imide and HOBt/HOAt for 10–60 min at 0°. Acetylation, when required, is done with acetic anhydride–DIEA (1 : 1, v/v, 30 equivalents) in the minimum amount of DMF. The ninhydrin test can be done on a few beads of the peptide–resin and analyzed with the help of a microscope.

Monitoring of Coupling Reaction

Monitoring of the coupling of protected peptides on solid supports is rather difficult. The ninhydrin test and amino acid analysis become less useful as the length of the peptide chain increases. The first is less sensitive, and the information that the second can provide is sometimes limited, as it can be difficult to judge the extent of incorporation of a new segment if it contains residues that are already present in the peptide sequence. We have used solid-phase automatic sequencing to calculate yields, by analyzing aliquots of the different peptide resins after each of the segment couplings.[17,22] Results from "one segment previews" will give the coupling yield, and those from "one amino acid previews" will assess the homogeneity of the protected peptide. Unfortunately, the availability of this technique in many laboratories is still limited. Finally, an indirect method for monitoring the coupling of protected peptides consists of removal of an aliquot of the resin after each coupling, cleavage of the peptide, and analysis of the free peptide by HPLC and MS.

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[16] Synthetic Peptide Libraries

By MICHAL LEBL and VIKTOR KRCHNÁK

Introduction

When two independent presentations at the 13th American Peptide Symposium in 1991 described the techniques for the synthesis and screening of synthetic peptide libraries,[1-4] only a few scientists were convinced that

[1] K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, and R. J. Knapp, *Nature (London)* **354,** 82 (1991).
[2] R. A. Houghten, C. Pinilla, S. E. Blondelle, J. R. Appel, C. T. Dooley, and J. H. Cuervo, *Nature (London)* **354,** 84 (1991).

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this technology may eventually change the approach to the discovery of new drugs in major pharmaceutical companies, and, moreover, that even small biotechnology companies and university laboratories will join in the quest for new drug leads. These techniques were considered an oddity, which, being limited to peptides, would never play a significant role in drug discovery.* However, several companies based on the combinatorial library concept were founded (Selectide, Houghten Pharmaceuticals, Pharmacopeia, Sphinx, and others), but when Ellman and colleagues[10] and a Parke-Davis group[11] showed that the same library principles can also be applied to small organic molecules, the real "gold rush" started. The plethora of scientific data accumulated in a relatively short time has already answered the question as to whether combinatorial chemistry will work. The scientific community has responded (Professor Arno Spatola organized the first combinatorial techniques course at The University of Louisville in spring of 1996), and it can be expected that new chemists will not be surprised when asked to synthesize half a million compounds for a screening project "next week."

The very origin of all multiple and combinatorial syntheses resides with the frustration of Merrifield with the tediousness of peptide synthesis in

[3] R. A. Houghten, J. H. Cuervo, C. Pinilla, J. R. Appel, C. T. Dooley, and S. E. Blondelle, *in* "Peptides: Chemistry and Biology" (J. A. Smith and J. E. Rivier, eds.), p. 560. Escom, Leiden, The Netherlands, 1992.

[4] K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, F. Al-Obeidi, W. M. Kazmierski, and R. J. Knapp, *in* "Peptides: Chemistry and Biology" (J. A. Smith, and J. E. Rivier, eds.), p. 492. Escom, Leiden, The Netherlands, 1992.

* This attitude has changed slightly after papers describing synthetic library techniques were published in *Nature*.[1,2] It is appropriate to mention here that the scientific community was ready to accept the library techniques in 1991. A study of multiple solid-phase peptide synthesis on glass chips using photolithography was already published,[5] as well as the techniques using genetic engineering for the display of modified proteins on surfaces of phage.[6–8] However, in 1986, when Mario Geysen submitted a paper describing synthesis and screening of peptide mixtures bound to plastic pins, several prestigious scientific journals refused to publish his paper (it was later published in *Molecular Immunology*[9]).

[5] S. P. A. Fodor, R. J. Leighton, M. C. Pirrung, L. Stryer, A. T. Lu, and D. Solas, *Science* **251,** 767 (1991).

[6] S. E. Cwirla, E. A. Peters, R. W. Barrett, and W. J. Dower, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 6378 (1990).

[7] J. J. Devlin, L. C. Panganiban, and P. E. Devlin, *Science* **249,** 404 (1990).

[8] J. K. Scott and G. P. Smith, *Science* **249,** 386 (1990).

[9] H. M. Geysen, S. J. Rodda, and T. J. Mason, *Mol. Immunol.* **23,** 709 (1986).

[10] B. A. Bunin and J. A. Ellman, *J. Am. Chem. Soc.* **114,** 10997 (1992).

[11] S. H. DeWitt, J. K. Kiely, C. J. Stankovic, M. C. Schroeder, D. M. R. Cody, and M. R. Pavia, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 6909 (1993).

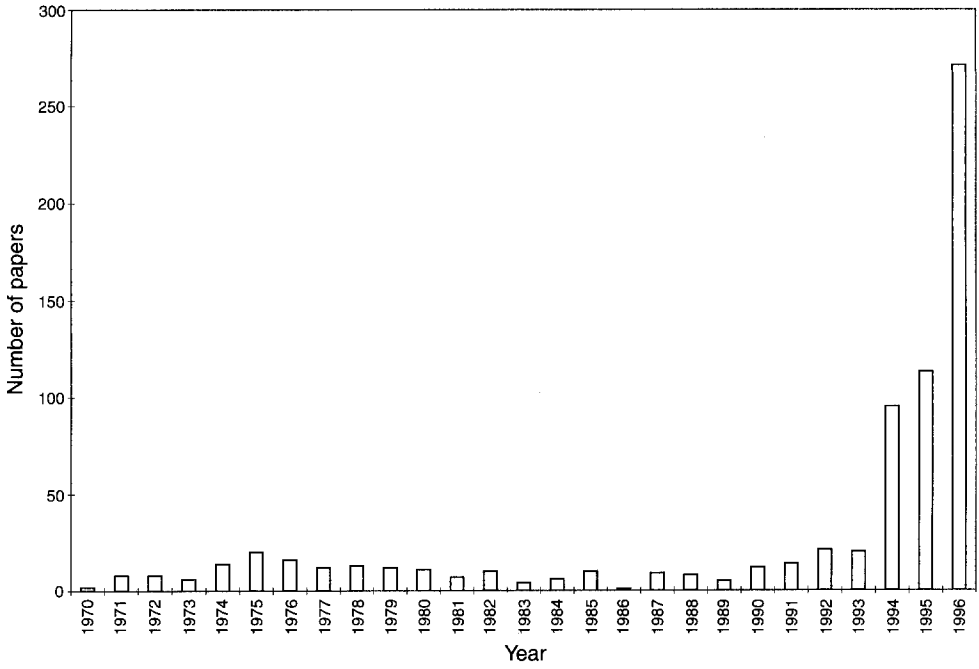

FIG. 1. Number of articles describing nonpeptide solid-phase chemistry.

solution.[12,13] The concept of synthesizing peptides on the solid phase seems obvious: a large excess of reagents is used to drive reactions to near completion, any soluble material is readily removed by filtration, and, owing to the fact that individual steps include either addition of liquid to the solid phase or separation of solid and liquid phases, the whole process can easily be automated. The advantages of the solid-phase concept are even more useful for multiple syntheses and the generation of combinatorial libraries. However, it took years to fine-tune all aspects of this fundamental synthesis concept. Merrifield's solid-phase synthesis was readily accepted by peptide chemists. Organic chemists, however, adopted it less enthusiastically. Only more recently has solid-phase synthesis become promoted among organic and medicinal chemists. Figure 1 illustrates the number of articles describing solid-phase synthesis of organic compounds (other than peptides) published between 1970 and 1996.

In this article we describe the currently available techniques for multiple and library synthesis of peptides. We focus on synthetic techniques that

[12] R. B. Merrifield, *J. Am. Chem. Soc.* **85,** 2149 (1963).
[13] S. Ostergaard and A. Holm, *Mol. Diversity* in press (1997).

can be immediately applied in most laboratories, that is, techniques that do not require major capital investment and instrumentation. All that is needed is the appropriate mind-set for the use of library techniques. We do not cover preparation of biological libraries, issues connected with library screening against particular targets, or results obtained in peptide library applications, and we also do not discuss various theoretical methods used for design of libraries.[14] We do not exhaustively review related literature, as this has been done by numerous authors.[15-26] Furthermore, a dynamic database of literature in the field of molecular diversity is available on the Internet.[27] Several reviews are dedicated to peptide libraries specifically (including peptide libraries generated by biological methods).[28-36] Practical

[14] W. A. Warr, *J. Chem. Inform. Comput. Sci.* **37,** 134 (1997).
[15] M. Rinnova and M. Lebl, *Collect. Czech. Chem. Commun.* **61,** 171 (1996).
[16] L. A. Thompson and J. A. Ellman, *Chem. Rev.* **96,** 555 (1996).
[17] J. S. Fruchtel and G. Jung, *Angew. Chem. Int. Ed.* **35,** 17 (1996).
[18] S. H. DeWitt and A. W. Czarnik, *Acc. Chem. Res.* **29,** 114 (1996).
[19] R. W. Armstrong, A. P. Combs, P. A. Tempest, S. D. Brown, and T. A. Keating, *Acc. Chem. Res.* **29,** 123 (1996).
[20] J. A. Ellman, *Acc. Chem. Res.* **29,** 132 (1996).
[21] W. C. Still, *Acc. Chem. Res.* **29,** 155 (1996).
[22] X. Williard, I. Pop, L. Bourel, D. Horvath, R. Baudelle, P. Melnyk, B. Deprez, and A. Tartar, *Eur. J. Med. Chem.* **31,** 87 (1996).
[23] V. Krchnák and M. Lebl, *Mol. Diversity* **1,** 193 (1996).
[24] C. Pinilla, J. Appel, C. T. Dooley, S. E. Blondelle, J. Eichler, B. Dorner, J. Ostresh, and R. A. Houghten, *in* "Peptide and Non-Peptide Libraries: A Handbook for the Search of Lead Structures" (G. Jung, ed.), p. 139. VCH, Weinheim, Germany, 1996.
[25] B. Dorner, S. E. Blondelle, C. Pinilla, J. Appel, C. T. Dooley, J. Eichler, J. M. Ostresh, E. Perez-Paya, and R. A. Houghten, *in* "Combinatorial Libraries: Synthesis, Screening and Application Potential" (R. Cortese, ed.), p. 1. de Gruyter, Berlin, 1996.
[26] J. M. Ostresh, S. E. Blondelle, B. Dorner, and R. A. Houghten, *Methods Enzymol.* 220 (1996).
[27] M. Lebl and Z. Leblova, Internet World Wide Web address: http://vesta.pd.com (1997).
[28] C. Pinilla, J. R. Appel, and R. A. Houghten, *in* "Immunological Recognition of Peptides in Medicine and Biology" (N. D. Zegers, W. J. A. Boersma, and E. Claassen, eds.), p. 1. CRC Press, Boca Raton, Florida, 1995.
[29] M. A. Gallop, R. W. Barrett, W. J. Dower, S. P. A. Fodor, and E. M. Gordon, *J. Med. Chem.* **37,** 1233 (1994).
[30] R. A. Houghten, *Trends Genet.* **9,** 235 (1993).
[31] J. K. Scott and L. Craig, *Curr. Opin. Biotechnol.* **5,** 40 (1994).
[32] C. Pinilla, J. R. Appel, S. E. Blondelle, C. T. Dooley, J. Eichler, J. M. Ostresh, and R. A. Houghten, *Drug Dev. Res.* **33,** 133 (1994).
[33] J. Eichler, J. R. Appel, S. E. Blondelle, C. T. Dooley, B. Dorner, J. M. Ostresh, E. Perez-Paya, C. Pinilla, and R. A. Houghten, *Med. Res. Rev.* **5,** 481 (1995).
[34] J. E. Fox, *Mol. Biotechnol.* **3,** 249 (1995).
[35] P. M. Dean, *Exp. Opin. Ther. Patents* **5,** 887 (1995).
[36] V. J. Hruby, *in* "The Practice of Medicinal Chemistry," p. 135. Academic Press, San Diego, 1996.

aspects of peptide library syntheses have also been covered in earlier review articles.[37–39]

Solid Support

Selection of the solid support depends on the type of library to be synthesized, as well as on the way it will be screened. The standard resin beads used for one-bead–one-compound libraries[1] are large enough to carry a sufficient amount of compound for bioassay; however, they should be homogeneously substituted. Furthermore, the resin beads should possess good mechanical properties; excessive fragility and tendency to form clusters (which may substantially lower the number of structures created) are the most common undesirable features. When bead-binding assays are used for the screening, the resin should swell both in organic solvents and aqueous media.

Polydimethylacrylamide or polyoxyethylene grafted polystyrene (TentaGel[40,41]) beads fulfill the above criteria. TentaGel, owing to its uniformity in size as well as its nonstickiness, is now the resin of choice for solid-phase library (both peptide and organic) synthesis based on the one-bead–one-compound principle. Polyethylene glycol–polystyrene (PEG–PS) resin[42] (Perseptive Biosystems, Bedford, MA) has a composition and properties similar to TentaGel. The major difference between the two resins is the location of the chemically reactive groups (anchor groups) in relation to the polystyrene matrix. Whereas TentaGel has the anchor groups at the end of long polyoxyethylene chains, far away from the polystyrene core, which promotes presentation of the library compounds for on-resin binding assays, PEG–PS may have the anchor groups in close proximity to the polystyrene core. In this case, the polyoxyethylene chains in PEG–PS do not serve as a spacer between the library compound and the polymer, but rather as a "modifier" of the polymer properties. ArgoGel (Argonaut

[37] M. Lebl, V. Krchnák, S. E. Salmon, and K. S. Lam, *Methods* (*San Diego*) **6,** 381 (1994).
[38] K. S. Lam and M. Lebl, *Methods* (*San Diego*) **6,** 372 (1994).
[39] R. A. Hougthen, *Methods* (*San Diego*) **6,** 354 (1994).
[40] W. Rapp, L. Zhang, R. Habich, and E. Bayer, *in* "Peptides 1988" (G. Jung and E. Bayer, eds.), p. 199. de Gruyter, Berlin, 1989.
[41] W. Rapp, *in* "Peptide and Non-Peptide Libraries: A Handbook for the Search of Lead Structures" (G. Jung, ed.), p. 425. VCH, Weinheim, Germany, 1996.
[42] G. Barany, F. Albericio, S. Biancalana, S. L. Bontems, J. L. Chang, R. Eritja, M. Ferrer, C. G. Fields, G. B. Fields, M. H. Lyttle, H. A. Sole, Z. Tian, R. J. Van Abel, P. B. Wright, S. Zalipsky, and D. Hudson, *in* "Peptides: Chemistry and Biology" (J. A. Smith and J. E. Rivier, eds.), p. 603. Escom, Leiden, The Netherlands, 1992.

Technologies, San Carlos, CA)[43] differs from TentaGel by having branched polyoxyethylene chains at the attachment point to the polystyrene core, resulting in a higher substitution compared to TentaGel (0.4 versus 0.2–0.3 mmol/g). Meldal's group[44–46] developed a polymer based on the copolymerization of acrylamide and polyoxyethylene that allows penetration of macromolecular targets (e.g., enzymes) into the interior of the beads (penetration of TentaGel by proteins is limited[47]). The more recently described highly cross-linked hydrophilic polymer CLEAR[48] may find its application in library synthesis soon. The compatibility of the resin with aqueous media and size uniformity are not critical for libraries that are cleaved from the resin and screened in solution, permitting the use of conventional resins, such as chloromethyl and benzhydrylamine polystyrene resins.

A variety of carriers in addition to resin beads have been used for library synthesis. The first peptide library was synthesized on polyacrylamide-grafted polypropylene "pins."[9] This support was found to be very useful for multiple peptide synthesis.[49] Larger scale synthesis was achieved by attaching higher substituted "crowns" to the pins.[50] Alternative solid support materials for peptide library synthesis include segmental planar carriers such as paper sheets[51–56] and cotton disks or squares.[57–64] Soluble

[43] O. Gooding, P. D. J. Hoeprich, J. W. Labadie, J. A. J. Porco, P. van Eikeren, and P. Wright, *in* "Molecular Diversity and Combinatorial Chemistry Libraries and Drug Discovery" (I. M. Chaiken and K. D. Janda, eds.), p. 199. American Chemical Society, Washington, D.C., 1996.

[44] M. Meldal, *Tetrahedron Lett.* **33,** 3077 (1992).

[45] M. Meldal, F. I. Auzanneau, O. Hindsgaul, and M. M. Palcic, *J. Chem. Soc., Chem. Commun.*, 1849 (1994).

[46] M. Meldal, F. I. Auzanneau, and K. Bock, *in* "Innovation and Perspectives in Solid Phase Synthesis" (R. Epton, ed.), p. 259. Mayflower Worldwide, Birmingham, UK, 1994.

[47] J. Vagner, G. Barany, K. S. Lam, V. Krchnák, N. F. Sepetov, J. A. Ostrem, P. Strop, and M. Lebl, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 8194 (1996).

[48] M. Kempe and G. Barany, *J. Am. Chem. Soc.* **118,** 7083 (1996).

[49] A. M. Bray, R. M. Valerio, A. J. Dipasquale, J. Greig, and N. J. Maeji, *J. Pept. Sci.* **1,** 80 (1995).

[50] N. J. Maeji, A. M. Bray, R. M. Valerio, and W. Wang, *Pept. Res.* **8,** 33 (1995).

[51] R. Frank and R. Doring, *Tetrahedron* **44,** 6031 (1988).

[52] R. Frank, *Bioorg. Med. Chem. Lett.* **3,** 425 (1993).

[53] R. Frank, *Tetrahedron* **48,** 9217 (1992).

[54] W. Tegge, R. Frank, F. Hofmann, and W. R. G. Dostmann, *Biochemistry* **34,** 10569 (1995).

[55] R. Frank, S. Hoffmann, M. Kiess, H. Lahmann, W. Tegge, C. Behn, and H. Gausepohl, *in* "Peptide and Non-Peptide Libraries: A Handbook for the Search of Lead Structures" (G. Jung, ed.), p. 363. VCH, Weinheim, Germany, 1996.

[56] R. Frank, *J. Biotechnol.* **41,** 259 (1995).

[57] J. Eichler and R. A. Houghten, *Biochemistry* **32,** 11035 (1993).

[58] M. Lebl and J. Eichler, *Pept. Res.* **2,** 297 (1989).

[59] J. Eichler, M. Bienert, A. Stierandova, and M. Lebl, *Pept. Res.* **4,** 296 (1991).

PEG carriers[65–67] have been proposed for library synthesis.[68,69] These supports are soluble in a variety of organic and aqueous solvents, enabling synthesis as well as screening of libraries in solution, but can be readily precipitated for removal of excess reagents by filtration.

Chemistry

This section focuses on the specifics of peptide library synthesis compared to the synthesis of individual peptides. In general, library synthesis requires greater emphasis on simplicity and reproducibility of the synthesis process.

Coupling

Typically, standard coupling reagents, such as active esters and carbodiimides, are used for library synthesis.[70–72] Difficult couplings can be driven to completion by more reactive coupling reagents such as TFFA[73] and *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-

[60] V. Pokorny, P. Mudra, J. Jehnicka, K. Zenísek, M. Pavlik, Z. Voburka, M. Rinnova, A. Stierandova, A. W. Lucka, J. Eichler, R. A. Houghten, and M. Lebl, *in* "Innovation and Perspectives in Solid Phase-Synthesis" (R. Epton, ed.), p. 643. Mayflower Worldwide, Birmingham, UK, 1994.

[61] M. Lebl, A. Stierandova, J. Eichler, M. Patek, V. Pokorny, J. Jehnicka, P. Mudra, K. Zenísek, and J. Kalousek, *in* "Innovation and Perspectives in Solid Phase Peptide Synthesis" (R. Epton, ed.), p. 251. Intercept, Andover, UK, 1992.

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[63] J. Eichler, M. Bienert, N. F. Sepetov, P. Stolba, V. Krchnák, O. Smekal, V. Gut, and M. Lebl, *in* "Innovations and Perspectives in Solid Phase Synthesis" (R. Epton, ed.), p. 337. SPCC, Birmingham, UK, 1990.

[64] J. Eichler, C. Pinilla, S. Chendra, J. R. Appel, and R. A. Houghten, *in* "Innovation and Perspectives in Solid Phase Synthesis" (R. Epton, ed.), p. 227. Mayflower Worldwide, Birmingham, UK, 1994.

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[71] J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis." Pierce, Rockford, Illinois, 1984.

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[73] L. A. Carpino and A. El-Faham, *J. Am. Chem. Soc.* **117,** 5401 (1995).

N-methylmethanaminium hexafluorophosphate *N*-oxide (HATU).[74] Couplings in peptide library synthesis are performed in the usual way (i.e., in a battery of bubblers, in closed plastic vials, in polypropylene syringes,[75] in tea bags,[76] or in microtiter plates[77]).

Once the length of a peptide exceeds about four amino acids, the tendency to acylate the amino groups is more influenced by the character of the acylated peptide than the acylating agent.[78–81] Because libraries are synthesized in most cases from 20 proteinogenic amino acids, the library will also contain the most difficult sequences. At the same time, the library will also contain sequences prone to side reactions, for example, alkylation of Trp nucleus during deprotection of 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc)-protected Arg-X-Trp peptides.[82] Such complications are unavoidable at the present time. Because we cannot know *a priori* whether a certain biological response is caused by desired peptide or a side product, the reproducibility of the chemical process is of ultimate importance.

Monitoring

Monitoring of acylation requires modified techniques compared to the synthesis of individual peptides. In the "split synthesis" method[1,2,83] different peptides are synthesized on each resin bead, and therefore coupling kinetics may be different for each bead. Whether the coupling reaction is complete in a resin sample is not relevant, as it is necessary to know whether all beads in the sample were coupled. The classic ninhydrin test[84] does not provide this information. However, the reaction can be followed at the level of individual beads using nondestructive methods, such as bromphenol blue monitoring.[85] Using this method, incompletely coupled (blue) individual beads can be identified among a vast majority of completely coupled (colorless) beads.

[74] L. A. Carpino, A. Elfaham, and F. Albericio, *Tetrahedron Lett.* **35,** 2279 (1994).
[75] V. Krchnák and J. Vagner, *Pept. Res.* **3,** 182 (1990).
[76] R. A. Houghten, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 5131 (1985).
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[78] V. Krchnák, Z. Flegelova, and J. Vagner, *Int. J. Pept. Protein Res.* **42,** 450 (1993).
[79] R. C. de L. Milton, S. C. F. Milton, and P. A. Adams, *J. Am. Chem. Soc.* **112,** 6039 (1990).
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[81] S. B. H. Kent, *Annu. Rev. Biochem.* **57,** 957 (1988).
[82] A. Stierandova, N. F. Sepetov, G. V. Nikiforovich, and M. Lebl, *Int. J. Pept. Protein Res.* **43,** 31 (1994).
[83] A. Furka, F. Sebestyen, M. Asgedom, and G. Dibo, *Int. J. Pept. Protein Res.* **37,** 487 (1991).
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Equally valuable is information about the total amount of 9-fluorenylmethyloxycarbonyl (Fmoc) group incorporated (and subsequently cleaved) relative to the entire mass of the resin used for the synthesis. We strongly recommend spectrophotometric quantitation of the cleaved Fmoc group after each step. The deprotection is usually performed after the resin is recombined, and one measurement can detect the major synthetic problems (premature cleavage of the peptide from the resin, use of improperly protected amino acids, etc.).

Protection, Deprotection, and Cleavage

Library synthesis involves handling of large numbers of reaction vessels at the same time. Because of the ease of manipulation and the simplicity of deprotection in multiple vessels in parallel, the Fmoc/*tert*-butyl (*t*Bu) protection strategy is mostly favored over the *tert*-butyloxycarbonyl/benzyl (Boc/Bzl) protection scheme. When a typical linker for either strategy is used to anchor the C-terminal amino acid, trifluoroacetic acid (TFA) or HF is applied to remove the side-chain protecting groups and at the same time cleave the peptide from the resin.

To facilitate the TFA cleavage process, reaction vessels, such as the wells of a microtiter plate, can be placed in an evacuated centrifuge, and the TFA can be evaporated simultaneously from all wells. Different multiple parallel processing methods must be applied when evaporation would harm the product. In this case, the TFA solution can be applied in small increments to reaction vessels, equipped at the bottom with a frit, and placed on top the grid of receiving vessels that contain a precipitating solvent (e.g., ether). This arrangement minimizes exposure of the products to TFA and cationic species generated from protecting groups and may, in some cases, eliminate the need to use scavengers.

Cleavage with liquid hydrogen fluoride (HF) is practical when an apparatus equipped with multiple reaction vessels is available.[86] Handling of HF is not very popular in many laboratories, but its low boiling point, combined with the well-understood chemistry of cleavage,[87] makes it a very flexible reagent. Furthermore, the very stable bond between the peptides and the polymeric carrier enables the use of a wide range of reagents for peptide and nonpeptide synthesis, as well as peptide modifications (see below).

In general, it is beneficial for library syntheses to remove the side-chain protecting groups prior to the peptide cleavage, thus avoiding contamina-

[86] R. A. Houghten, M. K. Bray, S. T. DeGraw, and C. J. Kirby, *Int. J. Pept. Protein Res.* **27,** 673 (1986).

[87] J. P. Tam, W. F. Heath, and R. B. Merrifield, *J. Am. Chem. Soc.* **105,** 6442 (1983).

tion of the library with cleaved protecting groups and scavengers. One way to separate side-chain deprotection from final cleavage of the peptides from the resin is the use of TFA–cleavable side-chain protecting groups in combination with a TFA-stable, HF-cleavable peptide–resin linkage.[75] Alternatively, the high/low HF procedure[87] can be used.

Application of gaseous reagents is an elegant method of multiple sample processing. Ester bonds between the peptides and the solid support can be cleaved by gaseous ammonia[88,89] or ammonia under high pressure.[90] Hydrogen chloride, hydrogen fluoride, and trifluoroacetic acid[91,92] can be applied in the form of gas as well. The use of gaseous reagents, as well as light, for peptide cleavage has one significant advantage; the cleaved peptides (or other compounds) remain physically located inside the resin beads, and individual beads can be handled either in the dry state or as a suspension in a nonextracting solvent (e.g., petroleum ether, ether) without cross-contamination of the cleaved compounds. The peptides can later be physically released from the beads for the bioassay (e.g., into the wells of the assay plate or onto the surface of an agar layer containing a cell culture) on addition of an extracting solvent, such as an aqueous buffer, methanol, or *N,N*-dimethylformamide (DMF), or by slow diffusion of the extracting solvent into the beads (e.g., on the surface of agar).[91,93]

Linkers for Library Synthesis

The decision whether to use a cleavable linker for the attachment of the first amino acid to the solid support depends on the intended use of the library. For on-bead binding assays, the use of linkers is not only unnecessary, but also contraindicated, as it may interfere with the binding of the macromolecular target to the peptide on the bead. Spacers between the peptide and the solid support, however, are beneficial in that they may improve the recognition of the peptide by the target molecule. Some resins

[88] A. M. Bray, N. J. Maeji, A. G. Jhingran, and R. M. Valerio, *Tetrahedron Lett.* **32,** 6163 (1991).

[89] A. M. Bray, R. M. Valerio, and N. J. Maeji, *Tetrahedron Lett.* **34,** 4411 (1993).

[90] M. Flegel, L. Lepsa, Z. Panek, I. Blaha, and M. Rinnova, *in* "Peptides: Chemistry, Structure and Biology" (P. T. P. Kaumaya and R. S. Hodges, eds.), p. 119. Mayflower Scientific, Kingswinford, UK, 1995.

[91] C. K. Jayawickreme, G. F. Graminski, J. M. Quillan, and M. R. Lerner, *Proc. Natl. Acad. Sci. U.S.A.* **91,** 1614 (1994).

[92] V. Krchnák, A. S. Weichsel, D. Cabel, and M. Lebl, *in* "Molecular Diversity and Combinatorial Chemistry. Libraries and Drug Discovery" (I. M. Chaiken and K. D. Janda, eds.), p. 99. American Chemical Society, Washington, DC, 1996.

[93] S. E. Salmon, R. H. Liu-Stevens, Y. Zhao, M. Lebl, V. Krchnák, K. Wertman, N. Sepetov, and K. S. Lam, *Mol. Diversity* **2,** 57 (1996).

are already equipped with long inert spacers (e.g., polyoxyethylene in TentaGel).

When the library compounds are intended to be screened in solution, the peptides have to be cleaved from the solid support. An ideal linker for combinatorial synthesis of peptide libraries should meet several criteria: (i) it must be stable to all chemical reactions during the library synthesis; (ii) it should enable quantitative cleavage of the peptides from the resin; (iii) the cleavage reagents should not alter the library compounds; and (iv) the cleavage conditions should not require complicated equipment and, preferably, should render the released compounds in a state ready for screening.

The linkers primarily used for standard peptide synthesis were designed in a way that the cleavage cocktail not only cleaves the peptides from the resin, but at the same time removes all side-chain protecting groups. For peptide libraries, however, where the intent is not to isolate the final products, but rather to test the library for various biological activities, the concurrent removal of protecting groups and cleavage of peptides from the resin is not necessarily beneficial. Therefore, several linkers were devised that enable deprotection of side chains while leaving the peptides on the resin. This step, which usually requires the use of scavengers, can eliminate any contamination of peptides by products of protecting groups, as the resin can be extensively washed prior to peptide cleavage. The peptides can then be cleaved under relatively mild conditions, preferably in aqueous media, providing solutions ready for the bioassay.

The library compounds can be cleaved from resin beads in one single step, or the release of compounds can be gradual, in several steps. Multiply cleavable linkers are particularly useful for one-bead–one-compound libraries, in that they enable the repeated release of library compounds for different levels of library screening, thus eliminating deconvolution steps.

Singly Cleavable Linkers

Linkers used most frequently for the synthesis of libraries were those developed for individual peptide synthesis. The most popular linkers for peptide acids and amides are acid-labile, and their core structures are based on benzyl structures (Fig. 2). Whereas benzyl esters yield carboxylates on cleavage from the resin, benzyl amide linkers were designed to generate C-terminal carboxamides. Benzyl (and other) esters can also be advantageously cleaved by various nucleophiles.

The original Merrifield resin[12] is still widely used for the generation of peptide acids using the Boc/Bzl protection scheme. Its acid lability was

FIG. 2. Benzyl-based linkers cleavable by acids.

increased for Fmoc/*t*Bu strategy by substituting the benzyl ring with electron donating groups (e.g., Wang[94] and Sheppard[95] linkers). Additional methoxy groups further increase the acid lability, as documented by the SASRIN[96] and 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid (PAL)[97] resins.

Because of the extremely high stability of benzyl amides to acids, benzhydrylamine type linkers[98] were developed for the synthesis of peptide amides. The *p*-methylbenzhydrylamine linker,[99] which requires liquid HF for peptide cleavage, is widely used for mixture libraries. Owing to its stability to a vast range of reagents, it is compatible with a variety of organic reactions used to modify resin-bound peptides. The acid stability of benzhydrylamine linkers was reduced by methoxy substituents,[100] which makes this linker cleavable by TFA. The most acid-labile linker is the trityl linker,[101,102] to which acids, alcohols, thiols, as well as amines can be attached. In addition to liquid cleavage cocktails, it can be cleaved by HCl gas or TFA vapors.[92]

Safety-catch linkers contain electron-withdrawing groups that increase their acid stability. Prior to the release of the peptides, these groups are chemically transformed into electron-donating groups, which makes the linker more acid labile, thus enabling the use of Boc-protecting groups in combination with TFA cleavage of the peptides from the resin. In the SCAL linker,[103,104] the electron-withdrawing methyl sulfoxide is reduced

[94] S. S. Wang, *J. Am. Chem. Soc.* **95,** 1328 (1973).
[95] R. C. Sheppard and B. J. Williams, *Int. J. Pept. Protein Res.* **20,** 451 (1982).
[96] M. Mergler, R. Tanner, J. Gosteli, and P. Grogg, *Tetrahedron Lett.* **29,** 4005 (1988).
[97] F. Albericio, N. Kneib-Cordonier, S. Biancalana, L. Gera, R. I. Masada, D. Hudson, and G. Barany, *J. Org. Chem.* **55,** 3730 (1990).
[98] J. P. Tam, R. D. DiMarchi, and R. B. Merrifield, *Tetrahedron Lett.* **22,** 2851 (1981).
[99] G. R. Matsueda and J. M. Stewart, *Peptides* **2,** 45 (1981).
[100] H. Rink, *Tetrahedron Lett.* **28,** 3787 (1987).
[101] K. Barlos, D. Gatos, I. Kallitsis, D. Papaioannou, and P. Sitiriou, *Liebigs Ann. Chem.*, 1079 (1988).
[102] K. Barlos, O. Chatzi, D. Gatos, and G. Stavropoulos, *Int. J. Pept. Protein Res.* **37,** 513 (1991).
[103] M. Patek and M. Lebl, *Tetrahedron Lett.* **31,** 5209 (1990).
[104] M. Patek and M. Lebl, *Tetrahedron Lett.* **32,** 3891 (1991).

to a methylthio group after the peptide has been assembled, prior to its release from the resin with TFA. A similar principle was applied to a benzyl ester linker by Kiso *et al.*[105]

Nucleophilic cleavage of ester bonds, for example, those formed from hydroxymethylbenzoic acid as a linker, can be used to release peptides directly into the screening solution. The formation of diketopiperazines (DKPs) from dipeptide esters, a well-documented side reaction in peptide synthesis, was used for the design of linkers that are cleavable under very mild conditions (e.g., pH 7).[106] Peptides cleaved in this manner, however, still have the DKP moiety attached to them. To overcome this limitation, Hoffmann and Frank developed a new type of safety-catch linker based on the intramolecular catalytic hydrolysis of an ester bond. This linker enables the release of free peptide acids into a neutral aqueous buffer.[107]

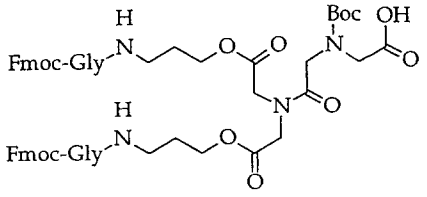
Multiply Cleavable Linkers

Peptide libraries often contain millions of individual peptides, which cannot be screened one by one. Although mixture libraries are screened in large pools of up to millions of peptides, a two-step procedure for the screening in solution of one-bead–one-peptide libraries was designed that involves the release of peptides from the beads in two separate steps.[37,108]

Three different types of multiply releasable linkers have been proposed. The first one, used at Selectide, is based on two different types of cleavage of an ester bond.[37,108,109] One ester bond is cleaved through DKP formation (see above), and the second by alkali or ammonia. These two reactions, however, do not yield exactly the same compounds, because the DKP moiety is still attached to the peptide cleaved in the first step.

To overcome this limitation, a second generation of multiply cleavable linkers was developed, in which the DKP remained on the resin. Peptides are attached to the linker via an ester bond of Fmoc-Gly-NH-$(CH_2)_3$-OH (Fmoc-Gly-HOPA), and after release to an aqueous solution, both copies of peptides have an identical C terminus, that is, the hydroxypropylamide of Gly (Gly-HOPA). The first linker of this generation was based on the Glu-Pro dipeptide. Glu provided a side-chain function for the attachment to the resin, and Pro enhanced the tendency to form the DKP. In another

[105] Y. Kiso, T. Fukui, S. Tanaka, T. Kimura, and K. Akaji, *Tetrahedron Lett.* **35,** 3571 (1994).
[106] A. M. Bray, N. J. Maeji, R. M. Valerio, R. A. Campbell, and H. M. Geysen, *J. Org. Chem.* **56,** 6659 (1991).
[107] S. Hoffmann and R. Frank, *Tetrahedron Lett.* **35,** 7763 (1994).
[108] S. E. Salmon, K. S. Lam, M. Lebl, A. Kandola, P. S. Khattri, S. Wade, M. Patek, P. Kocis, V. Krchnák, D. Thorpe, and S. Felder, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 11708 (1993).
[109] M. Lebl, M. Patek, P. Kocis, V. Krchnák, V. J. Hruby, S. E. Salmon, and K. S. Lam, *Int. J. Pept. Protein Res.* **41,** 201 (1993).



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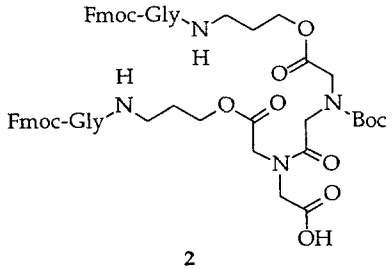


2

FIG. 3. Two doubly cleavable linkers (**1, 2**) based on the Ida–Ida motif.

linker, the core dipeptide was formed from two iminodiacetic acid residues (Ida).[110] The advantages of Ida include the presence of two chemically equivalent carboxyl groups, a high tendency to cyclize via DKP (a characteristic feature of N-substituted amino acids), and its low price. Two Fmoc-Gly-HOPAs are either coupled to both carboxyl groups of the C-terminal Ida (Fig. 3, linker **1**), or each Ida is linked to one Fmoc-Gly-HOPA (linker **2**). The remaining carboxyl group was used to attach the linker to the resin. The chemistry of both releases is shown in Fig. 4. A third, noncleavable copy of the peptide is attached to enable structure determination by sequencing from the resin beads. To avoid the presence of the hydroxypropylamide of Gly at the C terminus of released peptides, a modified linker (Fig. 5, linker **3**) was designed. It includes an additional ester linkage,[111] which is hydrolyzed after the release of the peptides from the resin beads.

Pharmacopeia scientists, who followed the Lam one-bead–one-compound library approach, used a photolabile linker of the *o*-nitrobenzyl type[112] (Fig. 6, linker **4**). The original linker, which yields a reactive resin-

[110] P. Kocis, V. Krchnák, and M. Lebl, *Tetrahedron Lett.* **34,** 7251 (1993).

[111] V. Krchnák, N. F. Sepetov, P. Kocis, M. Patek, K. S. Lam, and M. Lebl, *in* "Combinatorial Libraries: Synthesis, Screening and Application Potential" (R. Cortese, ed.), p. 27. de Gruyter, Berlin, 1996.

[112] J. J. Baldwin, J. J. Burbaum, I. Henderson, and M. H. J. Ohlmeyer, *J. Am. Chem. Soc.* **117,** 5588 (1995).

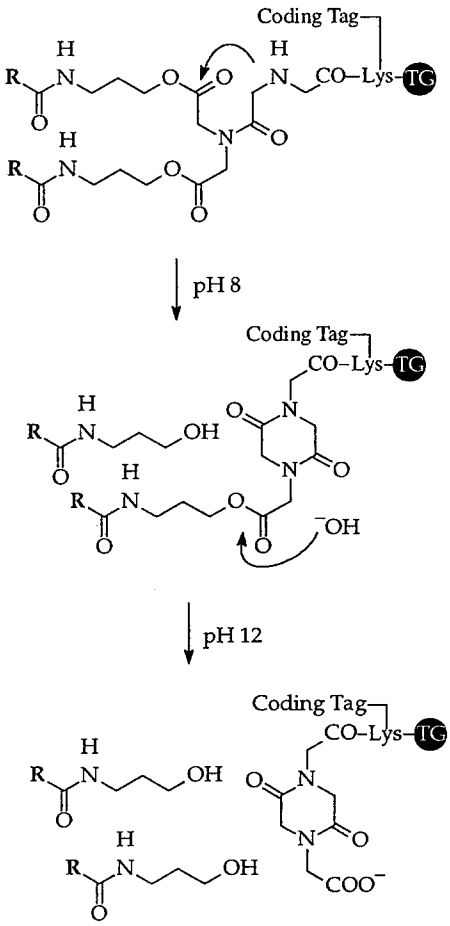
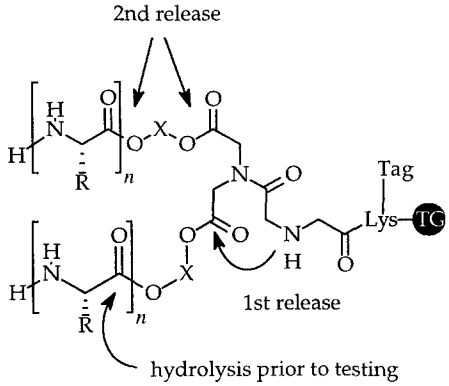


FIG. 4. Chemistry of both releases from a doubly cleavable linker. TG, TentaGel.

bound aldehyde, has been modified to produce less reactive ketone derivatives on cleavage (Fig. 6, linkers **5** and **6**).[113,114]

The third type of linkers for gradual release are based on the varying stability of different benzyl structures toward acids. Partial release of peptides from a benzhydrylamine linker by TFA vapors was used by Jayawickreme *et al.*[91] Cardno and Bradley[115] used a combination of benzyl linkers

[113] C. P. Holmes and D. G. Jones, *J. Org. Chem.* **60,** 2318 (1995).
[114] B. B. Brown, D. S. Wagner, and H. M. Geysen, *Mol. Diversity* **1,** 4 (1995).
[115] M. Cardno and M. Bradley, *Tetrahedron Lett.* **37,** 135 (1996).



3

FIG. 5. A doubly cleavable linker (**3**) that yields peptide acids.

(Fig. 7). The most acid-labile linker can be cleaved with 1% TFA, whereas 95% TFA is necessary to cleave the second linker. The third copy of the same compound remains attached to the resin for structure analysis or on-bead binding assays. It can, however, be cleaved by HF, if needed. Alternative designs of linkers allowing gradual release of library compounds has been reviewed.[116]

Multiple Synthesis Techniques

Solid-phase synthesis is amenable to parallel processing.[117] Individual cycles of peptide synthesis are in most cases composed of identical steps, the only difference being the kind of amino acid used for acylation of the growing peptide chain. Therefore, it was not surprising that most library synthesis methods were adapted from existing techniques for multiple parallel synthesis.

The surface of plastic (polypropylene) pins grafted with an easily functionalizable polymeric matrix (polystyrene or polyacrylamide) was used as the solid support for the first method of multiple peptide synthesis.[118] The pins are attached to a plate and arranged in the 96-well microtiter plate

[116] D. Madden, V. Krchnák, and M. Lebl, *Perspect. Drug Disc. Design* **2,** 269 (1995).
[117] G. Jung and A. G. Beck-Sickinger, *Angew. Chem., Int. Ed. Engl.* **31,** 367 (1992).
[118] H. M. Geysen, R. H. Meloen, and S. J. Barteling, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 3998 (1984).

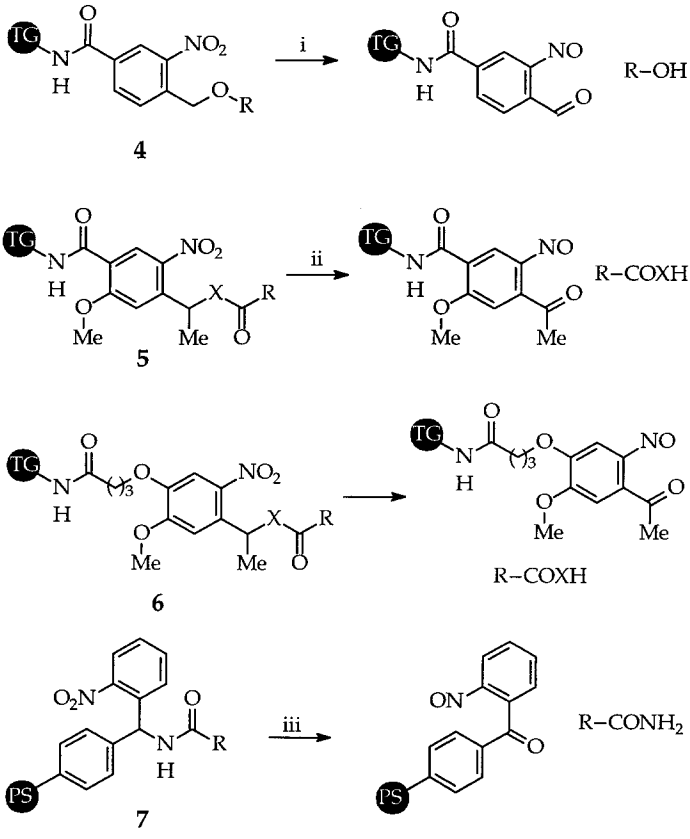


FIG. 6. Photocleavable linkers (**4–7**).

format, so that the wells of microtiter plates can serve as the reaction vessels for all synthesis steps. Originally, the peptides remained on the pins and were tested immobilized for binding to antibodies. Later, the substitution of the pins was increased to micromole amounts, and linkers were introduced, enabling cleavage and isolation of the peptides from the pins.[9,118–124]

[119] N. J. Maeji, A. M. Bray, R. M. Valerio, M. A. Seldon, J. X. Wang, and H. M. Geysen, *Pept. Res.* **4,** 142 (1991).

[120] R. M. Valerio, A. M. Bray, R. A. Campbell, A. J. Dipasquale, C. Margellis, S. J. Rodda, H. M. Geysen, and N. J. Maeji, *Int. J. Pept. Protein Res.* **42,** 1 (1993).

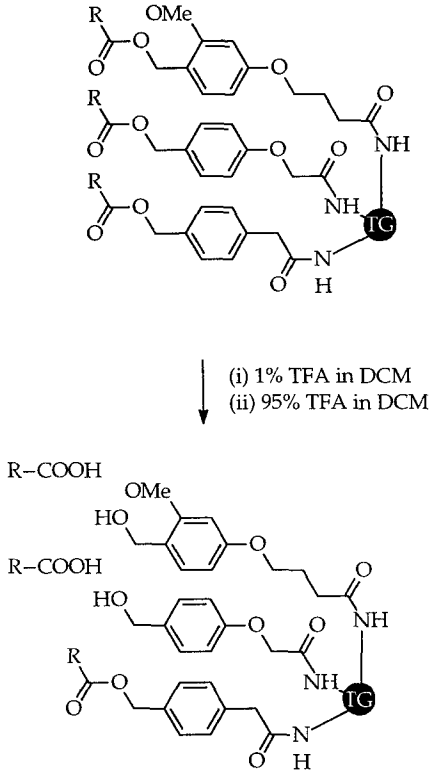


FIG. 7. Acid-sensitive multiply cleavable linker assembly.[115]

Another pioneering multiple peptide synthesis approach, termed the "tea bag" method, was introduced by Houghten.[76] The idea of this technique is very simple: If the solid support can be compartmentalized in separate packets (polypropylene mesh bags, Fig. 8), all common processes of peptide synthesis, such as wash and deprotection steps, can be performed simultaneously on many resin packets without cross-contamination. For the synthesis of peptides composed of the 20 proteinogenic amino acids, only 20 containers with activated amino acids, and two common containers

[121] J. X. Wang, A. M. Bray, A. J. Dipasquale, N. J. Maeji, and H. M. Geysen, *Int. J. Pept. Protein Res.* **42,** 384 (1993).
[122] H. M. Geysen and T. J. Mason, *Bioorg. Med. Chem. Lett.* **3,** 397 (1993).
[123] A. M. Bray, N. J. Maeji, and H. M. Geysen, *Tetrahedron Lett.* **31,** 5811 (1990).
[124] N. J. Maeji, R. M. Valerio, A. M. Bray, R. A. Campbell, and H. M. Geysen, *React. Polym.* **22,** 203 (1994).

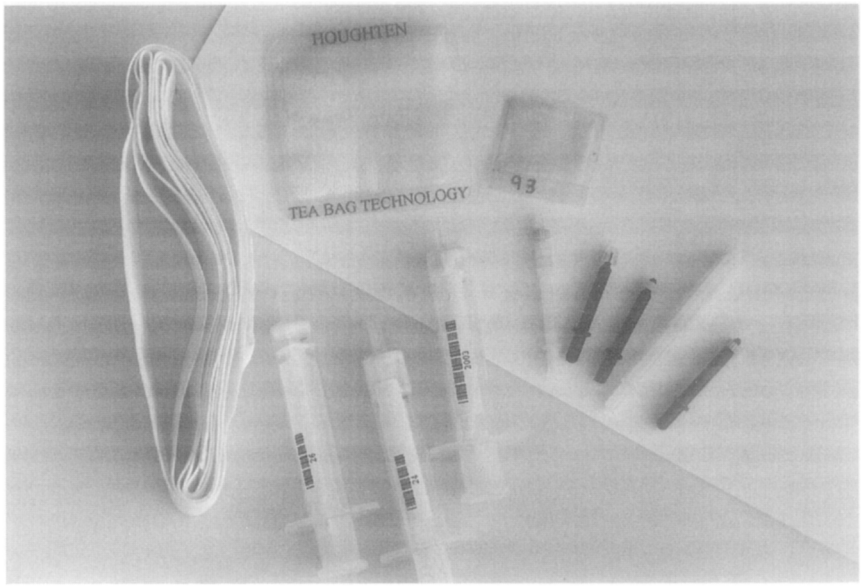


FIG. 8. Tools for simple multiple peptide synthesis: T-bag,[70] plastic syringe,[75] paper sheet,[52] cotton strip,[59] and plastic pin.[118]

for deprotection and wash steps, respectively, are needed. Up to several hundred labeled resin packets can be processed simultaneously. For common steps, they are placed in one big container, followed by sorting for individual couplings. Sorting of the bags can be facilitated by adding a radiofrequency tag to each bag.[125,126] This procedure is repeated several times, until the peptide sequences in all bags are completed. The resin used for this method is typically 4-methylbenzhydrylamine (MBHA) resin, and the peptides are deprotected and cleaved from the resin using the low/high HF method[87] in combination with multiple cleavage apparatus, which enables the simultaneous cleavage of up to 120 peptides.[86]

Polypropylene syringes equipped with a sintered polypropylene disks[75] are probably the simplest and least expensive disposable reaction vessel for solid-phase synthesis, enabling multiple peptide synthesis to be carried out in any laboratory. One 10-ml plastic syringe can hold up to 500 mg of resin. We have found the use of syringes as convenient reaction vessels for

[125] R. W. Armstrong, P. A. Tempest, and J. F. Cargill, *Chimia* **50,** 258 (1996).
[126] E. J. Moran, S. Sarshar, J. F. Cargill, M. M. Shahbaz, A. Lio, A. M. M. Mjalli, and R. W. Armstrong, *J. Am. Chem. Soc.* **117,** 10787 (1995).

the synthesis of peptide and especially nonpeptide libraries.[127] The synthesis protocol follows the common procedure for batchwise peptide synthesis using either Boc/Bzl or Fmoc/*t*Bu protection strategy. The syringes containing the resin are manually filled with the solvent or reagent of the particular synthesis step, shaken, and emptied. During library synthesis using the split/mix protocol, deprotection and all wash steps are done in one common reaction vessel (big syringe, classic glass bubbler, etc.), followed by distributing the resin slurry into as many syringes as amino acids are used in the next coupling. The syringes are typically kept on a tumbler during the couplings, which can be accelerated in a hot (70°) DMF bath[128,129] or by sonication.[130]

Manual parallel handling of a large number of syringes, although feasible, is not very convenient. Therefore, we constructed a block holding 42 syringes, termed MultiBlock (http://www.5z.com/csps.htm), to facilitate and accelerate multiple synthesis in syringes. The MultiBlock (Fig. 9) consists of five parts: a Teflon block that holds 42 polypropylene syringes equipped with a plastic frit; a vacuum adapter that connects each reactor to a vacuum line, enabling rapid washing of the resin under continuous flow; two Teflon plates with flexibly attached 42 stoppers to seal the syringes during reactions; and a glass cover to allow mixing of resin for split/mix library synthesis. The MultiBlock is made from Teflon, polypropylene, glass, and stainless steel. A number of manufacturers have introduced their own versions (http://www.charybtech.com),[131–133] most of them designed for general organic synthesis on the solid phase with the option to be coupled to an automatic liquid delivery station.

A method for the concurrent synthesis of 96 peptides in the wells of commercially available polypropylene deep-well microtiter plates was reported by Schnorrenberg and Gerhardt.[134] The wells of microtiter plates have no frits or filters at the bottom. Solvent and solutions are introduced by a pipetting device. For the removal of liquid the tip of their washing device is protected by a narrow stainless steel net. One well is handled at

[127] V. Krchnák, A. S. Weichsel, D. Cabel, Z. Flegelova, and M. Lebl, *Mol. Diversity* **1,** 149 (1996).

[128] K. Barlos, *Liebigs Ann. Chem.* **11,** 1950 (1986).

[129] J. P. Tam and Y. A. Lu, *J. Am. Chem. Soc.* **117,** 12058 (1995).

[130] J. Vagner, P. Kocna, and V. Krchnák, *Pept. Res.* **4,** 1 (1991).

[131] J. F. Cargill and R. R. Maiefski, *Lab. Robotics Automation* **8,** 139 (1996).

[132] J. R. Harness, *in* "Molecular Diversity and Combinatorial Chemistry: Libraries and Drug Discovery" (I. M. Chaiken and K. D. Janda, eds.), p. 188. American Chemical Society, Washington, DC, 1996.

[133] H. V. Meyers, G. J. Dilley, T. L. Durgin, T. S. Powers, N. A. Winssinger, H. Zhu, and M. R. Pavia, *Mol. Diversity* **1,** 13 (1995).

[134] G. Schnorrenberg and H. Gerhardt, *Tetrahedron* **45,** 7759 (1989).

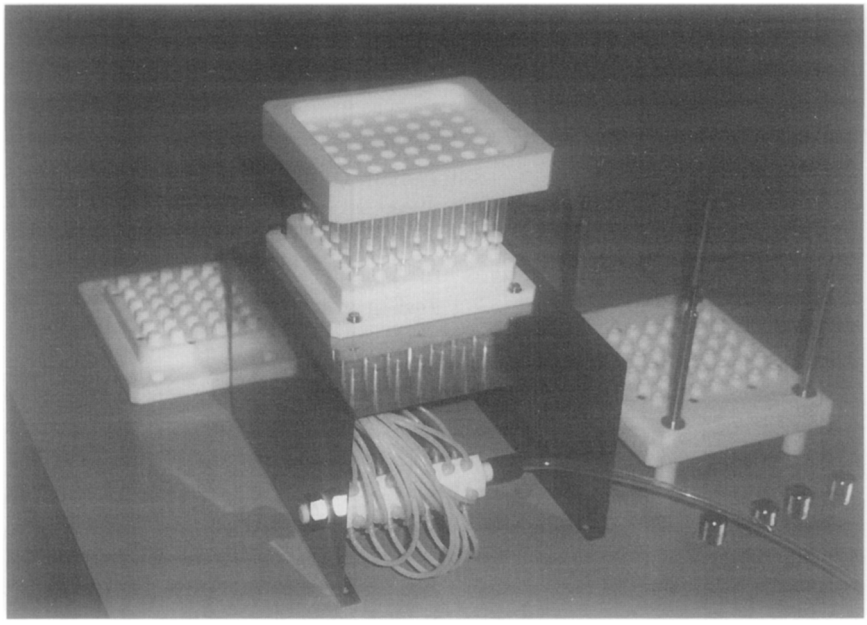


FIG. 9. MultiBlock (with permission of CSPS, San Diego, CA).

a time. An alternative approach has been described.[77] The solvent is aspirated by suction from the surface of the liquid after the resin beads have settled. Vacuum is applied to stainless steel needles that are slowly immersed into the wells. Solid-phase synthesis in modified microtiter plates where each well is equipped with a frit has also been reported.[133]

Planar segmental supports, such as cellulose membranes[51–55] and cotton,[58–64] have been shown to be versatile carriers for multiple synthesis. The substitution of these carriers must be sufficiently low, so that the amount of reagent solutions soaked into the carrier is high enough to ensure complete couplings. This principle of "inclusion volume coupling," which was later also tested on conventional resins in combination with applying centrifugation for liquid removal,[135] enables (i) the use of higher concentrations of activated amino acids, resulting in increased coupling rates, (ii) drastically decreased consumption of solvents, and (iii) the construction of multiple peptide synthesizers with virtually no reaction vessels, as the solid carriers themselves serve as the reaction vessels.[60]

[135] J. Eichler, R. A. Houghten, and M. Lebl, *J. Pept. Sci.* **2,** 240 (1996).

A synthesis method based on the attachment of the growing peptide chain to a glass surface, and using photolithographic techniques, was developed at Affymax.[5,136–139] A photolabile amino protecting group is cleaved on defined locations on the glass surface, and the whole surface is subjected to coupling with an activated amino acid. After completion of the coupling, other surface locations are deprotected, and the second amino acid attached. This process is repeated until all amino acids are attached to their particular locations, thus creating a surface covered with different peptides. That technology requires highly sophisticated instrumentation, which makes its widespread application unlikely. Various automated multiple peptide synthesizers have been constructed, some of which are commercially available.[34,134,140–150]

One-Bead–One-Compound Libraries

Having millions of peptides mixed together in one vessel, yet at the same time having each resin bead carry only one peptide, is achievable using the so-called split/mix, portioning/mixing, or divide–couple–recombine technique. This method was first reported in 1988 by Furka *et*

[136] C. Y. Cho, E. J. Moran, S. R. Cherry, J. C. Stephans, S. P. A. Fodor, C. L. Adams, A. Sundaram, J. W. Jacobs, and P. G. Schultz, *Science* **261,** 1303 (1993).

[137] L. F. Rozsynai, D. R. Benson, S. P. A. Fodor, and P. G. Schultz, *Angew. Chem., Int. Ed. Engl.* **31,** 759 (1992).

[138] J. W. Jacobs and S. P. A. Fodor, *Trends Biotechnol.* **12,** 19 (1994).

[139] C. P. Holmes, C. L. Adams, S. P. A. Fodor, and P. Yu-Yang, *in* "Perspectives in Medicinal Chemistry" (B. Testa, E. Kyburz, W. Fuher, and R. Giger, eds.), p. 489. VHCA, Basel, 1992.

[140] H. Gausepohl and R. W. Frank, *in* "Peptides 1992" (C. H. Schneider and A. N. Eberle, eds.), p. 310. Escom, Leiden, The Netherlands, 1993.

[141] R. N. Zuckermann, M. A. Siani, and S. C. Banville, *Lab. Robotics Automation* **4,** 183 (1992).

[142] H. Gausepohl, C. Boulin, M. Kraft, and R. W. Frank, *Pept. Res.* **5,** 315 (1992).

[143] G. Schnorrenberg, *Chim. Oggi.* **10,** 33 (1992).

[144] K. Nokihara and R. Yamamoto, *in* "Peptides: Chemistry and Biology" (J. A. Smith and J. E. Rivier, eds.), p. 507. Escom, Leiden, The Netherlands, 1992.

[145] J. E. Fox, *Biochem. Soc. Trans.* **20,** 851 (1992).

[146] V. Krchnák, D. Cabel, and M. Lebl, *Pept. Res.* **9,** 45 (1996).

[147] T. Luu, S. Pham, and S. Deshpande, *Int. J. Pept. Protein Res.* **47,** 91 (1996).

[148] J. A. Boutin, P. Hennig, P. H. Lambert, S. Bertin, L. Petit, J. P. Mahieu, B. Serkiz, J. P. Volland, and J. L. Fauchere, *Anal. Biochem.* **234,** 126 (1996).

[149] T. Geiser, H. Beilan, B. J. Bergot, and K. M. Otteson, *in* "Macromolecular Sequencing and Synthesis: Selected Methods and Applications" (D. H. Schlesinger, ed.), p. 199. Alan R. Liss, New York, 1988.

[150] H. H. Saneii and J. D. Shannon, *in* "Innovation and Perspectives in Solid Phase Synthesis" (R. Epton, ed.), p. 335. Intercept, Andover, UK, 1994.

al.[83,151,152] and independently used for the generation of peptide libraries by Houghten *et al.*[2] and Lam *et al.*[1] It is the method of choice for one-bead–one-compound libraries, which are based on the fact that each resin bead has only one peptide sequence attached to it.[1]

The first chemical reaction is performed in as many reaction vessels as building blocks (amino acids) are to be incorporated at the first position of the library. On completion of the coupling, all resin portions are combined, mixed thoroughly, and divided into as many reaction vessels as building blocks (amino acids) are to be incorporated at the second position of the library. This process is repeated until the entire library sequence is assembled. Because at each coupling only one amino acid is coupled to each bead, only one peptide is generated on each bead. One can drive any particular (condensation) reaction almost to completion without being concerned about the different coupling rate of amino acids because the different reactions are physically separated. This feature ensures the equimolarity of synthesized peptides.

Automation

Peptide libraries can be synthesized manually or by using an automated synthesizer.[148,153–155] The distribution of the resin is achieved either by volume distribution of a homogeneous (nonsedimenting) suspension of beads in an isopycnic solution,[154] by continuous stirring of the suspension during the distribution,[153] or by a combination of mechanical and gas stirring for creation of a homogeneous suspension.[148] Another design uses gas/mechanical stirring followed by sedimentation of the suspension in a symmetrical distribution vessel.[155]

When using amino acid mixtures for the coupling, standard multiple peptide synthesizers can be used for the synthesis of peptide libraries (see above). The only difference between the synthesis of individual peptides and libraries is the fact that amino acid mixtures, rather than individual

[151] A. Furka, F. Sebestyen, M. Asgedom, and G. Dibo, *in* "Highlights of Modern Biochemistry, Proceedings of the 14th International Congress of Biochemistry" p. 47. VSP, Utrecht, The Netherlands, 1988.

[152] A. Furka, F. Sebestyen, M. Asgedom, and G. Dibo, Poster presented at Tenth International Symposium on Medicinal Chemistry, Budapest, 1988.

[153] H. H. Saneii, J. D. Shannon, R. M. Miceli, H. D. Fischer, and C. W. Smith, *Pept. Chem.* **31,** 117 (1993).

[154] R. N. Zuckermann, J. M. Kerr, M. A. Siani, and S. C. Banville, *Int. J. Pept. Protein Res.* **40,** 497 (1992).

[155] Z. Bartak, J. Bolf, J. Kalousek, P. Mudra, M. Pavlik, V. Pokorny, M. Rinnova, Z. Voburka, K. Zenísek, V. Krchnák, M. Lebl, S. E. Salmon, and K. S. Lam, *Methods (San Diego)* **6,** 432 (1994).

amino acids, are incorporated in one, several, or all positions of the sequence.

Equimolarity

The most reliable way to ensure equimolarity of peptides in a library is using split synthesis.[1,2,83] Because the couplings of different amino acids are physically separated, differences in coupling rates between amino acids are not a problem. Owing to statistical distribution, however, in order for the library to contain all members with 99% confidence, the number of beads used for the synthesis should be at least five times higher than the number of peptides in the library. Some of the members will be underrepresented and some will be overrepresented, with five being the average occurrence of each member.

Although being optimal for the generation of completely randomized libraries, the split synthesis method may be less appropriate for the synthesis of peptide libraries composed of mixtures containing defined and mixture positions, such as iterative or positional scanning libraries (see below). Unless the defined positions are located exclusively at the N terminus of the sequence, the high number of reaction vessels needed for the synthesis makes the use of split synthesis impractical. (For example, the split synthesis of a library with one position defined with one of 20 amino acids, and 20 amino acids used for mixture positions, requires the use of 400 reaction vessels.)

Alternatives to split synthesis are coupling of mixtures of amino acids in a predetermined molar ratio, which compensates for the different coupling rates of the amino acids,[9,57,156–158] and repeated couplings of subequimolar amounts of equimolar mixtures of amino acids.[159–161] The goal of those techniques is the equimolar incorporation of all building blocks, which may be complicated by the fact that the coupling depends not only on the character of the incoming activated amino acid, but also on the sequence

[156] W. J. Rutter and D. V. Santi, U.S. Patent 5,010,175 (1991).

[157] C. Pinilla, J. R. Appel, P. Blanc, and R. A. Houghten, *BioTechniques* **13,** 901 (1992).

[158] J. M. Ostresh, J. M. Winkle, V. T. Hamashin, and R. A. Houghten, *Biopolymers* **34,** 1681 (1994).

[159] R. Frank, *in* "Innovation and Perspectives in Solid Phase Synthesis" (R. Epton, ed.), p. 509. Mayflower Worldwide, Birmingham, UK, 1994.

[160] A. Kramer, R. Volkmer-Engert, R. Malin, U. Reineke, and J. Schneider-Mergener, *Pept. Res.* **6,** 314 (1993).

[161] P. C. Andrews, J. Boyd, R. R. Ogorzalek-Loo, R. Zhao, C. Q. Zhu, K. Grant, and S. Williams, *in* "Techniques in Protein Chemistry V" (J. W. Crabb, ed.), p. 485. Academic Press, San Diego, 1994.

of peptide attached to the solid support. One library technique is based on the incorporation of mixtures that are depleted of or enriched in certain amino acids.[162]

Directed Libraries

Split synthesis results in a statistical distribution of library members, with most library members being represented more than once, which may bias the evaluation of the screening of one-bead–one-compound libraries. Progressively dividable materials, such as membrane-type carriers, are well-suited solid supports for the synthesis of libraries with nonstatistical distribution of library members.[163] The synthesis of this kind of library starts with n pieces of the carrier, to which n different building blocks are coupled (first randomization). Each of the n pieces is then divided into m parts, and these smaller parts are distributed into m reaction vessels in which m reactions are performed (second randomization). This process can be repeated as often as is practical regarding the size and number of the carrier segments. The result of this process is a library of as many support-bound compounds as pieces of the carrier have been produced during the synthesis, in which each library member is represented once and only once. Each part of the membrane can be prelabeled, and determination of structure can then be avoided. This approach has been applied by Pfizer scientists,[164] who used a classic beaded resin sealed between layers of porous polypropylene sheets as the solid support.

One-Bead–One-Mixture Libraries

Varying Complexity of Library Positions

Because the synthesis of complete one-bead–one-peptide libraries of longer sequences (e.g., >8 amino acids) is unrealistic owing to the large amount of resin required, coupling of amino acid mixtures rather than individual amino acids at selected positions of the library has been proposed as an alternative.[165] The least complex mixtures or individual amino acids were used for the N-terminal positions, and the C-terminal positions were the most complex ones, yielding a library with up to millions of peptides

[162] J. Blake and L. Litzi-Davis, *Bioconjugate Chem.* **3,** 510 (1992).
[163] M. Stankova, S. Wade, K. S. Lam, and M. Lebl, *Pept. Res.* **7,** 292 (1994).
[164] J. Steele, Second Annual Solid-Phase Synthesis Conference, February 6–7, 1997, Coronado, CA, 1997.
[165] V. Hornik and E. Hadas, *React. Polym.* **22,** 213 (1994).

per bead. Beads that reacted positively in a binding assay were sequenced, but only the N-terminal residues could be identified, and secondary libraries were synthesized in order to deconvolute the structure of the active peptides. The density of peptides on the bead surface can be an important factor in the binding assay. Wallace *et al.*[166] could detect binding only after the peptide library was displayed as an octamer complex.

Library of Libraries

The one-bead–one-motif library or "library of libraries" approach[167] represents a combination of the one-bead–one-compound and positional scanning (see below) library concepts. The idea is based on the assumption that a measurable biological signal can be obtained if a limited number of critical amino acids (usually three) are present at critical positions in the peptide sequence. The rest of the peptide sequence can be filled with an "average" amino acid, represented by a mixture of amino acids. The first of this type of library was a hexapeptide library prepared from the 20 proteinogenic amino acids with three defined and three mixture positions in all possible combinations, which yields a total of 160,000 mixtures. Because the defined positions were introduced through split synthesis, each peptide on a given bead had the same positions defined, and also the same amino acids at the defined positions, thus creating a one-bead–one-motif library. Consequently, each bead carried a mixture of $20^3 = 8000$ peptides. The principal advantage of this library format is the fact that it enables the identification of all possible combinations of (in this case three) key residues within a given sequence length, rather than single key residues in the positional scanning library format, which may not always be sufficient to be detected in the bioassay. The one-bead–one-"entity" library approach was the method of choice for the generation of this library, as the separate synthesis of 160,000 peptide mixtures would not be practical, unless ultra-high-throughput synthesis instrumentation is available.

An alternative is the library of libraries with variable length. At the beginning of synthesis and after each acylation, one-quarter of the resin is removed, the protecting group is cleaved off, and the mixture of amino acids is coupled to the remaining part. After this coupling, one-third of the resin is separated, and the remainder undergoes coupling with the mixture of amino acids. The next coupling is performed with half of the resin from the previous coupling. All removed portions of the resin are then combined

[166] A. Wallace, S. Altamura, C. Toniatti, A. Vitelli, E. Bianchi, P. Delmastro, G. Ciliberto, and A. Pessi, *Pept. Res.* **7,** 27 (1994).

[167] N. F. Sepetov, V. Krchnák, M. Stankova, S. Wade, K. S. Lam, and M. Lebl, *Proc. Natl. Acad. Sci. U.S.A.* **92,** 5426 (1995).

with the main part, and a randomization is performed. Synthesis of a library of libraries with a three-amino acid motif, by this method, consists of three randomization steps and four stages of multiple couplings of amino acid mixtures. As a result, each solid-phase particle of the library goes through three mandatory randomization steps (mixing the resin, separation into *n* parts, and coupling of individual amino acid) and as many as 12 acylations with the mixture of amino acids. This library, containing peptides of lengths from 3 to 5 residues, consists of 256 positional motif sublibraries. Among sublibraries of up to hexapeptides, all positional motifs are presented. The synthetic scheme of this example did not allow more than three successive acylations with the amino acid mixture, and therefore motifs in which "pharmacophore" positions are separated by more than three adjacent "structural unit" positions could not be represented. However, the scheme utilizing partial removal and separate processing of the resin from synthetic process can create unlimited possibilities of "library of libraries."

Libraries of Organized Mixtures

The characteristic feature of organized peptide mixtures is the presence of a single amino acid at certain position(s) in a sequence and a mixture of amino acids at remaining positions. This systematic array eliminates the need for any kind of structure determination at the library stage (i.e., sequencing of peptides or coding for nonpeptide compounds), as identification of the structures of the active compounds is inherent to the library deconvolution process (see below).

Iterative Libraries

The first iterative library was composed of 400 separate hexapeptide mixtures, represented as O_1O_2XXXX, in which the first two positions (O_1 and O_2) were individually defined and represented all possible combinations of two of the 20 proteinogenic amino acids (AA, AC, ..., through YW, YY).[2] The remaining four positions (X) were mixtures of 19 amino acids (Cys was excluded), so that each mixture was composed of $19^4 = 130{,}321$ individual peptides. This library was synthesized using the tea bag method in combination with the divide–couple–recombine synthesis.

In the initial screening of this type of library in a given bioassay, the most active peptide mixtures are identified, followed by an iterative process of synthesis and screening, during which all positions of the active mixtures are successively defined. If, for example, the mixture HKXXXX was the most active mixture in a given bioassay, His and Lys at positions one and two, respectively, would be kept unchanged, and the next set of 20 peptide

mixtures with the third position defined (HKOXXX) would be synthesized and tested in order to identify the most effective amino acids at the third position. After repeating this process twice more, in the last step of the iterative process, the sixth position of the active peptide mixtures would be defined by synthesizing and testing individual peptides.

Positional Scanning Libraries

The positional scanning (PS) library format[32,157,168,169] enables the identification of active compounds directly from the initial library screening data, thus avoiding the iterative synthesis and screening process associated with the above library format. A typical PS library is composed of n (n = number of diversity positions) sublibraries. Accordingly, a PS hexapeptide library is composed of six independent sublibraries (O_1XXXXX, XO_2XXXX, XXO_3XXX, $XXXO_4XX$, $XXXXO_5X$, $XXXXXO_6$), in which one position (O) is individually defined, and the other five positions are mixtures of amino acids. Thus, each of the sublibraries, while addressing a specific position, represents the same collection of peptides.

Screening of all sublibraries in a given bioassay provides information about the most effective amino acids at each position for the biological effect of interest, as well as about the relative specificity of each position (i.e., the fewer amino acids found to be active at a position, the more specific that position). The synthesis of all possible combinations of the most active amino acids at each position yields a range of individual peptides, which are then tested in order to determine their individual activities. If, for example, two amino acids were found to be highly effective at each position of the above hexapeptide PS library, $2^6 = 64$ individual peptides would be synthesized and tested based on that screening data.

Alternatively, each of the sublibraries can serve as the starting point for the iterative synthesis and screening process described above. It has to be kept in mind that amino acids identified in different positions need not be a part of the same motif, or they may represent only the most significant amino acid(s) of the same motif, which can be placed anywhere in the peptide sequence.[170–172] For example, if Arg is the key amino acid in the potential enzyme ligand (substrate, inhibitor), and the minimal length of

[168] C. Pinilla, J. R. Appel, and R. A. Houghten, *Methods Mol. Biol.* **66,** 171 (1996).
[169] J. R. Appel, S. Muller, N. Benkirane, R. A. Houghten, and C. Pinilla, *Pept. Res.* **9,** 174 (1996).
[170] S. M. Freier, D. A. M. Konings, J. R. Wyatt, and D. J. Ecker, *J. Med. Chem.* **38,** 344 (1995).
[171] D. A. M. Konings, J. R. Wyatt, D. J. Ecker, and S. M. Freier, *J. Med. Chem.* **39,** 2710 (1996).
[172] A. Wallace, K. S. Koblan, K. Hamilton, D. J. Marquis-Omer, P. J. Miller, S. D. Mosser, C. A. Omer, M. D. Schaber, R. Cortese, A. Oliff, J. B. Gibbs, and A. Pessi, *J. Biol. Chem.* **271,** 31306 (1996).

the ligand is a dipeptide, Arg can be identified as key residue in almost any position of hexapeptide library.[57]

Orthogonal Libraries

The characteristic feature of orthogonal libraries is the fact that each library member is present in two (or three) different mixtures, and any two (or three) mixtures of the library have one, and only one, peptide in common. This approach was first described by Deprez *et al.*[173] with the synthesis of a two-dimensional (2D, $n = 2$) orthogonal library consisting of two libraries representing the exact same set of peptides in two different arrangements. These libraries are prepared by coupling defined groups of amino acids as mixtures to each position of the sequence. The amino acids in each group are different for the two libraries and are arranged so that each group of amino acids used for the synthesis of library A contained one, and only one, amino acid of each group used for the synthesis of library B. Screening of both libraries in a bioassay of interest enables the identification of the most effective amino acid groups at each position of the peptide for that particular interaction. If, for example, the group containing Ala, Cys, Asp, and Glu would be the most effective at a given position in library A, these four amino acids would be present in a different group in library B, and screening of library B would reveal which of them is actually the best at that particular position.

Alternatively, by coupling groups of amino acids, orthogonal libraries can also be prepared through the synthesis of individual peptides, which are mixed as intermediates at certain stages of the synthesis. This is preferably done using an automated synthesizer in the 96-well microtiter plate format by alternatingly mixing the resins of rows or columns of the plate,[174] which greatly facilitates the generation of higher dimensional orthogonal libraries.

Libraries with Defined Structural Features

Cyclic Peptide Libraries

The conformational flexibility of peptides can be decreased by cyclization, which may increase the affinity of peptides to their biological receptors. Ligands for the gpIIbIIIa receptor were found using a cyclic disulfide peptide library, which owing to low concentration of library components, would

[173] B. Deprez, X. Williard, L. Bourel, H. Coste, F. Hyafil, and A. Tartar, *J. Am. Chem. Soc.* **117,** 5405 (1995).

[174] S. Felder and R. Kris, *Mol. Diversity* **2,** in press (1997).

not have been found in linear libraries.[108] Cyclization changing the preference of the biological receptor toward a particular target was shown in the study of streptavidin binding of cyclic libraries of various sizes.[175] Peptide libraries built around a cyclic peptide template, to which amino and other carboxylic acids were attached,[176] yielded an inhibitor of α-glucosidase.[177] Eichler *et al.*[177] studied an array of cyclic disulfide and lactam libraries and compared their activities with the linear analogs of the libraries. Only one lactam library was found to be active in an α-glucosidase inhibition assay.

Cyclic lactam peptide libraries have been intensively studied,[178–188] and an optimized strategy for their syntheses was developed. A cyclic pentapeptide library based on a known endothelin antagonist was synthesized, and the sequence of the known ligand was correctly identified.[187] The cyclic β-turn mimetic library by Virgilio and Ellman,[189] in which the ε-amino group of the C-terminal Lys was acylated with acrylic acid, the N terminus was acylated with iodobenzoic acid, and palladium-mediated cyclization provided clean products in high yield,[190] is closer to a nonpeptide than a peptide library.

[175] K. S. Lam, M. Lebl, S. Wade, S. Stierandova, P. S. Khattri, N. Collins, and V. J. Hruby, *in* "Peptides, 1994" (R. S. Hodges and J. A. Smith, eds.), p. 1005. Escom, Leiden, The Netherlands, 1994.

[176] J. Eichler, A. W. Lucka, and R. A. Houghten, *Pept. Res.* **7,** 300 (1994).

[177] J. Eichler, A. W. Lucka, C. Pinilla, and R. A. Houghten, *Mol. Diversity* **1,** 233 (1996).

[178] K. Darlak, P. Romanovskis, and A. F. Spatola, *in* "Peptides: Chemistry, Structure, and Biology" (R. S. Hodges and J. A. Smith, eds.), p. 981. Escom, Leiden, The Netherlands, 1994.

[179] D. Tumelty, D. Vetter, and V. V. Antonenko, *J. Chem. Soc., Chem. Commun.,* 1067 (1994).

[180] D. Winkler, A. Schuster, B. Hoffmann, and J. Schneider-Mergener, *in* "Peptides 94" (H. L. S. Maia, ed.), p. 485. Escom, Leiden, The Netherlands, 1995.

[181] C. G. Bradshaw, E. Magnenat, and A. Chollet, *in* "Peptides 94" (H. L. S. Maia, ed.), p. 485. Escom, Leiden, The Netherlands, 1995.

[182] D. Tumelty, M. C. Needels, V. V. Antonenko, and P. R. Bovy, *in* "Peptides: Chemistry, Structure and Biology" (P. T. P. Kaumaya and R. S. Hodges, eds.), p. 121. Mayflower Scientific, Kingswinford, UK, 1996.

[183] A. F. Spatola, Y. Crozet, P. Romanovskis, and E. Valente, *in* "Peptides: Chemistry, Structure and Biology" (P. T. P. Kaumaya and R. S. Hodges, eds.), p. 281. Mayflower Scientific, Kingswinford, UK, 1996.

[184] D. Winkler, R. D. Stigler, J. Hellwig, B. Hoffmann, and J. Schneider-Mergener, *in* "Peptides: Chemistry, Structure and Biology" (P. T. P. Kaumaya and R. S. Hodges, eds.), p. 315. Mayflower Scientific, Kingswinford, UK, 1996.

[185] A. F. Spatola and P. Romanovskis, *in* "Peptide and Non-Peptide Libraries: A Handbook for the Search of Lead Structures" (G. Jung, ed.), p. 327. VCH, Weinheim, Germany, 1996.

[186] J. J. Chen, L. M. Teesch, and A. F. Spatola, *Lett. Pept. Sci.* **3,** 17 (1996).

[187] A. F. Spatola and Y. Crozet, *J. Med. Chem.* **39,** 3842 (1996).

[188] A. F. Spatola, K. Darlak, and P. Romanovskis, *Tetrahedron Lett.* **37,** 591 (1996).

[189] A. A. Virgilio and J. A. Ellman, *J. Am. Chem. Soc.* **116,** 11580 (1994).

[190] M. Hiroshige, J. R. Hauske, and P. Zhou, *J. Am. Chem. Soc.* **117,** 11590 (1995).

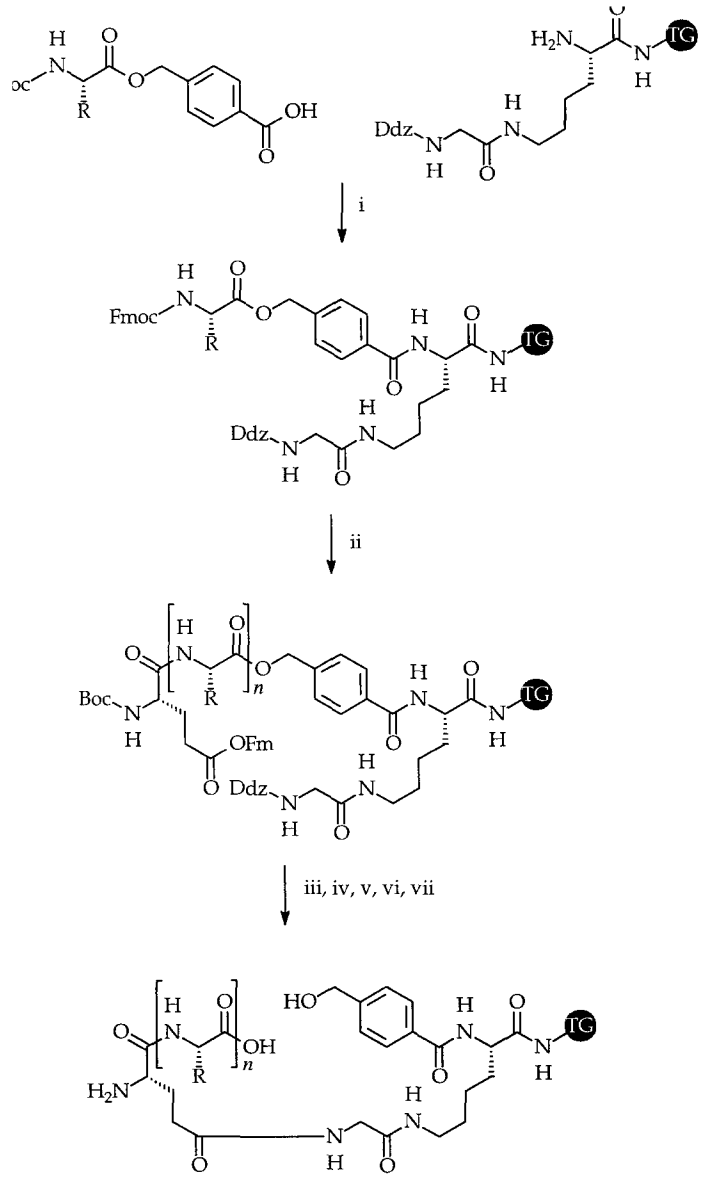
Conformationally Defined Peptide Libraries

A conformationally defined peptide library was designed and synthesized by replacing five positions of an amphipathic α-helical 18-mer peptide (Tyr-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Lys-Leu), either on the hydrophilic or the hydrophobic face of the helix, with one defined and four mixture positions. This library was tested for its catalytic activity in the decarboxylation of oxaloacetate. The catalytic activities of individual peptides identified were found to correlate well with their ability to fold into an α-helical conformation.[191–193] A similar approach was taken by Bianchi *et al.* by randomizing five positions in the α-helical portion of a 26-residue zinc finger motif.[194] Consensus sequences for the binding to an antilipopolysaccharide antibody were found through the screening of this library. Stabilization of the two-stranded α-helical coiled coil was achieved by incorporation of a lactam bridge.[195]

"Reversed" Peptide Libraries

Peptides are typically synthesized starting from the C terminus, because the synthesis of peptides in the N $\rightarrow$ C direction results in significant racemization at each coupling step. Biological receptors may recognize the C or N terminal or both ends of their peptide ligands. Although this is not an issue with respect to peptide libraries in solution (both termini are accessible), it becomes problematic when using support-bound libraries for the screening against receptors that recognize their ligands at the C terminus. Therefore, methods to "reverse" resin-bound peptide libraries have been developed.[196–198] This is done by on-resin cyclization of the peptides, followed by opening the ring in a manner that exposes the C terminus (Fig. 10).

[191] E. Perez-Paya, R. A. Houghten, and S. E. Blondelle, *J. Biol. Chem.* **270,** 1048 (1995).
[192] S. E. Blondelle, E. Takahashi, R. A. Houghten, and E. Perez-Paya, *Biochem. J.* **313,** 141 (1996).
[193] E. Perez-Paya, R. A. Houghten, and S. E. Blondelle, *J. Biol. Chem.* **271,** 4120 (1996).
[194] E. Bianchi, A. Folgori, A. Wallace, M. Nocotra, S. Acali, A. Phalipon, G. Barbato, R. Bazzo, R. Cortese, F. Felici, and A. Pessi, *J. Mol. Biol.* **247,** 154 (1995).
[195] M. E. J. Houston, A. Wallace, E. Bianchi, A. Pessi, and R. S. Hodges, *J. Mol. Biol.* **262,** 270 (1996).
[196] M. Lebl, V. Krchnák, N. F. Sepetov, V. Nikolaev, A. Stierandova, P. Safár, B. Seligmann, P. Strop, D. Thorpe, S. Felder, D. F. Lake, K. S. Lam, and S. E. Salmon, *in* "Innovation and Perspectives in Solid Phase Synthesis" (R. Epton, ed.), p. 233. Mayflower Worldwide, Birmingham, UK, 1994.
[197] C. P. Holmes and C. M. Rybak, *in* "Peptides: Chemistry, Structure, and Biology" (R. S. Hodges and J. A. Smith, eds.), p. 992. Escom, Leiden, The Netherlands, 1994.
[198] R. S. Kania, R. N. Zuckermann, and C. K. Marlowe, *J. Am. Chem. Soc.* **116,** 8835 (1994).



(i) DCC, HOBt, DMF, overnight
(ii) peptide synthesis, standard Fmoc protocol
(iii) 50% piperidine in DMF
(iv) 3% TFA in DCM
(v) DIPCDI, HOBt
(vi) 50% TFA in DCM
(vii) 0.5% NaOH, 30 min

FIG. 10. Reversing the peptide chain on the resin.

Increasing Diversity of Peptide Libraries

Libraries Containing Nonpeptidic Components

The diversity of peptide libraries is determined by the amino acid chains, while the peptide backbone is constant. The introduction of other than α-amino acids, however, enables the diversification also of the peptide backgone.[116] The peptide backbone can serve as a scaffold to which a variety of building blocks can be attached via coupling to various trifunctional amino acids, such as aminoglycine, diaminopropionic acid, diaminobutyric acid, ornithine, Lys, iminodiacetic acid, Asp, Glu, Ser, Thr, Hyp, and Cys.[196,199–201] Iminodiacetic acid was shown to be a suitable backbone unit for the construction of peptidelike libraries.[202] Iminodiacetic acid anhydride and similar symmetrical anhydrides were also used as templates for library synthesis[203] or for structural coding.[204] Attachment of carboxylic acids to the α- or ω-amino groups of various diamino acids was the basis for the construction of a so-called α,β,γ library (the scheme of the synthesis of this library is given in Fig. 11), in which both the peptide backbone and the amino acid side chains were diversified.[205] Figure 12 illustrates two extreme structures from this library.

Rivier and co-workers[206] prepared libraries of oligoamides, termed betides, in which one amino group of aminoglycine residues was used for the backbone, and the second amino group was derivatized (acylated, alkylated). In such "betidamino" acids, each *N'*-acyl/alkyl group can mimic naturally occurring amino acid side chains or introduce other functionalities. A potent gonadotropin-releasing hormone antagonist was discovered using

[199] M. Lebl, V. Krchnák, P. Safár, A. Stierandova, N. F. Sepetov, P. Kocis, and K. S. Lam, *in* "Techniques in Protein Chemistry V" (J. W. Crabb, ed.), p. 541. Academic Press, San Diego, 1994.

[200] M. Lebl, V. Krchnák, A. Stierandova, P. Safár, P. Kocis, V. Nikolaev, N. F. Sepetov, R. Ferguson, B. Seligmann, K. S. Lam, and S. E. Salmon, *in* "Peptides: Chemistry, Structure, and Biology" (R. S. Hodges and J. A. Smith, eds.), p. 1007. Escom, Leiden, The Netherlands, 1994.

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[202] P. Safár, A. Stierandova, and M. Lebl, *in* "Peptides 94" (H. L. S. Maia, ed.), p. 471. Escom, Leiden, The Netherlands, 1995.

[203] D. L. Boger, C. M. Tarby, P. L. Myers, and L. H. Caporale, *J. Am. Chem. Soc.* **118,** 2109 (1996).

[204] Z. J. Ni, D. Maclean, C. P. Holmes, M. M. Murphy, B. Ruhland, J. W. Jacobs, E. M. Gordon, and M. A. Gallop, *J. Med. Chem.* **39,** 1601 (1996).

[205] V. Krchnák, A. S. Weichsel, D. Cabel, and M. Lebl, *Pept. Res.* **8,** 198 (1995).

[206] J. E. Rivier, G. C. Jiang, S. C. Koerber, J. Porter, L. Simon, A. G. Craig, and C. A. Hoeger, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 2031 (1996).

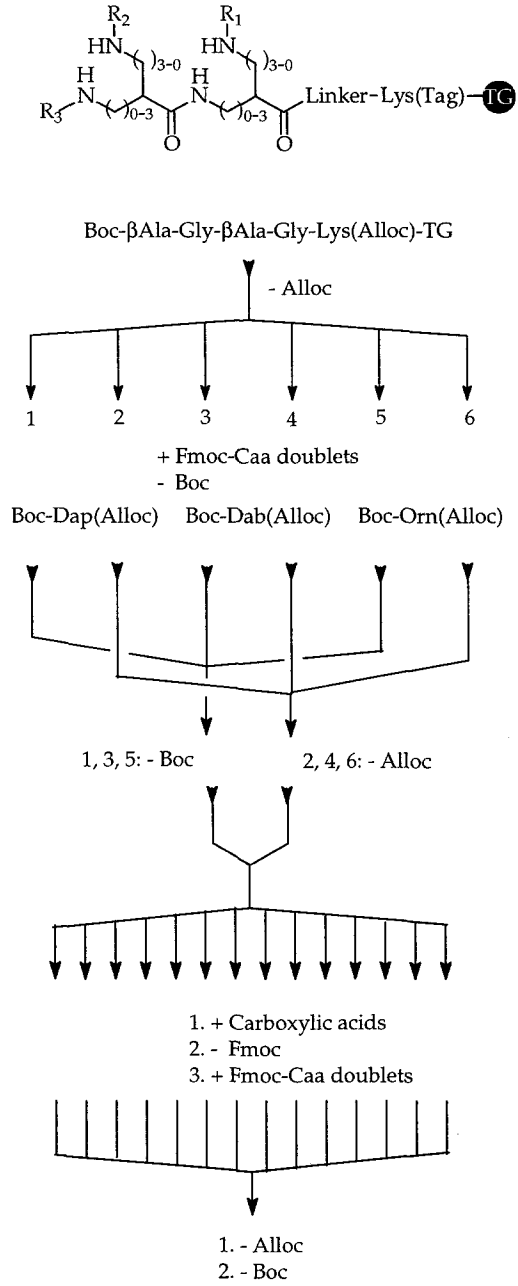

FIG. 11. Scheme of the synthesis of an α,β,γ library.[205]

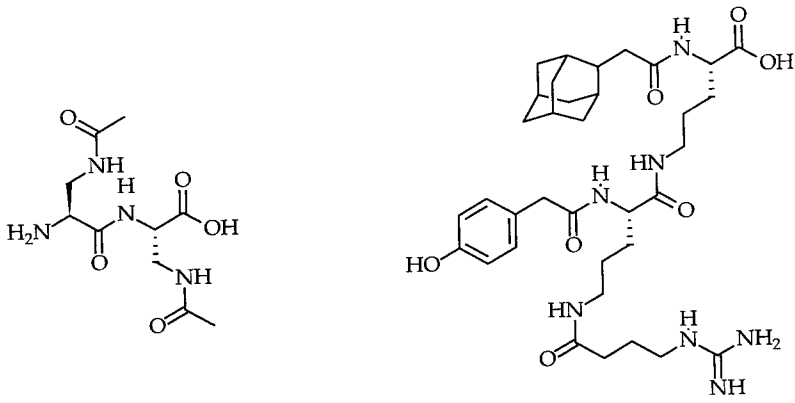

FIG. 12. Examples of "extreme" structures from an α,β,γ library.

a betide library. Interestingly, few differences in activities were found between the D- and L-nonalkylated betidamino acids.

Potent and specific zinc endopeptidase inhibitors were identified using a library of peptides modified at the N terminus with Z-Phe(PO_2CH_2).[207] In the Pfizer study of the endothelin antagonist developed by Fujisawa, Terrett *et al.*[208] kept N-terminal substitution intact and randomized all amino acids by an array of natural and unnatural α- and non-α-amino acids. The peptide chain was used as the "biasing element," targeting the binding pocket of Src SH3 domain in the library constructed by acylation of the peptide chain by the array of three building blocks containing amino and carboxyl functionalities and capped by a set of carboxylic acids.[209,210]

Peptide mimetics composed of α-aza-amino acids (termed azatides) have been applied to solid-phase synthesis.[211] A set of Boc protected alkyl hydrazines has been prepared, either by reduction of protected hydrazones or by alkylation of hydrazine with an alkyl halide followed by Boc protection. These hydrazine derivatives were converted to activated species by bis(pentafluorophenyl) carbonate and used in a stepwise manner to build

[207] J. Jiracek, A. Yiotakis, B. Vincent, A. Lecoq, A. Nicolaou, F. Checler, and V. Dive, *J. Biol. Chem.* **270,** 21701 (1995).

[208] N. K. Terrett, D. Bojanic, D. Brown, P. J. Bungay, M. Gardner, D. W. Gordon, C. J. Mayers, and J. Steele, *Bioorg. Med. Chem. Lett.* **5,** 917 (1995).

[209] A. P. Combs, T. M. Kapoor, S. Feng, J. K. Chen, L. F. Daude-Snow, and S. L. Schreiber, *J. Am. Chem. Soc.* **118,** 287 (1996).

[210] S. B. Feng, T. M. Kapoor, F. Shirai, A. P. Combs, and S. L. Schreiber, *Chem. Biol.* **3,** 661 (1996).

[211] H. Han and K. D. Janda, *J. Am. Chem. Soc.* **118,** 2539 (1996).

azatides. Vinylogous sulfonyl peptide libraries[212] were used for studies of synthetic receptors.[213] Libraries of synthetic receptors were generated by attaching randomized dipeptides to macrocyclic tetramine cyclen[214] or steroid scaffolds.[215]

Peptoid Libraries

Peptoids differ from peptides in the location of the side chains.[216–220] Whereas in peptides the side chains are connected to the α-carbon of the amino acid residues, in peptoids they are linked to the amide nitrogen of the backbone. Peptoids are resistant toward proteolytic degradation, and the diversity of peptoid libraries can be increased by introducing many amines as compared to the limited number of amino acids used for peptide libraries. Peptoids are easily synthesized from bromoacetic acid and amines,[221] or from preformed *N*-alkylamino acids.[216] Specific ligands for the α_1-adrenergic receptor and μ-opioid receptors were found using peptoid libraries.[219]

The combination of amino acids and N-alkylated Gly in the same molecule was successfully applied in several model studies.[164,222,223] Ostergaard

[212] C. Gennari, H. P. Nestler, B. Salom, and W. C. Still, *Angew. Chem., Int. Ed. Engl.* **34,** 1763 (1995).

[213] C. Gennari, H. P. Nestler, B. Salom, and W. C. Still, *Angew. Chem., Int. Ed. Engl.* **34,** 1765 (1995).

[214] M. T. Burger and W. C. Still, *J. Org. Chem.* **60,** 7382 (1995).

[215] R. Boyce, G. Li, H. P. Nestler, T. Suenaga, and W. C. Still, *J. Am. Chem. Soc.* **116,** 7955 (1994).

[216] R. J. Simon, R. S. Kaina, R. N. Zuckermann, V. D. Huebner, D. A. Jewell, S. Banville, S. Ng, L. Wang, S. Rosenberg, C. K. Marlowe, D. C. Spellmeyer, R. Tan, A. D. Frankel, D. V. Santi, F. E. Cohen, and P. A. Bartlett, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 9367 (1992).

[217] S. M. Miller, R. J. Simon, S. Ng, R. N. Zuckermann, J. M. Kerr, and W. H. Moos, *Bioorg. Med. Chem. Lett.* **4,** 2657 (1994).

[218] R. J. Simon, E. J. Martin, S. M. Miller, R. N. Zuckermann, J. M. Blaney, and W. H. Moos, *in* "Techniques in Protein Chemistry V" (J. W. Crabb, ed.), p. 533. Academic Press, San Diego, 1994.

[219] R. N. Zuckermann, E. J. Martin, D. C. Spellmeyer, G. B. Stauber, K. R. Shoemaker, J. M. Kerr, G. M. Figliozzi, D. A. Goff, M. A. Siani, R. J. Simon, S. C. Banville, E. G. Brown, L. Wang, L. S. Richter, and W. H. Moos, *J. Med. Chem.* **37,** 2678 (1994).

[220] S. M. Miller, R. J. Simon, S. Ng, R. N. Zuckermann, J. M. Kerr, and W. H. Moos, *Drug Dev. Res.* **35,** 20 (1995).

[221] R. N. Zuckermann, J. M. Kerr, S. B. H. Kent, and W. H. Moos, *J. Am. Chem. Soc.* **114,** 10646 (1992).

[222] V. Nikolaiev, A. Stierandova, V. Krchnák, B. Seligmann, K. S. Lam, S. E. Salmon, and M. Lebl, *Pept. Res.* **6,** 161 (1993).

[223] M. Lebl, V. Krchnák, N. F. Sepetov, B. Seligmann, P. Strop, S. Felder, and K. S. Lam, *Biopolymers (Pept. Sci.)* **37,** 177 (1995).

and Holm called the molecules composed partly from peptides and partly from peptoids "peptomers."[164]

Libraries from Libraries

Chemically modified peptide libraries have been generated through alkylation and/or reduction of the peptide bonds of existing peptide libraries,[224] thus dramatically changing the physicochemical properties of the peptides and greatly extending the range and repertoire of chemical diversity. The components of such transformed libraries, which have been termed "libraries from libraries," are stable toward proteolytic degradation, as they lack the characteristic peptide bond-CO-NH-. The chemical modification of peptide libraries is reflected by the library screening results, as illustrated by the following example.[225] A tetrapeptide library was divided into four aliquots. One aliquot remained untreated, one aliquot was reduced, the third aliquot N-benzylated, and the fourth aliquot N-benzylated and reduced, generating four different libraries from the same parent peptide library. These four libraries were screened in antimicrobial and receptor binding assays, yielding very different results depending on the character of the library backbone (see Fig. 13).

Library Analysis

Analysis of the synthesized library should make sure that all components are present and that they are present in the expected amounts. Important issues in the analysis of one-bead–one-compound libraries include examination of "statistically significant" sample sizes (how many beads should be analyzed), sensitivity of the analytical method (only picomolar quantities of compounds are available on the bead), and throughput of the method. Microsequencing and amino acid analysis are appropriate methods for peptide library characterization. Sequencing can detect incomplete couplings and incomplete side-chain deprotection. The sensitivity of modern automatic microsequencers (in the high femtomolar range) enables the detection of impurities in the range of 1 to 2%.

Drawbacks of an alternative technique, mass spectrometry, are (i) poor quantitation (signal intensity depends on ionizability of each component of the mixture, which can be dramatically different) and (ii) the fact that the compound has to be detached from the bead prior to analysis. The most

[224] J. M. Ostresh, G. M. Husar, S. E. Blondelle, B. Dorner, P. A. Weber, and R. A. Houghten, *Proc. Natl. Acad. Sci. U.S.A.* **91,** 11138 (1994).

[225] R. A. Houghten, S. E. Blondelle, C. T. Dooley, B. Dorner, J. Eichler, and J. M. Ostresh, *Mol. Diversity* **2,** 41 (1996).

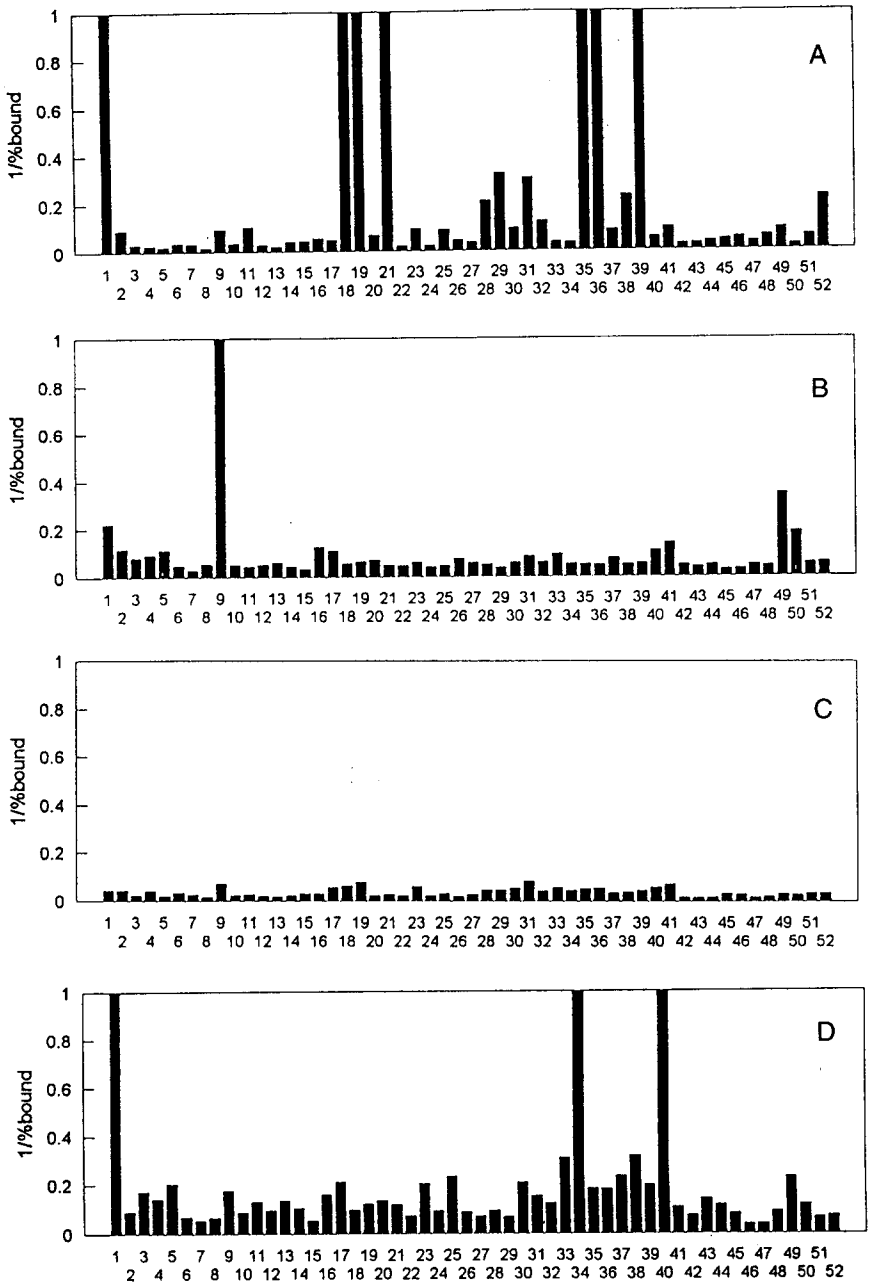

FIG. 13. Primary binding assay screening results from different "libraries from libraries." (A) Tetrapeptide library (mother library); (B) reduced library (pentamine library); (C) peptide perbenzylated library; (D) perbenzylated and reduced library.[225]

convenient methods for compound detachment are exposure to gaseous reagents (ammonia, hydrogen fluoride, cyanogen bromide, trifluoroacetic acid) or photolytic cleavage. In both cases, the beads do not have to be treated in separate vessels, because the detached compounds remain physically inside the bead until they are extracted directly prior to, or during, analysis.[226-230] Mass spectrometry can identify all types of impurities and, therefore, can be used to optimize the library synthesis.[231] Mass spectrometric analysis of hundreds of samples can be performed automatically.[232-234]

Drawbacks of an alternative technique, mass spectrometry, are (i) poor quantitation (signal intensity depends on ionizability of each component of the mixture, which can be dramatically different) and (ii) the fact that the compound has to be detached from the bead prior to analysis. The most convenient methods for compound detachment are exposure to gaseous reagents (ammonia, hydrogen fluoride, cyanogen bromide, trifluoroacetic acid) or photolytic cleavage. In both cases, the beads do not have to be treated in separate vessels, because the detached compounds remain physically inside the bead until they are extracted directly prior to, or during, analysis.[226-230] Mass spectrometry can identify all types of impurities and, therefore, can be used to optimize the library synthesis.[231] Mass spectrometric analysis of hundreds of samples can be performed automatically.[232-234]

Infrared spectroscopy is a valuable technique for the evaluation of the solid-phase transformation of functional groups, in particular techniques that work with individual beads.[235-237] Its application to the analysis of

[226] C. L. Brummel, I. N. W. Lee, Y. Zhou, S. J. Benkovic, and N. Winograd, *Science* **264,** 399 (1994).

[227] R. A. Zambias, D. A. Boulton, and P. R. Griffin, *Tetrahedron Lett.* **35,** 4283 (1994).

[228] R. S. Youngquist, G. R. Fuentes, M. P. Lacey, and T. Keough, *Rapid Commun. Mass Spectrom.* **8,** 77 (1994).

[229] B. J. Egner, G. J. Langley, and M. Bradley, *J. Org. Chem.* **60,** 2652 (1995).

[230] B. J. Egner, M. Cardno, and M. Bradley, *J. Chem. Soc., Chem. Commun.,* 2163 (1996).

[231] R. S. Youngquist, G. R. Fuentes, M. P. Lacey, and T. Keough, *J. Am. Chem. Soc.* **117,** 3900 (1995).

[232] S. W. Fink, W. L. Thompson, and J. R. B. Slayback, *Spectroscopy* **11,** 26 (1996).

[233] S. S. Smart, T. J. Mason, P. S. Bennell, N. J. Maeji, and H. M. Geysen, *Int. J. Pept. Protein Res.* **7,** 47 (1996).

[234] G. Jung, A. G. Beck-Sickinger, N. Zimmermann, J. Metzger, R. Spohn, S. Stevanovic, K. Deres, and K. H. Wiesmuller, *in* "Innovation and Perspectives in Solid Phase Peptide Synthesis" (R. Epton, ed.), p. 227. Intercept, Andover, UK, 1992.

[235] B. Yan, G. Kumaravel, H. Anjaria, A. Wu, R. C. Petter, C. F. Jewell, and J. R. Wareing, *J. Org. Chem.* **60,** 5736 (1995).

[236] B. Yan and G. Kumaravel, *Tetrahedron* **52,** 843 (1996).

[237] B. Yan, J. B. Fell, and G. Kumaravel, *J. Org. Chem.* **61,** 7467 (1996).

peptide libraries is rather limited,[238] as is the application of nuclear magnetic resonance (NMR) spectroscopy, which was shown to provide information about incorporation of individual amino acids into the mixtures.[148]

Amino acids analysis is also a useful method for the assessment of the "statistical purity" of peptide libraries, provided library samples with predictable properties are used. For example, five beads from a tetrapeptide library are essentially as likely to contain any amino acid only once as they are likely to not contain any particular amino acid at all. However, 5000 beads from the same library (about 5 mg of resin, assuming an average bead size of 130 μm) must represent equimolar amounts of all amino acids for the library to be "statistically pure." Using this method, major synthetic problems, such as omission of particular amino acids, or loss of the product from the carrier during synthesis or deprotection, can be easily detected. Amino acid analysis is quantitative and applicable even to peptides containing unnatural amino acids, or for the analysis of nonpeptide libraries containing amino acids as scaffolds or building blocks.

Similarly useful are multiple sequencing methods.[239] Sequencing by Edman degradation, however, is limited to peptides that have a free a N terminus and contain only α-amino acids.

The comparison of computer-generated mass distribution profiles[240] with experimentally obtained mass spectra of libraries[161,239] can be applied to samples of both one-bead–one compound, and mixture libraries. The theoretical profile may need to be corrected for the different proton affinities of amino acids. Peptide library evaluation by fast atom bombardment (FAB) mass spectrometry should take into consideration the high proton affinity of Arg.[148] Evaluation of mass distribution reveals incomplete couplings (shift toward lower molecular weights), incomplete deprotection, and unwanted library modification, such as oxidation, acylation, and alkylation (shift toward higher molecular weights) (see, e.g., Fig. 14). Information about classes of ions fragmenting into a common daughter ion, and about compound classes losing the same neutral mass, may be obtained using tandem mass spectrometry. This technique is also helpful in determining the completeness of the removal of specific protecting groups and for the detection of side products generated during the synthesis.[241]

[238] K. Russell, D. C. Cole, F. M. McLaren, and D. E. Pivonka, *J. Am. Chem. Soc.* **118,** 7941 (1996).

[239] J. W. Metzger, S. Stevanovic, J. Brunjes, K. H. Wiesmuller, and G. Jung, *Methods (San Diego)* **6,** 425 (1994).

[240] M. Lebl, V. Krchnák, and G. Lebl, Peptide Companion. Software, CSPS, P.O. Box 22567, San Diego, CA 92192-2567, 1995.

[241] J. W. Metzger, C. Kempter, K. H. Wiesmuller, and G. Jung, *Anal. Biochem.* **219,** 261 (1994).

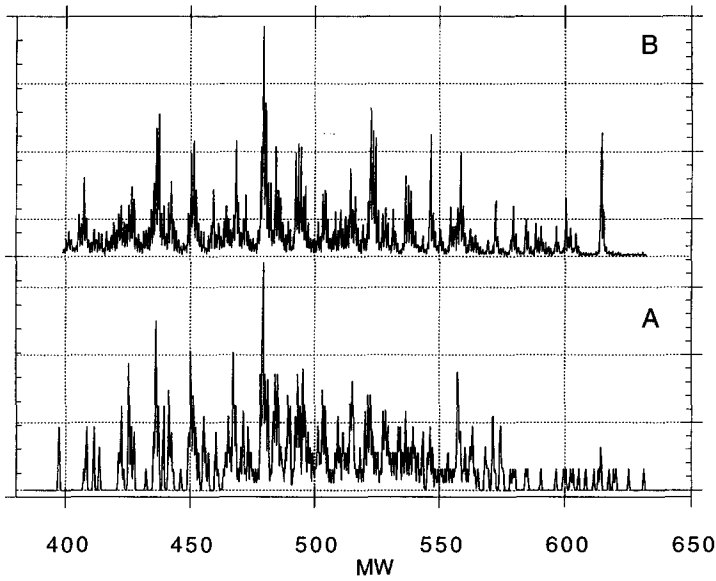

FIG. 14. Comparison of (A) calculated and (B) experimental mass spectra of a library of 480 members. (Data courtesy of Michael Griffith.)

Electrospray mass spectroscopy was used to characterize peptide libraries synthesized by various methods.[117,234,239,241-246] Boutin *et al.*[148] compared FAB mass spectrometry, capillary electrophoresis, and NMR for library characterization, and they concluded that existing analytical techniques can provide valuable information on the quality of synthetic libraries.

Structure Determination of Hits from Libraries

One-Bead–One-Compound Libraries

Direct Sequencing. The picomolar amounts of peptides contained in individual beads are a sufficient quantity for Edman degradation. Only

[242] J. W. Metzger, K. H. Wiesmuller, V. Gnau, J. Brunjes, and G. Jung, *Angew. Chem., Int. Ed. Engl.* **32,** 894 (1993).

[243] S. Stevanovic, K. H. Wiesmuller, J. Metzger, A. G. Beck-Sickinger, and G. Jung, *Bioorg. Med. Chem. Lett.* **3,** 431 (1993).

[244] T. Carell, E. A. Wintner, A. J. Sutherland, J. J. Rebek, Y. M. Dunayevskiy, and P. Vouros, *Chem. Biol.* **2,** 171 (1995).

[245] Y. M. Dunayevskiy, P. Vouros, T. Carell, E. A. Wintner, and J. Rebek, *Anal. Chem.* **67,** 2906 (1995).

[246] Y. M. Dunayevskiy, P. Vouros, E. A. Wintner, G. W. Shipps, T. Carell, and J. J. Rebek, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 6152 (1996).

minimal modification of the standard sequencing protocols of automated sequencers is required for using most solid support beads. Difficulties in sequencing peptides from Sepharose beads[247] may be overcome by mechanically disintegrating the beads and/or by applying the isothiocyanate solution in several cycles. A potential problem in bead sequencing is the uniqueness of the individual bead with respect to the always possible malfunctioning of the sequencer, or power failure; when the sequence information on one bead is lost, it cannot be recovered. A solution to this problem is cutting the beads in half and subjecting only one part to sequencing, while retaining the second half as a backup. The techniques of single bead manipulation are very simple. A good dissecting microscope, a petri dish, and an injection needle are needed. The needle is sharp enough to cut the beads, and its tip can be used lift the bead and transfer it to the sequencing support. "Packing" the bead into the fibers of a support filter prevents it from "jumping out." The originally used micromanipulator[1] was soon abandoned in favor of micropipette and needle techniques.

Peptides containing other than α-amino acids, or other nonsequenceable building blocks, can be sequenced until the first nonsequenceable building block is reached. Sequencing beyond that point can be enabled by coupling a subequimolar amount of the nonsequenceable building block, thus creating a sequenceable omission peptide on the same bead.[248,249] Alternatively, a sequenceable amino acid (typically Gly) can be cocoupled together with the nonsequenceable building block, thus creating a mixture of two peptides on one bead. The cocoupling of the second amino acid has also been used for the "tagging" of D-amino acids in the sequence, even though chiral sequencing is a possible alternative.[250]

For the sequencing of peptides containing more than the 20 proteinogenic amino acids, the chromatographic gradient for the separation of the phenylthiohydantoin (PTH)-amino acids can be modified, enabling the separation of unnatural and side-chain modified trifunctional amino acids,[223] as well as N-alkylated Gly.[13] Sequencing of cyclic peptides with a free α-amino group is feasible, because the amino acid at the N-terminal part of the cycle is not detected until the amino acid at the C-terminal side of the cycle is reached, which is detected as a modified amino acid (i.e., a cystine for disulfides and side-chain dipeptides for lactams). Sequencing of "reversed" peptides (see above) is more difficult.[196–198] Instead of applying

[247] V. Krchnák, A. Weichsel, S. Felder, and M. Lebl, unpublished results (1992).

[248] K. S. Lamand and S. E. Salmon, U.S. Patent 5,510,240 (1995).

[249] K. S. Lam, S. E. Salmon, V. J. Hruby, E. M. Hersh, and F. Al-Obeidi, WO Patent 546,845 (1992).

[250] M. Pavlok, Z. Voburka, T. Vanek, M. Rinnova, I. Bleha, L. Doleckova, and I. Kluh, *in* "Peptides 94" (H. L. S. Maia, ed.), p. 418. Escom, Leiden, The Netherlands, 1995.

C-terminal sequencing, the peptide can be attached (or reattached) to the resin via an amino acid side chain, thus exposing the α-amino group for sequencing (see Fig. 10).

The screening of one-bead–one-compound libraries often results in the identification of many positive beads. When a structural consensus can be expected (e.g., in small libraries, or libraries with fixed structural elements), it may be more efficient to simultaneously sequence groups of positive beads, rather than individual beads. Besides yielding information on specific positions of the sequence (only one or few amino acids are detected in that particular cycle of sequencing), as well as nonspecific positions (many or all amino acids are detected), this method provides structure–activity relationship information.[223,251] In a similar manner, sequencing can be used to detect mixture and defined positions in "libraries of libraries" (see above).

"Tagging" by addition of an unnatural amino acid has been used to identify mixtures composed of L- or D-amino acids by sequencing,[167] and nonsequenceable amino acids were "coded" by sequenceable amino acids.[165]

Coding and Decoding. Direct sequencing is not possible if other than α-amino acids and/or other nonsequenceable building blocks are used to synthesize a library. Therefore, several strategies for the coding of library compounds on single beads have been devised. The first, proposed by Brenner and Lerner,[252] was based on the coding of the structures of library components by oligonucleotides, which can be readily sequenced. Although nucleic acid coding has been successively used,[253–255] its application is limited owing to incompatibility of nucleic acid chemistry with many reaction conditions in organic synthesis. Two laboratories developed an alternative coding strategy for nonsequenceable one-bead–one-compound libraries[222,256] based on peptides as coding structures, which can be easily sequenced by Edman degradation. Coding subunits other than the building blocks used for library synthesis are attached to the solid support in a separate reaction. Other coding methods utilize different analytical techniques for the decoding of the coding tag, such as gas chromatography for

[251] M. Lebl, K. S. Lam, P. Kocis, V. Krchnák, M. Patek, S. E. Salmon, and V. J. Hruby, *in* "Peptides 1992" (C. H. Schneider and A. N. Eberle, eds.), p. 67. Escom, Leiden, The Netherlands, 1993.

[252] S. Brenner and R. A. Lerner, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 5381 (1992).

[253] J. Nielsen and K. D. Janda, *Methods (San Diego)* **6,** 361 (1994).

[254] J. Nielsen, S. Brenner, and K. D. Janda, *J. Am. Chem. Soc.* **115,** 9812 (1993).

[255] M. C. Needels, D. G. Jones, E. H. Tate, G. L. Heinkel, L. M. Kochersperger, W. J. Dower, R. W. Barrett, and M. A. Gallop, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 10700 (1993).

[256] J. M. Kerr, S. C. Banville, and R. N. Zuckermann, *J. Am. Chem. Soc.* **115,** 2529 (1993).

electrophoric tags (silylated halocarbons).[257,258] Ni *et al.*[204] used the very simple chemistry of polyamide formation in combination with protected iminodiacetic acid and a set of secondary amines.[203] Coding can be extended by applying the so-called digital coding principle, which is based on the use of more than one coding subunit for the coding of individual building blocks.[223,257,259]

A controlled ratio of stable isotopes in the tagging molecules (^{13}C and ^{15}N in Gly and Ala) was used as a very elegant method of encoding a combinatorial library.[259] Owing to the fact that the code can be constructed not only from different components, but that also their ratio can be changed and determined with high confidence, thousands of codes can be constructed from very limited numbers of coding blocks. The only limitation may be the price of isotopically labeled molecules.

Undesirable interaction between the coding structure and the biological target can be prevented by physically separating the coding from the library structures in the resin beads. This is done by isolating the coding structure in the bead interior, which is not accessible for most macromolecular targets (e.g., enzymes, antibodies), and assigning the library structure exclusively to the bead surface, where it can be recognized by the target molecule.[47,260] The method used to selectively address bead surface and interior volume is referred to as "bead shaving," because it involves the selective removal of protected amino acids from the bead surface using an enzyme, thus exposing a free amino group on the surface, while the bead interior remains amino-protected. Potential interference of the coding structures with the target molecule can be avoided completely by tagging the resin beads with a radio frequency transmitting chip.[126,261]

Mass Spectrometry. Sequencing of peptides by mass spectrometry was developed in several laboratories.[262,263] It can complement peptide sequencing[264] or be used to determine nonsequenceable components of peptides containing unnatural amino acids. The interpretation of sequencing data

[257] M. H. J. Ohlmeyer, R. N. Swanson, L. W. Dillard, J. C. Reader, G. Asouline, R. Kobayashi, M. Wigler, and W. C. Still, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 10922 (1993).

[258] H. P. Nestler, P. A. Bartlett, and W. C. Still, *J. Org. Chem.* **59,** 4723 (1994).

[259] H. M. Geysen, C. D. Wagner, W. M. Bodnar, C. J. Markworth, G. J. Parke, F. J. Schoenen, D. S. Wagner, and D. S. Kinder, *Chem. Biol.* **3,** 679 (1996).

[260] J. Vagner, V. Krchnák, N. F. Sepetov, P. Strop, K. S. Lam, G. Barany, and M. Lebl, *in* "Innovation and Perspectives in Solid Phase Synthesis" (R. Epton, ed.), p. 347. Mayflower Worldwide, Birmingham, UK, 1994.

[261] K. C. Nicolaou, X. Y. Xiao, Z. Parandoosh, A. Senyei, and M. P. Nova, *Angew. Chem., Int. Ed. Engl.* **34,** 2289 (1995).

[262] K. Biemann and S. A. Martin, *Mass Spectrom. Rev.* **6,** 1 (1987).

[263] K. Biemann, *Methods Enzymol.* **193,** 455 (1990).

[264] J. W. Metzger, *Angew. Chem., Int. Ed. Engl.* **33,** 723 (1994).

can be substantially simplified using deuterium exchange experiments,[265] as the determination of exchangeable protons can decrease the number of possible sequences by an order of magnitude.[240] Partial (~10%) capping of the growing peptide chain after each coupling is the basis of another approach to peptide sequence determination.[231] Thus, the beads in this library contained all N-terminal truncation analogs in addition to the complete library peptides, and the synthetic history could easily be assessed based on the mass differences between the truncated analogs. Residues with the same molecular weight can be differentiated by using a mixture of different capping reagents. If the capping reagent contains an isotope with a typical isotopic "signature," such as bromine, the individual shorter sequences can be easily identified even at a level close to the noise of an experiment.[266]

Libraries Composed of Separate Compound Mixtures

The identification of active individual compounds from libraries of this type (i.e., iterative, positional scanning, and orthogonal libraries) does not require any additional analytical techniques, because owing to the systematic arrangement of the mixtures making up these libraries (i.e., the implementation of defined and mixture positions), the identification of individual compounds that are responsible for an observed biological activity of a library is inherent to the library screening process (see above). This is one of the principal advantages of this class of libraries.

Experimental Procedures

Syntheses of One-Bead–One-Compound Peptide Libraries

Synthesis of Noncleavable Library. The library is synthesized[38] on 130-μm TentaGel (Rapp Polymere, Tubingen, Germany) resin beads. Alternatively, ArgoGel (Argonaut Technologies) polydimethylacrylamide beads, of Pepsyn Gel Resin (Cambridge Research Biochemicals, Northwitch, UK) can be used. In general, any resin that is compatible with organic solvents, as well as aqueous media, is adequate. Spacers, such as aminocaproic acid, aminobutyric acid, and/or β-Ala, may be attached to the resin prior to assembling the library. The resin beads are divided into 19 aliquots contained in 19 polypropylene vials or plastic syringes equipped with a plastic

[265] N. F. Sepetov, O. L. Issakova, M. Lebl, K. Swiderek, D. C. Stahl, and T. D. Lee, *Rapid Commun. Mass Spectrom.* **7,** 58 (1993).

[266] N. Sepetov, O. Issakova, V. Krchnák, and M. Lebl, U.S. Patent 5,470,753 (1995).

frit at the bottom (CSPS, San Diego, CA). (The number of amino acids we use in randomization, equal to the number of reaction vessels, is limited only by the patience of the chemist; the highest number that we have used is 384 syringes.)

Nineteen Fmoc-amino acids (all proteinogenic amino acids except Cys) are added separately into each of the resin aliquots using a minimal amount of DMF. The amino acids are added in 3-fold excess, and coupling is initiated by adding a 3-fold excess of benzotriazol-1-yl-oxytris-(dimethylamino)phosphonium hexafluorophosphate (BOP) and *N,N*-diisopropylethylamine (DIEA) [or *N,N'*-diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt). A trace amount of bromphenol blue is added to the reaction mixture.[85,267] The vials are tightly sealed (syringes are capped) and rocked gently for approximately 30 min at room temperature, or until all beads turn from blue to colorless. Completion of the coupling is confirmed by the ninhydrin test.[84] When the coupling is incomplete, the beads are allowed to settle, and the supernatant is gently removed; alternatively, in the case of synthesis in a syringe, it is expelled from the syringe. Fresh activated Fmoc-amino acid is added, and the reaction proceeds for 1 hr.

The resin pools are mixed in a siliconized cylindrical glass vessel fitted at the bottom with a frit. Dried nitrogen is bubbled through to mix the resin. After washing eight times with DMF, piperidine–DMF (1:4, v/v) is added. After 10 min of bubbling with nitrogen, the piperidine is removed and the resin washed 10 times with DMF. The amount of released dibenzofulvene–piperidine adduct is determined by measuring the absorbance at $\lambda = 302$ nm. A stable level of substitution determined in this manner throughout the library synthesis serves as one of the quality control criteria.

The resin is again divided into 19 aliquots for the coupling of the next 19 amino acids. After the coupling steps are completed, the Fmoc group is removed with piperidine–DMF (1:4, v/v), and the resin is washed with DMF and DCM. The side-chain protecting groups are removed by treatment with reagent K[268] (trifluoroacetic acid–phenol–water–phenol–ethanedithiol, 82.5:5:5:5:2.5; v/w/v/w/v) for 5 + 120 min. This treatment is performed in the common container (glass bubbler) or, in cases that separate pools of resin are required, in individual syringes. The resin is washed thoroughly with TFA, DCM, DMF, DMF–water (1:1, v/v), and 0.01% HCl in water, and stored in DMF at 4°. Again, small individual pools can be conveniently stored in the plastic syringes in which the whole

[267] V. Krchnák, J. Vagner, and M. Lebl, *Int. J. Pept. Protein Res.* **32,** 415 (1988).
[268] D. S. King, C. G. Fields, and G. B. Fields, *Int. J. Pept. Protein Res.* **36,** 255 (1990).

synthesis is performed. Larger library batches (up to 80 g) are stored with protected side chains in 0.2% HOBt/DMF at 4°.

To verify the quality of the library, several randomly chosen beads are sequenced, and the average amount of peptide per bead is determined. This value is confirmed by quantitative amino acid analysis of a random sample from the library (~1 mg). Whereas amino acid analysis is used to determine the overall amino acid composition of the library, sequence analysis confirms random distribution of amino acids at each position.

If the library is synthesized in MultiBlock (http:/www.z5.com/csps), the randomization of the resin is achieved by closing the block with glass plate, inverting it so that all resin is placed in the common area, shaking it mechanically, and inverting it again. Resin is uniformly distributed into the individual reaction compartments (syringes), and the next step of the synthesis can be performed. Any number of reaction chambers can be stoppered during the mixing, and therefore a number of different mixing schemes can be achieved in the MultiBlock.

Synthesis of Libraries for Two-State Solution Testing

TentaGel (5 g, 0.23 mmol/g, 130 μm average particle size) is swollen in DMF (swollen resin volume 25 ml), and Fmoc-Lys(Boc)/DIPCDI/HOBt (3 equivalents each) in DMF is coupled.[37] After 2 hr the resin is washed five times with DMF and once with DCM, and the Boc group is removed with TFA–DCM (1:1, v/v, 1 + 20 min). After washing with DCM (five times) and DMF (four times), the resin is neutralized with DIEA–DMF (1:49, v/v), washed with DMF (three times), and the linker **1** (Fig. 3, 3 equivalents) is activated by DIPCDI and HOBt (3 equivalents each) in DMF and coupled overnight. The resin is washed with DMF (five times), and the Fmoc group is removed with piperidine–DMF (1:4, v:v, 20 min). After washing with DMF (three times) and distribution of the resin into m reaction vessels (plastic vials or fritted syringes), individual Fmoc protected amino acids are coupled to each part of the resin using DIPCDI and HOBt (3 equivalents each). The reaction is monitored using bromphenol blue.[85,267] When complete coupling is observed in all reaction vessels (all resin particles are decolorized), completeness of the coupling is verified using the ninhydrin test.[84] All resin portions are combined, washed with DMF (five times), and the Fmoc group is removed as described above. This procedure (separate couplings and deprotection after combining the resin) is repeated $n - 1$ times (n = number of library positions). The side-chain protecting groups are cleaved by reagent K[268] for 2 hr, and washed with TFA (three times), DCM (five times), DMF containing 0.1% HCl (four times), and 0.1% HCl in water (five times). The library has to be stored in an acidic solution in order to prevent premature loss of peptides.

Quality Control of Doubly Releasable Library. Dried resin (5–10 mg) is shaken overnight in 2–5 ml of 0.1 *M* HEPES buffer (pH 8.5) in a polypropylene syringe equipped at the bottom with a polypropylene or Teflon frit and a polypropylene plunger. The absorbance of the solution (diluted, if necessary) at $\lambda = 280$ nm is measured, and the amount of released peptide is calculated according to the following formula:

$$\text{Release (mmol/g)} = \frac{\text{absorbance} \times \text{volume} \times \text{dilution}}{(1197n/x \pm 5559m/y)\text{mass}}$$

where mass is the quantity of library beads in grams, x is the number of amino acids in positions where Tyr is used, y is the number of amino acids in positions where Trp is used, n is the number of positions in the library where Tyr is used, and m is the number of positions in the library where Trp is used. If other amino acids with absorbance at $\lambda = 280$ nm are used in library construction, the above formula must be modified.

A solution of 0.2% NaOH is drawn into the syringe containing the library sample, and the syringe is shaken for 4 hr. The solution is expelled from the syringe and the absorbance measured at $\lambda = 280$ nm. The same calculation is performed using the formula shown above using coefficients 1507 and 5377 instead of 1197 and 5559, respectively. The amount of released peptide in each step should not differ by more then 10% from the theoretical value, which is calculated according to

$$\text{Theoretical release (mmol/g)} = \frac{\text{Subst.}}{1 + \text{Subst.}\,(3\text{MW} + 686)/1000}$$

where Subst. is the original substitution of the resin (in mmol/g), MW is the average molecular weight of the library peptides, and 686 is the molecular weight of the Ida linker (without Fmoc groups), plus one Lys residue, minus one molecule of water. The average molecular weight of a natural amino acid is 119.7 (19 amino acids, Cys excluded). Therefore, the average molecular weight of a pentapeptide library made from these 19 amino acids is 598.5. Starting with a resin substitution of 0.2 mmol/g, 0.134 mmol of pentapeptide should be released at each step using 1 g of dried library resin.

Two-Stage Release Assay in 96-Well Microassay Plates. Library beads are transferred into pH 4.5 buffer containing 1.0% carboxymethylcellulose (to retard sedimentation), shaken, and rapidly pipetted into the upper chambers of a vacuum-control 96-well filtration manifold (Model 09601, Millipore, South San Francisco, CA). Approximately 500 beads are placed in each filtration well, so that each plate contains approximately 48,500 unique peptides. The filtration plates serve as "master" plates for retaining subsets of peptides in unique locations. The transfer buffer is removed by

vacuum filtration, and the first stage release of peptides is accomplished by dispensing the appropriate buffer or tissue culture medium (neutral pH) to each well and incubating overnight. The released peptides are vacuum filtered into 96-well microassay test plates where the biological activity is determined. In some experiments the released peptides are distributed into several replicate plates for multiple simultaneous assay against different molecular targets. Wells identified as "positive" are marked, and the beads of origin are recovered from the corresponding well(s) of the filtration master plate with the aid of a low power stereomicroscope. The recovered beads are transferred one by one (one bead per well) into individual microwells of 96-well filtration plates. Cleavage of the ester (second) linker is then accomplished by addition of 0.2% NaOH and overnight incubation followed by pH adjustment. Alternatively, the second stage release may be achieved by overnight incubation in ammonia vapors in a desiccator or dedicated pressurized chamber. After drying, the appropriate buffer is added, and the plates are gently shaken for several hours. Thereafter, the peptide-containing buffer is filtered into the test plates for bioassay. The individual peptide beads corresponding to each positive well in the second stage assay are recovered and submitted for microsequencing.

Synthesis of Directed Libraries

Various procedures for synthesizing directed libraries have been developed.[163]

Synthesis of Directed Library on Cotton String. A cotton ribbon (5 m long, 3 cm wide) is treated for 1 hr in TFA–DCM (1:3, v/v), washed with DCM (three times), neutralized with DIEA–DCM (1:19, v/v, 5 min), and washed with DCM and DMF (three times). Fmoc-β-Ala (2 mmol) is coupled overnight by DIPCDI (2 mmol) and HOBt (2 mmol) activation with addition of *N*-methylimidazole (3.5 mmol). The cotton is washed with DMF, and the substitution level is determined by spectrophotometric determination of the cleaved Fmoc group [typical value is 0.4 mmol/g (68 nmol/cm)]. Five pieces of the cotton string (25 cm each) are placed in five polypropylene syringes, and Fmoc protected amino acids [Phe, Tyr(*t*Bu), Ala, Leu, Gly] are coupled by the DIPCDI/HOBt protocol. After the coupling is complete (monitored by bromphenol blue method[85]), the strings are washed with DMF, placed in one syringe (no frit needed), and the Fmoc groups cleaved. After washing with DMF and a 2% solution of HOBt in DMF, the mixture of 19 Fmoc-amino acids (Cys is excluded) with the molar ratios (determined in pilot experiments) adjusted for different reactivities[9,57,156–158] is coupled to all cotton pieces. After completion of coupling the cotton is washed, the

Fmoc groups cleaved, and the washed cotton string divided into five syringes in the following way. From each string 5 cm of the cotton is cut and placed in a different syringe. In this way all syringes have only one 5-cm piece of cotton cut from each 25-cm string and none have more than one. Coupling of five amino acid derivatives (same as above) is performed, the cotton washed, Fmoc groups removed, and cotton subdivided again. In this case 1-cm pieces are cut from all 5-cm pieces and placed in five syringes. Coupling of the same five amino acids is performed, the Fmoc groups are cleaved, and side-chain protecting groups are cleaved by TFA–DCM–anisole (50 : 45 : 5, v/v/v) for 2 hr. Cotton pieces are washed by DCM and methanol and dried. Quantitative amino acid analysis of a sample of one string has revealed a substitution level of 400 nmol/cm.

Synthesis of Directed Library on Functionalized Cross-Linked Teflon Membrane. Hydrophilic aminopropyl functionalized membrane (UV cross-linked aminopropylmethacrylamide, *N,N*-dimethylacrylamide, and methylenebisacrylamide on Teflon membrane, 16 × 16 cm, Perseptive Biosystems) with approximate 35 nmol/cm^2 substitution is placed into a 50-ml Falcon tube and acylated by Fmoc-β-Ala using the DIPCDI/HOBt procedure in DMF. Fmoc-Gly, Fmoc-β-Ala, and Fmoc-Gly are coupled consecutively. After deprotection, the membrane is divided into two parts, and Fmoc-Phe and Fmoc-Leu are coupled to them, respectively. After coupling completion (bromphenol blue monitoring[85]) and deprotection, the pieces are divided again into two halves and recombined for the coupling of Fmoc-Gln and Fmoc-Phe. For the next coupling the membrane is divided again into two pieces and recombined for coupling of Fmoc-Proc and Fmoc-Gly. The pieces resulting from these couplings (8 × 4 cm) are now divided into 19 strips (8 × 0.21 cm) and the strips are placed into 19 small polypropylene tubes. Nineteen natural amino acids (excluding Cys) are used for coupling in this stage. After coupling completion and Fmoc deprotection, the strips are cut into 19 pieces (4 × 2.1 mm) and divided into 19 vessels again. The same set of 19 Fmoc-amino acids is used for the last coupling. All pieces are combined, Fmoc groups are removed, and side-chain protecting groups are cleaved by reagent K.[268] Membrane pieces are washed with TFA (two times), DCM (five times), methanol (three times), and water (five times). The substitution level based on measurement of the absorbance of the last Fmoc release is 43.3 nmol/cm^2.

Synthesis of Dual Defined Iterative Hexapeptide Library

Nineteen (or any number corresponding to the number of used building blocks, depending on the capability of the chemist) individually labeled

porous polypropylene mesh packets are charged with 20 g of MBHA resin each.[269] Each of 19 of the 20 genetically coded Boc-amino acids (Cys excluded) is activated by DIPCDI and coupled to one of the 19 resin packets. The coupling reaction is monitored for completion by using bromphenol blue[85] or the ninhydrin test.[84] The resin packets are washed with DCM and dried; the resins of all packets are recombined and mixed thoroughly. This one-position resin is referred to as X-resin.

The X-resin is divided into 19 equal portions and placed into new polypropylene mesh packets. The Boc group is removed with TFA–DCM (11 : 9, v/v), and the resin is washed with DCM and 2-propanol, neutralized with DIEA–DCM (1 : 19, v/v), and washed with DCM. The 19 amino acids are activated by DIPCDI and coupled to the resin packets to generate 361 (19^2) dipeptides. These dipeptides are termed OX-resins. Mixing of all OX-resins affords XX-resin.

The coupling steps are repeated twice more to generate a 130,321 tetrapeptide mixture resin (XXXX-resin). The XXXX-resin is divided into 400 equal aliquots and placed in labeled polypropylene mesh packets. The Boc group is removed and the resin neutralized. Two amino acids are coupled to each of the packets using standard coupling procedures. The result is a hexapeptide mixture resin (O_1O_2XXX-resin) with two defined (O) and four mixture (X) positions.

The 400 separate peptide mixtures are deprotected and cleaved using the high/low HF method[87] in a multivessel apparatus.[86] Peptides are extracted from the resins with water or a mixture of acetic acid and water, the solution is lyophilized twice, and peptide mixtures are dissolved in water at 1 to 5 mg/ml. The peptide library is stored 1 to 2 weeks at 4°, or frozen for prolonged storage. Sonication facilitates the solubilization of peptide mixtures with hydrophobic amino acids at the defined positions.

Synthesis of Positional Scanning Hexapeptide Library

The positional scanning hexapeptide library[269] is composed of six sublibraries (O_1XXXXX-NH_2, XO_2XXXX-NH_2, XXO_3XXX-NH_2, $XXXO_4$XX-NH_2, $XXXXO_5$X-NH_2, and $XXXXXO_6$-NH_2), where one position (O) is individually defined with one of 19 amino acids and the remaining five positions (X) are mixtures of 19 amino acids. Thus, the entire library is made up of 114 (19×6) distinct peptide mixtures. Amino acids are mixed for coupling in a molar ratio that ensures equimolar incorporation

[269] C. Pinilla, J. R. Appel, and R. A. Houghten, *in* "Current Protocols in Immunology" (J. E. Coligan, ed.), Wiley, New York, 1994.

TABLE I
COMPOSITION OF AMINO ACIDS MIXTURES FOR COUPLING[a]

Amino acid	Molar ratio
Boc-Ala	1.18
Boc-Arg(Tos)	2.26
Boc-Asn	1.86
Boc-Asp(OBzl)	1.22
Boc-Gln	1.85
Boc-Glu(OBzl)	1.26
Boc-Gly	1.00
Boc-His(Dnp)	1.24
Boc-Ile	6.02
Boc-Lys(2-Cl-Z)	2.16
Boc-Leu	1.72
Boc-Met(O)	0.80
Boc-Phe	0.88
Boc-Pro	1.50
Boc-Ser(Bzl)	0.97
Boc-Thr(Bzl)	1.66
Boc-Trp(For)	1.32
Boc-Tyr(2-Br-Z)	1.44
Boc-Val	3.91

[a] Amino acids with different side-chain protection groups require an adjusted molar ratio.

of amino acids into peptides (Table I). One hundred fourteen polypropylene mesh bags are labeled and loaded with 400 mg MBHA resin each. Nineteen Boc protected amino acids are activated by DIPCDI and coupled to bags 96 to 114, whereas the mixture of 19 amino acids is coupled to bags 1 to 95. Resins bags 96 to 114 have a defined position at position 6. The other bags have mixture positions there. After Boc removal, 19 individual amino acids are coupled to bags 77 to 95, and the amino acid mixtures to the remaining bags. Resin bags 77 to 95 have a defined position at position 5. This procedure is repeated through the sixth coupling. The peptides are cleaved, extracted, and lyophilized as described in the previous protocol. Peptide mixtures are dissolved in water at 10 to 20 mg/ml and stored 1 to 2 weeks at 4° or are frozen for prolonged storage.

The higher final concentration of peptide mixtures in this library compared to the dual defined peptide library compensates for the presence of 19 times more peptides when compared to the latter peptide mixtures (five versus four mixture positions). The relative concentration of individual peptides within the mixtures decrease 19 times.

Synthesis of Library on Cellulose Paper Sheet

A sheet of chromatographic paper Whatman Chr1 (Maidstone, UK) is marked with a pencil (spot positions), and the sheet is dried under vacuum overnight.[53] A solution of 0.2 *M* Fmoc-amino acid (Pro or β-Ala), 0.24 *M* DIPCDI, and *N*-methylimidazole in DMF is soaked into the paper sheet, and the reaction is run for 3 hr in a closed container. The paper sheet is washed three times with DMF and treated with piperidine–DMF (1:4, v/v) for 20 min. After three washes with DMF and two washes with ethanol, the sheet is dried in a desiccator. Solutions of Fmoc-amino acid HOBt esters (0.5 ml, 0.3 *M*) are then spotted onto pencil marked spots, and reactions proceed for 20 min on a plastic tray covered with glass plate. The paper sheet is then washed twice with acetic anhydride–DMF (1:49, v/v) and treated with the same solution in the presence of 1% DIEA for 30 min. After washing with DMF (four times), deprotection by piperidine–DMF (1:4, v/v, 5 min), and washing by DMF (four times), the sheet is washed in bromphenol blue solution (0.01%) to reveal blue spots with available amino groups. The sheet is washed with ethanol (two times) and dried by cold air from a hair dryer between two layers of Whatman 3MM paper. Blue spots are used as a target for spotting activated solutions of amino acids (0.3 *M*, 0.5–1 ml). The paper sheet is optionally respotted after 15 min (if disappearance of blue color is slow). Final deprotection is performed by immersing the dry sheet into a solution of TFA–DCM–diisobutylsilane–water (50:453:2, v/v/v/v) for 2 hr. After washes with DCM (four times), DMF (three times), and ethanol (two times), the sheet is dried and ready for binding assay or storage (−20° in a sealed plastic bag). In the case of peptide synthesis on a linker cleavable by intramolecular DKP formation, the sheet has to be washed exclusively by acidic solutions so as to not lose the synthesized peptides prematurely. The spots can be cut or punched out of the dried sheet into polypropylene tubes or into wells of a microtiter plate, and after addition of neutral buffer the peptides are released into solution.

Synthesis of Peptide Library on Soluble Support

The library is synthesized on a poly(ethylene glycol) methylether (MeO-PEG) (molecular weight 5000) support.[69] The support is divided into four aliquots, and the first Boc-protected amino acid (Leu, Phe, Tyr, and Gly) is attached to the support by the *N,N'*-dicyclohexylcarbodiimide/4-dimethylaminopyridine (DCC/DMAP) procedure. Excess reagents are removed by precipitation with diethyl ether or ice-cold ethanol, or by ultrafiltration. Aliquots are taken from each portion and stored for later deconvolution, all portions are mixed, and the Boc protecting group is removed by a

TFA–DCM (1 : 1, v/v) mixture. The product is separated into four portions, and Boc-protected amino acids are coupled by HBTU in the presence of DIEA in a micture of DMF and DCM. Completeness of the coupling is followed by the ninhydrin reaction.[84] Uncoupled amino groups are acetylated by acetic anhydride. Recombination, separation into aliquots, and amino acid couplings are repeated another three times. After the last coupling, the aliquots are kept separate, and the final removal of Boc and BrZ groups is performed by iodotrimethylsilane.[270] Aqueous solution of aliquots are evaluated directly by biological assays.

Synthesis of Orthogonal Library

The synthesis of an orthogonal library is documented by an example of a tripeptide library composed of 12 amino acids in each step. Orthogonal mixtures are formed in the dipeptide stage, and each mixture contains 12 peptides. The synthesis is performed in a 96-well plate[77] according to the standard Fmoc/*t*Bu protocol.

RAM TentaGel resin is distributed into 144 wells of 2 plates. The Fmoc group is removed by piperidine–DMF, the resin is washed, and 12 Fmoc-protected amino acids (activated by DIPCDI/HOBt) are distributed by "rows," where each row contains one amino acid. The plate is left overnight with occasional shaking on an orbital shaker. The resin is washed with DMF, Fmoc groups are cleaved, and the resin is washed with DMF. To prepare all combinatorial dipeptides, the second amino acid (activated by DIPCDI/HOBt) is distributed by "columns," that is, each column receives one amino acid. After the condensation is complete, the resin beads are washed with DMF, Fmoc groups are cleaved, and the resin is washed with DMF.

The orthogonal mixtures are made the following way. One-half of the resin beads are withdrawn from all wells in the first row, and the mixture (of 12 dipeptides) is redistributed into 12 wells of the first row of a new 96-well plate. This operation is repeated for all 12 rows, resulting in 12 rows of wells filled with a mixture of dipeptides. The second half of the resin beads from all wells in the first column is mixed and redistributed into 12 wells of a new plate. Again, this operation is repeated for all columns. The result of this mixing is 2 times 12 rows containing mixtures of dipeptides, with the first 12 rows containing the "horizontal" mixtures and the next 12 rows "vertical" mixtures.

For the last condensation the first of 12 activated amino acids is distributed into the first row of both "horizontal" and "vertical" mixtures. After

[270] R. S. Lott, V. Chauhan, and C. H. Stammer, *J. Chem. Soc., Chem. Commun.*, 495 (1979).

acylation the resin beads are washed, Fmoc groups cleaved, and the resin beads washed with DMF and methanol and dried. Cleavage of peptides is accomplished by the standard TFA procedure.

Synthesis of α,β,γ Library

The library is synthesized on TentaGel resin using the following procedures.[205] The Fmoc group is removed with piperidine–DMF (1:4, v/v) 5 + 20 min, then the resin is washed with DMF six times. All washes are collected, the absorbance at $\lambda = 302$ nm is measured, and the Fmoc release is quantified. For coupling, 3-fold molar excesses of protected amino acid and HOBt is dissolved in DMF, DIPCDI is added, and the solution is drawn into a syringe with the resin. Completeness of each condensation reaction is checked by the ninhydrin test. The chloranil test is used in cases of coupling to secondary amino groups. Coupling time varies between 1.5 and 40 hr. For Z removal, the resin is washed three times with DMF, three times with DCM, and two times with thioanisole–TFA (1:9, v/v) for 1 min. The resin is left in thioanisole–TFA (1:9, v/v) overnight. The resin is then washed five times with DCM, neutralized with DIEA–DCM (1:19, v/v), and washed three times with DCM and DMF. For Boc deprotection, the resin is washed with DCM, treated with TFA–DCM–anisole (9:9:2, v/v/v) for 5 + 20 min, and washed with DCM six times. For Alloc deprotection, the resin is washed five times with DMF, a mixture of DMF–acetic acid–*N*-methylmorpholine (10:2:1, v/v/v) is added, argon is bubbled for 15 min, tetrakis(triphenylphosphine)palladium(0) is added, and the reaction is allowed to proceed for 3 hr. The resin is washed five times each with DMF, DCM, and DMF.

The library synthesis consists of 50 synthetic steps:

1–9. Synthesis of Boc-β-Ala-Gly-β-Ala-Gly-Lys(Alloc)-TentaGel.
10. Remove Alloc.
11. Divide resin into six aliquots.
12. Couple six coding pairs of Fmoc-amino acids.
13. Remove Boc.
14. Couple Boc-Dap(Alloc)-OH to portions 1 and 2. Couple Boc-Dab(Alloc)-OH to portions 3 and 4. Couple Boc-Orn(Alloc)-OH to portions 5 and 6.
15. Combine portions 1, 3, and 5.
16. Remove Boc.
17. Combine portions 2, 4, and 6.
18. Remove Alloc.
19. Combine all resin.
21. Divide into 46 portions.

22. Couple 46 acids.
23. Remove Fmoc.
24. Couple coding doublets of Fmoc amino acids.
25. Mix the resin.
26. Remove Alloc.
27. Remove Boc.
28. Divide resin into six portions.
29. Couple Boc-Dap(Alloc)-OH to portions 1 and 2. Couple Boc-Dab(Alloc)-OH to portions 3 and 4. Couple Boc-Orn(Alloc)-OH to portions 5 and 6.
30. Remove Fmoc.
31. Couple six coding pairs of Fmoc-amino acids.
32. Combine portions 1, 3, and 5.
33. Remove Boc.
34. Combine portions 2, 4, and 6.
35. Remove Alloc.
36. Combine all resin.
37. Divide into 46 portions.
38. Couple 46 acids.
39. Remove Fmoc.
40. Couple coding doublets of Fmoc-amino acids.
41. Mix resin.
42. Remove Alloc.
43. Remove Boc.
44. Divide resin into 50 portions.
45. Couple 50 acids.
46. Remove Fmoc.
47. Couple coding doublets of Fmoc-amino acids.
48. Remove Fmoc.
49. Mix resin.
50. Remove Z side-chain protecting group.

Acylation Monitoring by Bromphenol Blue

Couplings performed in neutral solution (DIPCDI/HOBt, preformed anhydrides, active esters) can be monitored by addition of trace amounts of bromphenol blue (BB).[85] The sensitivity of the method can be significantly diminished by application of large amount of bromphenol blue, and therefore no more indicator than the amount equal to 1% of available amino groups should be applied. Usually several drops of 0.1% solution of BB in DMF or *N*-methylpyrrolidone (NMP) (if the dissolution provides blue solution, it can be decolorized by the addition of HOBt) are added into

the last wash before coupling, or directly to the solution of activated acid. Blue-colored beads turn green, greenish yellow, and eventually yellow. The speed of some couplings can be puzzling. Most are complete within 2 to 5 min; in library synthesis, however, care should be taken about the slowest couplings, which may require much longer exposure. BB monitoring allows the evaluation of coupling at the level of individual beads. In this case a sample of the reaction slurry is placed on the petri dish and inspected under a microscope. It is easy to detect one incompletely coupled bead in the middle of tens of thousands of beads.

Successful application of BB monitoring requires the absence of quaternary ammonium salts on the resin, as resin containing these residual functionalities never becomes BB negative. Before using a new batch of solid support, the resin should be peracetylated (if it is not fully protected) and treated with BB solution. If blue coloration is observable, BB cannot be used for monitoring. In the presence of sulfonium salts (modified side chains of Met), the resin would be greenish even without the presence of free amino group.

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