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Perspective

Applications of Combinatorial Technologies to Drug Discovery. 2. Combinatorial Organic Synthesis, Library Screening Strategies, and Future Directions[1]

Eric M. Gordon,* Ronald W. Barrett, William J. Dower, Stephen P. A. Fodor, and Mark A. Gallop
Affymax Research Institute, 4001 Miranda Avenue, Palo Alto, California 94304

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Biographies

Ronald W. Barrett received his Ph.D. in Pharmacology from Rutgers University in 1983 and did postdoctoral work at the Addiction Research Foundation in Palo Alto, CA, in the field of opiate receptors. From 1986 to 1989, he worked in the Neuroscience Research Area at Abbott Laboratories in Abbott Park, IL. He joined Affymax in 1989 where he established the Receptor Pharmacology Group. Dr. Barrett is presently Vice President and Director of Receptor Pharmacology.

William J. Dower received his Ph.D. in Biology from the University of California, San Diego, and did postdoctoral work at Stanford University studying steroid control of specific mRNA metabolism and the amplification of genes mediated by chemotherapeutic agents. He joined Bio-Rad Laboratories in 1984 where as a member of the newly formed molecular biology unit, he introduced the electroporation technique for the high-efficiency transformation of bacteria. He joined Affymax in 1989 to establish a molecular biology research group. Dr. Dower is Director of Molecular Biology at Affymax.

Stephen P. A. Fodor received his Ph.D. in Chemistry at Princeton University and was an NIH postdoctoral fellow in Chemistry at the University of California, Berkeley. He joined Affymax in 1989 as a Staff Scientist in Optical Technologies, becoming Director of Physical Sciences. Dr. Fodor's group led the development of new technologies, merging photolithography with combinatorial solid-phase chemistry. In 1993 he joined Affymetrix as Scientific Director, where he is currently using oligonucleotide arrays to study a variety of DNA molecular recognition processes.

Mark A. Gallop received his Ph.D. from the University of Cambridge, England, for research in the area of organo transition metal chemistry and did postdoctoral work in the laboratories of Peter G. Schultz at the University of California, Berkeley. He joined Affymax in 1990 and is currently a Senior Scientist and leader of the Combinatorial Chemistry Group.

Eric M. Gordon received his Ph.D. in 1973 from the University of Wisconsin—Madison and did postdoctoral work at Yale University. His research interests include the rational design of enzyme inhibitors, the chemistry of amino acids, peptides, and natural products, and combinatorial chemistry. Dr. Gordon has authored 150 papers and U.S. patents in these areas. From 1974 to 1992 he worked at the Squibb Institute for Medical Research (subsequently the Bristol-Myers Squibb Pharmaceutical Research Institute) in Princeton, NJ, most recently as a Director of Medicinal Chemistry. In 1992, he joined the Affymax Research Institute where he is currently Vice President of Research and Director of Chemistry.

A. Combinatorial Organic Synthesis

The notion of creating huge, searchable libraries of small organic molecules is unprecedented in medicinal chemistry, and the possibility of doing so has recently captured the imagination of the drug–discovery community. The conventional paradigm of small molecule lead development, in which a compound undergoes many rounds of individualized, hand–crafted modification and biological testing en route to drug candidacy, will likely be dramatically accelerated by the application of combinatorial chemistry technologies to mass–produce and evaluate lead analogs. The ability to harness molecular diversity techniques as tools for lead discovery offers an unparalleled opportunity for medicinal chemistry to expand the breadth and scope of molecular structures that may be screened for biological activity. Widespread availability of collections of highly diverse small–molecule libraries should provide an opportunity to assess the impact of combinatorial organic synthesis on new–lead discovery. In this section of part 2, some of the issues which confront the practitioner of combinatorial organic synthesis, as they relate to the problems of molecular recognition in general and medicinal chemistry in particular, will be analyzed.

Issues in Practicing Combinatorial Organic Synthesis

Combinatorial organic synthesis (COS) presents somewhat of an intellectual inversion of the past 50 years of synthetic organic chemistry. The chemist of the Woodwardian era was interested in a masterful and carefully

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plotted natural product synthesis of a complex entity of known structure. Reactions were more often employed or developed to solve specific challenges rather than to provide generic methodologies. Rigorous control of reaction pathways, stereochemistry, and regiochemistry, and the exclusion of all but the desired diastereomer were obligatory in a faithful rendering of the technique. In contradistinction to natural product total synthesis, rather than generating a single, specific entity, the goals of COS are to create *populations* of molecular structures. Rather than exercising complete control, the combinatorial chemist, while maintaining high reaction efficiency and relative reactive compatibility, may actually seek to create situations and apply strategies in which stereochemical/regiochemical control is relaxed. This must be achieved while remaining cognizant of the impact these factors may have on the stoichiometry of the resulting libary and its design and ultimate use. Hence, the combinatorial chemist seeks to apply a series of Woodwardian reactions (reliable, high yielding) that operate generically on a diverse set of building blocks to provide a multitude of related products.

Criteria for Library Design. The primary objectives of producing small-molecule libraries by COS are to provide collections of compounds suitable for both drug-discovery screening and drug-development optimization. When complete, the combinatorial drug-discovery exercise should have created a stable population of low molecular weight entities, free of reactive and toxicity-causing functionality. While a paramount medicinal chemistry design criteria for small-molecule-libary construction might be that the *products* of diversity generation (individual library members) should "look" like drug leads, of still greater importance is that the library actually contains compounds capable of interacting at some detectable level with the biological target of interest. When small-molecule leads for a target have been previously defined (e.g., benzodiazepine ligands for a peptide or other G-protein-coupled receptor, transition-state inhibitors for a protease), the notion of searching for more potent derivatives among libraries combinatorially enriched in specific pharmacophore analogs is an obvious tactic to pursue. However, as the universe of well-defined macromolecular drug targets continues to expand through the impact of molecular cloning, the problem of identifying new pharmacophores capable of modulating the various interactions of peptides, proteins, carbohydrates, oligonucleotides, or lipids at these sites will also be intensified.

Will "rules" about the types of libraries that may prove generally useful in ligand discovery be discovered? Although the field of molecular diversity has not yet matured to the point where substantial insight into this question is forthcoming, it is intuitively obvious that small-molecule libraries, whose members structurally resemble historical leads, should provide a fertile reservoir of potential molecular diversity. Tangential to this, natural products aside, numerous historical drug leads were derived simply because synthetic routes to these molecules were readily available. It is likely that early-stage COS will be limited by applicable chemistry and that this will necessarily focus work toward traditional leads, whose syntheses are known and well-documented.

The successful identification from recombinant libraries of L-amino acid-based peptide ligands that inhibit protein–peptide, protein–protein, and protein–carbohydrate interactions suggests there is broad utility in screening large libraries of peptidic compounds. It remains to be seen whether collections of other random molecular structures that are quantitatively as diverse as existing peptide libraries prove in *de novo* ligand discovery to include the "pharmacophores of the future".

Ligand rigidity may be another important parameter to consider in the course of library design. The incorporation of conformational constraints into flexible lead molecules has emerged as a powerful strategy to enhance ligand potency and/or selectivity, particularly in the field of peptidomimetic medicinal chemistry.[2-10] Nevertheless, with regard to library design, conformational restriction may act as a two-edged sword: an inappropriate constraint is likely to abrogate the modest but perhaps detectable activity of a more flexible analog, which could, in a secondary library, be systematically constrained. From the point of view of random screening, it remains to be determined whether useful leads will arise more frequently from libraries of rigidified or flexible structures. Data from the evaluation of cyclic peptide libraries in both synthetic and recombinant systems may provide some important insights into this issue. A number of methods have been recently described for on-resin cyclization of peptides through both main-chain and side-chain functional groups.[6-10,68] At present, a portfolio of libraries containing both conformationally rigid and relaxed molecular diversity seems most appropriate. A longer range solution might be to moderate the high risk of conformational restriction by creating very large populations of semirigid molecular arrays, comprising structural families that collectively sample as completely as possible all regions of conformational space.

Characterization. The usual measures of evaluating success in organic synthesis may lose meaning in COS. The classical notions of such fundamental concepts as purity/homogeneity, yield, exact product structure, relative and absolute stereochemical control, specific physical properties are less relevant when applied to a broad population of molecules (of course they may become quite relevant as individuals emerge from a selection process). Additionally, the analytical mainstays of the synthetic organic chemist, such as NMR and IR, may become obviated. The NMR spectrum of a 10 000-component library mixture is not diagnostic. The loss of these powerful tools requires that compensating technologies be developed. A major dilemma of COS is the difficulty of confirming the degree to which the expected chemistry has proceeded on the entire population of substrate molecules. Several groups have recently reported on the use of electrospray mass spectrometry as a technique for evaluating the bulk composition of diverse peptide libaries.[11,12] Gross synthetic discrepancies, such as incomplete protecting group removal, may be detectable by mass analysis, providing an opportunity to optimize the library synthesis protocols. In the characterization of combinatorial products, the presence of "byproducts" (in COS, unexpected products), combined with the difficulty of detecting these compounds, will cause problems if one mistakenly concludes that a screening hit is the expected product. This section will conclude by offering a potential solution to this problem.

Efficiency/Automation. Among the chemical criteria relevant for small-molecule-library design is the efficiency of diversity creation. The assembly of most small molecules reduces to the intercombination of only three to five building blocks of molecular weight ~150 each. Synthetic reactions capable of combining numerous building blocks simultaneously constitute a highly efficient form of diversity generation. Thus the Ugi four-component reaction has a high combinatorial efficiency since building blocks of four families (amines, carbonyl compounds, isocyanides, and suitable acid components) are linked simultaneously to afford α-amino acid derivatives. In contrast, peptide chemistry traditionally links two building blocks at a time. In both the broad screening and the lead analoging modes, a longer range question pertains to the ability of the chemistry to eventually be automated. Once the key decisions and overall strategy have been determined, much of the actual chemistry is repetitive in nature. Machines will continue to be constructed to capitalize on this and libraries will be assembled under computer control.[13-15]

Quantity and Quality of Diversity. While the "quantity of diversity" that is experimentally accessible can be dictated by the number of building blocks in the basis set and by the number of synthetic operations applied, or able to be applied (see part 1[1]), the practical limitations on library size are most generally imposed by the format within which the diversity is created and evaluated. A small number of building blocks subjected to many synthetic steps will yield high (numerical) diversity; however the products of these reactions may be relatively large molecules, not well-suited for lead development as traditionally administered therapeutics. Thus, as the combinatorial process proceeds, an opportunity window may exist in which the bulk of the library possesses properties which standard medicinal chemistry usually seeks in small-molecule drug discovery (MW < 700, solubility, etc.). Continued application of the combinatorial process will lead to product libraries containing larger molecules (composed of more building blocks) wherein the individual library members have "outgrown" the classical criteria of a lead-drug molecule.

In surveying the historical landscape of drug discovery, there are particular pharmacophores or structural arrays which periodically surface far in excess of random chance (benzodiazepines, β-lactams, imidazoles, phenethylamines, etc.).[†] A review of recent successes in the era of "rational drug design" suggests that certain molecular concatenations—protein turn mimetics, conformationally restricted amino acids, transition-state analogs, dipeptide isosteres, molecular scaffolds, designed elements for enzyme inhibition—are often found in the medicinal chemistry of lead compound development. In consideration of the molecular structures which have left their mark on modern medicinal chemistry, one might conclude that the drug-discovery process is impacted not only by the sheer quantity of diversity surveyed, but additionally by the more subjective "quality" of diversity that is evaluated. Different organizations and individuals will certainly bring a wide variety of criteria to the subjective appraisal process, depending on style, experience, and bias. It may be speculated that the quality of diversity will be influenced by the sophistication of the building blocks originally introduced into the combining system (library bias on the part of the medicinal chemist) and the extent to which molecular substructures of the building blocks can be assembled in diverse, spatial (3-D) relationships. Thus the collected expertise of medicinal chemical knowledge may be used to "hyperevolve" or "bias" the libary by the planned introduction of commonly evolved elements; these elements are "retrocombinatorial synthons" of many known bioactive classes. Thus the building block basis set must be judiciously chosen and carefully attuned to the collected knowledge historically amassed in drug discovery.

Issues in the Selection of Building-Block Sets

The acquisition of a building-block library can be a major time and resource investment, and the eventual decision of which type of chemical building blocks to utilize places limits on the universe of structural diversity which ultimately can be explored. Depending on the specific objective, important building-block criteria include the availability of a large number of diverse, fairly complex, easily accessible starting materials. These may be either commercially available or prepared in a few steps from commercial materials. Members of a building block set should reflect a broad array of physicochemical properties, functionality, charge, conformation, etc. Building blocks may be chiral, achiral, or racemic. Certain building-block families have what may be termed a high "combinatorial potential". This relates to the high density per carbon atom of reactive functionality which can participate in new covalent combinations. For example, monosaccharides have high combinatorial potential since the high density of available hydroxyl groups leads to many potential connecting permutations. In addition to polymer formation, the high combinatorial potential of such types of building blocks may also be exploited as scaffolds for the generation of diversity (*vide infra*).

Synthetic Strategy

An important strategic element in combinatorial library synthesis is the degree of reliability of the ligand synthesis chemistry. What is the likelihood of general synthetic success with a particular reaction? The nature of combinatorial reactions, which must proceed in the face of a broad range of functionality on a multitude of substrates and where the products are difficult to analyze individually, demands that, in selection of synthetic methodologies, greater weight must be given to reaction sequences with reliable, predictable outcomes. A more subtle question revolves around the number of synthetic options available in the course of diversity generation. For example, a synthetic strategy structured in such a way that, as the process proceeded, new combinatorial possibilities opened up, would be preferable to having options narrow, especially if the goal was generating a maximum of structural diversity.

As previously noted, there are two distinct themes that must be considered for the successful application of combinatorial technologies to ligand discovery and optimization, *viz.* broad-based screening and directed chemical analoging. The issues underlying conceptual design, as well as the synthetic strategies utilized in construction of these different classes of libraries, are noteworthy and are summarized in Figure 1. Building block requirements for undertaking broad and narrow diversity searches differ markedly. The search for an initial lead molecule may be essentially a random screening exercise, where the em-

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[†] An interesting aside regarding these important substructures is that development of "generic" syntheses of key pharmacophores ultimately enabled facile generation of many analogs. Concurrently or subsequently, diverse biological activities were found among these compound classes. In a sense, this is suggestive of combinatorial chemistry, except the crucial molecules were made serially rather than in a parallel/combinatorial high throughput fashion.

Broad Screening	Chemical Analoging/Optimization
huge size library	modest size library
broadest structural diversity	relatively narrow structural diversity
no special initial structure goal	specific structural goal
any building blocks	specific retrocombinatorial building blocks
undefined order of reaction	specific order of combination
flexible synthetic strategy	well defined synthetic strategy
site of tether not crucial	tether crucial-build in redundancy
ligand possibly uncouplable	ligand should be releasable
single selection evolution	cumulative selection evolution

Figure 1. Combinatorial chemistry: comparison of two major themes.

phasis is on exposing a macromolecular drug target to the maximum possible structural diversity. The objective is to identify a ligand of significant affinity for the target, the exact ligand structure and its detailed characteristics at this point are not relevant: in fact any molecule will do. An approach to generating highly diverse libraries for use in medicinal chemistry might favor using building blocks which have distinguished themselves by appearing frequently in previous active leads (e.g., statine, hydroxyethylamines, Freidinger lactams[16]). On the other hand, once a lead is available, most drug discovery proceeds through a series of evolutions (optimizations) in order to meet a set of predetermined criteria. Since specific structural types are sought, searching in a very broad pool of diversity (as above) is unlikely to be successful (actually it could uncover a new lead but is less likely to optimize an existing one). Ideally, what is required in this type of diversity-generating strategy is to "explode" around the known lead, i.e., to create as highly diverse population as possible that bears close structural resemblance to the original hit, followed by a selection for desired criteria.

Clearly the subunits which lead to predetermined structures must be quite specific: from where should building blocks for known structural classes of pharmacophores arise? The answer, as in organic synthesis, lies in a retrosynthetic analysis or what we may term a *retrocombinatorial* approach to building-block selection. Lead structures should be retrosynthetically dissected in the maximum number of ways and upon these various possibilities imposed the needs of performing combinatorial chemistry. Inspection of the retrosynthetic tree invites the following key questions: By which modes of forward synthesis are the most building blocks available or obtainable? If the synthesis is allowed to proceed by that course, what is the scope and degree of reliability of the necessary reactions? Extending this line of reasoning should permit the maximum leverage to be applied combinatorially.

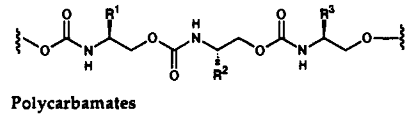


Figure 2. Structure of a synthetic oligocarbamate prototype.

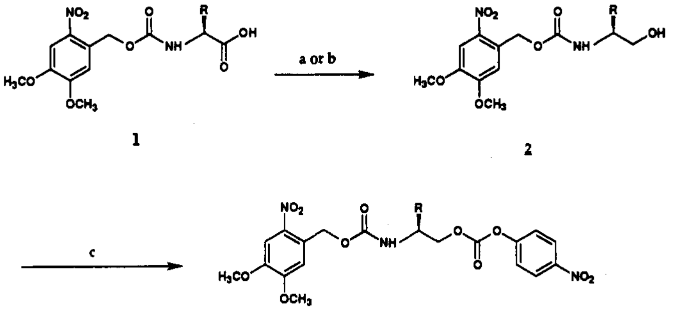


Figure 3. Synthesis of N-protected nitrophenyl carbonate monomers. Key: (a) BH_3, THF; (b) DCC, methylene chloride, *N*-hydroxysuccinimide, HOBt; then sodium borohydride, ethanol; (c) *p*-nitrophenyl chloroformate, methylene chloride, pyridine.

A common feature of both paradigms is likely to be a reliance on solid-phase-synthesis methods to facilitate the assembly of combinatorial libraries. Synthesis on a polymeric support greatly simplifies the problem of product isolation from reaction mixtures and also facilitates the partitioning of products into multiple aliquots for subsequent chemical elaboration. Moreover, the opportunity exists to take advantage of the support-tethered diversity in the design of convenient receptor binding assays for library evaluation. While there has been a long tradition of polymer-supported organic chemistry,[17-20] it is only in the areas of peptide and oligonucleotide synthesis that solid-supported chemistry has truly been optimized and become common-place. The advent of combinatorial organic synthesis will undoubtedly signal a renaissance in solid-phase organic chemistry, as workers attempt to adapt well-characterized homogeneous reactions to reliable solid-supported protocols.

Progress to Date: Synthetic Polymeric Diversity

The design and synthesis of novel synthetic monomers which, when assembled in a combinatorial fashion, could yield relatively low molecular weight polymeric materials is an approach that is well-suited to diversity generation and evaluation. Combinations of such monomers could lead to substances with novel backbones, possibly possessing desirable properties, such as metabolic stability, enhanced pharmacokinetic profiles, and cell and membrane permeability. Identification of these and other potentially modifiable parameters in such systems could facilitate drug discovery.

Schultz and co-workers have reported the synthesis of a library of oligocarbamates starting from a basis set of chiral aminocarbonates[21] (Figure 2). The monomeric units were readily obtained by the modification of amino acids via the intermediacy of the corresponding chiral amino alcohols (see Figure 3). The resulting nitrophenyl carbonate building blocks (*3*) were stable for several months at room temperature.

Oligocarbamates were synthesized on a solid support by deprotection of a resin-bound amine, protected with either the base-labile Fmoc or photolabile nitroveratryloxycarbonyl (Nvoc) group, followed by treatment with a nitrophenyl carbonate of type **3**. The deprotection/coupling cycle was repeated until an oligocarbamate of the desired length was attained (seven or eight cycles). Overall coupling yields were greater than 99% per step. Side-chain deprotection followed by resin cleavage afforded the desired oligocarbamates (Figure 4).

The VLSIPS photolithographic chip format, previously employed for oligopeptide synthesis, was used in the construction a spatially-addressable oligocarbamate library of 256 members. An anti-carbamate monoclonal antibody served as a model receptor for screening against this array. Antibody:oligocarbamate complexes were

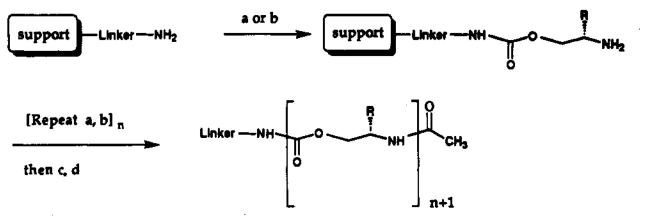


Figure 4. Solid-phase synthesis of oligocarbamates. Key: (a) nitrophenyl carbonate monomer, HOBt, diisopropylethylamine, NMP; (b) piperidine, NMP or $h\nu$; (c) acetic anhydride, NMP; (d) TFA, triethylsilane.

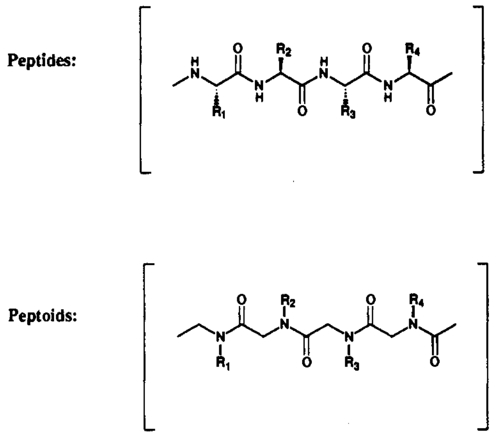
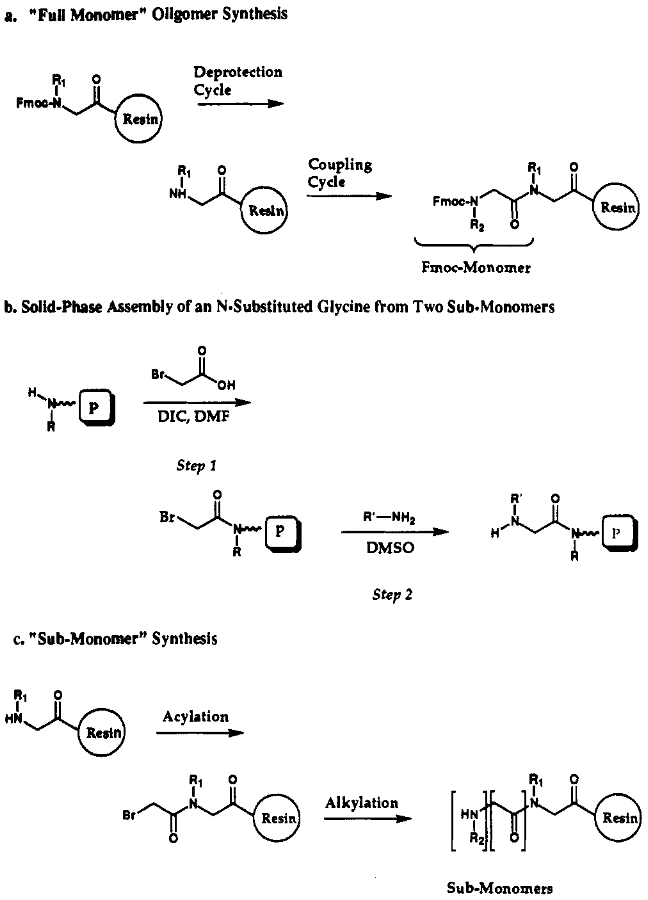


Figure 5. Comparison of peptide and peptoid backbones.

detected by treatment of the chip with a fluorescein-conjugated secondary antibody, followed by analysis using scanning epifluorescence microscopy. Because the location and structure of each different library member is defined by the synthetic strategy (binary masking) used in this technique, the necessity of sequencing the products is obviated. The binding activities of putative hits were confirmed by conventional assays using authentic material prepared by independent synthesis. A preliminary evaluation of the physicochemical properties of oligocarbamate molecules indicate that they are more hydrophobic than the corresponding peptide homologs, and their expected resistance to several proteases was confirmed.

Another type of synthetic polymeric diversity has been developed by Simon *et al.*[22,23] Through a variety of preparative routes, this group created a basis set of monomeric N-substituted glycine units, each bearing a nitrogen substituent similar to those of the natural α-amino acid side chains. The formal polymerization of these monomers results in a class of polymeric diversity which these workers have termed "peptoids" (Figure 5). Peptoids may be synthesized either manually or robotically following either a "full monomer" oligomer synthesis or via a "submonomer" synthesis, as reported by Zuckermann *et al.*[24] and illustrated in Figure 6. Various biological activities have been established for specific peptoid sequences, including inhibition of α-amylase and the hepatitis A virus 3C protease, binding to the tat RNA of HIV[22], and antagonism at the α_1-adrenergic receptor.[25] The peptoid approach to diversity generation has been extended to the preparation of encoded combinatorial libraries, in which natural amino acids code for the structure of the peptoid chain[26] (see part 1[1] and Figure 7).

An important variant of the synthetic polymeric diversity approach is directed toward construction of a chemical library in which the peptidyl backbone is conserved but a dipeptide unit is replaced at specific positions by a phosphonate dipeptide surrogate (see Figure 8). Such phosphonate pharmacophores are well-known as transition-state analogs for amide bond cleavage and have found wide usage in the inhibition of metalloproteases.[27-29] Campbell has described methods for the solid-phase synthesis of peptidylphosphonates that are compatible with the Fmoc/^{t}Bu protecting group strategy of standard peptide synthesis.[30] The key reaction step is formation of the phosphonate ester bond, which is achieved via a modified Mitsunobu condensation (Figure 9). Precursor lactic acid and protected amino phosphonate building blocks are prepared as shown in Figure 10.


a. "Full Monomer" Oligomer Synthesis

b. Solid-Phase Assembly of an N-Substituted Glycine from Two Sub-Monomers

c. "Sub-Monomer" Synthesis

Figure 6. Synthesis of peptoids.

When this process is applied to the combinatorial synthesis of peptidylphosphonates, the diversity product will be a metalloprotease enzyme inhibitor library. Enzyme-inhibitor libraries of this type and those focusing on other known inhibitory pharmacophores (thiols, hydroxamates, carboxyalkyldipeptides, etc.) may prove to be important tools in rapidly profiling novel proteases and for determining which pharmacophores are most effective at their inhibition. Using this knowledge, secondary inhibitory libraries can be constructed to optimize original leads. Through such a process it may be possible to dramatically accelerate the process of finding high-affinity enzyme-inhibitor ligands.

Another interesting type of polymeric diversity based upon a vinylogous polypeptide backbone has recently been reported by Hagihara *et al.*,[31] in which introduction of a trans olefinic linkage between the α-carbon and the carbonyl group of various amino acids is generalized. Additionally, Smith and colleagues have synthesized a nonamide polymer of (3,5)-linked pyrrolin-4-one oligomers which mimic the β-strand conformation of a normal peptide chain[32] (see Figure 11).

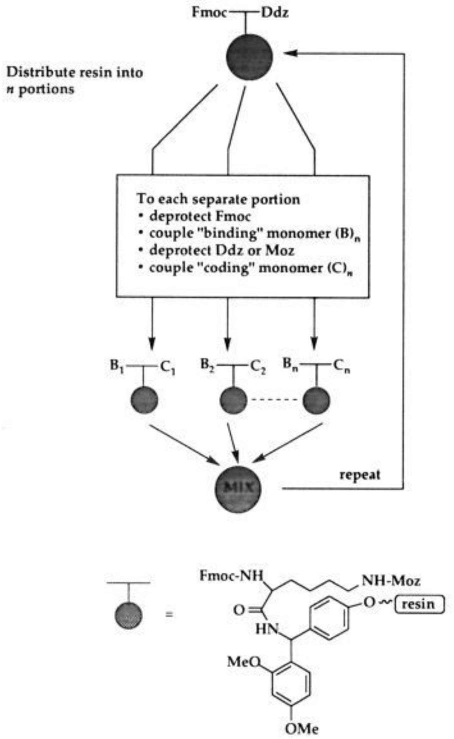


Figure 7. The synthesis of an encoded library consists of the following steps: (1) A bifunctional linker containing two orthogonally protected points of extension (Nα-Fmoc-Nε-Moz-Lys-OH) is attached to polystyrene resin via an acid-labile linker. (2) The solid support is divided into *n* equal portions at a mixture position. (3) A unique Nα-Fmoc-protected non-natural monomer (B) is coupled to the "binding" strand. (4) A series of Nα-Ddz-protected L-amino acids (C) are then coupled to the "coding" strand. (5) The solid supports are recombined.

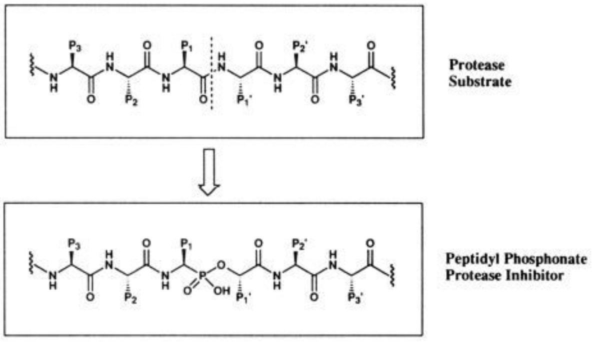


Figure 8. Peptidyl phosphonates.

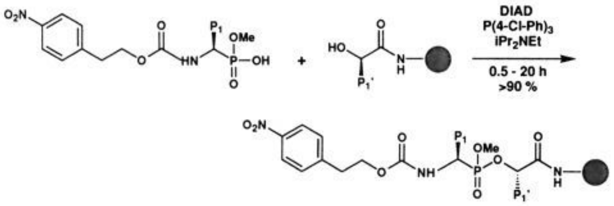
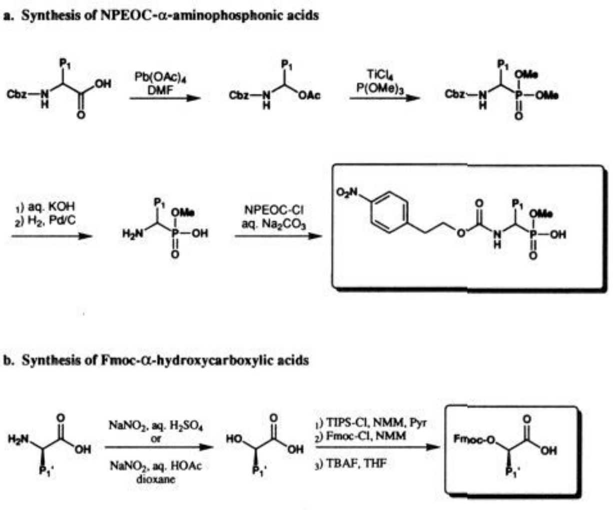


Figure 9. Solid-phase peptidyl phosphonate synthesis (SPPPS).


a. Synthesis of NPEOC-α-aminophosphonic acids

b. Synthesis of Fmoc-α-hydroxycarboxylic acids

Figure 10. Synthesis of peptidyl phosphonate building blocks.

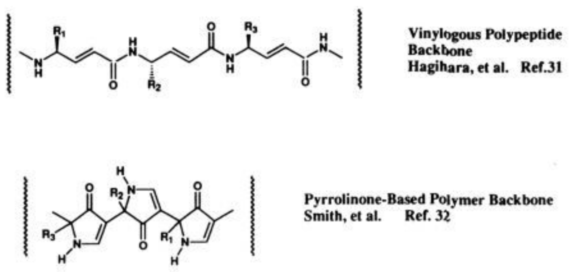


Figure 11. Novel polymeric backbones.

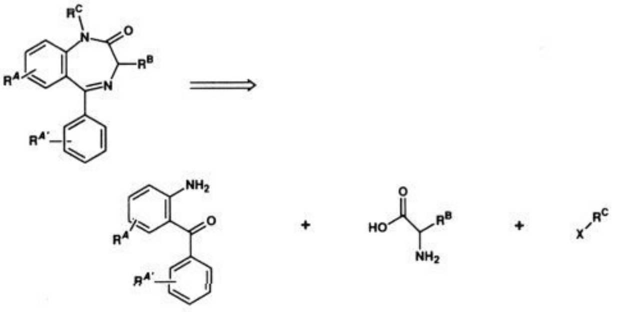


Figure 12. Components of a benzodiazepine library.

Nonpolymeric, Small-Molecule Diversity. The majority of chemical diversity generation discussed above concerns the preparation of linear molecules, in which the target structures are unambiguously specified by the order of building-block addition. In contrast, the great preponderance of organic synthesis proceeds rather differently, wherein building blocks interlock to give rise to nonpolymeric, three-dimensional arrays. The recent seminal work of Ellman on the solid-phase synthesis of 1,4-benzodiazepines lays the groundwork for creation of a small-molecule library of one of medicinal chemistry's most notable pharmacophores and represents one of the first examples of the application of combinatorial organic synthesis to nonpolymeric organic compounds.[33]

The benzodiazepines are synthesized on a solid support by the connection of three building blocks, each of different chemical families (Figure 12). Following the attachment of 2-aminobenzophenone hydroxy or carboxy derivatives to the support using an acid-cleavable linker, [(4-hydroxymethyl)phenoxyacetic acid], the N-protecting group is deblocked (piperidine/DMF), and the weakly nucleophilic amine is acylated with an α-Fmoc-protected amino acid fluoride, using 4-methyl-2,6-di-*tert*-butylpyridine as an acid scavenger (Figure 13). Fmoc deprotection, followed by treatment with 5% acetic acid in DMF, causes the general cyclization to the intermediate lactam. Capitalizing on the ability of lithiated 5-(phenylmethyl)-2-oxazolidinone to selectively deprotonate the anilide NH,

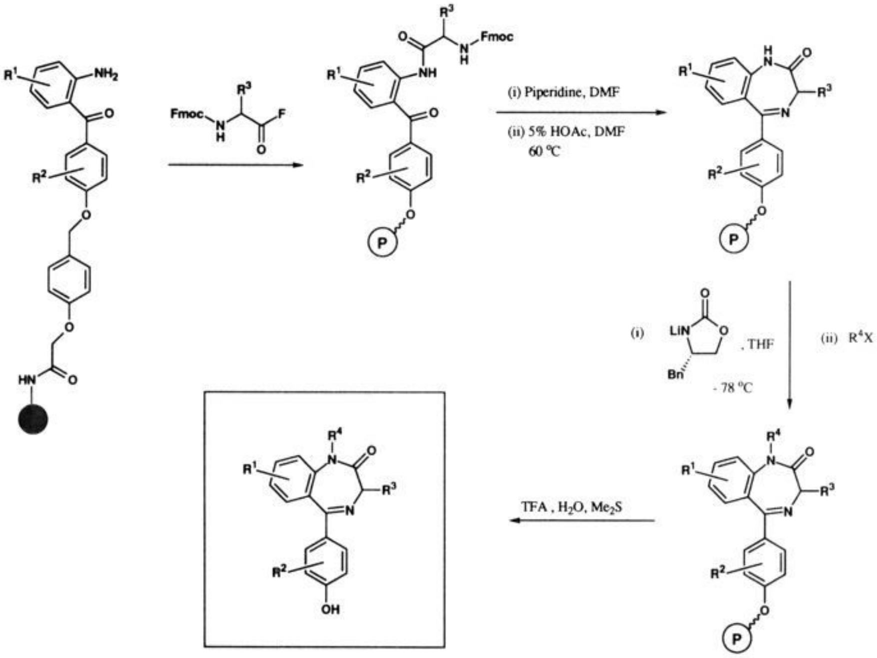


Figure 13. Combinatorial synthesis of the benzodiazepine pharmacophore.

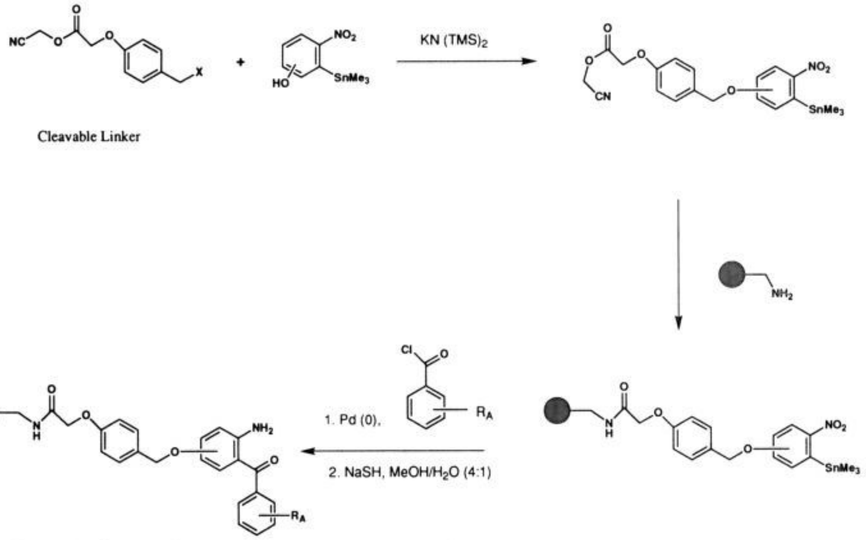


Figure 14. Synthesis of 2-aminobenzophenone derivatives on a solid support.

alkylation was achieved with a variety of alkylating agents. Aqueous acid cleaves the new benzodiazepine from the support in very high overall yields. The integrity of the chiral center was confirmed by a racemization test using chiral HPLC.

One of the limiting features of applying the above scheme to combinatorial library construction is that, though many alkylating agents and amino acid building blocks are commercially available, there is not a ready supply of appropriately functionalized 2-aminobenzophenones. Ellman addressed this problem directly by creating a general method for preparation of these materials on solid supports[34] (Figure 14). The stage is now set for the Ellman laboratory to create a benzodiazepine library.

Several other approaches to nonpolymeric molecular diversity have recently been published. In pursuit of small-molecule libraries, Nikolaiev *et al.* have used their amino acid encoding format (part 1[1]) with a building block basis set combining both amino acids and other synthetic units to prepare collections of nonpeptidic compounds and peptides refractory to Edman degradation (N-blocked peptides).[35] Representative examples of molecules which have emerged from such non-peptide libraries are shown in Figure 15.

A feature of several of the formats used in the display of synthetic diversity is that the potential ligands are tethered to a solid support. While screening strategies have been developed to exploit this feature, it is frequently desirable to screen compounds in solution. Many groups have engaged in developing releasable linker strategies to solubilize potential ligands. The issue has been addressed by a considerably different strategy by Hobbs DeWitt *et al.*, in which solid-phase chemistry, organic synthesis, and a designed parallel reaction apparatus were utilized for the generation of small-molecule libraries, the individuals of which, were termed "diversomers".[36] Target compounds which included dipeptides, hydantoins, and benzodiazepines were synthesized simultaneously but separately,

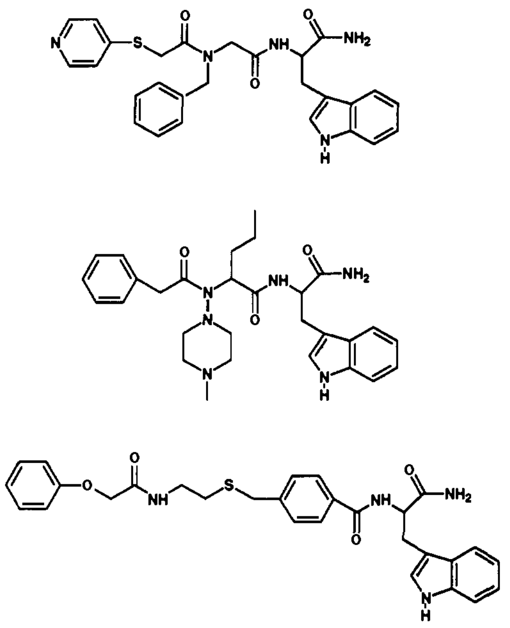


Figure 15. Structure of representative molecules from the Nikolaiev *et al.* nonpeptide library (ref 35).

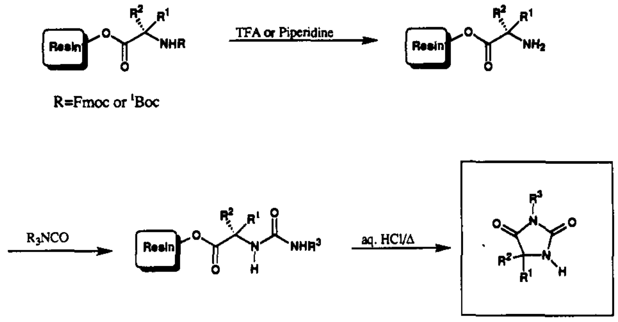


Figure 16. Synthesis of a hydantoin library.

on a solid support in an array format, to generate a collection of up to 40 discrete structurally related compounds. The preparation of hydantoins was carried out as shown in Figure 16. The synthetic strategy is directed through a resin-tethered penultimate product, in which the orchestrated revealing of distal functionality initiates attack on the resin-linking bond to eject the newly formed product into solution. Products which fail to react, should remain attached to the solid phase, and thus aid in product purification. The yields of hydantoins released from the resins in the final step ranged from 4 to 81% on a scale of 0.3–11 mg, which should be sufficient to support most preliminary *in vitro* biological testing. The resulting soluble, small molecules were characterized by traditional means. The authors also note the utility of ^{13}C gel-phase NMR to monitor reaction progress of the resin-bound intermediates.[37,38]

In a similar manner, a general method for multiple, simultaneous synthesis of soluble benzodiazepines was developed (Figure 17). Eight groups of five-amino acid resins were trans-imidated with five groups of eight 2-aminobenzophenone imines to form 40 resin-bound imines. Treatment with TFA liberated 40 discrete benzodiazepines from the resins. The products were obtained in 2–14-mg quantities, corresponding to 9–63% yields with estimated purities of >90%. Though the numbers of compounds involved in the diversomer methodology (~40) are significantly smaller than that which can be prepared by other library strategies (10^4–10^8), this interesting approach to parallel organic synthesis produces relatively pure materials on a preparative scale in the traditional, soluble format.

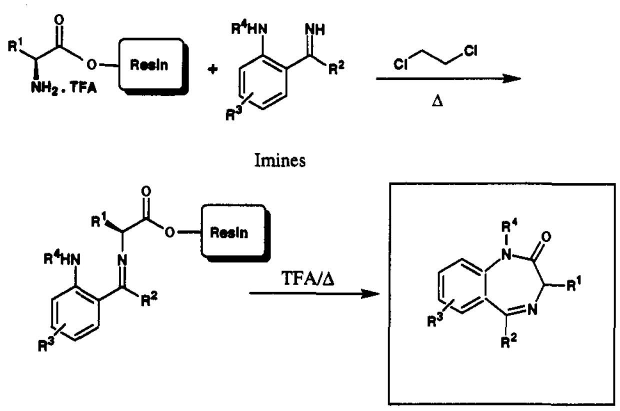


Figure 17. Preparation of a soluble benzodiazepine library.

Future Innovations

As the field of combinatorial chemistry receives increasing attention from the pharmaceutical establishment, it seems likely that the contents of chemical libraries will continue to evolve to look more and more like the type of compounds which have previously led to drugs. In spite of the complexity which early parts of the process may pose to the combinatorial chemist, a hidden advantage which combinatorially-derived molecules offer is that any "hit" will be readily synthesizable, by definition. This should be contrasted with a natural product driven approach to drug discovery and development, where often the structural complexity of the lead compound hampers the rapid preparation of analog molecules and the acquisition of SAR.

A previous point deserving further emphasis is that the vast universe of synthetic organic reactions are idiosyncratic transformations that fail to afford quantitative yields of unique products. Most synthetic chemistry procedures afford multiple products (regio- and stereoisomers, etc.) in variable yields. If diversity-generating chemistry proceeds ambiguously, how then are the results of small-molecule combinatorial organic syntheses to be understood and appropriate information extracted from library analysis? It may be speculated that encoding techniques will provide one method by which the combinatorial organic chemist can address the practical inefficiencies of chemical synthesis. Instead of envisioning an encoding tag as explicitly specifying the structure of an associated entity, one might consider the tag as *a record of the chemical history of individual library members*. Thus, after encoding the "recipe" or synthetic protocol used in the assembly of a combinatorial library, the library may be screened for active recipes. Once identified as "active", the synthesis would be replicated on a preparative scale, and the product mixture fractionated to identify active product(s). This strategy shifts emphasis from the criterion of singularity in a reaction outcome (a single predictable structure) to reproducibility and compatibility (orthogonality) with chemistry used in the synthesis of the encoding tag and in preparative scaling. The creation of encoded, small-molecule diversity, which can be released from a support (solubilized) while some type of link to the original tag is also maintained, is also likely to be an important area of investigation.

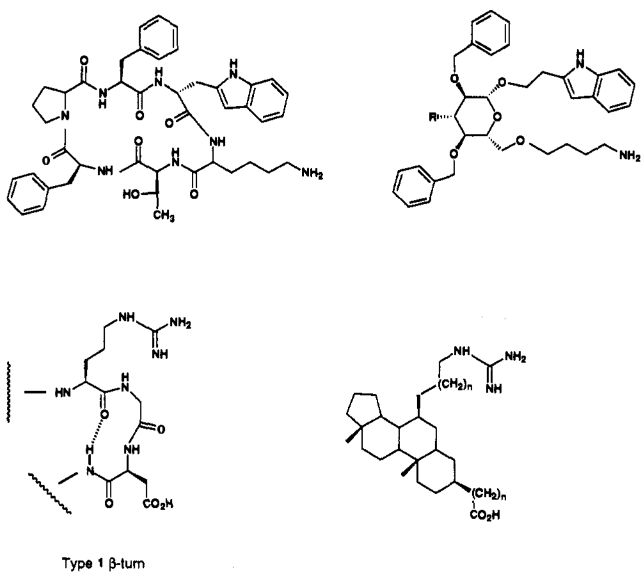


Figure 18. Scaffolds as templates for combinatorial chemistry.

One type of noteworthy chemical strategy which may have a bright future in the combinatorial realm rests on the conceptual extension of the work of Hirschmann, Nicolaou, and Smith and their co-workers into designing nonpeptidal peptidomimetics by the appropriate functionalization of designed scaffoldings (Figure 18). A specific example of this approach is the design and synthesis of a β-D-glucose-based nonpeptide mimetic of a potent cyclic hexapeptide somatostatin agonist.[39,40]

Hirschmann and co-workers have also used a functionalized steroidal template to serve as a backbone for mimicking a type 1 β turn.[41,42] The fact that such a remarkable job of molecular mimicry can be achieved with individual compounds bodes well for the application of this approach to combinatorial methods.

No library will ever be "complete" but instead will sample a subsection of a particular universe of molecular structure and space. In certain situations, libraries may be considered to be starting materials for the construction of new libraries of diversity. It is also useful to consider chemical libraries as collectable or archivable entities. Ideally, one seeks to preserve new compound libraries and use them for a variety of present and future screening needs. As time passes, the combinatorial chemist will be in possession of an accumulating collection of molecular diversity with which to challenge new drug targets. If chemical libraries are to become an item of commerce, a good deal more will have to be learned about their "shelf life" and how best to store them for future use. To date, the shelf life of chemical libraries is an open question.

B. Methods for Screening Combinatorial Libraries

The importance of distinguishing between the two principal applications of combinatorial technologies in ligand discovery, i.e., broad screening versus directed analoging, is particularly relevant to the design of assay methodogies for library evaluation. In searching large, highly diverse libraries for novel lead compounds, a premium is placed on the ability to detect rare ligands that may have modest affinity for the target receptor. The assay strategy may differ in screening analog libraries, since one is trying to develop quantitative SAR on a large number of compounds and to increase the potency of a lead. Regardless of the application, successful use of combinatorial libraries is highly dependent on the sensitivity and specificity of the assays that are used to identify and characterize ligands.

In this section, the various combinatorial library methods will be discussed in terms of the assays that are used. The assay formats are closely matched to the mode of presentation of the diversity. In broad terms, assay procedures can be grouped into three categories: (i) those that rely on affinity purification with an immobilized target receptor, (ii) those in which a soluble receptor binds to tethered ligands, and (iii) those in which soluble compounds are tested for activity, either directly or in competition assays. Each format presents different challenges with regard to the minimum affinity requirements for ligand detection, the demonstration of binding specificity, and the ability to discriminate among compounds in the library on the basis of their affinities for the target.

Isolation of Ligands by Affinity Purification

Recombinant Peptide Libraries. The various systems described in the first part of this series[1] for creating vast libraries of recombinant peptides (commonly referred to as peptide/nucleic acid complexes below) rely on affinity purification to select peptides that bind to a receptor. Two distinct methods have been employed to achieve affinity purification of peptide/nucleic acid complexes. The first involves incubation of a receptor in solution with the mixture of complexes. After allowing sufficient time for binding, the receptor is captured using immobilized streptavidin or an antireceptor antibody.[43,44] The second approach calls for preimmobilization of the receptor on beads, microtiter wells, or a chromatography support, followed by capture of the complexes.[45] In both cases, the use of a solid-support facilitates the separation and washing of receptor-bound complexes.

The method of receptor immobilization is a critical aspect of the successful use of recombinant peptide libraries. Because of the tremendous levels of ligand enrichment attainable through multiple rounds of selection and amplification, peptides that bind to any component of the solid support can be isolated from libraries. Peptides binding to streptavidin,[46,47] antireceptor antibodies,[48] or peptides that exhibit inherent nonspecific binding are readily isolated. Often, screening strategies employing subtractive methods and/or blocking ligands, are necessary to enhance the selection of ligands with desired binding specificity.

To enhance the probability of isolating peptide ligands with biological function, it is important to ensure that the receptor is active (for example, capable of binding its natural ligand) when immobilized. Immobilization of receptor proteins on microtiter wells or beads can be accomplished by passive adherence, covalent attachment, biotinylation and immobilization on streptavidin, or capture with high-affinity nonblocking antibodies. The first three processes often result in inactive proteins. The problem of immobilizing active protein can often be overcome by introducing into the receptor an immobilization handle through genetic engineering techniques. Peptide epitopes for a monoclonal antibody or a motif that allows for site-specific biotinylation of the protein[49] can be fused to proteins for this purpose. Generic immobilization strategies of this type greatly facilitate the creation of a high-density affinity matrix suitable for isolation of ligands.

Some drug-discovery targets may not be readily available as pure soluble receptors. Peptide ligands specific for the integrin IIb/IIIa have been successfully isolated from phage libraries by screening against platelets expressing a high density of this receptor.[50] It remains to be seen whether whole cells bearing receptors or other forms of impure macromolecular target will generally be successfully utilized to identify ligands. It can be anticipated that the problem of isolating non-receptor-binding sequences will be enhanced when dealing with impure forms of receptors.

The choice of using preimmobilized receptor rather than incubation with receptor in solution followed by receptor capture on a solid support may become important in one aspect of library screening. Because the phage and LacI/DNA complex systems are capable of displaying multiple copies of a peptide, multivalent binding can occur during affinity purification if receptors are immobilized at sufficient density. Multivalent binding effectively increases the avidity of the bound complexes and allows the isolation of complexes bearing peptides of lower affinity. Although it is possible that multivalent interactions may occur during the capture step of the two-step procedure, the stoichiometry of the phage or LacI complex, receptor, and the capture reagent need to be carefully controlled. Multivalent binding conditions may be more easily engineered with a high-density preimmobilized receptor.

The multivalency of the phage and LacI systems can be exploited to isolate peptides of modest affinities (K_d values of 1–1000 μM). This feature is particularly important in screening random libraries of peptides greater than six or seven amino acids in length. Libraries that can be routinely made have many fewer members (10^8–10^{10}) than the theoretical number of possible sequences for a given peptide length (for example, there are 1×10^{13} possible 10-mers). In general, peptide ligands for a receptor target consist of families of related sequences with few high affinity ligands in the family. Therefore, when a library is created, it is likely that only lower affinity members of the family will be represented. The ability to identify these low-affinity ligands then permits one to proceed to the secondary phase of discovery, the screening of mutagenesis libraries.

Methods for creating many variants of an initial sequence have been described in part 1. Such libraries will generally contain many low-affinity ligands and perhaps some high-affinity ligands in much lower abundance. A demand is therefore placed on the ability to selectively isolate the highest affinity ligands. Several methods have been described for efficient affinity selection. All are based on the prevention or disruption of multivalent interactions. The use of a low density of immobilized receptor to isolate high-affinity peptide ligands from a phage library containing many low-affinity ligands has been demonstrated.[44] Low receptor density reduces the possibility of multivalent interactions between phage particles and the receptor matrix. The "monovalent-phage" approach[45,51,52] has been successfully employed to isolate high-affinity mutants of human growth hormone displayed on phage. In this approach, phage particles with only a single chimeric pIII protein are created, thereby eliminating multivalent binding to immobilized receptor. Using this approach, mutants with K_d values of less than 5 pM have been identified.

An alternative method of affinity selection that may have advantages over other approaches has been developed.[48,53] Phage (or LacI particles) are allowed to bind in a multivalent fashion to a high density of immobilized receptor. For phage-bearing low-affinity ligands, the peptide on an individual pIII protein may be rapidly dissociating and reassociating, but the phage particle will not dissociate unless all the peptides on pIII are simultaneously in the unbound state. Dissociation of the phage can be initiated by addition of a competing ligand, which prevents rebinding of any individual peptide in the complex. Using a model system with peptides of known affinity, it was demonstrated that phage-bearing high-affinity peptides are retained for a greater length of time than phage with lower affinity sequences.[48] The concentration (and affinity) of the competing ligand, as well as the time and temperature of elution, can be varied to select for ligands of various affinities. This method has the advantage of using a high receptor density to ensure a full sampling of ligands of all affinity classes.

Achieving affinity selection is only part of the process of successfully screening recombinant peptide libraries. After selection, it is necessary to establish the binding specificity and, if possible, the affinity of individual peptides that result from the selection. Various assays have been described, including dot blots,[54] colony lifts,[55] and ELISA's with immobilized phage or immobilized receptor.[44] These methods differ in the minimum ligand affinity that is required for detection. In general, assays in which phage or LacI are immobilized (ELISA's, dot blots, colony lifts) require higher affinity (K_d values < 1 μM) peptides for detecting specific binding, and are therefore useful when such ligands are present in the selected pool. However, for reasons cited above, detection of the specific binding of lower affinity ligands is often necessary. In such cases, assays that use a high density of immobilized receptor are required to allow for multivalent binding and to increase the sensitivity of detection. If high-density receptor matrices are used for affinity purification and assay of individual clones, peptides with K_d values as high as 100–500 μM can be isolated with phage and LacI systems.[48]

An additional assay format has been described for estimating the affinity of peptides displayed by individually selected phage clones.[44] Radiolabeled receptor is first allowed to bind to the phage-borne peptides in solution. A high concentration of competing peptide is then added to prevent further binding, and the dissociation of radiolabeled receptor is followed with time. With a monoclonal antibody model system, a good correlation was observed between dissociation rates and the affinity of the peptide determined by solution methods. It has also been suggested that colony lifts with limited receptor concentration may allow discrimination of individual phage clones on the basis of their peptide's affinity.[55] However, this method may be confounded by differential levels of expression of phage by different colonies.

In summary, the successful identification of ligands from recombinant random peptide libraries depends not only on the nature and size of the libraries but also on effective screening strategies. Selection methods and assays of individual clones vary in their ability to select and detect lower affinity peptides and in the ease with which binding specificity can be determined. High-affinity ligands are most desirable, and initial conditions for screening of random libraries can be adjusted so that only high-affinity ligands are selected. However, for reasons stated above, engineering selection and assay conditions to allow isolation and detection of lower affinity ligands may be generally a more reliable strategy. These initial peptides can serve as starting points for creating secondary recombinant peptide libraries or as leads for refinement by synthetic chemical combinatorial methods.

Affinity Purification of Mixtures of Soluble Synthetic Compounds. Investigators have employed affinity purification methods to isolate ligands from mixtures of soluble peptides[26,56,57] and oligonucleotides (RNA or DNA).[58-60] In the case of nucleic acid libraries, one takes advantage of the ability to enzymatically amplify the molecules resulting from affinity purification, and as with the recombinant peptide systems, multiple rounds of selection and amplification are used. Theoretical considerations in optimizing conditions for the selection of high-affinity oligonucleotides have been described.[61] The authors illustrate (by way of computer simulation) the importance of nucleic acid and receptor concentrations as well as the efficiency of separating specifically bound molecules. Computer simulations show that, under ideal conditions, rare high-affinity molecules can be isolated from large libraries with relatively few rounds of selection and amplification. There have been a number of examples of successful identification of high-affinity oligonucleotides using this process.[62]

Relatively little work has focused on the affinity purification of ligands from soluble peptide libraries. The isolation of ligands for an anti-gp120 antibody from equimolar mixtures containing 19 or 32 peptides has been reported.[56,57] More recently, the same antibody was used to capture ligands from four mixtures, each of 50 peptides, comprised of unnatural amino acids fused to an encoding L-amino acid peptide strand.[26] After affinity purification, the resulting pool of peptides selected was resolved by HPLC and each peak subjected to Edman sequencing and mass spectrometry analysis. A major limitation of this approach is the sensitivity of these analytical methods. Sufficient peptide (>1–10 pmol) must be recovered in order to determine its sequence, requiring that each library member be present in relatively high amounts in the starting pool and that there be sufficient receptor available to isolate the requisite quantity of each of the high-affinity ligands. In addition, the method requires that selected peptide ligands be resolved chromatographically. While it seems unlikely that this methodology will be extended beyond libraries of modest size (less than a few thousand members), it may prove a useful technique for evaluating secondary (analog) libraries. The proposed approach of creating a library of soluble compounds with attached oligonucleotides tags may allow for the structural identification of minute quantities of compounds isolated by affinity purification.[63,64]

In theory, chromatography of compound mixtures using receptor columns should not only facilitate separation of nonbinding members of the library, but should also allow for the resolution of compounds on the basis of their receptor affinities. Work with various model systems has demonstrated that column retention time can be used as an index of affinity.[65,66] While columns of receptor target have been used in batch affinity purification methods, chromatography to resolve ligands of differing affinities has yet to be applied to screening combinatorial libraries. This method may be better suited to isolation of ligands of moderate affinity.[67] An additional limiting factor in the use of chromatography may be the large amount of receptor required to generate enough theoretical plates to effectively resolve compounds.

Binding of Receptors to Immobilized Ligands

Various methods for creating libraries of compounds attached to solid supports (pins, beads, chips, etc.) have been outlined in part 1.[1] Such libraries are screened by detecting the direct binding of a labeled receptor to an immobilized ligand. The identify of the ligand is either determined directly (by peptide sequencing or mass spectrometry), specified by its spatial location in an array, or deduced by reading an encoding tag.

There are a number of important issues related to solid-phase binding assays with immobilized ligands. First, the ability of a receptor to interact with a tethered ligand may be influenced by the site or nature of its covalent attachment to the solid support. In all of the methods published to date, peptide ligands are attached to a linker and support via the carboxy terminus of the sequence. An obvious example of the limitation imposed by this mode of immobilization would be in screening against the G-protein-linked receptors of various peptide hormones, many of which require a free C-terminal carboxamide for activity. In such a case, it is likely that many peptide analogs that would bind when free in solution would be missed in an assay where the same peptides were immobilized via their C-termini. To circumvent this problem, it is advantageous to have several alternative sites of ligand attachment to the surface. Methods for tethering peptides through their N-termini have been identified.[68] It is likely that the issue of how best to tether molecules to surfaces will become even more important when dealing with libraries of small nonpolymeric organic compounds.

The chemical nature of the linkage between the ligand and support may also affect the receptor-ligand interaction. One needs only to look at the variety of resins that are available for affinity chromatography to appreciate the importance of controlling the receptor–ligand interface. The types of linker groups that have been successfully employed in tethered library assays to date have been noted in part 1 of the series. Whether these linkers will generally provide for optimal presentation of compounds to other receptor systems remains to be seen.

Immobilized ligand assays require that the receptor be labeled in a way that allows for highly sensitive detection of receptor binding. The receptor can either be labeled directly or a secondary labeled reagent with high affinity for the receptor can be used. To date, colorimetric enzymes, radioisotopes, and fluorophores have been used in labeling receptors or secondary reagents. The reagents must be labeled in a way that maintains the activity of the receptor, for instance, its ability to bind a natural ligand. This can be greatly facilitated by creating chimeric recombinant receptors that incorporate peptide epitopes of antibodies or peptide sequences for site-specific radioactive phosphorylation[69] or site-specific biotinylation.[49]

Successful screening of libraries of immobilized synthetic ligands is dependent on the same types of issues as have been previously discussed with respect to evaluating recombinant peptide libraries: i.e., the affinity threshold for detection, the ability to discriminate ligands on the basis of their affinities, and the ability to distinguish specific binding from nonspecific binding. Methods development in this area is in its infancy. In principle, it should be possible to exploit multivalent binding to detect lower affinity ligands. Multivalent receptors can be created by a number of methods, including genetic fusions to generate bivalent receptor/Fc fusions[70] or through the use of monoclonal antibodies or streptavidin to create cross-linked receptors capable of interaction with more than one immobilized ligand. Optimization of the density of immobilized ligands may be required in order to allow for multivalent binding. As has already been noted, it may be important to be able to isolate relatively low-affinity ligands in the initial screening of random libraries. These compounds can then serve as the basis for further library construction in which the goal is to improve ligand affinity.

Affinity discrimination during the screening of either primary random libraries or secondary (analog) libraries is of obvious importance. There has been little published work on methodology in this area. In principle, low receptor concentrations, competing ligand-mediated dissociation, and/or stringent washing conditions can be utilized to identify the highest affinity ligands. Two issues complicate the use of such methods. The first is the likelihood that each pin, bead, or surface synthesis site does not contain the same amount of compound. With different compound loadings, one must be extremely cautious of using the absolute quantity of bound receptor as an index of a molecule's affinity. As new building-block and coupling chemistries are adapted to combinatorial formats, this may become a more significant problem than it is for high-yielding peptide chemistry. Another complicating feature of the immobilized ligand assay format is the fact that ligands of one particular kind are densely clustered on a surface. Both the association and dissociation rate constants of a receptor/ligand interaction are affected by surface ligand density. The binding of nearby ligands depletes the local receptor concentration and the association kinetics become diffusion limited. After dissociation, receptor rebinding is favored because of the high local-ligand concentration and the apparent dissociation rate is reduced. Theoretical and experimental analyses of these surface binding effects have been undertaken.[71] The impact that these surface binding kinetics will have on the ability to discriminate among library members on pins, beads, or glass surfaces remains to be seen.

The information generated by screening immobilized ligand libraries differs among the various library formats. In the case of bead-based technologies, compounds exceeding a threshold affinity are sampled from a large pool of ligands. Positive information is obtained, i.e., that a particular ligand binds to the receptor. One cannot, however, draw conclusions about the binding affinity of nonselected ligands. The sampling size may not have been large enough to include all high-affinity ligands, or a high-affinity bead may have been missed by the affinity selection method [for example, fluorescence-activated cell sorter (FACS) selection]. By contrast, the multipin and VLSIPS technologies allow one to perform a parallel assay in which data is obtained on every compound that is synthesized. In principle, both positive and negative binding information can be exploited in the design of second-generation compounds.

Incorporation of methods that assess the specificity of binding of ligands is an important aspect of screening random libraries. Screening immobilized ligands by direct receptor binding can lead not only to the identification of ligands of interest (for instance, ligands that compete with the natural ligand) but also to ligands that bind to undesired portions of the receptor or to secondary detection reagents. In the case of libraries of compounds on beads, it may be possible to remove undesired ligands in a subtraction step prior to screening for desired ligands. For compounds on pins or chips, it may be possible to make replicate arrays and test for total binding and nonspecific binding in parallel. Otherwise, sequential assays that first test for receptor binding of any kind, followed by an assessment of nonspecific binding will be required in order to correctly identify compounds that interact with the receptor in a desired manner.

Testing the Activity of Libraries of Soluble Compounds

The classical method of screening for a desired biological activity is to test soluble compounds one at a time in a competition binding assay, enzyme inhibition assay, or in a cell-based bioassay. Such approaches have been applied to library screening by releasing compounds synthesized on pins into microtiter wells, as described in section C of part 1 of this series.[1] A novel application of bead technology has recently been disclosed where compounds on individual beads are released locally onto a lawn of confluent mammalian cells and cause activation of cells in the area surrounding the bead.[72] The bead responsible for cell activation is isolated and a small amount of noncleaved peptide is sequenced to determine its structure. In both of these cases, the principal issue is whether enough compound is released to be detected by the assay. For pins, approximately 100 nmol of peptide can be released into a few hundred microliters of solution, while beads with diameters of $\sim 100\ \mu m$ can release on the order of 100 pmol of peptide.

Rather than assaying compounds individually, a second approach to screening soluble libraries is to assay compound mixtures. In addition to testing complex pools of soluble peptides (*vide supra*), libraries of oligonucleotides have been successfully screened as soluble mixtures.[73] The most frequently used strategy for screening mixtures of soluble compounds with the goal of ultimately identifying single active molecules is based on the "mimotope" approach, detailed in part 1. The essence of this strategy is that degenerate pools of peptides (or other compounds) are resolved into their most active constituents by an iterative process of testing and resynthesis until a single sequence is identified as having high activity. A variation of the methodology (termed "bogus-coin strategy") has also been described.[74]

There are a number of caveats to using this methodology for testing soluble compound mixtures. In practice, the results of each set of assays do not typically indicate a preference for a unique residue at any position within the sequence. Rather, comparable assay results may be obtained for several different amino acid substitutions and some decision must be made as to which of these partial solutions should be fully resolved. The number of peptide mixtures to be synthesized and tested in this protocol expands dramatically as the number of alternative sequences selected for complete resolution at each cycle is increased. Moreover, the deconvolution of different partial solutions may frequently produce divergent resolved sequences, in part because the contribution of each amino acid to the peptide–receptor interaction is typically dependent on other non-neighboring residues within the ligand. The problem of identifying the most potent ligand in a complex mixture by this type of iterative pathway is exacerbated by the relative abundance of lower affinity ligands that represent local binding optima.

Originally designed for identifying antibody ligands, the mimotope strategy has primarily been used for libraries of six to eight building blocks in length. It is not clear that ligands of this size will be optimal for other types of receptors (although success with opioid receptors[75] and other targets have been reported). As the length of the compounds in the library increases, resynthesis and testing of pools becomes more cumbersome.

Perhaps the greatest limitation of this methodology is the fact that the activity of a given pool is based on the cumulative activity of all the compounds in the pool; i.e., pools with the same activity may contain many low-affinity compounds or a few high-affinity compounds. For this reason, the methodology is greatly facilitated if the minimal fragment having activity is comprised of the same number of building blocks as used in constructing each library member (e.g., a uniquely active tetramer is more easily resolved from a tetrapeptide library than a hexamer library). Alternatively, the identification of active peptide(s) is facilitated if the receptor has specific requirements for a fixed position within a peptide ligand (e.g., the N or C termini). If neither of these conditions is true, it may be necessary to test many or all of the possible initial pools with two adjacent or nonadjacent fixed residues. This drastically increases the number of initial pools that need to be synthesized but increases the probability that a critical residue(s) is fixed in at least one pool to allow that pool to differentiate itself. It must be kept in mind that any pool identified as having the greatest activity may be composed of many moderately active compounds and that the most active compound(s) may reside in other pools.

Testing of mixtures of soluble compounds is also limited by the concentration of individual test compounds that can be achieved in the initial pools. Pools containing as many as 160 000 different peptides have been tested with each member being present at ∼10 nanomolar concentration.[76] Because of limitations on the solubility of the total pool, the concentrations of individual compounds present in increasingly larger libraries must be correspondingly diminished. This will ultimately limit the ability to identify the activity of compounds with modest potencies.

While the current methods for testing mixtures of soluble compounds have certain drawbacks, screening soluble libraries does have the decided advantage of avoiding the problems associated with assaying tethered molecules in other combinatorial technologies. Conventional binding and enzyme and cell-based assays (including those with poorly defined biochemical targets) can be used to test the activity of soluble compounds. It is likely that in the future, encoding strategies will be employed to allow more facile screening of soluble molecules. In the simplest format, single encoded beads can be dispensed into microtiter wells. The compounds can then be released from the beads and tested for activity, with the identity of the most active compound(s) being deduced by decoding the tag attached to the bead(s). To test large libraries of soluble compounds, mixed pools of encoded beads can be created. At each round of testing, only a fraction of the compound is cleaved from each bead. Active pools of beads are pursued by further subdividing the beads, partially releasing the compounds, and testing for activity. In the end, a single bead is identified with the greatest activity and the encoding tag is then read.[77]

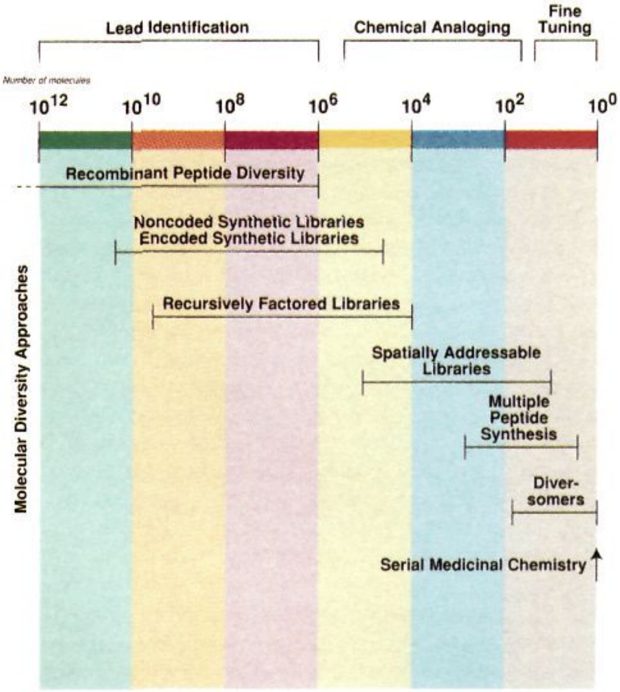


Figure 19.

C. Integration of Combinatorial Technologies for Drug Discovery and Future Directions of the Field

Regardless of whether the objective is a broad discovery search or analoging a known lead, a key aspect in the successful application of combinatorial technologies to drug discovery is the requirement for having a closely linked, coordinated process for the integration of synthesis and screening. The creation and evaluation of molecular diversity are two sides of the same coin. It is still premature to speculate on which type of format will prove most suitable for a particular type of discovery/development problem. Over time, a cataloging of successes and failures will clarify this issue. In all likelihood, command of a collection of combinatorial tools will be required for general success. One may imagine a "spectrum of molecular diversity" stretching from few to many molecules (see Figure 19). Serial synthesis (contemporary medicinal chemistry) operates on a few molecules (far right of chart). We expect that each individual combinatorial tool/format will turn out to be most valuable within proscribed regions of numbers of molecules. Hence, recombinant peptide diversity is particularly suitable for generating and screening large libraries of $>10^8$ compounds. The VLSIPS chip technology, although capable of displaying vast arrays, is primarily an analoging tool and is most useful for evaluating 10^2–10^4 compounds. The parallel organic synthesis methods of Hobbs DeWitt *et al.*[36] are applicable for tens of compounds. Encoded synthetic libraries appear to be relevant in broad diversity searching and may also prove useful in narrower optimization strategies. Given the repetitive nature of many of the manipulations required for library construction, an on-going priority will be to address the possibility of automating as many aspects of the generation/evaluation process as possible. Growth in library size also impacts directly on the physical size of compound collections and on the amounts of target receptor required for screening. These pressures will continue to drive the field toward minaturization and exploitation of nanotechnologies.

The power of combinatorial technologies in generating huge numbers of compounds suggests that in a lead-discovery mode, less preconceived bias need be brought to the process of making molecules. Another way of expressing this is as follows: let the numbers do the talking. Due to the time and effort required in serial approaches, each target molecule is selected with great care. Because of the relative ease in creating libraries, little risk is incurred, or effort expended, in allowing a wide variety of building blocks to participate in diversity generation. Since there is less up-front investment in any individual combinatorially created molecule, the combinatorial chemist can afford to take more risks. We can think in terms of a portfolio of libraries which might be routinely applied to the initiation of a drug-discovery search. This is not dissimilar to selecting a preliminary screening sample of diversity from a large database of individual molecules.

A related, but still immature, issue in combinatorial approaches to drug discovery revolves around the idea of "quantitation of diversity". An understanding of the concept of "measuring" molecular diversity could impact on designing libraries to contain maximal structural diversity. This notion has arisen previously in deciding which few representative, highly diverse compounds to select out of large database collections, when setting up groups of preliminary screening samples. The huge numbers involved in combinatorial approaches intensifies this issue. A number of interesting approaches to the diversity quantitation problem can be expected to emerge.

One working drug-discovery paradigm might be based on initially employing a portfolio of biological diversity (peptide libraries) together with standard chemical libraries (various-sized cyclic peptides, cysteinyl-linked cyclics, etc.), peptides with carboxyl or carboxamide display, synthetic polymeric diversity, as well as large libraries of semirigid and acyclic small molecules prepared by COS. Over time, favored libraries and directions would be expected to emerge. As the sophistication of combinatorial organic synthesis grows, the origins of a molecular structure as either "combinatorially or serially derived" will gradually become transparent.

Another area where considerable effort must be applied is in the registry of libraries and individual library members. It is unclear that library compounds should be registered and documented for testing in the same ways as serially produced compounds historically have been, but exactly what changes are necessary remain to be determined. Vast numbers of compounds have been and are being created; keeping track of these and their corresponding biological activities will require innovative database-management techniques. Additionally, nomenclature needs to be developed by which one can simply express the constitution, scope, and nature of chemical libraries. Legal issues, including the patenting and documentating of libraries and their component members, will need to be pioneered.

As repeatedly emphasized, it is obvious but imperative to have efficient means of evaluating the molecular diversity which is generated. Different assay techniques will be format specific. Assays must clearly discriminate specific from nonspecific binding. Since in a broad screening mode, one is almost always sampling a small percentage of the entire universe of diversity (10^{10} peptides are only 0.1% of the universe of 10-mers), it is crucial that appropriate assay techniques be competent to detect modest affinity ligands. The identification of weak binders in any of the aforementioned approaches is very important and should lead directly to preparation of secondary libraries in which original "hits" will become the centerpiece for more focused diversity creation. This is a consequential issue, since application of combinatorial technologies are best viewed as an iterative process and not a singular event. As the emphasis shifts to analog evaluation, assays must be capable of affinity discrimination between closely related library members. The tools of molecular biology have permitted the molecular engineering of targets to serve the purposes of screening. The rapid introduction of targets into a screening mode will require generic techniques for their handling, and manipulation of molecular targets by genetic engineering will continue to play a crucial role in marrying library evaluation and synthesis. Though combinatorial technologies may soon prove their worth in the drug-discovery process by delivering new leads quickly and cheaply, in order to completely fulfill the promise of "making drugs", an important question will be whether some of the common major obstacles to drug development (e.g., cell penetration, bioavailability, pharmacokinetics, metabolism) can be productively addressed through the application of combinatorial approaches (i.e., *combinatorial drug development*).

In the coming years, cloning and sequencing of the human genome promises that an unprecedented abundance of newly discovered proteins will become available as potential drug targets. Gaining even more prominence than it now assumes will be the issue of discriminating among a myriad of receptors and enzymes to identify valid targets for drug discovery. The ability to access potent and specific ligands for these targets will guide this process by untangling the physiological relevance of endogenous biochemical pathways. Combinatorial methods will be called upon to provide such molecules to quickly and cheaply drive target validation. In this manner, the identification of leads will benefit from a significant, but hidden, benefit which emerges from combinatorial screening; hits derived from chemical libraries should be readily amenable to combinatorial analoging.

Certain drug targets may present more or less of a historical precedent with respect to the likelihood of successfully identifying a tight binding ligand through the use of known pharmacophores. For example, the search for specific enzyme inhibitors may be facilitated by the intentional enrichment in the combinatorial synthetic process of building blocks containing known inhibitory pharmacophores. Particularly important or common types of drug targets may justify having on hand special libraries which are somewhat specific (i.e., a peptidyl hydroxyethylamine library for aspartyl- and metalloprotease inhibition[78–80]). On the other hand, in areas where there is less current information (e.g., antagonism of protein–protein or carbohydrate–protein interactions), a wider scope of diversity search should be taken until consistent patterns begin to emerge. In the case of newer, less explored target groups, combinatorial technologies can be expected to assist in unearthing new pharmacophore classes and to help establish an understanding of drug design for new types of targets.

Combinatorial technologies diverge sharply from historical precedent through a change in emphasis from the consideration of individual molecules to thinking in terms of populations of molecules. A common, but false, intuitive believe is that combinatorial chemistry is necessarily a random, screening search; the antithesis of rational drug design. In fact, all libraries are biased in some ways. All drug company compound files are biased by the historical programs of that institution, since a disproportionate share of compounds of particular types will have been deposited. *The notion of intentionally biasing a chemical library is a form of drug design,* but again not applied to individuals but rather to groups or populations of molecules. If a scientist hypothesizes on the basis of structural information that the current lead molecule contains a type II β-turn motif, then rather than performing two or three serial tests of this idea, the combinatorial chemist might create a library of narrow diversity utilizing a basis set of β-turn mimetics and thus interrogate many slightly different regions of conformational space simultaneously. The drug design of populations versus individuals is analogous to fishing with a net rather than just a hook. As more knowledge of workable strategies for combinatorial synthesis are understood, it is expected that structural and computational input and other rational design information will be integrated into a broad combinatorial medicinal chemistry approach.

Gaining a full appreciation of the issues and difficulties which must be surmounted in order to perform useful combinatorial organic synthesis will initially be a relatively slow process, especially because the important strategies and decision points differ so markedly from traditional organic synthesis. Retrocombinatorial analysis of existing pharmacophores and other important structures should assist in decision making; both in choosing routes of forward synthesis and in synthetic target selection. If combinatorial techniques are indeed to become a useful shortcut to new leads and optimized compounds, then one key implied goal of combinatorial organic synthesis is to intersect the pathway of modern medicinal chemistry upon which compounds move from the early discovery stages to clinical candidacy. Rich incentives await those who are able to mass produce important biologically active molecules quickly and cheaply. Not surprisingly, an aggressive, worldwide effort to understand and master this field has already begun.

This Perspective has been restricted to a consideration of the impact of combinatorial technologies on medicinal chemistry/drug discovery and development. From the point of view of applicability of the technologies, this is an artificially narrow view. Combinatorial processes will become important in diagnostic medicine,[81] agricultural chemistry, food chemistry, immunology, molecular biology, polymer studies, inorganic synthesis, and many other fields. Though the field of "combinatorial chemistry" is chronologically a new enterprise, the evolution of thought in this fertile area continues to outrace the experimental reduction to practice of many ideas. One may reasonably ask "why are combinatorial technologies happening now?". The answer is probably complex and beyond the scope of this Perspective. Nevertheless, the explosive recent interest in the application of combinatorial technologies to drug discovery is symptomatic of an idea whose time has come. Because the issues which confront the medicinal chemist differ so radically from historical approaches, the combinatorial field will no doubt continue to provide impetus and stimulation for the formulation of new concepts and ideas.

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