The Advantage of Covalent Capture in the Combinatorial Screening of a Dynamic Library for the Detection of Weak Interactions


Keywords: Combinatorial chemistry / Covalent capture / Chemical library / Molecular recognition / Amines

In this paper we address the advantage of screening a dynamic library by covalent capture in comparison with an approach in which the target is not covalently bound to the molecular receptor. The aim is the selection of recognition units for the binding of an anion (or polyanion) by relatively weak binding interactions, a situation typically found in supramolecular chemistry. To compare the two approaches, two model systems have been studied both based on the functionalization of a molecular platform by reversible imine formation. In the case of the noncovalently bound substrate, the platform $P_1$ is a trisubstituted benzene unit, 2,4,6-trimethylbenzene-1,3,5-tricarbalddehyde, to select three recognition arms for the binding of the trisodium salt of benzene-1,3,5-tricarboxylate. For the covalent-capture-based approach the platforms $P_2$ and $P_4$ are benzene derivatives with a tethered phosphonate target (tetrabutylammonium 2-formylphenyl ethylphosphonate) for the selection of a single recognition unit. The library of recognition elements comprises phenyl- and ammonium-functionalized amines. We show that the selection of recognition units for the binding of the substrate with weak to medium binding constants may encounter, by using a noncovalently bound substrate, serious problems. This is because the best conditions for the amplification of the library, that is, a large excess of variable recognition elements and target, lead also to competitive binding of the elements not bound to the platform with the target. This may result in negligible amplification of the best-fit members of the library. In contrast, upon tethering the target to the platform and using the covalent-capture strategy for the selection of the recognition elements, significant amplification is observed, even for systems with much lower binding constants. Although competition with excess recognition units may also become an issue in the case of the tethered target, there is a way to overcome the problem by working at low concentrations.

Introduction

Since its first introduction, dynamic combinatorial chemistry (DCC)[1,2] has shown its potential as a way to obtain collections of molecules without the hassle and complications related to the synthesis and deconvolution requirements of standard combinatorial chemistry. The possibility of screening in situ the dynamic combinatorial library (DCL) of molecules obtained by the spontaneous selection of the most suitable receptor for a specific molecular target has increased the interest in the study of these systems. Briefly, a DCL is a collection of molecules formed by a pool of common building blocks held together by reversible bonds.[3] Because of the reversibility in the bond formation the composition of a DCL is governed by the thermodynamic equilibrium between its components. Any alteration of this equilibrium such as the addition of a molecule (target) that binds selectively to one (or more) member(s) of the library will result in the shift of the equilibrium with a change in the composition of the library. This may lead to the selection of the member of the DCL with the highest affinity for the target. If this occurs, the concentration of this member is “amplified”. The implication for drug[4] or enzyme-inhibitor discovery,[5] just to give two examples, is obvious. Nonetheless, the fundamental limitations of DCC have been addressed in a series of theoretical contributions, mainly by Severin[6] and Sanders[7] and their co-workers, but also by others.[8] Under certain conditions,[6,8] and this has also been verified experimentally,[9] the addition of a target to a DCL does not necessarily lead to the amplification of the member of the DCL with the highest affinity for the target. Rules of thumb for a successful selection of library members with the highest affinity for the target have been suggested.[5–8] Although spectacular examples have demonstrated the possibility of isolating, also quantitatively, high-affinity artificial receptors from a DCL,[10] it seems that the application of DCC for detecting weak to medium binding events is much more cumbersome.[7d]

To make DCC attractive in the weak to medium binding regime, we recently suggested[11] covalent capture as a promising strategic improvement for the selection of the recognition unit. Briefly, this implies that the target is cova-
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lently bound to the receptor, which then “captures” molecular units that are able to interact with the target. Covalent capture,[12] in spite of the popularity it has gained recently, is not a new strategy as it is quite often used by natural systems for increasing the strength and selectivity of the interaction between them.[13] The conceptual difference between the covalent-capture approach and the traditional one in DCC is shown in Figure 1 for the recognition of a target T by a collection of potential recognition units that are assembled into a common platform P. In the standard DCC approach the library of receptors is first formed and, subsequently, they interact with the target to form the complex. In the specific case of Figure 1, for the sake of simplicity, only one of the members of the library binds to the target with a sizeable binding constant. The final outcome of the selection is the identification of a molecular receptor comprising the platform and the best-fit recognition units. In the covalent-capture strategy, for the purpose of selection, the target is covalently and irreversibly bound to the platform, and the recognition units that better interact with it are covalently captured by the platform. In this case the selected library component is the molecular receptor with the substrate covalently bound to it.

The covalent-capture process may be reversible or irreversible. In the first case, also referred to as dynamic covalent capture, the library composition will reflect the thermodynamic stability of the DCL members, whereas in the second case this is only true if the stabilities of the transition states towards product formation parallel those of the products. In this work we wish to address the advantage of dynamic covalent capture with respect to traditional (i.e., the target noncovalently bound to the receptor) dynamic combinatorial selection in detecting relatively weak binding events. This has been achieved by studying two model systems both based on the functionalization of a platform, a situation similar to that shown in Figure 1.

The platform is not involved in the molecular recognition process but allows the connection between the recognition elements and, in the case of covalent capture, also of the target. The presence of such a common building block is an advantage, particularly if this is the limiting species in terms of concentration, because it minimizes selection problems as demonstrated by Severin.[6b]

Results and Discussion

The Libraries Considered

The two libraries were designed to select recognition units for an anionic host by using imine formation as the reversible reaction.[14] Accordingly, the libraries considered were all under thermodynamic control. Platform P1 used in the classic DCC approach for selecting three recognition arms for an anion is 2,4,6-trimethylbenzene-1,3,5-tricarbaldehyde, whereas platform P2 is tetrabutylammonium 2-formylphenyl ethylphosphonate for the selection of a single recognition arm for the attached anionic phosphonate. It is well documented that the three substituents at alternating positions of a hexasubstituted benzene point in the same direction, either above or below the benzene plane.[15,16] Hence, the three aldehyde substituents upon imine formation will present three arms protruding from the same side.

Scheme 1. Synthesis of platforms P1–P4. Reagents and conditions: (i) HBr (33% in CH3COOH), (CH2O)n, ZnBr2, 90–100 °C, 93%; (ii) [(CH3)2CNO2] Na+, EtOH, 68%; (iii) NaH, CH3CH2P(=O)Cl2, NaHCO3, 12% (R = tBu), 15% (R = H); (iv) TBA acetate.

Figure 1. Schematic representation of the selection process of a recognition unit for a target T by using an assembling platform P. (a) Conventional DCL and (b) dynamic covalent capture. For the sake of simplicity it is assumed that only one of the library members binds to the target.
of the benzene ring, thus providing convergent recognition elements for the binding of the target.\textsuperscript{17} The synthetic strategies used to synthesize the two platforms are shown in Scheme 1.

The two selected platforms are quite different, not only in terms of their structure, but also for the number of recognition elements that each of them presents. Indeed, one may argue that platform \textit{P1} is greatly favored over platform \textit{P2} in the binding of a target due to the presence of three converging recognition units compared with the single unit present in \textit{P2}. However, one should consider that, having already connected the target anion (the phosphonate) in the case of \textit{P2}, this amounts to an infinite binding constant for the binding of the target to the platform, a situation impossible to achieve in the case of \textit{P1}. This is an important and critical factor that favors platform \textit{P2} over \textit{P1} in the detection of binding events. This difference makes it impossible to compare the composition of the library with \textit{P2} in the absence of the target. For this reason platform \textit{P3}, which bears a methoxy group in place of the phosphonate, was also considered.

The product distribution of the library obtained by reaction with \textit{P2} has been in all cases compared with that obtained with \textit{P3} to normalize the results for any intrinsic difference of stability of the formed imines not related to the stabilization due to the interaction with the phosphonate. In the case of platform \textit{P1} a comparison has been made between the libraries obtained in the presence and absence of the added target anion.

Because our aim was to prove the advantage of the covalent-capture strategy in DCC compared with the noncovalently bound substrate approach in the selection of recognition units for the binding of an anion, we limited the number of the components of the libraries to the minimum required, that is, two different types of amines, one bearing a phenyl group, which is unable to interact with an anion, and one comprising an ammonium ion supposed to interact with the target through a charge–charge interaction. Thus, the three-armed platform \textit{P1} leads to a four-membered library and the single-armed platform \textit{P2} to only a two-membered library. Charge–charge interactions do not have geometrical requirements\textsuperscript{18} unlike, for instance, hydrogen-bonding, and this removes most conformational issues in the recognition process. They are, however, sensitive to distance.

For this reason we have also explored platform \textit{P4} containing a bulky \textit{tert}-butyl group in the \textit{ortho} position with respect to the phosphonate. This should force the phosphonate group towards the ammonium group and thus strengthen the charge–charge interaction. The different libraries obtained by using the three platforms are presented in Figure 2, and the synthetic routes for the preparation of the platforms are shown in Scheme 1. It is crucial to understand that, whereas in the case of the conventional DCC approach based on platform \textit{P1} the selected library component will be a molecular receptor for the target, with platforms \textit{P2} and \textit{P4} the selection process will provide an indication of the best recognition unit for the target, and the pinpointed library member is not a molecular receptor. To obtain a molecular receptor the selected recognition elements should be used for the functionalization of a platform like \textit{P1}.

![Figure 2. Library components obtained with platforms P1–P4 by using amines A–C.\textsuperscript{19}](image-url)
Simulations for Recognition Site Selection by Using Conventional DCC

By mixing the two amines A and B with platform P1, the four products P1A3, P1A2B, P1AB2, and P1B3 are formed (see Figure 2). On a statistical basis, assuming an identical stability of the imines derived from A and B, the relative ratio of these four products should be 1:3:3:1. As will be shown below, however, amine A forms a more stable imine and, consequently, library members containing A are favored over those containing B. Addition of a generic target anion T should amplify library members forming stronger complexes with T, that is, P1B3, P1AB2, and P1A2B, in decreasing order. In the solvent used for our studies (CD3OD) the binding constants between an ammonium and a carboxylate or phosphonate anion are rather small, typically around 20–30 M⁻¹.[20] Simulations of the library composition were performed by assuming that each arm containing an ammonium and a carboxylate or phosphonate anion are rather small, typically around 20–30 M⁻¹.[20] Simulations of the library composition were performed by assuming that each arm containing an ammonium cation contributes one order of magnitude to the binding constant.[21] Accordingly, binding constants of 10, 100, and 1000 M⁻¹ for the binding of T by the three library members P1A3, P1AB2, and P1B3, respectively, were considered.[22] The expected amplifications for this system were calculated as a function of the ratio [amines]tot/[P1] and the amount of target T added (Figure 3).

These calculations indicate that maximum amplification should be observed when large excesses of amines and target are used (see Figure 3d for high [amines]/[P1] ratios).[23]

This is in full accord with the calculations performed by Severin using a similar, even though simpler model.[6b] In addition, these simulations confirm the advantage of having a common building block present at limiting concentrations (here P1). When no excess of amines (Figure 3a) is used, the expected amplification of P1B3 induces an amplification nearly as strong of the nonreceptor P1A3. Under these conditions, the final library composition does not reflect at all the attributed binding constants. On the other hand, a true reflection of the binding constants in the final library composition is observed when the platform P1 is present at very low relative concentrations (Figure 3d).

From these simulations we may conclude that in real experiments we should work under conditions of a large excess of amines and target with respect to the platform. We should also select a target anion with a very large binding constant with the best receptor to maximize its amplification in the DCL.[24]

Experiments Using a Conventional DCL

On the basis of the above simulations we were first interested in selecting a target anion showing a sizeable binding constant with receptor P1B3, the most promising candidate. The following anions were considered: The single-charged sodium bis(p-nitrophenyl) phosphate and sodium benzoate, the double-charged disodium p-nitrophenyl phosphate and...
disodium benzene-1,3-dicarboxylate, and the triple-charged trisodium benzene-1,3,5-tricarboxylate (the trisodium salt of trimesic acid). Binding constants for the 1:1 complexes were determined in [D₄]methanol by ¹H NMR spectroscopy and are reported in Table 1. As discussed in the Supporting Information, analysis of the data reveals that different binding stoichiometries do not occur under the experimental conditions.

Table 1. Binding constants $K_o$ of receptor P1B₃ with different target anions T determined by ¹H NMR spectroscopy in [D₄]methanol.

<table>
<thead>
<tr>
<th>T</th>
<th>$K_o$ [M⁻¹]</th>
</tr>
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<tbody>
<tr>
<td>Sodium benzoate</td>
<td>43 ± 8</td>
</tr>
<tr>
<td>Sodium bis(p-nitrophenyl) phosphate</td>
<td>61 ± 9</td>
</tr>
<tr>
<td>Disodium benzene-1,3-dicarboxylate</td>
<td>681 ± 41</td>
</tr>
<tr>
<td>Disodium p-nitrophenyl phosphate</td>
<td>650 ± 120</td>
</tr>
<tr>
<td>Trisodium benzene-1,3,5-tricarboxylate</td>
<td>2762 ± 519</td>
</tr>
</tbody>
</table>

Figure 4 shows the experimental data for the binding of the trisodium salt of trimesic acid to P1B₃ (1 × 10⁻³ M). The curve represents the best fitting of the data points with a 1:1 binding stoichiometry.

Analysis of the data in Table 1 shows that the largest binding constant is observed between P1B₃ and the sodium salt of trimesic acid, characterized by the number of charges matching that of the receptor. For each decrease in charge unit in the target anion, regardless of its nature, there is a roughly a decrease in the binding constant of one order of magnitude. Thus, the experimental results support the assumptions we have made in the calculations reported above. On the basis of these results, we selected the trisodium salt of trimesic acid as our anionic target.

Next we determined the equilibrium composition of the four-membered library to check whether it deviates from the statistical one (1:3:3:1 for P1A₃, P1A₂B, P1AB₂, and P1B₃) in case the four library members are not equally stable. The experiments were performed in [D₄]methanol by using ¹H NMR spectroscopy. It was immediately clear that not only were the library members containing the phenyl substituent A more stable, but they were also formed much faster than those containing the ammonium substituent B. Thus, the mixing of the three reagents under the conditions [P1]₀ = 1 × 10⁻² M and [A]₀ = [B]₀ = 4.4 × 10⁻² M at 65 °C in the absence of acid resulted in the relatively fast formation of P1A₃ (24 h) followed by a very slow equilibration to the final composition 4.6:3.5:4:1 of P1A₃ (2.25 × 10⁻³ M), P1A₂B (4.6 × 10⁻³ M), P1AB₂ (2.6 × 10⁻³ M), and P1B₃ (4.9 × 10⁻³ M), respectively, in around 60 d. To avoid this very impractical situation we first prepared P1B₃ by adding only B to P1 and subsequently added A. Following this protocol the same equilibrium composition was reached in 6 d (see the Supporting Information).

The next crucial experiment was the equilibration of the library by using the trianionic target to determine the extent of amplification of the library members containing the ammonium group. The addition of the trisodium salt of trimesic acid to the three reagents (P1, A, and B) sped up the equilibration process that nevertheless remained too slow for practical applications. Accordingly, as done before, we started from preformed P1B₃ and equilibrated the DCL by addition of A and T. Regrettably we were unable to use an excess of T because of solubility problems. Thus, we could not perform our experiment under the best conditions for maximum amplification. Under the conditions [P1]₀ = [T]₀ = 1 × 10⁻² M and [A]₀ = [B]₀ = 4.4 × 10⁻² M the equilibrium mixture had the composition 4.5:6:3.1:1 of P1A₃ (2.6 × 10⁻³ M), P1A₂B (4.4 × 10⁻³ M), P1AB₂ (1.8 × 10⁻³ M), and P1B₃ (5.8 × 10⁻⁴ M), respectively. For P1B₃ the amplification observed was only 1.2-fold greater than that observed in the absence of the target anion. Our expectation was of a 3.1-fold amplification for P1B₃ (see the Supporting Information). A summary of the observed and expected amplifications is reported in Table 2.

Table 2. Expected and observed amplifications of the different library members as a consequence of the binding of trimesic acid to the trisodium salt.

<table>
<thead>
<tr>
<th>Library member</th>
<th>P1A₃</th>
<th>P1A₂B</th>
<th>P1AB₂</th>
<th>P1B₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected amplification⁴</td>
<td>0.6</td>
<td>0.5</td>
<td>1.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Observed amplification⁵</td>
<td>1.2</td>
<td>1.0</td>
<td>0.7</td>
<td>1.2</td>
</tr>
</tbody>
</table>

[See text and Figure 3c for details.]

To explain these results it occurred to us that, under these conditions, the excess of the ammonium-functionalized amine B may actually compete with the three potential receptors of the library for the binding of the target. To verify whether this was a reasonable argument we re-ran simulations of the system introducing this time also a binding event between the free amine B and the target. These simulations are shown in Figure 5 and indicate that, although substantial amplification of P1B₃ should be observed in the case of negligible binding of B to T (top line of Figure 5) in the presence of a large excess of B, the amplification of P1B₃ diminishes significantly as a function of the strength of its interaction with T. In the extreme case of a binding constant for the complex B·T not much lower than that for complex P1B₃·T, even deamplification of the latter may be observed (lower curve of Figure 5). To verify whether B was actually a competitor of P1B₃ for the binding of T we monitored the formation of the complex be-
between T and P1B3 by 1H NMR spectroscopy and, after its formation, added increasing amounts of B. As shown in Figure 6, B competes with P1B3 for the binding of the target, and a 20-fold excess of B almost totally suppresses the formation of the complex P1B3·T. The apparent binding constant for the formation of the B·T complex is 460 ± 50 m−1. Clearly, if free B is involved in T complexation, this amine is subtracted from the library equilibrium, which results in an advantage for the library members containing A, as experimentally observed.

Although of little relevance in this work, one may argue that the chosen receptor has a considerable degree of conformational freedom such that the entropic gain in the binding of the target by P1B3 compared with the free amines is not high enough to offset their competition. Nevertheless, the results indicate the serious limitations of a conventional DCL approach in the selection of a target particularly under conditions of not particularly high binding constants, which represent the vast majority of the cases one meets in real selection experiments by using synthetic molecular receptors.

DCL with a Platform-Bound Target: Dynamic Covalent Capture

A possible way to avoid the above problem is to anchor the target to the platform used for the selection of the recognition element. In this way there is no unbound target in solution. The binding of the recognition element to the target results in its covalent capture by the platform (Figure 1b). Platforms P2–P4 upon mixing with amines B and C give two products for each platform: Comparison of the composition of the mixtures P2B, P2C and P4B, P4C with reference mixture P3B, P3C gives information on the amplifications obtained resulting from attractive interactions between the ammonium and phosphonate group. By using a five-fold excess of each amine with respect to the platform we first determined that P3B and P3C are formed in 1:1 ratio, which indicates that in this case the two library members are equally stable. Next, the ratios of the two imines formed with platforms P2 and P4 were determined. Products P2B and P2C were formed in a 2.1:1 ratio, whereas P4B and P4C are present in a 9:1 ratio. This amounts to a 2.1-fold amplification in favor of the ammonium derivative with platform P2 and a nine-fold amplification with platform P4 containing the tert-butyl substituent. The latter, in particular, is an impressive result considering that the noncovalent interaction is simply that between a phosphonate and ammonium group. By considering that the noncovalent interaction is simply that between a phosphonate and ammonium group, which under these conditions amounts to a binding constant of only 58 ± 19 M−1. Note that in the case of the conventional DCL discussed above, even taking advantage of a binding constant around 50-fold larger, we were unable to detect significant amplification. Thus, the dynamic covalent-capture approach resulting in intramolecular trapping for the formation of a DCL is significantly more sensitive in detecting weak interactions. The results obtained with platform P4 indicate how costly conformational freedom is in these systems: The introduction of the bulky tert-butyl group at the ortho position relative to the phosphonate results in a more than four-fold increase in the amplification without changing the functional groups directly involved in the recognition process.
As discussed before, competition between the free amine B and receptor P1B3 for the binding of target T is the main reason for the absence of observed amplification in a conventional DCL. Although much less sensitive, dynamic covalent capture is also subject to the same phenomenon.[27] As we have shown previously[27] (and we refer the reader to the simulations reported in our previous paper) using a related system based on hydrazones rather than imines, a large excess of hydrazides effectively caused a drop in amplification. This is because, when present in excess, the recognition element may bind intermoleurally to the platform-bound phosphate, a process that competes with the intramolecular interaction. Nonetheless, and this is the true advantage of dynamic covalent capture, the intermolecular competition can be effectively suppressed by working at low concentrations, which does not affect the intramolecular binding event. For the conventional DCL this is not an option because low concentrations affect all binding events.

Conclusions

The selection of recognition units for the binding of a substrate (in our case an organic anion or polyanion) with weak to medium binding constants by conventional DCC (substrate noncovalently bound to the potential molecular receptor) under experimental conditions typically encountered in the laboratory may come across serious problems. This is because, even by using the common platform approach (Figure 1a), the conditions leading to amplification of the library, that is, a large excess of variable recognition elements and target, also lead to competitive binding of the elements not bound to the platform with the target. This may result in negligible amplification of the best-fit members of the library. In contrast, when tethering the target to the platform and using the covalent-capture strategy for the selection of the recognition element, significant amplification is observed even with much lower binding constants. Although competition with excess recognition units may also become an issue in the case of the tethered target, there is a way to overcome the problem by working at low concentration.[27] The intramolecular recognition process involved in covalent capture is not affected by dilution, whereas the intermolecular process involved in the standard recognition process is greatly diminished. This indicates that, whenever synthetically possible, tethering followed by covalent capture is the strategy one should pursue whenever a common platform-based library is used for the selection of a recognition unit to be used in the synthesis of a molecular receptor. There are, however, conditions in which the tethering approach is not synthetically accessible and, accordingly, the system must be critically evaluated before choosing the most appropriate strategy. Nevertheless, we speculate that tethering followed by covalent capture of the best-fit recognition unit may be used to build complex molecular receptors with very high affinities for their target by using a protocol reminiscent of that used in “fragment-based drug discovery”,[28,29] that is, by building up the molecular receptor by independent optimization of its several fragments. Work in this direction is currently being pursued in our laboratory.[30]

Experimental Section

Materials: Solvents and reagents were purchased from commercial sources and used without further purification. Milli-Q® water was used throughout. The following buffers were used: 2-[Morpholino]-ethanesulfonic acid (MES) (pH = 6.0–6.8) and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (pH = 7.0–8.0). Platform P3 is commercially available. The synthesis of platform P2 has already been reported[27] 1H NMR spectra were recorded with Bruker AC 250 F or AM 300 spectrometers. ESI-MS data were recorded with an Agilent 1100 Series LC system.

1,3,5-Triethyl-2,4,6-triformaldehyde benzene (P1)[31,32] 1,3,5-Triethylbenzene (10.06 g, 60.8 mmol), acetic acid (100 mL), paraformaldehyde (16.42 g, 547 mmol, 9 equiv.), a 33% (5.7 m) HBr solution in HOAc (570 mmol, 100 mL, 9.4 equiv.), and ZnBr2 (41.08 g, 182 mmol, 3 equiv.) were introduced into a 250 mL pressure vial. The vial was sealed and the mixture stirred at 90–100 °C for 24 h. The solution turned reddish-brown in a few minutes, and TLC (EtOAc/petroleum ether, 1:20) revealed the disappearance of the starting material. The solution was poured into water (500 mL), and the solid precipitated was filtered and dried to give 26 g of crude 1,3,5-tris(bromomethyl)-2,4,6-triethylbenzene (97% yield) that was pure enough for the subsequent reaction. M.p. 158–160 °C. 1H NMR (250 MHz, CDCl3): δ = 1.34 (t, J = 7.68 Hz, 9 H, CH3), 2.94 (q, J = 7.68 Hz, 6 H, CH2), 4.58 (s, 6 H, CH2) ppm. 13C NMR (63 MHz, CDCl3): δ = 15.56, 22.71, 28.55, 132.62, 144.96 ppm. IR (KBr): ν = 2965, 1570, 1493, 1450, 1378, 1200, 1060, 1037, 956, 760, 702, 582 cm⁻¹. Subsequently, Na (3.21 g, 139.7 mmol) was added to a 500 mL round-bottomed flask containing dry ethanol (200 mL). After the disappearance of Na, 2-propyl nitrite (15.9 mL, 177 mmol, 6 equiv.) was added. After 1.5 h, 1,3,5-tris(bromomethyl)-2,4,6-triethylbenzene (13 g, 29.48 mmol) was added to the milky solution formed. The solution was stirred for 24 h. After this period, a precipitate of NaBr had formed, and TLC revealed the disappearance of the reagent. After evaporation of the solvent, the solid was taken up with diethyl ether and extracted with 10% NaOH (3 × 50 mL). The concentrated organic solution gave 5.8 g of a crude product (80% yield) that was recrystallized from ethanol. 1H NMR (250 MHz, CDCl3): δ = 1.30 (t, J = 7.53 Hz, 9 H, CH3), 3.00 (q, J = 7.53 Hz, 6 H, CH2), 10.61 (s, 3 H, COH) ppm. 13C NMR (63 MHz, CDCl3): δ = 16.48, 22.56, 134.09, 149.30, 194.25 ppm. IR (KBr): ν = 2976, 2875, 1696, 1553, 1458, 1421, 137 cm⁻¹. MS (ESI): ml/z = 247 [M + H]+.

General Procedure for the Synthesis of Homoinimes P1A3 and P1B3: Platform P1 (115 mg, 0.47 mmol) was introduced into a 5 mL vial containing CHCl3 (3 mL). Amine A or B (3.6 equiv.) and molecular sieves (4 Å) to remove the water formed were subsequently added to the solution. The mixture was stirred at 50–60 °C for 16 h. After this period, the solvent was evaporated and the excess A or B removed by distillation under reduced pressure. The following products were obtained.

P1A: 1H NMR (250 MHz, CDCl3): δ = 0.87 (t, J = 7.3 Hz, 9 H, CH3), 2.47 (q, J = 7.3 Hz, 6 H, CH2), 3.05 (t, J = 7.2 Hz, 6 H, PhCH2), 3.92 (dt, J = 1.1, 7.2 Hz, 6 H, NCH3), 8.48 (t, J = 1.1 Hz, 3 H, CH=N) ppm. C5H9N6 (543.79): calc'd. C 84.28, H 8.16, N 7.58; found C 83.97, H 8.31, N 7.38.

P1B: 1H NMR (250 MHz, CD3OD): δ = 1.11 (t, J = 7.4 Hz, 9 H, CH3), 2.82 (q, J = 7.4 Hz, 6 H, CH2), 3.32 (s, 27 H, CH3), 3.77 (t,
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J = 6.4 Hz, 6 H, NCH₃), 4.21 (dt, J = 1.3, 6.4 Hz, 6 H, NCH₃), 8.90 (t, J = 1.3 Hz, 3 H, CH=NH) ppm. C₆H₅CH₂ClN₂O (608.18) calc. C 59.25, H 9.45, N 13.82; found C 59.01, H 9.52, N 13.71.

2-tert-Butyl-6-formylphenyl Ethylphosphonate (P4): A solution of 2-tert-butyl-6-formylphenol in dry THF (1.01 g, 6.61 mmol) was slowly added to a suspension of NaH (11.2 mmol) under N₂ at 0 °C. Immediately afterwards, ethylphosphonic dichloride (1.65 g, 11.2 mmol) was added and the solution allowed to warm to room temperature. After 12 h, it was cooled again to 0 °C and 12 N HCl (2 mL) was added, which resulted in the immediate precipitation of a white solid. After filtration, the THF was evaporated and the crude product taken up with chloroform and extracted first with a pH = 8 aqueous solution of NaHCO₃ and next with a pH = 1 HCl. After drying, evaporation of the organic solvent gave 624 mg of a greenish-yellow solid. This material was triturated with ethanol and dried to give a white solid (590 mg). The above solution of HCl. After drying, evaporation of the organic solvent gave 624 mg of a greenish-yellow solid. This material was triturated with ethanol and dried to give a white solid (590 mg).

After drying, evaporation of the organic solvent gave 624 mg of a greenish-yellow solid. This material was triturated with ethanol and dried to give a white solid (590 mg). The above solution of HCl. After drying, evaporation of the organic solvent gave 624 mg of a greenish-yellow solid. This material was triturated with ethanol and dried to give a white solid (590 mg).

Combinatorial Screening for the Detection of Weak Interactions

General Procedure for the Synthesis of Imines P2B, P2C, P3B, P3C, P4B, and P4C: Equivalent amounts of platform and amine were dissolved in CD₂OD (600 μL) in a screw-cap NMR tube to form a 5 mM solution. The tube was kept at 50 °C for 2 d, and the reaction monitored by NMR spectroscopy (disappearance of the aldehyde proton signal). After cooling, the solvent was evaporated to give the desired compound.

P2B-Bu₄Cl: ¹H NMR (250 MHz, CD₂OD): δ = 8.92 (s, 1 H, CH=NH), 7.92 (dd, J = 7.79, 1.44 Hz, 1 H, Ar), 7.01–7.58 (m, 3 H, Ar), 4.10 (2 H, CH₂N), 3.70 (t, J = 6.00 Hz, 2 H, NCH₃), 3.19–3.26 (17 H, CH₃ and NCH₃), 1.59–1.83 (13 H, Ar), 1.41 (m, 8 H, CH₂), 1.21 (dt, J = 18.87, 7.97 Hz, 3 H, CH₃), 1.02 (t, J = 7.28 Hz, 12 H, CH₃) ppm. ¹³C NMR (63 MHz, CD₂CN): δ = 163.06, 133.49, 130.50, 127.85, 124.28, 123.37, 122.90, 67.84, 59.50, 55.79, 54.68, 30.75, 24.79, 20.73, 13.72, 8.76 (d, J = 6.3 Hz) ppm. ³¹P NMR (122 MHz, CD₂OD): δ = 26.79 ppm. MS (ESI; MeOH): negative mode: m/z = 269.2 [M – H]⁺.

General Procedure for the Combinatorial Screening by Using Platform P1

In the Absence of the Target Anion: A solution of amines A and B (A = [B] + 3[P1B₃]) was added to a solution of imine P1B₃ in CD₂OD (2 mL) to make the final concentrations reported in the experiments. This solution was kept at 65 °C until full equilibration of the library was observed as evidenced by the absence of changes in the ¹H NMR spectrum.

In the Presence of the Target Anion: A mother solution of imine P1B₃ in CD₂OD (2 mL) was split into two portions. Amines A, B (A = [B] + 3[P1B₃]), and the target anion were added to one of the two to make twice the final concentrations of these additives reported in the experiments. The two solutions were then mixed in different ratios, and the resulting solution was kept at 65 °C until full equilibration of the library was observed, as evidenced by the absence of changes in the ¹H NMR spectrum.

General Procedure for the Combinatorial Screening by Using Platforms P2–P4: The two amines B and C (5 equiv.) were added to a 5 mM solution (600 μL) of the appropriate platform in CD₂OD in an NMR tube, and the solution was kept at 50 °C until full equilibration of the library was observed, as evidenced by the absence of changes in the ¹H NMR spectrum.
Supporting Information (see footnote on the first page of this article): Library equilibration studies, additional simulations, and binding studies of receptor P1B, with different anions.

Acknowledgments

Support by the Ministry of University and Research (MUR) [contract 2006039071 (PRIN 2006)] to P.S. is kindly acknowledged.

[19] Amine A instead of C has been used for platform P1 to minimize steric hindrance during the recognition process.
[22] For the consistency of this assumption, see also the binding data reported in Table 1.
[23] This conclusion, valid for a platform-based library, cannot be generalized (see ref[39]).
[24] In a real system clearly one cannot know a priori what is the best target.
[25] As is well known, acid catalysis does accelerate the equilibration process. However, we have noticed that the addition of acid also alters the equilibrium composition, especially in the presence of a very low excess of amines. For this reason we have preferred to run our experiment without the addition of acid.

Received: December 30, 2009
Published Online: May 19, 2010

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