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The Challenge of Preparing and Testing Combinatorial Compound Libraries in the Fast Lane, at the Front End of Drug Development

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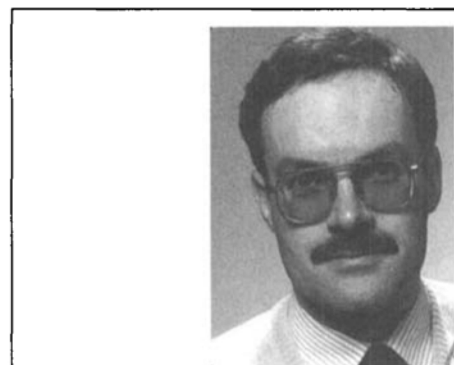
Abstract. Combinatorial Compound Libraries (CCL) are at the front end of the development of new chemical entities. They are the result of a pragmatic, goal-oriented attitude, which emphasizes the discovery side of research activities. The odds to bump into a discovery, however, are pushed to the extreme with rigorously planned rationales. The CCL field has its origin in peptide chemistry but is migrating into general organic chemistry. The incentive is the coveted goal to be able to prepare large libraries of 'small molecules'. Efforts are directed towards an expansion of the repertoire of high yield reactions on solid phase, in order to readily access semi-rigid globular ligands. The CCL approach comes with its own strategies to provide a massive increase of compound evaluations by taking full advantage of the integrated preparation process with the option to control the physical format of compound ensembles or to classify them into defined subpopulations. A noticeable impact on the drug discovery process is expected. The libraries' preparation and evaluation principles are briefly discussed in this essay.



Eduard R. Felder, born 1955 in Milano, Italy, studied Pharmaceutical Sciences at the Swiss Federal Institute of Technology (ETH) in Zürich. He attended a postgraduate program in the Molecular Biology Institute of the same school, where he also carried out his doctoral thesis work, with Prof. *Robert Schwyzer*, on new methods for the chemical synthesis of nucleic acids. His dissertation was awarded the ETH medal. In 1985, he joined the Department of Molecular Genetics at the *Beckman* Research Institute of the City of Hope (Duarte, California). In 1986, he accepted a position at the 'Eidgenössisches Institut für Reaktorforschung' in Würenlingen, where he studied effects of radiation on nucleic acids. In 1987, he moved to the Department of Chemistry at the University of California San Diego. There he worked with Prof. *Murray Goodman* on the synthesis of cyclic opioid peptide mimics and their characterization by NMR and molecular modeling. Since 1989, he is in the Biotechnology Ressort (Core Drug Discovery Technologies) of *Ciba-Geigy*, Basel.

1. Introduction

Combinatorial compound libraries (CCL) are *ensembles* of molecules generated simultaneously (or in a rapid sequence of steps) by combining structural elements from a set of building blocks or reactants with maximal use of parallel processes on the entirety of its components. Combinatorial compound libraries have become a topic of discussion with increasing consistency in the scientific community dedicated to the search for new ligands [1] of macromolecular targets. The technological progress of the past decade laid the ground for the ability to generate high molecular diversity rapidly and in a controlled manner, and also for the capacity to screen and evaluate the properties of complex preparations. Today, a multidisciplinary field of confluent science and technologies (biology, chemistry, automation, data processing *etc.*) has grown around the conceptually simple principle of reshuffling structural units in a combinatorial fashion. Recently, basic concepts for exploiting the dimension of large numbers have been brought up and adapted to an impressive array of different applications. The developments in biotechnology, immunology, and pharmaceutical research of specific binders (such as agonists, antagonists, and inhibitors), exploiting the knowledge of metabolic mechanisms rather than leads from natural products, demand a massive increase of compound evaluations in order to take full advantage of modern assays developed on the basis of newly explored molecular mechanisms and bearing a high throughput capacity. On the other hand, with the advent of combinatorial techniques, the number of available compounds leaps to orders of magnitude which cannot be handled by conventional screening techniques used for the measurement of single component series. Therefore, an integrated CCL approach must include innovative testing strategies. Pharmaceutical and agrochemical research organizations are aware of the enormous potential residing in CCL as a mean to identify new leads more efficiently as a result of a vast systematic search, *i.e.*, with a better chance to meet the requirements for a fast product development in the subsequent optimization effort. The underlying principles and relevant applications in the CCL field have been the subject of occasional summaries [2], which are excellent records of the progress achieved in this fast-moving area. At this point, it is critical for many organizations to chose a suitable overall strategy to exploit most efficiently the merits of the multifaceted CCL field. This is an organizational challenge with the necessity to assess the value of different approaches and the requirements for successful implementation, taking into account the available skills and means. Naturally, there are clashing opinions with regard to the prioritization of methodologies. Further points of discussion are definitions of such fundamental terms as 'library' and 'diversity', which are prone to personal interpretations. Clearly, these expressions are not linked to particular

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numbers: with good reasons one may call 'library' a set of a few dozen compounds or a phage culture with billions of structures. On the other hand, one may consider a simple array of different chemical functionalities a better representation of diversity than a large number of homologs. A compilation of frequently used terms is listed and explained in the *Appendix* [3].

2. Basic Principles of CCL Preparation

The basic principles of CCL generation are discussed extensively in a review by *Gallop et al.* [2a]. This section is a brief overview of combinatorial approaches for the preparation of libraries. It is important to keep in mind the fact that the assembly methods and the evaluation techniques (assay principles) are strongly interconnected by imposing restrictions on each other, raising the issue of format compatibility between library and assay in every planning phase of a CCL application.

The field of combinatorial libraries has its origin in the area of oligonucleotidic and peptidic biooligomers. The recombination of molecular subunits is the basis of immune systems and evolutionary mechanisms. Since the advent of efficient oligonucleotide chemistry, when it became possible to incorporate complex mixtures of synthetic oligonucleotides (random cassettes) into microbial expression systems, similar processes may be imitated *in vitro*: huge numbers (billions) of randomly permutated peptide sequences are expressed amidst the constant flanking sequences of a gene product. The role of selecting proteins with the desired binding properties from a vast pool of useless variants is assumed by an appropriate affinity enrichment step (*e.g.* by exposure to an immobilized antibody). The initial preference for biooligomeric CCL is explainable for essentially two reasons: the inspiration by naturally occurring mechanisms and the availability of the necessary genetic engineering tools. Furthermore, solid-phase chemical synthesis of peptides and oligonucleotides was the obvious choice upon engaging into fully synthetic libraries owing to their high degree of reliability and suitability for automation. Meanwhile, there is a justified eagerness to widen the repertoire of high-yield chemical reactions on solid phase as much as possible and gain access to many other structural classes. Beyond the known biooligomer chemistry, an ingenious procedure invented by *Furka* [4] (often quoted as split synthesis* or portioning/mixing*) contributed considerably to the fast development of chemically synthesized CCL. This particular protocol for handling solid-phase beads* ensures that each particle carries only one compound species and that a quantitatively even representation of the different library elements is maintained. The principle is illustrated in *Sect. 2.2.1* and in the *Appendix*.

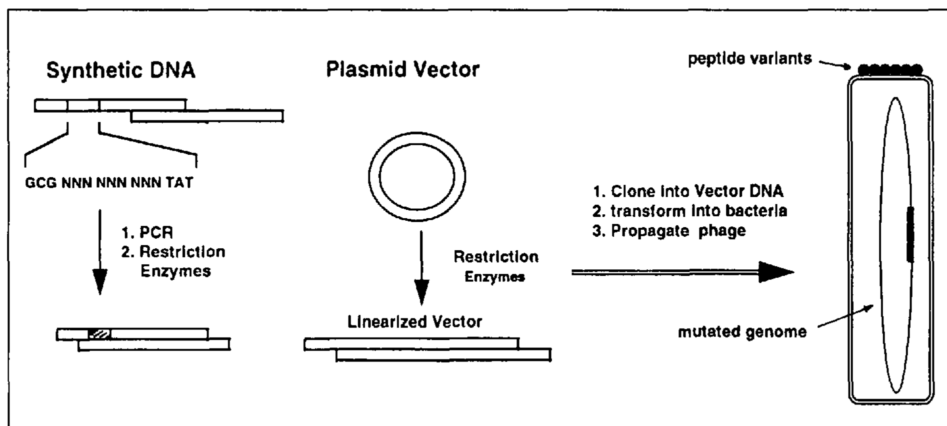


Fig. 1. *Phage display: Bacteriophages such as M13 are robust viral particles* (virions) *which infect bacteria such as* E. coli *and grow to high titers* (10^{12}/ml). They are suited as *in vitro* selection vehicles. Randomized oligonucleotides are inserted into a gene coding for a viral surface protein. The genomic manipulation translates into the display of peptide variants within the protein coat. A pooled phage stock is grown and subjected to selection, *e.g.* by binding to an immobilized target (enrichment for binders over non-binders).

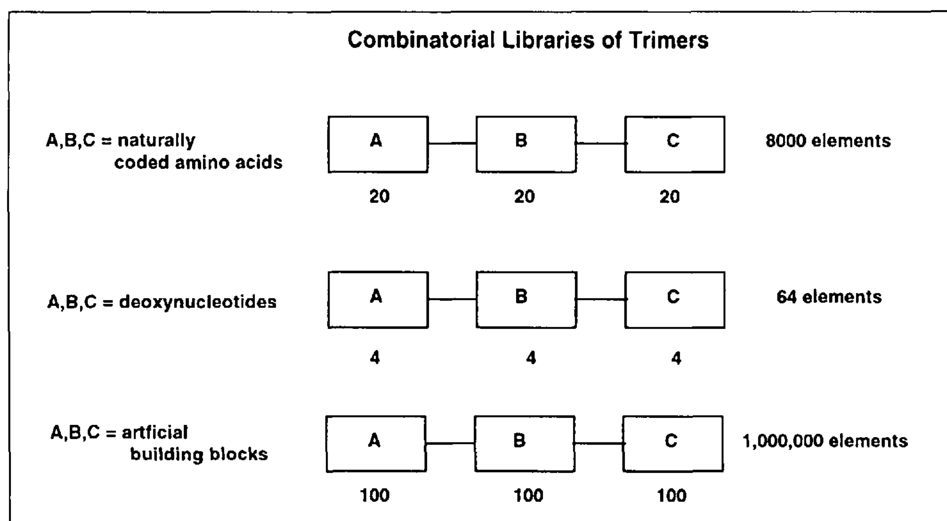


Fig. 2. *The impact of the number of building blocks on the number of library elements*

2.1. *Genetically Engineered Libraries*

As mentioned above, the number of molecular structures produced by biological expression systems bearing inserted stretches of randomized oligonucleotides may be enormous. Common features of these approaches are steps of variation (randomizing nucleotide sequences), selection (affinity enrichment), and amplification. The latter, while precluded for chemical libraries, is a prominent advantage of biologically oriented approaches allowing to handle minimal amounts of material with maximal diversity, and virtually eliminating any worries about detection limits. We discern among two basic options: nucleic-acid libraries and peptide/protein libraries. The latter have become more and more popular because of their ease of handling and their wide range of applications. Numerous examples are cited by *Gallop et al.* [2a] and by *Clackson* and *Wells* [5]. Peptides are presented either on the surface of cells or more often on bacteriophage envelops (phage libraries*). Proteins are physically anchored to the genes that encode them and which are co-selected and co-amplified. The DNA sequencing of resulting clones identifies the primary structure of the peptide responsible for the selection (flanked by the constant regions of the surface protein). This type of approach is often referred to as 'recombinant peptide diversity' (abbreviated RPD) (see *Fig. 1*).

A method for the systematic evolution of nucleic acid ligands has been developed

CHIMIA 48 (1994) Nr. 12 (Dezember)

by *Tuerk* and *Gold* [6] (SELEX procedure) based on *in vitro* transcription. Binding RNA transcripts are selected and amplified by the polymerase chain reaction (PCR).

The critical limitations of expression libraries are the preclusion from non-natural modifications and the high molecular weights of the library elements. Due to the limited number of building blocks, high diversity is only reachable with longer oligomer chains. The direct use of biooligomers as lead structures for developing drug candidates with enhanced bioavailability and pharmacokinetic properties is rather unattractive. Expression libraries will surely keep a prominent role in tool development (*e.g.* antibody surrogates [7]) and for broad screening purposes in order to gain insight in the structural elements which play a role in addressing a particular target. Such information may form the basis for designing smaller chemical libraries with a structural bias (thematic libraries*).

2.2. *Chemical Libraries*

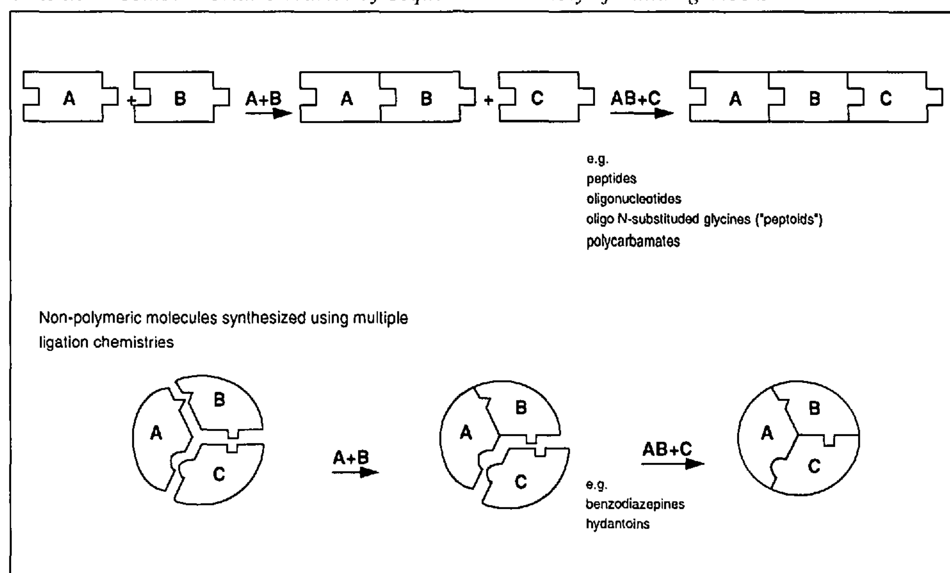
The well-established techniques of high-yield peptide and oligonucleotide synthesis have provided an excellent set of tools for the preparation of oligomer libraries from building blocks. Recent efforts are directed towards the rewarding goal of obtaining sufficient diversity with libraries of synthetic small molecules that more closely resemble attractive lead compounds in terms of size and rigidity, *e.g.* cyclic structures or variably modified templates (*Fig. 2* and *Scheme 1*).

2.2.1. *'One Bead One Sequence' Libraries and Encoded Synthetic Libraries (ESL)*

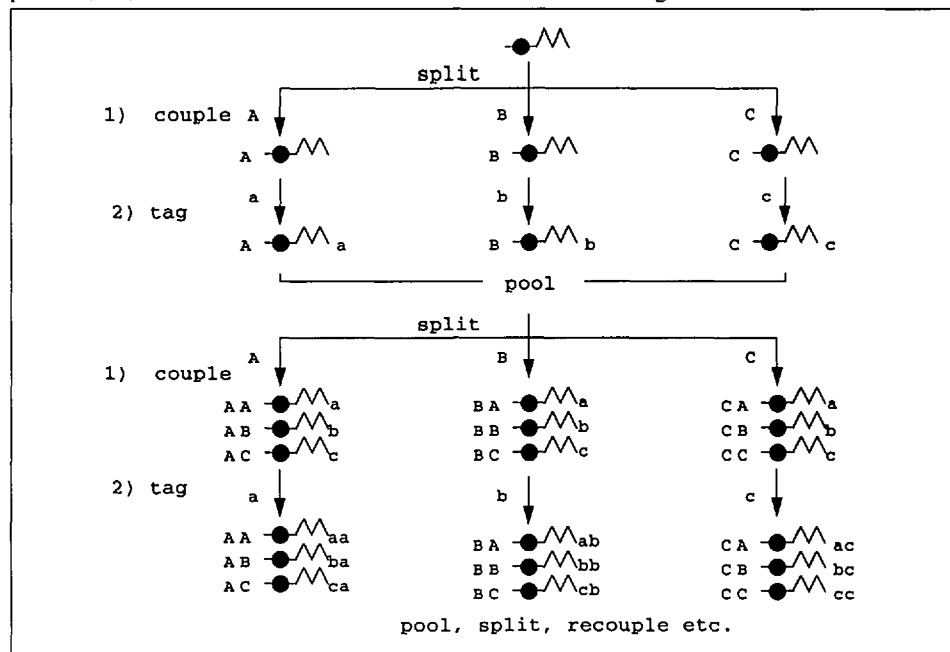
The bead handling procedure which ensures that only one 'sequence' [8], *i.e.*, one sequentially assembled compound, is present on any single bead, was first presented by *Furka* [4] (see portioning/mixing*). It is now broadly utilized also for libraries in solution, because it allows to randomize sequences while avoiding to use building block mixtures. Libraries on beads were first assembled with peptides [9] and tested in binding assays with the peptidic ligands grafted on the solid phase (see 'one bead one sequence' approach* and *Sect. 3.1.1*). The identity of peptides showing the desired binding properties can be determined by *Edman* degradation sequencing directly on the beads.

If the library elements are not sequenceable and cannot be analyzed by other general and straightforward methods, the application of an encoding strategy is necessary. *Scheme 2* is a schematic representation of the 'split synthesis' preparation protocol of a 'one bead one sequence' *encoded synthetic library*. Each synthetic step, carried out on a particular portion of the bead population, is accompanied or immediately followed by an independent coupling with a tag component coding for that step. The tags must be easily identifiable by standard analytical methods in lieu of the ligand they code for. In principle, the tag allows to retrace the history of the operations any particular bead has gone through and, therefore, to deduce the sequence of its corresponding ligand. The first tags employed were oligonucleotides [10], which have the advantage to be amplifiable by the polymerase chain reaction (PCR) and may be kept in minimal amounts or used for very small beads (< 50 μm) in high-diversity libraries. On the other hand, oligonucleotides are sensitive molecules unable to withstand the harsh reaction conditions that are necessary for the preparation of a broad variety of functionalized ligands (*e.g.* strong-acid treatments) and have a low coding capacity due to the maximum of only four subunit components. Soon, the more robust peptide tags were introduced [11] as well as a new class of non-sequential tag molecules in conjunction with a binary coding system [12] (see *Figs. 3* and *4*). In a binary coding strategy, defined

Scheme 1. *Combinatorial Libraries by Sequential Assembly of Building Blocks*



Scheme 2. *Split Synthesis: The Principle of a 'One Bead One Sequence' Library Preparation* (example of an encoded library). Capital letters stand for building blocks that are difficult to identify, lower-case letters stand for tag components coding for them. Tags must be suited for rapid, sensitive routine analysis. Each bead 'sees' only one reagent at a time. The randomization occurs by mixing the portions, *i.e.*, at the level of the *ensemble* and not at the level of single beads.



CHIMIA 48 (1994) Nr. 12 (Dezember)

mixtures of tag components are used to represent corresponding building blocks *and* their position within a sequence.

While 'one bead one sequence' libraries on beads have the merit to present each ligand as physically separate single compounds, they also impose heavy restrictions by limiting the choice of test systems essentially to binding assays with acceptors* bearing a reporter group. The alternative format is the release of the ligands into solution and to follow a sequential unrandomization approach as described below. In that case, the evaluation of libraries must usually be carried out on rather complex mixtures.

Bead-pooling* strategies (see also *Fig. 6* in *Sect. 3.1.1*) are a promising compromise, combining features of the solid and the soluble formats. The necessary prerequisites on the preparation side are the attachment of the ligands to the solid microparticles by a cleavable linker (*Scheme 3*) withstanding the deprotection reactions but suited for portionwise release of ligand aliquots into solution without reagent contaminations (*e.g.* by irradiation or with volatile reagents).

The advent of bead-pooling approaches has created the demand for larger beads with high loading capacity compatible with protocols for testing a library with multiple assays 'on' and 'off' the bead material. Working with large beads always raises the issue of the preparation scale in order to ensure a suitable ratio of bead number and possible number of combinations, and to assess the extent (governed by a *Poisson* distribution) of either redundancy or absence of certain sequences. An approximate redundancy of ten beads per combination yields a good margin of certainty that all sequences are represented. With that in mind, a library of 1 million elements may require between *ca.* 5–80 g solid support depending on the specifications of the bead materials (in the diameter range of 80–200 µm). For higher diversity libraries, a new strategy has been devised with the aim to deal with such material constraints [13]. In what is called the 'one bead one motif' approach, each bead carries several ligands all belonging to a *motif family* with fixed and randomized positions. After the identification of a binding motif, the best versions of ligands are detetermined in a second step with a more focussed library.

2.2.2. *Libraries for Sequential Unrandomization in Solution*

The methods illustrated above are 'bead oriented' in the sense that the physical individuality of each bead (carrying only

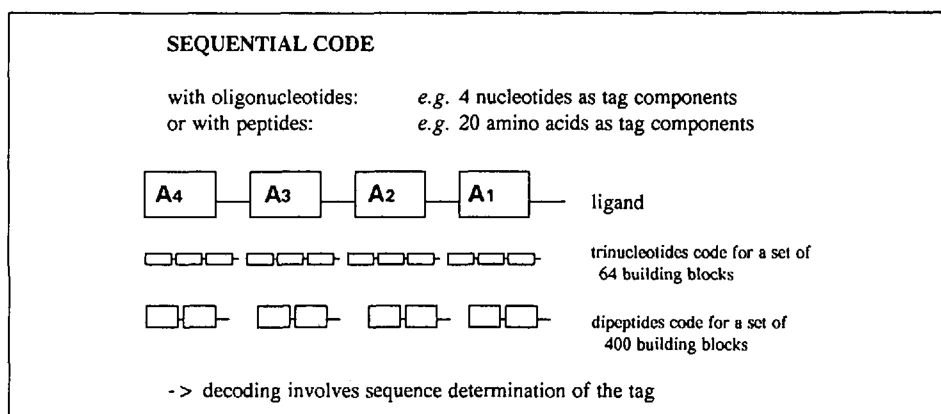


Fig. 3. *Code systems for encoded synthetic libraries* ('tagging'): *coding with oligomeric sequences*

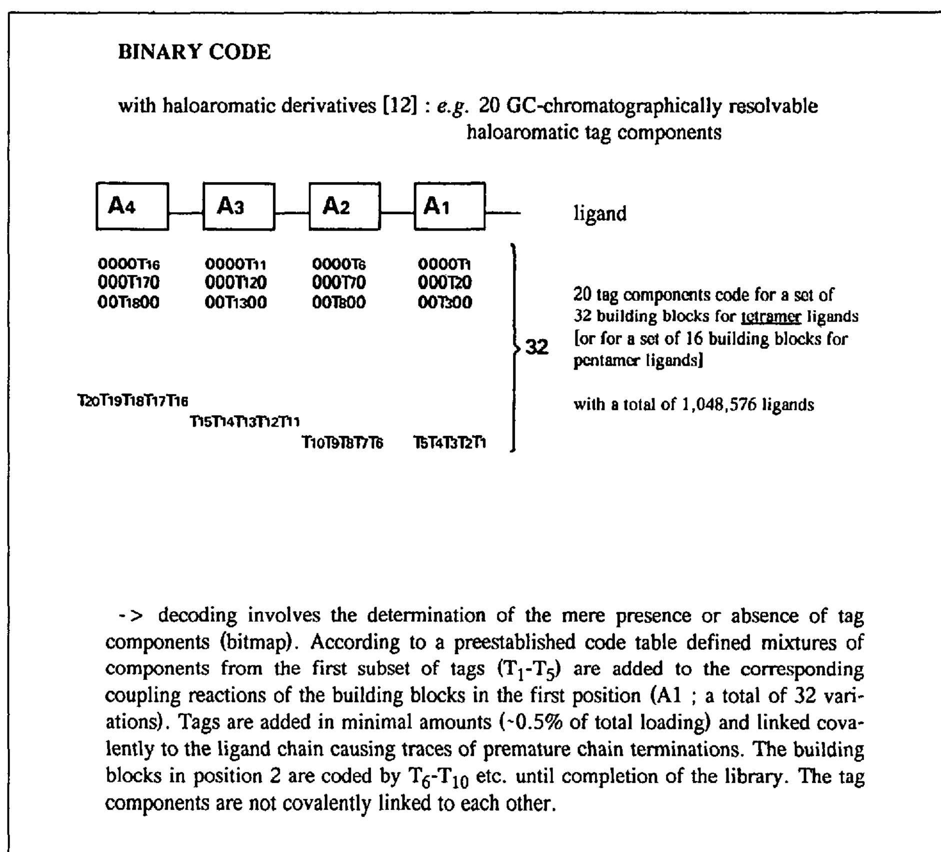
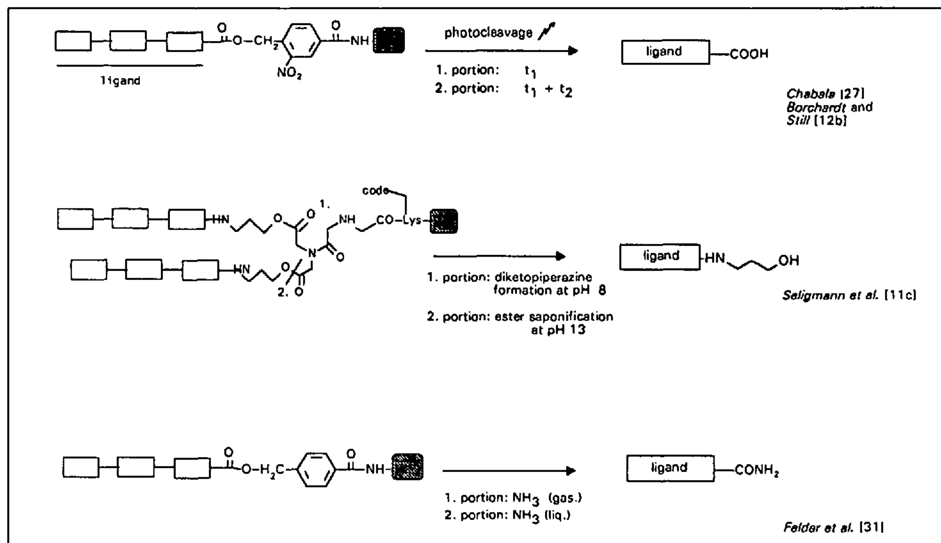


Fig. 4. *Code systems for encoded synthetic libraries* ('tagging'): *coding with a scattered multicomponent system*

Scheme 3. *Portionwise Cleavage of Ligands from Beads*



one type of ligand) is exploited in the screening process. This requires particular handling techniques for single-bead processing. A valuable complementation of such strategies is the evaluation of complex mixtures according to schemes devised for sequential deconvolution of information. The benefits of such approaches are the high throughput capacity, the format compatibility with virtually any type of test system (including functional and cellular assays) and the avoidance of microanalytical identification tasks (tags and coding systems for non-sequenceable building blocks are unnecessary). Clearly, the fact that complex mixtures are tested has its *caveats* in the form of potential artefact sources (*e.g.* physico-chemical associations, mutual ligand interferences a.o.). The sequential unrandomization* principle was first described by *Furka* [4] and usually makes use of the portioning/mixing* protocol (devised by the same author) for the sole purpose of achieving approximately equimolar representation of the ligands. The potential of this approach became evident after the report of successful applications by *Houghten et al.* [14]. The method, sometimes referred to as '*Houghten* approach'* is briefly outlined in the *Appendix* (see also 'sequential unrandomization'*, *Figs. 5*, *7*, and *8*).

The '*Houghten* approach' is an iterative process of alternating rounds of evaluation of sublibraries and synthesis of mixtures with decreasing complexity that ultimately evolves to the test of a single compound. It requires thorough planning for a coordinated effort of chemical and bioanalytical resources. Since the structural elements constituting the most active sequence are determined by virtue of the systematic evaluation of their contribution to the test performance of partially defined mixtures, the analytical structure determination is not necessary. For this reason, the '*Houghten* approach' is particularly suited for the generation of libraries from non-sequenceable building blocks, among which *N*-substituted glycines (NSG) play an important role. Protease-resistant oligomers thereof, called peptoids [15], can be assembled from a large variety of building blocks, be it from Fmoc-protected NSG or from primary amines and bromoacetic acid *via* a two step 'submonomer' coupling procedure [16] (see *Scheme 4*). Attention was recently drawn to the results of *Zuckermann et al.* [17] related to the discovery of nanomolar ligands for '7-transmembrane-G-protein-coupled receptors' from a biased peptoid library with 5000 members.

The concept of building up oligomers from building blocks has been applied also to carbamates [18] and oligophosphates [19]. Aside from sequential unrandomization, other strategies of sequence deduction from evaluation of partially defined mixtures have been described in attempts to increase efficiency. *Houghten*'s 'positional scanning' is a simplified short cut that avoids the lengthy deconvolution process by analyzing the contribution of each sequence position simultaneously with equally complex mixtures [20]. Recently, *Furka* and *Sebestyen* presented other variations of evaluation schemes [21]. Again, the purpose is reduction of the number of operations. All these strategies, though, are not expected to reduce the incidence of artefacts.

2.2.3. *Multiple Parallel Syntheses*

With the appropriate support from automatic instrumentation it is possible to synthesize a multitude of individual compounds in parallel with a throughput sufficient to satisfy the needs of a focussed project, *e.g.* with a thematic library* in a lead-optimization phase. Clearly, the lack of randomization steps decreases the number of synthesized compounds by orders of magnitude. Nonetheless, the attractiveness of individual syntheses resides in the possibility to monitor the quality more directly, to prepare dozens of milligrams and to keep track of the identity of the compounds simply by virtue of their position in the multivessel arrangement. Outputs of several thousand compounds per month can be envisaged. A typical set-up for parallel syntheses comprises a multiwell reaction block with *e.g.* 96 drilled cylindrical holes with glass filter bottoms suitable for simultaneous washes of solid phase resin portions. A robotic arm may be used for dispensing the reagents to each minivessel according to programmed protocols. A somewhat similar principle is used in the so called 'pin technology'* first described by *Geysen et al.* [22] for multiple peptide syntheses. Ligand assembly is carried out on polypropylene 'pin-heads' dipped into the wells containing reagent solutions. For simultaneous processing, the pins are arranged in a way to fit exactly into the wells. In a further extension of this principle *Hobbs De Witt et al.* [23] use an apparatus with rods, each containing a resin portion within a fritted glass filter at the lower extremity. The rods are lowered into the wells of a (heatable) reaction block. The suitability

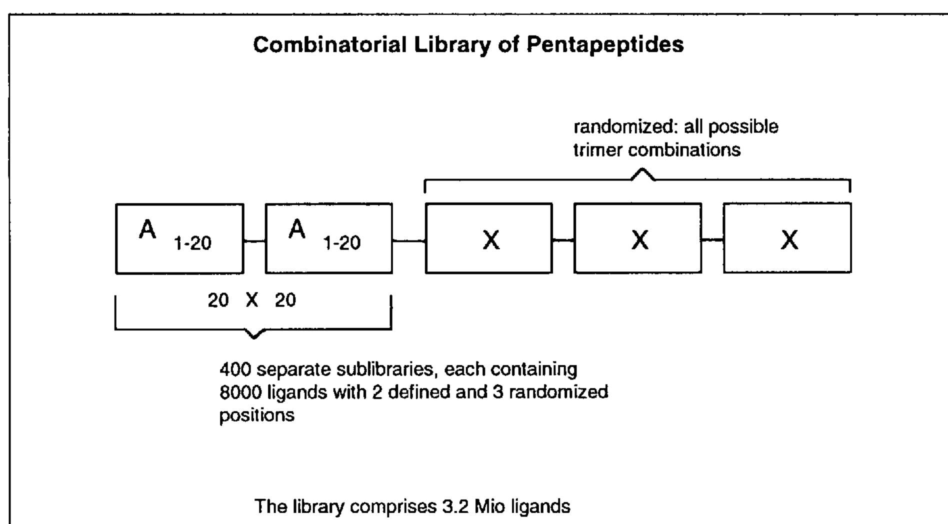
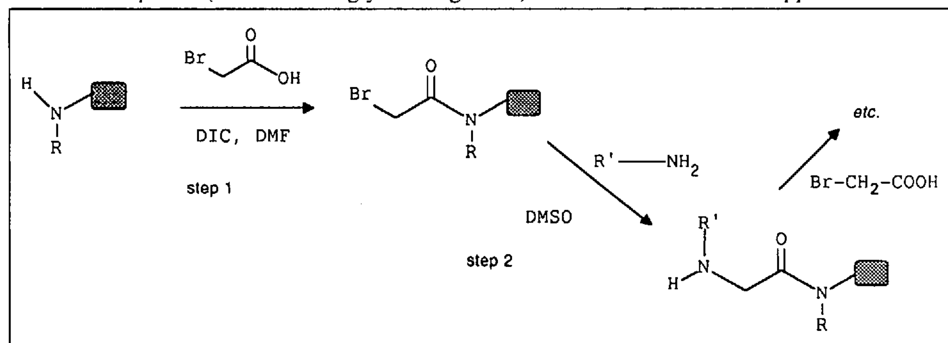


Fig. 5. *CCL as a set of sublibraries for testing mixtures in solution*

Scheme 4. *Peptoids* (*N*-substituted glycine oligomers) *and the 'Sub-Monomer' Approach*

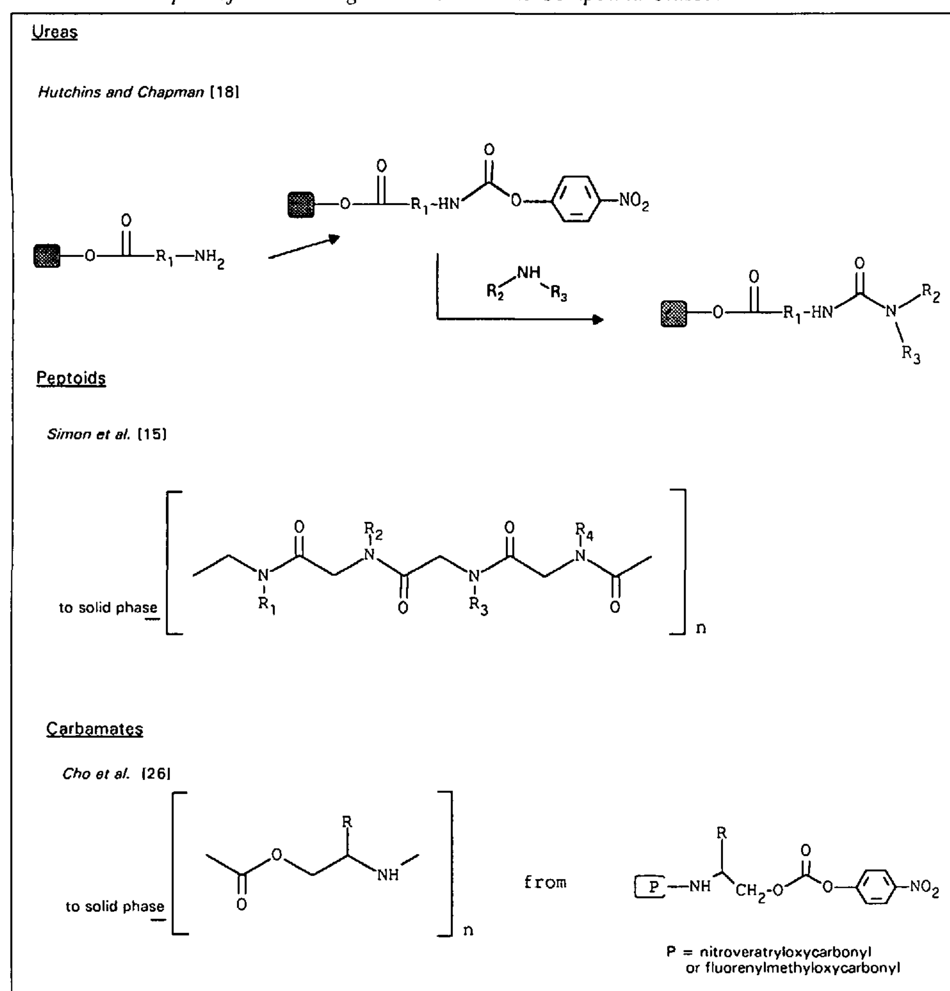


for synthesizing '*small molecule' libraries** was demonstrated by the preparation of a 40-membered benzodiazepine library. In a remarkable combination of solid-phase peptide synthesis and photolithography *Fodor et al.* [24] were able to carry out light-directed spatially addressable parallel chemical synthesis of peptides on surfaces. The photolabile nitroveratryloxycarbonyl *N*-protecting group is selectively cleavable from specific areas determined by the usage of stencils and light irradiation. The selected areas become accessible for the reaction with a particular building block for differential chain elongation. Ligand densities lie around 10^4–10^5/cm^2. Although conceptually elegant, this method has probably no lasting impact in the CCL field due to the restrictions imposed on the chemistry. Its value is better appreciated for possibly very important contributions to the biosensor technologies and microdiagnostics.

2.2.4. *Adaptation of Solution Chemistry to Solid Phase Chemistry*

As mentioned before, the CCL field has its origins in peptide chemistry but is migrating into general organic chemistry. The incentive is the coveted goal to be able to prepare large libraries of 'small molecules'. Ultimately, it would be desirable to obtain diversity also by means other than the linear chain elongation with bifunctional building blocks, *e.g.* on the basis of a rich repertoire of cyclizations, molecular bifurcations and modifications of templates, all carried out on solid phase in order to readily access globular semi-rigid ligands as well (the products of such approaches are sometimes called 'diversomers' [23] or '*small molecule' libraries*). Any selectant of this kind would be a more promising drug lead than a linear peptide. An obvious prerequisite for further progress is the adaptation of a broad array of chemical reactions to a solid-phase format. The achievement of reproduceable high yields with mild conditions and a large choice of reactants is going to be the crucial success factor. Note that the value of a combinatorial chemistry for preparing diverse libraries increases dramatically the more operations (insertions of variables) are carried out on solid phase. In preparing a 40-membered benzodiazepine library *Hobbs De Witt et al.* [23] introduced three variables in solution and one on solid phase. The potential of the method to widen the diversity is, therefore, more limited, in practical terms, if compared to *Ellman*'s approach [25], where two variables are introduced on the solid support. Progress in this sense will be essential for the ability to efficiently design thematic libraries around a hypothesis or a first generation lead obtained from a high diversity screen. *Scheme 5* and *6* review a few chemical structures of interest.

Scheme 5. *Examples of Non-biooligomeric Chain-like Compound Classes*



3. Evaluation of Libraries

It is an inherent characteristic of the combinatorial libraries approaches that it is most often neither possible nor sensible to control the quality of each member of a ligand population. For that reason, it is particularly important that the chemistry involved has been thoroughly tested previous to its use in application work. After that, it is recommendable to estimate the overall quality of libraries whenever possible: in the case of small soluble mixtures, electrospray MS allows a rough verification of the expected mass distribution [29]. In the majority of cases, the prepared libraries are directly evaluated with regard to their performance in a bioassay. Here, the goal is to actually correlate an interesting activity to one or more individual members of the library. This is a challenge deserving utmost attention, since it is obviously useless to rapidly produce millions of compounds without the capacity to deal with their evaluation within a similar time frame. Under these circumstances, innovative strategies were developed.

3.1. *Library Formats*

From the bioanalyst's perspective the library format is a critical factor for assessing the suitability of a CCL approach for a particular assay system or for planning the necessary adaptations of a system to achieve CCL compatibility. There are basically two different formats, namely the libraries on solid microparticles and the libraries in solution.

3.1.1. *Libraries on Beads*

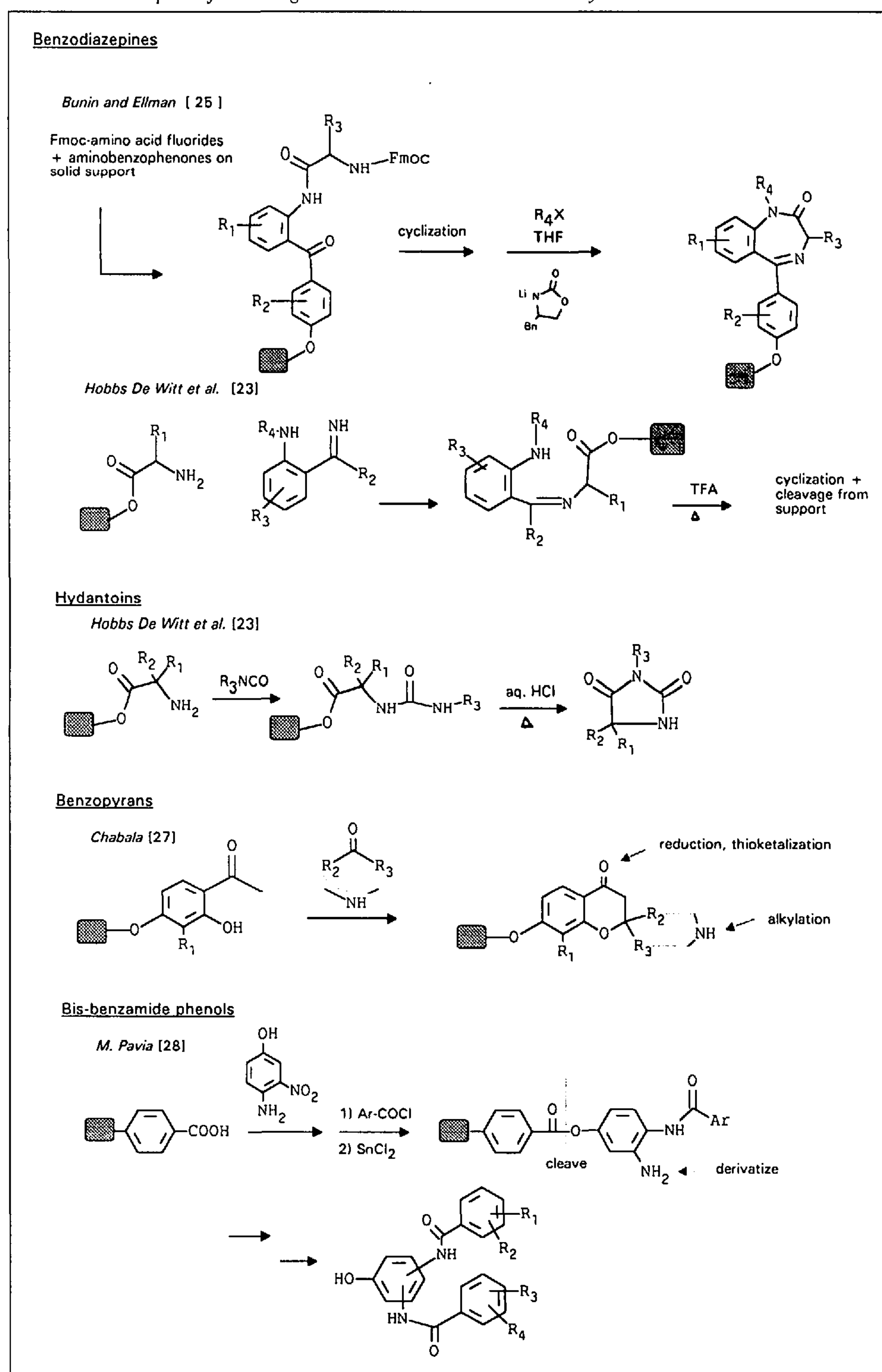
Some general considerations about binding assays on beads are mentioned in the *Appendix* and in *Sect. 2.2.1*. Due to the restrictions imposed by the presence of solid particles tethered to the ligands, there is a perceptible tendency to develop methods that, on one hand, take advantage of the option to physically separate ligands either individually or in groups and, on the

other hand, measure performances after cleavage in solution while keeping track of the associations between mother beads and their microeluates (see bead pooling*). Nevertheless there are justified efforts to improve the techniques for assays on solid beads considering aspects like bead-sorting speed, identification of artefact sources (*e.g.* bead matrices, linkers), and even compatibility with membrane receptor assays. *Stroud* [30] recently reported about the preservation of binding capability upon solubilization of three types of '7-transmembrane-G-coupled receptors'.

A wide range of bead sizes and loading capacities is available. 10 μm beads are used to achieve the highest diversity, when keeping an acceptably low volume is crucial. Beads with 2-nmol loading capacity were used in a combined approach, where a thrombin-binding assay on beads was followed up with a thrombin-inhibition assay in solution [31] in the multiwell titerplate format. *Rapp et al.* [32] developed PEG-grafted macrobeads with a particle size around 750 μm and loadings of 20–100 nmol.

In an original experimental set-up proposed by *Jayawickreme et al.* [33], beads are seeded on cell lawns or on dishes with gel layers provided with an appropriate reporter system that visualizes the diffusion of active ligands into the immediate surroundings of the beads. As mentioned in *Sect. 2.2.1* cleavable linkers make it possible to pursue a bead pooling strategy for high throughput evaluations in solution with relatively simple mixtures (see *Fig. 6*).

Scheme 6. *Examples of Non-oligomeric Combinatorial Chemistry on Solid Phase*



3.1.2. *Complex Libraries in Solution*

The evaluation of complex mixtures by means of sequential deconvolution of information has been discussed in *Sect. 2.2.2* (see also *Appendix*: *Houghten* approach*, sequential unrandomization*, *Figs. 5, 7*, and *8*). The interplay of different effects from individual library components has to be kept in mind but should not deter from applying deconvolution strategies in view of the proven usefulness in many examples, and considering that side effects shared by all sublibraries undergoing a comparative evaluation are often no hindrance to the identification of active ingredients.

It is important to note that in early stages of the deconvolution process, *i.e.*, when measuring and comparing activities of complex mixtures, combined effects arising from the contribution of whole families of homologous structures may be measured. This means that it is well possible to successfully detect and later identify components with moderate activity which would not be detectable, if measured as a single compound in the same extreme dilution it is represented in the context of a library. Fortunately, the two most critical steps of an iterative unrandomization are the two first rounds of assays. Two check-points must be fulfilled: detectable overall activity in the first round and increase of activity in the second round when testing the simpler follow-up mixture. An obvious complication that may arise with libraries in the 'soluble' format is the actual insolubility of certain components following the synthesis, during workup or in the test medium. The latter case is a common situation encountered in single compound screening as well and may be dealt with by applying proven expedients like *e.g.* addition of 1% dimethylsulfoxide or other adjuvants compatible with the assay system. The fact that library components usually differ widely in their physico-chemical properties has to be kept in mind during workup or any post-synthesis operation in order to avoid undesirable enrichments or losses of a subpopula-

tion at the expense of equimolar representation.

3.2. *Combinatorial Libraries (CCL) and High Throughput Screening (HTS)*

As discussed in the previous section, the combinatorial library approach comes with its own strategies to provide the massive increase of compound evaluations required for the balance of preparation and assay speed. Unlike conventional high-throughput screening of sample collections the CCL approach may take full advantage of the integrated preparation process, with the option to control the physical format (*e.g.* grafting on beads) or classify into defined subpopulations. These opportunities accelerate the evaluation by *e.g.* either exposing a bulk of physically separate samples simultaneously, in the case of a bead library, or by minimizing the number of measurements *via* iterative deconvolutions, in the case of a sequential unrandomization in solution. For the latter, a capacity of measuring *ca.* 10^2–10^3 samples per week is usually sufficient.

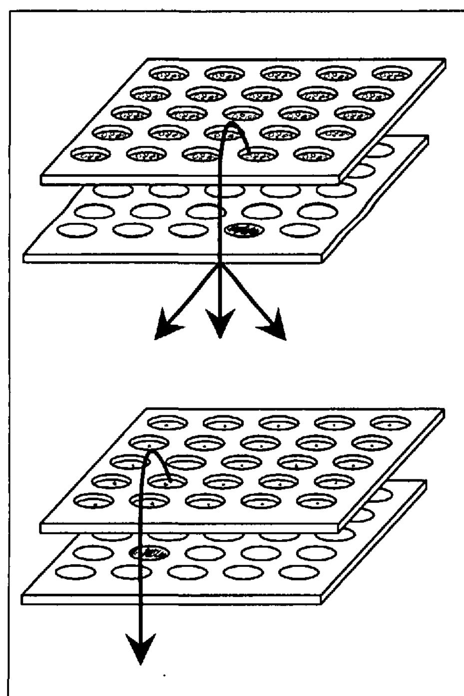


Fig. 6. *Bead pooling and redistribution in the multiwell titerplate format.* Bead pools are subjected to a partial cleavage. Pool microeluates are tested. The mother-beads of an active pool are redistributed to one bead per well. Residual portions of ligands are cleaved and eluted. The mother-beads of the best performers are decoded for structure identification.

CCL and HTS complement each other in a more powerful, combined lead finding effort. In the CCL era conventional HTS and its related technologies are far from obsolete. The ability to test large compound collections (corporate collections or acquired samples), whenever new targets become addressable by a high-capacity assay, is as crucial as ever. Innumerable basic structures of natural and synthetic origin will remain elusive for a combinatorial approach. In addition one may exploit high throughput systems for the evaluation of soluble libraries.

3.3. *Automation and Logistics*

Being a field of large numbers and multiple disciplines CCL are predestined to raise organisational challenges. Robotic support is optional in principle, but is virtually a must for broad application of the technology. Fully automated solid-phase synthesizers already belong to the standard equipment of peptide chemistry labs. Multiple peptide synthesizers for parallel processes are commercially available from various manufacturers (*e.g. Advanced Chemtech, Zinsser, MultiSyn-Tech, Applied Biosystems etc.*). The automation of the portioning/mixing* operation is the next obvious step and is available from *Advanced Chemtech* and *SynPep* (*Chiron Corp.* is alleging infringement on two US patents covering the instruments' technology). The main caution in acquiring commercial models is a thorough assessment of the level of flexibility that is offered. For instance, when working with non-peptidic building blocks, it may be necessary to have the ability to apply heat, vacuum or inert atmosphere *etc.* in a multiple parallel synthesis or to increase the number of portions in the portioning/mixing process. On the assay side, there is an even greater need for automation, specifically in the area of bead handling processes, *i.e.*, for bead sorting by a reporter signal (*e.g.* fluorescence) and bead pooling*. The data management and communication is a primary issue for the efficiency of sequential unrandomizations* where it is crucial that rounds of assays and follow-up syntheses can alternate each other without delay. This requires fast communication of results and readiness to resume synthesis when the information becomes available. Other logistical aspects concern the maintenance of stocks for building blocks, sublibraries and mother beads.

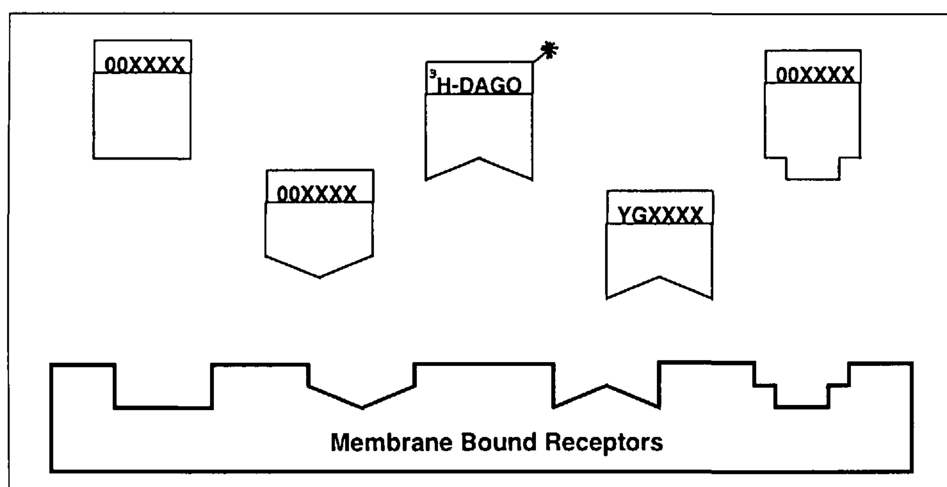


Fig. 7. *Opiate receptor-binding assay: The ability to inhibit the binding of a radioactively labeled μ-receptor-selective ligand [(^{3}H)DAGO] to the μ-site is measured.* The performance of sublibraries in this test is used as a criterion for sequential unrandomization (see also *Fig. 8*)

4. Discussion

Taken altogether, the strategies and technologies playing a role in the preparation and evaluation of combinatorial libraries are numerous and multidisciplinary. CCL are at the front end of the development of new chemical entities. They are the result of a pragmatic, goal-oriented attitude which emphasizes the discovery side of research activities. The odds to bump into a discovery, however, are pushed to the extreme with rigorously planned rationales. Former constraints to carry out high-throughput screening 'at random' are now overcome. It is up to the single organizations, from large industry branches to small academic groups, to work out concepts for exploiting CCL for their needs most productively. Where possible, the vastnesss of this field justifies widely coordinated efforts and suggests that more than a single type of approach should be pursued. On the other hand, it is well conceivable that a single laboratory, having isolated an enzyme or a receptor, may want to screen ligands for it and purchase a combinatorial library from a commercial vendor. Major pharmaceutical companies are likely to be in a position to cover all the technologies necessary for library screening. The collaboration with

CHIMIA 48 (1994) Nr. 12 (Dezember)

specialized start-up companies may be seeked in an initial phase for immediate 'state of the art' operation. The access to new, original targets is crucially important. This again may require a partnership with an organization focussing on the identification of desease genes and the molecular mechanisms involved in disease manifestation. On the other hand, those biopharmaceutical companies and academic institutes lacking chemistry support are the largest pool of potential customers for commercial CCL producers. In any case, it is reasonable to expect that the drug-discovery process will benefit considerably from the combinatorial techniques now available. The new situation makes it possible that lead finding in the initial phase of a project is carried out both faster and more broadly. This should ensure that only excellent leads are taken further into medicinal chemistry programs that refine the properties to the stage of product development. The unfortunate situation that time is wasted on the improvement of a prematurely chosen lead compound should be avoidable. The full integration of CCL into the drug discovery process is an upcoming challenge: a good balance with complementary technologies has to be established. Enough chemistry support for lead optimization is essential. In favorable cases lead refinement is approachable by combinatorial chemistry to a certain extent, with thematic libraries, but actual optimization will often go beyond the possibilities of modular chemistry. Furthermore, the design and synthesis of building blocks for thematic libraries tailored for the needs of particular projects must rely on strong chemistry resources with the ability to deliver the required tools before the lead structures become obsolete. On the assay side, at least one high-throughput system per project is needed together with an appropriate second system for deeper characterization of screening hits. Assays with read-outs that are not directly traceable to a known molecular mechanism should be backed up with a test system allowing to discriminate between modes of action.

In conclusion, it is hard to believe that the exploitation of combinatorial methods will not make a difference for the discovery of new chemical entities with useful properties. Putting it into practice is the worthwile task to pursue. Just how difficult this will turn out to be remains an open question but there is certainly a great demand for innovation.

Thanks are due to *Hans Rink* for valuable discussions.

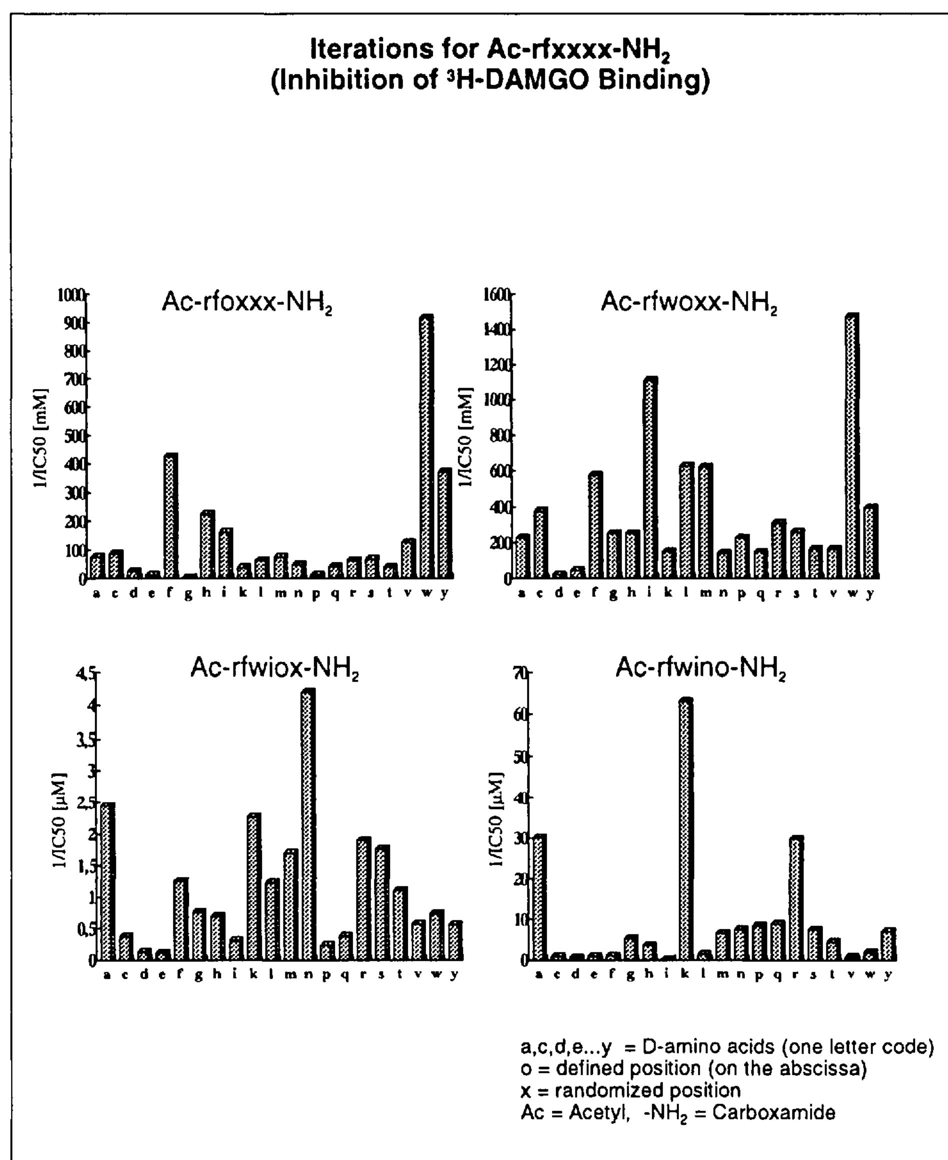


Fig. 8. *Sequential unrandomization: The deconvoluted structure is Ac-rfwink* (Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Lys). From *Houghten* [34].

Appendix

Along with the combinatorial libraries' techniques a jargon of typical terms and expressions has emerged. The following comments are not complete and are limited to simplified statements and definitions.

Some Terms and Expressions

Acceptor: A ligate of interest, typically a macromolecular entity (*e.g.* an enzyme, a receptor or a fragment thereof, an antibody *etc.*). A solution of labeled acceptors is used in the '*one bead one sequence*' *approach* in order to search for ligands.

Bead: A solid, insoluble, spherical microparticle, typically used for solid phase chemical synthesis. Diameters range from *ca.* 10 to *ca.* 800 μm. Materials are *e.g.* crosslinked polystyrene matrix, polyamide, Sepharose, glass, polystyrene grafted with polyoxyethylene *etc.* The term is also used for non-spherical particles. There are *ca.* 2.5 million '90 μm-beads' per gram resin. Beads may be used to synthesize potential **ligands** (library elements) and for displaying them to a ligate of interest (see also **acceptor**) in the ***'one bead one sequence' approach***.

Bead pooling: Bead-pooling strategies aim to efficiently test 'one bead one sequence' libraries beyond simple binding assays with labeled acceptors, *i.e.*, with functional assays in solution. Ligands are, therefore, cleaved from the beads (partial cleavage of a first portion). To achieve an acceptable throughput, it is necessary to test mixtures resulting from bead pools (*e.g.* 100 or 1000 beads) in a multiwell format. Interesting activities are then correlated to a particular pool of mother beads carrying a molecular tag. These beads are redistributed on a singular basis into new multiwell plates where a second portion of the ligands is released into solution and the activities of single compounds is measured. Bead pooling on a large scale requires advanced automation.

Diversomers: Non-oligomeric library elements. A core template is sequentially modified at discrete positions. Formally the same combinatorial and coding principles can be applied to these

types of molecules as for oligomeric sequences. Highly demanding in terms of selectively cleavable protecting groups.

Encoded libraries (= ESL = encoded synthetic libraries = encoded 'one bead one sequence' libraries): If the ligands assembled on a *'one bead one sequence' library* are not directly sequenceable molecules the identity of positive hits is determined by sequencing or determining the composition of a **'tag'** molecule residing on the same bead. The tag has to be built up in parallel with the potential ligand. Two classes of molecular tags have been described and applied: **sequential tags** and **bitmap (non-sequential) tags**. The former are typically oligonucleotides or peptides synthesized by alternating cycles of ligand and tag chain elongation with the help of orthogonal protection groups. For instance, an amino acid or a dipeptide unit may code for the identity of an 'artificial' building block which is neither an amino acid nor a nucleotide. Bitmap tags on the other hand are 'read' in a binary mode: the mere presence or absence of a series of tag building blocks (which donot need to be linked together) translates into *words* of information coding for both the presence and **the position** of a non-sequenceable ligand building block. The potential ligand is always present in vast excess over the tag sequence.

High diversity libraries: Libraries with maximal element variability. The latter is preferentially achieved by including a large number of building blocks in relatively short sequences rather than by sequence elongation.

The ***Houghten* approach**: The *Houghten* libraries consist of a set of separate sublibraries (compound mixtures) in solution. The evaluating assay may, therefore, be any test system applicable to compound solutions, including functional assays in cellular systems. The sublibraries are mixtures of compounds (typically oligomeric sequences) with a (known) constant, a randomized and a (known, defined) variable region. In analogy to the *'one bead one sequence'* approach the sublibraries are prepared with the portioning/mixing procedure in order to ensure equal representations of all (slow and fast reacting) building blocks. They are tested as if they were single compounds. In an iterative process the mixtures are compared with each other, the 'winning' variable regions are determined and incorporated as constant positions in a following round of synthesis which produces less complex mixtures. Repetitive cycles of this procedure lead ultimately to one defined molecule with the best performance in the test system used. Instead, the *'one bead one sequence'* approach may lead to a variety of lead structures. The *Houghten strategy* circumvents the necessity for analytical identification. Building blocks other than natural amino acids or nucleotides can be incorporated without additional complications, if they are suited for carboxamide coupling reactions. The first set of sublibraries comprises as many mixtures as the number of building blocks, if only one position is defined in the beginning, *e.g.* 20 sublibraries for 20 amino acids. If two positions are defined the number is (20 x 20 =) 400. For each subsequent round of tests 20 new sublibraries are prepared. If positions are defined contemporaneously, the number of sublibraries to be tested increases multiplicatively rather than additively.

Mimotopes: Small linear mimics of discontinuous epitopes.

Multiple peptide synthesizer: A machine for assembling a multitude of oligomeric sequences (typically peptides) in parallel. An experimental set-up which is sometimes applied in the *Houghten* approach for introducing defined variable sequence positions.

The ***'one bead one sequence' approach***: A library of potential ligands (*e.g.* oligomeric sequences) is synthesized on solid microparticles (*e.g.* resin beads) using a portioning/mixing procedure. Each particle is loaded with only one type of ligand (*ca.* 100 to 1000 pmol). The potential ligands are completely deprotected but remain grafted on the beads. They are screened by incubating the beads with a solution of labeled **acceptor** molecules (*e.g.* a fluorescent enzyme or receptor) which may be recycled and used in a separate experiment. After a number of wash operations with increasing stringency, the bead population is either visually inspected (*e.g.* while irradiating at excitation wavelength) or sorted with an adapted *fluorescence-activated cell sorter* (FACCS). The ligands of beads carrying the label are determined either by sequencing directly the ligand or by analyzing an 'identification tag' residing on the same bead (see **encoded libraries**). The library elements are on physically separate entities (beads). The observed bindings originate from interactions with single compounds in heterogeneous phase. The approach may lead to a variety of lead structures.

Peptoids: Oligomeric sequences of ***N*-substituted glycines**. Peptide surrogates preferentially used in CCL in order to display functionalities of peptides in a novel backbone. Resistant to peptidases.

Phage libraries: Consists of up to 10^{15} filamentous phage clones, each displaying an extraneous peptide sequence on the phage surface protein. The peptide is coded by a DNA sequence in the phage genome. An acceptor of interest (*e.g.* an antibody, receptor, enzyme *etc.*) is used to affinity purify phages that display binding peptides.

Pin technology: Peptides are synthesized simultaneously at the tip of plastic pins in the 96-multiwell plate format.

Portioning/Mixing: (= Split Synthesis, = Divide, Couple and Recombine). An ingenious procedure used for randomizing one or more positions within an oligomer and making sure that only one sequence (*i.e.*, a number of identical oligomers with the same sequence) is represented on any particular bead. The method involves distributing a pool of resin beads into separate reaction vessels (*portioning*) each reacting with (for instance) a different (unique) amino acid allowing the coupling reactions to go to completion (*couple*). This avoids competition between slow and fast reacting amino acids and makes sure that each bead 'sees' only one reactant. The portions are then recombined (*mixing*) leading to a randomization of the sequence position.

RPD: Recombinant peptide diversity: Peptides expressed on the surface of cells or phages, randomized by means of genetic engineering of the DNA coding for them.

Sequential unrandomization: An operation that is part of the iterative *Houghten* approach. Each time sublibraries are tested and compared with each other a 'winning' sublibrary is determined. Its superiority originates from the contribution of a unique variable that is defined for each sublibrary. The 'winning' variable will be constantly included in the next rounds of synthesis resulting in sublibraries with fewer and more defined elements, where the number of randomized positions is diminished by one and the number of constant positions increased by one. Each unrandomization step may be carried out at a single or at multiple positions simultaneously. For single positions, the number of sublibraries to be tested for a round of assays is equal to the number of building blocks *N*. An unrandomization step at two positions (simultaneously) requires $N \times N$ tests. A typical example is the simultaneous unrandomization of the two aminoterminal positions in a peptide library by testing (20 x 20 =) 400 sublibraries. Subsequently the positions are unrandomized sequentially at single positions. This requires 20 additional tests per position; the total number of tests increases additively.

'Small molecules' libraries: Libraries of diversomers or non-peptidic, biostable compounds with molecular weight below *ca.* 600 Da.

Tea bags: Solid-phase resin beads are divided in portions and packed into foraminated or porous bags which are permeable by solvents and reagent solutions. An experimental set-up which is sometimes applied in the *Houghten* approach for introducing defined variable sequence positions.

Thematic libraries (= intelligent libraries = biased libraries): Sizeable compound libraries for structural motif refinement comprising a comprehensive combinatorial reshuffle of an array of selected building blocks, whereby the selection relies on pre-existing information or hypotheses on what type of functionalities are important.

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