Dynamic Deconvolution of a Pre-Equilibrated Dynamic Combinatorial Library of Acetylcholinesterase Inhibitors

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A dynamic combinatorial library composed of interconverting acylhydrazones has been generated and screened towards inhibition of acetylcholinesterase from the electric ray Torpedo marmorata. *Starting from a small set (13) of initial hydrazide and aldehyde building blocks, a library containing possibly 66 different species was obtained in a single operation. Of all possible acylhydrazones formed, active compounds containing two terminal cationic recognition groups separated by an appropriate distance, permitting two-site binding, could be rapidly identified by using a dynamic deconvolution – screening procedure, based on the sequential removal of starting building blocks. A very potent bis-pyridinium inhibitor ($K_i = 1.09$ nM, $\alpha K_i = 2.80$ nM) was selected from the process and the contribution of various structural features to inhibitory potency was evaluated.*

KEYWORDS:

acetylcholinesterase · combinatorial chemistry · enzyme catalysis · hydrolases · inhibitors

Introduction

Dynamic combinatorial chemistry (DCC)[1–5] extends beyond static combinatorial chemistry (SCC; see ref. [6] and references cited therein) toward adaptive/evolutive chemical systems.[3, 7] It relies on reversible reactions or interactions between sets of basic components to generate continually interchanging adducts, thus giving access to virtual combinatorial libraries (VCLs) whose constituents are all possible combinations of the components available.[1–5, 8, 9] Such libraries allow for the target-driven generation or amplification of the active constituent(s) of the libraries, thus performing a self-screening process by which the active species are preferentially expressed and retrieved from the VCL.

This approach has been implemented recently in a number of instances concerning either the receptor-driven generation of a substrate/inhibitor or the reverse.[1–4, 7–9] In particular, in our laboratory, the anion-dependent generation of circular helicates led to the formulation of the DCC/VCL concept.[2, 7] The proof of principle was further substantiated by the induction of an inhibitor of carbonic anhydrase[8] and a bis-saccharide ligand of concanavalin A.[9] We herewith describe the application of the methodology to the generation of inhibitors of acetylcholinesterase and introduce a dynamic deconvolution procedure for the efficient screening of a VCL and the rapid identification of an active constituent.[10]

Acetylcholinesterase (AChE; see ref. [11] and references cited therein) is an enzyme whose function in the central and peripheral nervous systems is to terminate transmission at cholinergic synapses by hydrolyzing the cationic neurotransmitter acetylcholine (ACh), yielding acetate and choline. AChE has two distinct binding sites in proximity to each other. A catalytic site is located at the bottom of a deep (ca. 20 Å) narrow gorge, and a peripheral site is situated near the rim of this gorge. Both sites are lined with aromatic amino acid residues and are effective in hosting quaternary ammonium compounds. By bridging the two binding sites, very potent bis-quaternary ligands such as decamethonium, $(CH_3)_3N^+(CH_2)_{10}N^+(CH_3)_3$, have been identified. The crystal structure of the AChE – decamethonium complex gives a distance of ca. 12 Å between the two quaternary groups binding to the indole rings of Trp 84 at the active site and of Trp 279 at the peripheral site.[12]

Numerous inhibitors of AChE have been synthesized and studied, for example, mono- and bis-quaternary ammonium compounds, carbamates, and organophosphates.[13] Clinically AChE inhibitors are used in a number of situations, such as in the reversal of neuromuscular blockade, the treatment of myasthe-

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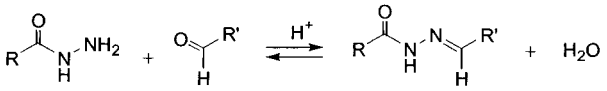
nia gravis and glaucoma, and it has been noted that they might be used for the treatment of Alzheimer's disease.[14] In addition, AChE inhibitors have found widespread use as insecticidal agents in agriculture and forestry. Of these inhibitors, bridging ligands have exerted particular interest. For example, series of ditopic ligands with different tether lengths between the two head groups were designed to achieve the best binding to both the active and the peripheral sites. The homologous bis-galanthamine,[15] bis-THA (THA = 9-amino-1,2,3,4-tetrahydroacridine),[16] and huperzine A – tacrine hybrid[17] were prepared and tested for enzyme activity; they displayed a significant enhancement of inhibitory potencies compared to the respective monomers that bind to a single site only. Moreover, the best inhibitors were the ligands having a suitable distance between the two head groups.

In this work the DCC/VCL concept has been implemented to identify new bridging inhibitors of AChE. A dynamic combinatorial library (DCL) was generated through reversible acylhydrazone connections between hydrazide and aldehyde building block precursors,[4p, 5h, 18] bearing quaternary ammonium functionalities. The recognition groups, the distance between these groups and their relative orientations as well as the rigidity of the spacers were varied. The resulting acylhydrazones were subsequently tested for inhibitory activity in the presence of the enzyme. Although the ultimate aim of the full dynamic DCC/VCL procedure is to amplify the active compound(s), this may be unfeasible due to limitations in availability and stability of the biological target as in the present case of the enzyme AChE. Hence the pre-equilibration alternative was applied,[9] and an enzyme assay was combined with the extension to DCC of a technique utilized in traditional combinatorial chemistry, providing a new approach termed dynamic deconvolution, for the efficient characterization of an active constituent in a dynamic library through identification of the building blocks which compose it.

Results and Discussion

Design of the pre-equilibrated dynamic combinatorial library

Library components and processes: In the present study, a dynamic combinatorial library of interchanging acylhydrazones was generated from an initial set of aldehydes and hydrazides under reversible reaction conditions in acidic aqueous medium (Scheme 1). Various hydrazide and aldehyde building blocks were selected (Figure 1), primarily chosen to contain substituted ammonium and pyridinium recognition groups since compounds of these types have been found to bind strongly to both the active site and the peripheral site of AChE.[19] In addition to using monoaldehydes, dialdehydes were added as linker units to further probe the effect of changing the structure of the spacer between the two binding sites. Building blocks with potentially low affinities were furthermore added for reference.



Scheme 1. *Reversible acylhydrazone formation.*

The reversible formation of an acylhydrazone is an advantageous reaction for generating a DCL.[4p, 5h, 18] Indeed, one can observe by proton NMR spectroscopy the acylhydrazone product in the mixture of aldehyde and hydrazide since it is sufficiently stable in aqueous media. Furthermore, the formation and component interchange processes are faster in acidic aqueous condition than in neutral and basic conditions so that the reaction can be controlled by adjusting the pH value.[18] These features are suitable for the generation of pre-equilibrated dynamic libraries to be used in conditions compatible with enzyme assays. In the present case, the pool of components was reacted in acetate buffer at pH 4.0, at which the formation is rapid and occurs within minutes, whereas the exchange is appreciably slower and may take from 15 min to 2 days depending on the acylhydrazone. The test of inhibitory activity was subsequently conducted in phosphate buffer at pH 7.2, at which the interconversion of the library constituents is essentially blocked and at which the assay conditions are optimal for the enzyme.

Evaluation of the inhibitory activity of the individual library constituents: At first, the various individual library constituents **X-Y′** and **X-Y″-X** (**X** = hydrazide building block, **Y′** and **Y″** = monoaldehyde and dialdehyde building blocks, respectively) were prepared separately by mixing appropriate amounts of **X** and **Y** at low pH and allowing the reaction to go to completion. The

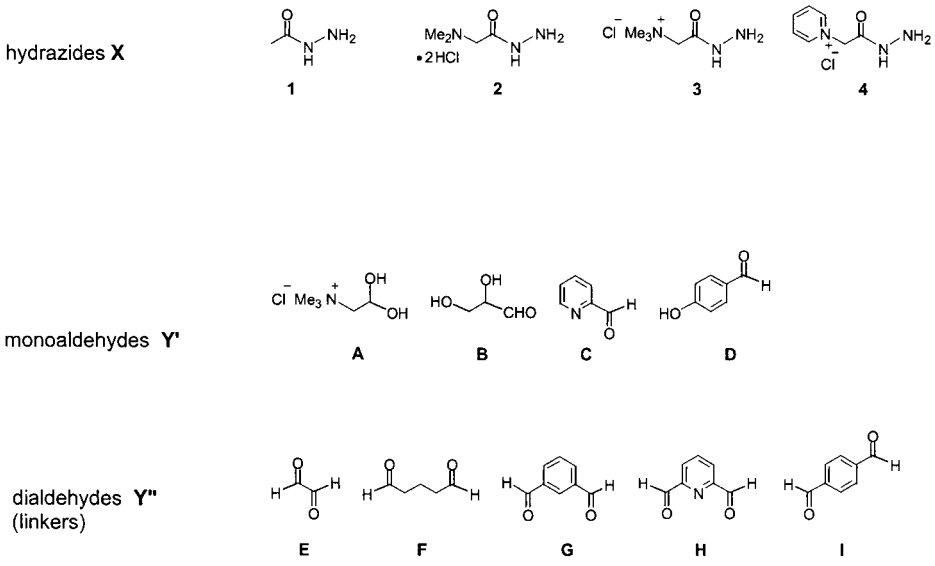


Figure 1. *Building blocks **X**, **Y** chosen as components for preparing the constituents of the pre-equilibrated dynamic combinatorial library of AChE inhibitors.*

inhibitory activity of these discrete compounds was then estimated by determining their effect on the enzymatic hydrolysis of acetylthiocholine, which was monitored by using dithiobisnitrobenzoic acid (DTNB, Ellman's reagent) to determine the production of free thiol in solution. These assays were performed without isolation of the products in order to be as close as possible to the conditions for the full-library generation (see below). The results obtained are shown in Figure 2.

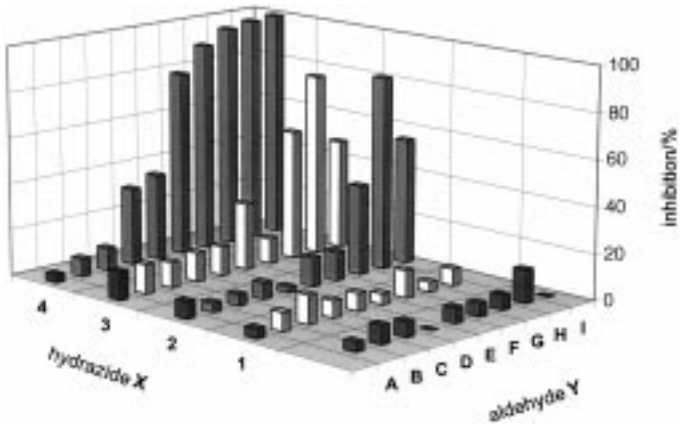


Figure 2. *Relative inhibition of AChE by the individual acylhydrazones* ***X-Y′*** *and* ***X-Y″-X****. The black bars show the activity of a single building block (aldehyde or hydrazide). The other bars show the activity of the combination of one hydrazide* ***X*** *and one aldehyde* ***Y*** *reacted in acetate buffer at pH 4.0. An inhibition value for* ***1-I-1*** *was not obtained due to the precipitation at the concentration used for pre-equilibration.*

The inhibitory activities were insignificant for the precursor hydrazides and aldehydes themselves (Figure 2, black bars). On the other hand, some of the **X-Y′** and especially **X-Y″-X** component combinations displayed remarkable inhibition of AChE. Upon examination of the results produced by using the various building blocks, several interesting effects could be distinguished:

- As expected, the condensation products of positively charged building blocks (methylammonium or pyridinium salts) proved highly efficient compared to their uncharged counterparts, which displayed no visible effects;
- of the positively charged hydrazide building blocks **2** – **4**, the pyridinium hydrazide **4** yielded the most potent inhibitors; this result is in agreement with previously reported inhibitors, indicating that pyridinium compounds are more potent than their methylammonium counterparts;[19]
- constituents with two positively charged termini derived from dialdehyde spacers displayed strong inhibition, whereas the monocondensation adducts with corresponding structures showed much lower affinity for the enzyme;
- activity proved to be very sensitive to the distance between the two positive charges and, other properties being equal, compounds with a longer linker gave generally stronger inhibition than the corresponding shorter adducts;
- the products composed of aromatic cores were more effective inhibitors than the related products with non-aromatic cores, this effect being more pronounced for the methylammonium compounds than for the pyridinium analogues under the conditions used;
- in summary, the most efficient inhibitors were the compounds containing two pyridinium groups and an aromatic core.

Evaluation of the pre-equilibrated DCL by dynamic deconvolution

After having ascertained the activity of each library component taken individually, the properties of the pool of all precursor components **X** and **Y**, under conditions of a pre-equilibrated dynamic library,[9] were investigated. Since it becomes increasingly difficult and time-consuming to find an effective compound by testing individual compounds when using large numbers of building block components, the evaluation of a pool library requires efficient procedures for characterizing the potent ligands among those formed in a dynamic mixture. In order to identify the active compound(s), we proceeded by sequentially omitting one of the building blocks from the pool library. This procedure amounts to a dynamic deconvolution strategy, taking advantage of the dynamic features of the library, since by removal of a given building block the remaining components will redistribute and all constituents which contain this unit will automatically be deleted from the equilibrating library. A decrease in inhibitory effect will indicate that the removed component is an important element in the generation of an active compound in the dynamic mixture.

The reaction of n hydrazides **X** with m_p aldehydes **Y** of functionality degree p (i.e., monoaldehydes: $p = 1$; dialdehydes: $p = 2$) yields a maximum library size N given by Equation (1), summing over all combinations n^p of n units p to p (with order, nonsymmetrical library).[3] The condensation of four hydrazides ($n = 4$) with four monoaldehydes ($m_1 = 4$), and five dialdehydes ($m_2 = 5$) thus results in 96 possible acylhydrazone combinations (not considering dialdehyde monoadducts).

$$N = \sum_p m_p n^p \qquad (1)$$

However, since the dialdehydes are symmetrical, some of these combinations are identical, so that this number is reduced to N_{sym} (combinations without order, symmetrical library), as given in Equation (2). With our figures, a total number of 66 different library constituents may be obtained starting from only 13 original building blocks.

$$N_{sym} = \sum_p \left[\frac{m_p}{p!} \prod_{i=0}^{p-1} (n+i) \right] \qquad (2)$$

Upon deconvolution, N_{sym} depends on whether an aldehyde or a hydrazide is removed. With our partially symmetrical library, this figure amounts to four or ten upon elimination of one aldehyde, whereas withdrawal of one hydrazide unit reduces the library size by a number of 24. Thus, more than one third of the library constituents can be removed simultaneously from the library. In a nonsymmetrical library, this number is even higher, and with our figures 39 out of 96 (40%) of the library species would be removed in one operation. Obviously, this fraction is

reduced with more extended libraries, but the actual number of removed compounds becomes increasingly larger at the same time. Dynamic deconvolution therefore allows the rapid identification of the components required for activity of the DCL constituent(s). It may point to a single constituent or, eventually, to a small group of active constituents representing leads for further elaboration.

The complete pool library (**all**) was generated by adding all building blocks (**1** – **4**, **A** – **I**) simultaneously under pre-equilibrating conditions in acidic buffer at ambient temperature. At the same time, 13 sublibraries were formed by mixing all components, except one specific **X** or **Y** building block, under the same conditions. Together with a reference sample (buffer) containing no building blocks, this series of 15 samples was enough to screen the entire library. In contrast, in the individual screening at least 50 samples have to be analyzed (9×4 combinations, 13 building blocks plus reference sample), clearly demonstrating the advantages offered by the dynamic screening method. In addition, potential **X-Y''-X'** inhibitors carrying two different **X** groups cannot be probed by only 50 samples, but further combinations have to be made. In the same situation, although dynamic deconvolution may also require additional experiments, it is nevertheless expected to allow a more efficient identification of **X** and **X'**.

The results obtained from this library generation – screening process are presented in Figure 3. The complete pool library (**all**) is composed of all possible condensation products in proportion to their relative thermodynamic stability. The inhibition of the AChE activity by a library indicates the presence of one or several active adducts in a given equilibrated mixture (see Figure 3, activity of **all** compared to that of the reference sample (buffer)). On sequential removal of each building block, one at a time, from the complete library, an increase in activity indicates that the component omitted contributed significantly to the inhibitory effect. The data in Figure 3 show that the largest effects are observed when either **4** or **I** have been removed from the pool. Consequently, the most active constituent must contain the fragments **4** and **I** (and is most likely **4-I-4**), in agreement with the results obtained from the separate investigation of individual compounds.

Such a procedure provides an efficient way to converge on the active constituent(s) of a DCL. In more complex cases with many more library members it may go through successive steps, the experiments with removal of a single component being followed by tests involving the removal of two or more components, thus rendering the procedure convergent. Simultaneous removal makes possible the identification of components that may contribute to activity, but less than the optimal one(s). This is illustrated in Figure 4, where it is seen that sequential removal of a component **X** or **Y** from a pool, which does not contain **I** (Figure 4, top), displays the less active constituent containing the fragments **4** and **H** (most

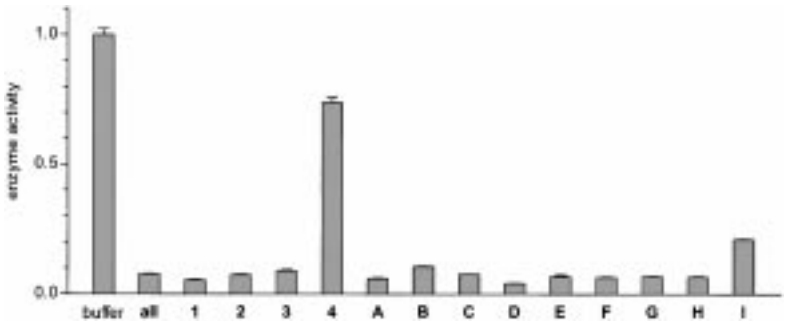


Figure 3. *Dynamic deconvolution of the full dynamic combinatorial library (**all**) formed in the pool of all **X** and **Y** components; each bar corresponds to the removal of a given component **1** – **4** or **A** – **I** from the full library (**all**).*

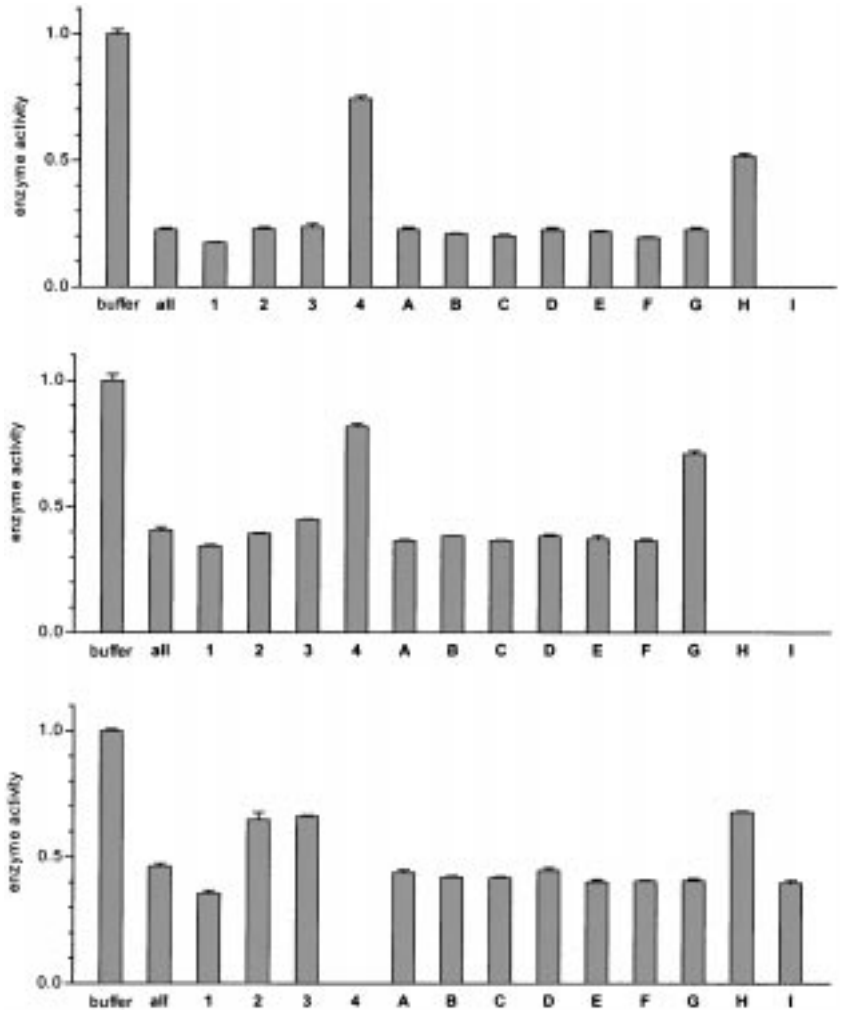
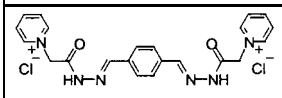
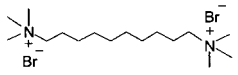
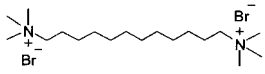
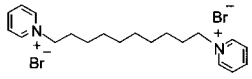
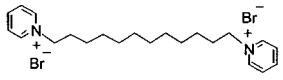


Figure 4. *Sequential dynamic deconvolution of the dynamic combinatorial library. Dynamic deconvolution of the initial full library containing **1** – **4** and **A** – **I**. Top: a library containing **1** – **4** and **A** – **H**; center: a library containing **1** – **4** and **A** – **G**; bottom: a library containing **1** – **3** and **A** – **I**.*

likely **4-H-4**). By omitting both **H** and **I** from the pool, the much less active combination of **4** and **G** is identified as producing the residual, but nevertheless significant inhibition (Figure 4, center). The combination(s) of **2**, **3**, and **H** make up the substantial activity remaining after removing **4** from the pool library (Figure 4, bottom).

The apparently most potent library component, **4-I-4**, was ultimately synthesized separately and its inhibitory effect further characterized and compared with other known bis-quaternary ammonium inhibitors. The results, shown in Table 1, indicate that

Table 1. *Inhibitory activities (IC_{50} values) of selected biscationic compounds.*

Compound	n[a]	IC_{50} [nM]
 **4-I-4**	14	2.30 ± 0.09
 decamethonium	10	1063 ± 42
 dodecamethonium	12	93.2 ± 2.3
 1,10-bis(pyridinium)decane	10	25.8 ± 1.4
 1,12-bis(pyridinium)dodecane	12	16.0 ± 1.3

[a] n = length of spacer (number of atoms) separating the cationic centers.

4-I-4 is indeed a very potent inhibitor, displaying a 50% inhibitory concentration (IC_{50}) in the low nanomolar range. Further inhibition studies yielded a mixed inhibition type with a competitive inhibition constant (K_i) of 1.09 ± 0.03 nM, and a noncompetitive constant (αK_i) of 2.80 ± 0.07 nM. It proved 500-fold more effective than decamethonium, and around tenfold more potent than the bis(pyridinium)decane and -dodecane analogues. Since the distance between the charged nitrogen atoms in **4-I-4** is approximately the same as in the bis(pyridinium)dodecane compound (ca. 16.7 Å in the fully extended forms), the results are indicative of an additional binding effect from the linker region. The role of the linker is also indicated in the comparative activities of **X-G-X**, **X-H-X** and **X-I-X** (**X** = **2** or **3**), where both angle (compare **G** and **I**) and central site (compare **G** and **H**) change.

Conclusion and outlook

It has been shown that acylhydrazone formation and exchange can be efficiently used to generate dynamic combinatorial libraries in aqueous media. Starting from a small set (13) of initial building blocks, a library containing possibly 66 different species could be generated in a single step in a short time. Among all possible acylhydrazones formed, active compounds of appropriate length containing potent recognition groups could be rapidly identified by using a dynamic deconvolution process. This methodology can in principle be extended to encompass much larger combinatorial libraries, allowing swift library generation and expeditious screening of active constituents.

Further exploration of the DCC approach is warranted, concerning in particular the reversible connecting reaction; indeed, faster exchange/equilibration would be very desirable. Furthermore, if several compounds of similar activity are formed, the dynamic deconvolution procedure may lead to a set of substances rather than to the identification of a single one, so that a series of experiments may be required. Studies along such lines are being pursued.

Experimental Section

General: Acetylcholinesterase from *Torpedo marmorata* (E.C. 3.1.1.7) was purified as described.[20] Acetylthiocholine iodide was purchased from Sigma. DTNB was obtained from Acros. Betaine aldehyde chloride was synthesized from dimethylaminoacetaldehyde diethyl acetal as described in the literature.[21] Dodecamethonium, 1,10-bis(pyridinium)decane, and 1,12-bis(pyridinium)dodecane were synthesized from the corresponding dibromoalkanes.[22] All other reagents were from commercial sources and used without further purification. Enzyme assays were carried out by using a Varian Cary 3 UV-Vis spectrophotometer. ^1H and ^{13}C NMR spectra were recorded with a Bruker AC200 spectrometer at 298 K. FAB mass spectra were determined by the Service de spectrométrie de masse at the Institut de Chimie, Université Louis Pasteur. Microanalysis was performed at the Service de microanalyse at the Institut de Chimie, Université Louis Pasteur.

Formation of discrete acylhydrazones: First, solutions of **A**, **B**, **1** – **4** (50 mM) and **E**, **F** (25 mM) were prepared in 100 mM sodium acetate buffer, pH 4.0. The solutions of **C**, **D** (50 mM) and **G**, **I** (25 mM) were prepared in 12.5% CH_3CN/100 mM sodium acetate buffer, pH 4.0. The solution of **H** (25 mM) was prepared in 16.7% CH_3CN/100 mM sodium acetate buffer, pH 4.0.

Solutions of hydrazide **X** (20 μL, 50 mM) and aldehyde **Y** (20 μL, 50 mM monoaldehyde or 25 mM dialdehyde) were added to sodium acetate buffer at pH 4.0 (460 μL, 100 mM). The resulting mixtures were equilibrated at ambient temperature for one week to ensure full reaction. However, much shorter times, from about 15 min to 2 days depending on the components, are sufficient. Aliquots of the equilibrated solutions were subsequently tested in an inhibitory AChE assay. Note that the concentration of **X-Y′** is twice that of **X-Y″-X**. This was done in order to observe some effect from the monofunctional products in comparison to the difunctional ones, which could be expected to be much more active, as was indeed found.

Generation of pre-equilibrated dynamic combinatorial libraries: First, solutions of **A**, **B**, **1** – **4** (5 mM) and **E**, **F** (2.5 mM) were prepared in 100 mM sodium acetate buffer, pH 4.0. The solutions of **C**, **D** (5 mM) and **G**, **I** (2.5 mM) were prepared in 1.25% CH_3CN/100 mM sodium acetate buffer, pH 4.0. The solution of **H**, (2.5 mM) was prepared in 1.67% CH_3CN/100 mM sodium acetate buffer, pH 4.0.

Full library (**all**): Solutions of each component **1** – **4** and **A** – **I** (20 μL, 5 mM for monofunctional and 2.5 mM for bifunctional compounds) were added to sodium acetate buffer at pH 4.0 (240 μL, 100 mM). The

resulting mixtures (500 μL) were equilibrated at ambient temperature for one week.

Partial library (**X** or **Y**): The procedure was identical to that for generating the full library except that buffer solution (20 μL) was exchanged for the component to be omitted.

An excess of aldehydes with respect to hydrazides was used so as to ensure full reaction and avoid interference from unreacted hydrazide components which contain the activity-bearing group. The experiments were also performed with a 1:1 aldehyde group/hydrazide ratio and gave very similar results; conditions used: **1** – **4** (20 μL, 3.5 mM), **A** – **I** (20 μL, 1 mM), in similar solutions.

Synthesis of 4-I-4: Terephthalaldehyde (109 mg, 0.81 mmol) was suspended in a solution of 1-(carboxymethyl)pyridinium chloride hydrazide (Girard's reagent P, 306 mg, 1.63 mmol) in acetic acid (10 mL). After heating at 60 °C for 20 min, the resulting precipitate was isolated by filtration, washed with acetone and dried. Recrystallization from water/acetone yielded 230 mg of pure product (54%). ^{1}H NMR (200 MHz, [D$_6$]DMSO, 25 °C): $\delta = 9.10$ (d, $J = 5.8$ Hz, 2H, Py-H2), 8.71 (br t,1H, Py-H4), 8.42 (s, hydrazone-H), 8.1 – 8.3 (m, Py-H3, hydrazone-H), 7.87 (s, Ph), 7.83 (s, Ph), 6.09 (s, CH_2), 5.69 (s, CH_2); ^{13}C NMR (50 MHz, [D$_6$]DMSO, 25 °C): $\delta = 166.59$, 146.36, 146.21, 144.32, 135.20, 127.49, 127.39, 61.36; MS (FAB, positive mode): *m/z*: calcd 437.2, found 437.2 [M − Cl$^-$]; elemental analysis (%): calcd for $C_{22}H_{22}Cl_2N_6O_2 \cdot 2H_2O$: C 51.87, H 5.14, N 16.50; found: C 51.71, H 5.06, N 16.26.

Enzyme assay:[23] The inhibitory activity of the equilibrated mixtures was determined by using the method of Ellman et al.[24] 10 μL of a 50 mM solution of acetylthiocholine iodide in water and 10 μL of a solution of an equilibrated mixture prepared as described above or of blank pH 4.0 solution were added to 980 μL of a solution of 1 mg mL^{-1} DTNB in 50 mM sodium – potassium phosphate buffer at pH 7.2 containing 0.06 – 0.07 units of AChE. The activity of AChE was monitored by following the linear change in absorbance ($V = \Delta A\,\text{min}^{-1}$) at 412 nm over 30 s at 25 °C. The relative inhibition was calculated according to Equation (3):

$$\text{inhibition [\%]} = \frac{V_0 - V_i}{V_0} \times 100 \quad (3)$$

where V_0 is the enzyme activity of the blank solution (100 mM sodium acetate buffer, pH 4.0, without inhibitor) and V_i is the enzyme activity of the solution in the presence of inhibitor. All experiments were repeated at least twice. The typical error range was ± 2.5% (standard error of the mean).

Sequential dynamic deconvolution: The libraries used for sequential deconvolution (Figure 4) were prepared similarly to the full library where one or more components (**I**, **H**, and **4**) were sequentially removed prior to library generation and deconvolution. Partial libraries where subsequently prepared in the same way as described for the full library. Aliquots of 20 μL (**I**, or **I** and **H** removed), or 50 μL (**4** removed) were added to the enzyme assays.

Inhibition: The 50% inhibitory concentrations (IC_{50}) were determined by adding inhibitor solutions ranging from 1 mM to 10 pM to the enzyme assay. The results were analyzed with the program GraphPad Prism (GraphPad Software, San Diego, CA, USA) using nonlinear regression analysis toward a one-site competition model. The inhibitory constants for **4-I-4** were estimated according to a mixed-inhibition model:[25] Samples were prepared containing 25 – 300 μM (seven concentrations) of acetylthiocholine, and 0 – 5 nM (four concentrations) of inhibitor, and the activity measured as mentioned above. Observed K_M and V_{max} values were determined for each of these series, and $K_{M,obs}/V_{max,obs}$, and $1/V_{max,obs}$ ratios relative to the noninhibited series calculated. The competitive inhibition constant (K_i), and the noncompetitive constant (αK_i) were then estimated by plotting the relative values versus the substrate concentrations, yielding straight lines with slopes $1/K_i$ and $1/\alpha K_i$, respectively.

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