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HIGHLIGHT

The rise, fall and reinvention of combinatorial chemistry

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Combinatorial chemistry provides a powerful tool for the rapid creation of large numbers of synthetic compounds. Ideally, these libraries should be a rich source of bioactive molecules, but there is the general feeling that the initial promise of combinatorial chemistry has not yet been realized. In particular, enthusiasm for conducting unbiased (non-structure-guided) screens of large libraries for protein or RNA ligands has waned. A central challenge in this area is to devise methods for the synthesis of chemically diverse, high-quality libraries of molecules with many of the desirable features of natural products. These include diverse functionality, a significant representation of chiral sp^3 centers that provide conformational bias to the molecule, significant skeletal diversity, and good pharmacokinetic properties. However, these libraries must be easy to make from cheap, readily available building blocks, ideally those that would support convenient hit optimization/structure reactivity relationship studies. Meeting these challenges will not be easy. Here I review some recent advances in this area and provide some thoughts on likely important developments in the next few years.

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Introduction

Combinatorial chemistry (combichem) encompasses a set of techniques that facilitates the synthesis of large numbers of compounds, commonly referred to as a "library", much faster and with far less effort than would have been the case if each compound were synthesized individually. Like many technological breakthroughs, combinatorial chemistry has gone through radical swings in perceptions of its utility. While I was not active in the field during its inception, I suspect that the initial reaction was something along the lines of "what a silly idea, that will never work", because my experience is that this is the typical reaction of most scientists to almost any novel concept. However, after the demonstration by the pioneers in the field that combinatorial chemistry was, in fact, feasible,[1–3] the mood turned to what Alan Greenspan, were he a chemist, might have termed "irrational exuberance".[4] Entire conferences, including an annual Combinatorial Chemistry Gordon Conference were devoted to this technique. There was a general feeling that the ability to make so many compounds at once, when combined with advanced robotics and informatics technology, would revolutionize the drug discovery process and make natural products obsolete. Unfortunately, but predictably, as time went by and the field evolved, many technical difficulties and limitations were encountered. The failure of the technology to meet the unreasonably high expectations resulted in something of a backlash against it. The Gordon Conference changed its name to High Throughput Chemistry and Chemical Biology, presumably because no one

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would be willing to come and be photographed at a Combinatorial Chemistry conference any more. In particular, the idea that very large, unbiased combinatorial libraries could serve as rich sources of lead compounds came under attack. During this period, many authors pointed out that, in retrospect, unbiased library synthesis and screening really had been silly after all, because if one calculated the total number of possible chemical compounds of molecular mass 500 containing certain atoms, this would exceed the number of atoms in the universe. This calculation was apparently meant to convey the idea that if one didn't have some idea of where in this enormous haystack one should look, one's chances of finding the needle were essentially zero. The resulting trend was for combinatorial chemistry to be relegated to the creation of small, highly focused libraries for the optimization of leads discovered by other means. For de novo discovery, most commercial and academic efforts turned to high-throughput screening of compound collections.

It would be a pity for this to be the end of the story. The rapid pace of discovery in molecular biology has suggested thousands of potential proteins and RNAs that represent attractive targets for pharmacological intervention in disease, but for which the rational design of an agonist or antagonist is not possible. There remains a clear need for an effective strategy for the unbiased discovery of drug leads and tool compounds. My own view is that combinatorial chemistry, when used imaginatively and appropriately, is likely to play a central role in the discovery of these species. Below I will highlight the most important issues regarding the effective use of this technology in discovery science and highlight some of the most interesting developments in the recent literature. Finally, I will provide some thoughts on significant challenges remaining and speculate on possible important developments that will occur in the near future.

What kind of libraries should we be making?

This is a central question in combinatorial chemistry and, not surprisingly, there are many points of view. The first issue is size. It is reasonable to suggest that if we are talking about unbiased screening, big is better and very big is much better, assuming that the library is truly diverse (see below). I spent many years in Texas, so my views on the desirability of "big" may be biased, but results from peptide library screening projects seem to provide clear evidence that, as one would expect, larger libraries provide more and higher affinity hits than do small libraries.[5] For example, ribosome display technology,[6] which can create peptide libraries of 10^{14} compounds, has tended to provide higher affinity protein ligands than phage display,[7] which provides "only" 10^7–10^9 compounds. The other, less obvious, issue has to do with the nature of the compounds that we should make and their diversity in "structure space". Everyone agrees that more diversity is good in theory, but practical issues also have to be considered and perhaps compromises must be made. For instance, many investigators have pointed out that a problem with the compound collections used in most high-throughput screening campaigns these days is that they are dominated by flat, hydrophobic, aromatic ring-rich molecules of limited three-dimensional diversity.[8,9] They argue that natural products or derivatives thereof have been developed into drugs, so therefore screening collections should more closely reflect the properties of natural products, including a greater richness in chiral sp^3 centers. Recently, experimental evidence favoring this view has been published.[10] The counterargument is that we have lots of excellent methods to make flat aromatic molecules, such as Pd-catalyzed coupling reactions, while, natural product-like, sterochemically rich molecules are typically difficult to make. This is true even using the full armamentarium of organic synthesis and for library construction one is generally restricted to highly efficient reactions compatible with solid-phase synthesis. Otherwise, the resultant library will be of poor quality, which was a major contributing factor to the souring of opinion on the technology in the first place. So the goal is to design diverse molecules that have many of the general features of natural products, but which can be made efficiently. This is no small challenge.

There has been impressive progress along these lines. One particularly interesting advance has been the development of schemes to achieve skeletal diversity through diversity-oriented synthesis (DOS).[11] While it is commonplace nowadays to make small libraries of, for example, a given heterocycle with many different substituents patterns, these libraries are highly concentrated in a small region of "shape space". Therefore, it is of great interest to devise schemes by which many differently shaped scaffolds can be accessed. The most powerful approach to skeletal diversity through DOS employs a "build/couple/pair strategy (BCP) developed by Schreiber and colleagues.[12] Solution phase organic synthesis is employed to create a series of building blocks with the desired properties. These are rigorously purified and characterized. They are then coupled together to generate further diversity, and then subjected to a "pairing" process to drive skeletal rearrangements that, depending on the building blocks employed, generate differently shaped products. A particularly elegant recent example is the work of Nelson and co-workers (Fig. 1).[13] They constructed two related "linkers" with fluorous tags[14] that terminated in either an allylic alcohol or a nosylated amine. These were coupled to one of several chiral "propagating" building blocks, all of which contained differentially functionalized alcohols. Finally, one of several "capping" building blocks, all of which contained a terminal alkene, was appended to the other end of the propagating moiety. Finally, in the "pair" phase, a metathesis catalyst was added.[15] It was assumed that the ruthenium carbene would initiate metathesis at the terminal alkene of the cap, and then initiate a series of cascade reactions. One of these is shown in all it's glory in Fig. 1A. A second example is shown in more abbreviated form in Fig. 1B and a third, where the metathesis cascade skipped one of the alkenes, in Fig. 1C. An ingenious feature of the design is that only molecules that completed a metathesis cascade, which results in cleavage of the double bond in the linker, would be decoupled from the fluorous tag. This enabled facile separation of the desired products from starting material or side products *via* binding to a

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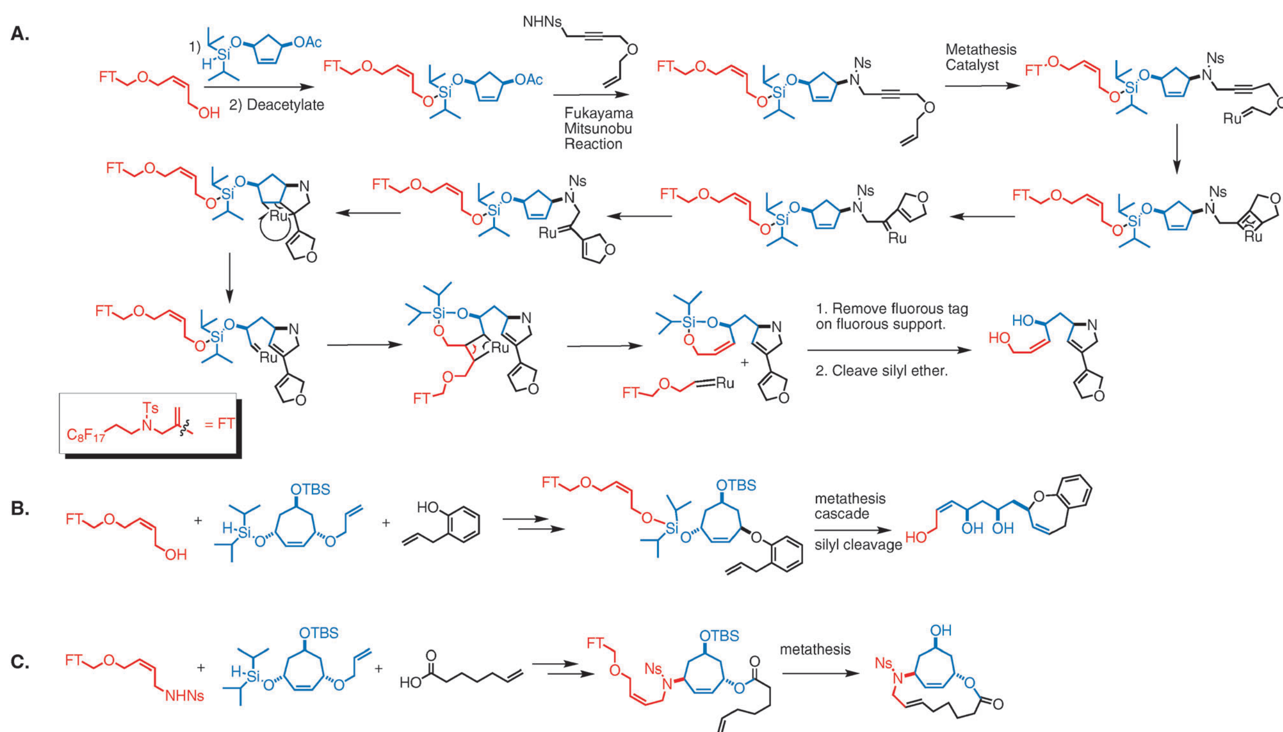

Fig. 1 Combinatorial synthesis of a diverse library of scaffolds by Nelson and co-workers.[13] See text for details. A. One of the metathesis cascades leading to a particular scaffold, including all of the presumed intermediates. B. A different scaffold showing only a key intermediate. C. A metathesis cascade that "skips" the endocyclic double bond leads to a different scaffold.

fluorous support. Using two linkers, eight propagating units and 15 capping units, the authors were able to achieve a remarkable 84 distinct scaffolds. While Nelson, and co-workers did not further elaborate their scaffolds, this certainly could be done, allowing for the synthesis of several thousand differentially substituted compounds of each skeletal type. These molecules can then placed into screening collections.

This kind of chemistry is quite beautiful and imaginative and is likely to increase the quality and diversity of screening collections. There have even been a few reports of bioactive species isolated from such libraries.[16] In its current form however, this methodology is not capable of creating the very large number (millions or more) of compounds that one would ideally like have in hand for unbiased screening campaigns. These strategies are most easily applied to split synthesis efforts, where there is a practical limit of perhaps 20–50 thousand compounds per library. This relates to the need for some sort of purification, or at least purity determination. The reactions employed do not proceed to completion uniformly and in many cases there is the need for compound characterization since the result of each reaction, especially the pairing process, cannot always be predicted with complete confidence (for example, see Fig. 1C).

Synthesis of huge combinatorial libraries

A far more powerful approach, in theory, is split and pool solid phase synthesis of combinatorial libraries (see Fig. 2). In this strategy, originally developed for the chemical synthesis of peptide libraries,[2] resin beads are split into as many different reactions vessels as one wishes to employ and all of the sites on the bead are derivatized with a given chemical. All of the beads are then pooled and mixed thoroughly. They are then re-split for the next chemical operation, for example the addition of a second unit of an oligomer. Very large numbers of compounds can be made this way. For example, if one had a skeleton with six differentially reactive "handles" and at each position appended 10 different substituents, then a library of one million (10^6) compounds would result. While this is very attractive, there are two important limitations inherent in the split and pool scheme. One is that the chemistry employed must be exceedingly efficient. There is no opportunity for purification or even analyzing the purity of more than a tiny fraction of the library.

Second, one does not know *a priori* what compound is on what bead and the scale of the synthesis is such that not enough compound is produced to employ NMR spectroscopy for this purpose. Mass spectrometry is the only technique sensitive enough to be used routinely but this clearly restricts the kind of functional diversity one can have. For example, enantiomers have the same mass. Given this restriction, it is not surprising that this technology, without encoding, has most commonly been applied to peptide-like compounds. For example, libraries of peptoids (oligo-*N*-substituted glycines)[17,18] and β-peptides[19] are readily made by this technique and the structure of the compound on a single bead can be determined by tandem mass spectrometry. As will be described below, these

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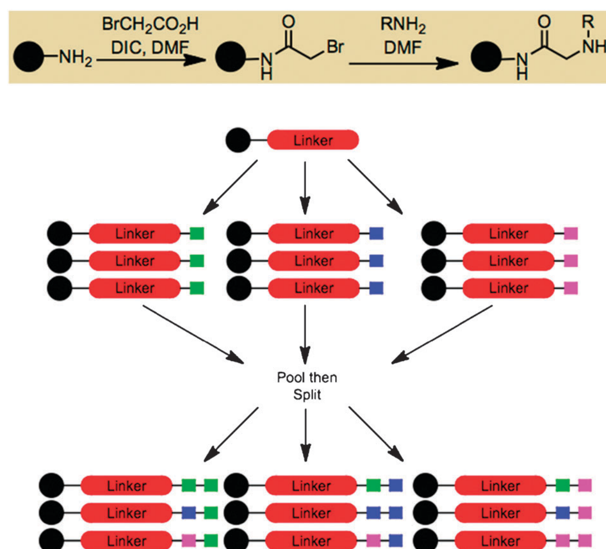

Fig. 2 The split and pool strategy for combinatorial library synthesis as applied to peptoids. A bead (black circle) is primed with a suitable linker terminating in a primary amine. The activated ester of 2-bromoacetic acid is then added to each bead. In this example where three different side chains are desired, the beads are split into three different flask and a different amine (colored squares) are added to each bead. The beads are then pooled and the process is repeated to provide a one bead one compound library of $3^2 = 9$ compounds.

libraries have proven to be reasonably good sources of protein ligands but have yet to produce truly useful probe molecules.

To escape the limitations of direct characterization from a single bead, many different encoding strategies have been developed.[20,21] Perhaps the most appealing of these is DNA encoding. This idea[22] is based on the powerful biological systems available for screening ribosome-synthesized peptide libraries. Phage display,[7] ribosome display,[6] *etc.*, all share the central feature that the peptide and the DNA that encodes it are linked physically. For example, in phage display, the peptide is displayed on the surface of the phage while the encoding DNA is encapsulated within the phage particle. In the screening process, one mixes soluble peptide-displaying phage, for example, with a soluble protein target and then remove that protein from solution, for example through immunoprecipitation. Any peptides bound to the protein will come along, dragging their encoding DNA with them. The binders can be amplified and the process is repeated. After several rounds of enrichment, the viruses are collected and the encoding DNAs are sequenced to reveal the identities of the peptide ligands.

The appeal of this kind of encoding is the unbelievable sensitivity and power of modern DNA sequencing technology. Even a few molecules of DNA associated with the target can be amplified easily using the polymerase chain reaction (PCR) and recent advances in massively parallel DNA sequencing allow tens of thousands of "reads" to be obtained in a single experiment, allowing facile evaluation of the entire population of DNA encoding tags associated with the target protein. Various strategies exist to export this approach to the world of synthetic libraries.[23,24] Perhaps the most relevant for the unbiased screening of large libraries are a "DNA routing" system from the Harbury laboratory[25] and a chemoenzymatic duplex DNA tagging strategy reported by Clark, Morgan and co-workers at Glaxo-Smith-Kline (GSK).[26] Both of these schemes employ split and pool chemistry to elaborate an amine-functionalized DNA, though the nature of the DNA tags and the methods of their construction are different[27] (see Fig. 3 and 4). Both have been shown to be effective. The Harbury study reported a DNA-encoded library of 100 million peptoids. They screened this against the SH3 domain of Crk and identified peptoids that bound with affinities in the low to mid micromolar range. The best peptoid hit ($K_D = 16\ \mu M$) bound about as well as a peptide selected from a display library.

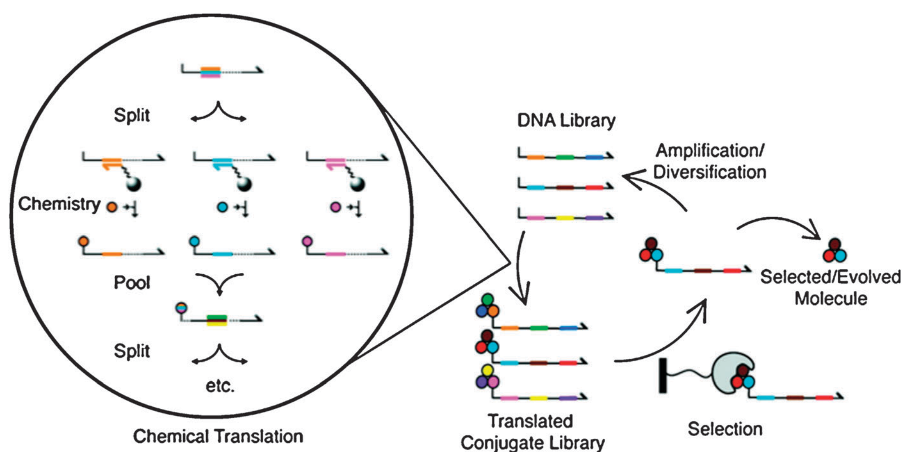

Fig. 3 The Harbury strategy for DNA-template routed split and pool, DNA-encoded library synthesis and screening. In this scheme, the sequence of the DNA tag routes compounds to the appropriate reaction vessels that contain the complementary sequence. Reprinted with permission from ref. 38.

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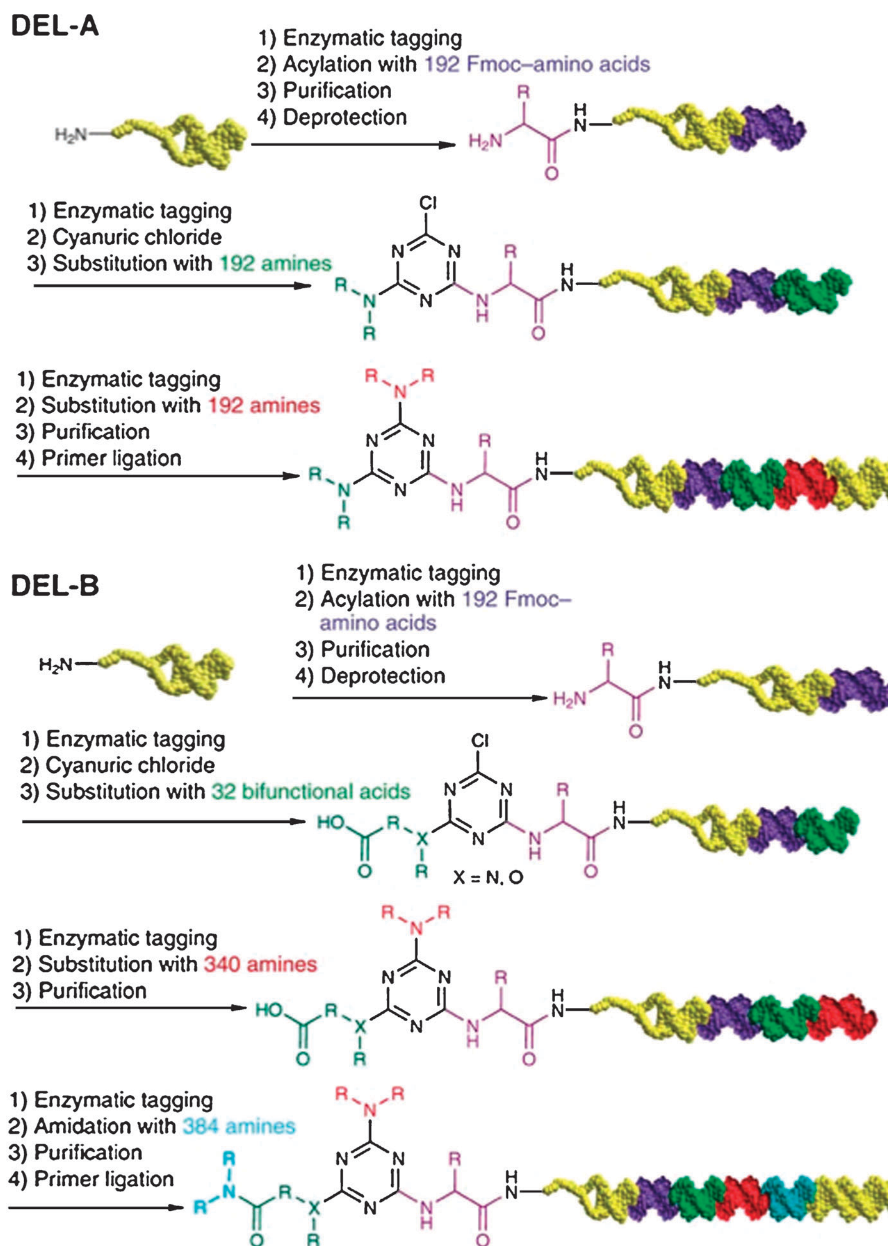

Fig. 4 A double-stranded DNA-encoded library of cyanuric acid-derived molecules. The different colored duplex DNA fragments encode the chemical being added in each synthetic step. Reprinted with permission from ref. 26.

Clark, Morgan and co-workers constructed a library of 800 million cyanuric acid-derived molecules and screened against the Aurora A and p38 MAP kinases. Low nM inhibitors of the kinases were obtained. In all of these experiments, the soluble small molecule-DNA tag conjugates are incubated with a protein target that is then precipitated, bringing along some DNA-small conjugates with it. Several rounds of such a protocol must be done before the level of enrichment of such hits is sufficient to allow for productive analysis. At this point however, deep sequencing methods can be used to identify many thousands of the bound molecules,[28] providing an "instant SAR" data base. This is a unique advantage of extremely large libraries decoded by deep sequencing.

It is reasonable to speculate that, in the future, the combination of some of the more imaginative chemistry discussed above with a DNA-encoded library format will provide researchers with a previously unheard of number of interesting compounds with which to carry out screening campaigns. While it is true that not all reactions that a chemist would like to use are compatible with the presence of an encoding DNA, the advantages of this technology far outweigh the limitations in most cases.

Screening combinatorial libraries for bioactive compounds

While this review focuses on combinatorial chemistry and not screening per se, the

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two are difficult to separate completely and, as mentioned above, how one screens influences the design of the library one makes.[29] The most common screening modality employed currently is functional screening. In this strategy, one or more compounds are dissolved in the wells of a microtiter plate along with the biological target of interest. An assay is then carried out in each well that (ideally) reveals if the compound(s) present engenders the desired activity. In some cases, the target may be a known protein and the assay measures its activity. My own view is that this kind of screening will be displaced over the next several years by binding screens using large encoded libraries. Functional screens carried out in 384 or 1536 well plates are quite expensive and are practically limited to, at best, one million or so compounds. They require much specialized equipment and expert staff. Binding screens can be done in batch format and are much less expensive. By using libraries designed to allow direct determination of the structure of the molecule on a bead, several hundred thousand to a few million compounds can be screened at once,[30–32] while the DNA-encoded libraries allow tens to hundreds of millions of compounds to be analyzed. Finally, binding screens can be designed to ensure high selectivity of a hit, whereas this is often difficult to do in functional screens. A binding screen using a soluble protein target can be contaminated with a huge excess of highly diverse competitor proteins to block binding of relatively non-specific "greasy" compounds in the library to the protein of interest. We have found this to be extremely important in obtaining high selectivity ligands.[33] If the target protein is an integral membrane receptor, one can screen for molecules that ignore all other cell surface proteins in favor of the target by including differentially labelled cells that are identical except for the fact that they do or do not display the target receptor.[34] However, microtiter plate-based functional screening will not go away, since it is the only way to carry out phenotypic screens. Live animals such as zebrafish or worms, in addition to cell lines, can be used in such efforts, so this mode of screening can select for favorable pharmacokinetic properties in addition to simple activity, which binding screens cannot do.

Hit optimization

Perhaps the single biggest problem with how most high-throughput screens are done today is that hit optimization is often not incorporated into the overall design of a screening campaign. This is especially a problem with compound collections in which little thought has gone into issues downstream of obtaining screening hits. It is very important to understand that the compounds that arise from a primary, unbiased screen are unlikely to be of great utility as either a tool compound or a drug lead. Almost always, many derivatives must be analyzed for improved potency, selectivity or pharmacokinetic properties. Too often, derivatives of hits from compound collections are not trivial to synthesize and their optimization must await the attention of several skilled organic chemists, creating a fatal bottleneck in the development of truly useful bioactive compounds. Thus, libraries must be designed with this in mind. They should be modular and composed of pieces where analogues can be made form commercially available or very easily synthesizable pieces. Peptoids (Fig. 2) are a good example of this. They are easily made (even by molecular biologists) using a simple two-step solid phase synthesis protocol. The diversity comes from primary amines of which there are thousands available commercially. Thus, if a hit contains, for example, a critical benzyl side chain, one can buy or make many analogues that have a methyl here, a chlorine there, *etc*. One can then imagine synthesizing a library of compounds that resemble the hit, but have side chains altered relatively conservatively to begin to optimize the fit of each important side chain to the protein target.[35,36] Peptoids are perhaps not the ideal scaffold for this kind of work in the long run since they are "floppy" and contain relatively limited chemodiversity. But more elaborate libraries of oligomers with the favorable qualities of peptoids, but incorporating more conformational constraints and greater chemodiversity would be extremely interesting, particularly if combined with DNA-encoding technology.

The need for compound optimization also raises the issue of a facile method by which to obtain quantitative data regarding the potency of derivatives of a given hit. Auer and co-workers have described an approach in which each of the molecules in a OBOC library is equipped with an alkyne tag that allows fluorescent labelling of molecules after a screen.[37] They have shown that when tagged and released from the bead, there is enough compound from a single 90 micron bead to support several fluorescence polarization experiments, allowing the K_D of the hit-protein complex to be determined without the need for re-synthesis. My own laboratory has described a different system in which all of the hits from a OBOC library screen are cleaved and printed onto glass slides without re-synthesis.[31] Titrations using the target protein on the slides can reveal the relative binding affinity of each hit. These sorts of assays will be extremely useful in optimization efforts, since they will both identify the best binders in a derivative library and provide quantitative information regarding the effects of various substituents on the K_D of the complex.

Summary

Combinatorial chemistry is a powerful tool for the creation of compound libraries as a potential source of bioactive compounds. While it has undergone a roller coaster history, particularly with regard to their utility in unbiased screening efforts, I believe that some of the advances discussed above have poised this area for explosive growth in the near future. We are not quite there yet. Not all of the cutting edge technologies have achieved general acceptance and the creation of truly interesting natural product-like libraries is still in its infancy. Putting all of the pieces together to create technology platforms that will allow the routine screening of tens to hundreds of millions of interesting molecules, followed by equally routine hit optimization, will require more work. But, in my opinion, within five years it will be possible for the leading laboratories in the field to routinely identify highly selective nM compounds for most protein targets in a highly efficient fashion. This is likely to have a significant impact on biological research and drug development.

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