Discovery of a novel tetrahydroacridine acetylcholinesterase inhibitor through an indexed combinatorial library

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Background: Methods for the rapid and efficient preparation of drug candidates through combinatorial chemistry are of increasing interest. We have previously reported an indexed combinatorial library method that allows both the preparation and testing of compounds in solution. We set out to apply this method to develop more effective analogs of the known, marketed drug tacrine, an acetylcholinesterase inhibitor.

Results: A one-step condensation of cyclohexanones with cyanoanilines to generate tetrahydroacridine pools was developed. The resulting library of (formally) 72 tetrahydroacridines was screened against acetylcholinesterase, and a compound 10-fold more potent than tacrine, 7-nitrotacrine, was discovered. Its increased potency could be readily explained by examining the known structure of the complex of acetylcholinesterase with tetrahydroacridine.

Conclusions: In this work, we have provided a relatively rare example of carbon–carbon bond formation in a pool synthesis, and have discovered a potentially useful acetylcholinesterase inhibitor.

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Introduction

Methods for the efficient preparation of libraries of drug candidates in a format that can be readily screened are of increasing importance [1,2]. Much previous work in combinatorial chemistry has focused on oligomeric compounds, but methods for the preparation of non-oligomeric chemical diversity will clearly be crucial to the widest application of combinatorial principles to drug discovery [3,4]. We recently described the principle of indexed combinatorial libraries [5], which permits any straightforward chemical linking chemistry to be used, provides compounds in a soluble format ready for screening, and does not require the iterative synthesis used in other deconvolution methods. We applied the indexing method to the discovery of carbamate inhibitors of acetylcholinesterase [6]. Another group has used essentially the same method to discover an inhibitor of matrix metalloproteinase-1 and a ligand for the neurokinin NK3 receptor [7]. A similar concept called 'orthogonal' chemical libraries has recently been used to discover a novel tripeptide ligand for the vasopressin V2 receptor [8].

The indexing method relies on the following principle, exemplified for the simplest case of two different molecular subunits that are united to form a drug candidate. Variants of each subunit (in the example shown, **A–H** and **1–6**) are used to generate molecular diversity. The complete library of all possible combinations is never actually synthesized in an indexed combinatorial library experiment; instead, many sub-libraries or pools are prepared. The pools can be considered to form a two-dimensional matrix in which the first subunit varies along one axis and the second varies along the other axis (Fig. 1), although of course the concept is not limited to two dimensions. The synthesis of the pools of compounds is organized in such a way that two pools on different axes always have a single component in common. Pools on the same axis have no components in common. Pools with these characteristics are obtained by reacting a single representative of one subunit class with all members of the other subunit class. When these pools are tested for a biological activity, the identification of the two most potent pools thereby identifies the single compound they have in common as the most potent in the

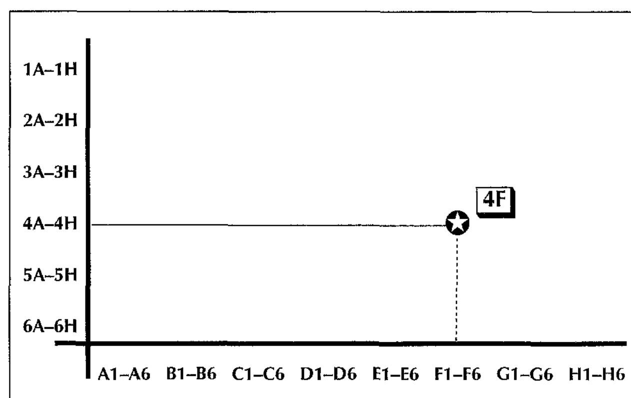

Fig. 1. An indexed combinatorial library can be viewed as a two-dimensional matrix. The identification of the two most potent pools (in this case pool **4**, containing members **4A–4H** and pool **F**, containing members **F1–F6**) immediately identifies the most potent compound in the library as the compound that these two pools have in common, which in the example shown is compound **4F**.

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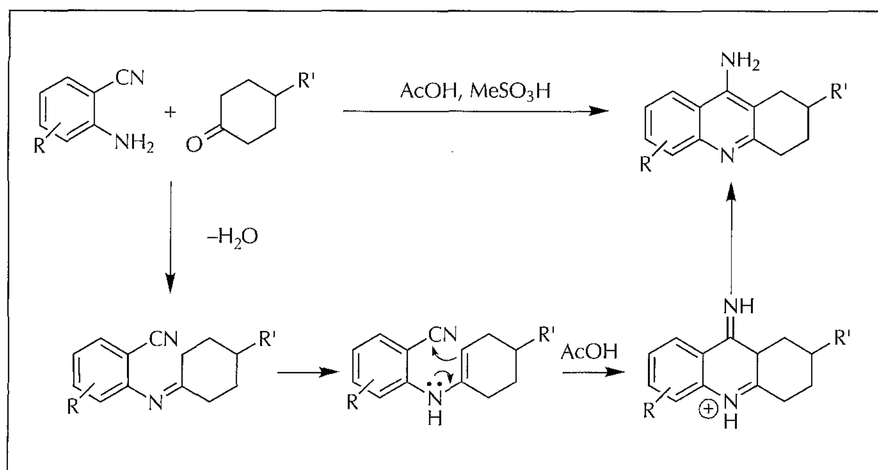

Fig. 2. One-step C–C bond-forming reaction to generate tetrahydroacridines. The presumed mechanism of reaction is also shown (below).

library, provided that there is no synergism or antagonism between pool members. In the example shown, of the six pools that are formed from **1–6**, pool **4** is the most potent. Of the twelve pools formed from **A–H**, pool **F** is most potent. The only component they have in common is combination **4F**, which must therefore be the most potent in the library. This method offers the advantage that the sub-libraries are prepared simultaneously and tested simultaneously, and the assay results directly point to the compound of most interest. No iteration is required, unlike the situation with 'deconvolution' methods, where active pools are first found, and the individual components within active pools must then be resynthesized for further testing.

We have directed our further efforts to develop the indexed combinatorial library method toward novel heterocyclic inhibitors of acetylcholinesterase. Such compounds are used in the treatment of *inter alia*, myasthenia gravis and glaucoma [9–11]. We have focused on tacrine (tetrahydroacridine), a cognition-enhancing drug used in Alzheimer's disease, whose molecular basis of action is thought to rely on inhibition of acetylcholinesterase [12]. Symptomatic treatment of conditions such as Alzheimer's disease, which are characterized by an acetylcholine deficit at cholinergic synapses, can be achieved by controlled inhibition of acetylcholinesterase. The indexed library method is particularly applicable to tetrahydroacridines because it uses solution chemistry and provides soluble compounds for testing. The solid-phase synthesis of a library of tetrahydroacridines would be difficult because they do not have a site available for attachment to a support. Furthermore, testing the drug candidates in solution permits one to assay the desired effect, enzyme inhibition, directly. When the library is tested while immobilized on a solid support, a surrogate endpoint, such as binding to the biological target, must be used instead.

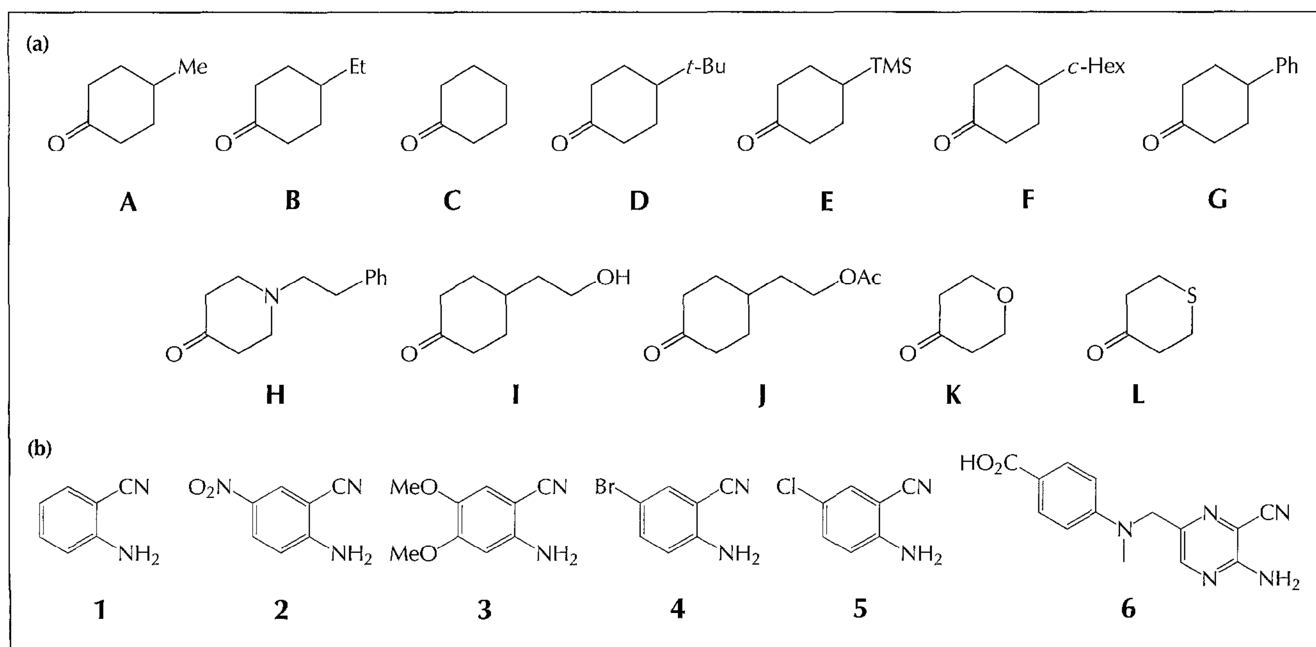

Fig. 3. Building blocks for the tetrahydroacridine combinatorial library. **(a)** Cyclohexanone building blocks (compounds **A–L**); **(b)** *o*-cyanoaniline building blocks (compounds **1–6**).

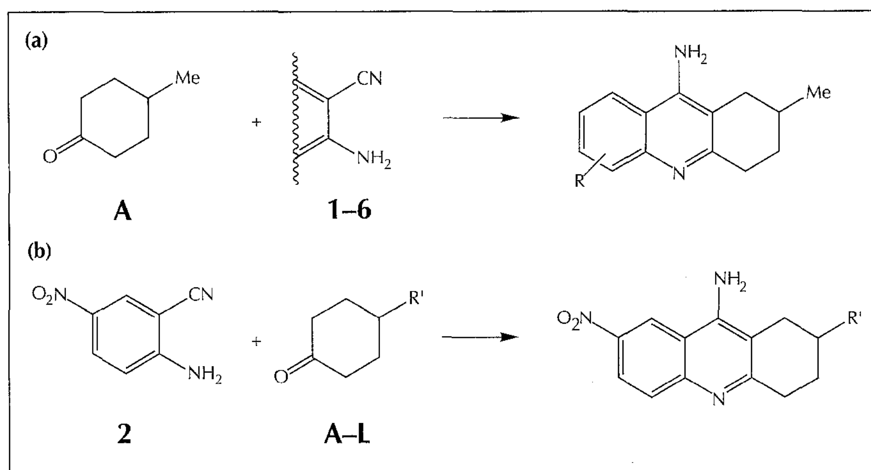

Fig. 4. Combinatorial reactions for the preparation of pools. **(a)** Preparation of pools in which a cyclohexanone is the unitary reagent (example shown for compound **A**). **(b)** Preparation of pools in which an *o*-cyanoaniline is the unitary reagent (example shown for compound **2**).

A one-step condensation of cyclohexanones with cyanoanilines to generate tetrahydroacridine pools was developed in our laboratory based on earlier reactions of cyanoanilines with ketones [13], and the resulting library of (formally) 72 tetrahydroacridines was screened against acetylcholinesterase. A novel inhibitor was discovered whose increased potency could be readily explained through analysis of the structure of the known acetylcholinesterase–tetrahydroacridine complex. This combinatorial library is a rare example in which carbon–carbon bond formation has been accomplished.

Results

A library of tetrahydroacridines consisting of 18 subpools was prepared based on the indexing principle. An acid-catalyzed, one-step, C–C bond-forming reaction of aminobenzonitriles and cyclohexanones to form the basic tetrahydroacridine structure (Fig. 2) was developed. The utility of a variety of aminobenzonitriles (all commercially available) was investigated for this reaction. The 3,5-dinitro and 3-chloro-5-nitro substitutions were shown to have very low reactivity, and were consequently omitted from the library synthesis. The reaction was applied to basis sets of the 12 cyclohexanones in Figure 3a (symmetrical so as to avoid the possible production of positional isomers) and 6 *o*-cyanoaniline derivatives in Figure 3b. The 18 'row' and 'column' reactions each use one fixed reagent from one of the two classes of reactants (the unitary reagent) and a mixed reagent containing all of the members of the other basis set (Fig. 4). Each of the basis set molecules is the unitary reagent in only one reaction of the 18; in the mixed reagent, all the members of the appropriate basis set are equimolar. Reactions were run to completion and the 'yields' of the pools were nearly quantitative. A selection of the individual components in these pools was prepared and the presence of the expected members in each row and column reaction was verified by high-pressure liquid chromatography (HPLC). The resulting 18 pools were used to determine aggregate IC_{50} values against

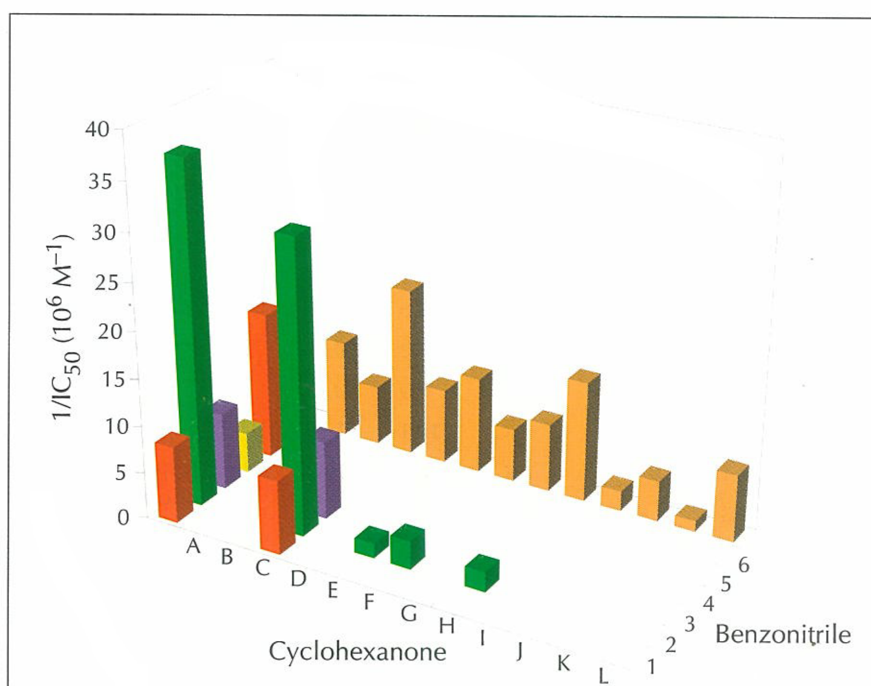

Fig. 5. Inhibition of acetylcholinesterase by tetrahydroacridines displayed as $1/IC_{50}$ (10^6 M^{-1}), so that higher bars represent more potent inhibition.

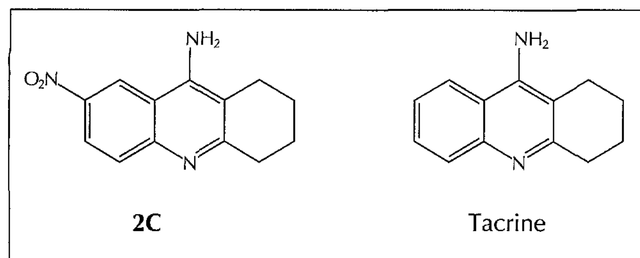

Fig. 6. Structure of the most potent compound in this library, nitrotetrahydroacridine (**2C**), and that of tacrine.

acetylcholinesterase (Fig. 5). Select compounds in the active rows and columns were individually synthesized and assayed. The most potent inhibitor in this library is 1,2,3,4-tetrahydro-7-nitro-9-aminoacridine (**2C**, Fig. 6), a heretofore unknown compound [1]. The statistical criterion we have developed for pool screening (the hit is significant if $R_{hit} - R_{ave} > \sqrt{2}t\sigma\sqrt{n}$, where R is the response function, t is Student's t, σ is standard deviation and n is the number of compounds in the pool [5]) could be applied to the assay values and standard deviations (Table 1) for the active column to demonstrate that the superior activity of **2C** is significant at the 99.9% confidence level. When tested in pure form, **2C** shows 10-fold greater potency (K_i = 10 nM, Fig. 7) than the parent compound, tacrine. To validate the discovery of this compound and test the ability of the method to find potent compounds present in small amounts in multicomponent mixtures, novel versions of column **C** were prepared using log serial dilutions of only cyanoaniline **2**, with all other cyanoanilines maintained at their usual level. This experiment formally tests pool sizes equivalent to 60 and 600 compounds without actually changing the constituents. The $1/IC_{50}$ values are 38 (in units of 10^6 M^{-1}) (undiluted), 12.6 (10x diluted) and 10.4 (100x diluted). These data compare well to a statistical analysis that suggests that the discovery (at the 95 % confidence level) of an inhibitor only one log unit more potent than the lead compound, tacrine, would only have been possible within a pool size of 25 members.

Table 1. Acetylcholinesterase inhibition by rows (**A–L**), columns (**1–6**) and by individual members of the library.

	$1/IC_{50}$	S.D.		$1/IC_{50}$	S.D.
A	10.4	0.6	**1**	8.2	0.4
B	6.4	0.3	**2**	36.4	3.0
C	18.1	0.9	**3**	8.2	0.5
D	8.0	0.8	**4**	4.3	0.3
E	10.3	0.5	**5**	15.8	0.9
F	5.7	0.1	**6**	3.2	0.3
G	7.3	0.3	**1C**	7.9	0.3
H	12.9	1.0	**2C**	31.1	1.6
I	2.1	0.2	**5C**	8.3	0.4
J	4.3	0.3	**2E**	1.57	0.05
K	1.1	0.1	**2F**	3.0	0.1
L	7.2	0.3	**2H**	2.1	0.1

$1/IC_{50}$ is given in units of 10^6 M^{-1}
S.D., standard deviation.

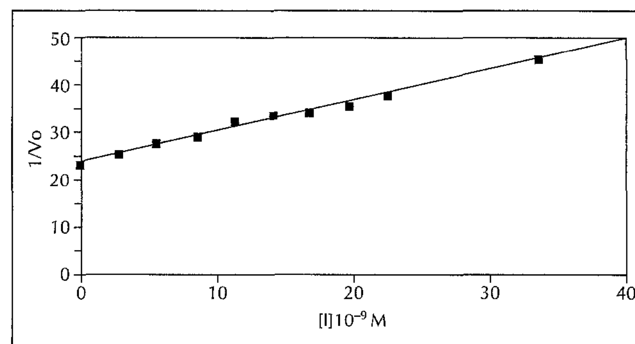

Fig. 7. Inhibition data for **2C** presented as a Dixon plot. [I], inhibitor concentration.

Discussion

The average potency of all of the compounds in these pools is fairly high, as evidenced by the statistics showing that the 10-fold more potent nitrotacrine could be found in only a relatively small pool size. Lower average potency would cause the potent molecules to stand out more clearly, and so be detectable in larger pool sizes. In such situations, combinatorial methods are more useful to elucidate structure–activity relationships than to select one potent compound from a multitude of drug candidates. That is, the results of the pool screenings themselves provide useful information about potency enhancement. These data also provide insight into the limits of pool screening for developing lead compounds in situations where the activity is relatively unresponsive to a major structural variation (e.g., changes in the substituents at the 4-position of the cyclohexane ring) within the library. In general, the screening of large pools will be most effective when there is a significant distinction among the activities of the constituents, which can be best achieved by incorporating the greatest molecular diversity into the building block sets.

Because the structure of the complex between acetylcholinesterase and tacrine has been reported [14], it can be used to aid interpretation of the results of this study. Figure 8 shows the active site region of the X-ray crystal structure, modified by the inclusion of the nitro group at the appropriate position on the tetrahydroacridine ring. The molecular interactions believed to be responsible for the high affinity of tacrine for acetylcholinesterase include a hydrogen bond between the ring nitrogen and the main-chain carbonyl of His440 and between the amino nitrogen and a structural water molecule. A stacking interaction has been postulated between Phe330 (not shown) and the aromatic part of tacrine, as well as between tacrine and Trp84, making the drug the middle of a 'sandwich' formed by these

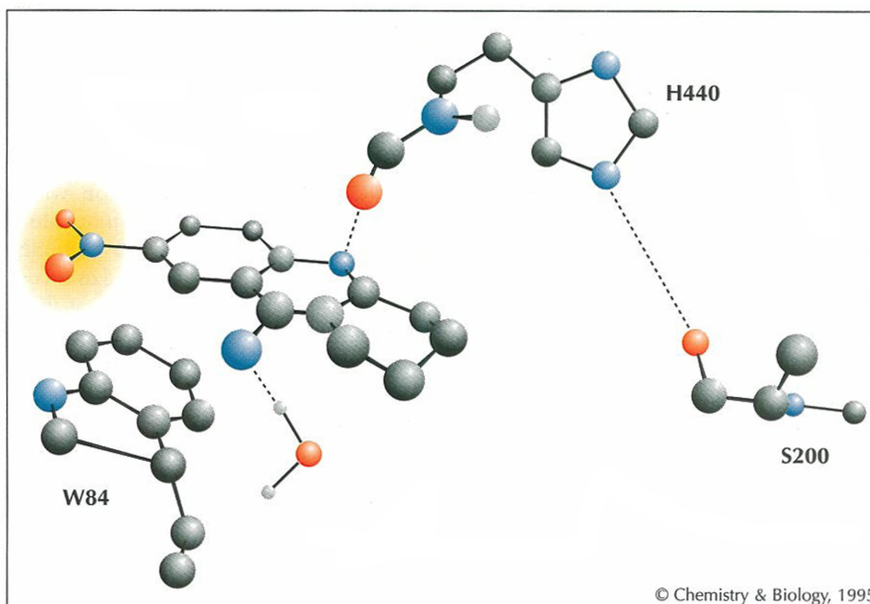

Fig. 8. Postulated structure of the complex between acetylcholinesterase and nitrotetrahydroacridine (**2C**). This structure is based on that reported in the crystal structure of Harel *et al.* [14], modified only by the addition of an NO_2 group (highlighted in yellow) on the tetrahydroacridine ring. Atom colors: carbon, black; hydrogen, white; nitrogen, blue; oxygen, red.

two residues. An important role in the interaction of the aromatic ring of tacrine with the enzyme may therefore be π-stacking and/or charge-transfer interactions, which would very likely be enhanced in a complex with the nitroaryl compound **2C**.

Significance

This study exemplifies several of the key principles necessary for application of modern combinatorial chemistry to the discovery or development of novel drug candidates. First, it uses an efficient chemical reaction, which was optimized on a selection of single compounds before the pool synthesis was conducted, that greatly increases molecular complexity through C–C bond formation. Second, it uses a direct method for deconvolution of the results of pool screening, and a statistical model to validate the results. Finally, it integrates structural information with the library screening results to generate a model of the key molecular interactions and potentially inform further rounds of synthesis and screening.

A major challenge for combinatorial chemistry systems is to provide a straightforward way to identify the active compound(s). After all, the preparation of a large conglomeration of molecules has never been difficult for chemists; the challenge, which has been very effectively met during many decades of natural products chemistry, has been determination of the structure of the active few among the many. Several strategies have been used to identify active compounds in combinatorial libraries. Microsequencing can be applied to those natural biopolymers for which methods already exist. Solid-phase chemistry, linking the molecule to a microscopic bead or surface location, permits molecular identification by tagging. Chemical, biochemical, or positional codes or tags can be used to identify active compounds in these libraries, but often limit the range of chemistry and diversity of assays that can be used. To conduct solution-phase assays, yet still gain the higher throughput associated with parallel synthesis of the library, assays must be performed on pools. Deconvolution (synthesis and testing to find active pools, re-synthesis of active sub-pools) is one way to discover active compounds in this type of pool screening, but it can be laborious. The indexing method offers the advantages of solution-phase assays without the iteration step.

Materials and methods

Pool condensation of cyclohexanones and o-cyanoanilines

A solution of 6 mmol of a cyclohexanone and 1 mmol each of six 2-cyanoanilines in 2 ml of acetic acid was heated in an Ace pressure tube at 125 °C for 24–48 h. The solvent was removed under reduced pressure. The protocol was similar for the other dimension except the mixed reagent was 1 mmol each of 12 cyclohexanones and the unitary reagent was 12 mmol of a 2-cyanoaniline. HPLC of the resulting tetra-hydroacridines was performed at 35 °C using a Nucleosil C-18 reverse phase column (150 x 4.6 mm) with a 4:1 ratio of a 0.02 M NaH_2PO_4 (pH 3) to acetonitrile as eluent, a flow rate of 0.9–1.7 ml min^{-1}, and 240 nm detection.

Characterization of 1,2,3,4-tetrahydro-7-nitro-9-aminoacridine

Melting point (mp) (MeOH) 197–198 °C. Infrared absorbance (IR) (film): 3326, 3228, 1643, 1502 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.38 (d, *J*=3 Hz, 1H), 8.22 (dd, *J*=3, 9 Hz, 1H), 6.80 (d, *J*=9 Hz, 1H), 5.13 (br s, 2H, exchangeable), 2.60–1.10 (m, 8H). Fast-atom bombardment mass spectroscopy (FAB MS) (*m/z*+H): 244. Analysis: calculated for $C_{13}H_{13}N_3O_2$: C, 64.19; H, 5.39; N, 17.27. Found: C, 64.10; H, 5.43; N, 17.14.

Characterization of 1,2,3,4-tetrahydro-7-nitro-2-(trimethylsilyl)-9-aminoacridine

Mp (MeOH) 199–200 °C. IR (film): 3327, 3224, 1648, 1496 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.38 (d, *J*=3 Hz, 1H), 8.22 (dd, *J*=3, 9 Hz, 1H), 6.80 (d, *J*=9 Hz, 1H), 5.13 (br s, 2H, exchangeable), 2.61–1.00 (m, 7H), 0.13 (s, 3H), 0.01 (s, 6H). FAB-MS (*m/z*+H): 316. Analysis: calc'd for $C_{16}H_{21}N_3O_2Si$: C, 60.93; H, 6.71; N, 13.32. Found: C, 60.98; H, 6.66; N, 13.40.

Characterization of 1,2,3,4-tetrahydro-7-nitro-2-(cyclohexyl)-9-aminoacridine

Mp (MeOH) 194–196 °C. IR (film): 3327, 3224, 1646, 1504 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.57 (d, *J*=3 Hz, 1H), 8.22 (dd, *J*=3, 9 Hz, 1H), 6.79 (d, *J*=9 Hz, 1H), 5.12 (br s, 2H, exchangeable), 2.65–0.90 (m, 18H). FAB-MS (*m/z*+H): 326. Analysis: calc'd for $C_{19}H_{23}N_3O_2$: C, 70.13; H, 7.12; N, 12.99. Found: C, 70.08; H, 7.22; N, 12.88.

Characterization of 1,2,3,4-tetrahydro-7-nitro-2-(phenethyl)-9-amino-2-aza-acridine

Isolated as a yellow oil. IR (film): 3332, 3319, 1649, 1499 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.60–6.95 (m, 8H), 5.11 (br s, 2H, exchangeable), 2.85–2.65 (m, 6H), 2.47-2.12 (m, 4H). FAB-MS (*m/z*+H): 349. Analysis: calc'd for $C_{20}H_{20}N_4O_2$: C, 68.95; H, 5.79; N, 16.08. Found: C, 68.80; H, 5.88; N, 16.22.

Characterization of 1,2,3,4-tetrahydro-6,7-dimethoxy-9-aminoacridine

Isolated as a yellow gum. IR (film): 3320, 3305, 1652, 1500 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.85 (s, 1H), 7.64 (s, 1H), 6.15 (br s, 2H, exchangeable), 3.85 (s, 3H), 3.79 (s, 3H), 2.02–1.02 (m, 8H). FAB-MS (*m/z*+H): 259. Analysis: calc'd for $C_{15}H_{18}N_2O_2$: C. 69.74; H, 7.02; N, 10.84. Found: C, 69.70; H, 7.11; N, 10.96.

Preparation of stock solutions from reaction pools

An appropriate amount of a sub-library was weighed out and dissolved in methanol to prepare a 1 mM or 2 mM stock solution. The calculation of concentration was based upon the aggregate molecular weight of the sub-library. The stock solution was diluted with de-ionized water as required for inhibition studies.

Inhibition of acetylcholinesterase

A stock solution of electric eel acetylcholinesterase (Sigma, AChE) was prepared in deionized distilled water containing 1 mg ml^{-1} ammonium sulfate and stored at −20 °C. A 75 mM substrate solution of acetylthiocholine iodide was prepared in a potassium phosphate buffer, pH 8.0. A 0.01 M dithionitrobenzene (DTNB) solution was prepared in 0.1 M potassium phosphate buffer, pH 7.0 containing sodium bicarbonate. The AChE stock was thawed on ice before use, 5 μl of which was withdrawn and mixed with 995 μl of deionized distilled water to make a 1:200 AChE solution. The enzymatic reaction was initiated at 25 °C by addition of 10 μl of 1:200 AChE solution into an assay buffer of 0.1 M potassium phosphate, pH 8.0 containing 20 μl of 75 mM acetylthiocholine iodide (500 μM), 100 μl of 0.01 M DTNB (333 μM), and serial dilutions of inhibitors (a tetrahydroacridine mixture). The reaction was followed at 25 °C by UV–Vis at 412 nm for 3 min. The rate (ΔA per min) at 25 °C under the specified conditions was determined by the increasing absorbance, which derives from the thionitrobenzene product released (ϵ = 11 400) from DTNB by reaction with thiocholine. The IC_{50} value was obtained from Dixon plots, is defined as the inhibitor concentration required to inhibit control enzyme activity by 50 %, and is expressed as a concentration based on the stock solution concentration of the sub-library. The concentration for each pool in the assay was calculated using the combined molecular weights of the expected pool members.

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