Preparation and Screening against Acetylcholinesterase of a Non-Peptide "Indexed" Combinatorial Library

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Abstract: A combinatorial library composed from nine alcohols and six isocyanates to formally generate 54 carbamates has been designed, prepared, and screened against acetylcholinesterase from the electric eel. In order to deduce the most active member of the library, it was prepared as 15 sublibraries in which one of the reacting components was fixed and the other reactants were used as an equimolar mixture. The product mixtures were tested and their activities used as "indices" to the rows or columns of a two-dimensional matrix reflecting the activities of individual carbamates. A number of carbamates in the most active row and column were synthesized and assayed, demonstrating that the most active cell in the matrix could be identified by the sublibrary synthesis procedure. Other methods for generating large libraries of molecules for biological screening that have recently been developed have relied on a covalent attachment between library members and a label to identify the active components. Indexed libraries offer the advantage that they can be prepared from any class of compounds composed from multiple subunits and that any type of assay (binding, enzyme inhibition, agonism/antagonism, cell-based, or even whole organism assays for biological activity) can be used because all compounds are generated in a free form.

Introduction

Chemical diversity methods for the preparation of large libraries of molecules to screen for biological activity have recently become a significant subject of research.[2] Almost all rely on the approach of block synthesis, wherein molecules are assembled from a single set of building blocks that possesses common linking chemistry and diverse "side chain" functionality. Peptides,[3] despite their known disadvantages as pharmaceuticals, or peptide-like molecules[4] have generally been used as the building block set in chemical diversity experiments. This has been the case for two principal reasons: they are relatively easy to prepare on solid supports, which can further facilitate their identification. To discover compounds with optimal biological activity and the ability to resist endogenous degradative pathways, it is necessary to expand beyond the genetically-coded amino acids to unnatural residues and linking chemistries.[5] Biological methods to create diversity do not offer this capability, though they do produce libraries of significantly greater size than can currently be accessed with chemical methods. This report describes a novel method to prepare and screen libraries that does not depend on solid-phase chemistry.

A major challenge in any effort to screen large ensembles of compounds (natural products, fermentation extracts, synthetic compound collections, or combinatorial libraries) for function is identification of the "actives". Engineering the chemical diversity offers the opportunity to link an identifier to each molecule in the library. Means of identification of elements in libraries include physical isolation on macroscopic pins,[6] microscopic localization on a surface,[7] sequencing of the peptides themselves,[8] sequencing of polymers coding for peptide sequences (including DNA[9] or peptides[10]), and sequencing by organic identifying groups.[11] A disadvantage of linking an identifier to each molecule in the library is that it can prevent the ensemble from penetrating macromolecules, organelles, cells, organs, or organisms. With many libraries, the assays are consequently limited to binding, but efforts to overcome this limitation have been made. "Range-finding" or "deconvolution" methods have been reported wherein pools of peptides are synthesized on solid phase, cleaved from the support, and tested and then individual components within active pools resynthesized on solid phase for further testing.[12] Methods have also been devised that combine a linked identifier with a solution-phase assay. Here, only a portion of a support-bound peptide is cleaved for assay, so that those supports that produce "hits"

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can be retrieved for sequencing; a recent example used an *in situ* cell-based color assay.[13] The discovery of active peptide ligands through sublibrary synthesis procedures has been reported by Houghten.[14] In this method, which does not require iterative synthesis, hexapeptide pools are prepared with one position fixed and the rest randomized (20 pools of YXXXXX, where X = 20 amino acids and Y = one of the 20 amino acids, 20 pools of XYXXXX, and so on). These 120 pools are synthesized, cleaved from the support, and tested, thereby discerning the most potent residue at each position of the hexapeptide.

New techniques are required both to expand the structural diversity available in synthetic libraries and to permit more diverse assays. We report an advancement toward this goal, *indexed combinatorial libraries*, that is applicable to any molecule that can be assembled in a simple chemical process from multiple subunits. Using this technique, we have prepared and screened pools containing candidate carbamates[15] as inhibitors of acetylcholinesterase.[16] Inhibitors of this enzyme are used in the treatment of myasthenia gravis and glaucoma and have been proposed as possible therapeutics for Alzheimer's disease.[17] This method differs from the deconvolution or range-finding methods in that all of the synthetic chemistry is performed simultaneously and in solution, followed by parallel assays of the resulting pools. Less effort is required to adapt the preparation of a pharmacophore of interest to pool synthesis than to solid-phase synthesis, thereby making combinatorial principles applicable to a wider range of targets. Iterative syntheses and assays are also avoided, providing a significant reduction in the effort needed to synthesize and test the large numbers of compounds that can be composed from N different building block sets. This work also extends the principles of combinatorial synthesis outside the rather narrow area of peptide chemistry, where they have been best applied. This method is applicable to compounds (specifically, carbamates in this work) that may not be amenable to synthesis on solid phase or within oligomeric structures, as is required by most other combinatorial synthesis procedures.

Theory

The combinatorics in the preparation of a library can be conceptually represented as an N-dimensional matrix, wherein each axis has as many elements as are present in each set (n). Consider a library of molecules composed from two sets of substructures **A** and **B**, each of which has 10 structural variants (the number of elements in each building block set, a and b = 10). They can be envisioned to compose a 10 × 10 grid (Figure 1). Each cell contains, for the combination ($\mathbf{A}_x$, $\mathbf{B}_y$), its assay value. To examine all as pure compounds would require 100 experiments. One cell possesses the maximum response function ($\mathbf{R}_{x,y}$) in the grid; the task is to find it without actually looking at them all. Were the contributions of **A** and **B** to the response function completely independent, the best combination could be discovered by choosing any **B** for testing with all **A**'s, and any **A** for testing with all **B**'s. When **A** and **B** are not independent variables in the response function, indexing permits all combinations to be tested. By screening the rows and the columns, which are indices to the cells at their intersections, *as mixtures*, only 20 reactions/assays are required to find the

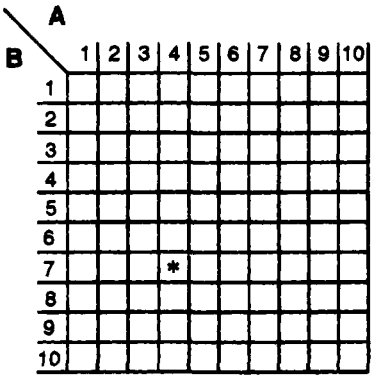

Figure 1. Conceptual matrix for a combinatorial synthesis.

maximum response.[18] Each compound is tested twice, once each as a component of an **A** mixture and a **B** mixture (100 compounds × 2 assays = 200 = 20 row/column reactions × 10 compounds in each). The index to the maximum cell in this example is its *row* reaction, composed of one reagent $\mathbf{B}_7$ and an equimolar mixture of **A** reagents $\mathbf{A}_1$–$\mathbf{A}_{10}$, and its *column* reaction, composed of the reagent $\mathbf{A}_4$ and an equimolar mixture of **B** reagents $\mathbf{B}_1$–$\mathbf{B}_{10}$. Because all combinations are tested, an assumption that parameters **A** and **B** do not interact is not required. This example shows a 5-fold improvement in the synthesis and data collection efficiency (the parallelism advantage[19]) for the library compared to one-at-a-time processing. Clearly, this process can be conducted with more elements in each set and with more sets, leading to higher-dimensional arrays and to higher efficiency in data collection.

In general, assays of pooled compounds are limited by the precision and sensitivity of the assay, the potency of the "hits", and the number of elements in each pool. Consider a worst-case scenario, a single potent compound that is diluted by mediocre combinations, for row $\mathbf{B}_7$. If its combination with $\mathbf{A}_4$ has a value ($\mathbf{R}_{4,7}$)[20] of 10^9 in the assay in question and all of its other **A** combinations are 10^6, the response function for the row ($\mathbf{R}_{A,7}$) should be their average (eq 1), or 10^8. The increase of the row average due to the inclusion of the "hit" must be greater than the error in the assay to reliably identify an active row (eq 2).[21] Characteristics of the screening of pools consistent with intuition are demonstrated by eq 3, which is derived from eq 2 by straightforward algebra. Equation 3 is also a generalization of eq 2 to pools of n members. Greater potency ($\mathbf{R}_{hit}$ increases) will obviously make "hits" more likely to be detected. A significant population of mediocre compounds ($\mathbf{R}_{av}$ large) will have the opposite effect, raising the background and making it more difficult to identify actives. Precise assays (σ small) will make actives easier to find. Lone active compounds are more likely to be missed with larger building block sets ($n \rightarrow \infty$) because they are "diluted" by inactive components,[22] but larger building block sets are also expected to increase the probability that at least one active is present in the library. It is

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(20) The response function (=assay value) for each cell is indexed by its A column and B row ($\mathbf{R}_{A,B}$). The assay value for a row is given by $\mathbf{R}_{A,y}$ where y is the row number, and for a column is given by $\mathbf{R}_{x,B}$, where x is the column number.
(21) This is simply the comparison of the experimental means (t-test) with and without the potent compound. The same criterion is applied to columns. Of course, the only experimental values accessible at the stage of library screening are the row and column averages with the potent compound, as well as their standard deviations. As was done in this work, once actives have been identified and individual pure compounds synthesized for testing, "hits" can be validated statistically using eq 3.

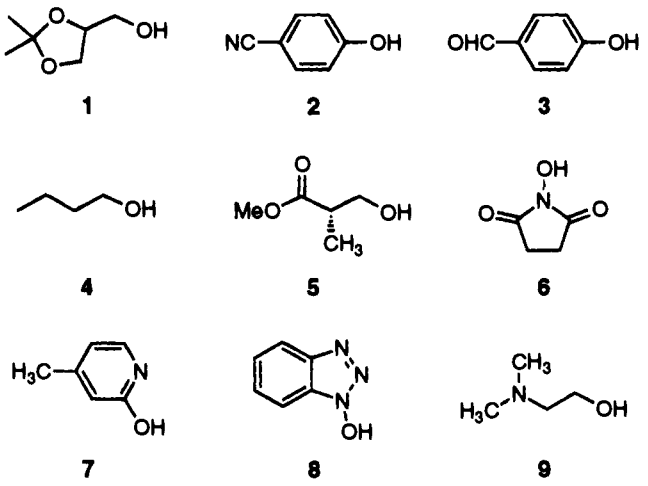


Figure 2. O building blocks.

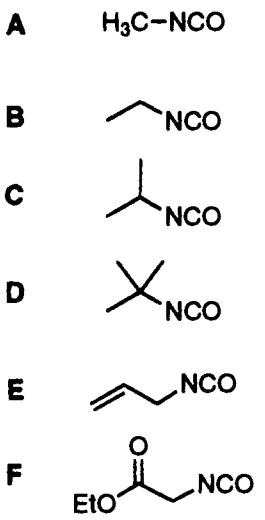


Figure 3. N building blocks.

impossible to quantify the relationship between set size and the likelihood of finding a "hit" *a priori*; it will be unique to each possible library chemistry and assay.

$$R_{A,7} = \frac{1}{a}\sum_{i=1\to a} R_{i,7} \quad (1)$$

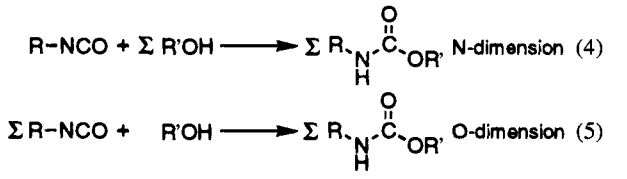
$$R_{A,7} - \frac{aR_{A,7} - R_{4,7}}{a-1} > t\sigma\sqrt{\frac{a+(a-1)}{a(a-1)}} \quad (2)$$

or more generally, for n building blocks in the Nth dimension

$$R_{hit} - R_{ave} > t\sigma\sqrt{\frac{(2n-1)(n-1)}{n}} \quad (3)$$

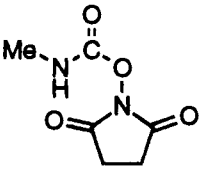
Results

A library of carbamates (which should inhibit acetylcholinesterase by carbamoylation of the active site serine[16]) was prepared using indexed combinatorial synthesis. A basis set of the nine alcohols in Figure 2 was used in reactions with the basis set of six isocyanates in Figure 3. These building block set sizes were chosen so that the library would be large enough to demonstrate the principle but small enough to permit the composition of (members of) the library to be verified by analytical methods. Each of the 15 row and column reactions utilized a *unitary reagent* and a *mixed reagent* (eqs 4 and 5).



Each of the basis set molecules was the unitary reagent in only one reaction of the 15; it was present in a mixed reagent equimolar with the members of its basis set in all reactions where the other basis set members were unitary reagents. To eliminate kinetic effects, reactions were conducted with a stoichiometric quantity of the unitary reagent relative to the total of the mixed reagents, so reactions were forced to completion for each component in the reaction. The resulting 15 mixtures were used to determine aggregate IC_{50} values against acetylcholinesterase. Select compounds in the active rows and columns (*vide infra*) were individually synthesized and characterized, and their presence in row and column reactions was verified by HPLC.

The data are presented graphically in Figure 4 and listed in Table 1. In the N-dimension, the inhibitory potency decreases in the order Me > *i*-Pr > Et > *t*-Bu. The same order was found when the carbamates from column 6 were individually prepared, purified, and analyzed. In the O-dimension, the potency decreases in the order succinimide > benzotriazole > benzaldehyde. Synthesis and assay of both active and inactive components from row A showed potencies that closely reflected those obtained in the mixed assays.[23] It also permitted the presence in the sublibrary of low-activity components to be established by HPLC as a control that the row reaction indeed possesses all imputed members. The most potent inhibitor in this library is *O*-succinimidyl *N*-methylcarbamate (**6A**), a heretofore unknown compound.



6A

Table 1

O-dimension	$1/IC_{50}$ M^{-1}	N-dimension	$1/IC_{50}$ M^{-1}	compd	$1/IC_{50}$ M^{-1}
1	18	**A**	1414 ± 135	**1A**	0
2	573 ± 82	**B**	360 ± 15	**2A**	330
3	1055 ± 125	**C**	431 ± 30	**3A**	527
4	71	**D**	161 ± 15	**5A**	0
5	37 ± 6	**E**	422 ± 16	**6A**	1497
6	1623 ± 209	**F**	224 ± 25	**6B**	156
7	717 ± 67			**6C**	168
8	1168 ± 152			**6D**	51
9	0				

(22) The right-hand side of eq 3 is only a mildly increasing function of n because it explicitly scales with the square root of n and t gently decreases with increasing n. The value for t is based on $2n - 3$ ($=n + (n - 1) - 2$) degrees of freedom. At the 95% confidence level, t is ~2 for > 10 degrees of freedom, corresponding to $n > 6$.

(23) The parallels between the assay results with pure compounds and sublibraries suggest that the contributions of the O-residue and the N-residue are independent.

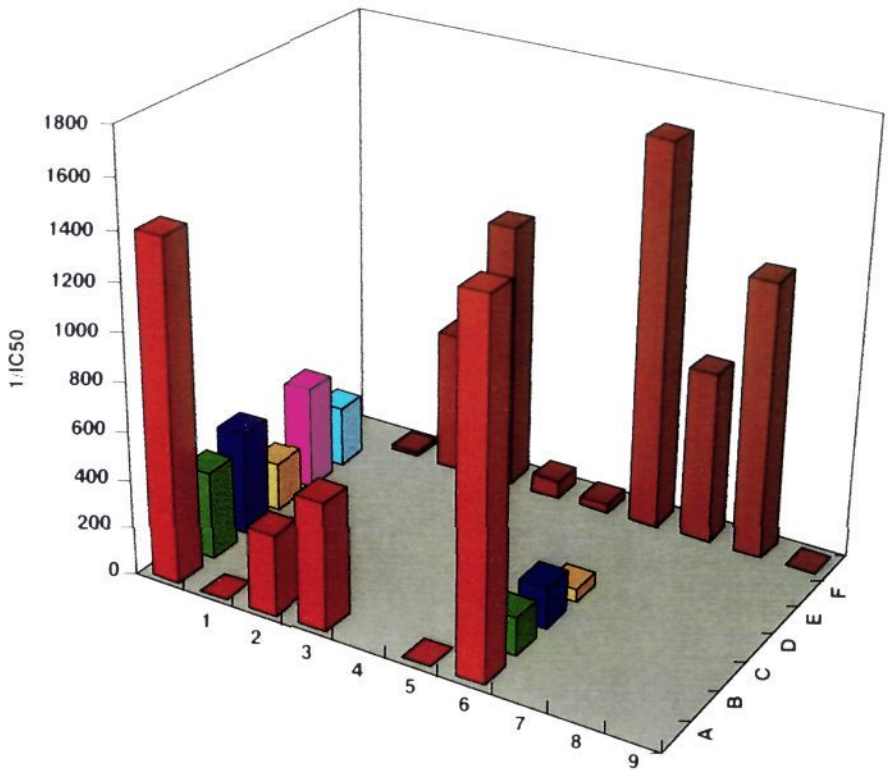


Figure 4. Response function for the inhibition of acetylcholinesterase by carbamates displayed as $1/IC_{50}$ (M^{-1}). The back row gives the results of mixture assays in the O-dimension, and the left-most column gives the results of mixture assays in the N-dimension. Individual compounds within the grid in both the most active row and the most active column were chosen for synthesis and assay as pure compounds. Their assay values are given at the intersections of the row for the O-dimension of their alcohol component and the column for the N-dimension of their isocyanate component. Some structure−activity relationships can be perceived in the data. For example, it is clear that *N*-methylcarbamates are superior to all other *N*-alkyl groups, consistent with knowledge that propionylcholine is a much poorer substrate for acetylcholinesterase than acetylcholine. Relatively acidic alcohols that can be good leaving groups in the acylation of the active site serine are superior. The IC_{50} value for **6A**, 0.7 mM, primarily reflects the rate of acylation of the serine hydroxyl under these assay conditions, not its binding potency. Time-dependent inhibition (data not shown) was shown by compounds in column 3.

Discussion

Approaches to the chemist's task of providing molecules with specific functions have varied through time. Random screening was an early focus, but its low intellectual appeal and low success rate provided impetus to develop more rational approaches, a trend most easily seen in rational drug design. While providing a much more directed path for the development of compounds optimized for a given pharmaceutical target, an advance of unquestioned value, there are still many factors controlling the interactions of macromolecules with "micromolecules" that are incompletely understood. Furthermore, many of the interactions dictating function are not between molecules but between a molecule and a complex system which cannot be analyzed by solely chemical principles, making design one of the most challenging problems facing organic and medicinal chemists. As a consequence, the Edisonian approach is enjoying a resurgence in the form of combinatorial chemistry or combinatorial libraries. Increased interactions between chemistry and biology have allowed chemists to see more clearly nature's only acknowledged mechanism for achieving function: evolution. The principle that one object among a large number of possible variations may have a desired property and can thereby be selected is at the core of efforts in combinatorial chemistry. It is also the antithesis of nearly two centuries of preparative chemistry. The synthesis of a single molecule of predictable structure has long been the prime goal; function has been secondary. In combinatorial chemistry, the premium is placed on function and structural determination follows, an approach reminiscent of natural products screening. A novelty in library synthesis is engineering to make identification of the "actives" straightforward; it is also designed to be fast and inexpensive, in counterpoint to the pure Edisonian approach to achieving function, which can be time-consuming and costly. Parallelism in synthesis, so that the number of compounds prepared is greater than the number of chemical steps required, is also common.

An important consideration in the design of a library synthesis is the number of building block sets. It is natural to think in terms of one set, such as the genetically-coded amino acids, but a library can also be prepared from multiple (overlapping or nonoverlapping) sets, usually linked in a predefined order. The use of multiple sets is analogous to the grade school math problem, "How many outfits can you make from five shirts and four pants?", while a single set is analogous to a deck of playing cards from which hands of varying size can be drawn; the larger the hand, the more possibilities. This distinction is reflected in the size of the library and the protocol for synthesis, and such syntheses can be designated as *combinatorial* or *permutational*. The former produces compounds of defined order (basic amino acids at position 1, hydrophobic amino acids at position 2, etc.), while the latter can produce compounds of variable length, ordered in all possible sequences within the set. The naturally-occurring polymers used in biological methods for library production are directional and composed from one set and therefore constitute permutational syntheses. One advantage of combinatorial syntheses is that compounds from chemically-distinct building block sets can be united to form substances that could never be obtained by a permutational synthesis, no matter how diverse the set. Because oligomeric molecules must have common linking chemistry, much of their structure is

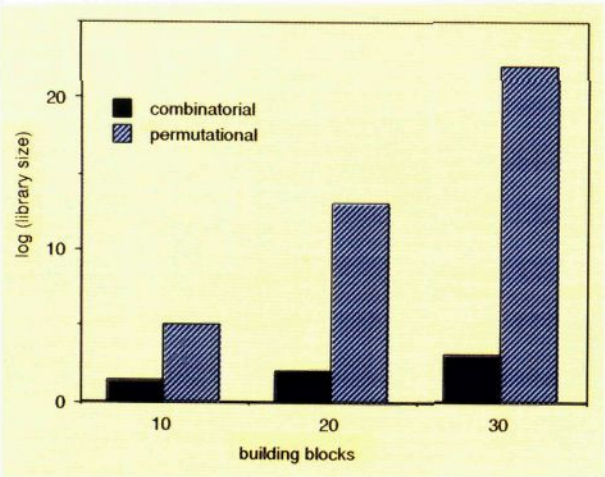


Figure 5. Quantity of diversity. In order to make a fair comparison between combinatorial and permutational syntheses, this graph assumes that the combinatorial synthesis is performed with the building blocks divided into two sets of equal size and that the permutational synthesis produces a molecule of length one-half the set size. It also shows the expected exponential dependence of library size on the number of building blocks.

constant, not diverse, and the same must be true of the libraries derived from them. While oligomeric libraries can be made in either way, most organic compounds (*e.g.*, the carbamates studied here) are not prepared using a repeated linking chemistry, so only combinatorial libraries can be prepared from them.

The power of these two different library methods can be easily seen in their *parallelism advantage*, defined as the number of compounds made per chemical step. Distinction between them can be made on the basis of the scaling of the parallelism advantage or the library size with the number of members in the building block set(s). The number of steps in a combinatorial synthesis is the sum of the number of members in each building block set ($N_1 + N_2 + ... + N_x$), and the number of compounds generated is the product of the number of members (N_x) in each building block set ($N_1 \times N_2 ... \times N_x$). The number of steps in a permutational synthesis is the product of the number of members in the building block set and the length of the sequence (Nl), and the number of compounds generated is the number of members in the building block set raised to the power of their length (N^l). The parallelism advantage for permutational syntheses is the quotient of a power function and a product function of the number of building blocks, whereas for combinatorial syntheses, it is the quotient of a product and a sum. The size of a permutational library is most sensitive to the length of the molecule, while the size of a combinatorial library is most sensitive to the set size. The chemical diversity in a library can be measured by both its quantity (Figure 5) and quality (Figure 6). Permutational synthesis provides a large size in few steps, like the many words that can be composed from a single alphabet. Combinatorial synthesis can incorporate more structural variety, like some novel language composed from the Roman, Greek, Cyrillic, Hebrew, Sanskrit, and Kanji alphabets. The distinction between combinatorial and permutational synthesis is thus related to the number of building block sets, ordering, and length.

One of the key issues in the use of combinatorial methods based on pooled synthesis is the maximum pool size in which a "hit" can be reliably found. The statistical formalism of eq 3 can be applied to the data (Table 1) obtained in this library synthesis. This analysis suggests that subpools formed from as many as 70 building blocks or a total library size of over 800 would still have permitted a single active to be reliably

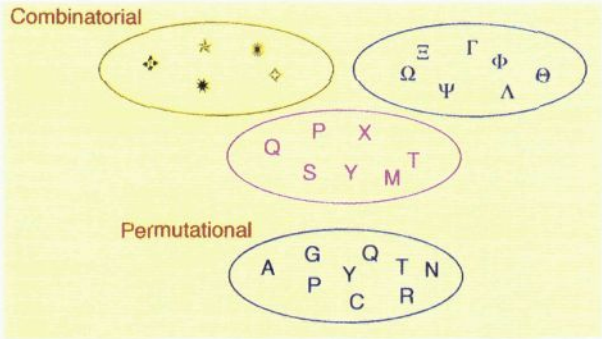


Figure 6. Quality of diversity: Examples of set notations for combinatorial and permutational syntheses.

identified using our assay. This quantity of diversity will sufficiently explore the structure–activity relationships in an interesting class of compounds to make the indexed library approach a valuable addition to the many applications of combinatorial chemistry. Reactions that combine more than two components might be used to make larger pools and libraries, but assay precision will still directly limit the pool size that can be reliably screened.[24] To increase library size without increasing assay precision would require increasing the number of pools.[25]

Conclusion

Indexed combinatorial libraries not only address one of the motivations behind the development of library or molecular diversity methods, the preparation and testing of *all* possibilities to eliminate assumptions about structure–activity relationships, but may also provide insight into structure–activity relationships. Indexing permits the preparation and identification of active non-oligomeric compounds and the use of any type of assay. It is worth emphasis that the goals of library methods such as this one are not so much synthetic as analytic. Their purpose is to gain information on as wide a universe of structures as possible in the shortest time and with the smallest cost. Once interesting molecules are identified from among the many in the library, conventional macroscopic synthesis techniques can be used to obtain them in quantities necessary for further study. It is important to recognize that the testing of mixtures makes possible interactions between compounds that could lead to either an increase (synergism) or decrease (antagonism) of the measured potency compared to that of the pure compounds. The latter is a "false negative" possible in any screening effort. Provided that the library is diverse enough to find a sufficient number of interesting leads, such antagonism can be tolerated.

Comparison between the methods of permutational and combinatorial synthesis shows that there are advantages unique to each. Generally, the number of reactions required to prepare a combinatorial library scales with the sum of the number of elements in each building block set ($a + b + ... + z$), while the number of compounds in the library scales with their product ($a \times b \times ... \times z$).[19] For the library prepared in this study, the size was a relatively modest 54 and the parallelism advantage in the synthesis and screening was only 3.6. However, it is worth emphasis that the quantity of diversity in a library (=size) is not the only measure of its value in the discovery of active

(24) For example, pools in a three-dimensional library could be formed from one unitary reagent and two mixed reagents. A $10 \times 10 \times 10$ synthesis would constitute 30 100-element subpools in a 1000 element library. Greater pool sizes and greater library sizes would require more precise assays than in this example.

(25) This could be accomplished by splitting the synthesis into further subpools of the mixed reagent(s), but an increase in the number of pools results in a corresponding decrease in the parallelism advantage.

compounds; the quality of the diversity is also important. The virtue of combinatorial synthesis lies in its ability to unite compounds from chemically-distinct building block sets to form substances that could never be obtained by a permutational synthesis. The approach described here may also provide a general strategy to optimize processes in which the variables are not continuous. Principles of combinatorial chemistry should be applicable in many areas beyond drug discovery.

Experimental Section

General Procedures. Acetylthiocholine iodide, DTNB (5,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent), and acetylcholinesterase (EC 3.1.1.7, Type V-S from the electric eel) were obtained from Sigma. Isocyanates were purchased from Aldrich. An Ace pressure tube was bought from Ace Glass, Inc. Benzene and triethylamine were distilled from calcium hydride. THF was distilled from sodium/benzophenone. All chemical reactions were carried out under a nitrogen atmosphere in the Ace pressure tube. NMR spectra were recorded at 300 and 75 MHz for ^1H and ^{13}C, respectively, on a Varian XL-300 spectrometer. Proton chemical shifts are reported in parts per million (ppm) relative to $CDCl_3$, as are carbon chemical shifts. High-resolution mass spectra were recorded on a JEOL JMS-SX 102A mass spectrometer. Analytical HPLC was performed on an HP 1090 liquid chromatograph with a diode array detector and recorded on an HP 3392A integrator. Enzyme assays were conducted on a Shimadzu UV-160U UV−visible recording spectrophotometer.

Preparation of Carbamate Mixtures in the O-Dimension. A THF solution (5−10 mL) of an alcohol or a phenol (6 mmol) and six alkyl isocyanates (1 mmol each) containing 10 mol % of triethylamine (based upon alcohol/phenol) was sealed in an Ace pressure tube. The reaction mixture was heated at 60−80 °C for 4 h and then allowed to stand at room temperature overnight. The solvent and triethylamine were then removed by a rotary evaporator.

Preparation of Carbamate Mixtures in the N-Dimension. A THF solution (5−10 mL) of an alkyl isocyanate (9 mmol) and nine alcohols/phenols (1 mmol each) containing 10 mol % of triethylamine (based upon alcohol/phenol) was sealed in an Ace pressure tube. The reaction mixture was heated at 60−80 °C for 4 h and then allowed to stand at room temperature overnight. The solvent and triethylamine were then removed by a rotary evaporator.

Preparation of Carbamates. Similar to the preparation of carbamate mixtures, a THF solution (10 mL) was prepared containing an alcohol (or a phenol) (10 mmol), an alkyl isocyanate (10 mmol), and 10 mol % of triethylamine. The reaction solution was heated at 60−80 °C for 4 h and then left at room temperature overnight. The product was obtained after removing the solvent and triethylamine. The yield was near quantitative. When necessary for high-resolution mass spectrometry (HR-MS), the carbamate was further purified via a silica gel flash column (hexanes/ethyl acetate). The ^1H, ^{13}C NMR, and HR-MS data of these carbamates are tabulated below.

***O*-Succinimidyl *N*-Methylcarbamate (6A).** ^1H NMR (300 MHz, $CDCl_3$): δ 2.80 (s, 4H), 2.86 (d, J = 5.1 Hz, 3H), 5.61 (br, 1H). ^{13}C NMR ($CDCl_3$): δ 25.47, 28.24, 152.00, 170.16. HR-MS: calcd for $C_6H_8N_2O_4$ 172.0484, found 172.0483.

***O*-Succinimidyl *N*-Ethylcarbamate (6B).** ^1H NMR (300 MHz, $CDCl_3$): δ 1.18 (t, J = 7.2 Hz, 3H), 2.81 (s, 4H), 3.27 (m, 2H), 5.91 (br, 1H). ^{13}C NMR ($CDCl_3$): δ 14.65, 25.47, 36.98, 151.27, 170.35. HR-MS: calcd for $C_7H_{10}N_2O_4$ 186.0640, found 186.0643.

***O*-Succinimidyl *N*-Isopropylcarbamate (6C).** ^1H NMR (300 MHz, $CDCl_3$): δ 1.20 (d, J = 6.6 Hz, 6H), 2.79 (s, 4H), 3.80 (m, 1H), 5.48 (br, 1H). ^{13}C NMR ($CDCl_3$): δ 22.50, 25.48, 44.78, 150.42, 170.17. HR-MS: calcd for $C_8H_{12}N_2O_4$ 200.0797, found 200.0791.

***O*-Succinimidyl *N*-*tert*-Butylcarbamate (6D).** ^1H NMR (300 MHz, $CDCl_3$): δ 1.36 (s, 9H), 2.80 (s, 4H), 5.42 (br, 1H). ^{13}C NMR ($CDCl_3$): δ 25.48, 28.52, 52.15, 148.5, 170.25. HR-MS: calcd for $C_9H_{14}N_2O_4$: 214.0953, found 214.0956.

***O*-(4-Formylphenyl) *N*-Methylcarbamate (3A).** ^1H NMR (300 MHz, $CDCl_3$): δ 2.90 (d, J = 3 Hz, 3H), 5.06 (br, 1H), 7.29 (d, J = 9 Hz, 2H), 7.87 (d, J = 9 Hz, 2H), 9.95 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 27.0, 122.43, 131.20, 133.35, 154.22, 155.94, 191.15. HR-MS calcd for $C_9H_9NO_3$ 179.0582, found 179.0583.

***O*-((*S*)-2-(Methoxycarbonyl)propyl) *N*-Methylcarbamate (5A).** ^1H NMR (300 MHz, $CDCl_3$): δ 1.11 (d, J = 9 Hz, 3H), 2.70 (d, J = 3 Hz, 3H), 2.74 (m, 1H), 3.62 (s, 3H), 4.11 (m, 2H), 4.79 (br, 1H). ^{13}C NMR ($CDCl_3$): δ 13.54, 27.35, 39.30, 51.73, 65.80, 156.69, 174.48. HR-MS: calcd for $C_7H_{13}NO_4$ 175.0844, found 175.0845.

***O*-((2,2-Dimethyl-1,3-dioxolan-4-yl)methyl) *N*-Methylcarbamate (1A).** ^1H NMR (300 MHz, $CDCl_3$): δ 1.34 (s, 3H), 1.41 (s, 3H), 2.78 (d, J = 3 Hz, 3H), 4.16 (dd, J = 3 Hz, 12 Hz, 1H), 3.69 (dd, J = 6 Hz, 9 Hz, 1H), 4.03 (m, 2H), 4.28 (m, 1H), 4.69 (br, 1H). ^{13}C NMR ($CDCl_3$): δ 25.29, 26.65, 27.54, 65.33, 66.10, 73.98, 109.74, 156.69. HR-MS: calcd for $C_8H_{16}NO_4$ (MH^+) 190.1079, found: 190.1083.

High-Performance Liquid Chromatographic Analysis of *N*-Methylcarbamates. Analysis of the A-series carbamates was performed on an HP 1090L liquid chromatograph with a diode array detector operating at 220 nm. A 250 mm × 4.9 mm i.d. Econosil 5 μm C18 column (Alltech) was used. The mobile phase was an acetonitrile−water mixture, programmed from 30% acetonitrile−70% water (v/v) to 50% acetonitrile-50% water (v/v) over 10 min and then back to 30% acetonitrile−70% water (v/v) over 10 min. The flow rate was 0.5 mL/min, and the temperature was ambient. The mixture of nine *N*-methylcarbamates which is the most potent in the O-dimension was analyzed, with the traces provided in the supplementary material. Four *N*-methylcarbamates were individually analyzed under the same conditions. The mixture consists of nine peaks in which the above four single *N*-methylcarbamates were clearly identified at the retention times of 5.34, 10.82, 12.52, and 14.27 mim for *O*-succinimidyl, *O*-((*S*)-2-methoxycarbonyl)propyl), *O*-(4-formylphenyl), and *O*-(4-cyanophenyl) *N*-methylcarbamate, respectively.

Acetylcholinesterase Assay.[26] Acetylcholinesterase (AChE) was obtained from Sigma as a lyophilized powder and was dissolved in 2 mL of deionized distilled water containing 1 mg/mL ammonium sulfate and then stored at −20 °C in the freezer, which was referred to as 1:1 AChE stock solution. The substrate solution of acetylthiocholine was prepared as a 3 mM solution in a potassium phosphate buffer, pH 8.0. Typically, 4.334 mg of acetylthiocholine iodide was dissolved in 5 mL of potassium phosphate, pH 8.0. A 0.01 M DTNB solution was prepared in 0.1 M potassium phosphate buffer, pH 7.0, containing sodium bicarbonate. Typically, 39.6 mg of DTNB was dissolved in the 10 mL of 0.1 M potassium phosphate, pH 7.0, with addition of 15 mg of sodium bicarbonate. AChE was thawed on the ice before use, 75 μL of which was withdrawn and mixed with 450 μL of deionized distilled water to make 1:7 AChE solution. The enzymatic reaction was initiated by addition of 10 μL of 1:7 AChE stock solution into an assay buffer of 0.1 M potassium phosphate, pH 8.0, containing 200 μL of DMSO, 100 μL of 3 mM acetylthiocholine iodide, and 100 μL of 0.01 M DTNB at 25 °C. An appropriate amount of inhibitor (a carbamate mixture) was in the assay solution during the inhibition study. The enzymatic reaction was followed by UV−vis at 412 nm for 5−10 min. The rate of the enzymatic reaction (ΔA/min) at 25 °C under the specified conditions was determined by the increasing absorbance which derives from the TNB product released from DTNB by reaction with thiocholine. The IC_{50} value was obtained from Dixon plots and is defined as the inhibitor concentration required to inhibit control enzyme activity by 50%.

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Supplementary Material Available: Figure showing HPLC traces for the row 6 mixture and for the individual carbamates in that mixture (1 page). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the Journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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