

GENERATION AND SCREENING OF DITOPIC DYNAMIC COMBINATORIAL LIBRARIES

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Abstract

Dynamic Combinatorial Chemistry (DCC) is a new supramolecular concept that extends beyond static combinatorial chemistry towards adaptive chemical systems. This concept relies on reversible interchange between sets of basic components to generate continually interconnecting adducts, giving access to dynamic combinatorial libraries (DCLs) comprising all possible combinations of the components available. Such libraries allow for the target-driven generation 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 DCL. In this study, the implementation of such libraries on biological interactions was examined and dynamic libraries, generated from small arrays of initial precursors, were probed against binding to biological receptors.

modern drug development, notably in initial lead generation and refinement.

Common approaches in combinatorial chemistry are however built upon sequential and irreversible syntheses, performed in parallel or concertedly, and all constituents of the library are more or less robust molecules [1, 2]. While this methodology offers satisfactory control, its flexibility in library generation is inherently limited inasmuch as all structures have to be designed distinctly, and are produced separately. If however, dynamic features can be introduced in the generation process, a new dimension of the combinatorial procedure can be envisaged. In this case, the library maintains the flexibility to self-adjust to the chosen target macromolecule at a given time in a certain environment, and by virtue of reversible molecular and supramolecular interchange processes, it can adapt to the system constraints.

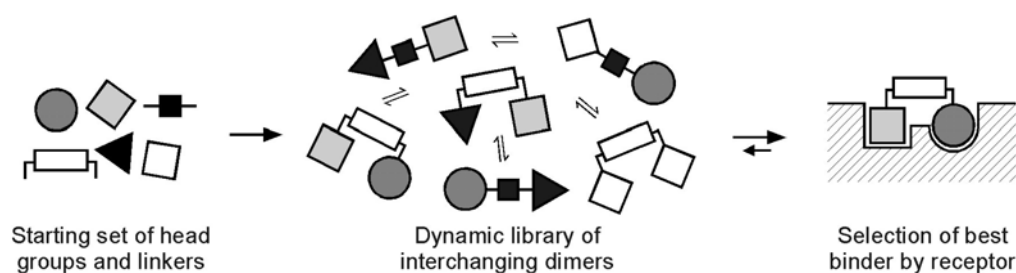


Figure 1. Schematic representation of the generation and screening of a ditopic dynamic combinatorial library.

1 INTRODUCTION

1.1 Dynamic Combinatorial Chemistry

Over the last two decades, combinatorial chemistry has been extensively developed to produce vast substance libraries, composed of combinations of given structural motifs, in the pursuit for new drug candidates. Numerous techniques and methodologies have been designed to produce chemical combinatorial libraries, and library generation has become an important tool in many steps in

Dynamic combinatorial chemistry (DCC) is a recently developed approach that gives access to such self-adjusting libraries (Figure 1) [3-8]. It is based on libraries consisting of rapidly interchanging species, each formed or broken *in situ*, through a variety of reversible connection processes involving non-covalent interactions or reversible covalent reactions.

1.2 Reversibility in aqueous phase

In order to set up versatile, yet robust dynamic libraries, reactions capable of being operated under mild conditions had to be selected. In addition, the reactivity needed to be

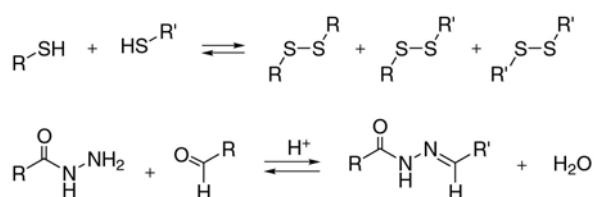


Figure 2 Reversible covalent bonds compatible with aqueous systems. Top: disulfide redox interchange; bottom: reversible acyl hydrazone formation.

compatible with the binding of the ligands to the target molecules. The disulfide redox interchange [9, 10], and the acyl hydrazone formation [11], fulfil these prerequisites (Figure 2). Disulfides undergo rapid interchange with thiols at moderate to high pH, but the bond formed is stable at low pH, thus allowing to lock/unlock the dynamic process by a simple change in pH. In addition, the reactivity of non-aromatic disulfides is reasonably similar, and the equilibrium constant is close to unity.

The reversible formation of an acyl hydrazone behaves similarly. It is sufficiently stable in aqueous media, and 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. Both reaction types are also highly chemoselective.

1.3 Biological target species

In order to demonstrate the generation and screening of ditopic DCLs, biological receptors with two sites in close proximity were selected. The first of these, Concanavalin A (Con A) is a plant lectin specific for a branched trimannoside core unit, located in *N*-glycosidic carbohydrate-peptide linkages of glycoproteins [12, 13]. The Con A binding site has been mapped by crystallography and has been shown to be rather shallow,

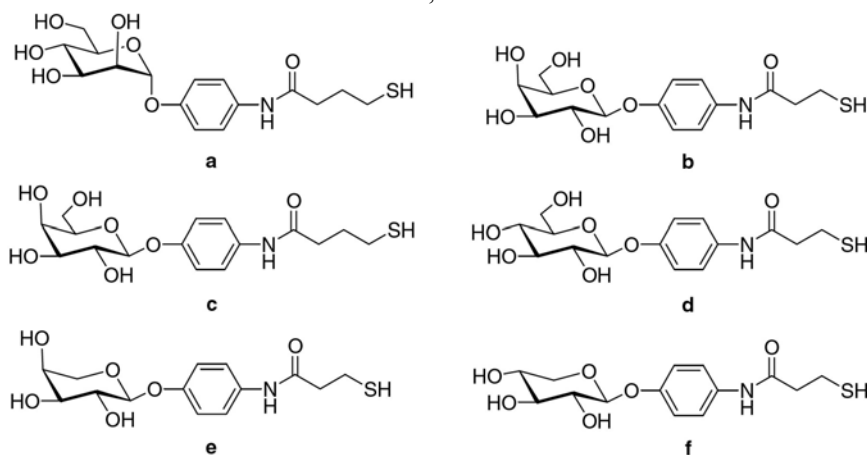


Figure 3 Carbohydrate thiol components. The headgroups were composed of D-mannose (a), D-galactose (b, c), D-glucose (d), L-arabinose (e), and D-xylose (f)

composed of a network of hydrogen bonds interacting with the natural substrate [14]. The majority of these interactions are formed with the non-reducing, peripheral mannosides of the trimannoside unit, rather than with the central mannose group, thus implying that the latter acts more or less as a linker between the interacting parts.

The second target, acetylcholinesterase (AChE), is an enzyme whose function in the central nervous system (CNS) is to terminate transmission at cholinergic synapses by hydrolysing the neurotransmitter acetylcholine [15, 16]. AChE has two distinct binding sites close to each other. A catalytic site is located at the bottom of a deep gorge, and a peripheral site is situated near the rim of this gorge [17].

2 RESULTS AND DISCUSSION

2.1 Library design

The design of the carbohydrate building blocks was built on considerations concerning the mimicking of the native trimannoside unit. Thus, the thiol components **a-f** (Figure 3) were prepared (synthesised as the more stable disulfide homodimers, **a-a**, **b-b**, etc.) [9]. A phenylamido group was also introduced, primarily because it provided a chromophore for the subsequent analyses by high performance liquid chromatography (HPLC). Three hexopyranosides (D-mannose, D-glucose, D-galactose), and two pentopyranosides (L-arabinose, D-xylose) were used as carbohydrate head groups, and two different linkers, varying in length by one methylene group were examined.

Various hydrazide and aldehyde building blocks were selected for the AChE system (Figure 4), 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 [11]. 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.

2.2 Library size

The library size that can be generated varies within the two libraries. A ditopic disulfide library theoretically composed of all homo- (e.g., A-SS-A) and heterodimers (e.g., A-SS-B) amounts to a maximum size of n^2 . In the case of a library composed of heterodimers that are symmetrical, such that species A-SS-B equals B-SS-A, the size is reduced, consisting of $n(n+1)/2$ components. In the present case, $n = 6$, thus resulting in a final library size of 21 constituents.

In the acyl hydrazone case, the reaction of n hydrazides with m_p aldehydes of functionality degree p (i.e. monoaldehydes: $p = 1$; dialdehydes: $p = 2$) yields a maximum library size summing over all combinations n^p of n units p to p (with order, non-symmetrical library) [7].

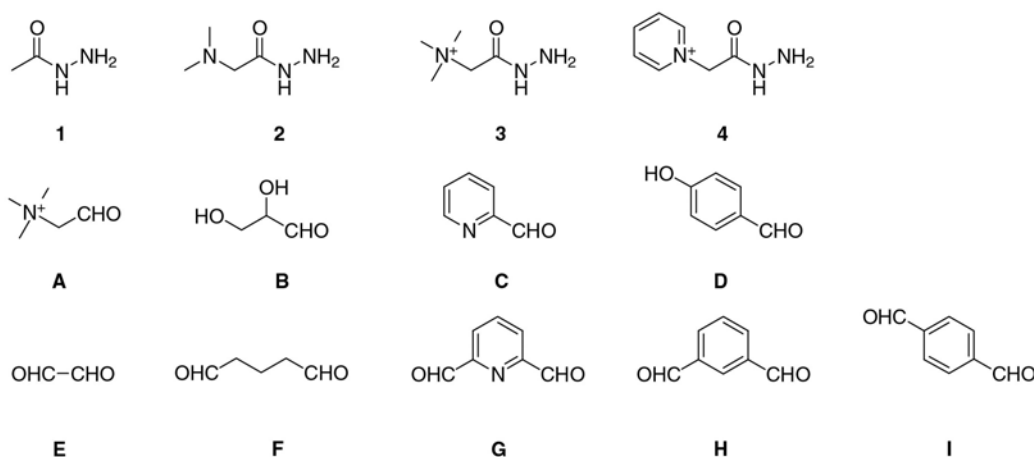


Figure 4 Building blocks for acyl hydrazone library. Top: hydrazides; middle: monoaldehydes; bottom: dialdehydes.

The condensation of four hydrazides ($n = 4$) with four monoaldehydes ($m_1 = 4$), and five dialdehydes ($m_2 = 5$) results in 96 possible acyl hydrazone combinations. Since the dialdehydes are symmetrical, some of these combinations are identical, so that this number reduces to some extent. With our figures, a total number of 66 different library constituents. may be obtained from only 13 original building blocks.

Although these numbers are fairly small, compared to “classical” combinatorial libraries, the principle can easily be extended to larger arrays.

2.3 Disulfide library

The libraries resulting from scrambling of the original building blocks were generated by mixing the

homodimers of **a-f** together with the initiating reagent dithiothreitol (DTT), capable of reducing some of the disulfides to the corresponding thiols. Upon initiation, interconversion between the disulfides occurred, the rate of which was highly dependent upon the pH of the solution. At high pH (>8), scrambling was achieved reasonably rapidly (within hours), whereas at low pH (<5) no scrambling could be detected. A similar pH-dependence was recorded for the binding of the carbohydrates to Con A, where a near neutral pH was preferable. A pH of 7.4 was chosen as a level at which a reasonable rate of scrambling could be obtained, while receptor binding was not significantly affected. When analysing the generation and selection events in the presence of the receptor, a method for its separation from the equilibrating library was required. For this reason, Con A immobilised on Sepharose beads was used, thus allowing for simple filtration of the unbound species.

Scrambling occurred smoothly at pH = 7.4 from the original species generating all 21 expected ditopic combinations as analysed by RP-HPLC (Figure 5 top).

When Con A was added to the equilibrating pool, a shift in the concentrations of the different constituents present unbound in solution was recorded. The fraction of some of the free species decreased, notably the D-mannose-containing homo- and heterodimers. In order to characterise the species actually bound to the receptor, acidifying the solution to pH 4 eluted the Con A-Sepharose beads and the composition of the eluate was analysed (Figure 5 bottom). Clearly, the D-mannose homodimer (**a-a**, Figure 6) was most efficiently bound to the lectin, and to a lesser extent the D-mannose containing heterodimers. All other species in the equilibrating pool did not bind to Con A-Sepharose and remained in solution. Thus, the receptor could be used to “fish out” the best-bound species from the equilibrating pool, while leaving the others behind.

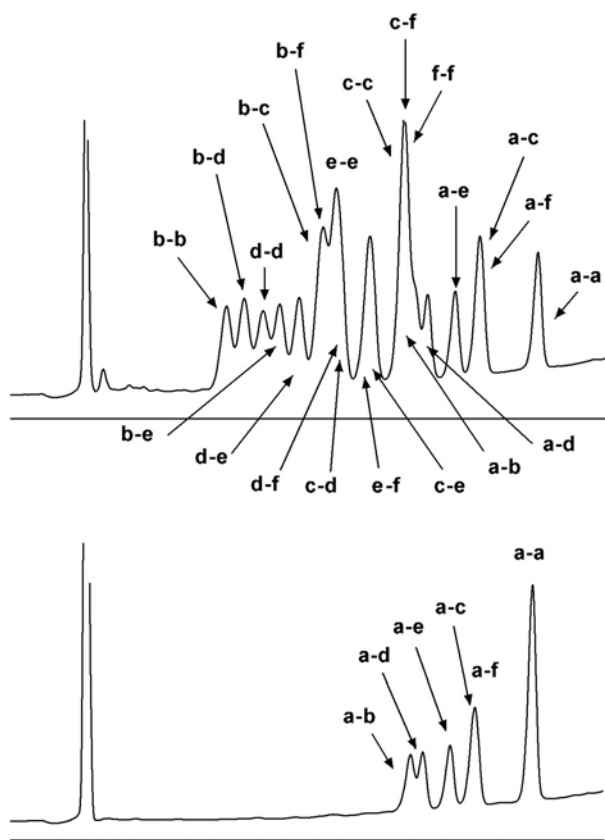


Figure 5 HPLC chromatograms of carbohydrate disulfide library. Top: generation of 21 different ditopic constituents; bottom: elution of bound ligands to the receptor. Compound **a-a** (Man-Man) represents the best binder.

The results indicate that it is possible to generate a dynamic library of bis-carbohydrates based on reversible covalent disulfide bond formation between thiol-derivatised carbohydrate components in aqueous solution under mild conditions. The utilisation of the disulfide bond proved to be highly useful, since it allowed for comfortable initiation of equilibration, and exchange could be stopped by simple acidification of the solution. This provides a simple start/stop mechanism that is easy to control.

The results also show that the DCL generated can be directly screened *in situ*, by adding the targeted receptor to the equilibrating pool of library components, and that preferential binding of some constituents takes place. As long as the chemical interchange does not interfere with the structure or function of the receptor, and the generation can be performed under sufficiently mild conditions, then this offers a very convenient method for the rapid screening of a large number of compounds directly in one step. In addition, a shift in equilibrium can

be expected resulting in amplification of the species most strongly bound to the receptor at the expense of the others.

2.4 Acyl hydrazone library

In the case of the acyl hydrazone library, a different methodology was used. Because of the low availability and stability of AChE, a two-step approach was adopted. At first, 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. Subsequently, screening was conducted at conditions optimal for the enzyme. 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 characterising the potent ligands among those formed in a dynamic mixture. In order to identify the active compound(s), one of the building blocks was sequentially omitted 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.

Thus, 13 sub-libraries were formed by mixing all components, except one specific hydrazide or aldehyde building block, under the scrambling conditions. Together with a reference sample (**buffer**), containing no building blocks, this series of 15 samples (incl. **all**) was enough to screen the entire library. In contrast, using individual screening at least 50 samples have to be analysed (9 x 4 combinations, 13 building blocks plus reference sample) clearly demonstrating the advantages offered by the dynamic screening method.

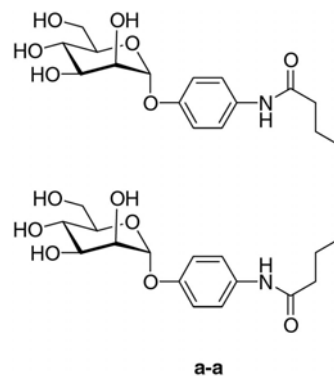


Figure 6 Compound **a-a** (dimannoside) selected from the disulfide library

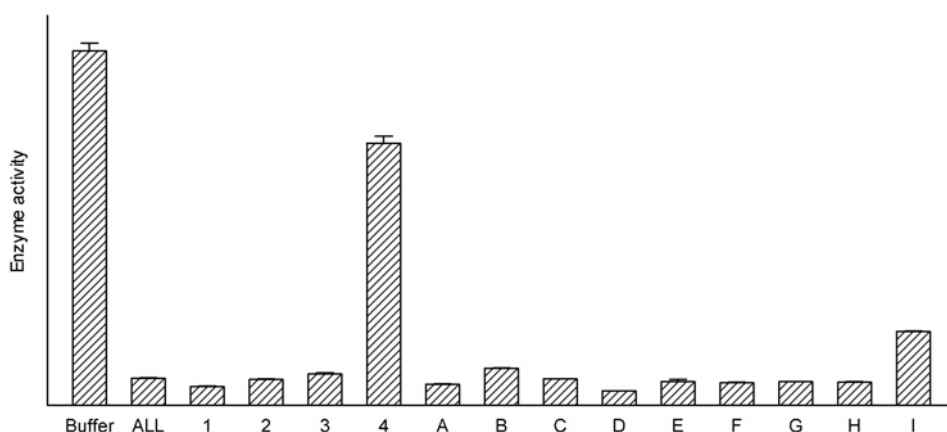
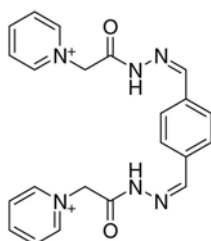


Figure 7 Dynamic deconvolution of a ditopic acyl hydrazone library. Compounds **4**, and **I** most active.

The results obtained from this library generation/screening process are presented in Figure 7. 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. On sequential removal of each building block from the complete library, an increase in activity indicates that the component omitted contributed significantly to the inhibitory effect. The data in Figure 7 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**, most likely **4-I-4** (Figure 8).



4-I-4

Figure 8 Ditopic compound **4-I-4** with high affinity for AChE

Component **4-I-4** was ultimately synthesised separately and its inhibitory effect further characterised and compared with other known bis-quaternary ammonium inhibitors. The results indicate that **4-I-4** is indeed a very potent inhibitor, displaying inhibition in the low nanomolar range ($K_i = 1.09$ nM, $\alpha K_i = 2.80$ nM). It proved 500-fold more effective than decamethonium, and around ten-fold more potent than the bis(pyridinium)-decane and -dodecane analogues. Since the distance between the

charged nitrogens in **4-I-4** is approximately the same as in the bis(pyridinium)-dodecane compound (~ 16.7 Å in the fully extended forms), the results are indicative of an additional binding effect from the linker region.

Dynamic deconvolution 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.

3 CONCLUSION

In comparison to other combinatorial techniques, such as parallel/multiple synthetic protocols and encoded solution phase techniques, the DCC methodology provides access to easily controlled solution phase libraries. The size of the libraries can easily be augmented so that trimers, tetramers, etc, can be envisaged, resulting in library sizes well on a par with existing static chemical libraries. In conjunction with the *in situ* receptor binding technique, such libraries can easily be screened, and the resulting bound antagonists/inhibitors identified. It has been shown that disulfide and acyl hydrazone formation and exchange can be efficiently used to generate dynamic combinatorial libraries in aqueous media. Among all possible constituents formed, active compounds of appropriate length containing potent recognition groups could be rapidly identified using a *dynamic deconvolution* process.

4 ACKNOWLEDGEMENTS

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