Combinatorial Chemistry: A Powerful Tool for Basic Research and Drug Discovery

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This article is an introduction to the area of combinatorial chemistry. Although it is by no means a comprehensive survey of the subject, it can serve as a source of basic information about this field. It discusses several issues including chemical diversity measurements, synthesis methods (solid-phase synthesis and synthesis in solution), number of compounds needed for a particular screening project, use of mixtures vs single compounds in the screening, design of flexible vs rigid compounds, importance of application of purified or crude products, and amount of each compound needed for a particular project.
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Introduction
Philosophy and Diversity of High-Throughput Chemistry

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What is the purpose of high-throughput chemistry (HTC)? The answer to this question may be one of the few points in this introductory chapter all scientists in the field will agree on: The purpose of HTC is to speed up the discovery, or optimization, of new chemical entities (i.e., compounds, compositions, or materials) with specific desired properties, such as biological or catalytic activity, or mechanical strength.

In fact, HTC is just a tool used by chemists in lieu of appropriate means to rationally design chemical structures, or compositions with specific properties, when the available information on structural requirements for the desired property of the target compounds is not sufficient to predict their optimal structure a priori. This can be compared to the situation of alchemists in past centuries, which were trying to "transmutate" lead into gold without knowing the atomic structure of the elements [1]. Those who later believed they understood the atomic structure, ridiculed the alchemists, and postulated "transmutation" was impossible. However, the alchemist's experiments yielded numerous novel materials, and, most importantly, furthered our understanding of nature. And today it is well known that one atom can be "transmutated" into another, it "just" requires some reshuffling of protons and neutrons in the atomic nucleus.

Likewise, we ought to remain humble in judging our understanding of the principles of molecular interactions in terms of our ability to rationally design compounds with specific properties "from scratch", in particular when not much information on structural requirements for the desired properties is available. While there are some examples of the computational design of new drugs in the literature, the reality of those studies is often more prosaic in that they merely describe what was found earlier through experiments.

And this is where HTC comes in. Because chemists, like most people, are ambitious and want to be successful. And while they may not be able to achieve such sensational scientific accomplishments like accelerating tiny particles to a speed faster than light, they do want to discover new compounds that can cure cancer, or at least stimulate hair growth. However, making one new compound per week, which until recently was considered respectably productive within the pharmaceutical industry, chemists can expect to generate some 1,500 novel structures during their careers. Statistically, the chances of any of these compounds to become a new worldwide-approved drug are rather slim. But what if they can make 1,500 compounds in one day? Well, they would just increase their probability of success by a factor of 7,500. This is the simple reason why high-throughput chemistry methods have been developed, and are rapidly being introduced into virtually all strategies for the discovery of new chemical entities.

But not only do we want to make as many compounds as possible, as quickly as possible, these compounds should also be as different from each other as possible in order to cover as much as possible of what is referred to as chemical space. This space stretches over all theoretically possible conformations of all compounds within a given range of size. When the structures of a set of compounds made by HTC are evenly distributed over the respective chemical space, each of the compounds has a better statistical chance of being identical or at least similar to the "optimal" conformation for a desired property (e.g., biological activity), as compared to a set of compounds that cover only a fraction of the chemical space, even if the total number of compounds is the same in both sets. (Figure 1)

In other words, preparing a set of compounds differing solely in the number of methylene groups in one particular substituent is not likely to cover much of the chemical space. It should be noted, however, that one single methylene group can make all the difference, as for example in the hormone oxytocin, where the replacement of an asparagine by a glutamine residue, which is identical to asparagine except it has one more methylene group in the side chain, results in complete loss of biological activity (Figure 2).

The most popular format of HTC is that of combinatorial libraries. These are collections of synthetic compounds ranging in complexity from merely some dozens to up to millions of compounds. The central feature of combinatorial libraries is that all compounds making up the library represent combinations of two or more "building blocks" which are connected by chemical reactions. Typical combinatorial libraries have a common structural scaffold or backbone with two or more "attachment points" (e.g., amino, hydroxy, carboxy, sulfhydryl groups), to which "building blocks" (e.g., alcohols, carboxylic acids, amines, aldehydes, anhydrides) are attached in a combinatorial fashion. A complete combinatorial library is composed of all possible permutations of the building blocks at their respective positions. If the scaffold of a library has three attachment points (prospective diversity positions), and ten different building blocks are used for each diversity positions, then the complete combinatorial library is composed of $10^3 = 1000$ compounds. (Figure 3). The complexity and structural diversity of a combinatorial library are thus proportional to the number of diversity positions and building blocks, and the chemical dissimilarity among the latter, respectively.

Combinatorial libraries can be classified based on their chemical character, or the synthesis strategy used to make them, as illustrated in Figure 4. Oligomer libraries (see e.g. in a review [2]) are generated through stepwise assembly of similar building blocks by repetition of the same (or similar) reaction. Scaffolded libraries [3] are based on a molecular scaffold having functional groups, to which the different building blocks are attached in a regio-selective fashion. The generation of most small molecule libraries [4], on the other hand, involves a series of different reactions, during which the scaffold is being formed simultaneously with the introduction of variable positions.

Synthesizing a combinatorial library can be rather straightforward, as the same protocol is typically used for all compounds, so that the synthesis method has to be worked out only once. That, however, is not always as easy as it may sound, as the optimal reaction conditions can vary greatly among the different building blocks used for a particular step.

Some may argue that, because of the similar origin of all compounds in a given library, a combinatorial library can only cover a more or less large fraction of the chemical space. This potential shortcoming of combinatorial libraries can be addressed either by using flexible scaffolds, which cover more chemical space than rigid structures, or by varying not only the combination of building blocks, but also the structure of the scaffold within the library (e.g., different ring sizes, or linear and cyclic scaffolds), while the synthesis method still remains the same for all compounds.

We should keep in mind, however, that not all products of HTC are of combinatorial nature. Using HTC techniques, one can make 1000 completely unrelated compounds that have no common structural motifs. While these compounds may cover a larger fraction of the chemical space than a typical combinatorial library, their synthesis is by far more sophisticated in that it most likely involves several different reactions to generate the various types of compounds.

HTC methods are increasingly utilized for various scientific and industrial projects requiring the availability of large numbers of novel chemical entities, as evidenced by the growing database of literature on molecular diversity, library generation and screening, and solid phase chemistry, which is available on the Internet[5]. This introductory chapter is not intended to cover all topics of scientific interest in this field, but it rather touches on several interesting areas. The following questions continue to be the subject of sometimes controversial discussions among HTC scientists[6], as they address central issues of the subject:

1. How many compounds should be made for a particular project?

The more the better, it's simple statistics. In reality, however, there are practical limitations in terms of both synthesis and screening capacities. As a general rule, the number of compounds made should be reversely proportional to the amount of knowledge available about the studied interaction. If key structural features for the desired properties of the sought after compound are known, it would not make much sense to generate compounds not having these important structural elements, unless the aim of the project is the discovery of novel compounds completely unrelated to already known structures.

On the other hand, if nothing is known about what the desired compounds should look like, as is the case in the search for ligands to a newly discovered protein, then as many and chemically diverse compounds as possible should be made.

Reflecting these considerations, compound libraries are often classified as either lead discovery or lead optimization libraries, with lead discovery libraries being much larger and covering more chemical space compared to the less complex, more focussed lead optimization libraries.

The size and complexity of a particular compound library also depends on the decision as to whether or not to use stereochemically pure chiral building blocks for the synthesis. Again, if it is known that one or the other enantiomer of a particular building block is important for the desired property of the molecule, this enantiomer should be used. If the stereochemical preferences are not known, however, one could either use only one set of enantiomers, reducing the chances of finding the correct molecule by 50% (supposed only one isomer of the molecule is active), or include both enantiomers of chiral building blocks (if available), which doubles the synthesis effort. Alternatively, chiral building blocks can be used as racemic mixtures, and the active isomeric mixtures of compounds subsequently either separated, or re-synthesized as pure isomers in order to identify the active isomer of the compound. That, of course, requires the general acceptance of working with compound mixtures, which brings us to the next question.

2. Mixtures or single compounds? (And, if mixtures, then how complex should they be – 10, 100, 1000, or even millions of compounds per mixture?)

It has been shown by numerous studies that both single compounds arrays, as well as compound mixtures with varying complexity, are suitable tools for the discovery of novel compounds with specific properties.

HTC methods were first established and used for the generation of synthetic peptide combinatorial libraries [2,7]. Such peptide libraries were composed of several millions of peptides, which were either prepared as organized peptide mixtures [8,9], or as one-bead-one-compound libraries [10] (see below). The concept of testing vast compound mixtures was actually taken from nature, and is therefore not as far-out as it is sometimes discussed. Virtually all of the highly specific interactions in biological milieus between ligands and their acceptor molecules take place in a rather heterogeneous environment, i.e., in the presence of numerous other molecules, without interference. Furthermore, fairly complex mixtures of natural products in the shape of plant or animal extracts, fermentation broths, and the like, continue to be an important source of novel bioactive compounds. Therefore, it is not surprising that numerous novel compounds could be identified through the screening of highly complex mixtures of synthetic compounds. Compared to the rather laborious process of isolating and identifying an unknown compound from a natural product mixture, the deconvolution (i.e., the process of identifying individual active compounds within synthetic compound mixtures by synthesizing less complex mixtures and eventually individual compounds) is a straightforward process.

However, there is a persisting skepticism towards the generation and screening of large synthetic compound mixtures, and that is why the current trend in HTC is towards single compound arrays, or, if mixtures at all, then very small, i.e., less than 100 compounds per mixture. The major concerns about mixtures are (i) that the relative concentration of active compounds within the mixture is too low to be detected in the bioassay, (ii) that agonists and antagonists within the mixture cancel each other out, and (iii) that the detected activity of mixtures is caused by artifacts such as impurities or side products, which may not be reproducible in the re-synthesis of individual compounds from active mixtures.

While these concerns may be a valid reason for not using mixtures for one or the other project, they are not a general counter argument against mixtures, as discussed in detail by Houghten et al. [11]. In general, mixtures have to be screened at a much higher concentration than single compounds with the total screening concentration being reversely proportional to the complexity of the mixture. In functional assays, a potent antagonist may indeed mask the activity of an equally potent agonist present in the same mixture, not so in binding assays, where both agonists and antagonists create the same signal. And a well worked-

out, repeatedly rehearsed, and reproducible synthesis method is an essential prerequisite for the synthesis of any combinatorial library, mixtures and single compounds alike.

Moreover, working with mixtures is by far more economical in terms of time and money, in particular for projects involving the generation and screening of large (i.e., > 10,000) numbers of compounds, as illustrated by the following example. A single screening of a library of 50,000 compounds formatted as single compounds, at a throughput of 2500 compounds per day, would take about one month. If the screening cost per compound were $ 1, screening the whole library would cost $ 50,000. When formatted in mixtures of 20 compounds, on the other hand, screening the same library could be done in one day, at a total cost of $ 2,500. Provided the synthesis method had been worked out reproducibly, re-synthesizing and testing individual compounds from the active mixtures should not take more than a week, so that the overall saving in time is at least 75%. If individual compounds do not have to be resynthesized, the savings are even more pronounced. Furthermore, much less target molecule is used up when working with mixtures, which is particularly important when the screening target, or other assay reagents, are expensive and/or available only in very small amounts (e.g., a newly isolated or expressed protein).

For relatively small (i.e., < 50,000 compounds) libraries, it may be a good idea to synthesize all compounds separately, then mix aliquots of each compound for screening, leaving enough of each compound for follow-up studies using single compounds. An alternative is to synthesize singles and mixtures at the same time. These approaches eliminate the need to re-synthesize single compounds from active mixtures, they are, however, less practical for larger libraries.

3. Flexible or rigid compounds?

This is a fairly controversial question. Some argue that rigid molecules have a greater propensity for high-affinity binding than more flexible structures, in which each rotatable bond uses up potential binding energy. Such rigid molecules have to be presented in exactly the correct structure and conformation in order to fit into the respective binding site. The *a priori* discovery of novel rigid high-affinity binders is a rather risky game of "all or nothing", because such ligands won't be found in the most complex and diverse library, if this library happens to lack that one perfect compound, even if very similar analogs of it are present.

On the other hand, the chances of finding a ligand to a particular target molecule in a library of more flexible compounds are higher, since such flexible structures are able to adopt a range of conformations, with any luck including one that fits into the binding site of the target. For some interactions, that "binding conformation" is only formed when the ligand is brought into spatial proximity to the binding site of the target (induced fit).
Such flexible molecules, however, are often only modest or low-affinity binders, and require a fair amount of structural optimization in order to turn them into acceptable ligands.

In conclusion, even if we are after rigid high-affinity binders, we should be flexible in our approach to finding them.

4. Should the compounds be purified, or how pure should crude products at least be?

This is again an issue of balancing risks and resources. Testing pure compounds is no doubt the safest way to generate unambiguous data, as testing crude compounds always bears the risk of the observed activity being due to side products, rather than the compounds themselves. Moreover, an interesting compound may be barely or not at all present in the synthesis product, and can therefore not be found in the screening. However, the elimination of these risks does often not justify the time spent to purify each compound, unless the fractionation of synthesis products is the final element of generating the library, which will then include all side products separately, without even knowing the structures of most of them.

Purity standards for crude products depend largely on the efficiency of the synthesis method, as well as the robustness of the bioassay, and are typically in the range of >70-80%.

When working with unpurified compounds it is absolutely essential that the synthesis method is reproducible, so that any possible active side products can be readily re-generated. Such side products may actually be more interesting new drug leads than the intended compounds, as they may present completely new structures.

5. How much of each compound should be made?

Historically, organic chemists liked to make "decent" amounts of compounds, i.e., >100 milligrams. In HTC, such relatively large-scale syntheses become uneconomical in terms of both synthesis cost and storage space. Besides, 100 mg of a compound with a molecular weight of about 500 D yield 2 L of a 100 μM solution, which is far more than needed, even if the compounds were to be tested in quadruplicates at different concentrations in hundreds of bioassays. In view of these considerations, HTC is now being miniaturized down to the picomole scale in order to make the synthesis more economical [12]. In fact, there are several approaches to the synthesis at the scale of single beads of solid support (see below) with a diameter of approximately 100 μm. A typical capacity of such beads is approximately 100 pmol, so that the compound on a single bead can theoretically yield 10 μl of a 10 μM solution. While this amount may be enough to test the compound in one bioassay, maybe even in duplicate, it will not suffice to establish dose-response curves, or to test the compound in more than one bioassay. On the other hand, binding to the target molecule can be observed even on smaller beads - 60,000 beads of 3 μm diameter can be immobilized on 1 mm^2 of an etched optical fiber bundle, and specific interaction with the target can be observed on the level of individual beads [13]. In any case, highly miniaturized synthesis approaches always bear the potential need to subsequently re-synthesize all or at least some of the active compounds for follow-up studies or additional screening projects.

In general, the decision as to how much of each compounds should be made in a particular HTC project should depend on (i) the financial and space resources available for that particular project, (ii) the capacity and scale of the synthesis instrumentation used, (iii) if the compounds are purified, the expected yield of purified compounds, and (iv) the number of bioassays the compounds will be tested in, as well as the volumes and concentrations of compound solutions needed for each bioassay.

6. Solid-phase synthesis or synthesis in solution?

As discussed before, HTC techniques were first developed for the multiple parallel synthesis of peptides and peptide combinatorial libraries. The majority of these methods utilize Merrifield's concept of solid-phase peptide synthesis [14], which is based on the stepwise assembly of peptide chains after covalent attachment of the C-terminal amino acid to a polymeric solid support. Unlike in solution synthesis, where intermediates and final products are typically isolated and purified by extraction and precipitation, in solid-phase synthesis such procedures are replaced by simply washing the solid support, to which the growing peptide remains attached until it is typically cleaved after the synthesis is completed. Apart from greatly facilitating and accelerating the synthesis process, solid-phase synthesis is also readily amenable to automation due to the highly repetitive character of the process (i.e., repetition of the cycle: coupling-wash-deprotection-wash).

The classical support materials for solid-phase synthesis are functionalized polymer resin beads based on crosslinked polystyrene, polyethylene glycol, or other polymers. Today, support materials used for solid-phase synthesis come in many shapes and forms, such as plastic pins, cellulose disks, as well as spatially addressable membranes or slides, as reviewed comprehensively by Hudson [15,16].

While solid-phase synthesis became very popular and refined in peptide chemistry during the 1980ies, it did not receive much attention from organic chemists at that time. It was only after the striking success of synthetic peptide combinatorial libraries that the solid-phase principle was rapidly adopted for high-throughput organic chemistry [17]. In fact, the essence of many of the initial reports on the generation of "non-peptide combinatorial libraries" was the adaptation of general, well established organic chemical reactions to solid-phase synthesis with the aim of "getting organic chemistry on the solid support". Today, solid-phase synthesis is the method of choice for the majority of HTC projects [18-23].

A modification of solid-phase synthesis has been referred to as "resin-capture", in which the synthesis is performed in solution, and the desired molecule is trapped selectively on the solid support, while excess of reagents is removed by washing the solid support. This strategy is only feasible if neither starting material, nor side products have any functional groups that could react with the solid support [24]. A reverse approach, in which the solid phase support interacts with excess of starting materials and side products, leaving only the desired product in solution, is called "polymer-supported quench"[25].

Notwithstanding the growing success of solid-phase synthesis in organic chemistry, quite a few chemists remained faithful to conventional synthesis in solution, also for HTC syntheses[26]. This may be beneficial for syntheses involving reactions that are not easily performed on solid phase, or when the products of solid-phase synthesis are considerably less pure than the same compounds made in solution. In general, however, highly parallel HTC in solution requires by far more synthesis instrumentation compared to HTC on solid phase (e.g., for parallel liquid transfer and liquid/liquid extractions).

A hybrid of solid-phase and solution synthesis has been devised with the aim to combine the advantages of both methods [27]. As in solid phase synthesis, the molecules are assembled while bound to a polymeric support, which, unlike in solid-phase synthesis, is soluble in the reaction medium, so that the reaction kinetics are comparable to solution synthesis. After the reaction, the polymeric support can be precipitated for removal of excess of reagents. The solvent and reaction conditions for the precipitation have to be chosen and optimized carefully in order to prevent inclusion of remaining reagent, which could interfere with the next synthesis step after the polymer is dissolved again, within the precipitated particles. This approach was also originally developed for peptide synthesis [28], and has now been revived for HTC.

Another emerging technology aimed at combining the benefits of solid and liquid phase synthesis is fluorous phase synthesis[29,30], in which the growing molecule is attached to a chemical "tag" with a high proportion of fluorine. The synthesis is carried out in a three phase liquid system (i.e., an organic solvent, an aqueous phase, and a fluorocarbon solvent), with the tagged compound preferentially soluble in the "fluorous" phase, thus enabling extraction of excess reagent and side products either to the organic, or the aqueous phase.

7. Is synthesis automation essential for efficient HTC?

It is not essential, but it can make life much easier - if one can afford it. Various instruments for the automated parallel synthesis of up to hundreds of compounds are now available [31-33], and many companies are dedicating much effort to in-house programs for the development of their own systems for automated HTC (see e.g [34]). Using the right methods, however, large numbers of compounds can also be prepared without the need for synthesis automation, even without hiring an army of lab workers.

One approach for manual solid-phase-HTC is based on the segmentation of the solid supports for multiple parallel synthesis (one segment per compound) [35,36], and is particularly suited for combinatorial syntheses. A membrane-like support [35], for example, can be easily divided into as many segments as compounds are to be synthesized. Synthesis steps that are identical for several or all compounds can thus be performed in common reaction vessels without cross-contamination between solid support segments, which can be sorted and re-distributed between synthesis steps as often as necessary (Figure 5). The sorting of solid support segments can be further facilitated using bar codes or radio frequency tags [37-39].

A powerful, yet simple method for the manual or semi-automated solid-phase synthesis of mixtures of up to millions of compounds is the "one-bead-one-compound" approach [10](Fig. 5). It has also been referred to as "split-and-mix" [40] or "divide-couple-recombine"[8] approach, and is based on coupling each building block to separate portions of the solid-phase resin, followed by combining and mixing all resin portions, before dividing the resin again for the next synthesis step (Figure 6). By repeating this procedure three more times, and using 20 different building blocks for each synthesis step, a library of 160,000 (20^4) compounds can be readily prepared. This process yields libraries containing an individual, unique compound on each resin bead. When 1 g of 130 μm resin beads (approx. 1,000,000 beads) are used for the synthesis for a library of 1,000 compounds, each compound is synthesized on an average of 1,000 beads. If only 1,000 beads were used, the statistical probability of having any particular structure present in the library would be only about

70%. After assembling the library on the resin, it can be either cleaved for bioassays in solution, or left on the resin for solid-phase assays. The bio-assays are typically performed on single beads, so that the screening format of one-bead-one-compound libraries is that of single compounds, rather than compound mixtures[41].

Perspective

The various approaches and philosophies of combinatorial and high-throughput chemistry have been discussed, advocated, and dismissed at numerous occasions. We believe the most appropriate approach to these issues is to be open to new ideas and concepts, even if they seem to negate previously held theories, and to utilize the technology most suitable for solving the problem, which may not always be the most popular technology at the time.

This brief discussion of questions regarding HTC is intended to evoke the interest of the reader with scientific ambitions. Curious and creative minds are needed to push the technology of HTC further, and unanswered questions should be understood as a challenge to newcomers to this exciting field.

Figure captions

Figure 1. Coverage of the chemical space (square) by synthetic compounds (stars). A: Compounds are evenly distributed over the entire space; three compounds are similar to the optimal structure (circle). B: Compounds are more similar and cover only a fraction of the chemical space; none of the compounds comes close to the optimal structure. Note: The chemical space is actually not two-, but multi-dimensional with the axes being defined by various molecular descriptors, such as structure, molecular weight, lipophilicity, charge, etc.

Figure 2. Structures of oxytocin (top, active) and Gln^5-oxytocin (bottom, inactive).

Figure 3. Combinatorial library of nine compounds on a five-membered ring scaffold with one constant (R) and two varied (A and B) positions, using three building blocks for each of the two varied positions (A_1 through A_3 and B_1 through B_3, respectively). Accordingly, if the library had three varied positions, and 10 different building blocks were used for each varied position, then the complete library would be composed of 1000 (10^3) individual compounds.

Figure 4. Different library types: oligomeric libraries (top), scaffolded libraries (center), and condensed libraries (bottom). R: Variable library positions. A,B: Functional groups used for reactions during library generation. Oligomeric libraries are built by connecting similar building blocks through repetition of the same or similar reactions - peptides and oligonucleotides are typical examples. Scaffolded libraries are generated through regio-selective coupling of building blocks to different sites of the molecular scaffold. In condensed libraries, it may be difficult to trace the character of building blocks used for library generation.

Figure 5. Process of sorting, combining, and re-sorting of support segments for multiple parallel solid-phase synthesis.

Figure 6. Principle of one bead one compound library synthesis (see text for details).

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