Lebl M., Krchnak V., Salmon S. E., Lam K. S.: Methods: A Companion to Methods in Enzymology *6*, 381 (1994).
216. Salmon S. E., Lam K. S., Lebl M., Kandola A., Khattri P. S., Wade S., Patek M., Kocis P., Krchnak V., Thorpe D., Felder S.: Proc. Natl. Acad. Sci. U.S.A. *90*, 11708 (1993).
217. Kocis P., Krchnak V., Lebl M.: Tetrahedron Lett. *34*, 7251 (1993).
218. Bradley M.: *Fourth International Symposium on Solid Phase Synthesis and Combinatorial Chemical Libraries, Edinburgh, September 12–16, 1995*, poster P18.
219. Bradshaw C. G., Chollet A. R., Wells T. N. C.: Ref.[100], p. 318.
220. Darlak K., Romanovskis P., Spatola A. F.: Ref.[154], p. 981.
221. Lam K. S., Lebl M., Krchnak V., Lake D. F., Smith J., Wade S., Ferguson R., Ackerman-Berrier M., Wertman K.: Ref.[154], p. 1003.
222. Lebl M., Krchnak V., Sepetov N. F., Nikolaev V., Stierandova A., Safar P., Seligmann B., Strop P., Thorpe D., Felder S., Lake D. F., Lam K. S., Salmon S. E.: Ref.[123], p. 233.
223. Holmes C. P., Rybak C. M.: Ref.[154], p. 992.
224. Kania R. S., Zuckermann R. N., Marlowe C. K.: J. Am. Chem. Soc. *116*, 8835 (1994).
225. Lam K. S., Hruby V. J., Lebl M., Knapp R. J., Kazmierski W. M., Hersh E. M., Salmon S. E.: Bioorg. Med. Chem. Lett. *3*, 419 (1993).
226. Lam K. S., Lebl M., Krchnak V., Wade S., Abdul-Latif F., Ferguson R., Cuzzocrea C., Wertman, K.: Gene *137*, 13 (1993).
227. Lam K. S., Lebl M.: Immunomethods *1*, 11 (1992).
228. Lam K. S., Lebl M., Wade S., Stierandova A., Khattri P. S., Collins N., Hruby V. J.: Ref.[154], p. 1005.
229. Lam K. S., Zhao Z. G., Wade S., Krchnak V., Lebl M.: Drug Dev. Res. *33*, 157 (1994).
230. Lam K. S., Lebl M., Wu J., Wade S., Lou Q., Zhao Z. G., Chen M. L., Chang M. P., Smith M., Grimes W. J.: *Peptides 1994. Proc. 23rd Eur. Pept. Symp., Braga 1994* (H. L. S. Maia, Ed.), p. 453. ESCOM, Leiden 1995.
231. Seligmann B., Abdul-Latif F., Al-Obeidi F., Flegelova Z., Issakova O., Kocis P., Krchnak V., Lam K. S., Lebl M., Ostrem J., Safar P., Sepetov N., Stierandova, A., Strop P., Wildgoose P.: Eur. J. Med. Chem. 30 (supplement); *Proceedings of the XIIIth International Symposium on Medicinal Chemistry* (J. C. Müller, Ed.), p. 319s, 1995.
232. Meldal M., Svendsen I., Breddam K., Auzanneau F. I.: Proc. Natl. Acad. Sci. U.S.A. *91*, 3314 (1994).
233. Meldal M.: Ref.[100], p. 61.
234. Meldal M., Auzanneau F. I., Bock K.: Ref.[123], p. 259.
235. Ator M. A., Beigel S., Dankanich T. C., Echols M., Gainor J. A., Gilliam C. L., Gordon T. D., Koch D., Koch J. F., Kruse L. I., Morgan B. A., Krupinski-Olsen R., Siahaan T. J., Singh J., Whipple D. A.: Ref.[154], p. 1012.
236. Gainor J. A., Gordon T. D., Morgan B. A.: Ref.[154], p. 989.

237. Lam K. S., Wu J.: Methods: A Companion to Methods in Enzymology *6*, 401 (1994).
238. Wu J., Ma Q. N., Lam K. S.: Biochemistry *33*, 14825 (1994).
239. Lam K. S., Wu J., Lou Q.: Int. J. Pept. Protein Res. *45*, 587 (1995).
240. Till J. H., Annan R. S., Carr S. A., Miller W. T.: J. Biol. Chem. *269*, 7423 (1994).
241. Bartak Z., Bolf J., Kalousek J., Mudra P., Pavlik M., Pokorny V., Rinnova M., Voburka Z., Zenisek K., Krchnak V., Lebl M., Salmon S. E., Lam K. S.: Methods: A Companion to Methods in Enzymology *6*, 432 (1994).
242. Zuckermann R. N., Kerr J. M., Siani M. A., Banville S. C.: Int. J. Pept. Protein Res. *40*, 497 (1992).
243. Zuckermann R. N., Kerr J. M., Siani M. A., Banville S. C., Santi D. V.: Proc. Natl. Acad. Sci. U.S.A. *89*, 4505 (1992).
244. Kerr J. M., Banville S. C., Zuckermann R. N.: Bioorg. Med. Chem. Lett. *3*, 463 (1993).
245. Saneii H. H., Shannon J. D., Miceli R. M., Fischer H. D., Smith C. W.: Ref.[123], p. 335.
246. Saneii H. H., Shannon J. D., Miceli R. M., Fischer H. D., Smith C. W.: Ref.[154], p. 1018.
247. Eichler J., Pinilla C., Chendra S., Appel J. R, Houghten R. A.: Ref.[123], p. 227.
248. Lam K. S., Wade S., Abdul-Latif F., Lebl M.: J. Immunol. Methods *180*, 219 (1995).
249. Needels M. C., Jones D. G., Tate E. H., Heinkel G. L., Kochersperger L. M., Dower W. J., Barrett R. W., Gallop M. A.: Proc. Natl. Acad. Sci. U.S.A. *90*, 10700 (1993).
250. Jayawickreme C. K., Graminski G. F., Quillan J. M., Lerner M. R.: Proc. Natl. Acad. Sci. U.S.A. *91*, 1614 (1994).
251. Sebestyen F., Dibo G., Kovacs A., Furka A.: Bioorg. Med. Chem. Lett. *3*, 413 (1993).
252. Sebestyen F., Szalatnyai T., Durgo J. A., Furka A.: J. Pept. Sci. *1*, 26 (1995).
253. Sebestyen F., Dibo G., Furka A.: Ref.[100], p. 63.
254. Furka A., Sebestyen F., Campian E.: Ref.[154], p. 986.
255. Furka A., Sebestyen F., Campian E.: Ref.[123], p. 385.
256. Campian E., Sebestyen F., Furka A.: Ref.[123], p. 469.
257. Erb E., Janda K. D., Brenner S.: Proc. Natl. Acad. Sci. U.S.A. *91*, 11422 (1994).
258. Stevanovic S., Jung G.: Anal. Biochem. *212*, 212 (1993).
259. Stevanovic S., Wiesmüller K. H., Metzger J., Beck-Sickinger A. G., Jung G.: Bioorg. Med. Chem. Lett. *3*, 431 (1993).
260. Biemann K.: Methods Enzymol. *193*, 455 (1990).
261. Falk K., Rötzschke O., Stevanovic S., Jung G., Rammensee H. G.: Nature *351*, 290 (1991).
262. Stevanovic S., Gnau V., Malcherek G., Falk K., Rötzschke O., Rammensee H. G., Melms A., Jung G.: Ref.[123], p. 327.
263. Sepetov N. F., Issakova O. L., Lebl M., Swiderek K., Stahl D. C., Lee T. D.: Rapid Commun. Mass Spectrom. *7*, 58 (1993).
264. Sepetov N. F., Issakova O. L., Krchnak V., Lebl M.: U.S. Patent Application 07/939,811, (1992).
265. Metzger J. W., Wiesmüller K. H., Gnau V., Brünjes J., Jung G.: Angew. Chem., Int. Ed. *32*, 894 (1993).
266. Brummel C. L., Lee I. N. W., Zhou Y., Benkovic S. J., Winograd N.: Science *264*, 399 (1994).
267. Youngquist R. S., Fuentes G. R., Lacey M. P., Keough, T.: Rapid Commun. Mass Spectrom. *8*, 77 (1994).
268. Egner B. J., Langley G. J., Bradley M.: J. Org. Chem. *60*, 2652 (1995).
269. Youngquist R. S., Fuentes G. R., Lacey M. P., Keough T.: J. Am. Chem. Soc. *117*, 3900 (1995).
270. Chu Y. H., Avila L. Z., Biebuyck H. A., Whitesides G. M.: J. Org. Chem. *58*, 648 (1993).
271. Fassina G., Lebl M., Chaiken I.: Collect. Czech. Chem. Commun. *53*, 2627 (1988).

272. Tjoeng F. S., Towery D. S., Bulock J. W., Whipple D. E., Fok K. F., Williams M. H., Zupec M. E., Adams S. P.: Int. J. Pept. Protein Res. *35*, 141 (1990).
273. Geysen H. M., Mason T. J.: Bioorg. Med. Chem. Lett. *3*, 397 (1993).
274. Ostresh J. M., Winkle J. M., Hamashin V. T., Houghten R. A.: Biopolymers *34*, 1681 (1994).
275. Dooley C. T., Houghten R. A.: Life Sci. *52*, 1509 (1993).
276. Houghten R. A., Appel J. R., Blondelle S. E., Cuervo J. H., Dooley C. T., Eichler J., Pinilla C.: Ref.[154], p. 971.
277. Pinilla C., Appel J. R., Houghten R. A.: Ref.[100], p. 65.
278. Pinilla C., Appel J. R., Chendra S., Houghten R. A.: Ref.[100], p. 903.
279. Houghten R. A., Appel J. R., Blondelle S. E., Cuervo J. H., Dooley C. T., Pinilla C.: Peptide Res. *5*, 351 (1992).
280. Pinilla C., Appel J. R., Milich D., Houghten R. A.: Ref.[154], p. 1016.
281. Pinilla C., Appel J. R., Houghten R. A.: Gene *128*, 71 (1993).
282. Pinilla C., Buencamino J., Appel J. R., Hopp T. P., Houghten R. A.: Mol. Diversity *1*, 21 (1995).
283. Houghten R. A., Dooley C. T.: Bioorg. Med. Chem. Lett. *3*, 405 (1993).
284. Dooley C. T., Houghten R. A.: Ref.[154], p. 984.
285. Dooley C. T., Chung N. N., Schiller P. W., Houghten R. A.: Proc. Natl. Acad. Sci. U.S.A. *90*, 10811 (1993).
286. Blondelle S. E., Simpkins L. R., Houghten R. A.: Ref.[100], p. 761.
287. Blondelle S. E., Simpkins L. R., Houghten R. A.: Ref.[123], p. 163.
288. Eichler J., Houghten R. A.: Ref.[100], p. 320.
289. Eichler J., Houghten R. A.: Biochemistry *32*, 11035 (1993).
290. Houghten R. A.: Gene *137*, 7 (1993).
291. Houghten R. A.: Trends Genet. *9*, 235 (1993).
292. Houghten R. A.: Curr. Biol. *4*, 564 (1994).
293. Hortin G. L., Staatz W. D., Santoro S. A.: Biochem. Int. *26*, 731 (1992).
294. Owens R. A., Gesellchen P. D., Houchins B. J., DiMarchi R. D.: Biochem. Biophys. Res. Commun. *181*, 402 (1991).
295. Kramer A., Volkmer-Engert R., Malin R., Reineke U., Schneider-Mergener J.: Peptide Res. *6*, 314 (1993).
296. Kramer A., Schuster A., Reineke U., Malin R., Volkmer-Engert R., Landgraf C., Schneider-Mergener J.: Methods: A Companion to Methods in Enzymology *6*, 388 (1994).
297. Volkmer-Engert R., Erhardt B., Hellwig J., Kramer A., Hohne W., Schneider-Mergener J.: Lett. Pept. Sci. *1*, 243 (1994).
298. Kramer A., Vokalopoulou E., Schleuning W. D., Schneider-Mergener J.: Mol. Immunol. *32*, 459 (1995).
299. Frank R., Kieß M., Lahmann H., Behn C., Gausepohl H.: Ref.[230], p. 479.
300. Tegge W., Dostmann W., Hofmann F., Frank R.: Ref.[230], p. 481.
301. Wallace A., Altamura S., Toniatti C., Vitelli A., Bianchi E., Delmastro P., Ciliberto G., Pessi A.: Peptide Res. *7*, 27 (1994).
302. Tam J. P., Zavala F.: J. Immunol. Methods *124*, 53 (1989).
303. Blake J., Litzi-Davis L.: Bioconjugate Chemistry *3*, 510 (1992).
304. Zhang W. J., Hortin G. L., Panek R. L., Lu G. H., Hsu F. F., Marshall G. R.: Ref.[154], p. 978.
305. Buettner J. A., Hudson D., Johnson C. R., Ross M. J., Shoemaker K.: Ref.[123], p. 169.
306. Cass R., Dreyer M. L., Giebel L. B., Hudson D., Johnson C. R., Ross M. J., Schaeck J., Shoemaker K. R.: Ref.[154], p. 975.
307. Wong W. Y., Sheth H. B., Holm A., Irvin R. T., Hodges R. S.: Ref.[154], p. 995.

308. Green M., McGeehan G. M., Anderegg R. S., Bickett D. M., Kassel D., Aquino C. J., Sugg E. E., Millington S., Norwod D. L., Wiseman J. S., Bermann J.: Ref.[123], p. 239.
309. Ullmann D., Jakubke H. D.: Ref.[123], p. 693.
310. Wiesmüller K. H., Beck-Sickinger A. G., Ihlenfeldt H. G., Wieland H. A., Udaka K., Walden P., Jung G.: Ref.[154], p. 998.
311. Sepetov N. F., Krchnak V., Stankova M., Lam K. S., Wade S., Lebl M.: Proc. Natl. Acad. Sci. U.S.A. *92*, 5426 (1995).
312. Markiewicz W. T., Adrych-Rozek K., Markiewicz M., Zebrowska A., Astriab A.: Ref.[123], p. 339.
313. Case-Green S. C., Elder J. K., Mir K. U., Maskos U., Southern E. M., Williams J. C.: Ref.[123], p. 77.
314. Beattie K. L., Hurst G. D.: Ref.[123], p. 69.
315. Latham J. A., Johnson R., Toole J. J.: Nucleic Acids Res. *22*, 2817 (1994).
316. Brenner S., Lerner R. A.: Proc. Natl. Acad. Sci. U.S.A. *89*, 5381 (1992).
317. Nielsen J., Brenner S., Janda K. D.: J. Am. Chem. Soc. *115*, 9812 (1993).
318. Ohlmeyer M. H. J., Swanson R. N., Dillard L. W., Reader J. C., Asouline G., Kobayashi R., Wigler M., Still W. C.: Proc. Natl. Acad. Sci. U.S.A. *90*, 10922 (1993).
319. Nestler H. P., Bartlett P. A., Still W. C.: J. Org. Chem. *59*, 4723 (1994).
320. Borchardt A., Still C. W.: J. Am. Chem. Soc. *116*, 373 (1994).
321. Chait B. T., Wang R., Beavis R. C., Kent S. B. H.: Science *262*, 89 (1993).
322. Kerr J. M., Banville S. C., Zuckermann R. N.: J. Am. Chem. Soc. *115*, 2529 (1993).
323. Nikolaiev V., Stierandova A., Krchnak V., Seligmann B., Lam K. S., Salmon S. E., Lebl M.: Peptide Res. *6*, 161 (1993).
324. Vagner J., Krchnak V., Sepetov N. F., Strop P., Lam K. S., Barany G., Lebl M.: Ref.[123], p. 347.
325. Lebl M., Krchnak V., Stierandova A., Safar P., Kocis P., Nikolaev V., Sepetov N. F., Ferguson R., Seligmann B., Lam K. S., Salmon S. E.: Ref.[154], p. 1007.
326. Stankova M., Issakova O., Sepetov N. F., Krchnak V., Lam K. S., Lebl M.: Drug Dev. Res. *33*, 146 (1994).
327. Flegelova Z., Krchnak V., Sepetov N. F., Stankova M., Issakova O., Cabel D., Lam K. S., Lebl M.: Ref.[230], p. 469.
328. Safar P., Stierandova A., Lebl M.: Ref.[230], p. 471.
329. Lebl M., Krchnak V., Safar P., Stierandova A., Sepetov N. F., Kocis P., Lam K. S. in: *Techniques in Protein Chemistry V* (J. W. Crabb, Ed.), p. 541. Academic Press, San Diego 1994.
330. Lebl M., Krchnak V., Sepetov N. F., Nikolaev V., Stankova M., Kocis P., Patek M., Flegelova Z., Ferguson R., Lam K. S.: *Proceedings of 10th International Conference on Methods in Protein Structure Analysis* (M. Z. Atassi and E. Appella, Eds), p. 335. Plenum Publishing, New York 1995.
331. Krchnak V., Weichsel A. S., Cabel D., Lebl M.: Peptide Res. *8*, 198 (1995).
332. Cuervo J. H., Weitl F., Ostresh J. M., Hamashin V. T., Hannah A. L., Houghten R. A.: Ref.[230], p. 465.
333. Ostresh J. M., Husar G. M., Blondelle S. E., Dörner B., Weber P. A, Houghten, R. A.: Proc. Natl. Acad. Sci. U.S.A. *91*, 11138 (1994).
334. Young J. K., Nelson J. C., Moore J. S.: J. Am. Chem. Soc. *116*, 10841 (1994).
335. Simon R. J., Kaina R. S., Zuckermann R. N., Huebner V. D., Jewell D. A., Banville S., Ng S., Wang L., Rosenberg S., Marlowe C. K., Spellmeyer D. C., Tan R., Frankel A. D., Santi D. V., Cohen F. E., Bartlett P. A.: Proc. Natl. Acad. Sci. U.S.A. *89*, 9367 (1992).
336. Cambell D. A, Bermak J. C.: J. Am. Chem. Soc. *116*, 6039 (1994).
337. Gennari C., Nestler H. P., Salom B., Still W. C.: Angew. Chem., Int. Ed. *34*, 1763 (1995).
338. Hebert N., Davis P. W., DeBaets E. L., Acevedo O. L.: Tetrahedron Lett. *35*, 9509 (1994).

339. Campbell D. A., Bermak J. C., Burkoth T. S., Patel D. V.: J. Am. Chem. Soc. *117*, 5381 (1995).
340. Zuckermann R. N., Kerr J. M., Kent S. B. H., Moos W. H.: J. Am. Chem. Soc. *114*, 10646 (1992).
341. Zuckermann R. N., Martin E. J., Spellmeyer D. C., Stauber G. B., Shoemaker K. R., Kerr J. M., Figliozzi G. M., Goff D. A., Siani M. A., Simon R. J., Banville S. C., Brown E. G., Wang L., Richter L. S., Moos W. H.: J. Med. Chem. *37*, 2678 (1994).
342. Miller S. M., Simon R. J., Ng S., Zuckermann R. N., Kerr J. M., Moos W. H.: Bioorg. Med. Chem. Lett. *4*, 2657 (1994).
343. Pei Y., Moos W. H.: Tetrahedron Lett. *35*, 5825 (1994).
344. Yedidia V., Leznoff C. C.: Can. J. Chem. *58*, 1144 (1980).
345. Zambias R. A., Boulton D. A., Griffin P. R.: Tetrahedron Lett. *35*, 4283 (1994).
346. Chen C., Randall L. A. A., Miller R. B., Jones A. D., Kurth M. J.: J. Am. Chem. Soc. *116*, 2661 (1994).
347. Crowley J. I., Rapoport H.: Account. Chem. Res. *9*, 135 (1976).
348. Leznoff C. C.: Account. Chem. Res. *11*, 327 (1978).
349. Frechet J. M. J.: Tetrahedron *37*, 663 (1981).
350. Leznoff C. C.: Chem. Soc. Rev. *3*, 65 (1974).
351. Forman F. W., Sucholeiki I.: J. Org. Chem. *60*, 523 (1995).
352. Kuhn H., Neumann W. P.: Synlett *1994*, 123.
353. Deshpande M. S.: Tetrahedron Lett. *35*, 5613 (1994).
354. Frenette R., Friesen R. W.: Tetrahedron Lett. *35*, 9177 (1994).
355. Backes B. J., Ellman J. A.: J. Am. Chem. Soc. *116*, 11171 (1994).
356. Yu K. L., Deshpande M. S., Vyas D. M.: Tetrahedron Lett. *35*, 8919 (1994).
357. Schultz P. G., Lerner R. A.: Science *269*, 1835 (1995).
358. Richter L. S., Gadek T. R.: Tetrahedron Lett. *35*, 4705 (1994).
359. Krchnak V., Flegelova Z., Weichsel A. S., Lebl M.: Tetrahedron Lett. *36*, 6193 (1995).
360. Krchnak V., Cabel D., Weichsel A., Flegelova Z.: Lett. Pept. Sci. *1*, 277 (1995).
361. Randolph J. T., McClure K. F., Danishefsky S. J.: J. Am. Chem. Soc. *117*, 5712 (1995).
362. Goff D. A., Zuckermann R. N.: J. Org. Chem. *60*, 5744 (1995).
363. Goff D. A., Zuckermann R. N.: J. Org. Chem. *60*, 5748 (1995).
364. Moon H., Schore N. E., Kurth M. J.: Tetrahedron Lett. *35*, 8915 (1994).
365. Kick E. K., Ellman J. A.: J. Med. Chem. *38*, 1427 (1995).
366. Wang G. T., Li S., Wideburg N., Krafft G. A., Kempf D. J.: J. Med. Chem. *38*, 2995 (1995).
367. Kurth M. J., Randall L. A. A., Chen C., Melander C., Miller R. B., McAlister K., Reitz G., Kang R., Nakatsu T., Green C.: J. Org. Chem. *59*, 5862 (1994).
368. Goebel M., Ugi I.: Tetrahedron Lett. *36*, 6043 (1995).
369. Keating T. A., Armstrong R. W.: J. Am. Chem. Soc. *117*, 7842 (1995).
370. Boojamra C. G., Burow K. M., Ellman J. A.: J. Org. Chem. *60*, 5742 (1995).
371. Green J.: J. Org. Chem. *60*, 4287 (1995).
372. Hiroshige M., Hauske J. R., Zhou P.: Tetrahedron Lett. *36*, 4567 (1995).
373. Hutchins S. M., Chapman K. T.: Tetrahedron Lett. *36*, 2583 (1995).
374. Virgilio A. A., Ellman J. A.: J. Am. Chem. Soc. *116*, 11580 (1994).
375. Gordon D. W., Steele J.: Bioorg. Med. Chem. Lett. *5*, 47 (1995).
376. Scott B. O., Siegmund A. C., Marlowe C. K., Pei Y., Spear K. L.: Molecular Diversity *1*, in press.
377. Bray A. M., Chiefari D. S., Valerio R. M., Maeji N. J.: Tetrahedron Lett. *36*, 5081 (1995).
378. Hutchins S. M., Chapman K. T.: Tetrahedron Lett. *35*, 4055 (1994).

379. Look G. C., Holmes C. P., Chinn J. P., Gallop M. A.: J. Org. Chem. *59*, 7588 (1994).
380. Fitch W. L., Detre G., Holmes C. P.: J. Org. Chem. *59*, 7955 (1994).
381. Rano T. A., Chapman K. T.: Tetrahedron Lett. *36*, 3789 (1995).
382. Burbaum J. J., Ohlmeyer M. H. J., Reader J. C., Henderson I., Dillard L. W., Li G., Randle T. L., Sigal N. H., Chelsky D., Baldwin J. J.: Proc. Natl. Acad. Sci. U.S.A. *92*, 6027 (1995).
383. Patek M., Drake B., Lebl M.: Tetrahedron Lett. *36*, 2227 (1995).
384. Murphy M. M., Schullek J. R., Gordon E. M., Gallop M. A.: J. Am. Chem. Soc. *117*, 7029 (1995).
385. Wipf P., Cunningham A.: Tetrahedron Lett. *36*, 7819 (1995).
386. Meutermans W. D. F., Alewood P. F.: Tetrahedron Lett. *36*, 7709 (1995).
387. Dankwardt S. M., Newman S. R., Krstenansky J. L.: Tetrahedron Lett. *36*, 4923 (1995).
388. Hirschmann R., Nicolaou K. C., Pietranico S., Salvino J., Leahy E. M., Sprengeler P. A., Furst G., Smith A. B., III, Strader C. D., Cascieri M. A., Candelore M. R., Donaldson C., Vale W., Maechler L.: J. Am. Chem. Soc. *114*, 9217 (1992).
389. Nowick J. S., Powel N. A., Martinez E. J., Smith E. M., Noronha G.: J. Org. Chem. *57*, 3767 (1992).
390. Meyers H. V., Dilley G. J., Durgin T. L., Powers T. S., Winssinger N. A., Zhu H., Pavia M. R.: Mol. Diversity *1*, 13 (1995).
391. Dankwardt S. M., Phan T. M., Krstenansky J. L.: Mol. Diversity *1*, in press.
392. Boyce R., Li G., Nestler H. P., Suenaga T., Still W. C.: J. Am. Chem. Soc. *116*, 7955 (1994).
393. Patek M., Drake B., Lebl M.: Tetrahedron Lett. *35*, 9169 (1994).
394. Kocis P., Issakova O., Sepetov N. F., Lebl M.: Tetrahedron Lett. *36*, 6623 (1995).
395. Carell T., Wintner E. A., Bashir-Hashemi A., Rebek J. J.: Angew. Chem., Int. Ed. *33*, 2059 (1994).
396. Carell T., Wintner E. A., Rebek J. J.: Angew. Chem., Int. Ed. *33*, 2061 (1994).
397. Anderson R. C., Jarema M. A., Shapiro M. J., Stokes J. P., Ziliox M.: J. Org. Chem. *60*, 2650 (1995).
398. Anderson R. C., Stokes J. P., Shapiro M. J.: Tetrahedron Lett. *36*, 5311 (1995).
399. Moon H., Schore N. E., Kurth M. J.: J. Org. Chem. *57*, 6088 (1992).
400. Yan B., Kumaravel G., Anjaria H., Wu A., Petter R. C., Jewell C. F., Wareing J. R.: J. Org. Chem. *60*, 5736 (1995).
401. Dörner B., Ostresh J. M., Husar G. M., Houghten R. A.: Ref.[230], p. 463.
402. Houghten R. A., Ostresh J. M., Husar G. M., Dörner B., Blondelle S. E.: Ref.[230], p. 459.
403. Eichler J., Lucka A. W., Houghten R. A.: Peptide Res. *7*, 300 (1994).
404. Eichler J., Lucka A. W., Houghten R. A.: Ref.[230], p. 461.
405. Ecker D. J., Vickers T. A., Hanecak R., Driver V., Anderson K.: Nucleic Acids Res. *21*, 1853 (1993).
406. Wyatt J. R., Vickers T. A., Roberson J. L., Buckheit R. W. J., Klimkait T., DeBaets E., Davis P. W., Rayner B., Imbach J. L., Ecker D. J.: Proc. Natl. Acad. Sci. U. S. A. *91*, 1356 (1994).
407. Freier S. M., Konings D. A. M., Wyatt J. R., Ecker D. J.: J. Med. Chem. *38*, 344 (1995).
408. Kauffman S.: Ber. Bunsenges. Phys. Chem. *98*, 1142 (1994).
409. Levitan B., Kauffman S.: Mol. Diversity *1*, 53 (1995).
410. Kauffman S.: Perspect. Drug Disc. Design *2*, 319 (1995).
411. Burgess K., Liaw A. I., Wang N.: J. Med. Chem. *37*, 2985 (1994).
412. Martin E. J., Blaney J. M., Siani M. A., Spellmeyer D. C., Wong A. K., Moos W. H.: J. Med. Chem. *38*, 1431 (1995).