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Exploring the Relation between Amplification and Binding in Dynamic Combinatorial Libraries of Macrocyclic Synthetic Receptors in Water

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Abstract: Herein we describe an extensive study of the response of a set of closely related dynamic combinatorial libraries (DCLs) of macrocyclic receptors to the introduction of a focused range of guest molecules. We have determined the amplification of two sets of diastereomeric receptors induced by a series of neutral and cationic guests, including biologically relevant compounds such as acetylcholine and morphine. The host–guest binding affinities were investigated using isothermal titration calorimetry. The resulting dataset enabled a detailed analysis of the relationship between the amplification of selected receptors and host–guest Gibbs binding energies, giving insight into the factors affecting the design, simulation and interpretation of DCL experiments. In particular, two questions were addressed: Is amplification by a given guest selective for the best receptor? And does the best guest induce the largest amplification of a given receptor? Our experimental results and computer simulations showed that the relative levels of amplification of hosts by a guest are well-correlated with their relative affinities, and simulations have confirmed previous observations that amplification can be selective for the best receptor when only modest amounts of guest are used. In contrast, the correlation between guest binding and the extent of amplification of a given receptor across a wide range of guests tends to be poorer, because every guest has its own unique set of affinities for competing receptors in the DCL. This implies that the results of screening a DCL for selective receptors by comparing the response of the mixture to two different guests should be interpreted with caution. DCLs are complex mixtures in which all compounds are connected through a set of equilibria. Obtaining quantitative information about all host–guest binding constants from such systems will require the explicit and simultaneous consideration of all of the main equilibria within a DCL.

Keywords: disulfides · dynamic combinatorial chemistry · macrocycles · molecular recognition · water

Introduction

The design of successful synthetic receptors requires being able to balance i) the strength of the non-covalent interactions formed between the host and the guest; ii) the cost or benefit of desolvating the host and the guest; iii) the entropic penalty associated with placing the host and guest in conformations suitable for binding; and iv) the benefit or cost of any non-covalent intra-host[1] or intra-guest interactions made or broken during binding. With our current imperfect understanding of all these interactions this balancing act is not always as successful as one would wish, prompting the exploration of alternative approaches to synthetic receptors that are less dependent on a detailed understanding.[2] One such alternative is provided by dynamic combinatorial chemistry.[3] In this method a set of relatively simple building blocks are designed, prepared and combined. A reversible reaction is used to link the building blocks together to produce an equilibrium mixture of potential receptors (a dynamic combinatorial library or DCL). The binding energy between host and guest can then be harnessed to drive the synthesis of (ideally) the best receptor by shifting the equilibrium of receptors towards those with the highest affinities (Figure 1).

Dynamic combinatorial chemistry has spread into many different areas, using an increasing number of reversible reactions to generate not only hosts but also guests for a wide range of targets[3e,4] as well as sensors,[5] and catalysts.[6] In some cases, spectacular levels of amplification of receptors with exceptionally high affinity[4a] or remarkable structure[7]

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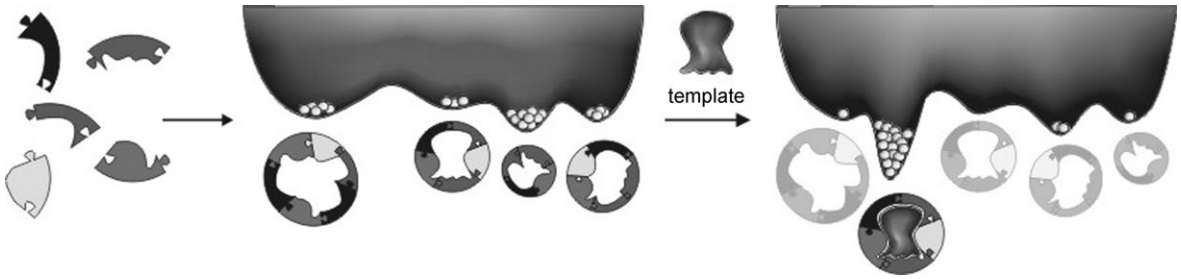


Figure 1. Bifunctionalized building blocks combine to form a dynamic combinatorial library of macrocycles that are potential receptors. The amounts of the individual macrocycles depend on their thermodynamic stabilities as represented by the depth of the corresponding well in the free energy landscape. Addition of a guest or template that selectively binds to one of the macrocycles induces a shift in the equilibrium towards this species at the expense of all other library members.

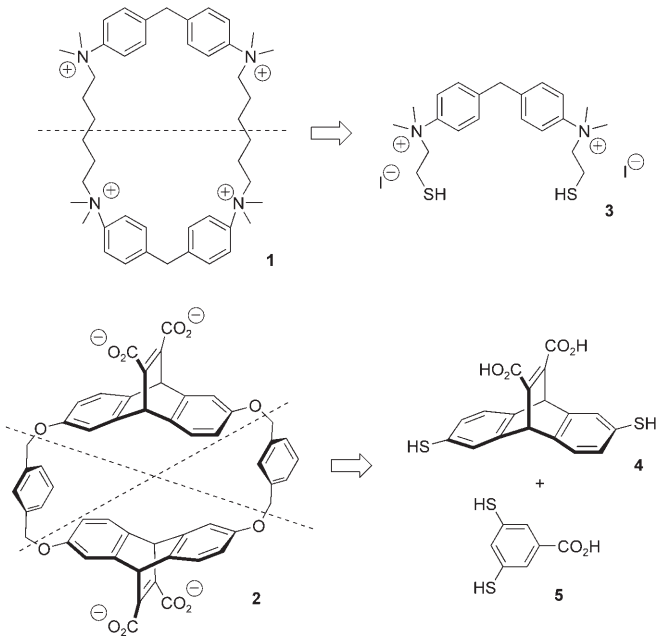
have been observed. A fundamental understanding of the complex and sometimes counterintuitive behavior of DCLs in response to the introduction of the template has started to emerge as a result of theoretical investigations by Severin and co-workers[8] and ourselves.[9]

Key to most applications of DCLs is the presumption that library members that bind strongly to a template get amplified efficiently. This paper describes a detailed study of the binding and amplification of two pairs of diastereomeric receptors from a DCL by several different but structurally related guests, including biologically and pharmaceutically relevant compounds such as acetylcholine and morphine alkaloids. It represents the most extensive dynamic combinatorial dataset to date, allowing us, for the first time, to probe the relationship between binding strength to a particular host in a DCL and the guest-induced amplification of this host across a wide range of guests. From the data obtained in this study of selected hosts in DCLs of moderate complexity, we can see where the results of DCL experiments can be correctly analyzed and interpreted using relatively simple techniques, and identify situations where more sophisticated methods are required.

In particular, two key questions will be addressed: Do the best guests induce the strongest amplifications? Are the best hosts the most amplified? Some results of these studies have previously been communicated.[6a,9c,10] The paper is structured as follows: We first describe the design and synthesis of the building blocks, followed by the analysis of the DCLs, their response to guest molecules, and the characterisation of selected host molecules. We then describe the thermodynamics of host–guest binding. Finally, we come to the essence of the investigation where we explore the relation between binding and amplification.

Results and Discussion

Design and synthesis of building blocks: Building blocks were designed to contain structural elements of previously known successful synthetic receptors for specific guests. These receptor fragments were equipped with suitable reversible covalent attachment points. We reasoned that exposing a DCL made from such building blocks to the original guest should then lead to re-formation of the corresponding receptor, while different guests may lead to new receptors. We chose reversible disulfide chemistry for constructing the DCLs. We[11] and others[12] have demonstrated that disulfide DCLs can be generated by mixing thiol building blocks in aqueous solution at close to neutral pH. Oxygen from the air is sufficient to irreversibly oxidize the thiols into the desired disulfides, producing water as the only by-product. While oxidation is taking place, the mixture contains disulfides and thiols, allowing for equilibration through nucleophilic attack of thiolate anions on the disulfides, displacing a new thiolate anion in the process. Thus, exchange is catalytic in thiolate anion; it can be switched off by allowing oxidation to go to completion or by addition of acid to protonate the thiolate.

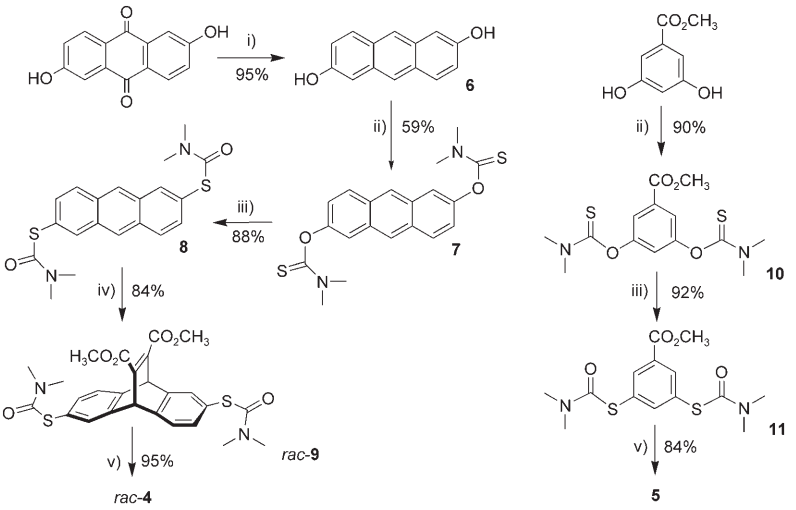


Scheme 1. Design of the dithiol building blocks was inspired by the Koga/Breslow (**1**)[13] and Dougherty (**2**)[14] cyclophane receptors.

We selected two previously described water soluble hosts **1**[13] and **2**,[14] which were known to bind strongly to anionic and cationic guests, respectively. The hosts were built up from subunits that would allow the introduction of two thiol groups with minimal effect on the overall structure of the receptor (Scheme 1). Both receptors were known to be tolerant to minor structural variations such as changes to the length of the alkyl spacer in **1**[13b] and the nature of the phenyl spacer in **2**.[14e,n] We started our experiments on the Koga/Breslow receptor **1**, and successfully synthesized the required dithiol building block **3**. Unfortunately, when exposed to the conditions required for disulfide oxidation and exchange (pH 7–9), this building block decomposed.[15] We were more successful with the building blocks **4** and **5** that were inspired by the Dougherty cyclophane **2**.[6a,9c,10]

Dithiol building block **4** was synthesized as a racemic mixture as outlined in Scheme 2. The key step is a Diels–Alder reaction between the protected precursors **8** and dimethylacetylenedicarboxylate which proceeded in 84% yield. The thiol groups were introduced starting from aromatic alcohols through a Newman–Kwart rearrangement on the O-thiocarbamate intermediate **7**, following methods described by Field and Engelhardt.[16] A similar strategy was used to synthesize building block **5**. Both compounds can easily be obtained in 10–30 g scale.

Guest-induced amplification of hosts from DCLs of macrocyclic disulfides: In previous studies using disulfide DCLs made from dithiol building blocks including **4** and **5** we have observed that introducing templates **T2**–**T4** and **T13** (Table 1)



Scheme 2. Building block synthesis. i) $NaBH_4$, RT, aq Na_2CO_3; ii) Me_2N-C(S)-Cl, DABCO, 0 °C→RT, DMF; iii) 3 h at 240 °C, Ph_2O; iv) dimethyl acetylenedicarboxylate, 1.25 h at 190 °C, Ph_2O; v) 1.75 M KOH in diethyleneglycol, 30 min at 105 °C.

Table 1. Experimentally observed binding constants and free energies, enthalpies and entropies of binding of templates **T1**–**T13** to hosts **12a** and **13a** and amplification factors (AFs) for hosts **12a** and **b** and **13a** and **b** induced by the same templates (5 mM) in libraries A and B.[a]

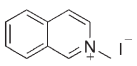
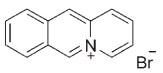
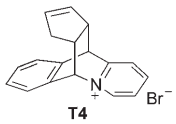
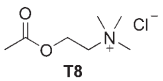
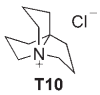
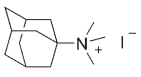
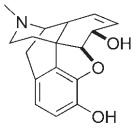
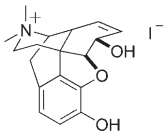
Guest	Host	K[b] [M^{-1}]	ΔG^o[b] [kJ mol^{-1}]	ΔH^o[b] [kJ mol^{-1}]	$T\Delta S^o$[b] [kJ mol^{-1}]	AF(A)[a]	AF(B)[a]
 T1	**12a**	2.1×10^3 (0.3)	−19.0 (0.3)	−15.1 (7.6)	4.2 (2.1)	5	4.5
	12b					3.2	3.4
	13a	2.10×10^3	−18.9	−22.4	−3.6		5.7
	13b						1.6
 T2	**12a**	4.5×10^4 (0.5)	−26.5 (0.3)	−23.8 (0.8)	2.8 (1.1)	4.2	1.8
	12b					2.6	1.4
	13a	2.0×10^5 (0.0)	−30.2 (0.1)	−38.3 (1.7)	−8.1 (1.7)		16.2
	13b						2.6
 T3	**12a**	1.30×10^5[c,d]	−29.1[c,d]	−23.7[c,d]	5.4[c,d]		
	12b						
	13a	6.40×10^5[c]	−33.1[c]	−40.6[c]	−7.5[c]		
	13b						
 T4	**12a**	3.6×10^5 (0.3)	−31.7 (0.2)	−19.0 (0.9)	12.7 (1.1)	10.6	6.2
	12b					3.2	2.2
	13a	3.3×10^5 (0.0)	−31.5 (0.0)	−30.8 (1.1)	−1.0 (0.7)		10.9
	13b						3.2
 T5	**12a**	8.5×10^2 (1.0)	−16.7 (0.4)			0.6	0.7
	12b					0.5	0.7
	13a	6.5×10^3 (0.5)	−21.7 (0.2)				5.5
	13b						1.2
 T6	**12a**	5.6×10^4 (0.2)	−27.1 (0.1)	−18.5 (0.4)	8.7 (0.5)	11.2	9.1
	12b					4	3.9
	13a	3.2×10^4 (0.3)	−25.7 (0.2)	−30.6 (1.5)	−4.9 (1.7)		14.9
	13b						1.3
 T7	**12a**					7.1	3
	12b					5.4	2.8
	13a	9.9×10^4 (1.0)	−28.5 (0.3)	−12.6 (0.9)	16.0 (1.2)		9.7
	13b						5.3

Table 1. (Continued)

Guest	Host	K[b] [M^{-1}]	ΔG^{o}[b] [kJ mol^{-1}]	ΔH^{o}[b] [kJ mol^{-1}]	$T\Delta S^{o}$[b] [kJ mol^{-1}]	AF(A)[a]	AF(B)[a]
 T8	**12a**	3.9×10^{3} (0.9)	−20.4 (0.6)	−31.6 (0.7)	−11.1 (1.2)	2.1	2
	12b					1.6	1.9
	13a	6.5×10^{3} (2.0)	−21.7 (0.6)				7.1
	13b						2.3
 T9	**12a**	7.9×10^{4} (0.2)	−28.0 (0.1)	−18.8 (0.6)	9.2 (0.5)	9.2	10.8
	12b					4.7	6.7
	13a	4.9×10^{4} (0.0)	−26.8 (0.0)	−26.2 (0.2)	0.6 (0.2)		22
	13b						3.6
 T10	**12a**	2.4×10^{5} (0.0)	−30.7 (0.1)	−19.5 (0.3)	11.2 (0.4)	11.4	12.1
	12b					5.5	6.9
	13a	4.0×10^{4} (0.0)	−26.3 (0.0)	−25.4 (1.8)	0.9 (1.7)		9.7
	13b					1.1	
 T11	**12a**	1.1×10^{6} (0.2)	−34.5 (0.6)	−19.5 (0.6)	15.0 (1.1)	15.5	20.8
	12b					3.8	6.1
	13a	9.3×10^{4} (1.0)	−28.3 (0.4)	−26.5 (1.3)	1.9 (1.6)		6.9
	13b						0.4
 T12	**12a**	2.7×10^{4} (0.2)	−25.3 (0.2)	−33.6 (3.6)	−8.3 (3.4)	5.7	6.6
	12b					2.8	3.4
	13a					1.3	
	13b						1.4
 T13	**12a**	5.4×10^{5} (0.4)	−32.7 (0.2)	−46.4 (0.7)	−13.7 (0.9)	11.2	18.1
	12b					7.2	13.9
	13a	2.6×10^{4} (0.6)	−-25.1 (0.6)	−42.9 (4.0)	−17.8 (4.5)		3.7
	13b						0.8

[a] The DCLs in set A contained 5 mM **4**, and the libraries in set B contained 3.33 mM **4** and 1.67 mM **5**. Errors are shown in parentheses and are based on the reproducibility of the data over 2 or 3 separate experiments. [b] Determined using isothermal titration calorimetry using 10 mM borate buffer pH 9.0 at 298 K. [c] Data taken from ref. [6a]. [d] Data for a mixture of **12a** and **12b**.

induced dramatic changes in the library compositions, leading to the amplification of hosts **12** and **13**.[6a,10] The structure of the selected dynamic combinatorial receptors differs from the expected disulfide analogue of the Dougherty receptor **2**. This difference may be due to the more flexible nature of the -CH_2-O- units in **2** as compared to the -S-S- units in the disulfide analogues. Flexibility is important as host **2** is reported to bind guest **T2** in a partially collapsed conformation[14] and it is likely that the more rigid disulfide analogue of **2** is unable to adopt a similar collapsed conformation. Alternatively, or additionally, the increased length and different bond angles of the disulfide linkages may cause a difference in binding.

Since building block **4** was used as a racemic mixture hosts **12** and **13** were obtained as mixtures of stereoisomers. Later we reported the unexpected observation that template **T5** amplifies receptor **14** containing four units of building block **4**.[17] Remarkably, a strong preference for one out of four possible diastereomeric products was observed. This observation prompted us to return to receptors **12** and **13** and investigate the stereoselectivity of their amplification for a wide range of guests. We succeeded in separating all isomeric receptors from each other and from the remaining library members using an isocratic HPLC method as shown in Figure 2.

The assignments of the diastereomers of hosts **12** and **13** were conducted by ^{1}H NMR spectroscopy. In case of host **12** the analysis was straightforward: the diastereomeric products have different symmetries and give rise to different numbers of signals in the ^{1}H NMR spectrum. For the diastereomer in which all three subunits have the same chirality (**12b**) a simple NMR spectrum very similar to that of the building block is expected, whereas the trimer in which the subunits have different chiralities (**12a**; *RR*,*RR*,*SS* or *SS*,*RR*,*RR*) all three subunits are inequivalent. Isolation of the predominant stereoisomer by preparative HPLC and subsequent analysis by NMR showed three, rather than one set of signals per macrocycle, proving it to be **12a**.

For host **13** a similar analysis is inconclusive as both diastereomers will give two sets of signals. However, since one of the diastereomers (**13b**; *RR*,*SS*) is a *meso* compound while the other (**13a**) is produced as a racemic mixture of *RR*,*RR* and *SS*,*SS*, addition of a suitable chiral shift reagent should give separate signals for the two enantiomers of the racemate without producing additional signals for the *meso* isomer. We have isolated a mixture of both diastereomers of **13** by preparative HPLC and analyzed this material by ^{1}H NMR in D_2O. Addition of homochiral ammonium salt **15** induced marked changes in the NMR spectrum as shown in Figure 3. The signals due to the bridgehead protons (5.5–5.1 ppm) are most revealing, as only two peaks are expected per diastereomer. Thus, four signals are expected in the absence of the chiral guest (cf. Figure 3a), and six in the presence of the guest. Although some of the signals in Figure 3 overlap, this is indeed what is observed. Integration of the signals indicates that **13a** is the predominant diastereomer.

Having established the stereochemistry of the diastereomers of hosts **12** and **13** we set out to study the selectivity in the amplification of these four compounds using a large

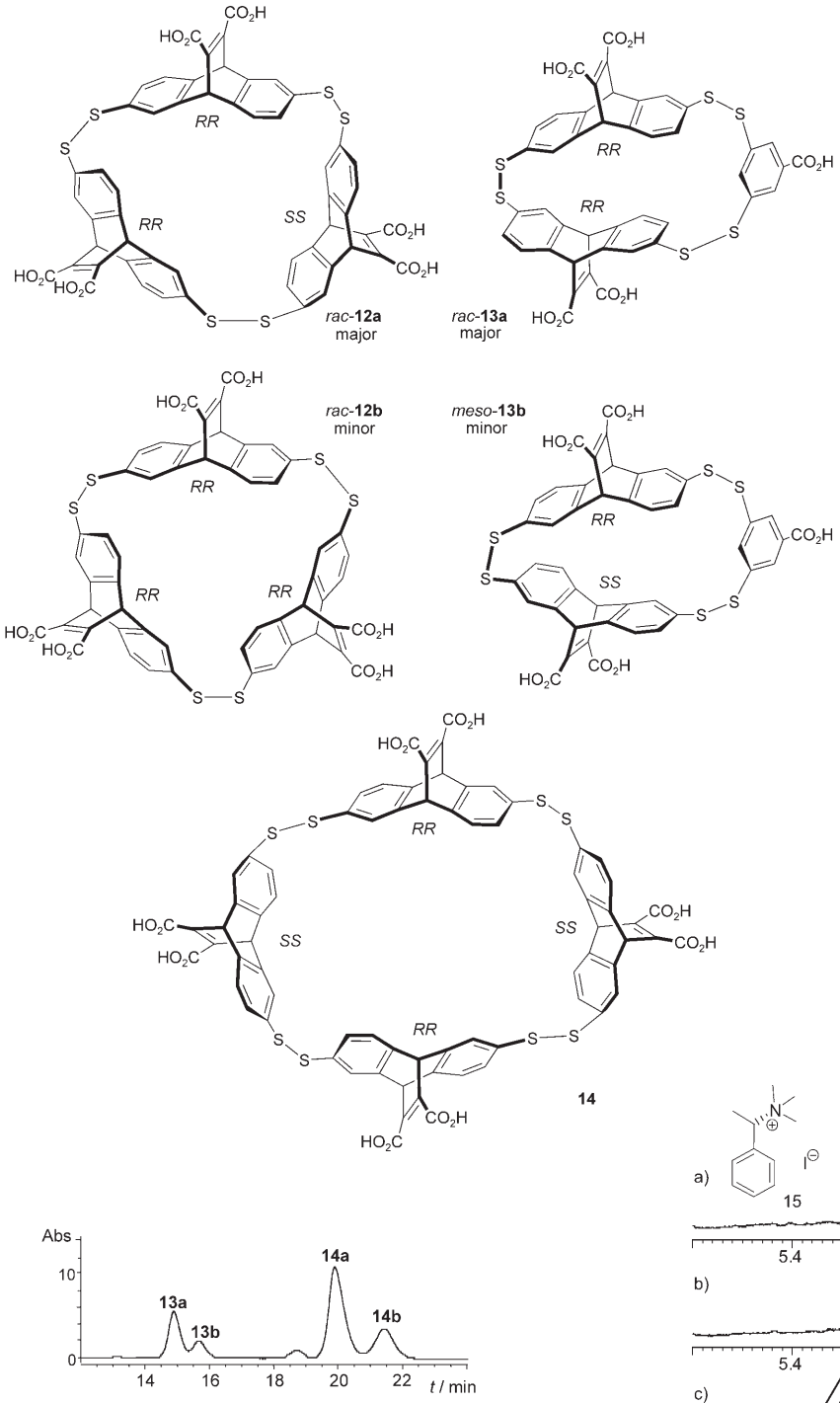


Figure 2. Part of the HPLC analysis (Waters Symmetry reversed phase C18 column 25.0 cm × 4.6 mm, 5 μm particle size, 50:32:18:0.1 H_2O/MeCN/IPA/TFA) of a DCL made from **4** (3.33 mM), **5** (1.67 mM) and 5 mM quinuclidinium iodide (**T9**) showing separation of the diastereomers of hosts **12** and **13**. Additional minor library members can be observed at 18.7 min and elsewhere in the trace.

series of different but structurally related templates. The choice of the templates was partially motivated by the extensive studies by the Dougherty laboratory,[14] which had demonstrated that quaternary amines and imines were good guests for receptor **2**. Following this precedent we selected guests **T1**–**T11** (Table 1). We also included a number of biologically relevant templates: acetylcholine (**T8**) and two morphine alkaloids: morphine itself (**T12**), and the N-methylated morphine derivative **T13**.

Two sets of 14 DCLs were made: one biased towards the formation of **12** (set A) and one biased towards the formation of **13** (set B). The libraries in set A contained 5 mM **4**, and those in set B contained 3.33 mM **4** and 1.67 mM **5**. The libraries were prepared by dispersing the building block(s) in water and adjusting the pH to 8.0. In each set, 13 DCLs contained a template (**T1** to **T13**, respectively; at 5 mM concentration), while no template was added to the 14th DCL. The libraries were allowed to oxidize in capped HPLC vials and stirred at 25 °C for three weeks. Precipitation occurred in the libraries to which **T3** had been added, and these libraries were therefore not analyzed further. All of the other libraries remained clear

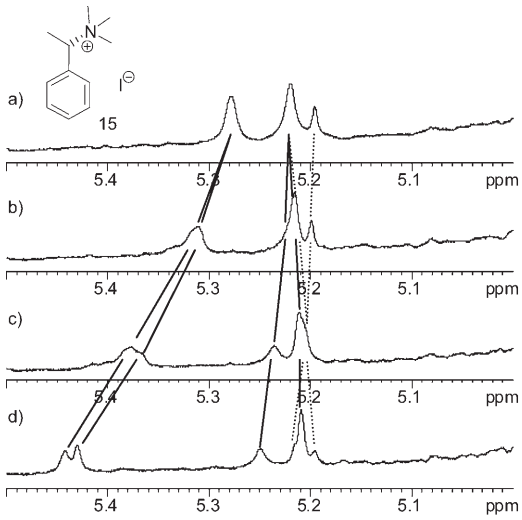


Figure 3. Part of the ^{1}H NMR spectra of a mixture of stereoisomers of host **13** (in 49 mM pD 8.9 sodium borate buffer) in the presence of increasing amounts of homochiral ammonium ion **15**, showing the signals of the racemic mixture of *RR*,*RR* and *SS*,*SS* **13a** split in two while the signals of the meso stereoisomer **13b** are shifted but otherwise unaffected. a) [**15**] = 0 mM; b) 0.13 mM; c) 0.63 mM; d) 1.18 mM.

solutions. The library compositions were analyzed by HPLC and the amplification factors for receptors **12a** and **b** and **13a** and **b** were computed. Amplification factors are defined as the concentration of a library member in a templated library, divided by its concentration in the corresponding untemplated library, and can be calculated by dividing the areas of the HPLC peaks corresponding to these compounds in the templated libraries and the untemplated libraries. The results are summarized in the last two columns of Table 1.

These four receptors represent the major amplified species in most of the libraries. The tetramer **14** was the major host in libraries templated by **T5**,[17] and a significant minor constituent in libraries of set A templated by **T9**,[9a] and to a lesser extent **T10** and a few others. It was not possible to identify **14** in the untemplated libraries of set B. Other hosts were visibly amplified as minor constituents of the libraries, accounting for a few percent of the total peak area in various experiments. However, as the aim of the present study is to compare binding and amplification across a wide range of templates, we here only report results on the two most widely amplified hosts **12** and **13**.

Table 2 shows the selectivity of the amplification of hosts **12** and **13** induced by the templates in the libraries in set B. Modest diastereoselectivities were observed: In almost all cases, **12a** is more strongly amplified than **12b**, and **13a** is more strongly amplified than **13b** suggesting that **12a** and **13a** are better hosts than their respective diastereomers, irrespective of the guest. In the untemplated libraries, the ratios of **12a** to **12b** and **13a** to **13b** are 1.3:1 and 0.4:1, respectively. In general, the preference for the amplification of **13a** over **b** is more pronounced than the preference for **12a** over **b**. This difference may be due to hosts **13** presenting a smaller, more elongated cavity, leading to a tighter fit, where steric interactions between the hosts and guests are likely to be more important. In hosts **12**, the cavities are larger and more spherical, leading to a looser fit. Amongst the templates that were analyzed the bulky and rigid **T11** was the most diastereoselective, particularly in the case of **13**. However, in all cases the diastereoselectivities were significantly less than the more than 30-fold selectivity we previously observed for host **14**.[17] We also analyzed the selectivity in the amplification of receptor **12a** versus **13a** and found that bulky guests preferred the larger host **12a**.

In addition, we have studied the selectivity in the amplification of receptor **12a** vs **12b** in the DCLs of set A (cf. Table 1). As the ratio of the amplification factors for a pair of diastereomers should be proportional to the ratio of their binding constants (see the Supporting Information), this ratio should remain the same across different DCLs. Indeed, we observe a good correlation between the diastereoselectivities in the amplification of **12** in libraries A and B (Figure 4). This can interpreted as evidence that the DCLs have all reached equilibrium and also provides an indication of the accuracy of the experimental data.

In an attempt to probe whether enantioselective amplification was occurring in the presence of homochiral template **T13**, we equilibrated 10 mM of *rac*-**4** in the presence of 1.6 mM **T13**. We deliberately used a sub-stoichiometric quantity of the template. If amplification would be enantioselective, the preferred enantiomer of the building block would be recruited by the template to give one enantiomer of the trimer. No template would remain to amplify the remaining building block, which should therefore form mostly macrocycles other than trimer. A ^{1}H NMR spectrum of the resulting mixture was taken with **T13** acting as a chiral shift reagent, giving separate peaks for the enantiomers of **12a** and **b** in approximately 1:1 ratio, indicating that the amplification of **12** was not significantly enantioselective.

Table 2. Experimentally observed selectivity in the amplification of hosts **12a** vs **12b**; **13a** vs **13b** and **13a** vs **12a** by various templates (5 mM) in DCLs of set B made from **4** (3.33 mM) and **5** (1.67 mM).

Template	AF(**12a**)/AF(**12b**)	AF(**13a**)/AF(**13b**)	AF(**13a**)/AF(**12a**)
T1	1.3	3.6	1.3
T2	1.3	6.2	9.0
T4	2.8	3.4	1.8
T5	1.1	4.6	7.8
T6	2.3	11	1.6
T7	1.1	1.8	3.2
T8	1.0	3.1	3.6
T9	1.6	6.1	2.0
T10	1.7	8.7	0.75
T11	3.4	16	0.33
T12	2.0	0.90	0.20
T13	1.3	4.5	0.20

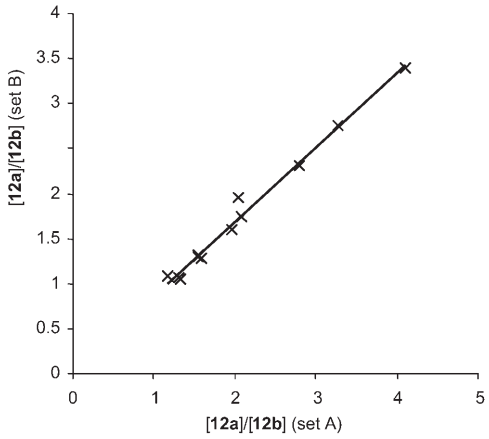


Figure 4. Ratios of the diastereomers of **12** in experimental DCLs of set A (5 mM **4**), vs those of set B (3.33 mM **4** and 1.67 mM **5**) for various templates (5 mM).

Host–guest binding: The binding of templates **T1**–**T13** to the hosts **12a** and **13a** was studied using isothermal titration microcalorimetry (ITC) in 10 mM borate buffer pH 9.0.[18] The results are shown in Table 1 from which a number of trends are apparent. Firstly, the unquaternised templates (**T1** and **T12**) both have binding constants more than an order of magnitude lower than those of their quaternised counterparts (**T2** and **T13**). Similar observations have been reported by Dougherty et al.[14f,j] and are evidence for the involve-

ment of cation–π interactions in host–guest binding. Second, large guests tend to prefer the larger host **12a**, while small templates tend to prefer the smaller host **13a**. Third, templates with rather elongated aromatic structures such as **T2** and **T3** are bound particularly strongly by **13a**.

Binding by **12** and **13** can be expected to be driven by two major contributors: cation–π and hydrophobic interactions, each with their own enthalpic and entropic components. Whereas cation–π interactions are most likely enthalpy driven, hydrophobic interactions have a more complex thermodynamic signature which can vary from entropy driven (small curved solutes) to enthalpy driven (planar solutes or cavities).[19]

For most of the templates measured, the enthalpies of binding to **13a** are around $-10\,kJ\,mol^{-1}$ stronger than the corresponding enthalpies of binding to **12a** (Figure 5). The more favorable enthalpy contribution is almost completely offset by an entropic penalty of around $10\,kJ\,mol^{-1}$. This pattern is consistent with **13a** presenting a tighter, more elongated cavity than **12a**, with greater conformational constraints on binding but also with the prospect of forming a more intimate host–guest contact. The fact that the flatter, aromatic guests tend to have more enthalpy-driven binding, in particular to **13a**, supports this notion.

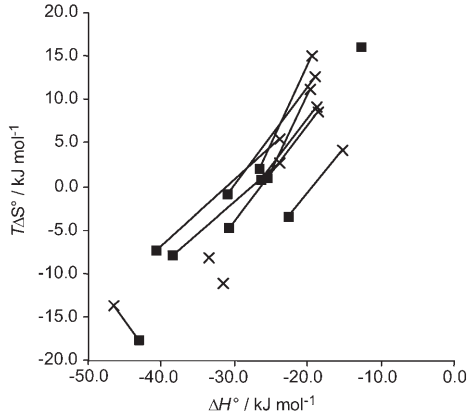


Figure 5. Correlation between experimentally determined enthalpy and entropy of binding of guests **T1**–**T13** to hosts **12a** (×) and **13a** (■). The lines connect data points corresponding to the same guest.

Correlation between guest binding and amplification: The original naïve conception of dynamic combinatorial chemistry was that the best hosts in a DCL would be most amplified upon addition of a guest. Conversely, when exposing a given DCL to a variety of guests, one would expect that the guest that elicits the strongest response from the library is the one that is most strongly bound. Recent investigations by Severin[8] and ourselves[9a,c] have demonstrated that the first of these two presumptions is not always correct and depends on experimental conditions in a predictable way. Below we will present further evidence that supports this notion. With the present dataset we are also, for the first time, able to experimentally test the validity of the second presumption by investigating the correlation between the guest induced amplifications for a selected host and its binding to these guests.

Are the best hosts the most amplified? It is well established that a breakdown of the correlation between binding and amplification can occur when two or more library members compete for a scarce building block.[8,9a,c] Library members that use relatively small numbers of that building block per host have a competitive advantage over those members that contain larger numbers of the particular building block. Thus, low oligomers tend to have an advantage over high oligomers, and hetero oligomers over homo oligomers. An intuitive way of rationalizing this behavior is illustrated by the examples in Figure 6. Given a fixed amount of building block a DCL can produce twice the number of cyclic dimers than it can produce cyclic tetramers. Thus, for every four building blocks the system can gain twice the guest–dimer binding energy against only one guest–tetramer binding energy, provided there is sufficient template available. Hence, the system may prefer forming dimers even in cases where the tetramer is actually the strongest binder. Similar arguments apply in a mixed building block library, when guest binding affinity is associated with one of the building blocks. For example, if only the white building blocks in Figure 6 bind the guest, the system tends to form heterodimers even though the homodimer may be the strongest binder. These effects will only occur in the presence of excess template. When the template concentration is reduced to substoichiometric levels the system will revert to selectively producing the best receptor.

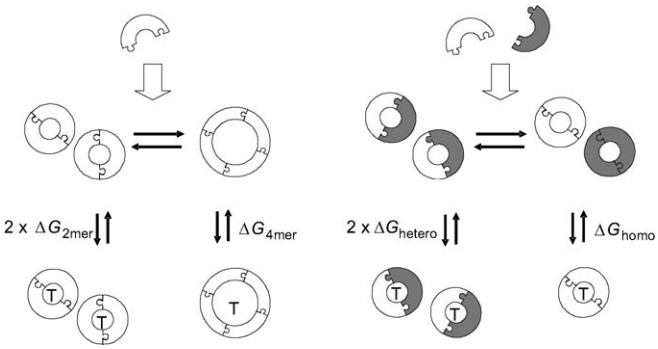


Figure 6. In the presence of excess guest (T) there is an inherent preference for the formation of small oligomers over large oligomers and for the formation of hetero oligomers over homo oligomers because, with a fixed amount of building blocks, the system is able to produce more of the small or hetero oligomers than of the large or homo oligomers.

The mixed building block DCLs of set B contain homotrimers **12** in competition with heterotrimers **13**. In a previous study on the amplification induced by guest **T9** at different concentrations ranging from 0.5 to 10 mM we have shown that at high concentrations of this guest the DCL "misbehaves" and heterotrimer **13a** is somewhat more amplified than homotrimer **12a** despite the latter having a

somewhat higher affinity for the guest.[9c] Upon reducing the template concentration to a sub-stoichiometric level the better receptor **12a** is the most amplified.

The present dataset allows a more elaborate investigation of the competition between **12a** and **13a** encompassing guests **T1**–**T13** and comparing binding affinities with amplification factors. In an ideal case of two isomeric receptors R1 and R2 a plot of $\log(AF_{R1}/AF_{R2})$ vs. $\log(K_{R1}/K_{R2})$ for the various guests will be a straight line passing through the origin (when binding constants are equal, amplification factors should be equal) and having a slope of 1 (the ratio of amplification factors equals the ratio of binding constants).[20] Our experimental DCLs, in which we used building block and template concentrations of 5 mM, show a clear deviation from this ideal behavior (Figure 7a). Data points falling in the white quadrants of the plot represent DCLs where the best binder of the two receptors has the higher amplification factor. In the grey quadrants, the DCL selects the "wrong" library member. This undesired behavior occurs only for a relatively narrow range of binding events, where $K_{13a} < K_{12a} < 3.2 \times K_{13a}$.

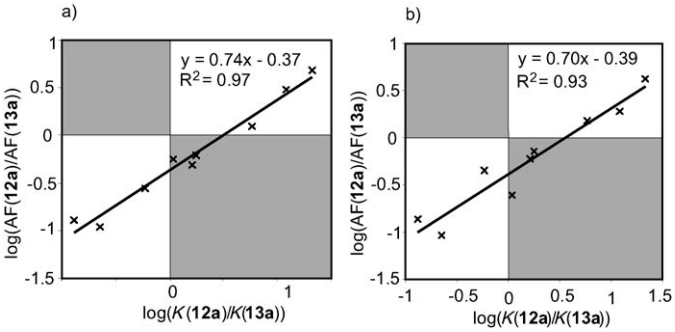


Figure 7. Correlation between ratios of amplification factors and binding constants of **12a** and **13a** for a range of guests in (a) experimental and (b) simulated DCLs of set B (3.33 mM **4**; 1.67 mM **5** and 5 mM template). The shaded parts of the graph signify "misbehaving" DCLs where the most amplified of the two receptors is not the strongest binder.

Inspection of Figure 7a reveals that we still obtain an essentially linear relationship between $\log(AF_{12a}/AF_{13a})$ versus $\log(K_{12a}/K_{13a})$ but the y intercept (−0.37) is clearly not zero and the gradient (0.74) is significantly less than 1. The non-zero intercept reflects a systematic bias towards the amplification of the heterotrimer **13a** over the homotrimer **12a** by a factor 2.3 (i.e. $10^{0.37}$), in line with the inherent preference for the amplification of hetero oligomers over homo oligomers. The reduced slope of the line indicates that the sensitivity of the DCL (i.e., the extent to which a difference in guest binding between **12a** and **13a** is reflected in a difference in amplification) is suboptimal. However, the correlation between relative amplification and relative binding is good ($R^2 = 0.97$), suggesting that if the binding constants for a few host/guest combinations are known, it should be possible to deduce the relative affinities for other guests with reasonable accuracy.

We have conducted computer simulations of these libraries using our DCLSim software[9a,b] with the aim of investigating how the values of the slope and intercept depend on experimental conditions. Simplified versions of the DCLs were simulated, where only the two species under consideration (**12a** and **13a**) were allowed to bind to the template, using the experimentally observed binding constants from Table 1. No other experimental values were incorporated in the model, thus excluding any other host–guest interactions which undoubtedly occur. This methodology has previously been shown to reproduce the main trends in experimental DCLs, and allows us to rapidly explore the key aspects of their behavior.[9] Figure 7b shows the results of the simulations (full details are given in the Supporting Information). Comparing these data with that in Figure 7a shows that, while the individual simulated data points deviate from their experimental counterparts, the trends in the simulated data closely match those of the experimental data. This agreement is all the more remarkable for the fact that the model does not include other major binding constants, such as those to the tetramer **15** and to the minor diastereomers **12b** and **13b**, and serves to further validate the robustness of simplified DCL simulations.

We have performed similar simulations for different guest concentrations and found that, as expected, at lower guest concentrations the bias towards hetero-oligomers disappears and the intercept of plots of the type of Figure 7 approach zero (Figure 8). Also the correlation between amplification factor and binding constant (as reflected in the value of the correlation coefficient R^2) improves upon reducing the guest concentration, in line with previous observations.[8,9a,c] However, the guest concentration appears to have little effect on the slope of the lines (i.e., the sensitivity of the DCL). The main experimental parameter that determines the slope turned out to be the ratio of the building blocks **4** and **5** in the DCL.

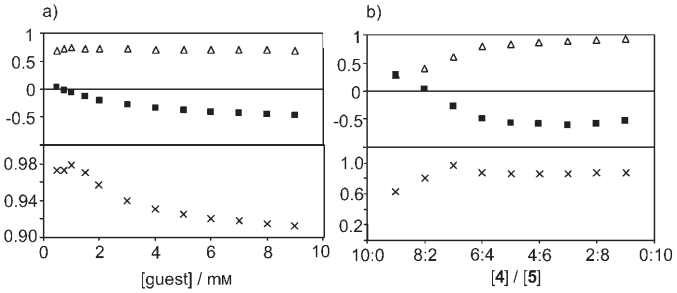


Figure 8. Dependence of the slopes (△), intercepts (■) and correlation coefficients (×) of the plots of $\log(AF_{12a}/AF_{13a})$ vs $\log(K_{12a}/K_{13a})$ on a) the concentration of the guests at a fixed 2:1 ratio of **4** and **5**, and b) the **4**:**5** ratio at a fixed guest concentration of 5 mM in simulated DCLs.

In our experimental mixed building block DCLs we used building blocks **4** and **5** in a 2:1 ratio and also the simulations shown in Figure 8a were performed using this library composition. Varying the ratio of **4** to **5** has profound effects

on the slope, intercept and correlation coefficient of the relationship between $\log(AF_{12a}/AF_{13a})$ and $\log(K_{12a}/K_{13a})$. Increasing the fraction of **5** brings the slope close to unity, while the correlation between binding and amplification reaches a maximum close to [**4**]/[**5**] ratio of 7:3 and the intercepts is nearly zero at a [**4**]/[**5**] ratio of 8:2. Thus, the optimum [**4**]/[**5**] ratio for a close to zero *y* intercept is *not* the optimum for R^2, which in turn is different from the optimum ratio that gives a slope close to unity. These results suggest that there are no simple guidelines for choosing building block ratios in mixed libraries when competing receptors are present. Thus, when screening mixed building block libraries it may be beneficial to set up and analyze several experiments at different building block ratios.

Do the best guests induce the strongest amplifications? This question is key in assessing the potential of DCLs for the development of selective receptors that specifically favor a certain guest (X) over another (Y). Ideally one should be able to identify selective receptors by comparing the product distributions in a DCL exposed to X with that exposed to Y. Inspection of the data in Table 1 reveals that a large difference in amplification factors between two guests correctly predicts a large difference in binding constants in some cases, but not in others. For example in library B guest **T1** amplifies host **12a** 6.4 times better than guest **T5**, while the difference in binding constant is only 2.5-fold. In contrast, in the same library guest **T4** amplifies host **12a** 8.9 times better than guest **T1**, while the difference in binding constant is more than two orders of magnitude larger (a factor of 424). We have analyzed the selectivity in the amplification for all possible guest combinations for hosts **12a** and **13a** in libraries A and B. The results are shown in Figure 9 and demonstrate that, while a correlation between selectivity in binding and amplification is evident, considerable scatter exists. Nevertheless, the instances where large selectivities in amplification are observed are invariably associated with highly selective receptors, although the reverse may not necessarily be true.

In an attempt to understand the origin of the scatter observed in Figure 9 we have analyzed the relationship between the guest-binding strength and the amplification factor of a given receptor in more detail. In the absence of complicating factors such as competition from other library members, this relationship is expected to be sigmoidal: For weakly binding guests the host will be essentially unamplified, and so small changes in the binding energy will have little effect, producing a flat region of the curve. For strongly binding guests, the amplification of the host will be limited by the availability of the building blocks, again producing a flat region. Only at intermediate binding energies will the amplification factor vary significantly with the guest binding energy.

Figure 10a shows the experimental results for the amplification of **12a** induced by guests **T1**–**T13** (where available) as a function of the host–guest binding constant in DCLs of set A. Figure 10b and c show the corresponding data for

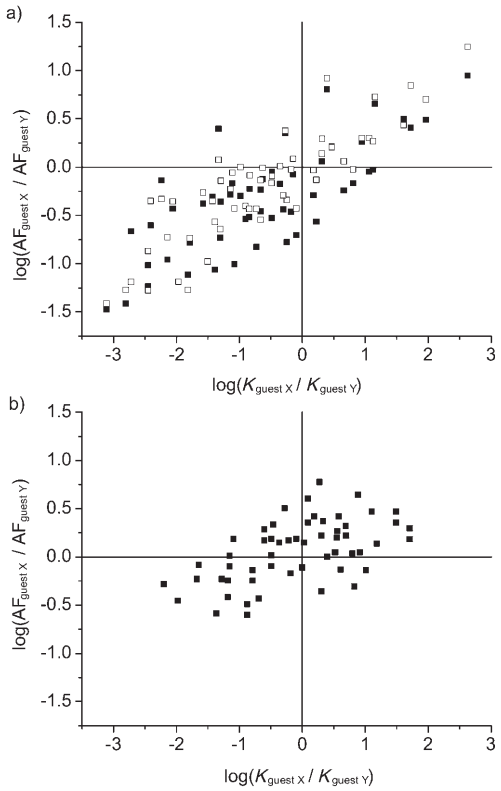


Figure 9. Correlation between relative binding constants and relative amplification factors of receptors a) **12a** and b) **13a** comparing two guests X and Y corresponding to any combination of **T1**–**T13** in the experimental DCLs of class A (□) and B (■).

hosts **12a** and **13a** in the DCLs of set B. Amplification of host **12a** in the simple DCLs of set A, which contain only one building block, appears to correlate fairly well with the host–guest binding energy. Amplification of the same host in the more complex DCLs of set B shows a somewhat increased amount of scatter, while the amplification of host **13a** in the same libraries is hardly correlated at all to the guest binding energy. Thus, under the conditions of our experiments, the correlation between host–guest binding energy and amplification factor is different for different libraries and different for different receptors.

Although some scatter is expected through experimental error, there is another more plausible cause for the variability of the correlation: competition for the guest by other library members. Figure 11 shows the results of a simulation of a simple model DCL made from a single building block, consisting of one non-binding dimer, two trimers and one non-binding tetramer (full details are given in the Supporting Information). The solid line shows how the amplification of one of the trimers varies as a function of the affinity towards a variety of guests, when the other trimer essentially does not bind. As expected, a sigmoidal relationship is observed. The dashed lines show the results of giving the competing trimer a progressively higher but constant binding energy. Thus, competition from a single library member causes the sigmoidal curves to shift further left as the com-

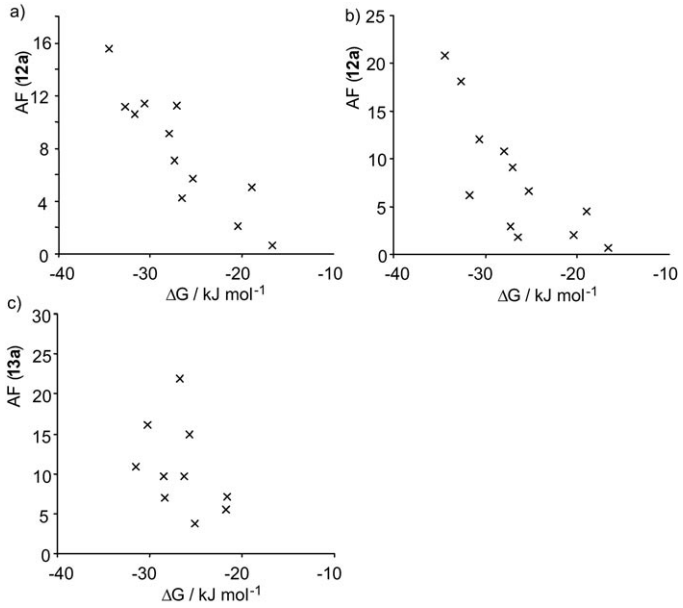


Figure 10. Experimentally observed amplification factors (AFs) as a function of the host–guest Gibbs binding energy of a) host **12a** in DCLs of set A (5 mM **4**), b) host **12a** and c) host **13a** in DCLs of set B (3.33 mM **4** and 1.67 mM **5**).

petitive binding becomes stronger. These shifts are caused by a competitor which, rather unrealistically, binds all guests with the same affinity. In experimental DCLs, the binding strength of any competing species is likely to vary from one guest to the next, resulting in considerable scatter.

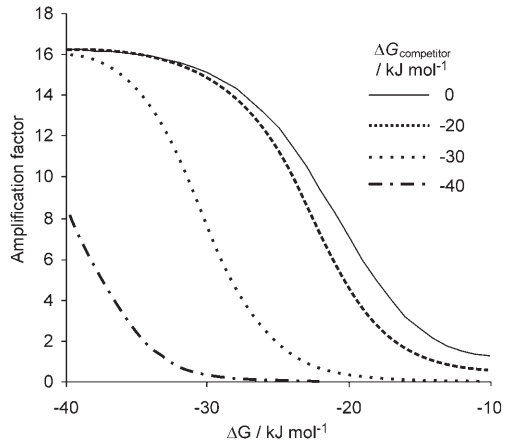


Figure 11. Amplification of a trimeric receptor in simple simulated DCLs, in the presence of competitors with fixed free energies of binding. Full details of the simulations are provided in the Supporting Information.

In our experimental systems, there are many library members that may compete with **12a** and **13a**: the diastereomers **12b** and **13b**, the homotetramer **14** and its diastereomers, and in fact any other library member. Interestingly, the minor diastereomers **12b** and **13b** do not appear to be a major source of scatter. Treating the pairs of isomers as a single species, with a single amplification factor, and weighted average binding constants did not substantially improve the correlations.

Reducing the template concentration can be expected to improve the correlations of the type shown in Figures 9 and 10 to some extent by removing the competition from relatively weakly binding small and/or hetero oligomers. However, using lower guest concentrations is unlikely to lead to perfect correlations as the disruptive effect of truly competitive binders in the DCL will be unaffected by reducing the template concentration. These expectations were confirmed by performing simulations of the DCLs at a reduced substoichiometric template concentration of 1.5 mM. A comparable drop in template concentration has been shown to be sufficient to cause **T9** to go from preferentially amplifying its weaker binder **13a** to preferentially amplifying the stronger binder **12a**.[9c] The simulations were based on a minimal model system in which only a homo- and a heterotrimer had affinities for the guests, using the free energies of binding in Table 1 (see Supporting Information for details), which varied only from the model used to generate Figure 7b in the concentration of the template. The results for the DCLs of set B (Figure 12) show only marginally better correlations between selectivities of amplification and binding, as compared to the experimental data in Figure 9, which was obtained using a template concentration of 5 mM.

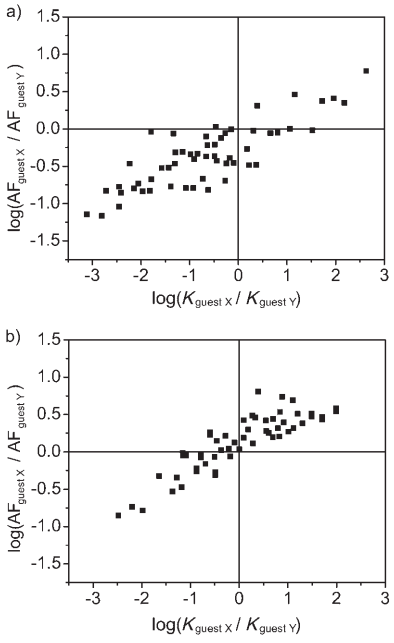


Figure 12. Correlation between relative binding constants and simulated relative amplification factors of receptors a) **12a** and b) **13a** comparing two guests X and Y corresponding to any combination of **T1**–**T13** in DCLs of class B using a template concentration of 1.5 mM.

Conclusion

We have made dynamic combinatorial libraries (DCLs) from dithiols **4** and **5** in water and analyzed the amplification of diastereomeric receptors **12a** and **b** and **13a** and **b** in response to the introduction of guests **T1**–**T13**. Nearly all guests induced larger amplifications of receptor **12a** as compared to **12b**. Similarly receptor **13a** was preferred over its diastereomer **13b**, essentially irrespective of the guest. In contrast, the selectivity in amplification between **12a** and **13a** depended on the nature of the guest, with larger spherical guests preferring **12a**, while smaller more elongated guests preferred the smaller receptor **13a**. We have investigated the thermodynamics of binding of guests **T1**-**T13** to receptors **12a** and **13a**. Affinities span three orders of magnitude, ranging from $8.5\times10^{2}\,\text{M}^{-1}$ for binding of **T5** to host **12a** to an impressive $5.4\times10^{5}\,\text{M}^{-1}$ for binding of **T13** to **12a**. By comparing binding and amplification data two key questions were addressed: Is amplification by a given guest selective for the best receptor? And does the best guest induce the largest amplification of a given receptor? We performed computer simulations to further explore the behavior of the DCLs under different "experimental" conditions. The results of the experimental and theoretical investigations show a good—albeit biased—correlation between relative amplification and relative affinity for different hosts for the same guests. Furthermore, the theoretical investigations were in line with other studies[8,9a,c] that showed that this bias could be alleviated, leading to the reliably selective amplification of the best binder, by using a modest amount of guest. However, the correlation between host–guest binding and the amplification factor for a given host across a wide range of guests (i.e., the selectivity of the host for the guests) was less satisfactory, largely as a result of every guest having its own unique pattern of affinities for the various competing receptors in the mixture. Thus, a plot of the ratio of the amplification factors induced by guests X and Y versus the ratio of the underlying host–guest binding constants showed considerable scatter. Nevertheless the highest guest selectivities were characterized by the biggest difference in amplification factor.

In conclusion, assessing the absolute binding affinity of a certain guest for a certain host from the extent to which the guest induces the amplification of this host is not necessarily reliable in a quantitative sense, but is in most cases still qualitatively useful. Most importantly, when strong amplifications or strongly selective amplifications are observed the selected receptors are indeed strong or highly selective binders, even though the reverse is not necessarily true. To further extend the information that can be obtained from DCLs, we are currently developing methodology that allows also quantitative information on host–guest binding to be extracted from the amplification factors by analyzing the behavior of the entire DCL, instead of focusing on selected amplification events.[21]

Experimental Section

Materials and methods: ^{1}H NMR spectra were recorded on Bruker DRX-500, DRX-400 or DPX-250 instruments or on a DRX instrument fitted with a cryoprobe. ^{13}C NMR spectra were recorded on Bruker DPX-400 (100 MHz) or DRX-500 (125 MHz) instruments. Chemical shifts are quoted in parts per million with reference to solvent signals. All chemicals were purchased from Acros, Aldrich, Avocado or Fluka in reagent grade quality or better and used without further purification. All solvents used in synthesis were distilled prior to use and dry solvents were freshly distilled from CaH_2 under argon. Ultrapure water was obtained from a Millipore water purification system, and when used for HPLC, passed through a 0.45 μm Millipore filter. HPLC grade MeCN, MeOH and 2-PrOH (Fisher, Romil) were passed through a 0.45 μm Millipore filter and used without further purification.

Analytical HPLC was carried out on Hewlett Packard 1050 and 1100 systems coupled to UV analyzers and the data were processed using HP Chemstation software. Separations were performed on reversed phase Waters Symmetry C18 columns (25.0 cm × 4.6 mm, 5 μm particle size) for analytical HPLC. Except where otherwise stated, the chromatography was carried out at 45 °C using Jones Chromatography or Anachem column ovens and using UV detection at 320 nm.

LC-MS was conducted on a Hewlett Packard 1050 system, coupled to a diode array detector and a Micromass Platform LC quadrupole mass analyser, controlled using a combination of MassLynx and OpenLynx software. Separations were achieved using reversed phase columns in the same manner as the analytical HPLC analyses. Mass analysis was conducted in the negative ion mode.

Preparative HPLC was conducted on a Gilson preparative HPLC system, equipped with a Gilson 305 pump, two Gilson 306 pumps, a Gilson 806 manometric module, a Gilson 811C dynamic mixer, a Gilson model 231 sample injector, a Gilson FC204 fraction collector, and a Gilson 401 dilutor. UV detection was performed originally using a Gilson 115 UV detector, and later using a Shimadzu SPD-6 A UV detector. The data were originally collected using a HP3395 integrator, and later a computer running Gilson Unipoint software. Separation were performed using a Nucleodur C18 preparative column (25.0 cm × 2.1 cm, 100 Å, 5 μm), with a Nucleodur C18 guard column (5.0 cm × 2.1 cm, 100 Å, 5 μm). Except where otherwise stated, a Grant water bath was used to heat the column, and a length of tubing between the pump and the column, to 45 °C. This arrangement ensured that the mobile phase was at the correct temperature before it entered the column.

Microcalorimetry: Isothermal calorimetry measurements were conducted by using a MCS-ITC calorimeter from MicroCal, LLC, Northampton, MA, USA. A single 3 μL aliquot and 29 aliquots of 10 μL were titrated into the calorimetric cell every 3 minutes. The data were analyzed using the customized ITC module of the Origin 5.0 software package and a least squares fitting procedure to fit the data to the appropriate binding model. All measurements were carried out at 298 K.

Guests T1, **T5**–**T8**, **T10** and **T12** are commercially available. Guests **T2**,[14a] **T3**,[6a] **T4**,[6a] **T9**,[22] **T11**,[22] and **15**[23] were prepared as reported previously.

***N*-Methylmorphinium iodide (T13)**: The compound was prepared by reacting morphine (0.50 g, 1.75 mmol) with methyl iodide (10 mL, 160 mmol) in acetonitrile (100 mL) for 72 h at room temperature followed by recrystallization from acetonitrile. ^{1}H NMR (500 MHz, D_2O): $\delta = 1.98$ (d, 1H), 2.47 (dt, 1H), 2.83 (dd, 1H), 3.22 (s, 3H), 3.23–3.34 (m, 3H), 3.30 (s, 3H), 3.37 (t, 1H), 3.44 (d, 1H), 4.02 (t, 1H), 4.27 (m, 1H), 4.98 (dd, 1H), 5.30 (dt, 1H), 5.65 (dm, 1H), 6.56 (d, 1H), 6.65 ppm (d, 1H); ^{13}C NMR (125 MHz, D_2O): $\delta = 23.49$, 29.30, 33.32, 41.34, 50.21, 54.15, 56.08, 65.41, 69.89, 90.03, 117.90, 120.20, 122.50, 125.55, 129.01, 133.13, 138.44, 145.31 ppm; elemental analysis calcd (%) for C 50.60, H 5.19, N 3.28; found: C 50.39, H 5.19, N 3.27.

Anthracene-2,6-diol (6):[24] Sodiumborohydride (9.6 g, 0.25 mol) was dissolved in a 1 M solution (240 mL) of sodium carbonate in water. 2,6-Dihydroxyanthraquinone (4.8 g, 20 mmol) was added in portions while stirring at room temperature. After the initial gas evolution had stopped the so-

lution was gently refluxed for 15 min. The reaction mixture was transferred to a large beaker and acidified using 250 mL 3 M HCl (caution: extensive foaming). The precipitated product was filtered and taken up in acetone and filtered over a short pad of celite. The solvent was evaporated and the compound dried under vacuum overnight, yielding 3.99 g (19 mmol, 95%) anthracene-2,6-diol. The product was used without further purification in the next step. ^{1}H NMR ([D$_6$]DMSO, 400 MHz): $\delta =$ 7.06 (dd, 2H), 7.12 (d, 2H), 7.80 (d, 2H), 8.11 (s, 2H), 9.63 ppm (s, 2H).

Dimethylthiocarbamic acid *O*-(6-dimethylthiocarbamoyloxyanthracen-2-yl) ester (7): Crude anthracene-2,6-diol (**6**; 9.0 g, 42.8 mmol) was dissolved in 80 mL anhydrous DMF under a nitrogen atmosphere. The solution was cooled to 0°C and DABCO (28.8 g, 257 mmol) was added in portions. To the resulting suspension a solution of *N*,*N*-dimethylthiocarbamoyl chloride (suspect carcinogen!) (31.8 g, 257 mmol) in anhydrous DMF (40 mL) was added dropwise at 0–5°C. In cases where the suspension appeared to "solidify", more DMF was added. The suspension was allowed to warm to room temperature in a melting ice bath overnight and stirred for a total of 24 h. The reaction was monitored by TLC (silica gel, 1% methanol in chloroform; R_f starting material: 0.6; R_f product: 0.2). The reaction mixture was filtered and the residue was washed thoroughly with water and finally with a small amount of ethanol to give 9.65 g (25.1 mmol, 59%) of a yellow-brown powder. The product was pure enough to be used in the next step, but can be recrystallized from chloroform/acetone (m.p. 282–284°C). ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta =$ 3.41 (s, 6H), 3.49 (s, 6H), 7.26 (dd, 2H), 7.61 (d, 2H), 7.97 (d, 2H), 8.37 ppm (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta =$ 41.41, 45.88, 121.42, 125.90, 128.86, 131.63, 132.78, 134.03, 153.69, 190.31 ppm; LC-MS: *m*/*z*: calcd: 407.0864; found: 407.0859 [M+Na$^+$].

Dimethylthiocarbamic acid S-(6-dimethylcarbamoylsulfanylanthracen-2-yl) ester (8): Crude *O*-thiocarbamate **7** (8.0 g, 20.8 mmol) was suspended in diphenyl ether (100 mL) under a nitrogen atmosphere and heated to 230°C for 3 h. The reaction mixture was allowed to slowly cool to room temperature. The product was isolated by filtration (gentle heating to melt the diphenyl ether may be necessary) and washed extensively with hexane to afford 7.0 g (18.2 mmol, 88%) of beige crystals. The product was pure enough to be used in the next step, but can be recrystallized from chloroform/acetone (m.p. 277–279°C). ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta =$ 3.06 (br s, 6H), 3.14 (br s, 6H), 7.50 (dd, 2H), 7.98 (d, 2H), 7.97 (d, 2H), 8.37 ppm (s, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta =$ 36.69, 126.23, 126.27, 128.48, 130.98, 131.34, 131.83, 135.11, 166.58 ppm; LC-MS: *m*/*z*: calcd: 407.0854; found: 407.0859 [M+Na$^+$].

Dimethyl 2,6-bis(dimethylcarbamoylsulfanyl)-9,10-dihydro-9,10-ethenoanthracene-11,12-dicarboxylate (9): A mixture of the S-thiocarbamate **8** (7.0 g, 18.2 mmol) and dimethyl acetylenedicarboxylate (6.58 g, 46.3 mmol) in diphenyl ether (40 mL) under a nitrogen atmosphere was heated for 75 min at 190°C. The reaction was monitored by TLC (silica gel, 10% acetonitrile in chloroform; R_f starting material: 0.35; R_f product: 0.15). The reaction mixture was allowed to cool to room temperature and hexane was added and the solvents were decanted to give an oily product that solidified upon standing. The product was purified by chromatography (silica gel, gradient elution 5 to 10% acetonitrile in chloroform) yielding 8.08 g (15.3 mmol, 84%) of a yellow solid. Note: use of ethanol-stabilized chloroform gives poor separation. An analytical sample was obtained by recrystallization from acetone/petroleum ether (60–80) (m.p. 195–196°C). ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta =$ 3.06 (br s, 6H), 3.14 (br s, 6H), 7.50 (dd, 2H), 7.98 (d, 2H), 7.97 (d, 2H), 8.37 ppm (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta =$ 38.48, 53.50, 53.97, 77.61, 125.99, 127.23, 132.32, 134.65, 145.57, 146.10, 148.10, 167.05, 168.37 ppm; LC-MS: *m*/*z*: calcd: 549.1130; found: 549.1129 [M+Na$^+$].

2,6-Dimercapto-9,10-dihydro-9,10-ethenoanthracene-11,12-dicarboxylic acid (4): Under a nitrogen atmosphere S-thiocarbamate **9** (2.0 g, 3.8 mmol) was suspended in a 1.75 M solution (70 mL) of KOH in diethyleneglycol that had been purged with argon for 2 h. The solution was heated at 105°C for 30 min. After the solution had cooled down to room temperature 500 mL of water (purged) was added followed by rapid addition of 10% HCl (55 mL). The very fine precipitate was centrifuged, washed extensively with water and dried under vacuum overnight to give 1.15 g (18.8 mmol, 85%) of a beige powder (m.p. decomp). ^{1}H NMR (CD_3OD, 400 MHz): $\delta =$ 5.53 (s, 2H), 6.94 (dd, 2H), 7.24 (d, 2H), 7.33 ppm (d, 2H); ^{13}C NMR (CD_3OD, 100 MHz): $\delta =$ 53.33, 125.23, 125.77, 126.83, 129.83, 142.52, 146.59, 148.99, 168.61 ppm; LC-MS: *m*/*z*: calcd: 379.0075; found: 379.0060 [M+Na$^+$].

3,5-Bis(dimethylthiocarbamoyloxy)benzoic acid methyl ester (10): Methyl 3,5-dihydroxybenzoate (5.0 g, 29.7 mmol) was dissolved in anhydrous DMF (20 mL) under a nitrogen atmosphere. The solution was cooled to 0°C and DABCO (13.3 g, 119 mmol) was added in portions. To the resulting suspension a solution of *N*,*N*-dimethylthiocarbamoyl chloride (14.7 g, 119 mmol) in DMF (20 mL) was added dropwise at 0–5°C. Where the reaction mixture "solidified" more DMF was added to enable efficient stirring. The suspension was allowed to warm to room temperature and stirred for 24 h. The reaction mixture was poured into water (200 mL), filtered and the residue was washed with ethanol to give 9.22 g (26.9 mmol, 90%) a white crystalline powder. The product was pure enough for most purposes, but can be recrystallized from ethanol (m.p. 135–136°C). ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta =$ 3.34 (s, 3H), 3.44 (s, 3H), 3.88 (s, 3H), 7.05 (t, 1H), 7.63 ppm (d, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta =$ 39.84, 44.33, 53.38, 122.66, 123.86, 134.25, 154.92, 166.41, 187.96 ppm; LC-MS: *m*/*z*: calcd: 365.0606; found: 365.0605 [M+Na$^+$].

3,5-Bis(dimethylcarbamoylsulfanyl)benzoic acid methyl ester (11): O-Thiocarbamate **10** (8.0 g, 23.3 mmol) was suspended in diphenyl ether (80 mL) and heated under a nitrogen atmosphere on a sand bath to 230–240°C for 3 h. The reaction was monitored by TLC (silica gel, $CHCl_3$/CH_3CN 9:1, R_f starting material: 0.4, R_f product: 0.15). After the reaction mixture was allowed to cool to 30–40°C it was poured into 160 mL hexane and slowly allowed to cool to 4°C. The product (7.37 g, 21.5 mmol, 92%) was obtained after filtration and extensive washing with warm hexane as light beige crystals (m.p. 140–141.5°C). ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta =$ 3.05 (br d, 6H), 3.88 (s, 3H), 7.82 (t, 1H), 8.16 ppm (d, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta =$ 36.42, 51.81, 129.64, 130.81, 136.65, 145.78, 165.07, 165.27 ppm; LC-MS: *m*/*z*: calcd: 365.0606; found: 365.0616 [M+Na$^+$].

3,5-Dimercaptobenzoic acid (5): Under a nitrogen atmosphere S-thiocarbamate **11** (7.0 g, 20.4 mmol) was suspended in a 1.75 M solution (70 mL) of KOH in diethyleneglycol that had been purged with argon for 2 h. The solution was heated at 105°C for 30 min. After the solution had cooled down to room temperature 500 mL of water (purged) was added followed by rapid addition of 10% HCl (55 mL). The precipitate was filtered (or centrifuged in cases where the precipitate is fine), washed extensively with water and dried under vacuum overnight to give 3.50 g (18.8 mmol, 92%) of a white powder. The product can be recrystallized from degassed ethanol/water (2 g acid in a refluxing mixture of ethanol (28 mL) and water (48 mL) under an inert atmosphere (m.p. 221–223°C). ^{1}H NMR (CD_3OD, 400 MHz): $\delta =$ 7.40 (s, 1H), 7.64 ppm (s, 2H); ^{13}C NMR (CD_3OD, 100 MHz): $\delta =$ 126.97, 132.69, 133.06, 134.87, 168.13 ppm; LC-MS: *m*/*z*: calcd: 185.9809; found: 185.9808 [M^+].

Dynamic combinatorial libraries: The relevant building blocks were dissolved in water, with sufficient 2.5 M aqueous NaOH solution to fully deprotonate the thiols and carboxylic acids on the building blocks, using sonication where necessary. The pH was then adjusted to 8 using 1 M aqueous HCl and 2.5 M aqueous NaOH solutions. Aliquots of 500 μL of building block solutions were added to 2 mL HPLC vials, and mixed with 500 μL of a template solution and a magnetic stir bar. The vials were then capped, and stirred for at least three weeks at 298 K.

Receptor 12a was isolated by preparative HPLC from DCLs prepared using 10 mM **4** and 10 mM **T13** or **T11**. Aliquots of 500 μL were chromatographed (20 mL min^{-1} 50:50:0.1 H_2O/MeCN/TFA, ambient temperature). For each run, fractions were collected at 0.25 minute intervals, typically from 14–17.75 minutes. Examination of the chromatograms of this region revealed two overlapping peaks. Fractions containing pure **12a** were combined and the solvent was removed in vacuo. The material obtained from 20 injections was re-constituted in 5 mL 1:1 MeCN/H_2O, and re-chromatographed (20 mL min^{-1} 50:50:0.1 H_2O/MeCN/TFA, ambient temperature) in one injection. The collected fractions corresponding to the main peak were dried in vacuo, redissolved in 50 mM borate buffer using the minimum number of 1.5 mL aliquots to achieve complete dissolution (typically 1 mg mL^{-1}), and separated into 1.5 mL centrifuge tubes. Ali-

quots of 100 μL 2 M HCl was added to each tube, the contents of the tubes were vortexed, and the resultant suspensions were centrifuged for 15 minutes at 13,000 rpm. The pellets were washed twice by addition of 500 μL 40 mM HCl, resuspension by vortexing, and recentrifugation for 15 minutes at 13000 rpm. The final pellets were dried in vacuo overnight. This procedure typically gave 4 mg of **12a** per 10 mL of DCL solution. ^{1}H NMR (500 MHz, CD_3OD, 300 K): $\delta = 7.57$ (s, 2H), 7.47 (s, 2H), 7.38 (s, 2H), 7.26 (d, 2H, $J = 7.6$ Hz), 7.20 (m, 4H), 7.01 (d, 2H, $J = 7.9$ Hz), 6.89 (m, 4H), 5.82 (s, 2H), 5.81 (s, 2H), 5.72 ppm (s, 2H); elemental analysis calcd (%) for **12a** + $6H_2O$: C 55.37, H: 3.61, N 0.00; found: C 55.37, H 3.46, N 0.00.

Receptor 13a was isolated by preparative HPLC from DCLs prepared using 6.66 mM **4**, 3.33 mM **5** and 10 mM **T2**. The DCLs were diluted 1:1 with MeCN + 0.4% TFA, and 5 mL aliquots were chromatographed (20 mL min^{-1} 55:30:15:0.1 H_2O/MeCN/IPA/TFA, retention time typically 15.5–20 min). The collected fractions from four such injections were combined, dried in vacuo and redissolved in 10 mL 1:1 MeCN/H_2O. Aliquots of 500 μL of this solution were chromatographed again to separate **13a** from **13b** (20 mL min^{-1} 50:50:0.1 H_2O/MeCN/TFA). For each run, fractions were collected at 0.25 minute intervals, from 12.5–20.0 minutes. Examination of the chromatograms of this region revealed two overlapping peaks. Fractions containing pure **13a** were combined and the solvent was removed in vacuo. The material obtained from 20 injections was re-constituted in 5 mL 1:1 MeCN/H_2O, and rechromatographed a third time (20 mL min^{-1} 50:50:0.1 H_2O/MeCN/TFA) in a single injection. The collected fractions corresponding to the main peak were dried in vacuo, and reprecipitated as described for **12a**. This procedure typically gave 1 mg of **13a** per 10 mL of DCL solution. ^{1}H NMR (500 MHz, CD_3OD): $\delta = 5.62$ (s, 2H), 5.67 (s, 2H), 7.11 (d, 2H), 7.19 (d, 2H), 7.24 (m, 4H), 7.56 (s, 2H), 7.61 (s, 2H), 7.91 (s, 2H), 8.07 ppm (s, 1H); elemental analysis calcd (%) for **13a** + $4H_2O$: C 53.51, H 3.34; found: C 53.27, H 3.39; LC-MS: m/z: calcd for: 914.9591; found: 914.9602 [M+Na$^+$].

The mixture of 13a and 13b for the experiments described in Figure 3 was obtained using only the first chromatographic step described above.

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