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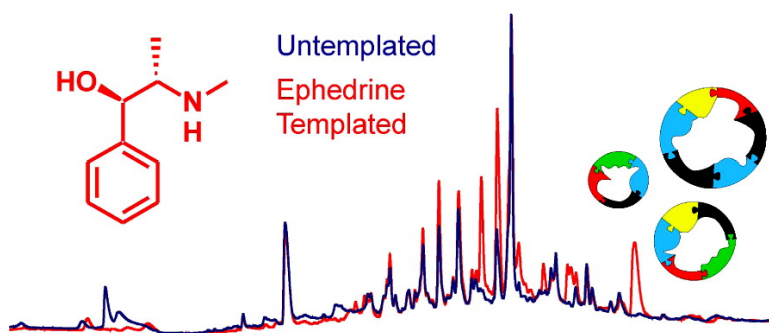
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Two-Vial, LC−MS Identification of Ephedrine Receptors from a Solution-Phase Dynamic Combinatorial Library of over 9000 Components

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Dynamic combinatorial chemistry is a powerful technique for exploring molecular recognition.[1] New synthetic receptors have been discovered,[2] often with exceptional properties,[3] and new ligands for biomolecules have been identified.[4d,5] With few exceptions,[4d,5] the size of the dynamic combinatorial libraries has been small, often based on only one building block forming less than ten detectable library members. We now report that it is feasible to use much larger libraries, effectively screening more than 9000 compounds in a single analysis. This has led to the identification of new receptors that bind ephedrine in water.

Dynamic combinatorial libraries (DCLs) are equilibrium mixtures of oligomers made by linking building blocks through reversible bonds. The concentration of each oligomer depends on its stability relative to the other library members. If a guest molecule is added to a library of potential hosts, those hosts which bind strongly will be stabilized and tend to be amplified.

As pointed out in a recent critical review by Ladame,[1a] an important unanswered question in dynamic combinatorial chemistry relates to library size: it is as yet unclear what size is optimal and it is also unclear whether screening larger libraries in solution is even feasible, which has until now discouraged the use of large libraries. In theory, the probability of generating strong binders increases with library size, yet so does the probability that the concentration of individual library members drops below detection limits.[6]

Experimental studies featuring large DCLs are scarce. Miller has recently reported a resin-bound dynamic combinatorial library with a theoretical library size of more than 11000 members.[4d] A number of studies of potentially large solution-phase DCLs have been published, but the number of library members that was effectively screened was invariably small, typically not more than 10−100 compounds.[5,7] Reasoning that modern analytical techniques should be able to cope with much larger libraries, we set out to study a solution-phase disulfide DCL made from eight different thiol building blocks **1−8**. While assessing the effective size of such a DCL is difficult, our results (vide infra) suggest that we are sampling the cyclic oligomers up to tetramer in this system, which amounts to at least 9000 unique compounds (see Supporting Information, SI).

We screened this DCL for binding to ephedrine (**9**), a member of the phenylethylamine group of compounds, that also includes dopamine, adrenaline, and amphetamine, many of which act upon the sympathetic nervous system. Ephedrine itself interacts with adrenergic receptors; its precise mechanism of action depends on the particular receptor subtype.[8] Ephedrine contains both hydrophobic and hydrophilic functionality and is protonated under the DCL conditions (pH 8.0). We envisaged that recognition of this compound may take place by the hydrophobic and anionic groups of building blocks **1−8**.
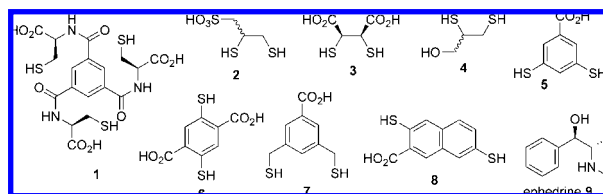
Two libraries were prepared by mixing equimolar amounts of building blocks **1−8** (5.0 mM total), with and without ephedrine (5.0 mM) in water at pH 8.0.[9] After stirring the mixtures for one week, a comparison of the HPLC analyses of the two mixtures revealed a number of peaks that had either appeared or increased in size (Figure 1).
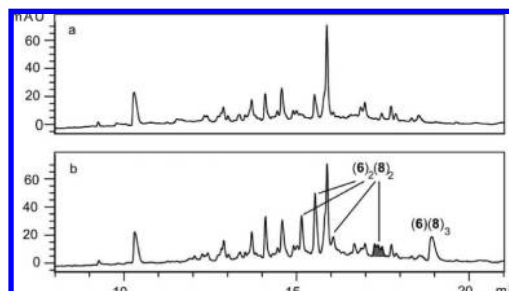

Figure 1. HPLC chromatograms of DCLs made from building blocks **1−8** (5.0 mM total concentration in 50 mM pH 8.0 borate buffer) (a) in the absence of template; and (b) in the presence of 5.0 mM ephedrine **9**.

As expected for a library of this size, almost every compound coelutes with many others. For example, LC−MS analysis of the small peaks at 17.5 min (shaded in Figure 1) reveals the simultaneous presence of several library members. A comparison of the mass spectra of the templated and untemplated libraries at this retention time (Figure 2) suggests that the amplified species has a mass of 922.7. The compounds that may be present in the DCL that are within 0.5 amu of this mass are: (**6**)$_2$(**8**)$_2$, (**4**)$_2$(**7**)(**8**)$_2$, (**2**)$_2$(**6**)$_3$, (**2**)$_4$(**3**). LC−MS−MS experiments allowed us to identify (**6**)$_2$(**8**)$_2$ as the amplified compound: We observed the loss of fragments with a mass corresponding to that of **6**, **8**, and (**6**)(**8**) (see SI). Using a similar combination of LC−MS and LC−MS−MS experiments on the other amplified signals in the templated DCL revealed that these correspond to a series of isomers of (**6**)$_2$(**8**)$_2$ and a single tetramer with composition (**6**)(**8**)$_3$ (Figure 1b).
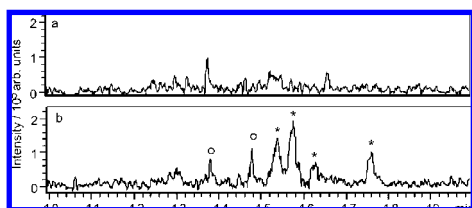
Notably, compounds (**6**)(**8**)$_3$ and (**6**)$_2$(**8**)$_2$ were only detectable when the template was present (Figure 3). This demonstrates that it is not necessary for library members to exist in detectable
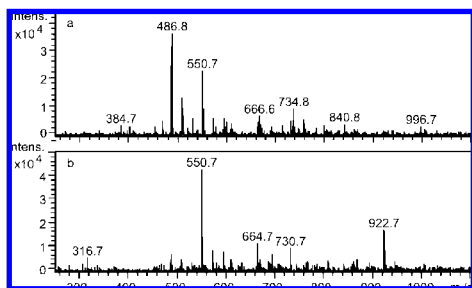
Figure 2. Mass spectrum of the HPLC peaks at 17.5 min of the DCL of Figure 1 in the absence (a) and presence (b) of ephedrine **9**.

Figure 3. Extracted ion chromatograms for $[(6)_2(8)_2]^-$ ($m/z = 922.8$) from LC−MS analysis of the libraries shown in Figure 1 in the absence (a) and presence (b) of **9**. The peaks labeled with an asterisk have isotopic distributions consistent with that predicted for $(6)_2(8)_2$, while those labeled with a circle are due to the tails of compounds with m/z 920.8.

Table 1. Thermodynamic Parameters for the Interaction of Ephedrine with Receptors $(6)_2(8)_2$ and $(6)(8)_3$ [a]

	$(6)_2(8)_2$	$(6)(8)_3$
K (M^{-1})	1.3×10^4	1.5×10^4
$\Delta G°$ ($kJ \cdot mol^{-1}$)	−23.5	−23.8
$\Delta H°$ ($kJ \cdot mol^{-1}$)	−24.2	−23.6
$T\Delta S°$ ($kJ \cdot mol^{-1}$)	−0.7	0.2

[a] Determined using isothermal titration calorimetry in 50 mM borate buffer pH 8.0 at 298 K.

quantities in the untemplated library as long as their amplification is efficient enough to push their concentrations over the detection limit when the template is present.

To confirm binding by the selected receptors and facilitate their isolation, biased libraries were prepared using only building blocks **6** and **8**, and their amplification as mixtures of isomers was again observed (see Figure S3 in SI). There are two possible sequence isomers for $(6)_2(8)_2$ (**6**−**6**−**8**−**8** and **6**−**8**−**6**−**8**), while the directionality of block **8** creates the possibility of regioisomers. Of the five possible isomers of $(6)_2(8)_2$, four are observed. For compound $(6)(8)_3$, four regioisomers are expected, all of which are observed. Approximate amplification factors[10] for all isomers in the biased libraries range from 1.2 to 2.0 for $(6)_2(8)_2$ and from 3.2 to 6.7 for $(6)(8)_3$. As no clear selectivity in the amplification of the isomers was observed, no attempts were made to separate these; both receptors were isolated as mixtures of isomers by preparative HPLC.

The thermodynamics of binding of ephedrine to the hosts in water was quantified using isothermal titration calorimetry (Table 1). The binding is enthalpy driven, similar to the binding of hydrophobic ammonium ions by structurally related receptors.[2a,3b] The affinities are among the highest reported thus far for synthetic receptors for this class of molecules in water[11] and within the range of affinities of adrenergic receptors for ephedrine.[8b,c] However, the affinities are modest compared to some of our previous results obtained through dynamic combinatorial chemistry using other guests.[3] It is gratifying that the amplifications are nevertheless clearly detectable.

In conclusion, we have demonstrated that solution phase dynamic combinatorial chemistry can be used to identify receptors from a mixture of at least 9000 theoretical compounds, using only two vials and two LC−MS−MS analyses. This has led to the discovery of a set of new hosts for ephedrine. While in traditional combinatorial chemistry libraries consisting of mixtures of compounds have fallen out of favor because of the frequent occurrence of false positives during screening, our results have not shown any indications of similar problems in DCLs. The compounds that were significantly amplified all turned out to be strong binders.[9] These results demonstrate the feasibility of screening DCLs that are much larger than the solution-phase libraries reported thus far.

Supporting Information Available: Detailed descriptions of library preparation and analysis; synthesis, isolation and characterization of the receptors $(6)_2(8)_2$ and $(6)(8)_3$; ITC data; details of estimation of library size; amplification data for other templates. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) In ref 5a a large number of imines can potentially form based on a difunctional scaffold. However, the binding pocket on the target is only large enough to accommodate monofunctionalized compounds. Moreover, the imine equilibrium lies on the side of the building blocks, so that there is very little difunctionalized material present in the DCL. In ref 5b the screening method relied on the UV chromophore of one of the building blocks, so that all library members not containing this building block remained undetected.

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(9) The choice of the experimental conditions was arbitrary. We are currently conducting a theoretical investigation aimed at identifying the optimum conditions for screening large libraries, also addressing the probability of finding the best binders in such large libraries.

(10) The amplification factor of a library member is the ratio of its concentrations in the templated and untemplated libraries.

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