Dynamic Combinatorial Chemistry

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Abstract: Dynamic combinatorial chemistry is based on the reversible combination of initial building blocks to form dynamic combinatorial libraries. It has recently emerged as an efficient strategy to detect and to evaluate affinity between the library products and a target molecule. In this review, examples from various fields of chemistry and biochemistry are presented and extensively discussed. The last section deals with the practical aspects for implementing this approach.

1. INTRODUCTION

High throughput techniques and especially the use of robots allow the pharmaceutical industry to screen an ever-increasing number of compounds for drug activity. At first, compounds for testing may be selected from the large, readily available collections of products accumulated over years of synthetic effort in industrial or academic research laboratories. A second step consists of synthesizing new libraries, either as individual isolated compounds, or as mixtures.

A prerequisite for a substance to be pharmaceutically active is to have some *affinity* for a specific biological target which, most often, is a protein. Affinity may not always lead to activity, but it is legitimately the prime property sought. Affinity arises when, among the various shapes and distributions of polarities accessible to the potential drug, some are complementary to the biological target in the sense of molecular recognition. Consequently, the perspective of finding a hit from a given pool depends on the relevance of that pool with respect to the target: does the "interactional space" covered by the substances of the pool comprise the interactional features of the target?

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In this context, combinatorial synthesis has developed as being the simplest and most efficient way to synthesize libraries of compounds [1]. The method is economical from the synthetic perspective: instead of increasing the number of steps, it relies on increasing the number and variety of building blocks, the combination of which quickly leads to *large* pools of compounds. The method also provides a handle on the *relevance* of the pools for covering a particular "interactional space". The interactions in which the library products can engage reflect the combination of the interactions accessible to the building blocks. Using structurally very diverse building blocks will lead to the coverage of a vast space of interactions, whereas selecting structurally analogous building blocks will focus the library around a specific point of interest.

This review presents a new area of combinatorial chemistry termed *dynamic combinatorial chemistry* which makes use of *reversible* connections between the initial building blocks. As will be shown, this approach stems from the field of supramolecular chemistry. It is specially suited for screening affinity and may thus be of great interest to drug research. The basic concept, outlined in Fig. (**1**), has been presented in detail in the literature [2-6], and will simply be summarized here. It rests on the reversible bonding of the initial building blocks, and the consequent equilibria which establish between the formed library components. Reversible bonding can be covalent, leading to libraries of molecules as, for

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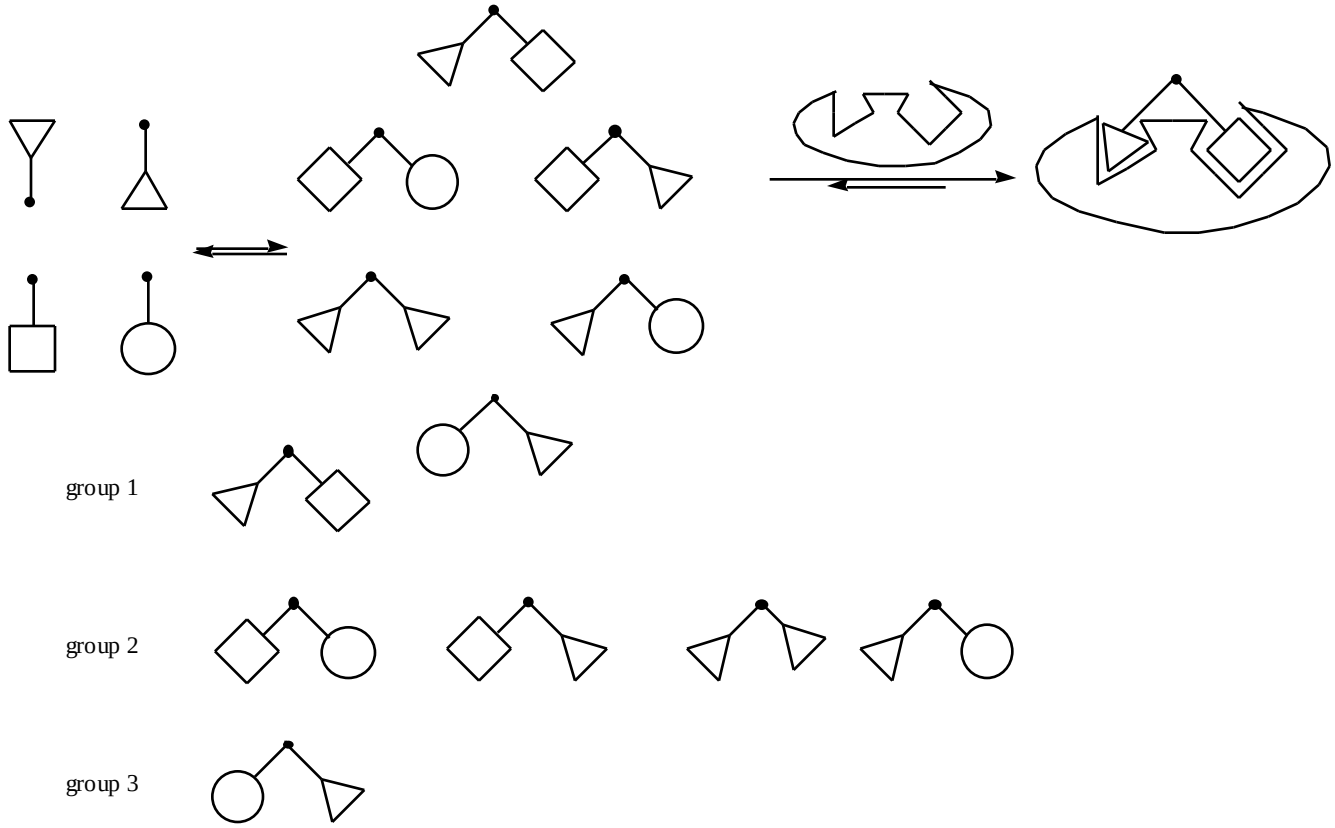


Fig. (1). Target recognition-directed selection and casting of a ligand from a dynamic library based on reversible connections between a set of building blocks. Three groups of products may identified. Group 1 products strongly bind to the target (both subunits bind), group 2 products bind weakly (one out of two subunits bind), and group 3 products do not bind (neither subunit binds).

example, imines, borates or disulfides. It can be of non-covalent nature leading to libraries of supermolecules built from hydrogen bonds, ionic bonds, or coordination to transition metals.

Reversibility allows a continuous variation of the proportions between the library products in response to a particular assay. The presence of a given target substance for example, may result in an increase of the mole fraction of the products which bind to it. The target substance -in most biological assays, this would be a protein- recognizes the library components for which it has the highest affinity. These associations lead to the selective stabilization of those strongly bound products and consequently favor their formation. Thus, the proportions between library products are shifted towards the formation of the desired compounds. Such *dynamic* amplification contrasts with the static character of "classical" libraries based on irreversible connections between the building blocks. In the latter, each product represents a small fraction of the library, whereas in the former, a product with desired properties may become a major component, and thus be easily detected.

Screening a dynamic mixture amounts to the molecular recognition induced molding/casting of the 'fittest' library component(s) by the target. It consists of using the target as a template to build its best complement(s) from the available building blocks [7]. In this respect, it may be compared to polymer imprinting techniques [8] with the difference that the template usually operates under kinetic control in the latter case, and under thermodynamic control in a dynamic combinatorial system. In both cases however, binding is the driving force for shifting the proportions between possible products implying that affinity (not necessarily activity) will be detected.

As shown in the following, examples of dynamic processes have been studied in very

diverse areas of chemistry and biochemistry. Section 2 gives an overview of the forces which govern the expression of diversity in a dynamic combinatorial mixture. Archetypical reversible processes such as conformational equilibria and non-covalent bonding are presented in sections 3 and 4. Reversible covalent reactions have often been discarded by chemists as being the source of product instability. Examples of their application to the dynamic generation of macrocycles are presented in section 5. In section 6, examples of dynamic combinatorial chemistry involving biomolecules are treated extensively. Last but not least, a number of practical aspects of the preparation and screening of dynamic mixtures are discussed in section 7.

2. POTENTIAL DIVERSITY IN REVERSIBLE COMBINATORIAL SYSTEMS

Open and Closed Systems

A set of buildings blocks that can reversibly bond each other defines an ensemble of products which may potentially be formed. The structural diversity expressed in this ensemble depends both on the number of products, and on the structural variety of the precursor functional groups. With respect to the number of possible products, two scenarios may be envisaged depending on whether the system is closed or open. In a closed system (Fig. (**2**)), a limited number of building blocks interconnect to form the library components. The number of possible products is thus predetermined by the number of building blocks involved. A set of **n** amines and **m** aldehydes for example, may reversibly form **n** × **m** imines. In an open system, the number of building blocks which may connect is unlimited. A set of **n** amino-aldehydes for instance may form $\mathbf{n^2}$ different dimers, $\mathbf{n^3}$ trimers, $\mathbf{n^4}$ tetramers, etc. Without even considering cyclic species or the possibility of cross-linking, the number of potential products is basically infinite, including in the limit case of a single building block. Of course, the number of species actually present in the mixture is practically limited by contextual factors such as the shortage of building blocks and competing reverse reactions. The diversity of an open system cannot be fully expressed, and may for this reason be termed potential diversity or virtual [3] diversity.

A nice illustration of a large number of products obtained from the open reversible combination of a small number (one !) of components is the condensation of $Si(OH)_4$ in basic aqueous medium [9-11]. As shown in Fig. (**3**), silicate ions reversibly condense into multiple linear, cyclic and polycyclic oligomers, most of which have been identified by ^{29}Si NMR. These products form a dynamic ensemble in which specific reversible pathways lead from one species to another.

Proportions of Products

In a dynamic combinatorial system, the mixed building blocks are continuously competing for reaction. The proportion of products may thus be strongly biased by the intrinsic reactivities of the building blocks. Some products may represent a major fraction, whilst others may be practically absent from the mixture. For this reason as well,

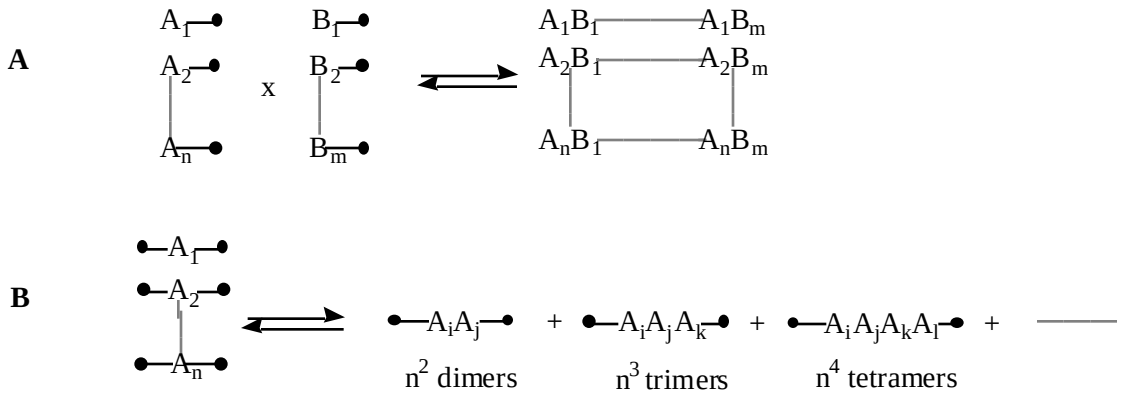


Fig. (2). Closed (A) and open (B) reversible combinatorial systems.

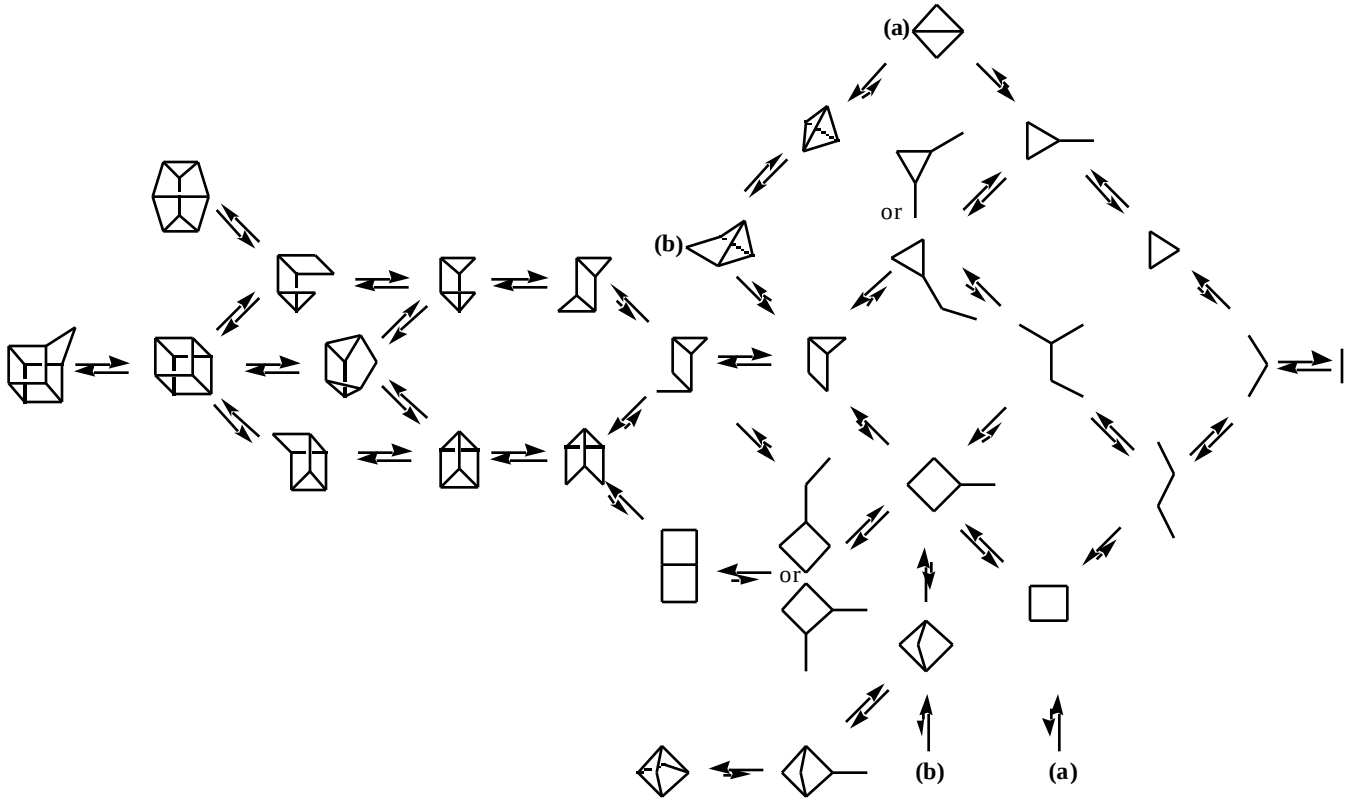
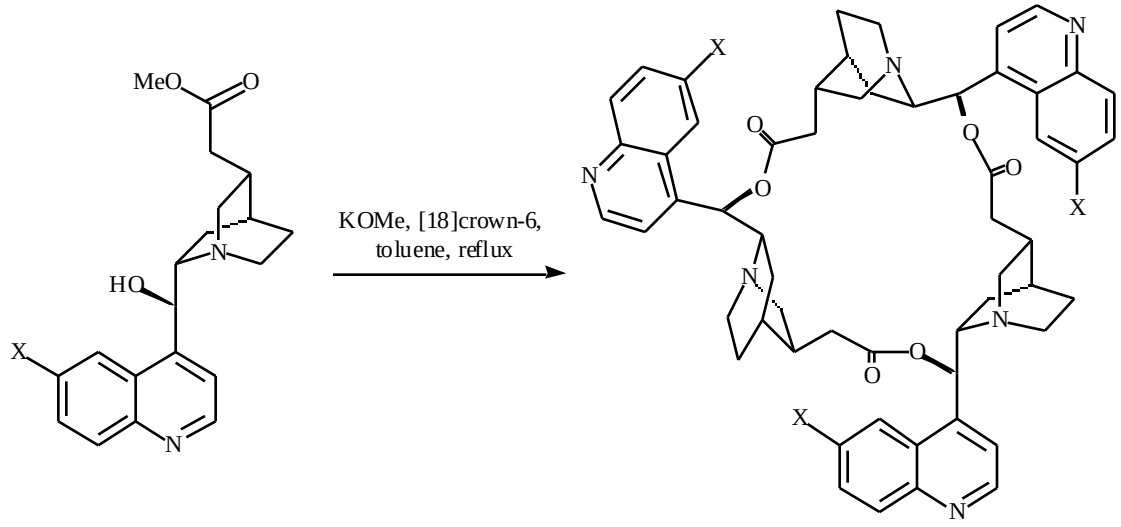


Fig. (3). Schematic representation of a dynamic mixture of silicate oligomers obtained by iterative condensation of $Si(OH)_4$ in water. Each linkage represents a Si-O-Si-bridge. many of the species shown have been characterized by ^{29}Si NMR, some species are hypothesized intermediates that have yet to be identified.

the ensemble of possible products may not be actually expressed in the mixture, and may be termed a potential diversity or virtual [3] diversity.

Besides the intrinsic reactivities of the building blocks, structural factors may play a role in the product distribution. The example shown in Fig. (**4**) illustrates how a potentially diverse system can yield almost quantitatively one product. It consists of the reversible transesterification of the cinchonidine derived hydroxy-ester **1** under thermodynamic control using KOMe in refluxing toluene. The system is open and may in principle



1a X=OMe
1b X=H

X=OMe
X=H

Fig. (4). Quantitative reversible formation of a cyclic trimer of cinchonidine derived hydroxy-ester **1**.

produce oligomeric or polymeric mixtures. However, the cyclic triester is formed quantitatively [12]. This strong bias was attributed to a predisposition of the system: not that the formation of the cyclic trimer is faster than that of the other oligomers (reactive species are all identical, and the linear trimer is flexible and not particularly preorganized for cyclization) but rather that its degradation is disfavored compared to the degradation of the other products.

Several other factors influence the proportions of products among a pool of equilibrating species: intramolecular attractive or repulsive interactions, conformational preferences, solvation terms all contribute to the energetic state of each species. These factors may increase or decrease either of the (or both) kinetic constants k_i and k_{-i} of each equilibrium. Overall these factors determine the intrinsic thermodynamic preference of the system. This sometimes leads to an even distribution of species (Fig. (**3**)) and sometimes to a complete bias towards one entity. The trimerization of **1** (Fig. (**4**)) is an unusual such example. Other well-studied processes such as protein folding or metal coordination based self-assembly (see section 4 below) also exemplify how a unique product may be expressed from a potentially diverse system.

Strongly unbalanced proportions of products are considered a major drawback in a library based on irreversible connections. This is usually avoided by the use of the "divide-couple-and-recombine" method (see refs. 13-19 in ref. 1). As will be shown in the following sections, an unbalanced distribution of products does not necessarily constitute a problem in a dynamic system, as long as the expression of minor species remains possible (e.g. that the associated energy difference is not overwhelming).

Template Effects

Thus, a mixture of reversibly reacting species under thermodynamic control generates an ensemble of potential products the proportions of which reflect the interplay of a number of parameters characteristic of the system. The point of interest is that these proportions may vary from this initial state upon interaction of some of the products with a target substance introduced in the mixture. The target substance acts as a template to stabilize the products which bind most strongly to it [7].

In the case of the dynamic equilibria of silica oligomers shown in Fig. (**3**), the initial proportion of products reflect the intrinsic preference of the system at a given concentration and pH. The addition of alkali metals notably affects the distribution of products. Ion pairing between metal cations and silica anions decreases repulsions between anions within oligomers and thus favor polymerization [10]. More specifically, alkali metals stabilize oligomers with labile appendages and large ring structures such as the tricyclic octamer and the substituted cyclic trimer which would otherwise rearrange to more rigid structures (the cubic octamer and the tetrahedral tetramer, respectively). The change in the composition of the mixture allows for the identification of those silica oligomers which bind best to alkali metal ions. This, and examples in the following sections, show that a variation of the distribution of products upon interaction with a target compound can be used to recognize those products that exhibit affinity for the target.

3. CONFORMATIONAL AND CONFIGURATIONAL EQUILIBRIA : INDUCED FIT

When screening a library for affinity, a hit should both bear a combination of functional groups complementary to those of the target, and allow a proper positioning and orientation of these groups in space for associating optimally with the target. Typically, the first aspect is explored when reacting different building blocks in a combinatorial library: from the reaction of A_1 and B_1, A_1 and B_2 etc., one obtains the products combining these functions A_1B_1, A_1B_2 etc… The question of how these building blocks are oriented within each product is often neglected. It refers to conformational and configurational isomerizations which are typical reversible processes (Fig. (**5**)). Internal rotations, ring inversions, isomerization of double bonds for example are thermal or photochemical equilibria.

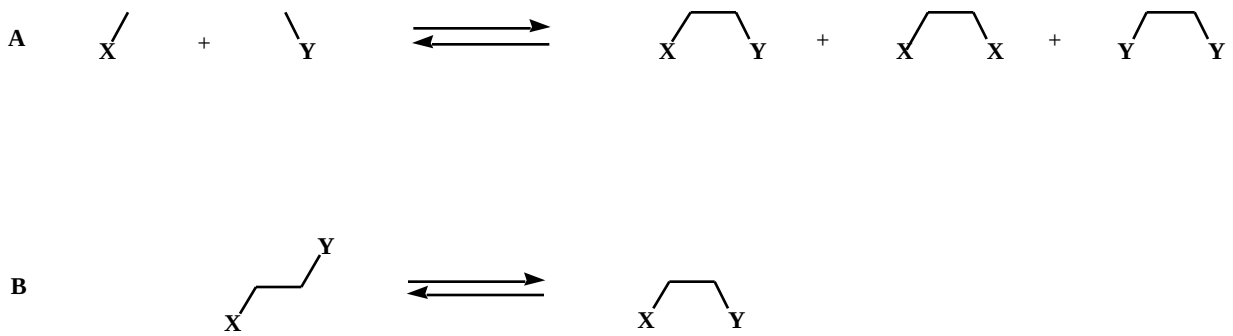


Fig. (5). The distinction of reversibly combining the nature of the recognition function (A), and their relative positioning in space (B).

Recent work by Eliseev *et al.* illustrates how photochemical equilibria between configurational isomers may be driven by the recognition of arginine (Fig. (**6A**)) [13,14]. In this case, the relative orientation of two recognition functions such as carboxylates is modulated through light induced isomerization of two double bonds. The pool is composed of three species: the *Z/Z*, *E/E* and *E/Z* isomers, of which one (*Z/Z*) has a much stronger affinity for arginine than the two others. The mixture is alternatively exposed to arginine, which retrieves the best arginine binder, and to irradiation, which brings the remaining isomers back to the initial equilibrium. After a few cycles, the HPLC analysis of the mixture shows a strong enrichment in the *Z/Z* isomer.

An analogous system was described by Berl *et al.* involving the thermal isomerization of two hydrazone double bonds (Fig. (**6B**)) [15]. These equilibria determine the relative orientation of two pendent pyridine rings, again generating a mixture of three isomers, along with some tautomers. Upon addition of dibutyl barbiturate as a target, the equilibria are shifted almost quantitatively towards the *Z/Z* isomer. This compound possesses two converging 2-aminopyridine moieties which can simultaneously bind to the barbiturate, forming four hydrogen bonds, whereas the other isomers may associate to barbiturate with only two hydrogen bonds.

In a system developed by Hayashi et al., the four conformers αααα , αααβ , ααββ, αβαβ of a *meso*-tetrakis(2-hydroxyphenyl)porphyrin (Fig. (**6C**)) can be spectroscopically characterized as they equilibrate slowly on the NMR time scale [16]. In $CDCl_3$ at room temperature, these conformers are in the ratio 1.0:4.4:2.2:0.8 respectively. Upon adding tetramethoxy-*p*-benzoquinone, the fraction of the αααα isomer shifts from 12.5 to 80% as it strongly complexes the guest.

These examples demonstrate that intermolecular attractive interactions may shift conformational or configurational equilibria towards the species with the highest complementarity to a given target, a phenomenon well known to biochemists as induced fit. They also show how each species may be described as a combination of different possible conformations or configurations. For each combination, the recognition functions have a specific relative orientation, and determine a distinct point in the interactional space available to the molecule.

Chemistry is rich with many different kinds of reversible isomerizations, and the playground for tuning recognition group orientations is vast. A recent publication by Sugasaki *et al.* makes use of the equilibria between isomers of a cerium(IV) double decker porphyrin [17]. Pernía *et al.* and Moraczewsky *et al.* showed that recognition can stabilize the disfavored *cis* C-N rotamer of secondary amides [18] and carbamates [19], illustrating how a minor (but potentially present) component of a pool can become a major component. Along this line, a survey of 399 protein structures found only 0.03% the peptide bonds adopt a *cis* conformation, all of which are stabilized by intramolecular forces.[20]

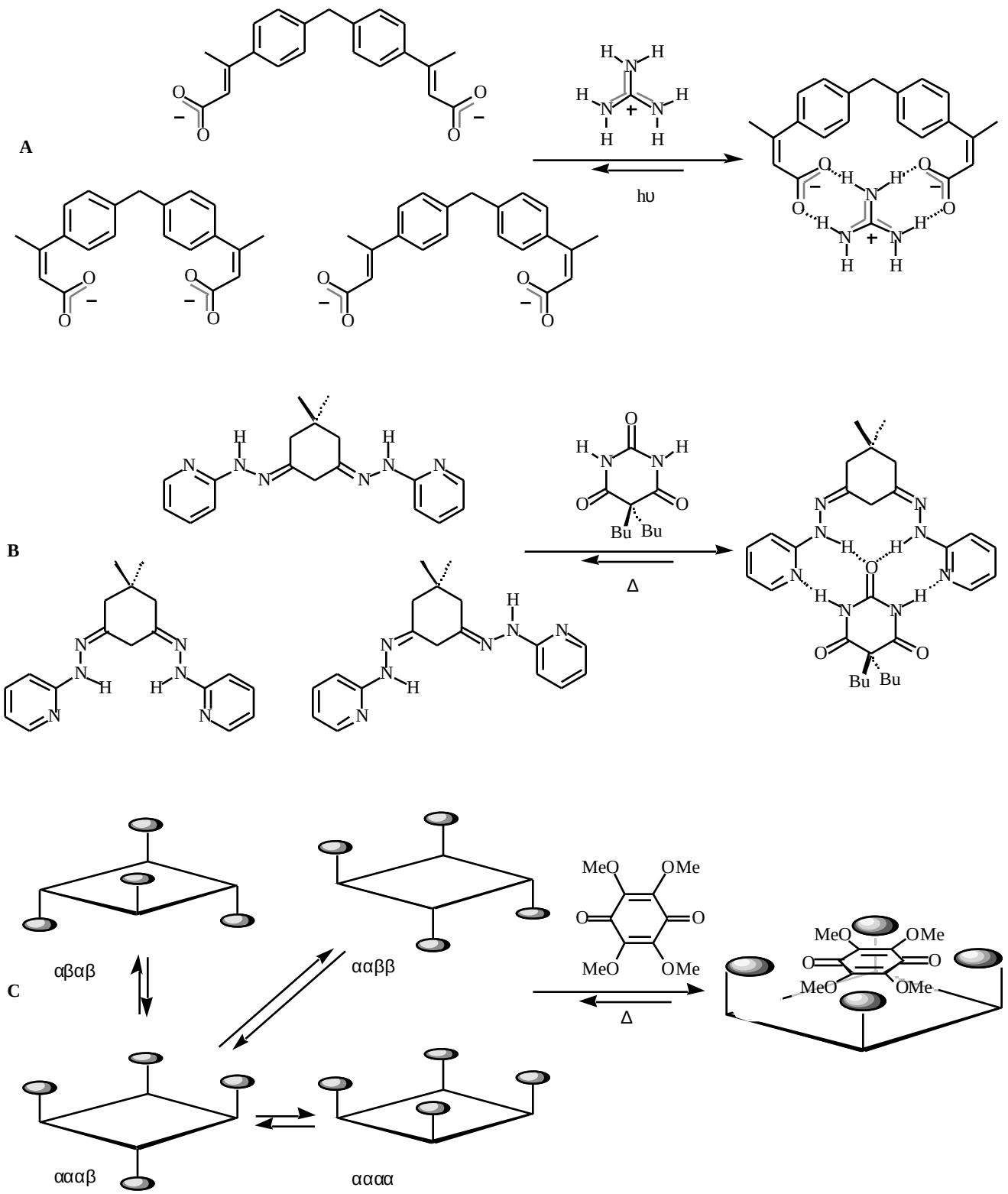


Fig. (6). Three examples of the target recognition driven selection of receptors from thermal or photochemical equilibrating mixtures of configurational/conformational isomers. In (C), the square frame schematizes a porphyrin plane. Each of the four *meso*-2-hydroxyphenyl substituents (spheroids) can adopt two distinct conformations.

The few examples presented so far deal with a small number of well-characterized combinations. The oligoamide **2** contains a sequence of four 2,6-diaminopyridine donor-acceptor-donor (DAD) hydrogen-bonding subunits, linked by three isophthalate groups (Fig. (**7**)). Its conformational

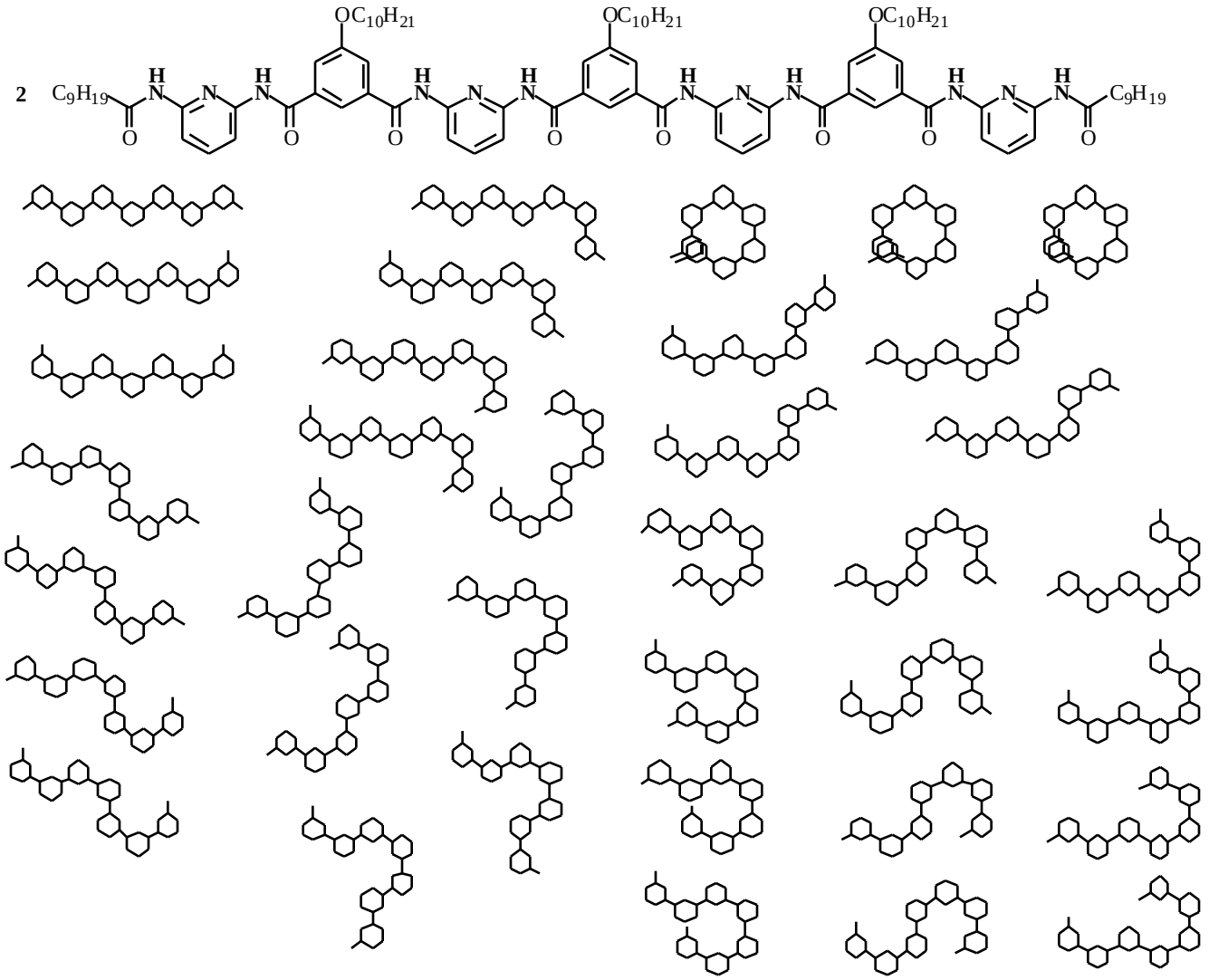


Fig. (7). Structure and possible rotameric forms of the molecular strand **2**. The linkages between the hexagons represent the CO-NH-fragments. The thick linkages indicate those within which 180° rotation was performed about the CO-aromatic ring bond. All 36 possible rotamers are represented, corresponding to the combinations obtained by rotations about the 6 linkages.

behavior is expected to display many more combinations of orientations of these four recognition moieties [21]. If one considers only rotation about aryl-CO bonds, dynamic conformational equilibria generate a library of 36 different quasi-planar/conjugated rotamers (Fig. (**7**)). These 36 species have occurrences of 1 or 2 depending on their symmetry. Their energies and therefore the population of each conformer may differ due to long-range effects. The outcome of a selection of this library through binding of an effector depends on the template employed for deconvolution. With an ADA imide template, binding may occur at any site without interfering with the various rotations (Fig. (**8**)). In contrast, upon the binding of a double-faced, ADA/ADA cyanurate template, curvature is introduced into the backbone of **2**. Binding of two cyanurates leads to the stabilization of three conformers of C, S and helical shape, out of the 36 possible (Fig. (**8**)) [21].

Thus, equilibrating mixtures of conformers can actually be used to probe interactions. Molecules can be designed the conformers of which present various orientations of recognition functions relevant to a given context. The system may be tuned so that these structures have similar energies, leading to an even distribution of the population of conformers. A flexible system offers innumerable combinations of orientation, whereas a rigid system may be purposely biased towards particular geometries.

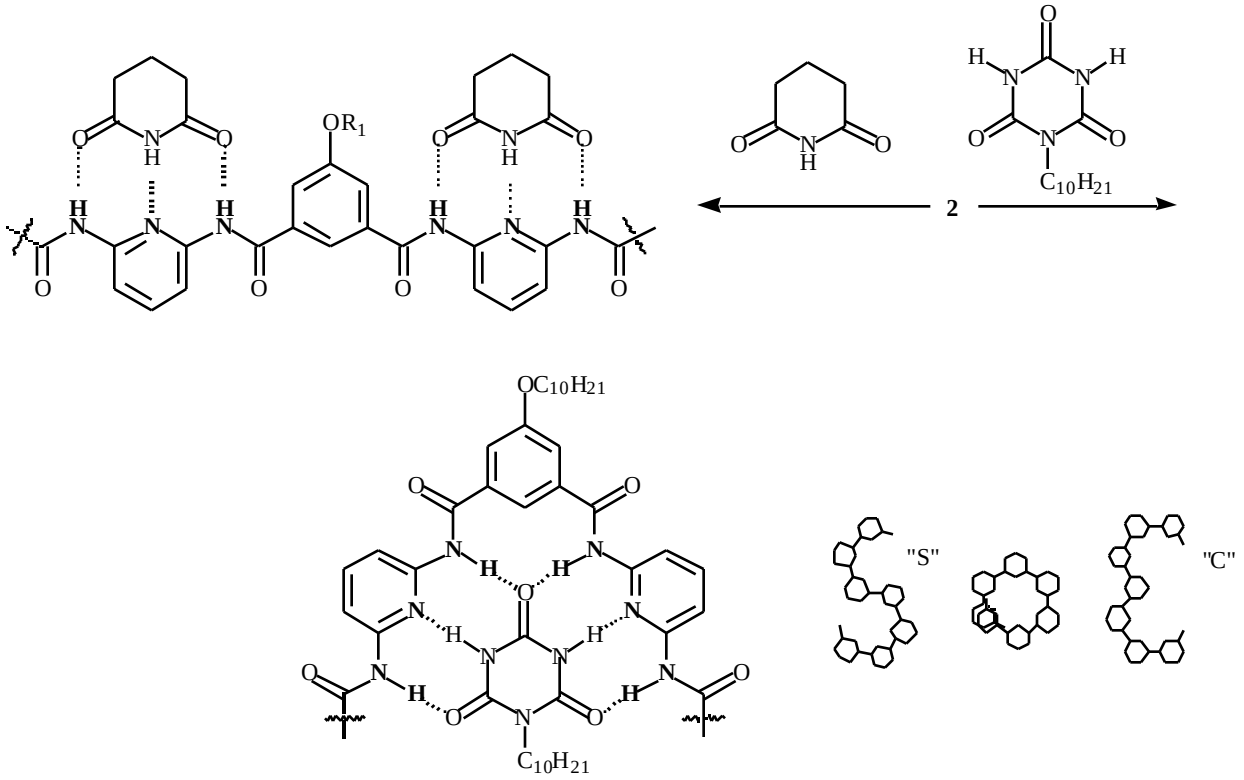


Fig. (8). Template dependent expression of the combinations of orientations of the four DAD hydrogen-bonding subunits of **2**. The three conformers stabilized upon binding to two cyanurate templates are shown on the right.

Other recognition-induced deconvolution of mixtures of conformers may be cited here. For example, the ten tertiary amide groups of a peptide nucleic acid (PNA) decamer presumably result in 2^{10} possible conformers. Binding to the complementary DNA sequence leads to a single conformer within the duplex.[22,23] Similarly, tertiary amides of proline residues within peptides or proteins all generate two almost isoenergetic conformers which are resolved under the effect of intramolecular interactions upon folding to secondary and tertiary structures.

4. NON-COVALENT BONDING. MOLDING OF SUPRAMOLECULAR RECEPTORS

Supramolecular synthesis has emerged as a powerful method for building large structures by spontaneous self-assembly of non-covalently linked components. In these systems, coordination bonds to transition metals, hydrogen bonds, or other reversible connections are often involved in an open way (Fig. (**2**)) and may potentially generate innumerable combinations. This is the case when multivalent metal ions are mixed with multidentate organic ligands. However, the components may be designed so as to lead to the quantitative formation of a well-defined species from the pool of possible combinations, reflecting the thermodynamic preference of the system for the expression of this species. In the following four examples, the presence of a guest species conditions the assembly. The guest serves as a template for the formation of a complementary entity possessing a binding cavity -a receptor- which may otherwise be little expressed, or not expressed at all.

Ma *et al*. showed that the tartrate derived bis(pyridine) bidentate ligand **3** and Pd(II) in D_2O spontaneously form two equilibrating species: a macrocyclic dimer, and a macrocyclic trimer (Fig. (**9A**)) [24]. At millimolar concentrations, dimer and trimer are both present in significant amounts, and no other species is detected by NMR. The addition of small hydrophobic guests (e.g. sodium *p*-toluate) favors the formation of the dimer as they fit in its smaller hydrophobic cavity. On the other hand, larger hydrophobic guests (e.g. sodium

cholate) stabilize the trimeric structure. Adamantane dicarboxylate is too large to fit in the dimer, and too small to fill the trimer cavity, and does not template any of these species.

The system described by Hiraoka *et al.* (Fig. (**9B**)) also involves the reversible coordination of pyridine groups to Pd(II) in D_2O [25]. Unlike the preceding case however, in the absence of any guest, the tridentate ligand **4** and Pd(II) lead to the formation of a complex mixture of oligomers, indicating that well defined species require a template to be formed in significant amounts. Thus, in the presence of $CBrCl_3$ as a guest species, a plane symmetrical dimer of **4** forms quantitatively. The addition of 1,3,5-

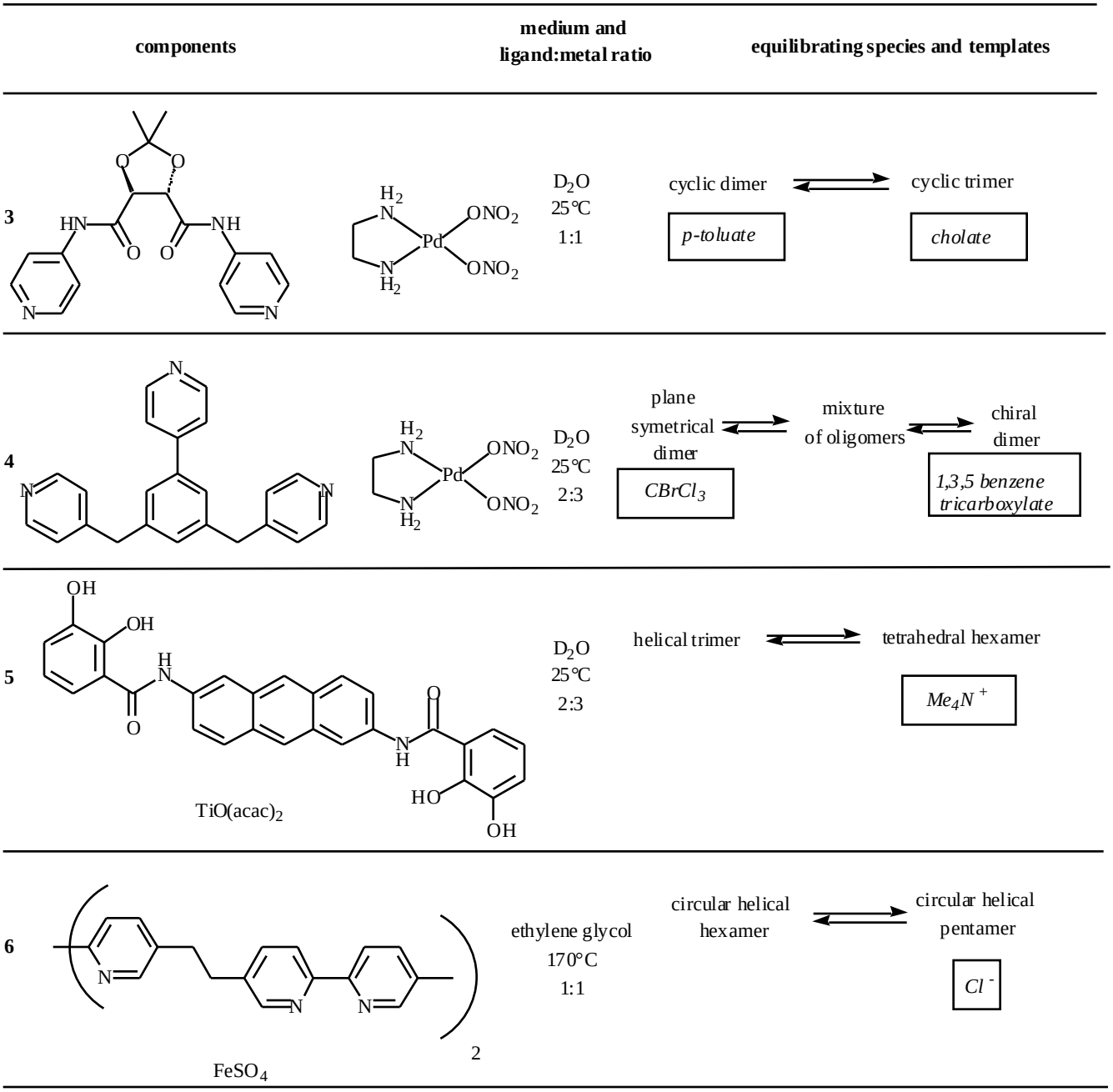


Fig. (9). Four examples of the guest induced stabilization of a receptor reversibly formed upon combining a set of non-covalently bound components. The initial components are represented on the left, the conditions in which diverse combinations interconvert and in which the guest recognition takes place are mentioned in the middle. The equilibria involved are represented on the right, along with the guests used to specifically stabilized some of the species (in italics).

benzenetricarboxylate templates the formation of a different chiral dimer, under the form of a racemate.

The presence of a guest can thus serve as a template for the formation of a receptor, be it a component already present in significant amounts, or a presumed minor component of an ill-defined mixture. The amplitude of the template effect is pushed one step further in the two following examples where the equilibria shift from a unique species quantitatively formed in the absence of the guest, to another species, quantitatively formed in the presence of the guest. Thus, Scherer *et al.* describe the quantitative formation of a helical trimer formed by three bis(bidentate) catecholamide ligands **5** and two Ti or Ga ions [26]. The tetramethyl ammonium cation induces a reversible but quantitative transformation of the helix to a tetrahedral hexamer of **5**, which includes the guest in its cavity (Fig. (**9C**)). Circular helicates formed by tris-(bipyridine) ligand **6** and Fe(II) also undergoe such a dramatic equilibrium shift [27,28]. Whilst $FeSO_4$ leads to the quantitative formation of a circular hexanuclear hexamer of **6**, exchanging the sulfate against chloride anions leads to the quantitative conversion of the hexamer to a circular pentanuclear pentamer of **6**, having one chloride ion bound to its central cavity.

Numerous other systems may be cited here. They all register in the category of the guest induced formation of non-covalently assembled receptors [29-32]. To summarize, they show to what extent a strong molecular recognition may drive an equilibrium to the quantitative formation of a species complementary to the added guest/target substance. This may lead to the expression of a unique combination out of the various components of the system, which may not have been present in significant amounts in the absence of the guest. The identification (the screening) of this combination results directly from its expression as a major component of the mixture in the presence of the guest. These examples also demonstrate how structurally very different entities may be generated upon combining a small number of components. Complex systems can indeed arise from one ligand and one transition metal, leading to the formation of assemblies having cavities of various sizes and shapes.

However, it may be argued that these systems remain poor with respect to the number of recognition functions that are combined. As a second step, other components bearing various recognition functions may be deliberately introduced with the aim of increasing the number of possible products, as well as their structural variety. Recent publications show that this approach is now being followed [33,34]. As shown in Fig. (**10**), these systems take advantage of the assembly as a scaffold to reversibly combine and organize in space the recognition functions displayed by the ligands.

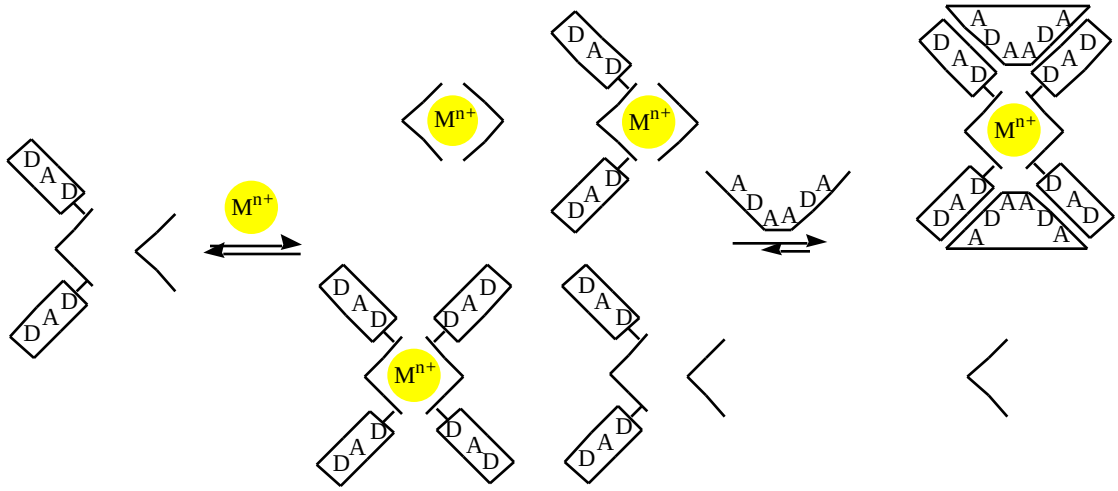


Fig. (10). Dynamic mixture of ligands and complexes formed upon coordination of two different ligands to a transition metal cation; stabilization of a receptor complex with a complementary substrate molecule. One ligand bears two recognition groups with DAD hydrogen-bonding patterns; the substrate presents two complementary ADA patterns.

Thus, 2,2'-bipyridines bearing hydrogen bonding functions can be combined in pairs upon coordination to Cu(I) or Pd(II). In the complexes formed, the relative orientation of the hydrogen-bonding function is preorganized according to the coordination preference of the metal (tetrahedral and square planar respectively). Introducing a substrate species leads to the stabilization and the identification of the complex bearing complementary functions in a proper orientation for recognition [33].

Interesting alternatives to transition metal coordination are hydrogen-bonded assemblies. Large and stable structures held by multiple hydrogen bonds may spontaneously assemble from complementary units. These may be endowed with diverse pendent functions leading to numerous combinations of assemblies, all having the same frame, but differing by the combination of pendent groups that they display [34,35]. Such libraries of non-covalent assemblies have been compared to libraries of antibodies [36]. Indeed the self-assembled frame is shared by all library components and is analogous to the constant part of the heavy chains and light chains of antibodies. The pendent groups grafted on the assembly may be compared to the variable parts of the antibodies where recognition of the antigen takes place. A recent publication by Calama *et al.* showed that selection and amplification by a target substance could be performed in these hydrogen-bonded dynamic assemblies [37].

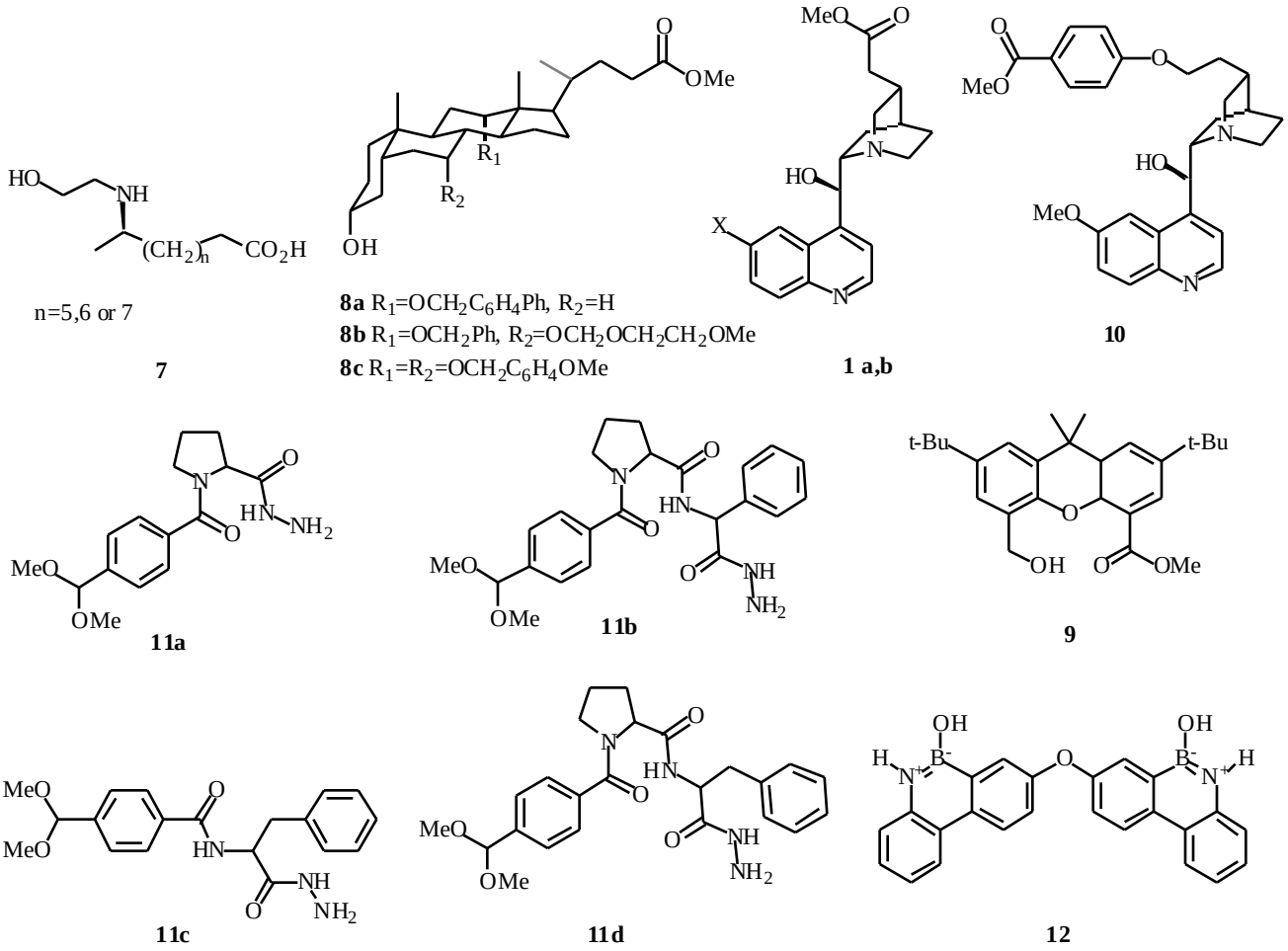
5. DYNAMIC LIBRARIES OF MACROCYCLES

Reversible covalent linkages are often associated with product instability and tend to be avoided by synthetic organic chemists. However, a number of recent publications show that their use is increasing in the context of dynamic combinatorial mixtures, and particularly in the context of libraries of macrocycles. Macrocycles define cavities and are good candidates for the recognition of small molecules. As shown below and in the previous section, their spontaneous formation is rather common in open systems. Additionally, synthetic macrocycles may prove to be biologically active substances. Libraries of natural macrocycles have indeed been identified as the active constituents of defensive secretions by insects [38,39]. These are very large ring poly-lactonic structures which incorporate in a random sequence two to eleven monomers selected from a small set of the (2-hydroxyethylamino)-alkanoic acids **7** (Fig. (**11**)). Although the distribution of these products does not reflect thermodynamic equilibrium, they clearly derive form each other, and their biosynthesis has some dynamic character.

Extensive reports on the reversible macrocyclic oligomerization of synthetic hydroxy esters have appeared in recent years. Brady *et al.* described the reversible transesterification of the cholate derived hydroxy esters **8** induced by the methoxide ion which leads to a distribution of dimeric to pentameric macrocycles (Fig. (**11**)) [40]. Upon addition of alkali metal ions, the mole fraction of some cycles increases suggesting association of the ions to specific cavities [41].

In the alkaloid and xanthene series however, reversible transesterification of the hydroxyesters **1** and **9** quantitatively yields to the respective trimer and dimer. Mixing the two precursors leads to their complete segregation in different macrocycles [12,42]. Higher oligomers of **1** may be generated under kinetic conditions, but they all transform into the trimer under thermodynamic control [43]. The more flexible quinine derivative **10**, however, forms oligolactones of different ring sizes which eventually incorporate monomers of **1** [44].

These lactonizations require relatively harsh reaction conditions (base in boiling toluene), in which hardly any recognition may take place. Milder reversible reactions have been explored more recently, such as the transimination of hydrazones by various hydrazides at room temperature using acid catalysis,[45,46] or the condensation of borazaaromatic acids to anhydrides [47]. The hydrazido-aldehydes **11** form a mixture of macrocyclic oligohydrazones up to the undecamer, in which different monomers are incorporated [45]. Compound **12** forms quantitatively a dimer [47].



precursors	medium	oligomeric macrocycle(s) formed under thermodynamic control
7	(biosynthesis)	cyclic dimer to undecamer in a random sequence
8 a,b,c	KOMe, Dicylohexyl-18-crown-6, toluene, reflux	distribution of cyclic dimer to pentamer and linear intermediates
1 a,b	KOMe, [18]crown-6, toluene, reflux	cyclic trimer
9	KOMe, [18]crown-6, toluene, reflux	cyclic dimer
10	KOMe, [18]crown-6, toluene, reflux	cyclic dimer to tetramer / colactonizes with **1**
11 a,b,c,d	CH_2Cl_2, CF_3CO_2H, RT	colactonization of cyclic dimers up to undecamers
12	acetone, water, RT	cyclic dimer (kinetic products include larger cycles)

Fig. (11). Precursors of oligomeric macrocycles, and the products formed under thermodynamic control.

These result show that the distribution of products obtained from the formation of oligomeric macrocycles is extremely system dependent. Two distinct scenarios may be envisaged. Firstly, when different monomers spontaneously form a series of macrocycles of variable sizes, a diverse dynamic mixture emerges. The distribution of products is likely to be uneven,

but the presence of significant amounts of various species show that the energy difference between them is not excessive, and that their mole fraction may be increased upon interacting with a complementary substance. In a second scenario, one species is quantitatively formed, reflecting a predisposition or a preorganization of the system towards this substance. Other products are possible, but their formation energy is significantly higher. Such systems are not particularly prone to the expression of diversity. However, they may constitute frames for combining elements of the same family. For example, in the case of **1** which forms a trimer quantitatively, mixing the two analogs **1a** and **1b** leads to a mixture of trimers incorporating the monomers in a random sequence [42]. Macrocycles may thus be used for grafting combinations of recognition groups in the same way as transition metal complexes [33] or hydrogen-bonded assemblies [37] (see section 4). As long as these recognition groups do not interfere with the self-assembly processes, the reversibility will ensure their statistical distribution among the various species formed.

6. TARGETING BIOMOLECULES

The systems reviewed so far may be considered as models. The relatively simple recognition schemes and limited number of components allows to extensively characterize the products, and to understand the ins and outs of the phenomena. Most of the examples presented involve the guest templated formation of receptors. What about the receptor templates facilitating the formation of guests, what about the molecular recognition of biomolecules, and what are their perspectives for drug discovery?

Nucleic Acids

At least three examples exist in the literature of dynamic mixtures involving the molecular recognition of nucleic acids. Each of these was developed in a specific context. Goodwin et al described the template directed condensation of a 3'-aldehydo-trinucleotide and a 5'-amino-trinucleotide to the corresponding imine in the presence of the complementary hexameric strand [48]. The equilibrating species were covalently linked imines, and the driving force for shifting equilibria was base pairing within the hexanucleotide duplex. In a different approach, Miculka et al used reversible base-pairing to combine various oligonucleotides which differed not through their sequence but through the nature of pendent oligopeptides [49]. The library thus consists of various combinations of peptides attached to a constant DNA duplex frame. Recognition directed selection might be performed to detect specific binding to a combination of peptides. This system has much in common with the libraries of hydrogen-bonded supramolecular assemblies combining various appended recognition residues described by Calama et al [37].

Dynamic libraries of DNA-binding Zn(II) complexes have been prepared, based on the reversible condensation of a set of six amines to salicylaldehyde, and the subsequent reversible coordination of the salicylaldimine products and of the amine building blocks to Zn(II) [50,51]. The reversibility of these processes and the library product distribution were not fully characterized in this case. Yet, screening against resin bound oligo(A×T) led to the identification of a DNA-binding Zn(II) complex. Independent experiments determined its K_d to be 1.1 μM.

Peptides

Peptide-recognition directed selection from dynamic mixtures of disulfides was developed and reported independently by two groups [52,53]. In both cases, two thiols A-SH and B-SH are combined to form three disulfides A-SS-A, A-SS-B, and B-SS-B using reversible thiol-sulfide exchange conditions. In the work of Hioki and Still, A and B both possess hydrogen bond donors and acceptors. Under equilibrium conditions, the mixture obtained by disproportionation of A-SS-B contains 35mol% of A-SS-B and 65mol% of A-SS-A and B-SS-B. In the presence of the immobilized peptide Ac(D)Pro(L)Val(D)Val, the equilibrium is shifted up to 95mol% of A-SS-A bound the peptide-containing resin, and the remaining B-SS-B

[52]. Shibakami et al combine two anionic lipids which differ through the length of their hydrocarbon chain. Thus, A-SS-A, A-SS-B, and B-SS-B are lipid dimers each possessing two pairs of hydrophobic chains of 14 or 18 carbons [54,55]. In aqueous solution, these compounds form vesicles and the equilibrium takes place within the bilayer membrane. Upon insertion of the ion channel peptide gramicidin in the membrane, the relative proportions of the peptide dimers are shifted in favor of the homodimers A-SS-A and B-SS-B, indicating nearest neighbor recognition between the transmembrane peptide and its surrounding lipids [53].

These two examples involve the reversible covalent combination of the building blocks. Cha et al have investigated non-covalent combinations in the binding of aqueous dipeptides onto monolayers of diakyl oligoglycyl amphiphiles [56]. The reversible combinations then consist of the numerous relative positions of the amphiphiles within the monolayer. In the presence of the aqueous dipeptides GlyX (X = Phe and Leu), specific binding sites are induced upon rearrangement of the lipids, resulting in an association constant of 35 M^{-1} for GlyLeu. Aqueous peptides of the type XGly were not bound at all. Since the combinations involve the relative positions of the binding oligoglycyl head groups rather than combining their nature, this approach may be compared to induced fit processes in dynamic mixtures of conformational or configurational isomers (see section 3).

Proteins

Molecular recognition of proteins is most challenging both because of its complexity and because of its relevance to drug discovery. Again, we find in the literature several independent examples of the use of the reversible bonding of building blocks for the identification of small molecules binding to proteins. In the earliest example we could find so far, the phenomenon was termed 'episelection' [57,58]. The tripeptideboronic acid inhibitor of trypsin (BOC)Ala-Val-Lys-boronate **13** (K_i = 7 nM) was incubated in a water-methanol solution (Fig. (**12**)). Reversible esterification of the boronic acid by methanol leads to an equilibrium between the acid, two diastereomeric monoesters, and the diester. In the presence of trypsin, the component(s) with the highest affinity are selected and bound by the protein. Using X-ray crystallography to solve the structure of the protein-inhibitor complex, Katz *et al.* were able to show that trypsin had selected exclusively and quantitatively one of the two possible mono-esters [57].

In a related work, Katz et al used the reversible coordination of two poor inhibitors of the same enzyme trypsin to construct a very strong inhibitor [59,58]. Specifically, the combination of a weak trypsin inhibitor such as amidinobenzimidazole **14** (K_i = 87.5 μM) and of Zn(II) leads to a K_i value of 5.3 nM. The complex formed by the two components is thus a much stronger inhibitor than the components taken separately. X-ray structure analyses show that the complex formed by Zn(II), **14** and trypsin involves chelation of the metal both by the imidazole nitrogens of **14** and by a histidine and a serine residue of the protein [59]. Although this work was not extended to a combinatorial context where several ligands and metal ions could be combined, it shows that self-assembled entities are eligible enzyme inhibitors, and opens the perspective of screening dynamic mixtures using enzyme recognition to direct the formation of complementary species. Coordination biochemistry represents a plausible domain of application of dynamic combinatorial chemistry, and the binding properties of numerous metal complexes enzyme inhibitors may be explored in this way [60].

Transition metals might directly coordinate to enzyme residues [59,60]. They might also provide a frame for assembling and positioning recognition moieties in space [33]. Thus, the coordination of three bipyridine ligands having one pendent 2-acetamido-2-deoxy-α-D-galactopyranose to Fe(II) yields a dynamic mixture of four isomeric complexes Λ-*fac*, Δ-*fac*, Λ-*mer* and Δ-*mer*, each displaying three galactoses in a specific arrangement [61,62]. Upon incubation with lectins which recognize multiple GalNAc residues, the

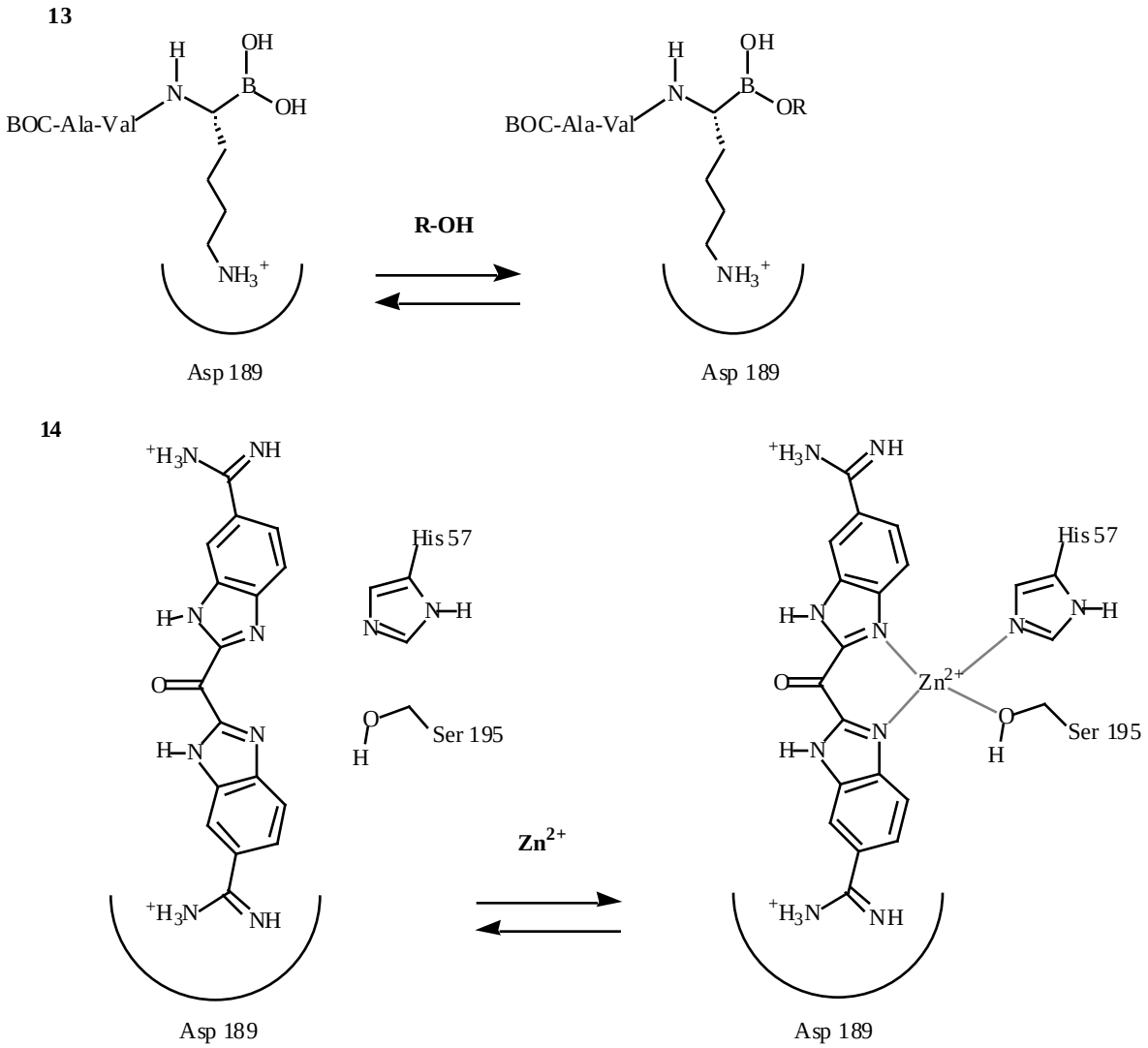


Fig. (12). The structure of two reversibly assembled trypsin inhibitors.

proportions in the mixture are shifted. *Vicia villosa* B_4 lectin leads to an enrichment of the Λ-*mer* isomer, whilst *Glycine max* lectin shows a strong binding preference for the Δ-*fac* and Δ-*mer* isomers [61].

Swann et al showed that not only chemical reversible processes but also enzyme catalyzed pathways may be used to generate dynamic combinatorial libraries [63]. Indeed, enzymes allow to accelerate the formation and breakdown of otherwise kinetically inert compounds such as peptides. Specifically, mixing an initial stock solution of two peptides YGG and FL with the protease thermolysin under conditions in which both peptide hydrolysis and formation occur leads to the scrambling of amino acids within numerous peptides of variable length [63]. Among the identified products was the pentapeptide YGGFL known to bind the monoclonal antibody 3E7 with a K_d of 7.1 nM. When the peptides were allowed to diffuse from the equilibrating mixture through a dialysis membrane to a compartment containing 3E7, a 12% amplification of the peptide YGGFL was measured, showing that the antibody acts as a molecular trap for YGGFL and favor its formation from the equilibrating species. The small amplitude of the effect was assigned to the low quantity of antibody used, which may have been saturated with the YGGFL already present in the unbiased mixture.

The ability of an enzyme to mold its inhibitor from a pool of reversibly assembled building blocks was tested using the well-characterized enzyme carbonic anhydrase II (CAII) as a model [64]. A small dynamic library of twelve imines was built from four amines and three aldehydes

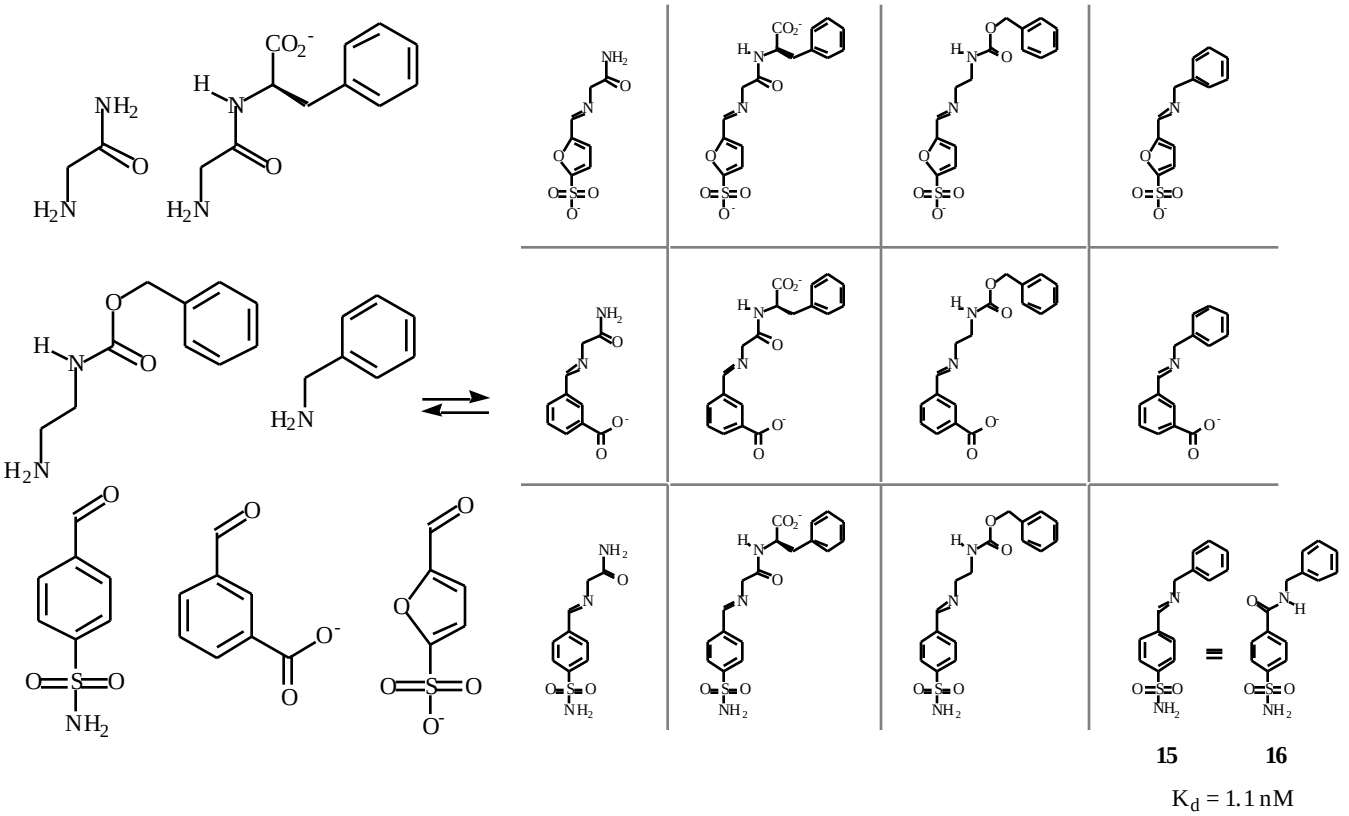


Fig. (13). A dynamic library of imines for screening carbonic anhydrase inhibitors.

(Fig. (**13**)). These were chosen so that the imines display different combinations of polar, non-polar and charged subunits. Two building blocks, namely benzylamine and 4-sulfamoylbenzaldehyde, were introduced as precursors of imine **15**, the structure of which is very similar to the strong CAII inhibitor amide **16** (K_d = 1.1 nM). Based on previous knowledge of CAII inhibitors, only imines derived from 4-sulfamoylbenzaldehyde were expected to bind to CAII, with **15** being the strongest inhibitor by two orders of magnitude. Indeed, when CAII was introduced in the dynamic mixture, the amount of imine **15** was shown to increase by 150 %. The increase was done at the expense of other 4-sulfamoylbenzaldimine products, the proportions of which decreased in the presence of CAII. In competition experiments between two amines and one aldehyde, CAII was shown to enhance the formation of its best inhibitor up to a factor of 21. These experiments did not aim at discovering new inhibitors, but they clearly illustrate the feasibility of the concept: cavities at an enzyme surface may template the formation of a complementary ligand from a pool of building blocks, and thus facilitate their identification as being prominent species in the mixture.

7. PRACTICAL ASPECTS

The number of examples presented in this article clearly establish that the dynamic combinatorial approach has already proven to be useful in a great variety of contexts for identifying specific ligands or receptors. However, the actual experimental tuning and the possible difficulties do not show through the short descriptions we gave. This section focuses on the various aspects of practical use when implementing such an approach.

Reactivity

In a dynamic combinatorial system, all building blocks are mixed and are continuously competing for reaction. Thus, the proportion of products may be significantly biased by intrinsic reactivities of the building blocks (see section 2). Template effects may be strong enough to counterbalance this bias and induce the formation of another

species [26-28]. Nonetheless, such effects are not always possible, and an even distribution of products is justifiably desirable. Compounds with similar reactivities may be selected. This is the case for instance in the library of imines of ref 64: the four amines on the one hand and the three aldehydes on the other hand were chosen for their comparable reactivities; aliphatic aldehydes and simple alkyl amines were excluded, because of their excessively high electrophilicity or low nucleophilicity, respectively. The proportions between the library building blocks may also be adjusted so as to compensate for slightly different reactivities. Another way to circumvent this problem is to choose building blocks whose recognition moieties are remote from and do not interfere with the reactive bonding functions. All building blocks then bond with similar affinities. Illustrations of this are found in the various systems which use a self-assembled frame to combine remote pendent recognition groups. Almost statistical distributions are then obtained [33-37,42,49,61].

Reactions

Various types of chemical reversible processes have been presented: conformational and configurational isomerizations, non-covalent bonding and covalent bonding such as thiol-disulfide exchange, imine formation, boronate, silicate, and carboxylate esters formation. Metathesis using Grubs catalyst might lead to dynamic libraries of olefins.[65] Libraries of borates from boric acid and 1,2-diols are also envisaged [66]. Other reversible reactions such as Diels-Alder or Michael reactions are yet to be explored in this context. In most cases, the reversible process occurs in the same medium as the recognition events to be identified. Thus, although some recognition might be achieved in boiling toluene [41] or even boiling ethylene glycol [27], reactions requiring mild conditions will often be preferred. For most biomedical applications, recognition takes place in water at 20 to 40°C under close to neutral pH. Reactions reversible under these conditions include some metals coordination [24,25], thiol-disulfide exchange [52,53], bor(on)ate ester formation, and imine formation [48,64]. Kinetic factors and competing reactions may sometimes be troublesome. Imines from aliphatic aldehydes and amines for example are kinetically stable and do not exchange in basic medium. When equilibrium does take place under neutral conditions, however, competing hydrolysis then strongly shifts the equilibrium towards dissociation, which precludes a direct analysis of the mixture. Imines from aldehydes and O-aryl and O-alkyl oximes remain stable even a low pH, but exchange necessitates acidic catalysis [67]. There is no doubt that more chemistry will be discovered or rediscovered for achieving equilibrium under mild conditions, and efforts are in progress in this direction [67]. An interesting alternative is to separate the recognition step and the equilibrium step into two different chambers. This renders compatible a mild medium for recognition and more drastic conditions for equilibration such as proteases [63] or strong UV irradiation [13,14].

Reversibility

The question of reversibility reaches further than simply establishing an equilibrium between the library components. Reversibility implies that a hit may not be stable in the absence of the target entity. This will affect the identification of the said hit from the library components if dissociation from the target is required for screening. It might also limit practical applications: what if the hit spontaneously decomposes back in the initial building blocks ? For the purpose of screening, most of the equilibria mentioned in this review are strongly pH and temperature dependent. They can be frozen at will upon changing conditions. Chemical quenching is sometimes possible, for example in the case of imines which can be irreversibly reduced to amines using $NaBH_3CN$ [48,64]. The methathesis reaction is also efficient at capturing a dynamic assembly as an irreversible covalent form [68]. Such treatments allow the use of chromatography to analyze the mixtures without dissociation of the products during elution. Nevertheless, whilst these tricks facilitate screening, they do not solve the dilemma of using identical conditions for both reversibility and

recognition and then facing eventual decomposition of a hit when isolated for practical use. For drug research, a hit selected from a dynamic library is unlikely to be useful as such. Instead it provides a starting point for developing stable analogs ("lead structure"). For example, the imine function is a reversibly formed analogue of the stable peptide bond: they both consist of an sp2 carbon rigidly connected to a monosubstituted sp2 nitrogen. The reader should note that these latter remarks do not apply when recognition and equilibrium take place in separate chambers. The library products might then be kinetically stable where the recognition takes place and equilibrate under more drastic conditions [13,14,63].

Stoichiometry and entropic factors

The recognition directed stabilization of some library products upon association to the target is a stoichiometric event. The amount of bound product(s) is generally not expected to be larger than the amount of target in the mixture. In order to induce a maximal amplitude of the stabilization, it is desirable to use a stoichiometric amount of target, that is as much target as there may be of one product. This rule was followed in a majority of examples cited here. If only a small quantity (less than one equivalent) of the target is available, small effects are expected and actually observed [63]. When the target is a protein, one equivalent might represent a relatively large and expensive sample. Micromolar scale assays, and recovery of the target using dialysis or ultrafiltration help limit the cost of such experiments.

These remarks concern the ratio between the target compound and the amount of each library product that might potentially be formed. However, they do not point to the number of different products or address the question of how large a dynamic combinatorial library can be. Many of the examples presented above are limited to a prototypical equilibrium between two species (A)×(B), or to a minimum number of combinations: (A)+(B) ×(AA)+(AB)+(BB) [13-15,33,42,52-55]. In these cases, the ensemble of products was termed a mixture rather than a library. In fewer systems, numerous (and sometimes innumerable) combinations are available, whether they are actually expressed or not [21,27,45,63]. Larger libraries should in principle cover vaster interactional spaces that might interact with the target, and give more opportunities to find a hit. However, as in any combinatorial approach, there are drawbacks to inconsiderately increasing the number of library components. In the case of a dynamic combinatorial library, the difficulty arises from entropic limitations to the stabilization of a library product by the target. Coming back to Fig (**1**), the library products may be divided in three categories: those that bind strongly (both subunits bind simultaneously) to the target, those that bind weakly (one out of two subunits bind), and those that do not bind (neither subunit binds). In the presence of the target, the proportion of compounds of the first category increases -this constitutes the molding and allows the detection of the hit. This is done at the expense of products of the second category due to the shortage of at least one of their building blocks. A side effect is a relative increase of the proportions of products of the third category, this effect being significant in small libraries. The free energy used for stabilizing the hits (e.g. the amplitude of equilibrium shift) is the binding energy of the hit relative to the binding energy of the products of the other categories (e.g. its selectivity). Using simple words, the amplitude of the equilibrium shift upon binding of a hit to the target is linked not only to how strong the hit binds, but how much stronger than the other library components. Consequently, a library based on a large number of building blocks will include many products of the second category which compete with the hit(s) for binding to the target, and limit the amplitude of the equilibrium shift. This entropic limitation has been quantitatively estimated for a hypothetical library composed of *n* components all equilibrating with each other: if a single library product has an affinity for the target *n* times larger than any other product, then the target induced amplification could lead to the transformation of 50% of the overall library material to form the effective product [14]. A theoretical model was also proposed by Moore et al to evaluate the extent of template effects over mixtures of equilibrating polymer sequences [69].

For practical purposes, a large library may be envisaged when strong and specific recognition is expected. Selectivity may then lead to significant equilibrium shifting despite the number of possible combinations. On the other hand, small libraries will be useful to assess small energy differences. As for other combinatorial libraries, a dynamic combinatorial library should be carefully elaborated. The relevance of each building block in terms of the recognition potential it brings should be evaluated by computational means, and redundant information avoided. Large mixtures are not necessarily meaningful, and relatively small sets of well-chosen building blocks might carry as much interaction potential.

CONCLUSION AND PERSPECTIVES

Most of the references cited in this article are less than five years old. In this short time span, the dynamic combinatorial approach has developed rapidly and has already proven useful in a great variety of contexts: organic, inorganic and biochemical. The concept has been put in practice by numerous authors, often on an independent basis. The multiple examples presented here show how the underlying principle of the method, namely reversibility, defines both its strengths and its limits. Not so long ago, a "mixture of unstable species" would have been regarded by chemists as a most inconvenient system. Yet, it now emerges as an efficient means to detect and to evaluate affinity and as such, it carries great potential for the discovery of pharmacophores. Another foreseen development is to direct dynamic mixtures not only through recognition, but also through other functions. This may lead to the generation and screening of dynamic libraries of catalysts and materials [3,70,71].

As emphasized by Eliseev and Lehn, dynamic combinatorial chemistry participates in the emergence of an adaptative chemistry [2,3]. Along with polymer-imprinting techniques, transition state analog-induced catalytic antibodies, or function-directed evolution of nucleic acids, molecules are not only considered for what they are, but also for how they may evolve and adapt to imposed selection schemes.

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