Dynamic diversity in drug discovery: Putting small-molecule evolution to work

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From oligonucleotides to orangutans, nature has found darwinian evolution to be the most efficient means of optimizing populations of organisms – or molecules. Recently, several research groups have begun adapting darwinian evolution to the identification of small molecules with specific properties. Although still at an early stage, this new field of 'dynamic diversity' shows promise as a method for the identification of high-affinity ligands for biomolecules.

With the advent of combinatorial chemistry and high-throughput screening, the field of drug discovery has undergone a tremendous upheaval in the past decade[1,2]. Rather than relying on the screening of legacy collections of compounds derived from natural sources, or through the use of frequently laborious medicinal chemistry, academic researchers and pharmaceutical corporations can now (at least in theory) generate and screen a vast number of compounds for a particular target in only a few days. However, such a fundamental change in the way that lead compounds are found prompts the question 'are there any techniques remaining to be discovered for drug discovery that have the potential to be even more efficient than current combinatorial and high-throughput methods?'

The roots of the dynamic combinatorial chemistry field

In the 'early days' of small-molecule combinatorial chemistry, several researchers explored the possibility of generating solution-phase mixtures of compounds and screening them as a mixture for affinity to a receptor. For example, the Rebek group examined libraries prepared from reacting one of three core tri- or tetra-acid halides [compounds (1)–(3), Fig. 1] with a mixture of amines to produce libraries of up to 97,461 members[3–5]. Although HPLC traces of these crude libraries indicated that many compounds had been produced, the main difficulty with this technique was the need to develop a workable assay and analytical format to determine active components. Rebek and coworkers used an iterative deconvolution protocol to identify compound (4) as an effective trypsin inhibitor[6]. More recently, affinity chromatography or capillary electrophoresis coupled to mass spectral analysis has emerged as a partial solution to this analytical problem[7–10]. However, all published examples so far have been limited to relatively small libraries.

By contrast, molecular biologists routinely use libraries containing millions of compounds as mixtures in their work. Techniques such as PCR (Ref. 11) enable the amplification of individual components of these libraries, solving the analysis and assay problems. Similarly, processes such as *in vitro* selection of oligonucleotides (SELEX: systematic evolution of ligands by exponential enrichment)[12,13] and phage display[14,15] are currently widely used as tools for the selection and amplification of oligonucleotides or polypeptides with desired ligand-binding or catalytic properties. In this review, we will discuss an emerging field of research known as 'dynamic diversity' that uses concepts derived from nature to provide a new method of ligand identification. Essentially, this method is

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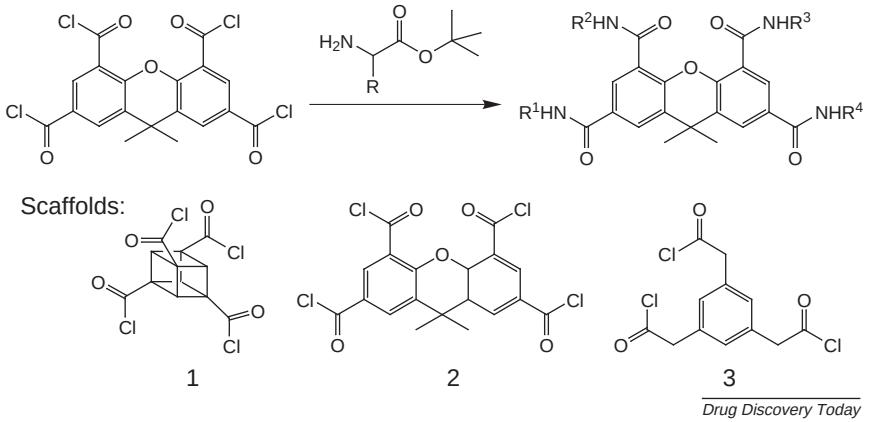

Figure 1. *Reactive scaffolds employed in the preparation of solution-phase mixture libraries[3–5].*

the first step towards a selection and amplification process applicable to solution-phase small-molecule mixture libraries. These authors suggest that this method is complementary to current drug discovery methods and might offer several advantages. Dynamic combinatorial libraries comprise mixtures of compounds constructed under conditions in which a selection process can alter the composition of the mixture. With respect to the drug discovery process, the selection is based on the affinity of the compound for a target receptor.

In addition to borrowing from the biological field, many of the ideas driving the dynamic combinatorial chemistry (the rapid interconversion of library constituents, without modifying the receptor) field have come from the sciences of template-directed synthesis[16–18], molecular imprinting or template polymerization[19], self-assembly (thermodynamic templating)[20] and molecular recognition, areas that have been the subject of intensive research by physical and synthetic organic chemists for the past few decades[21]. Self-assembly involves noncovalent forces such as hydrogen bonding, coordination around a metal ion, or hydrophobic interactions. In each case, there is a template that is involved in the synthesis of a well ordered supramolecular structure based on the most favorable thermodynamic interactions with the template. Other biological antecedents for the dynamic combinatorial chemistry concept include the spontaneous resolution of enantiomers under equilibrating conditions[22], templated polymerization[23] and nonlinear asymmetric catalysis[24].

The main goal of a dynamic equilibrium strategy is to have a receptor template for the synthesis of a compound (either a transition-metal complex or a covalently linked assembly of components) under reversible conditions[25]. It is here where concepts derived from self-assembly and combinatorial chemistry intersect with the biological concepts of mutation and selection to provide a framework for small-molecule evolution. Although the field of dynamic combinatorial chemistry is still young, several different strategies have been explored in proof-of-concept experiments that suggest that many dynamic processes might be incorporated into drug discovery.

Proof-of-concept demonstrations of dynamic diversity

Thermolysin-mediated peptide equilibration

One of the first proof-of-concept demonstrations of dynamic diversity used reversible proteolytic digestion of peptides catalyzed by thermolysin to scramble peptide libraries in the presence of a receptor (Fig. 2)[26]. To demonstrate the reversability of the proteolysis reaction, this group first examined the products obtained after incu-

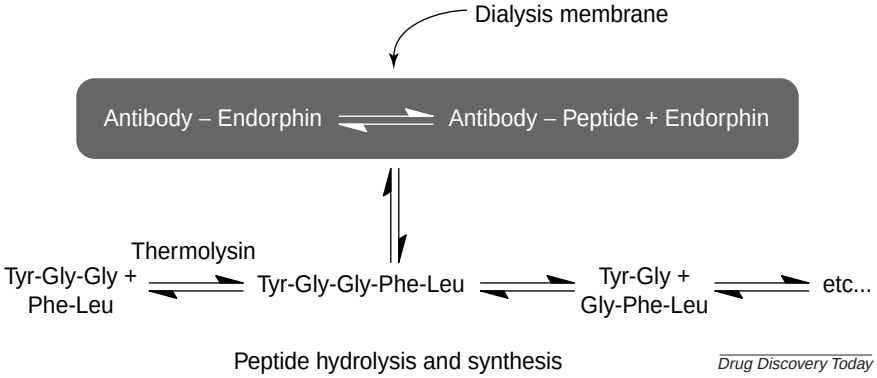

Figure 2. *Peptide scrambling and amplification experiment carried out in Ref. 26. Thermolysin-mediated reversible proteolysis was physically separated from antibody binding by a molecular weight-selective dialysis membrane.*

bation of Tyr-Gly-Gly and Phe-Leu with thermolysin. In addition to the starting materials, Tyr-Gly-Gly-Phe-Leu and several additional products were obtained, thus, establishing the ability of thermolysin to mutate the Tyr-Gly-Gly and Phe-Leu peptide mixture. This thermolysin-mediated peptide equilibration was then carried out in the presence of an antibody known to bind to Tyr-Gly-Gly-Phe-Leu. Indeed, increases in the quantity of Tyr-Gly-Gly-Phe-Leu present in the reaction were observed relative to the quantities found in the antibody-free experiment.

Photoisomerization

The Eliseev group[27,28] has used *cis-/trans*-photoisomerization to interconvert three isomeric α,β-unsaturated dicarboxylates. This photoisomerization, coupled to repeated cycles of affinity chromatography in the presence of a resin-bound guanidinium group, leads to the enrichment of the highest-affinity binding compound (Fig. 3). The use of physical separation (pseudo-batch processing) of selection and mutation events in these experiments should be noted as this enables the use of a scrambling mechanism (irradiation) that would otherwise be potentially damaging to the receptor.

Eliseev and coworkers found that exposing a solution containing the dicarboxylate compound (5) (Fig. 3) to UV light provided a mixture of isomers (Fig. 3) with the ratio of compounds (7):(6):(5) = 3:28:69. This mixture was then passed over an affinity chromatography column derived by immobilizing guanidinium groups on silica gel. After the eluent was subjected to 30 cycles of this treatment, the ratio of isomers in solution was (7):(6):(5) = 48:29:23. The compounds that were retained on the guanidinium resin, comprising approximately 11% of the starting concentration, had a ratio of (7):(6):(5) = 85:13:2. As a control, the equilibrium ratio of the compounds was measured after eight hours of prolonged exposure to UV irradiation; in this case, the ratio was (7):(6):(5) = 52:31:17. When the experiment was run in the absence of a guanidinium receptor (with unfunctionalized silica as the affinity matrix), approximately 12% of the mixture of isomers was absorbed with a ratio of (7):(6):(5) = 55:31:14. Finally, it was found that 12% of the total material was retained on the guanidinium matrix in the absence of UV irradiation, with an isomer ratio of (7):(6):(5) = 5:20:75. Although the library examined in this case consisted of only three compounds, Eliseev effectively demonstrated how the addition of a receptor to an equilibrating mixture in solution can

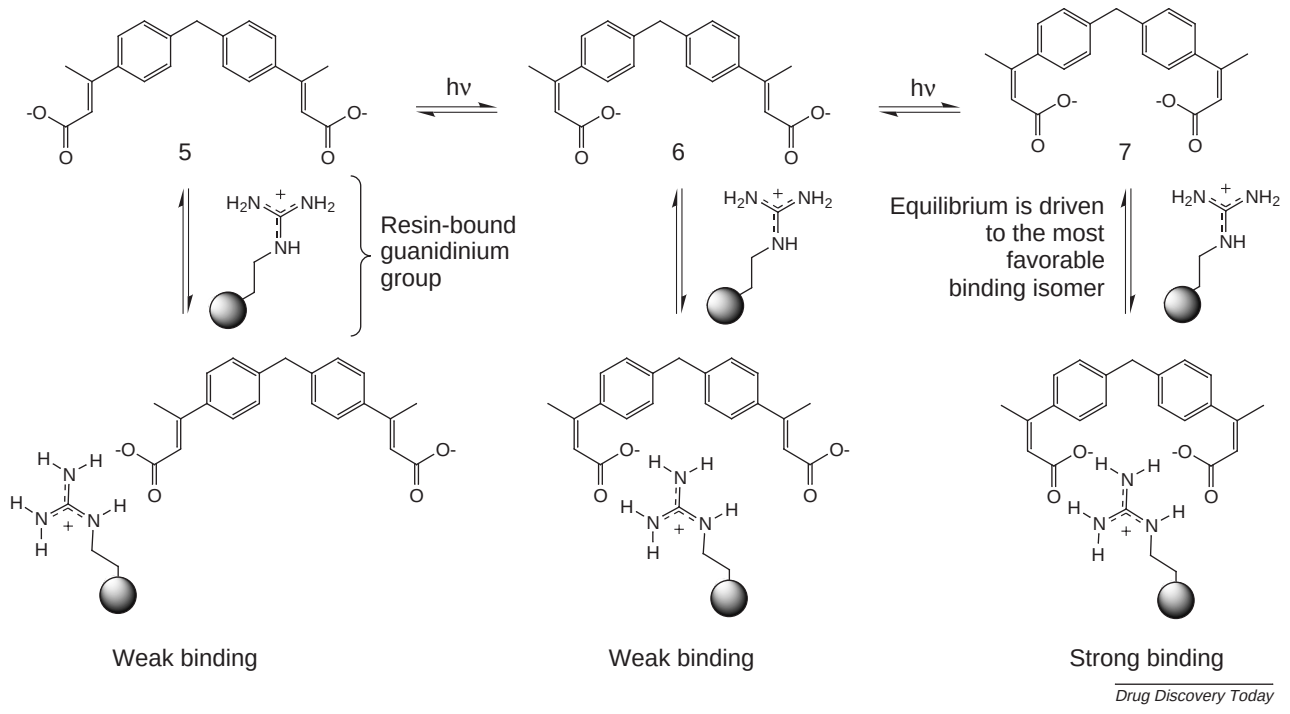

Figure 3. *Photochemical equilibration of three isomeric dicarboxylates followed by cycles of affinity chromatography results in enrichment of compound (7), a selective guanidinium receptor.*

increase the formation of a high-affinity compound.

Reversible formation of imines using carbonic anhydrase

Lehn and coworkers have examined the ability of carbonic anhydrase (CA) to reversibly form imines in a dynamic combinatorial library created from aromatic aldehydes and primary amines[29]. After allowing the mixture to reach equilibrium, an excess of sodium cyanoborohydride was added, thereby fixing the equilibrium concentration by reducing all imines to amines (Fig. 4). Surprisingly, crude recovery of library constituents was 20–30% lower in the presence of CA. This is potentially a consequence of aldehydes reacting with amino functionality (i.e. lysine side-chains) on the periphery of the enzyme, indicating one hurdle that must be surmounted in the process of converting dynamic combinatorial chemistry from an intellectual curiosity to a viable drug discovery method (that of allowing for a rapid interconversion of library constituents, without modifying the receptor).

One solution to this, of course, is the flow-through apparatus described by Eliseev and coworkers as already described. Analysis of the product mixture by HPLC indicated an enhancement of some library members relative to the mixture obtained in the absence of the enzyme, suggesting that the enzyme was able to influence the product distribution. In particular, the presence of CA caused the concentration of compound (8) (Fig. 4), which was derived from the condensation of benzylamine and *p*-sulfonamide benzaldehyde followed by reduction with sodium cyanoborohydride, to double. Meanwhile, production of other library products decreased by >80%. Although no actual inhibition constants for this compound were reported, the similarities of this compound with those known to inhibit CA (Refs 30,31) suggests that the observed results are consistent with a selection process occurring based on CA-inhibitory ability. As a control experiment, a library lacking the components of compound (8) (Fig. 4) was incubated with CA. In this case, CA had no effect on the distribution of amines in solution. Finally, selection experiments were run with a library formed from only the components of compound (8) and one additional amine present. This experiment provided a selectivity ratio for the production of compound (8) of 21:1. Interestingly, when hexyl-4-sulfamoyl-benzoate, a known inhibitor of CA, is added into the reaction, the ratio changes to 2:1.

Receptors for solid-supported tripeptides

Another reversible reaction that has been used to generate a dynamic equilibrium is the formation of disulfide (SS) bonds. Recently, solid-supported peptides have been used to drive the formation of disulfide-linked macrocyclic compounds[32]. Prior to attempting the dynamic diversity experiment, a solid-supported combinatorial library of tripeptides was used to identify a sequence with high affinity for two molecules of compound (9) linked by a disulfide bond [(9)-SS-(9); Fig. 5]. The polymer-bound tripeptide Ac-(D)-Pro-(L)-Val-(D)-Val was found to bind to (9)-SS-(9) with a millimolar binding constant.

With an already known ligand–receptor interaction, the next area to be investigated was the degree to which the original disulfide could be scrambled in the absence of a receptor. Exposure of Ph-SS-(9) to thiophenol (HS-Ph) and triethylamine provided an equilibrium distribution of 35% (9)-SS-Ph, and 65% Ph-SS-Ph + (9)-SS-(9) (K_{eq} = 3.8). When the same experiment was performed in the

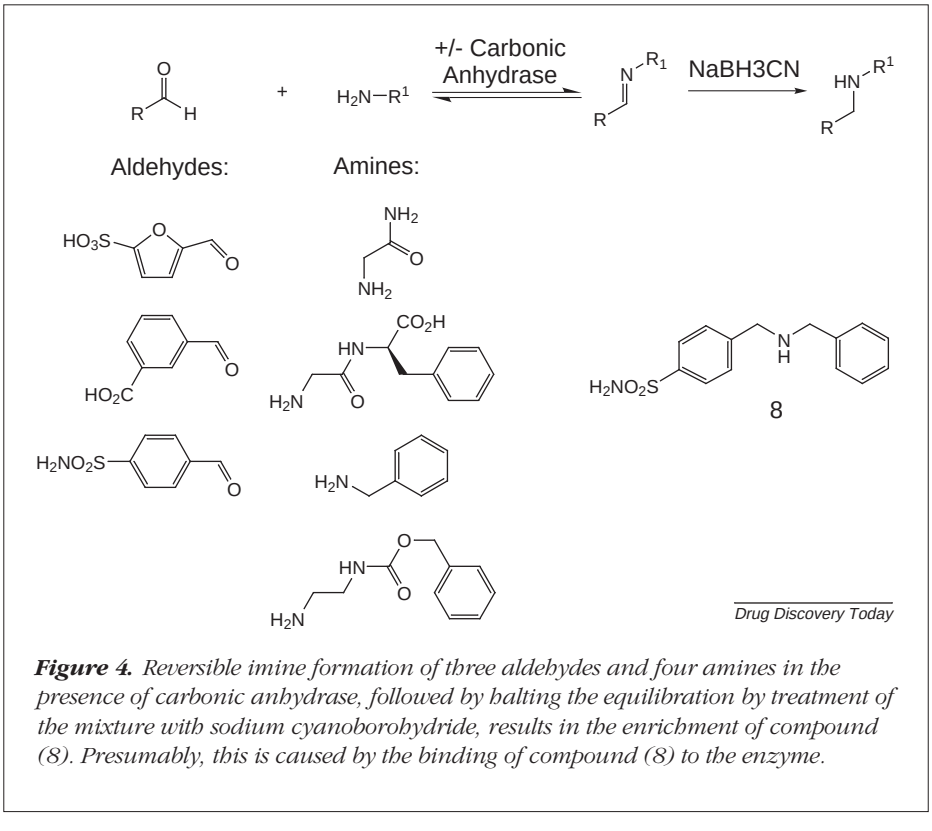

Figure 4. *Reversible imine formation of three aldehydes and four amines in the presence of carbonic anhydrase, followed by halting the equilibration by treatment of the mixture with sodium cyanoborohydride, results in the enrichment of compound (8). Presumably, this is caused by the binding of compound (8) to the enzyme.*

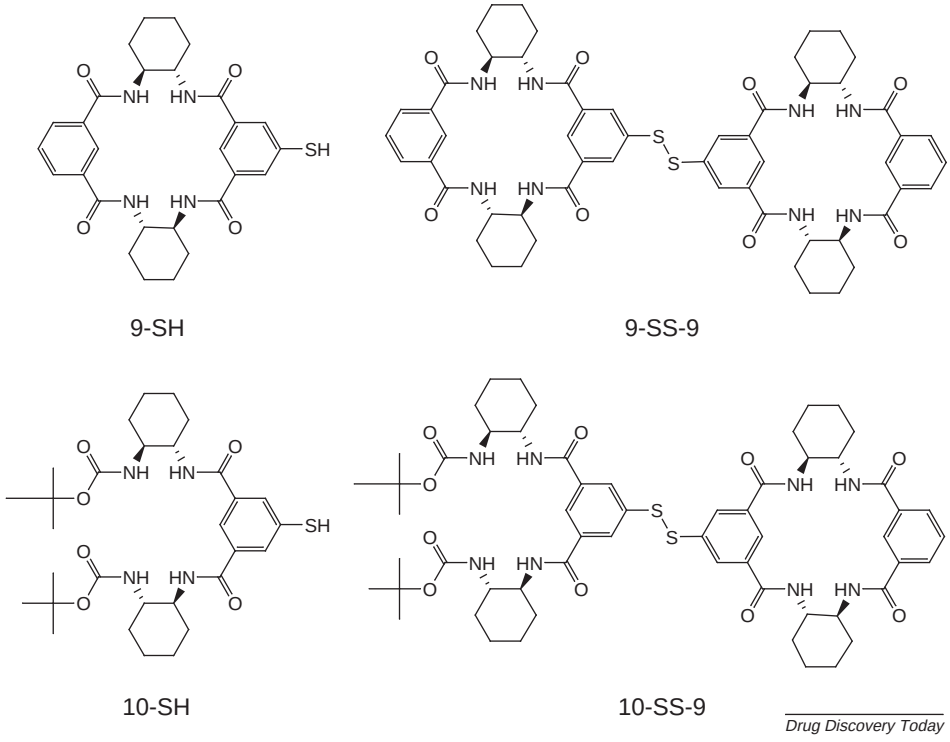

Figure 5. *Compounds used in Ref. 32 in their study of library generation via reversible disulfide-bond formation.*

presence of the tripeptide receptor, K_{eq} = 360, with only 5% of the material remaining as (9)-SS-Ph. After further investigation, it was found that (9)-SS-(9) was the only compound bound to the resin. This simple experiment demonstrated the principles of chemical evolution. However, as Ph-SS-(9) does not bind to the target peptide at all, the system has to evolve in favor of (9)-SS-(9). To provide a more finely tuned selection, the mixed disulfide (10)-SS-(9) [where compound (10) (Fig. 5) is a slightly modified peptide binder] was treated with (9)-SH and (10)-SH. The K_{eq} value between the unsymmetrical disulfide and the two symmetrical disulfides was found to be 1.8 in the absence of a receptor, and 32 in the presence of the tripeptide. The resin contained only a trace amount of (10)-SS-(9), with the remainder of the material being (9)-SS-(9).

Self-selecting macrocycles

Of course, a question that always arises when examining an equilibrating mixture of species is how the relative thermodynamic stabilities of the various compounds affect their concentration. In the presence of a macromolecular receptor, one presumption in dynamic diversity experiments is that (favorable) receptor-binding energy can more than compensate for any differences in stability between the various library members.

Sanders and coworkers have extensively examined reversible transesterification reactions in the absence of a receptor, to determine the ability of such mixtures to 'self-select' certain compositions based on their thermodynamic stability[33]. For example, if the cholic acid-derivative compound (11) is subjected to thermodynamic transesterification conditions, it might be expected that a wide range of cyclic [such as compounds (12)–(15)] and linear esters would be formed (Fig. 6). It was found that the precise ratio of these compounds formed when compound (11) is subjected to reflux conditions in the presence of sodium methoxide in toluene changed significantly depending on the identity of the R^1 and R^2 substituents. For example, compound (16) (Fig. 7) gave isolated yields of 0% (dimer), 17% (trimer), 17% (tetramer) and 10% (pentamer), while compound (17) (Fig. 7) gave isolated yields of 11% (dimer), 31% (trimer), 21% (tetramer) and 0% (pentamer). This self-selection of macrocycles based on favorable intramolecular interactions is directed at a somewhat different goal than studies aimed at selection based on intermolecular binding energies, but depends on similar concepts.

The Sanders group has described similar studies using cinchonidine, quinidine and xanthate monomers[34–36]. In each case, thermodynamic self-selection was observed that provided enrichment of specific macrocycles. This work has been expanded to include the reversible reaction of hydrazide and aldehyde functional groups[37]. Likewise, a conceptually related approach to the self-selection of supramolecular receptors has been described that relies on hydrogen bonding[38]. In an interesting parallel to Sanders' work, Schröder and coworkers have recently described a naturally occurring dynamic combinatorial library of

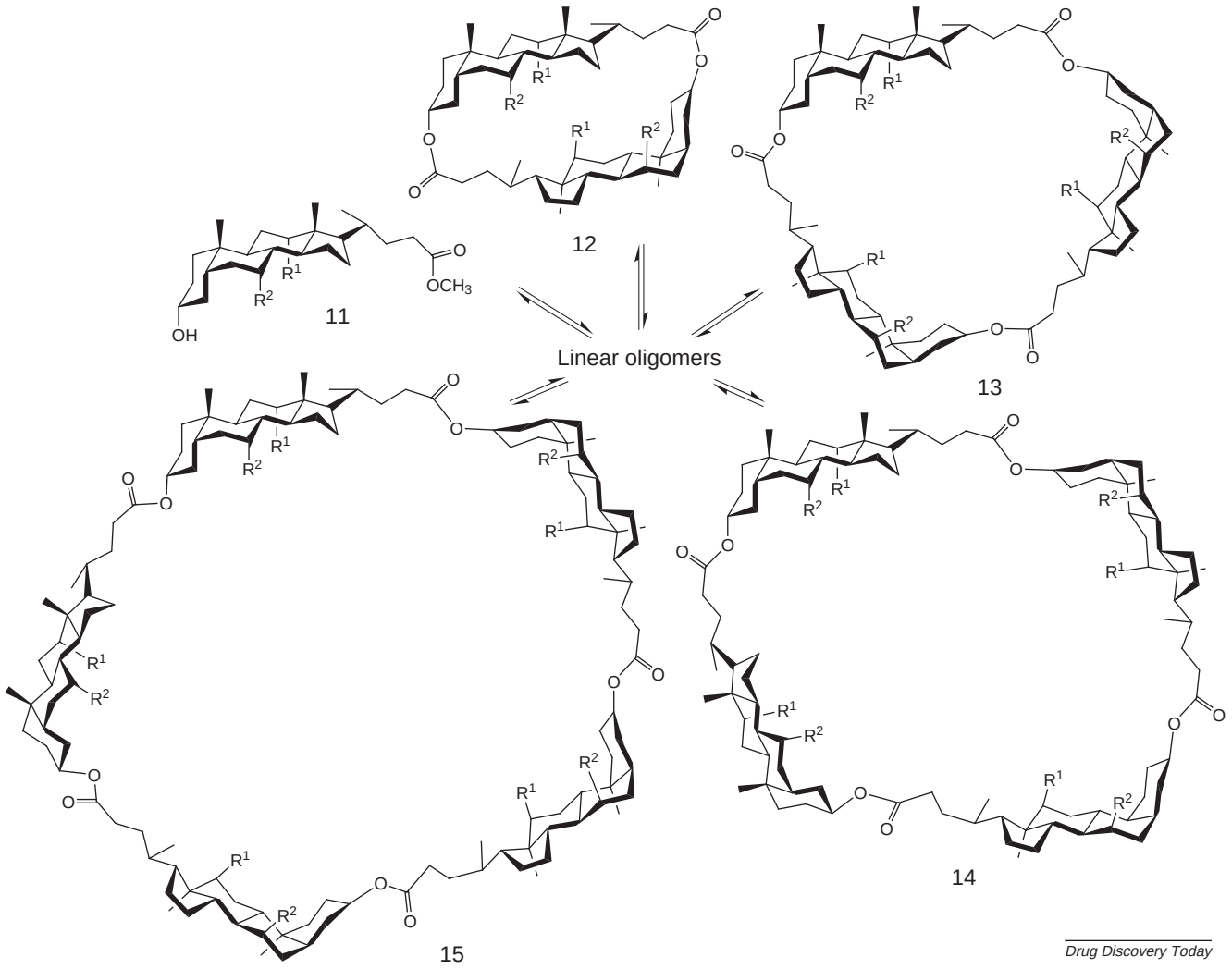

Figure 6. *Subjecting the cholic acid-derivative, compound (11) to conditions conducive to transesterification results in the formation of a series of cyclic oligomers [compounds (12)–(15)] via linear intermediates. A process of 'self-selection' causes the relative proportions of the individual macrocycles to change based on the substitution (R^1 and R^2) of compound (11).*

macrocyclic compounds secreted by the pupal form of *Epilachna borealis*, which are probably used as a chemical defense mechanism[39].

Dynamic libraries of DNA-binding coordination complexes

Reversible imine formation coupled to transition-metal coordination in a multi-step equilibrium has been reported as a method for the generation of DNA-binding compounds[40,41]. Initial studies used a series of six salicylaldimines as library components, and addition of a solution of Zn^{2+} was assumed to initiate equilibration of the 36 unique bis(salicylaldiminato) zinc complexes (Fig. 8). Incubation of this solution with an oligo(dA·dT) constituted the selection process; subsequent trifluoroacetic acid-mediated hydrolysis of the complexes eluted from the affinity resin, followed by derivatization with 2-naphthoyl chloride and HPLC analysis enabled deconvolution of the library. This resulted in the identification of complex (18) (Fig. 9), which was found to have an approximate affinity (K_D) for oligo(dA·dT) of 1.1 μM in a solution-phase assay.

This study proceeded on the supposition that the salicylaldimines were hydrolytically stable in the presence of zinc. However, during spectroscopic characterization (NMR and UV–visible light) of complex (18), this was not found to be the case, suggesting that a significantly more complex equi-

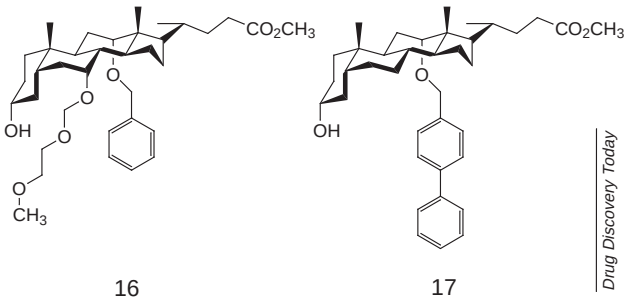

Figure 7. *Two cholic acid-derivatives used in Refs 34–36. As compounds (16) and (17) have different substitution patterns, they 'self-select' for different macrocyclic structures.*

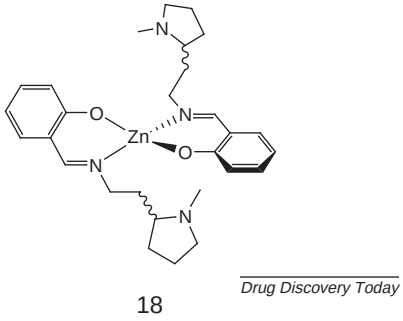

Figure 9. *This DNA-binding bis(salicylaldiminato)zinc complex was selected and amplified both from a library generated from pre-formed salicylaldimines, and from a library in which imine formation was carried out* in situ.

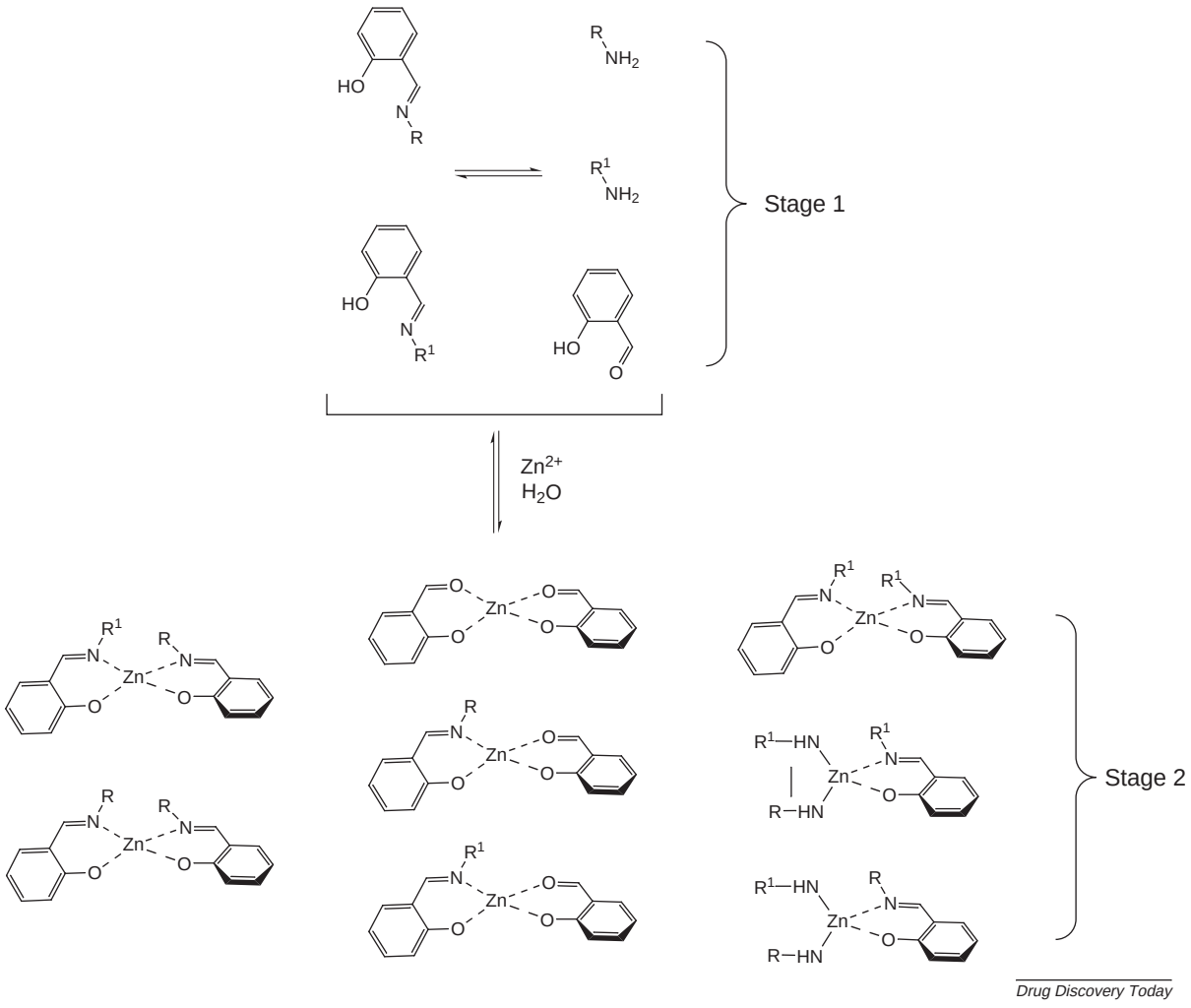

Figure 8. *Multistage equilibration. Free amines, salicylaldehyde and zinc equilibrate with a large number of coordinated species.*

librium was operant (Fig. 8), providing a correspondingly more complex library. Furthermore, this realization prompted the possibility that commercially available amines could just be mixed with salicylaldehyde in a Zn^{2+} solution, the mixture then being allowed to equilibrate in the presence of DNA before selecting for a high-affinity complex. If such a multi-equilibrium process is indeed under thermodynamic control, the point at which the equilibration is started (for example, in this case, as salicylaldimines or as amines plus salicylaldehyde) should not affect the final result. Klekota and Miller found that this was in fact the case[41].

Conclusions

To date, all published examples of dynamic combinatorial libraries have essentially been proof-of-concept studies, and much work remains if this laboratory concept is to be converted into a truly useful tool for drug discovery. Firstly, the range of reactions that can be used in dynamic combinatorial libraries must be increased. Secondly, while synthetic organic chemistry has almost exclusively been directed towards the development of synthetic methodologies that provide the highest degree of irreversability and product selectivity, many synthetic reactions are known that proceed reversibly (under thermodynamic control).

Some reactions that might potentially be adaptable to dynamic combinatorial chemistry including aldol condensations, the Baylis–Hillman reaction[42], olefin metathesis, palladium pi-allyl chemistry, acetal formation and some cycloadditions. As many of these reactions are optimally performed in an organic solvent, a method is required that can enable the separation of library scrambling (the 'mutation' or reversible bond-forming process) from the selection of high-affinity library members. Eliseev's photochemical flow-through reactor is one example of such a separation, and others are certainly conceivable.

Finally, a relatively large quantity of receptor is required for each experiment. This is because of both the sensitivity limits of current analytical instrumentation, and of the fact that all implementations of the dynamic library concept have so far provided (at best) a linear amplification of the highest-affinity ligand yield, rather than the exponential increase observed in

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Collaborations…

Pharmacopeia (Princeton, NJ, USA) and **Hoffman-La-Roche** (Basel, Switzerland) have enetered into a research collaboration focusing on small-molecule agonists for Melanocortin-4 as a novel pathway for the regulation of body weight and late-onset obesity. Under the terms of the agreement, Roche will provide funding for Pharmacopeia to pursue chemical optimization of the lead compounds isolated by Pharmacopeia. Any clinical candidate discovered will then be commercialized by Roche. Joseph A. Mollica, the Chairman and CEO of Pharmacopeia said 'This program is the first of our later-stage internal programs to be partnered. With this new relationship, we look forward to moving out MC-4 project forward without incurring the negative financial impact of a self-funded program.'