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Invited review

Combinatorial chemistry: a rational approach to chemical diversity

X Williard, I Pop, L Bourel, D Horvath, R Baudelle, P Melnyk, B Déprez, A Tartar*

Chimie des biomolécules, URA CNRS 1309, Faculté de pharmacie et Institut Pasteur de Lille, 1, rue du Professeur-Calmette, 59019 Lille Cedex, France

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Summary — Although knowledge-based de novo design of high affinity ligands for receptors or enzymes appears to be the most rational approach to the discovery of new pharmacologically active substances, high throughput random screening of compounds with no known structural similarity to the natural ligand has proved to be a highly efficient method. In order to increase the number of new molecules, methods of combinatorial synthesis have been considerably developed in recent years. In this review we will examine some of the main strategies for generating large arrays of diverse molecular entities, the screening of such libraries, the methods used to identify an active compound, and the chemical technologies used in the development of this very promising source of new drugs.

combinatorial synthesis / diversity / library / medicinal chemistry

Introduction

For many years the discovery of biologically active compounds likely to become new drugs was essentially based on serendipity. Although the structure-based design of ligands for macromolecular targets of known 3D structure appeared to be promising, relatively few successes can be attributed to this approach. Meanwhile, pharmaceutical companies have never given up their efforts in the systematic random screening of compounds from various sources for drug discovery. The large number of compounds undergoing such a screening procedure counterbalances the low probability of finding an active compound. Both the capacity and the diversity of the screening procedures have been gradually improved in recent years. Due to the development of molecular biology, an ever-increasing number of promising targets for the development of new therapies have been identified and included in the screening programs. Simultaneously, the robotization and miniaturization of the tests have gradually increased the number of compounds likely to be screened daily from tens to thousands.

Due to the implementation of these high throughput screening procedures, the availability of a large number of compounds to feed to the screening robots has eventually become a major bottleneck. Initially, these compounds came from natural sources such as plants, animals or fermentation broth or from libraries of chemical compounds accumulated over the years by the chemists working in the companies. Although these remain major sources of new leads, they present some severe drawbacks. Natural compounds, which still represent an unmatched source of molecular diversity, require sophisticated purification and structural elucidation and may eventually lead to molecules of excessive structural complexity and limited availability. On the other hand, chemical libraries reflect more the history of each pharmaceutical company than a real chemical diversity. The recent discovery of a series of lead compounds related to benzodiazepines interacting with a wide variety of biological targets could be explained, at least in part, by their over-representation in existing chemical libraries. All these factors prompted chemists to design rational strategies for one cheap and easy synthesis of a large number of structurally diverse compounds. The common features of these strategies are that they involve combinatorial synthesis [1, 2] and that the most time-consuming steps of organic synthesis, purification and structural characterization are only undertaken once a promising activity has been detected. The great interest in these methods is illustrated in figure 1 by the sharp increase of the number of papers devoted to chemical diversity between 1990 and 1995.

*Correspondence and reprints

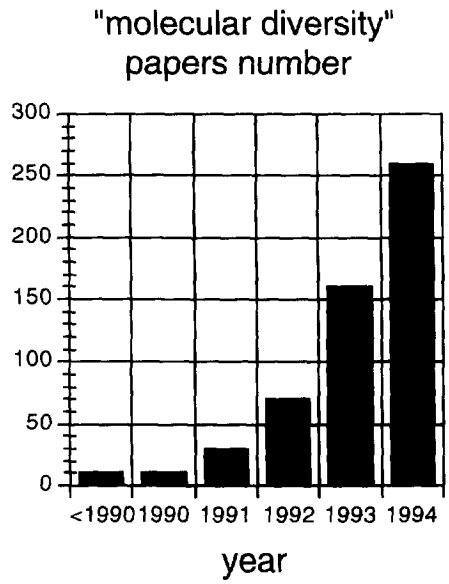

Fig 1. Evolution of the number of publications devoted to molecular diversity.

Principle of combinatorial chemistry

The general principle of combinatorial chemistry is illustrated in figure 2. Classically, chemists perform reactions using one molecular species of each reagent (A and B) and expect to obtain if not a single, at least a major, product (A–B). In the case of combinatorial chemistry, instead of a single molecular species, groups of building blocks are reacted together. Using simultaneously a group of n building blocks (A_1 to A_n) with another group of n' building blocks (B_1 to $B_{n'}$) leads to a mixture of all combinations (A_1B_1 to $A_nB_{n'}$).

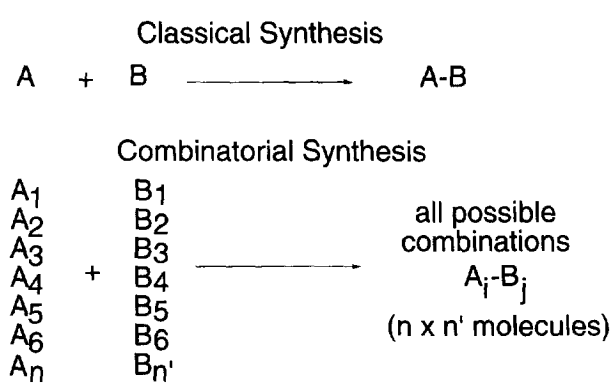

Fig 2. General concept of combinatorial chemistry.

Thus starting from $n + n'$ building blocks leads to $n \times n'$ compounds. The repetition of several steps of combinatorial synthesis quickly leads to a considerable number of compounds (fig 3). Although such figures may appear very promising, they must be considered with great care (it is difficult to detect the presence of an active compound in the most complex mixtures).

Once a biological activity has been detected, the next step consists of the structural identification of the compound responsible for this activity in the mixture. More so than in classical organic synthesis, the success of combinatorial chemistry depends on the robustness of the chemical reactions involved. These reactions must be generally suitable for a large variety of reagents leading to the expected compounds in high yield. Otherwise intractable mixtures, that will hinder any structural elucidation, would be obtained. In this context, the methods involving solid-phase synthesis pioneered by Merrifield [3] provide a major advantage for combinatorial chemistry. According to the general principle illustrated in figure 4, one of the building blocks A (or family of building blocks A_1 to A_n) is covalently linked to an insoluble support. The second building block, B (or family of building blocks B_1 to $B_{n'}$) is then added in a soluble form. The reaction of A with B leads to a compound A–B covalently linked to the support, allowing excess B, any catalyst and solvent to be easily separated by simple filtration at the end of the reaction. This cycle can be easily repeated and automated. A clear advantage of solid-phase synthesis is that, due to their facile elimination, large excesses of reagents can be used to force reactions to go to completion, reducing the complexity of final mixtures. Another advantage is that all the synthesized compounds remain linked to the polymer until the end of the synthesis, avoiding losses during extraction or precipitation procedures (which are typically used in classical solution synthesis).

	Combinatorial positions	Number of compounds formed
Basis Set of 10	2	10^2
	3	10^3
	4	10^4
Basis Set of 100	2	10^4
	3	10^6
	4	10^8

Fig 3. Number of compounds susceptible to be generated using different combinatorial strategies.

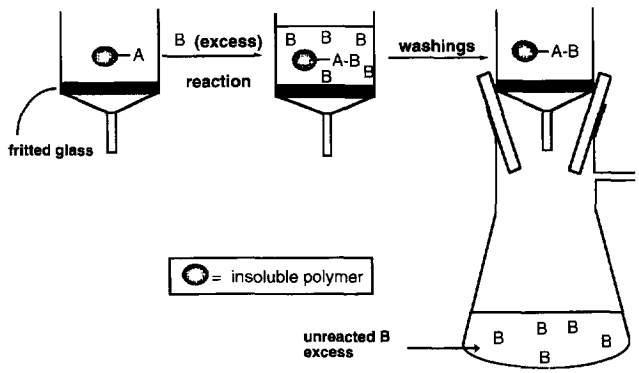

Fig 4. General principle of solid-phase synthesis.

Chemistry

The major elements that validate a combinatorial approach are essentially the structural diversity and the uniqueness of the compounds generated, and not just their number. Different approaches have been followed to generate structural diversity [4] (fig 5). Dimerization relies on a single reaction suitable for accommodation of a wide variety of building blocks. Oligomerization, template strategy and heterocyclic chemistry involve more sophisticated successions of steps.

Dimerization

Dimerization represents the simplest form of combinatorial synthesis. In the case of monofunctional building blocks, soluble-phase synthesis can be used, if the coupling reagents do not interfere with the biological screenings. A recent example is a library of 1600 dimers partitioned into two series of 40 sublibraries (fig 6). In the first series, 40 different acyl chlorides were reacted individually with the same mixture of 40 nucleophiles (alcohols or amines),

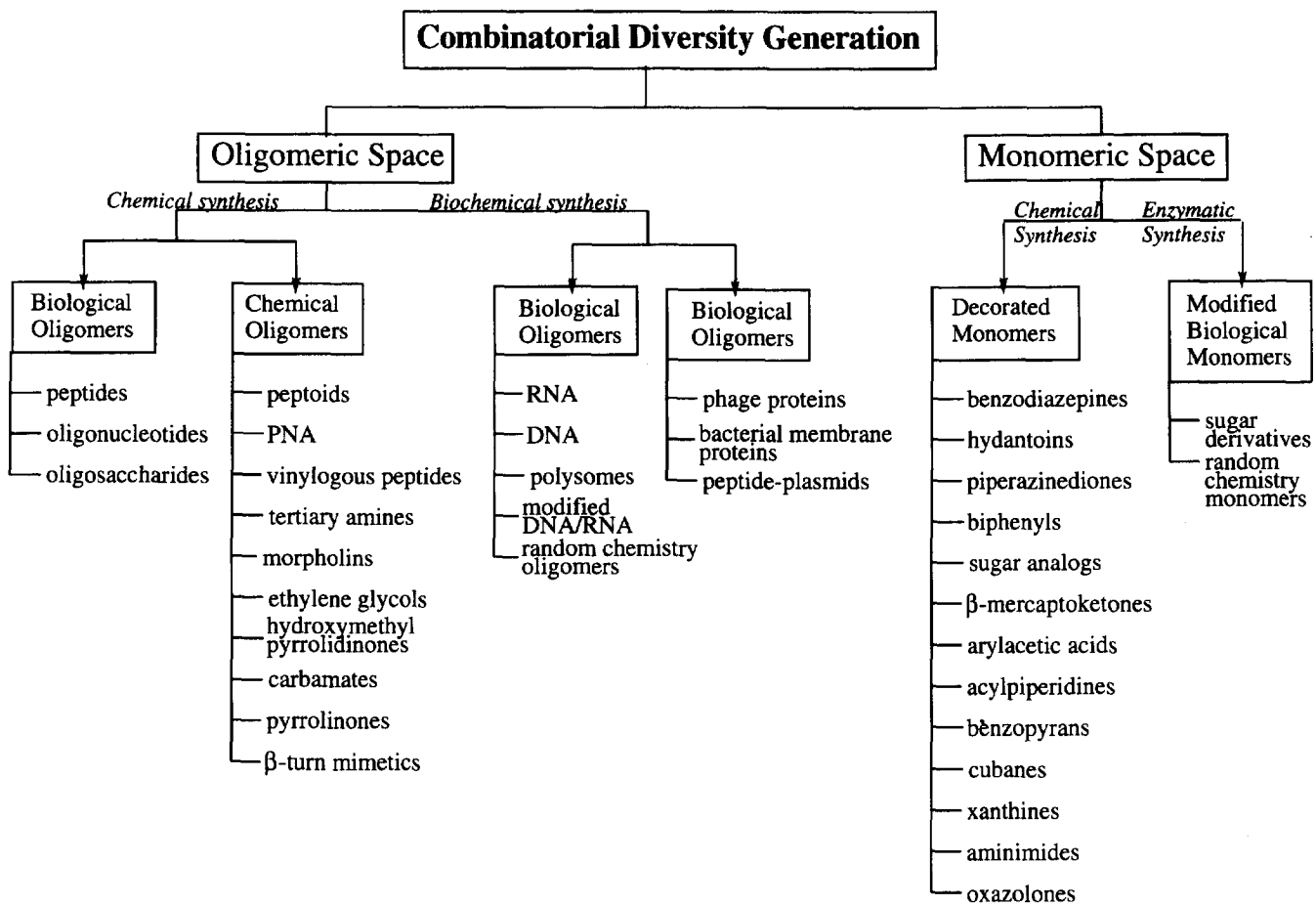

Fig 5. Combinatorial diversity generation (reproduced with permission from reference [4]).

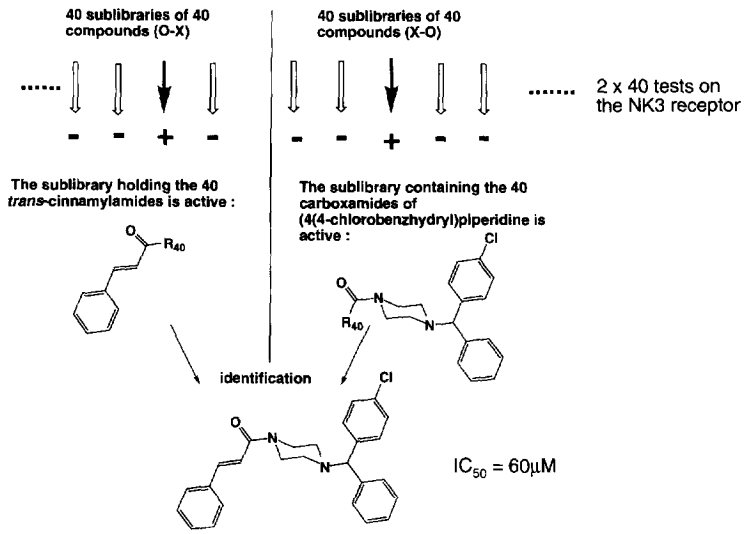

Fig 6. Library of dimers.

leading to 40 sublibraries of 40 dimers each. In the second series, the sames 40 nucleophiles were reacted individually with the mixture of the 40 acyl chlorides also leading to 40 sublibraries of 40 dimers each. The 80 mixtures were tested and allowed the identification of a weak NK3 inhibitor (IC_{50} = 60 µM) [5].

Oligomerization

Unlike dimerization, this strategy requires a series of at least bifunctional building blocks. As soon as the number of combinatorial steps increases, the use of solid-phase methods becomes inevitable. Early achievements in combinatorial oligomerization involved peptides, taking advantage of the robust and well-documented peptide synthesis methods. Furthermore, the use of commercially available unnatural amino acids gave access to a relatively large structural diversity. However, due to their poor stability in biological fluids and to limited bioavailability, peptides generally do not represent very promising lead compounds. This led to new methods of oligomerization involving the formation of chemical bonds, which, in contrast to the peptide bond, displayed significant stability towards proteolysis. Typical examples of this approach are illustrated in figure 7. Peptoids [6] consist of a repetition of *N*-substituted glycine residues. Their synthesis was described by Zuckerman et al [6], who took advantage of the huge variety of commercially available primary amines. Their almost complete stability in physiological liquids has recently been confirmed. Other chemical polymers like oligocarbamates [7] or polypyrrolinones [8] have also been described.

Template strategies

In these strategies, a basic polyfunctional scaffold is modified sequentially by a variety of building blocks introduced in a combinatorial manner [9]. The role of this scaffold is both to define the general geometry of the molecular space to be explored and, by the nature of the chemical groups attached, to recruit pertinent families of building blocks (fig 8).

Heterocyclic synthesis

Considering the large number of existing heterocyclic drugs, significant effort has been devoted to extending

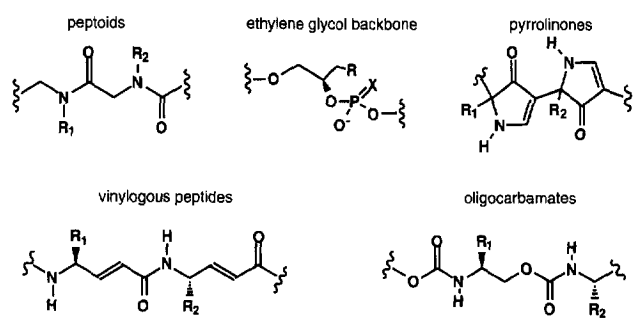

Fig 7. Chemical oligomers.

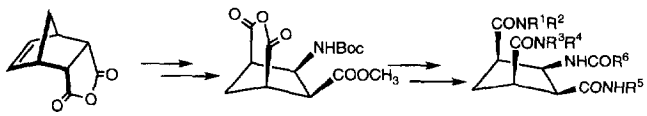

Fig 8. Decoration of a norbornene template.

the scope of combinatorial synthesis to such structures, particularly using polymer-supported strategies. The earliest version produced relatively small arrays (about 40) of discrete heterocycles resulting from the parallel assembly of two or three components [10]. Several expedient routes to the 1,4-benzodiazepines (Diversomer™) were described, a similar approach has been devised for the synthesis of hydantoins (fig 9). As the technology has improved, the number of pharmacologically interesting synthesized heterocycles has increased together with the size of the libraries. More sophisticated solid-phase syntheses are regularly published, using more and more elaborate chemical reactions, including the Mitsunobu [11] reaction, the Wittig–Horner reaction [12], enolate formation [13] and Suzuki cross-coupling [14].

Structural elucidation of active compounds

Combinatorial synthesis yields a wide array of different chemical structures which can be directly screened for various biological activities. In some cases, this screening is performed on compounds which are still linked to the support used for their synthesis. In other cases a soluble form is tested after cleavage of the link to their support. Once an activity has been detected in a mixture, the next step consists of identifying the structure of the compound responsible for the activity.

Depending on whether they rely on partition of the library into sublibraries followed by evaluation of various formats of the sublibraries, or analytical methods, strategies for the identification of active compounds from combinatorial mixtures can be divided into two major groups.

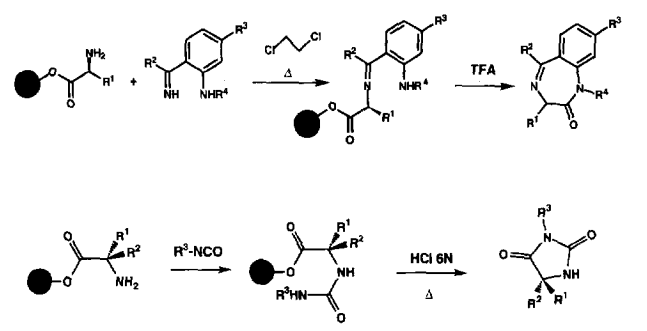

Fig 9. Synthetic schemes of benzodiazepine and hydantoine libraries.

Strategies involving sublibraries

These strategies are used when the mixtures of compounds are released from the support and screened in a soluble form. Their purpose is to take advantage of the possibility of controlling the format under which the library is synthesized so that structural elucidation can be achieved without having to rely on purification and analytical characterization. Three major methods have been reported. While in the first (deconvolution) the sublibraries are only synthesized once an activity has been detected within a mixture, in the other two cases (positional scanning and orthogonal partition) sublibraries are devised and prepared to allow a direct identification.

Deconvolution procedures [15]

This strategy was initially proposed by Houghten [16, 17] and is a reliable but time-consuming method. It involves an iterative procedure in which libraries containing a decreasing number of compounds are prepared successively until a unique structure is identified. A simplified example of this strategy is given in figure 10. In this example, the initial library is produced by a three-step combinatorial synthesis involving a mixture of two building blocks at each step. Thus, it consists of a mixture of eight trimers ($A_1B_1C_1$, ..., $A_2B_2C_2$) resulting from the coupling of the two-component mixtures [A_1, A_2], [B_1, B_2] and [C_1, C_2]. When an activity is detected of a mixture, the identification of the active compound is performed according to the following three-step procedure. In order to iden-

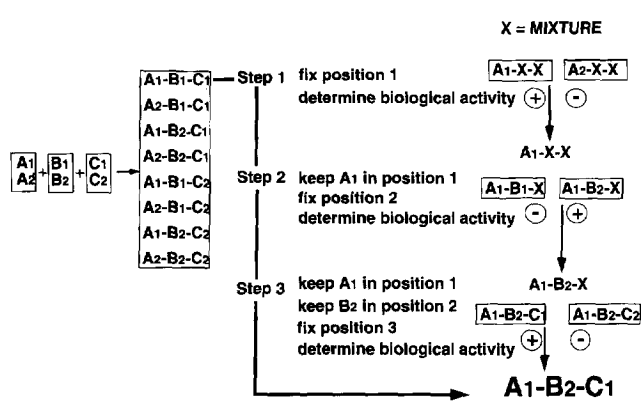

Fig 10. Identification of an active compound by deconvolution.

tify which of the two building blocks A_1 or A_2 is required for the activity in the first position, two sublibraries A_1-X-X and A_2-X-X are synthesized, in which a unique building block is used in the first position while the remaining two positions still consist of mixtures (X). Biological evaluation of these two sublibraries can be used to determine which of A_1 or A_2 is responsible for the activity initially detected in the mixture. Starting from this information, the first position is fixed and the same operation is performed for the second position, involving the synthesis of two other sublibraries. If A_1 has been identified as the active building block, the two next sublibraries will be A_1-B_1-X and A_1-B_2-X, allowing the identification of B_1 or B_2 as the building block required for the activity. Repeating this operation for the third position eventually allows the identification of a unique active structure. The efficiency of this method (the ratio of compounds tested/synthetic steps involved in the deconvolution) sharply increases when a large number of building blocks and combinatorial steps are involved. In the previous example, the procedure requires 3 x 2 deconvolution steps for a library of only eight trimers. In contrast, for a library of 160 000 tetrapeptides (20^4), only 4 x 20 deconvolutions will be required.

This strategy was initially used with peptides, allowing the identification of several active compounds, including potent antimicrobial peptides [16] and opioid receptor antagonists [17, 18]. More recently, it was used for the identification of highly potent ligands for several trans-membrane receptors from combinatorial libraries of peptoids (*N*-substituted polyglycines) [14].

Positional scanning

This involves the partition of the library into sublibraries from the beginning of the synthesis. A major difference from the above approach is that the number of compounds per sublibrary and the concentration of the active species remain constant throughout the procedure. Positional scanning of the library described above involves the preparation of six sublibraries in which a single building block is present in one of the three positions, while mixtures (X) are used in the remaining two positions. The six libraries A_1-X-X, A_2-X-X, X-B_1-X, X-B_2-X, X-X-C_1 and X-X-C_2 are tested simultaneously to determine which individual building block confers the highest activity in each position. The most active compound is then defined as resulting from the combination of each of these most potent building blocks (fig 11). This method has allowed the discovery of potent opioid receptors antagonists from a library of acetylated hexapeptides derived from D-amino acids [19, 20] and a potent antimicrobial peptide from a permethylated peptide library [21].

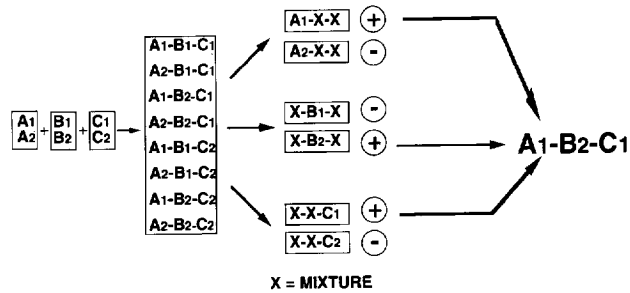

Fig 11. Identification of an active compound by 'positional scanning'.

Orthogonal libraries

This strategy was described by Déprez et al [22] and also allows a straightforward identification of active compounds without requiring any additional synthetic steps. A simplified example is shown in figure 12, for a library containing nine compounds. Using an appropriate solid-phase method, two libraries (A and B) containing the same nine compounds are prepared. Both libraries are partitioned into three sublibraries (A_1, A_2, A_3, B_1, B_2, B_3). An important property of these sublibraries is that when three compounds are present together in one sublibrary of library A (for instance, **4**, **5** and **6** in sublibrary A_2), they will be partitioned in the three different sublibraries of B (**4** in B_1, **5** in B_2, and **6** in B_3). The partition is designed so as to maximize structural diversity within each sublibrary so that a single species of a sublibrary should be responsible for the biological activity. Thus, the screening of the two libraries allows easy identifi-

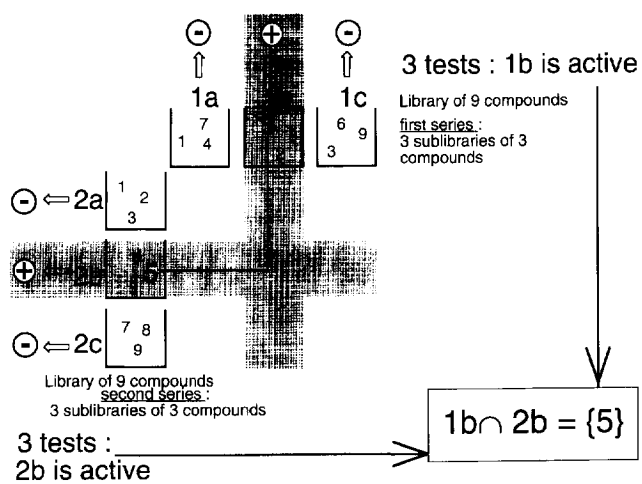

Fig 12. General concept of orthogonal partition.

cation of active compounds. In the example in figure 12, an activity detected in A_2 and B_2 leads to the identification of compound **5** as the active compound.

This strategy was applied with a library containing 15 625 compounds partitioned into 125 sublibraries and allowed the identification of a low nanomolar V2 vasopressin antagonist (IC_{50} = 60 nM) [20].

Direct or indirect analytical methods: the 'one bead–one compound' strategies

These methods are well suited to the structural identification of compounds still linked to their polymeric support.

One bead–one compound'

This concept was initially reported by Furka et al [23] as an unexpected consequence of the split methodology in solid-phase synthesis. As indicated below, the use of large excesses of soluble reagent is a general strategy used to accelerate reactions and force them to go to completion. However, when the soluble reagent consists of a mixture of several building blocks, each with a different reaction rate, there is a major risk that the most reactive building blocks will be over-represented in the final polymer-bound mixture. A simple method to avoid this, known as the split synthesis, consists of dividing the resin before each combinatorial step and reacting each building block individually in a different vessel (fig 13). After the coupling (which can thus be performed with large excesses of reagents), the different samples of resin are pooled and mixed before being submitted to the next combinatorial step. The repetition of the splitting/pooling operations has an important consequence on the microscopic compositions of the resulting polymers. Polymers used for solid-phase synthesis generally consist iof small beads of 50–100 microns in diameter. During a splitting step, each individual bead will be directed to one, and only one, of the different reacting vessels and will react with only one of the building blocks. Consequently, at the end of a synthesis, each bead will carry a unique chemical entity (care must be taken to avoid that the number of expected chemical entities will not exceed the number of beads which typically falls in the range of 10^7 to 10^8 per gram of resin). This observation was initially referred to as the 'one bead, one peptide' [24] and later as the 'one bead, one compound' strategy. It has led to a series of interesting developments due to the fact that the quantity of compound present at the surface of an individual bead, in the range of several nanomoles, is sufficient to allow both biological screening and structural identification.

Initial reports of this method, illustrated in figure 14, involved combinatorial peptides screened for their

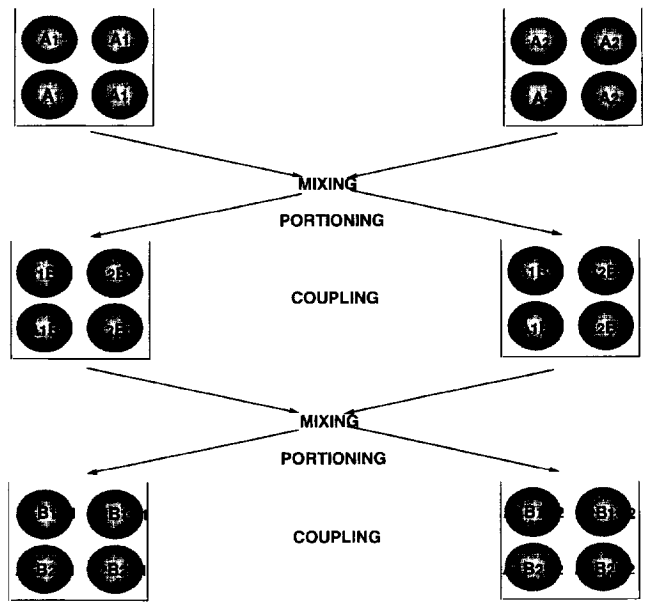

Fig 13. General principle of 'split synthesis'.

capacities to bind monoclonal antibodies. Beads displayed as monolayers were incubated with the labelled antibody allowing the localization of positive beads. These were isolated, washed and underwent recurrent Edman degradation, which allows a direct identification of the active peptide.

Several improvements of this method have been reported in recent years. Some of them offered the possibility of releasing a part of the peptide and screening it in solution. The remaining part was used for the structural identification. Other improvements helped increase the structural diversity accessible by this method.

Molecular tags

One of the major drawbacks of the initial methods was that their applicability was limited to polymers which could be analyzed by Edman degradation. In order to increase the diversity, methods of molecular tagging were developed. Their general principle relies on the separation between the binding function (interaction with biological targets) and the tagging function (structural identification). While the binding function requires the largest structural diversity, the tagging function should rely on a simple and robust method. Although this concept of associating binding and tagging structures has known its widest developments in the case of strategies involving polymer-linked compounds, it was initially proposed for

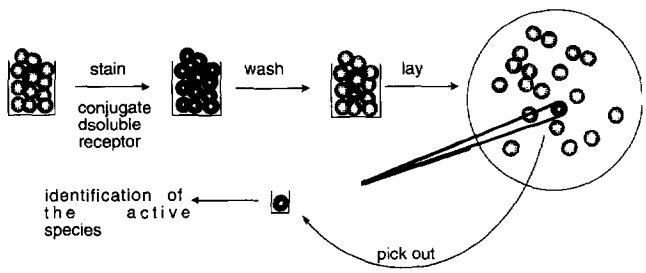

Fig 14. Screening of polymer-linked compounds.

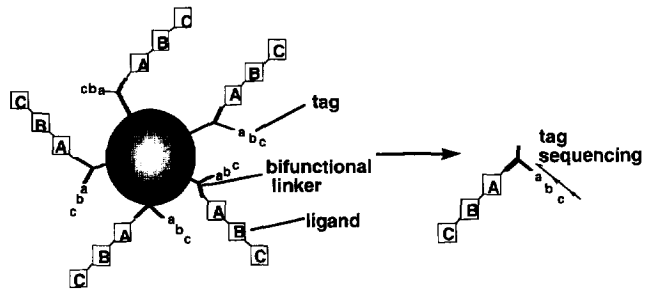

Fig 15. Stoichiometric and sequential tagging.

soluble compounds. In this case, instead of taking advantage of the size of a polymer bead to provide the quantity of compound required to match the sensitivity of analytical methods, the power of genetic amplification was used [25]. During the synthesis, each building block in the binding sequence was correlated with an oligonucleotide in the tagging sequence. In this case, molecules in which the binding and tagging sequences were covalently associated were cleaved from the support. Immobilized macromolecular targets were used to select the active species, whose oligonucleotide tags were then amplified by the polymerase chain reaction until they reached a level at which DNA sequencing could be used.

The main drawback is that the chemical methods used for their synthesis and the intrinsic low chemical stability of oligonucleotide drastically limit the scope of chemical reactions which can be used to assemble the binding sequence.

In contrast, as the quantities of compounds can be easily adjusted by controlling the loading of the beads, the polymer-supported strategy does not require amplification and allows easy access to a wide variety of tagging procedures. The 'one bead, one compound' strategy must be slightly modified to suit this method. During each splitting of the resin into separate vessels, two compounds instead of one are assembled at the surface of the beads: one provides the binding sequence while the other, the tagging sequence, codes for the structure of the binding sequence and allows its identification. In its initial form, molecular tagging still relied on peptide sequencing; each building block of the binding sequence was associated with a different amino acid in the tagging sequence (fig 15). Thus, once a bead is identified by its ability to bind a biological target, the nature of the binding compound is easily determined by sequencing the tagging peptide. The second generation of molecular tags relies on a much faster and sensitive non-sequential molecular tagging strategy. In this case, the complete history of the synthesis is recorded on each bead using a chemical binary code (fig 16). Using the presence or absence of N different chemical labels means that 2^N events can be recorded (the nature of the building blocks and the position of incorporation must be described). This tagging method is comparable to classical bar code identification [26]. Once a positive bead has been isolated, all the chemical tags present at its surface are released simultaneously by cleavage (generally photolytic) of their link to the polymer and analyzed. This analysis is usually performed by gas phase chromatography coupled to capillary electrophoresis which allows the tagging sequence to represent less than 1% of the compounds present at the surface of the polymer.

Spatially addressable libraries

Combinatorial libraries can also be generated by extensive parallel processing of discrete compounds. The identity of any compound in such a library will be given by its location on a synthesis support (which can, in the simpliest case, be a well in a microtiter plate). A basic requirement for the parallel synthesis of large number of individual compounds is miniaturization. With this aim, two major techniques were

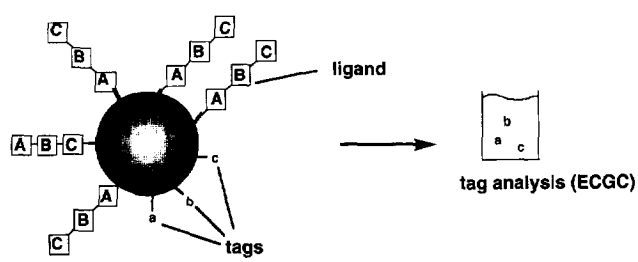

Fig 16. Non-stoichiometric and non-sequential tagging on polystyrene beads with electrophoric compounds.

introduced: the 'multipin' approach and light-directed parallel synthesis.

Combinatorial libraries by the multipin method

The solid support for the multipin method, introduced by Geysen and coworkers in 1984 [27], consists of amino-functionalized polyacrylic-acid-grafted polyethylene rods, called 'pins'. The synthesis is usually performed under a format of 96 pins fixed into a 8 x 12 format plastic holder (fig 17) designed to fit exactly into the wells of a microtiter plate containing the reagent solutions. As a unique compound is synthesized on each individual pin, the structure of any component of the pin-supported library is easily determined by its spatial location. Polyethylene pins are mostly employed in the synthesis of peptides. The syntheses are performed by repeatedly reacting the pins with activated amino acid solutions distributed in fitted microtiter plates. Manual distribution of the amino acid solutions in the microtiter plate wells, the most time-consuming step of a synthetic cycle, has been partially automated [28] by using a pipetting robot. The multipin combinatorial libraries are subsequently used in bioassays in either tethered or soluble forms. Therefore, to obtain compounds in a soluble form, different labile anchors are inserted between the first building block and the rods [29, 30]. In this case, the treatment of the arrays of pins carrying the synthesized molecules with a suitable cleavage reagent generates a soluble library, which can be used directly in any given bioassay. Recently, the multipin method was reported to have been successfully applied to non-peptidic structures, such as benzodiazepines [31], β-turn mimetics [32] and 4-aminoproline analogues [33].

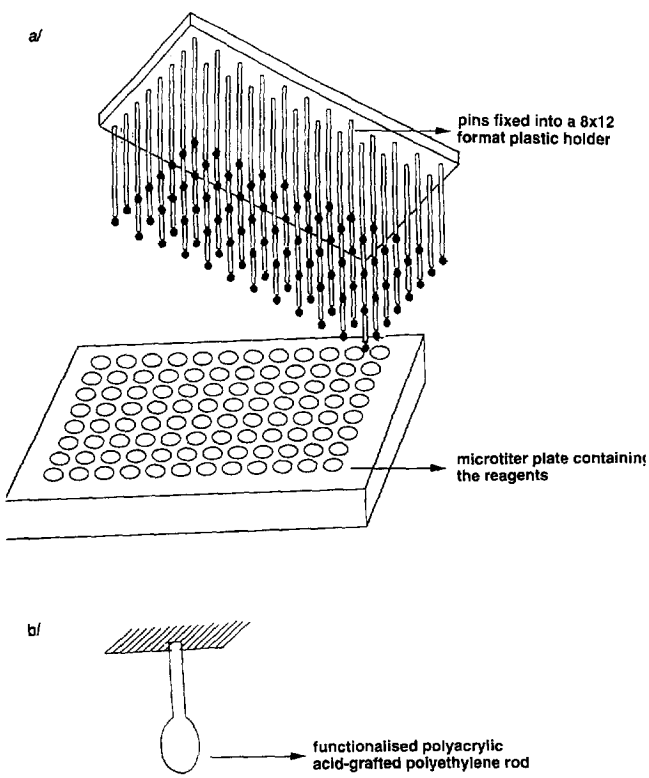

Fig 17. Schematic representation of a) a block for the pin synthesis and b) a pin.

Combinatorial libraries by light-directed parallel synthesis

Light-directed spatially addressable parallel chemical synthesis takes advantage of the spatial resolution of photolithography and the possibility of its miniaturization. The merging of these two technologies is based on the use of photolabile amino-protecting groups in the Merrifield synthetic procedure [34]. The support used for light-directed synthesis is an amino-functionalized borosilicate glass (microscope slides). Photolabile amino-protecting groups are first covalently anchored to a functionalized slide surface (fig 18). Irradiating the slide surface through a suitable mask allows selective deprotection of the light-exposed parts of the slide while the masked part remains protected. The first set of building blocks bearing photolabile protecting groups is then exposed to the entire surface. Coupling will only occur in the regions that were exposed to light in the preceding step. Irradiation of the surface through a second mask allows the deprotection of a different region for coupling with another protected building block. The pattern of masks and the sequence of building blocks define the structure and precise location of each member of the library. The number of different compounds which can be synthesized is limited only by the resolution achieved in addressing two contiguous sites; 2^n synthesis sites are created for n synthetic steps employing a so-called binary masking strategy. This strategy always delivers a building block to one half of each existing synthesis region and therefore doubles the number of compounds in each subsequent step (fig 19).

Both natural (peptides and oligonucleotides) and unnatural (carbamates) biopolymer libraries were generated using light-directed combinatorial synthesis [7, 35, 36]. Bioassays have been performed by treatment of the oligomer arrays with a solution of labelling reagents. Identification of the active compounds is effected with a scanning epifluorescence microscope set to the excitation and emission wavelengths

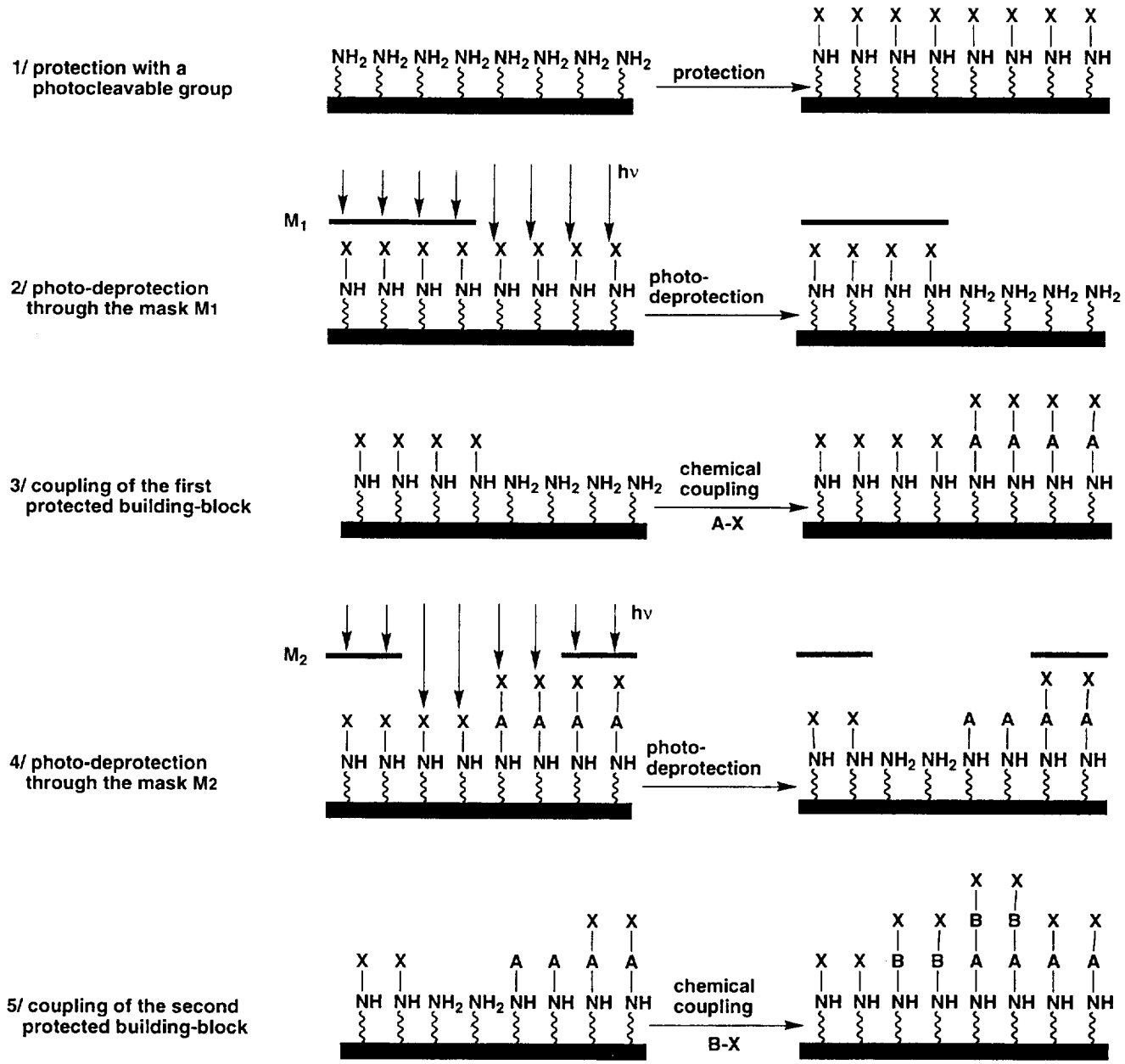

Fig 18. Concept of light-directed spatially addressable parallel chemical synthesis.

of fluorescein. The data collection and image processing require approximately 15 min.

Conclusion

Within a few years, the scope of combinatorial synthesis has extended from peptides to a wide variety of different chemical structures. Considering the broad structural diversity accessible and the rapidity of screening, it is likely that the discovery of a lead compound will progressively cease to be a bottleneck in the multistep process of drug design. In addition to giving rise to new chemical strategies, which will add new classes of chemical compounds to those available by combinatorial methods, the task of medicinal

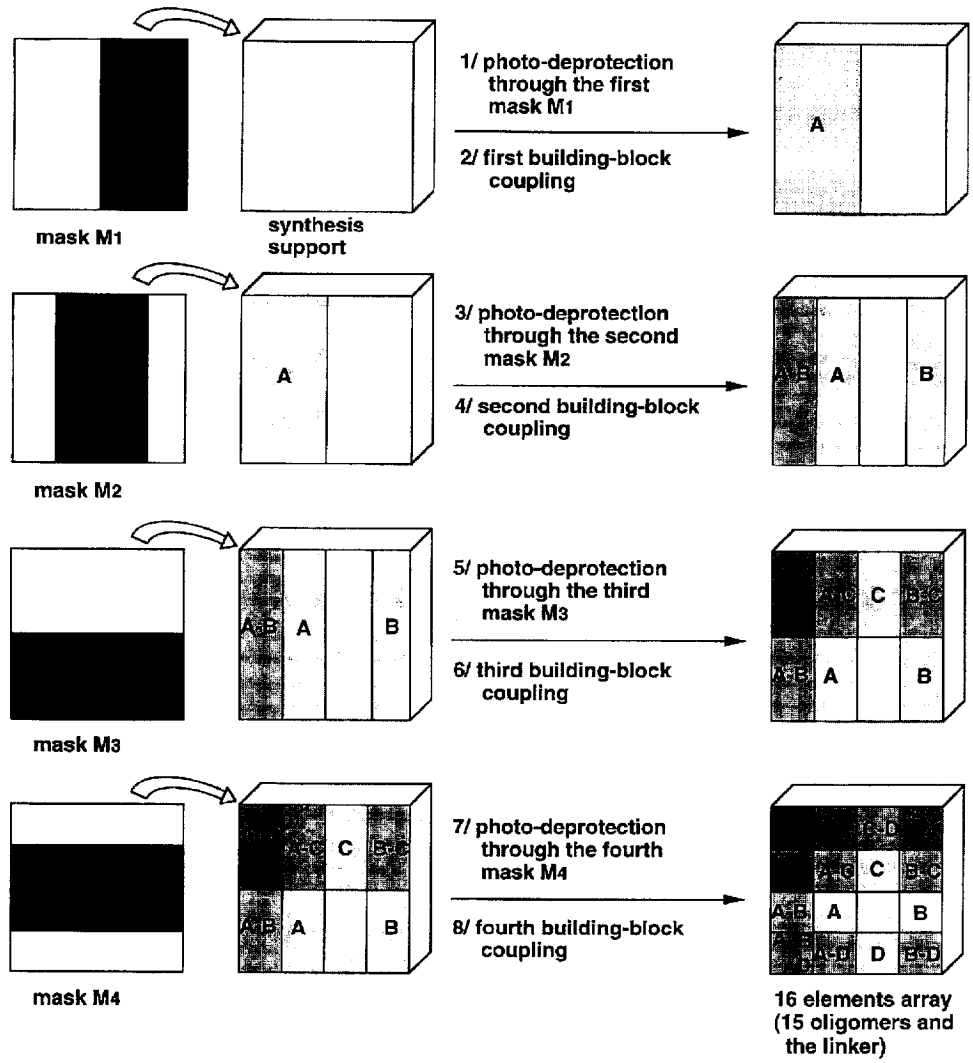

Fig 19. Illustration of a four-step synthesis with generic monomers A, B, C and D, using the binary strategy in a light-directed approach.

chemists will be increasingly devoted to the fine tuning of the lead compounds discovered to rationally optimize their initial pharmacological profile.

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