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Synthetic library techniques: Subjective (biased and generic) thoughts and views

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Summary

Various aspects of synthetic diversity generation and screening are discussed. Controversial issues are raised and different points of view are presented. We hope the article will stimulate thinking about the utilization of library techniques and start a discussion about questions concerning their application.

Introduction

The application of library techniques for drug discovery is growing rapidly. Due to this growth and due to the number of different laboratories participating in the development of these techniques, different philosophies of library application exist. The answers to many questions are not available and reviews about these techniques are influenced heavily by the experience of the individual researcher [1–19]. We have tried here to summarize our numerous discussions (internal and external) and address most of the issues concerning the application of synthetic library techniques. Do not be surprised that we are not discussing multiple-synthesis techniques. They were the inspiration for the development of the library techniques, and some of them are actually very similar to libraries, such as the ingenious synthesis of peptide or nucleotide arrays on glass chips [20], synthesis on plastic pins [21], synthesis in tea-bags [22], and multiple-synthesis on paper [23–25]. Some of these techniques have been applied to library synthesis [26–28]. Intentionally, we do not discuss biological (phage, plasmid, etc.) libraries, since we have no hands-on experience in this area (but you can find the relevant information in several review articles [5,9,11,29,30]). Sometimes we might seem schizophrenic for arguing the same issue from several points of view, but this is, in our view, the appropriate approach until some questions are answered definitively. (But even then, we think, it is appropriate to ask again and again: what if?)

Since it is not important for the purpose of this discussion who has expressed a certain view or idea (and, besides, the expressed view is in a number of cases not shared by the authors), we decided to present our thoughts as a discussion between two imaginary persons: T.U. Tor (A) and C.H. Allenger (B). Sub-headings specify the subject of controversy and are included only for the reader's orientation. Do not expect exhaustive referencing on the subject discussed; only the most illustrative papers are mentioned. Reviews including a complete literature background we have published elsewhere [2,4,16,31]. A complete listing of papers dealing with combinatorial chemistry compiled by us [32] is available on Internet as part of the 'home page' of this journal (http://vesta.pd.com). Our manuscript should be viewed more as a 'Focus Point' (similar 'Focus Points' by Ellman, Pirrung, Czarnik and Mitscher on combinatorial chemistry have been published recently [33–36]), than as a typical review.

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On synthetic strategy

T.U. Tor (A):

"There are in principle two approaches for building a library: the one-bead–one-compound (Selectide) technology [37] using split/mix synthesis [38], and the iterative synthetic approach [27,39], first described for synthesis of peptide mixtures on pins by coupling a mixture of amino acids in several positions of the sequence [28].

The one-bead–one-compound technique consists of three basic steps: (i) library synthesis; (ii) screening, by either an on-bead assay or solution assay; and (iii) structure determination. The key features of this technique are the existence of only one chemical entity per bead and the fact that the identity of the compound is not traced during synthesis, but determined only after identifying the bead of interest. The first aspect, in particular, has a number of consequences that we will address.

The iterative approach is based on a synthetic algorithm, according to which a number of sub-libraries are synthesized, each bearing a common structural feature; e.g., a particular amino acid at a specific position. The most active sub-library is identified and a new set of libraries is synthesized having as a common feature the previously identified structural motif. Since mixtures of amino acids (generally building blocks) are coupled at nondecisive positions, approximately equimolar, but tiny quantities of all compounds are prepared."

C.H. Allenger (B):

"The one-bead–one-compound approach has several clear advantages. Equimolar mixtures of compounds are always prepared. It is not so labor-intensive, since only one library is synthesized. There is no need to track compound identity, since the structure of interest is determined only after the bead has been shown to contain a compound with desirable properties. It also allows for a rapid on-bead binding assay."

A: "The advantage of having one compound per bead is relative. Firstly, you must realize that one can synthesize only as many compounds as beads. In practice it is less, but I will come to this point later. Secondly, each one-bead–one-compound library provides a unique set of compounds that is in principle not reproducible in the case of more complex libraries; we can discuss this later also. The fact that you do not care about the structure of a compound until the compound is selected is elegant in peptides which can be easily sequenced, but becomes a problem in non-peptide libraries. The often claimed disadvantage that the iterative method cannot discover all active motifs is more or less propaganda; it just takes longer to explore all possible iteration pathways.* Anyway, how many different peptides that kill cancer cells does one need?"

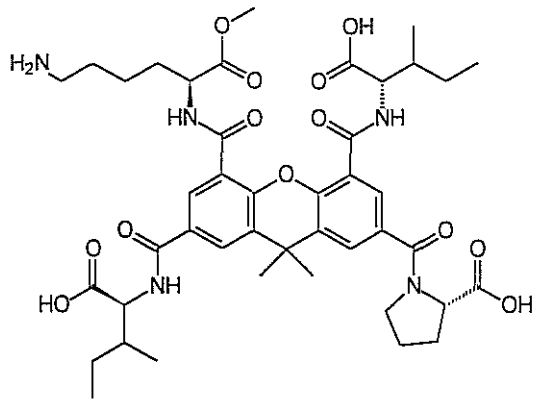


Fig. 1. Structure of the trypsin inhibitor ($K_i = 9$ μM) identified by Carell et al. [46].

B: "Just one. But since it is hard to believe that a hit from a generic library will become a drug without further modification, it would be helpful to know all possible alternative structures that interact with the receptor. Every computational chemist would appreciate this kind of input for rational drug design."

A: "You are right, but in fact the iterative technique provides you with information of that kind. Since mixtures of iterative sub-libraries are not random, but contain compounds with a characteristic feature, the result of testing such mixtures provides us with information regarding the importance of certain amino acids at particular positions. In positional scanning libraries [41–43], one or two amino acids are defined in a certain arrangement at all positions. Compounds resulting from the combination of all amino acids identified as important for biological activity at all positions are then resynthesized and tested as individual peptides, after which the compound with highest activity is found."

B: "Yes, this is an ingenious, but still time-consuming task. However, it is probably not as time-consuming as the alternative iterative technique based on the synthesis of several libraries using differently modified (enriched or depleted) mixtures of amino acids for

*It is illustrative to study the paper of Dooley et al. [40]. The authors followed several pathways and they were able to identify a new series of μ-selective opioid peptides. They would probably have found other structures had they started their iteration from the middle of the molecule or from the carboxy terminus.

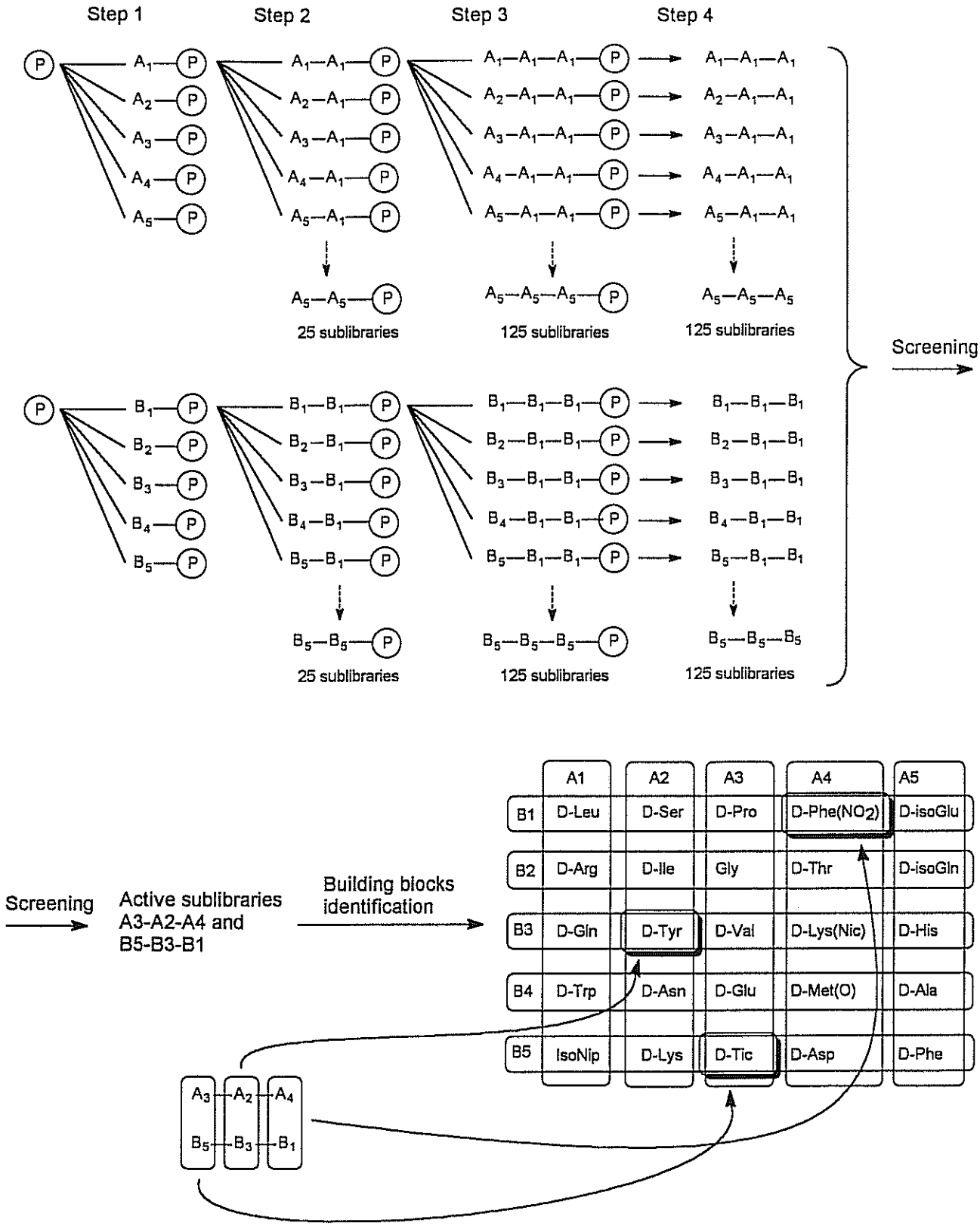


Fig. 2. Scheme of the synthesis of orthogonal libraries [48]. (Step 1) coupling of mixtures A1 to A5 to five aliquots of solid carrier, and mixtures B1 to B5 to another five aliquots of the solid phase (the composition of the mixtures is shown in the figure); (step 2) splitting of each aliquot into five portions and coupling the mixtures A and B to each respective sub-aliquot; (step 3) repetition of step 2; (step 4) cleavage of all sub-libraries. Screening of libraries A and B identified as positive: sub-libraries A3-A2-A4 and B5-B3-B1. The overlap of mixtures A3 and B5, A2 and B3, and A4 and B1 identified the building blocks of the active compound (marked in the scheme).

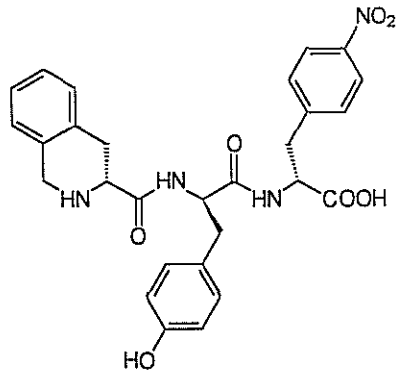


Fig. 3. Structure of the V2 vasopressin inhibitor [48]. $IC_{50} = 63$ nM (V2 receptor).

coupling at defined positions [44].* The biological activity of a particular sub-library is evaluated and compared with the activity of the original (unchanged, full) mixture. A decrease, increase, or no change in activity may indicate the group of building blocks most important for activity. The process is repeated until individual building blocks in the library are identified."

A: "So, if you insist on something really fast, you should consider one more method for rapid library synthesis that was applied for the synthesis of a non-peptide library based on a scaffold structure. Functional groups at the scaffold are of the same type, they are not differentially protected and they are treated with a mixture of suitable building blocks in a one-pot reaction. If the resulting mixture is active, the next library is synthesized omitting certain building blocks, until the blocks responsible for the activity are found [45–47]. This method of library synthesis is fast; however, the deconvolution process may be cumbersome."

B: "This one-pot library synthesis represents the most brutal chemical approach. Coupling a mixture of more than 10 compounds at the same time is alchemy, not chemistry. You don't know whether the building blocks may be coupling to at least some functionalities on the resin. A positive result is a matter of luck; a negative result says nothing at all. Even if the first library synthesis is fast, the deconvolution process may take longer than the synthesis of an orthogonally protected scaffold and the synthesis of a real library."

A: "The opposition of an old-fashioned chemist is understandable. However, even this brute-force method provided ligands with reasonable affinity [45,46]. The structure of a trypsin inhibitor ($K_i = 9$ μM) deduced from this library is given in Fig. 1. So why criticize something we do not like, just because we have not invented it? What about splitting the differences and making a library from a scaffold having two types of functionalities (or two types of protecting groups), and instead of coupling the whole mixture of building blocks at the same time, dividing the resin and coupling mixture of only two (or a limited number of) building blocks in each reaction vessel? In this case the library synthesis will still be fast and the soul of the chemist would not get so frustrated."

B: "I know what you mean. You obviously like the approach of so-called 'orthogonal libraries' [48], in which two sets of libraries are synthesized at the same time. In every step of a library synthesis a limited mixture of building blocks (in the quoted example, a mixture of five amino acids) is coupled to the solid support, the support is divided into aliquots and then another limited mixture is coupled. The solid support is never mixed together and therefore one ends up with a number of sub-libraries (mixtures of compounds). In the given example (Fig. 2), five groups of five amino acids were coupled in three steps, thus forming 125 sub-libraries each containing 125 compounds. The total number of synthesized compounds was 15 625, and since the second orthogonal library was synthesized in the same way, the only difference being the composition of amino acid mixtures, identification of active pools from both libraries allowed the authors to recall the active structure. A nanomolar ligand of the vasopressin V2 receptor identified in this way is shown in Fig. 3."

A: "The advantage of this approach is that no deconvolution steps are required for the identification of the active compound. Moreover, the result from screening orthogonal libraries is internally validated, since a positive signal in the first library must be confirmed by a signal from the orthogonal library, reducing the danger of false-positive results. Also, instead of testing 'families' of compounds as in the iterative approaches, in the orthogonal libraries the positive compound is tested within a mixture of unrelated structures. A similar approach was reported in the case of carbamates [49], amides and ester libraries [50]."

B: "But even this approach may provide false positives. The authors of the paper you quoted [48] found the intersection of two libraries to be inactive and attributed this result to an unwanted byproduct. However,

*This technique is based on the 'bogus-coin strategy', a strategy for finding the coin of different weights in the group of many similar coins using the minimum number of weighings. Library building blocks are divided into three groups. The proportion of the first group is decreased, the proportion of the second group is increased and the proportion of the third group is unchanged.

as they mentioned, this technique can be applied to the rapid screening of arrays of individual compounds synthesized by various multiple-synthetic methods, since individual compounds may be organized as orthogonal mixtures.

However, this laborious synthesis is applicable only to small libraries with limited complexity. Can you even imagine to synthesize a tetrapeptide library with 50 amino acids at each position?"

A: "Of course I can. I would not like to do it by hand (even not by my technician's hand), but there are robots, and some companies are already developing library synthesizers that will take care of this task. Once this technical problem is solved, such an approach will probably represent the future of combinatorial library techniques."

B: "I would agree, this might be the future for large pharmaceutical companies that can afford to invest half a million dollars to purchase this instrumentation. However, there will still be number of laboratories 'fooling around' with bare hands and ingenious ideas."

On completeness of libraries

A: "In the one-bead–one-compound strategy the completeness of libraries simply refers to the theoretical number of compounds that can be generated by combinatorial synthesis. In the iterative approach you can probably make all possible structures in every case, but the concentration of these compounds will be so low, that the result would be as if they had not been present anyway. Now the question arises how complete the library should be, or how concentrated the compounds available for testing should be."

B: "There is no definitive answer for all libraries and targets. There will always be a big difference between designing a *generic* library, which should provide us with a new structural type for a given target, and a *dedicated* library, which beforehand has inherent features reflecting the designer's knowledge about the structure of the ligand and that should improve the properties of an already-existing lead compound."

A: "Yes, I do agree. Let's talk about generic libraries that are designed without any structural bias. In the one-bead–one-compound technique the researcher is always working with incomplete libraries, since the synthesis of a library is driven by statistics and populations of beads follow the Poisson distribution. To test all possible permutations, one would have to screen a many-fold redundancy of beads.*

In addition, the split-synthesis method, which was designed to synthesize equimolar mixtures of peptides, is producing equimolarity only in cases where the number of beads used for the synthesis is significantly (several times) higher than the number of possible structures. If one wants to synthesize a mixture of 64 million peptides (all hexapeptides composed of 20 amino acids) using only 20 million beads (20 g of resin of 100 μm average particle size), he must eventually miss approximately 44 million peptides, since every bead carries only one peptide entity. Therefore, to synthesize complete mixtures, it is better to use methods in which the individual coupling steps are performed using a mixture of amino acid derivatives."

B: "And what are the consequences? Since the practical size of libraries prepared by the one-bead–one-compound technology is millions of beads; that is, up to millions of different compounds, the large library will therefore never be complete and the chance that an important compound could be missed is significant. For example, a library of hexapeptides (a hexapeptide cannot be considered as a long peptide) may contain 64 million different peptides if only 20 natural amino acids are used for randomization. Upon increasing the number of building blocks (aa) used by including the unnatural and D-amino acids, the number of possible structures grows to the extent that only an extremely small fraction of all possible structures can be present in a real library. The chances that this library will contain a structure that is close to the active compound is therefore small. Moreover, whenever the library is resynthesized, it will contain a completely different mixture of compounds, so the experiment is, in principle, not reproducible. To multiply the number of compounds by using smaller beads would not solve the problem. Peptides will not be sequencable (too small amounts), and coding by nucleic acids to overcome the sensitivity problem, though possible, brings with it synthetic problems."

A: "However, to synthesize and screen a library in which we expect only one hit would be risky. This single positively reacting molecule might be missed easily for a number of practical reasons. It is more realistic to expect a number of hits for the given macromolecular acceptor, all sharing similar structural fea-

*To synthesize a library covering 95% of all permutations with 99% confidence, one would have to use three times excess of beads over the number of potential structures in the case that the total number of structures is higher than 10^5 [51]. For smaller libraries, the redundancy must be higher.

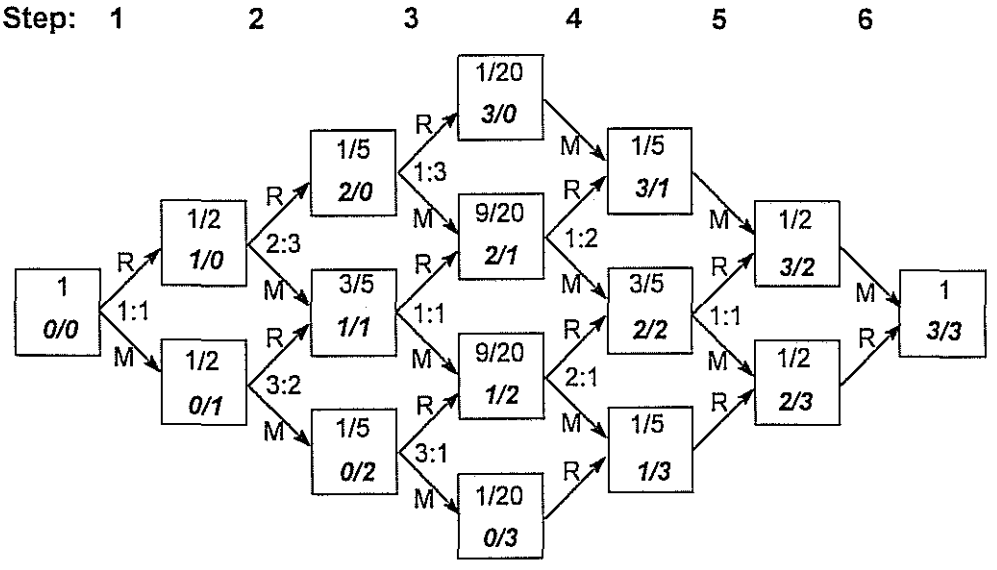


Fig. 4. Synthesis scheme of a 'Library of Hexapeptide Libraries'. Numbers in the boxes specify the amount of the resin and the status of the resin (number in bold italics). The status is defined as the number of defined positions/the number of positions containing the building block mixture. Note that the ratio into which the resin is split for the next synthetic step (for the R or M process) is equal to the ratio of the remaining steps using the R and M process. For example, the resin with status *0/2* (no defined position and two positions containing a mixture of building blocks) has to undergo three randomizations (R processes) and one coupling of the mixture (M process); therefore the resin must be split into a ratio of 3:1.

tures, but at the same time differing in others. In other words, the *motif* required for binding or any other biological function is more important than the individual *molecule*. For example, in a peptide molecule we can identify critical residues – those which cannot be replaced without significant loss of activity – and noncritical residues, which more or less only serve as a structural unit displaying the critical residues for interaction.* Therefore, screening of even an incomplete library, e.g. a fraction of a library of decapeptides, can provide a reasonable number of positive beads if only three to four residues in the sought-after peptide are critical for binding under the screening conditions."

B: "I enjoyed your excercise. However, I think that completeness of a library is not at all critical. The maximum number of building blocks should be used in library synthesis, without taking into account the final complexity. Since not all building blocks are equally important or contribute to the same extent to binding, exclusion of some building blocks, just to prevent increasing the complexity but without knowing the importance of such blocks, would decrease the probability of identifying a hit. The library does not need to be complete; what is more important is that it contains the most crucial building blocks. Then, the optimal structure does not have to be contained in the library; however, the most significant features responsible for the activity must be present in the hit."

A: "I am glad that I have pleased you. Therefore, I will add some more food for thought: combination of two methods, screening individual compounds and sreening mixtures, might provide the best results. In this approach, the split synthesis is combined with the coupling of amino acid mixtures using the algorithm described in Fig. 4. The resulting beads carry structures for which a given number of positions in the sequence are defined, thus defining the particular motif (in the example shown the motif is composed of three amino acids), and the remaining positions are occupied by mixtures of amino acids. Each bead thus contains AA^n different peptides with one defined motif (AA = the number of amino acids in the mixture used

*The number of expected hits from the peptide library depends on the number of critical residues in the binding structure, the number of beads screened, and the number of amino acids randomized at each position, but does not depend on the length of the library or total number of permutations in the library. It can be calculated by the formula: $n = (x)(P_f)[S/(A_n)^{n_{crit}}]$. In this equation, n is the number of expected positive hits, x is the number of different binding motifs, P_f is the 'placement' factor (i.e. the number of possible placements of each motif in the peptidic chain), S is the number of beads screened, A_n is the number of amino acids (subunits) used for randomization, and n_{crit} is the number of critical residues.

for coupling; n = the number of positions at which the mixture was coupled).

For example, a tripeptide motif can be arranged in 20 different ways in a hexapeptide molecule (e.g., ABCXXX, XABCXX, XAXXBC, ... where A, B, and C denote defined amino acid residues and X represents mixtures of amino acids). Since there are 8000 (20^3) tripeptide motifs composed of 20 amino acids, the full representation of a library of tripeptide motifs in hexapeptide libraries, using 20 amino acids for randomization and 20 amino acids in coupling mixtures ($6_{20}(3_{20})$LofL), will be composed of 160 000 beads. A complete library of hexapeptides (6_{20}) would be composed of 64 000 000 beads. Results using this library format have been published recently [52].

There is also a method that allows directed synthesis of all members of a library [53]. If someone needs to be cautious, this technique may be applied and all compounds can be tested. The method is based on the application of a divisible carrier (like a membrane, thread, or tea-bag array). In every step of the synthesis in which randomization is performed, the carrier from the previous synthesis step is divided into n parts (n = the number of building blocks used in the particular step), appropriate pieces are assembled and the next reaction is performed in n reactors. Every part is then divided again for the next randomization. The synthesis of a model library of 2888 pentapeptides as shown in Fig. 5 may illustrate this approach."

B: "To synthesize a complex generic library in this way would be extremely difficult. Just imagine dividing 160 000 pieces of a membrane into 20 aliquots for the last synthesis step of the pentapeptide."

A: "The technique is not designed for the synthesis of large generic libraries. Dedicated libraries of non-peptide structures in which only 20 building blocks are used for randomization in three consecutive steps would contain 8000 compounds. A library of this size is easily achievable using the manual method. However, automated versions of the technique, using robots sorting the carriers and lasers for cutting, may handle much bigger libraries."

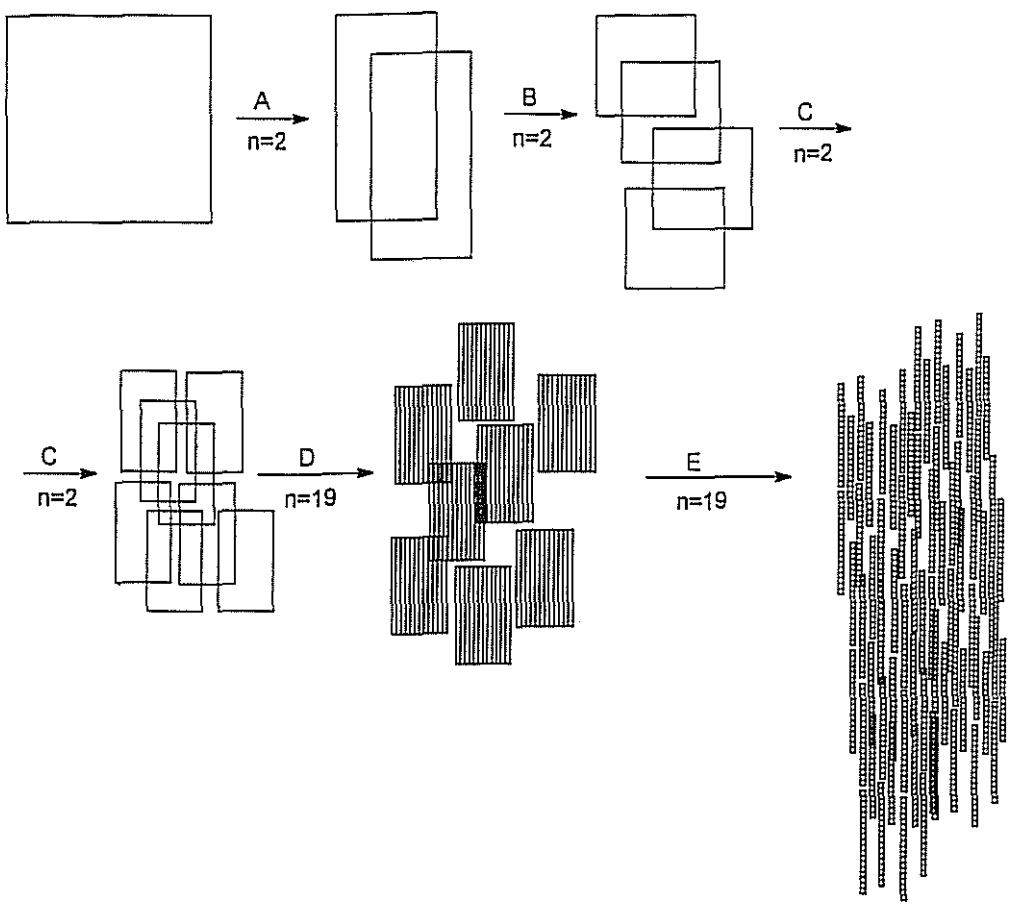


Fig. 5. Synthesis of a library on a divisible carrier (membrane, thread), assuring that all members of the library will be present and none will be synthesized twice [53]. (Step A) The carrier is divided into two parts and two building blocks are coupled; (step B) the carrier pieces are divided into two pieces and two building blocks are coupled to the appropriate pieces; (step C to E) the pieces are further divided into n pieces; each piece is transferred to the appropriate coupling solution, and the process is repeated with the smaller pieces in the next steps.

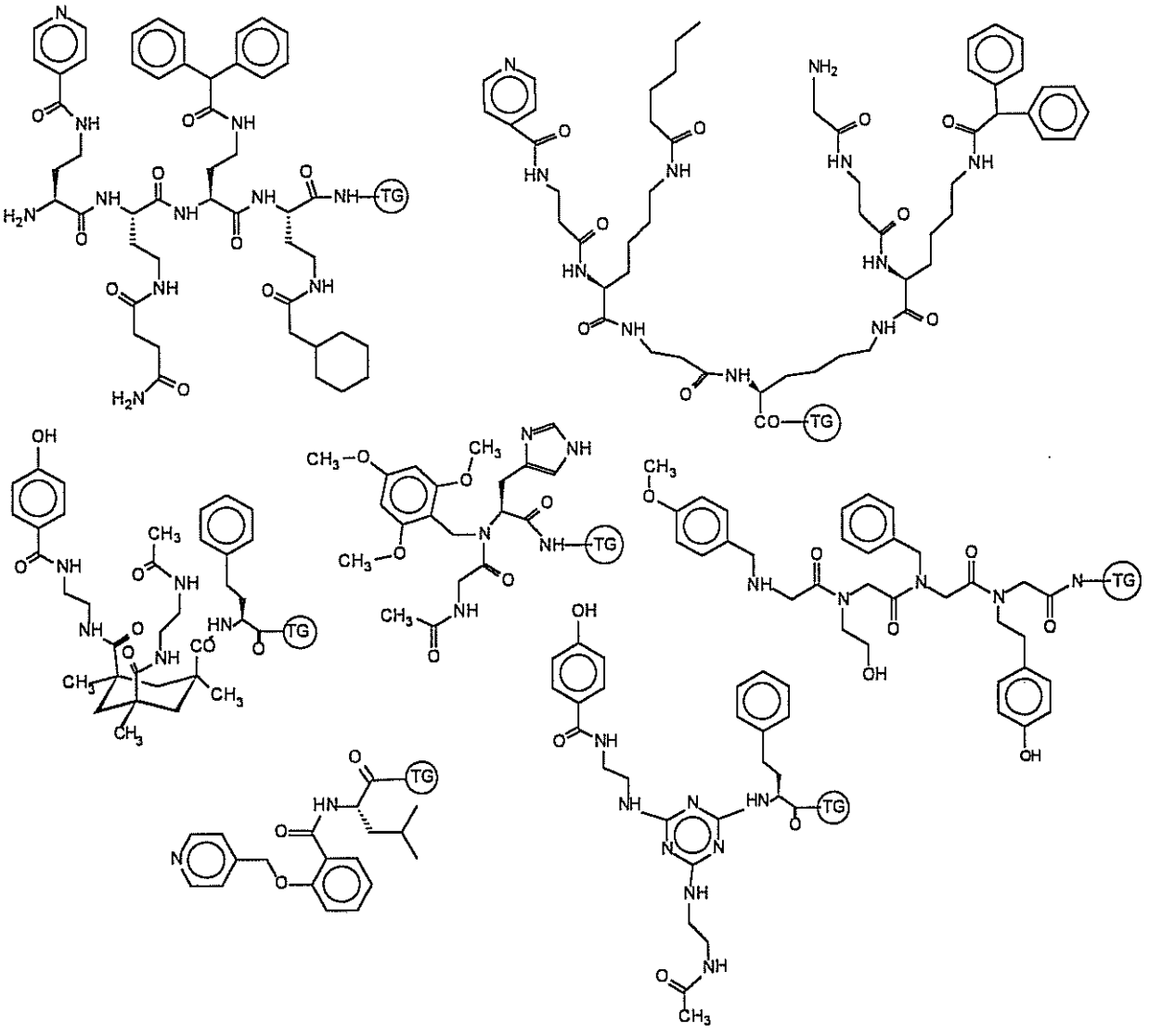


Fig. 6. Examples of streptavidin ligands from various libraries.

On structural diversity

B: "In contrast to the complexity of libraries, the structural diversity, reflecting the dissimilarity of compounds in a library, cannot easily be quantified. I would prefer not to have a very diverse library, not even a generic one. A much better approach is to have a number of less-diverse generic libraries that densely cover a given conformational space. Hits from such libraries will not only be more potent, but also potentially closer to the final structures. The more diverse a library, the lesser the coverage of conformational space. The chances of having a hit from a very diverse library are low. If we do not have a hit, it is difficult to design the next libraries, since we do not know if a real hit may closely resemble certain library structures or whether the target needs a completely different structural type."

A: "The greater the diversity, the greater the chance of discovering an initial hit. However, individual members of a library should possess reasonable conformational rigidity, they should be neither very rigid (low chance of attaining the correct conformation) nor very flexible (weak binders due to the entropy factor). If there is no information available regarding the structural requirements for interaction with a given target, the highest possible diversity and moderate flexibility in a library should be applied. Initial screening should then give us a hint of the potent structure. It is too much to expect that a compound from an initial screening will be close to a drug (again, this applies when little or nothing is known about the ligand). Another argument for using very diverse library structures, even though for medicinal chemistry not very appealing, is that knowledge of different structures interacting with the same macromolecular target might help signifi-

cantly in the application of computational techniques to the design of optimal ligands. An example of this may be the streptavidin ligands found in various peptide and non-peptide libraries (see Fig. 6) [4]."

B: "Whatever method is used for generating synthetic diversity, one can never match the diversity generated by nature. In addition, compounds extracted from various life forms obviously had a biological function in the mother species. It is, therefore, reasonable to expect some biological activity of them in a different system. Hence, screening natural sources of diversity will always be more successful."

A: "The identification of a hit obtained from a synthetic library has an important advantage over compounds isolated from natural sources. Even before we determine the structure of a hit from a synthetic library, we know that it can be synthesized and that the synthesis should be reasonably simple, since complicated chemistries are not likely to be used for library construction. For example, taxol will never be discovered in a synthetic library, but we don't have to spend years developing a reasonable synthesis for new drugs."

On the kind of structures

B: "Since one has to spend a considerable amount of time (and money) on developing chemistry useful for the synthesis of combinatorial libraries, a careful design of structures and corresponding chemistry is almost inevitable. Combinatorial solid-phase chemistry should enable us to directly synthesize libraries of structures known to be of pharmacological interest. Benzodiazepines may serve as an example [54–56]."

A: "In other words, you want to say that it makes no sense to explore peptide libraries, since it is difficult to transform a peptide lead into a drug. However, even though it is difficult to develop a peptide-based drug, there are numerous examples of successful peptide transformations. In some cases a minor structural modification provides the peptide molecule with satisfactory stability and even oral bioavailability [57–62]. Several peptides have been commercially available as medicines for years (oxytocin, vasopressin, LHRH analogs, calcitocin, ACTH) and if we would include in this list insulin and the artificial sweetener, Aspartam, the market share of peptides could be considered more than marginal. However, in general, it would be advantageous to find primary leads in non-peptidic libraries, but can we expect high-quality (i.e., high-affinity, synthetic simplicity, low-toxicity) leads in non-peptidic libraries?

Natural ligands for macromolecular targets (enzymes, receptors, antibodies) are usually of peptidic character, so leads from peptide libraries of various length and complexity can be expected with reasonable certainty. The same expectations can be fulfilled in the case of peptide-like, i.e., linear flexible and more or less conformationally restricted libraries. However, rigid small organic molecules might not interact properly, so binding might not be observed.

Moreover, peptides are ideal if fast onset or offset of the action is required (the half-life of some peptides, such as ANF, is measured in seconds). On the other hand, deamino-D-arginine vasopressin (DDAVP) should be taken only twice a day as a nasal drop, which completely compensates for the insufficient production of vasopressin."

A: "Nevertheless, the majority of the drugs today are relatively small molecules, the structures of which are far from resembling those of peptides. Therefore, I think that combinatorial chemistry should explore structural types that were not previously studied. Only investigating structures known to be pharmacologically active limits the possibilities of the combinatorial approach. New structural types are welcome, not only from the point of view of novelty (which may be crucial in some projects). Completely new structures could be discovered that may successfully compete with or replace existing drugs. In addition, patenting a new drug is much easier."

B: "Considering the structures of today's drugs, one has to look into the history of their discoveries. Most drugs were not found by 'rational' methods, i.e., methods based on the knowledge of particular biological mechanisms, the structure of the enzyme or receptor, or the structure of the natural effector. The majority of drugs were discovered through screening of various sources of diversity, ranging from extracts from plants and animals, to the extreme of chemical compounds synthesized in former Eastern Bloc chemistry laboratories. Some prominent structural classes, which are now considered as drug-like structures, were discovered by accident. Prior to verifying their activity, most investigators never believed these compounds would have a chance of displaying biological activity. Compounds illustrating this situation are benzodiazepines, discovered in the 1950s by Sternbach [63]. The discovery of antiproliferative activity of *cis*-platinum due to a leaking electrode, or the discovery of penicillin thanks to the contamination of a fermentation plate, are other examples of this kind.

If we consider the limited number of structures systematically tested for biological activity, the potential of a library approach becomes apparent. Any medicinal chemist synthesizing new compounds at a

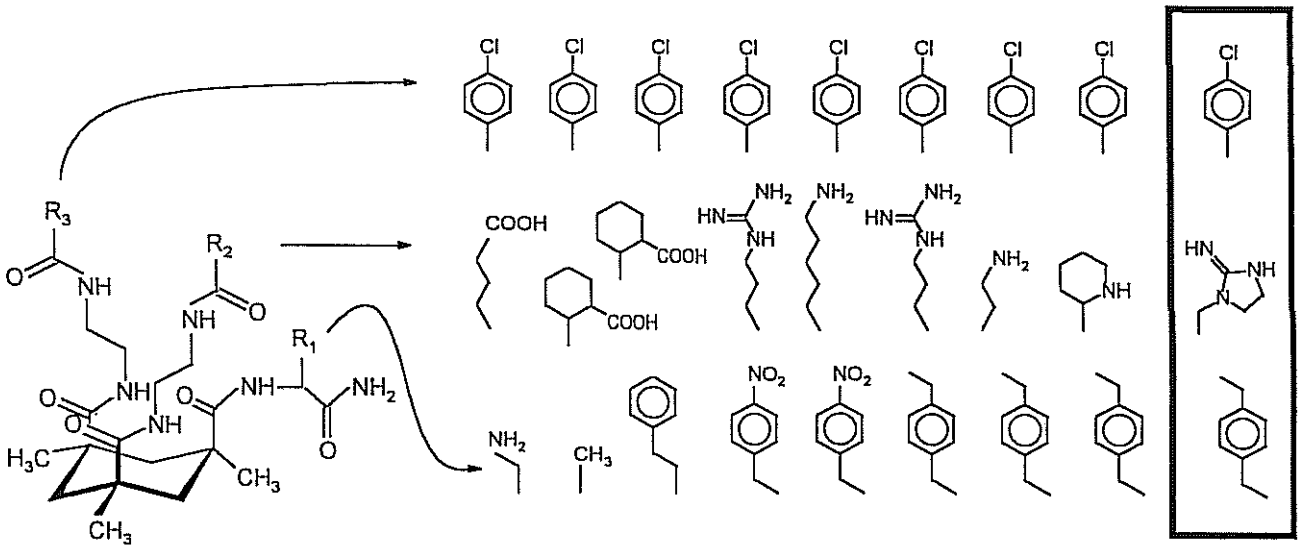


Fig. 7. Consensus between thrombin inhibitors selected from a non-peptide library. The best combination of building blocks (K_i=4 μM; chromogenic assay) is marked by the box.

pace of one compound per week can be considered as productive. However, this productivity means that he may prepare only 2000 compounds in his career. Since every medicinal chemist wants to be successful, he or she won't prepare any compounds that by his or her 'gut feelings' are not likely to be biologically active.

The library approach dramatically changes this philosophy. Even if 'gut feelings' are not favorable towards a new class of compounds expressed in a library, the synthesis and screening of a new collection of hundred of thousands (or millions) of structures, now taking only several weeks, may seem an attractive approach. The probability of finding an active compound may be low, but if it is found it may be a breakthrough discovery. The synthesis of a library in which known structures are contained may seem like an attempt to 're-invent the wheel' or 'rediscover benzodiazepines'. Companies seeking an edge over a competitor would probably be willing to explore structures that the competition would not even dream about. An illustration of this situation may be given here.

Construction of a library based on Kemp's triacid and its screening for enzyme inhibitors was met with a high level of skepticism, since the accepted knowledge of enzyme substrate and inhibitor structure excluded the possibility that a structure generated in this library could be a good inhibitor. In other words: 'Everyone who understands the enzymatic mechanism must clearly see the nonsense of this library design'. Nevertheless, the library was synthesized, screened and ligands with an inhibitory activity better than ligands from the peptide library were found [4,64]. The structure of the best ligand selected from the primary library is given in Fig. 7.

We should be aware of our extremely limited experience with novel classes of structures that may be explored in a library format and should open our minds to completely new ideas, unbiased by the experience of medicinal chemistry in this century. We should always keep in mind that we only have scratched the surface of an enormous potential opened by synthetic chemistry. We can paraphrase Gregory Grant's saying about developments in peptide chemistry [65]: "Synthetic chemistry opened the door to the discovery of new pharmaceuticals, but library techniques blew the doors off the hinges".

But let us return to the question of why peptides are not more widely sought as drugs. The first reasonably simple method for the synthesis of peptides and peptide analogs was discovered only 30 years ago [66], and was not immediately accepted by the scientific community. Peptides, therefore, had to do a lot of 'catching up' with other classes of successful drugs. However, even with this drawback, it seems quite strange that, with a market share of several billion US dollars, peptides are sometimes treated as 'the compounds that never made it'."

A: "Combinatorial chemistry is a useful tool for finding out what kind of biological activities a certain new structural type may possess. This approach has recently been applied to a cubane-1,3,5,7-tetracarboxylic acid and 9,9-dimethylxanthene-2,4,5,7-tetracarboxylic acid scaffold, but has general applicability [45–47]. Four carboxyl groups on a xanthene scaffold have been converted to acid chlorides and treated with a mixture of primary and secondary amines. This one-pot reaction yielded a library that presented potential pharmacophores on a rigid xanthene scaffold. An assay for trypsin inhibitors revealed that this mixture

contains positive compound(s). The deconvolution process then resulted in determination of amines essential for a given biological effect. The selection of building blocks based on their similar reactivity with activated carboxylic acids does not seem substantiated. Various reactivities could be overcome by adjusting the concentrations of individual amines."

B: "Another example might be peptoid structures with high affinities for the α-adrenergic receptor [67], small ligands for streptavidin [68], an ACE inhibitor from highly functionalized pyrrolidines [69], carbonic anhydrase inhibitors from dihydrobenzopyran library [70], or a thrombin inhibitor based on Kemp's triacid-based scaffolding structure [71]. In all given examples, the classical understanding of structure–activity relationships did not help to predict success of the libraries. On the other hand, hits from an acylpiperidine library [70], a substituted sulfonamide library [72], or different libraries of transition-state analogs [73,74] had been expected with certainty."

On the type of chemistry

A: "Synthetic combinatorial chemistry obviously started with amide bond formation. The synthesis of peptides on a solid phase has been mastered for approximately 30 years and was ready for library synthesis. However, the scientific community soon realized (see e.g. Refs. 54, 75–78]) that one chemical reaction in one type of compound (peptide) is a good start, but offers only limited diversity of chemical structures. The use of amide bond formation has been extended to the first generation of non-peptide libraries, including peptoids, small scaffold-based libraries, etc. (see e.g. Refs. 54, 75–80). However, there are many other chemical reactions that were proven to be working on a solid phase (for reviews, see e.g. Refs. 81–83). The most recent chemical literature illustrates the explosion of interest in solid-phase organic chemistry by an increase in the number of papers describing a variety of chemical reactions on a solid phase, which can be used in combinatorial chemistry (more than 50 papers in 1994 and part of 1995; for a detailed compilation see e.g. Refs. 16, 19, and 84 or WorldWideWeb Internet dynamic database [32]). Let me just illustrate some types of chemistry by the examples given in Fig. 8.

There are several criteria for the selection of suitable chemistry for future use in library design and synthesis. As the most relevant features I consider: (i) a high yield and purity of the products; (ii) a variety of available building blocks; (iii) compatibility with other chemistry; and (iv) user-friendly reaction conditions. One would not like to perform a reaction in 50 vessels under an inert atmosphere at –78 °C. Combinatorial chemistry will in the near future use a battery of compatible chemical reactions, fine-tuned for a large variety of building blocks. By combining these reactions, one will be able to create an almost infinite set of structural variations."

B: "You may change your mind once you have a fully automatic instrument capable of running organic reactions under an inert atmosphere at –85 °C. However, you forgot to mention one very important aspect. The selection of chemistry for library design and construction must also be guided by the ability or possibility to perform such chemistry in the pharmaceutical industry. It is depressing to have an active compound with perfect pharmacological properties that cannot be prepared on a large scale or whose costs prevent its use a priori."

A: "The design of libraries does not need to take into account the feasibility and easiness of future syntheses of compounds. There are at least three good reasons for this: (i) it is naive to expect that the first hit from a library will be a final drug; (ii) there are alternative ways to synthesize any compound; and (iii) the chemistry used in a library synthesis must be compatible with the resin, linkers and building blocks. This compatibility limits the array of chemical reactions applicable to library construction. Alkyl aryl ethers may serve as an example: they can be prepared by the Williams reaction on industrial scale, but for library synthesis the reaction of choice would be the Mitsunobu reaction, since it is performed under mild conditions (room temperature, reaction time in hours, resin-friendly solvents) and it is compatible with, for example, ester linkages used in releasable linkers. Industrial production of a small-molecule drug is unlikely to be based on solid-phase chemistry and, therefore, the selection of strategies for its synthesis may not be limited. However, you should not be too optimistic. It will not be easy to convert the process according to the wish of the industrial chemist. We may quote here Prof. Cornforth (see Ref. 89) who said about the ideal of the industrial chemist: 'It is no good offering an elegant, difficult, and expensive process to an industrial chemist, whose ideal it is something to be carried out in a disused bath tub by a one-armed man who cannot read, the product being collected continuously through the drainhole in 100% purity and yield."

B: "Various chemistries may be applied to combinatorial library design in two different ways: (i) scaffold-based libraries, in which a small scaffold (e.g. cyclopentane, cyclohexane, benzene) contains three to four functional groups and each of this group is used independently for attaching a different set of building

blocks (we call this the 'glucose approach' after Hirschman's scaffolded mimicks of somatostatin [90,91]); or (ii) chemical reactions can be combined in a 'linear' fashion, e.g., a set of aromatic hydroxy acids is coupled via an amide bond to a set of amino acids; hydroxyl groups are then used in a Mitsunobu reaction with a set of alcohols [92]. At the end of this synthesis, the structure of a randomized molecule may be similar to a scaffold-based compound ('benzodiazepin approach', named after Ellman's combinatorial synthesis [54,55])."

On building blocks

A: "The selection of building blocks for a certain library depends on whether there is any information available regarding structural requirements for a small ligand, or whether nothing is known about the types of interactions involved in binding the potential ligand to a given target. If the library design considers such information, the selection is biased and the building blocks should contain whatever seems to be important for binding. A generic library, not biased towards certain types of interactions, should contain a variety of building blocks. The selection can be made by the naked eye (by intellect alone), or it can be done with the help of a computer, the rationale being the selection of the most dissimilar structures. Chiron's 'flower plots' is one example [93,94].*"

B: "Dissimilarity is not the best criterion for the choice of generic building blocks. The selection should primarily be based on the type of interaction. All interactions known to play a critical role in the binding of two molecules should be considered. This includes hydrophobic, aliphatic as well as aromatic interactions, and charge interactions. Building blocks should contain positively and negatively charged groups, the ability to form hydrogen bonds (the presence of both hydrogen donors and acceptors), and, last but not least, chelating groups. Not until all types of interactions are covered, the criterion of dissimilarity, in this case different positions in conformational space, should be considered. This means, for example, that in a generic library one would not include building blocks that differ only by one methylene group."

A: "Well, there are a number of examples in which one methyl or methylene group makes the difference between binding and no binding, or between agonism and antagonism. Just try to insert one methylene into a backbone of any peptide hormone (see e.g. Ref. 95), or try to guess the activity of a peptide analog in which valine is replaced by isoleucine or leucine."

B: "Firstly, a backbone is not a building block; it predetermines the position of a building block and it is, therefore, a more crucial element. You remember that we have already shown how one methylene group can influence the diversity if added to the backbone or to the side chain. The second point concerns the verdict 'active' or 'not active'. This always reflects a particular activity and assay. If you have a sensitivity of the assay in the micromolar range and your hit is present in just a little less than a micromolar concentration, any unfavorable change will turn the weakly active compound into an inactive one."

A: "I agree: it is extremely difficult to guess what the influence of a particular structural feature will be. However, it is clearly not possible to include all possible building blocks in a library. The question is whether it is better to fish using a net with large openings at sea, or using a dense net at a small pond.

We have recently compared the selection of building blocks used by four different laboratories [67,68,96,97] in the synthesis of combinatorial libraries [15]. Successful library design was achieved in the cases of dedicated or biased libraries [67,96]; however, the optimal design (if at all possible) of a 'generic' library is still being sought. The most promising approach in this direction is probably the already mentioned 'flower-plots' method of the Chiron's scientists [94]. The success of a dedicated library does not depend on the library size. For example, an inhibitor of ACE with K_i ~ 160 pM (Fig. 9) was found in a library of functionalized pyrrolidines constructed by randomization of a mixture of four amino acids, four aldehydes, five olefins and three mercapto acids (the library contained probably more than 480 compounds due to the low stereo- and regioselectivity of the reactions)."

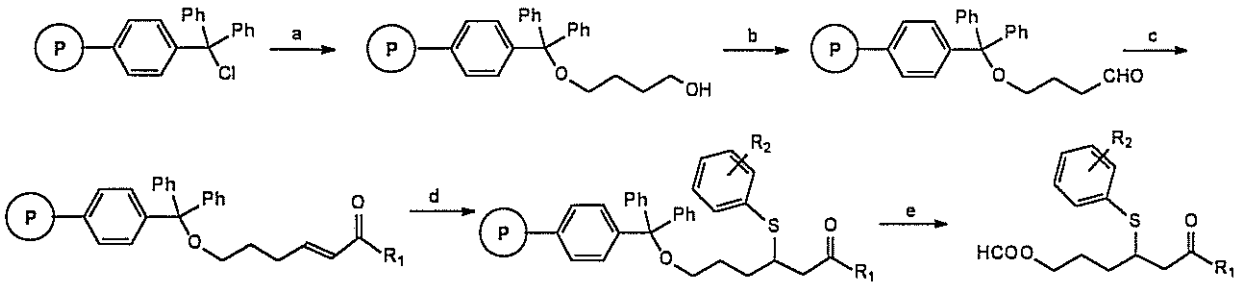
B: "You should add that these building blocks have been selected with specific structural features in mind. It is relatively easy to catch fish with a very dense net in a small pond if you a priori know that there are fish in the pond."

On clean chemistry

A: "Library techniques depend to a significant extent on chemistry that can be performed on a solid sup-

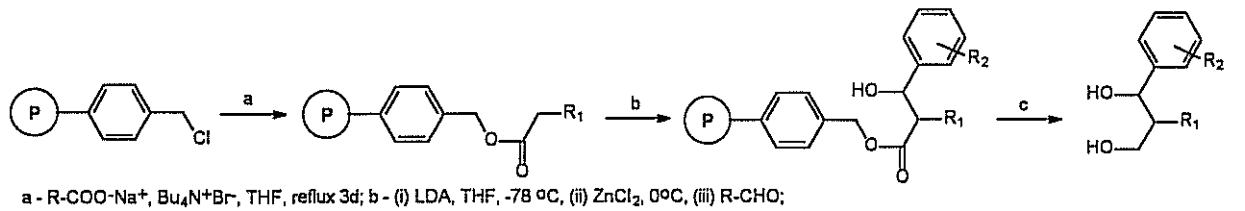
*This method evaluates a number of properties of building blocks (lipophilicity, topological indices, chemical functionality descriptors, and receptor-recognition descriptors) and compares them in the form of circular plots. 'Petals' of the 'flower' represent individual properties and the color of the center represents the similarity to a given prototypical block.

port. It will be very difficult to transform some reactions to solid-phase reactions. Moreover, reactions with immobilized components cannot be monitored for their completion. Intermediates cannot be purified and products of side reactions will accumulate in the final product. This problem is for any good organic chemist very hard to accept. Therefore, the selection of non-amide-bond chemistry for future use in libraries has to be based on the possibility of driving all reactions to completion. If we are not sure whether a



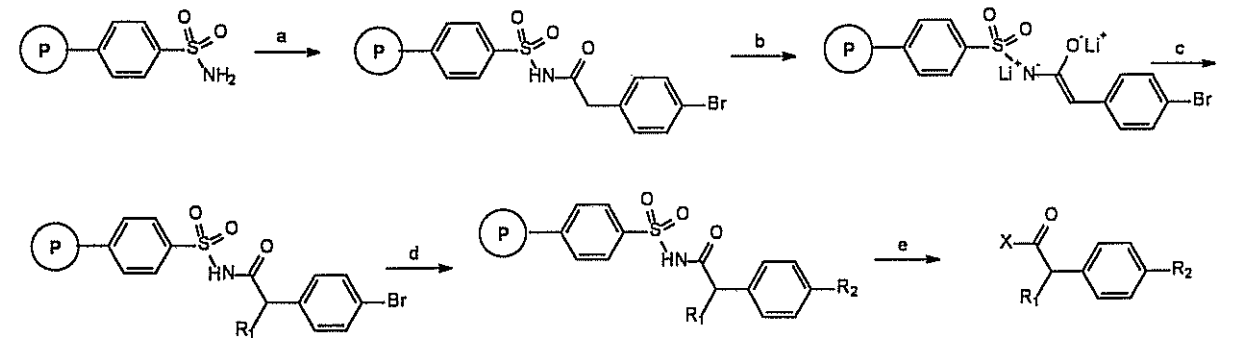
a - 1,4-butanediol, pyridine, 2d, r.t.; b - Pyr.SO_3, DMSO, Et_3N, 2h, r.t.; c - Ph_3P=CHCOR$_1$, THF, 60°C, 2d;
d - R_2-C_6H_4SH, NaOMe (cat.), THF, 2d, r.t.; e - HCOOH, THF, 2h, r.t.

Chen et al., 1994



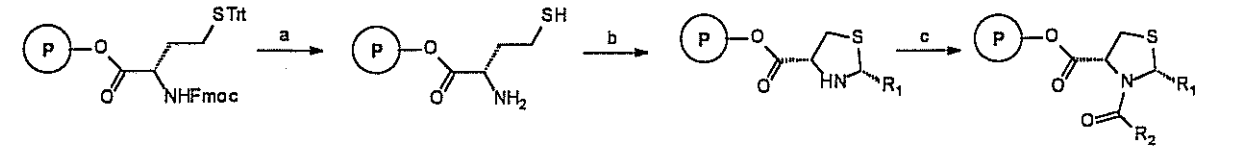
a - R-COO$^-$Na$^+$, $Bu_4N^+Br^-$, THF, reflux 3d; b - (i) LDA, THF, -78 °C, (ii) $ZnCl_2$, 0°C, (iii) R-CHO;
c - DIBAL-H, toluene, 0°C

Kurth et al., 1994



a - Br-$C_6H_4CH_2$COOPFP/DMAP; b - LDA, THF, 0°C; c - R-X, 0°C; d - alkyl-9-BBN or aryiboronic acid,
Pd(PPh_3)$_4$, Na_2CO_3, THF, 65°C; e - (i) CH_2N_2, (ii) OH$^-$ or amine

Backes and Ellman, J.Am.Chem.Soc., 116, 11171 (1994)



a - 20% piperidine/DMF; 5% TFA/DCM/iBu_3SiH; b - R_1CHO/AcOH; c - R_2COOH/DIC/DMF

Patek et al., 1995

Fig. 8. Examples of syntheses of non-peptide libraries. (A) Chen et al., 1994 [85]; (B) Kurth et al., 1994 [86]; (C) Backes and Ellman, 1994 [87]; (D) Pátek et al., 1995 [88].

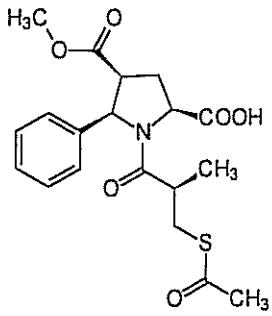


Fig. 9. Structure of the ACE inhibitor from a non-peptidic library [69]. $K_i \sim 160$ pM (ACE inhibitor).

certain step provides a desirable product, it is not possible to include this reaction and/or building block in a library. It is essential to know that a library contains all the structures that we intended to synthesize."

B: "Your first assumption is not correct. For a one-bead–one-compound library, the solid-phase synthesis cannot be replaced by anything. However, iterative (or deconvolution) methods can make use of solution synthesis, which may be simplified by the use of soluble polymers, that allow easy workup of reaction mixtures by precipitation [98].

The reaction monitoring on the solid phase is being addressed (besides specific colorimetric techniques [99–101]) by the use of FT-IR spectroscopy [102–105] and gel-phase NMR [106–109]. Even though the monitoring is not as convenient as in the liquid phase, certain transformations can be followed very simply.

The acceptance of dealing with mixtures instead of pure components is a major mental block for a number of researchers; however, our goal is generating diversity. In theory then, the more complex the reaction, the better. The important topic in this technique is not the purity, but the reproducibility. If we find a positively reacting bead in our one-bead–one-compound library, it does not necessarily mean that we are dealing with a unique structure. As we explained earlier, even beads containing thousands of structures (in a library of libraries) can provide valuable information. Moreover, even conventional peptide chemistry does not provide 100% pure compounds every time. We have encountered situations in which the major component of the synthetic mixture was not active and the activity was expressed by the minor component (partially protected peptide; the result of incomplete cleavage of the protecting group). If we were able to recall the structure on the positive bead, or even better said, to recall the synthetic history of the positively reacting bead (the blocks used for randomization, the conditions applied for their attachment, the methods used for deprotection, etc.), then we would be able to reproduce exactly this history with a larger batch of the same solid support and reconstruct the compound in this way (if we were lucky and all reactions proceeded in 100% yield producing only one single product), or the mixture of compounds on the bead originally selected from the library. The next step would be application of modern separation techniques, testing all components of the mixture in the appropriate biological test and selecting the one responsible for the positive response of the library bead. The presence of side products resulting from incomplete reactions may help in structure elucidation using mass spectroscopy [68]. This does not mean advocating dirty chemistry, it is only a pragmatic standpoint towards the reality of combinatorial chemistry."

A: "Nice talk. I would just like to know what you would do if you were not able to reproduce the activity of a mixture. What if it is a false-positive result, or if the minor compound responsible for the activity – being therefore very active, because it is a minor component – does not appear in reasonable amount in the resynthesized mixture?"

B: "It is always better having to solve a problem than having nothing to worry about."

On libraries for the discovery of inactive compounds

A: "It is always desired that a new library will yield novel active structures for the studied target. However, the knowledge of what is not active is also very important. This awareness is particularly appreciated when a patent application is written. Since it is fortunately not possible to cover the whole universe of compounds, we must limit our structural types that exhibit certain activity into a reasonable frame. Now the question of what not to cover arises, and nobody wants to leave an uncovered hole where others can dig and find activity. The use of combinatorial chemistry in dedicated libraries, designed to find out what is essential for activity, is a valuable tool."

B: "Since most libraries cannot be complete, there is always a risk that the particular preparation will not contain the critical structures, and a negative result may be misleading. It is too risky to base any important decision on an experiment designed for a negative answer."

On screening methods

A: "The synthesis of a library without its subsequent biological evaluation will only be an end in itself.

There are two different assays that can answer the question whether there is an active compound in a library: the on-bead binding assay and the solution assay. The on-bead assay is fast and easy to perform, whereas the solution assay can provide functional evaluation of a potential hit; however, in the case of a one-bead–one-compound library a solution assay requires more sophisticated chemistry that includes specially designed linkers allowing the release of a compound into the solution."

B: "The third possibility combines the ease and quickness of the on-bead assay, with the reliability and the ability to functionally evaluate the solution assay. Hybrid screening starts with the on-bead binding assay. After selection of potential candidates, the compound is released from the bead and its activity in solution is evaluated [4]."

On on-bead binding assays

B: "The on-bead binding assay is applicable to almost all soluble targets and it is fast, allowing high-throughput assays. Lam's original histochemical procedure [37] has been successfully applied by many laboratories. Bead-bound peptide libraries were screened against an acceptor molecule (e.g., natural or artificial receptors, enzymes, antibodies, or even small molecules) using an ELISA-type assay [37,110–112].* Its principle is very simple: the assay is based on the interaction of molecules available on the surface of the bead with a target in the solution. The on-bead assay is not necessarily limited to binding. Phosphorylation of the compound on the bead represents an elegant method of evaluating the function of the compound while still bound to the bead [122]. Another application of solid-phase-bound libraries is the search for enzymatic substrates. In this method, the library of peptides, the structure of which included internally quenched fluorophors, was incubated with the appropriate enzyme. When the peptide was cleaved, internal quenching was eliminated and the bead carrying the substrate sequence was identified by its fluorescence [115]."

A: "There is no doubt that on-bead binding is simple and fast. However, each library contains many compounds (peptides) that will lead to nonspecific interaction (highly charged peptides, very hydrophobic molecules, etc.). This fact alone makes the discrimination of real and false-positive hits problematic, and a reliable on-bead binding assay may be so difficult to establish that some targets have to be eliminated."

B: "To eliminate unwanted interactions, each bead that reacts positively in the first screen goes to the second round for a specificity test. Beads are pre-incubated with a soluble ligand and then the binding assay is repeated. If the bead reacts positively, its interaction can be regarded as nonspecific and such beads are discarded and not considered as real hits. An example of the importance of specificity testing is illustrated by the following example. One million beads from a pentapeptide library were incubated with the gpIIb/IIIa receptor and 7480 positive beads were detected. These beads were stripped of the coloration and reincubated in the presence of a high concentration of G4120, the specific ligand for this receptor. Non-stained beads in this step may be considered as specific, but they were stripped again and reincubated in the presence of a low concentration of the competitor to define the highest binders. Only eight beads were selected in this way and five of them did contain the expected sequence Arg-Gly-Asp."

A: "Too much work. I like an alternative technique, which does not require the tedious selection of hundreds or thousands of positive beads in the first steps of screening, but uses different color reactions for discriminating between specific and nonspecific interactions [123]. Only beads containing the proper color combination are picked from the library and sequenced.**"

B: "Nevertheless, what counts is the real hit. We have recently analyzed the success rate of several projects in the on-bead binding assay. It ranges from two weeks of screening to obtain the first hits, which were for more than 95% real (after resynthesis the activity is verified in a solution assay), to several months before the hits are found. In the worst case, only 3% of the hits were real."

On solution assays

A: "Iterative methods based on the screening of mixtures of compounds in solution [27] can be adapted to any screening strategy, starting with binding assays in microtiter plates and ending with testing in animals. Rumor says that Richard Houghten was testing (well, it would be better to say *tasting*) the peptide mixtures

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*This approach was subsequently expanded to include a fluorescence-based assay, using for example a fluorescence-activated cell sorter (FACS) [113], fluorescence microscopy [114,115], measuring fluorescence in solution [116], radio-ligand binding [117,118], or magnetic bead binding [119]. (To be correct, the on-bead binding assay has originally been described by Modrow et al. [120,121] in the pre-library era).

**A similar technique named 'Pelican' was presented by scientists of Arris at the American Peptide Symposium [124].

himself in an attempt to find artificial sweeteners. One-bead–one-compound libraries synthesized by the use of a doubly (in principle multiply) cleavable or partially cleavable linker may be applied to solution screening in all cases where the test could provide a reasonable result for limited volumes of compounds at limited concentrations [125]. Using beads of 130 μm diameter with 0.2 mmol/g substitution, one can release 100 pmol of compound in two distinct steps. If the release volume is 100 μl, the highest achievable concentration is 1 μM. This is then the sensitivity limit of the assay. However, bigger beads with higher substitution can also be used for library synthesis, and concentrations of up to 50 μM may be achieved. Moreover, the screen is performed in two steps and completely independent structures may be discovered."

B: "It does not seem to be reasonable to increase the amount of material released from one bead. The probability of finding a hit may be increased by performing the synthesis on bigger and/or more highly loaded beads, but this would only complicate the synthesis (slower couplings, washings) and increase the possibility of discovering weak compounds. A more promising way is to increase the diversity of the library, or to synthesize a new library containing different structural types. Optimizing a two- or three-digit micromolar hit may take longer if compared to the synthesis of new libraries, or may not be possible at all."

A: "The synthesis of libraries containing a multiply cleavable linker is more difficult than the synthesis of iterative or 'orthogonal' libraries or 'positional scanning' libraries. Moreover, the structure of a hit must be identified by only using the material available on one bead. Iterative libraries provide the structure of the active compound by following the synthetic and screening algorithm, while orthogonal libraries directly provide information about the structure from the screening."

B: "Yes, the chemistry of multiply cleavable libraries is more difficult (considering also the chemistry of coding, see below). However, only one library of the one-bead–one-compound type has to be prepared, and this library is not influenced by the choice of a defined partial structure. There is always substantial synthetic work needed to follow-up every potential lead in the iterative libraries and, besides that, the issue of test sensitivity to detect weak ligands is very serious. Orthogonal libraries are limited in their size; the synthesis of an orthogonal library randomizing 25 building blocks in five steps would require handling 6250 sub-libraries.

It is not necessary to use multiply cleavable linkers. The alternative is kinetic cleavage of the compound from the bead. Different possibilities of kinetic release have been discussed elsewhere [15]. Scientists at Pharmacopeia are using photolytic cleavage in two stages to test the libraries in solution [70,72]: the libraries are divided over the wells of microtiter plates (10 beads per well), and the first portion of the compound is released by a short exposure to UV light after which the mixture is tested. Positively responding mixtures are then identified and the corresponding beads are individually placed in the wells. A second exposure to UV light releases the rest of the molecule from the bead and the active bead can be detected.

The solution assay of a one-bead–one-compound library may be performed using a different format: the compound may be cleaved from the linker in the dry state, i.e. the library compound is still inside the beads, but already as a free compound. Diffusion of the compound from the bead into a medium in which the activity is evaluated will detect the bead of interest [126]. In this assay a staged release from a relatively stable attachment to the bead can be used [126], or multiply cleavable linkers recently developed may be applied [125,127,128]."

On pooling strategies

A: "Solution assays in combinatorial chemistry suffer from one inherent difficulty: the testing of a vast number of compounds. In principle it would be possible to screen all the compounds prepared by the mix/split approach seperately, but this is not realistic in complex libraries. In practice, we face a problem of pooling samples and screening mixtures of compounds. There is substantial evidence suggesting it may not be possible to find the best compound by this strategy. The obstacle arises from the fact that a pool may contain one very active compound or many less active compounds."

B: "The screening outcome of pooled samples depends on the library. If the library has been designed to be highly diverse; i.e., to contain very dissimilar structures, the probability that this library will contain many hits is quite low (unless the target is very promiscuous). In this case, the screening results are not very sensitive to the pooling strategy, since the library contains only a limited number of hits. However, if the library contains compounds which are structurally similar, a pooling strategy is of the utmost importance. It is worthwhile to mention that peptides are structurally similar (all of them only differing by a side chain attached to alpha-carbon atoms of a monotonous backbone) and a peptide library may either

contain no hit, or several to many hits sharing one (or more) common motif(s)."

A: "Even though the theoretical examination of the pooling problem has been published [129–131], the practical value of this analysis is questionable until we know what hits to expect in a library. In a real library, we can speculate about how many hits we may expect, but there is a simple way to find out how rich a particular library is. Before starting any pooling, we should perform a so-called bead-load experiment. Compounds are released from a sample of the library (the sample contains tens up to hundreds of beads), and the biological response of the released mixture is evaluated [132]. The criterion for a number of compounds in one pool for real-library screening is dictated by statistically significant differences in the activity of pools."

On assay expectations

B: "In the iterative technique, one is always guided toward one structure, since a selection is made in every step, and for practical reasons it is not possible to follow all alternatives or to start from different starting points. Let us consider some examples from our laboratory. The screening for binders to streptavidin would never show any signal in a library in which two amino acids were defined at the N-terminus of a hexapeptide. However, one would very likely see strong signals in a library in which the H and P were defined in the middle of the molecule (XXOOXX library), or the H and Q defined in a different arrangement (XXHXQX library). We certainly would consider Y and G as the best combination at the amino terminus of a hexapeptide library (OOXXXX) in the screening for ligands of the anti-β-endorphin antibody. However, the next iteration (YGOXXX library) might give us a headache in deciding what amino acid residue we should define for the next step. Screening for the anti-insulin antibody would give us a nice FN signal in the OOXXXX library, but following this lead we would certainly miss the much stronger binding motif W_GF located at the C-terminus of the molecule [110,111, 133,134]."

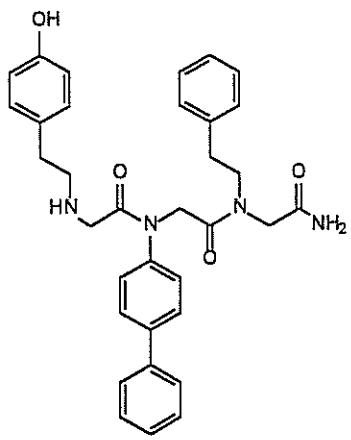
A: "This speculation may be correct; however, the positional scanning will clearly show how each position is sensitive towards changes and will determine the most active amino acids at each position. The anti-β-endorphin-antibody case at the third position will simply reflect that this position is not crucial. Once you have the whole picture, it is easy to synthesize all peptides representing all combinations of selected amino acids. Even in the case of two motifs, both would probably be found, while peptides having amino acids from two different motifs will simply not show any binding. By the way, how many motifs with affinity for the same receptor can one expect?"

B: "Well, quite a number. For example, peptide sequences exhibiting binding to the insulin antibody are quite different, depending on the length and conformational restriction of the peptide [134]. Another example may be sequences that bind to the opiate receptor discovered by solution-screening methods [14,40]. If peptides with free and protected amino termini are present in one library (as may be the case for the one-bead–one-compound libraries), the consensus would be hard to define."

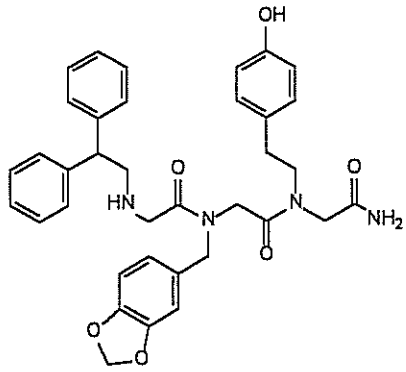
On structure determination

A: "At the end of a screening process using iterative methods testing mixtures of compounds, the structure of the ligand is known based on the synthetic algorithm of the library. In the case of 'positional scanning libraries' several possible combinations of identified building blocks have to be resynthesized and evaluated. However, library techniques that do not track the identity of compound(s) during the synthesis require structure determination at the end of the process (for a review, see e.g. Ref. 4). Structure determination of peptide ligands is straightforward. Edman degradation can be applied to the analysis of structures available in low picomolar or even high femtomolar amounts. Complications start with noncoded amino acids; nevertheless, more than one hundred unusual amino acids of different configuration can be determined by HPLC techniques. Determination of the structure of non-peptidic ligands is much more difficult. Direct methods for organic structure determinations cannot be used, due to their limited sensitivity. The only technique available is mass spectroscopy, but its application is also limited to reasonably small libraries (probably up to 100 000 entities) [135–138]. Modification of direct mass spectroscopic analysis is Youngquist's approach, in which part of the growing molecule is capped in each synthetic step, building a 'ladder' which documents the history of the synthesis of a molecule on a particular bead [135, 139].

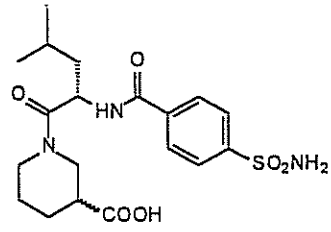
The alternative to direct structure determination is coding. In this case the synthesis of the screening structure is accompanied by the parallel synthesis of a coding structure, which may be easily sequencable (such as polynucleotides [140] or peptides [75,141]), or by randomly attaching coding blocks to the bead matrix which can be easily detached and analyzed by sensitive analytical techniques [112]. The last approach



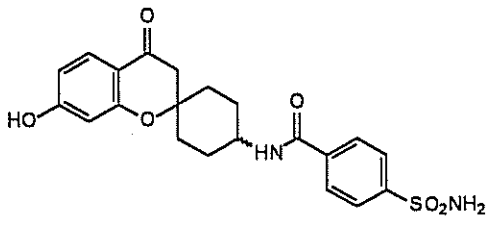
Ki = 5 nM (alpha1-adrenergic receptor)
Zuckermann et al., 1994



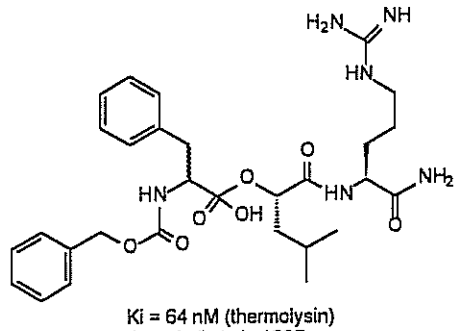
Ki = 6 nM (mu-specific opiate receptor)
Zuckermann et al., 1994



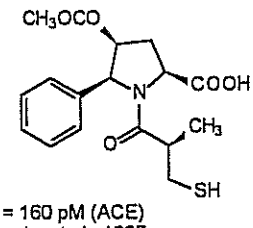
Kd = 4 nM (carbonic anhydrase)
Burbaum et al., 1995



Kd = 15 nM (carbonic anhydrase)
Burbaum et al., 1995



Ki = 64 nM (thermolysin)
Campbell et al., 1995



Ki = 160 pM (ACE)
Murphy et al., 1995

Tyr-Pro-Phe-Gly-Phe-Arg-NH_2 IC_{50} = 13 nM
Trp-Trp-Pro-Lys-His-Gly-NH_2 IC_{50} = 9 nM
Ac-Phe-Arg-Trp-Trp-Tyr-Met-NH_2 IC_{50} = 33 nM
Ac-Arg-Trp-Ile-Gly-Trp-Arg-NH_2 IC_{50} = 5 nM (agonist)

Dooley et al., 1995 (mu opioid receptors)

Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Lys-NH_2 Ki = 16 nM

Dooley et al., 1994 (mu opioid receptors)

D-Trp-Phe-D-Arg-Pro-D-Phe-NH_2 Ki = 3.5 uM (thrombin)

Seligmann et al., 1995

Fig. 10. Structures of several leads from combinatorial libraries [40,67,70,73,145,146].

was mastered by Pharmacopeia [70,72] and is used for the screening of non-peptide libraries. The question of whether the screening or coding structure interacts with the receptor in bead-binding screening has been solved by physically separating the surface and inner part of the polymeric bead; the screening structure is then only synthesized on the surface, while coding inside the bead hidden from macromolecular target molecules [142]."

B: "Obviously the best way for coding would be the combination of chemistry with an other, 'orthogonal', type of coding; optical, mechanical, or electronic. Something like having a bar code on every bead."

A: "Well, this is not just fantasy anymore. Miniaturized tea-bags containing a microchip capable of storing information about the frequency of electromagnetic radiation have already been used in the synthesis of a model library [143,144]. The new company based on this technology, Irori, was started recently. The technique can certainly be miniaturized to the level of a single bead containing a chip inside."

B: "This is really an exciting technique. However, it will take some time before it will become available to everybody, and, even then it will probably be very expensive. Other coding techniques are elegant, anyone can enjoy reading articles describing them, but

not synthesize the coded libraries. These techniques create a hurdle in the synthesis. To account for this disadvantage and still being able to recall the structure, you may combine three different kinds of information: (i) the first building block can be pre-coded on the resin and this code will be read after the bead of interest is found. The pre-code may be an amino acid, a pair of amino acids, a color, or anything that unambiguously links the first building block to the code; (ii) if the library is not pooled after the last randomization, the second set of information (the last building block) can be obtained by knowing from which sublibrary the bead of interest originated; and (iii) finally, the molecular weight of the compound of interest can be determined by mass spectroscopy. Since in most cases there are three to four randomizations in non-peptide libraries, and the first and last building blocks are already known, the third and/or the second building block can be determined from the remaining molecular mass."

On results

A: "The utility of each technique is proved by results. However, even though library techniques have been applied by a number of laboratories, there are not yet so many results reported in the literature which clearly show the success of the combinatorial techniques. The structures found in the libraries showing binding to various antibodies (most popular being the antibody against β-endorphin), or to streptavidin, are not that impressive. Most of the reported results were obtained in various model systems and their practical value is limited."

B: "The problem here is very simple. There is an extremely large number of scientists working in this field, both at universities and in the industry. Combinatorial science has been the topic of at least one international conference per month in 1995. But the practical results which might be used in the development of a new drug will not be shown in the near future, until the patents which are being filed right now become publicly available. However, some of the results are already coming into the world (such as inhibitors of the α-adrenergic receptor [67], carbonic anhydrase [70,72], angiotensin-converting enzyme [69] (see Fig. 9), opioid receptor [40,145], the vasopressin receptor [48] (see Fig. 3), thermolysin [73], or thrombin [146]) (see Fig. 7). Structures of several leads selected from libraries are given in Fig. 10. Furthermore, Selectide will present the structure of a potential clinical candidate (an inhibitor of factor Xa) selected from a combinatorial library as soon as the patent describing it has been published."

On the history of molecular diversity

A: "Who may be called the 'Father of Diversity'?"

B: "There is no 'Father of Diversity', even though there are some researchers claiming this title. However, there is a 'Mother of Diversity': Mother Nature. Well, she did not publish Her findings anywhere, but maybe this is due to the fact that the experiment is not finished yet. What can be a better way of creating a mixture of everything, than putting all elements in one pot and heating it, cooling it, compressing it, and whatever ... for several billion years. Some results are known, for example: you and me."

A: "I agree. We don't have to argue about the priorities. Anybody can look at the list of published papers and patents and try to draw his own conclusion. Besides that, it is really not that important who was the first. The time was ripe for a new approach and a number of scientists were thinking along the same lines. It is still happening today and I am sure that we will witness publications of the same idea or experiment by several laboratories at the same time. It is exactly as the late professor Rudinger said: 'No idea is so stupid that two people may not be working on it at the same time in neighboring labs or on opposite sides of the globe'."

On social aspects

B: "The new field of combinatorial chemistry represents a real breakthrough, not only in the number of compounds synthesized and tested (one 'librarian' easily synthesizes more compounds than the whole chemical community in the pre-library era), but in the way of thinking as well. Handling and testing unpurified compounds without even knowing their structure may serve as an example. Even the publication policy has witnessed some changes: it became possible (and probably important) to publish not only the results of your research, but also new ideas about it (see e.g. Ref. 147), because if you do not publish it now, somebody else will."

A: "There is no doubt that this field is growing exponentially and if you want to be the first, you have to be fast. But this fact alone does not entitle authors to publish ideas without any experimental data. A similar rule should be applied for citation. If somebody has already published an article on a similar subject, the old-fashioned way of citing his work should still be applied."

B: "I do not think that authors are deliberately not citing relevant work of others. It may not be easy to

follow all newly emerging articles and it is probably much more difficult or even impossible to attend all conferences dealing with combinatorial chemistry to keep updated."

A: "You definitively do not need to attend all conferences, since the majority of speakers are the same and they cannot present new results each time. It seems to me that the organizers of conferences about diversity or combinatorial chemistry realized that in this hot field you cannot afford to miss such a conference (what if...) and they are just profiting from it."

B: "Do you think that we will ever be invited again to speak at these conferences if we would agree on this last point?"

A: "Why not? Remember, we are only fictional."

Conclusions

There may be different views on almost every aspect of combinatorial library techniques. Nevertheless, we will never argue the usefulness of such techniques. To demonstrate this fact, we compiled our thoughts and views in a written dialog to share our mindset with other enthusiastic 'librarians'.

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