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Molecular amplification in a dynamic system by ammonium cations

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Abstract—Using hydrazone chemistry, a simple dynamic system of macrocyclic pseudo-peptides was prepared which contains a cyclic structure that resembles a known receptor for ammonium cations. A series of ammonium salts were tested as templates and the expected receptor was amplified in different degrees according to the template used. Competitive non-covalent interactions with a crown ether were used to demonstrate in situ that the templated system is still dynamic. Formation of host–guest complexes was proven by ESI-MS and ^{1}H ROESY experiments. Relative affinities of the various templates for the isolated receptor correlate well with the effect they produce on the library composition.

1. Introduction

Dynamic combinatorial chemistry (DCC) has attracted increased interest over recent years as a new strategy for the preparation and identification of new host and guest compounds.[1] The approach combines the merits of combinatorial chemistry with molecular evolution, and in principle integrates preparation and screening of libraries in one single process.[2]

Dynamic combinatorial libraries (DCLs) are generated by the assembly of building blocks through reversible bonds. Since every member in the mixture contains one or more reversible connections, the library product distribution is thermodynamically controlled and may be influenced by template effects.[3] Stabilization of one particular member of the library by addition of a template molecule will shift the equilibria leading to an increased concentration of the selected compound at the expense of the other members. In this way, a guest can be used as a template to select and amplify its preferred host from a library of potential receptors (Fig. 1).

Several DCLs have been prepared so far using either covalent[4] or non-covalent[5] bonds to reversibly connect the building blocks.[6] Amplification of selected receptors has been observed in libraries in which the building blocks are held together by hydrogen bonds[5a,b] or metal coordination.[5c–e] However, amplification of covalently assembled receptors seems to be more elusive and not many examples have been reported so far.[7]

We have recently developed the use of hydrazone exchange to prepare DCLs.[4f] A range of interconverting macrocycles can be generated from bifunctionalized building blocks containing one hydrazide and one protected aldehyde linked to a central unit (Fig. 1). The presence of acid catalyses (a) hydrazone formation that produces the assembly of the macrocycles, and (b) hydrazone exchange that leads to the interconversion of the macrocycles. Neutralization of the reaction medium switches off the exchange converting the library into a 'static' mixture from which the individual members can be isolated.

Here we report molecular amplification from a very simple dynamic mixture of macrocyclic pseudo-peptides. The selected macrocycle binds selectively to different ammonium salts used as templates for its amplification. The affinities of the various templates for the macrocycle

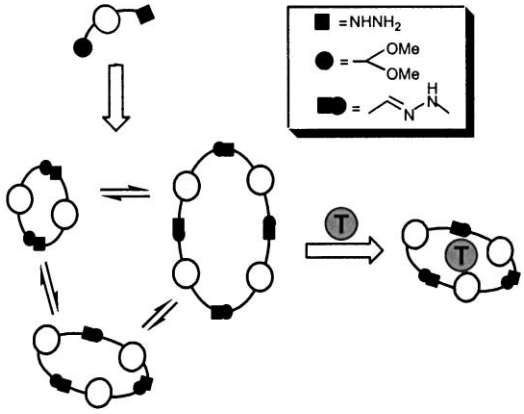

Figure 1. Generation and templating of a dynamic combinatorial library.

Keywords: ammonium cations; host–guest complexes; dynamic system.

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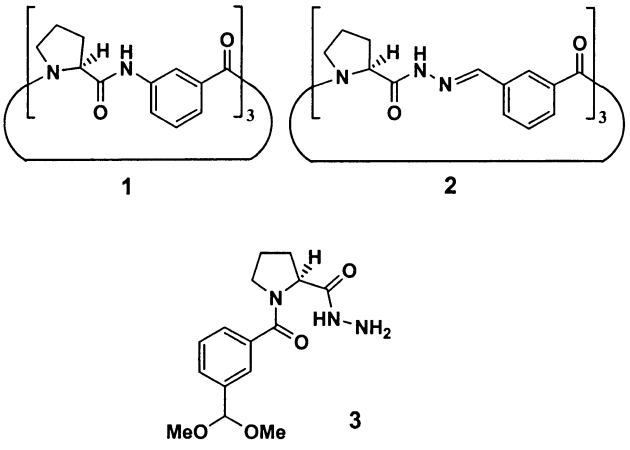


Figure 2.

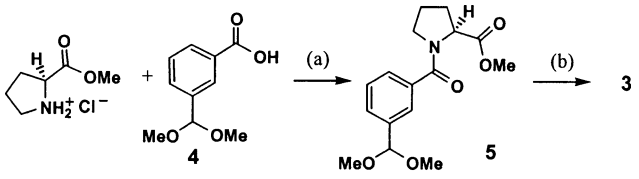
are in accordance to the response that each salt exerts on the system.

Kubik et al. have reported the synthesis and receptor properties of the cyclic peptide **1** composed of L-proline and 3-aminobenzoic acid in an alternating sequence (Fig. 2).[8] Macrocycle **1** is able to bind ammonium cations in chloroform with stability constants between 11 000 and 42 000 M^{-1}. If the peptide bonds that connect the N-terminus of 3-aminobenzoic acid with the C-terminus of proline in **1** are replaced by hydrazones, a similar macrocycle **2** is obtained, which contains three additional C=N imine bonds. Pseudo-peptide **2** could be a member of a DCL prepared by acid cyclization of building block **3**. Our expectation was that ammonium salts would bind the cyclic trimer **2** strongly enough to template its formation leading to molecular amplification.

2. Results

Building block **3** was prepared by standard EDC coupling of L-proline methyl ester hydrochloride with 3-carboxybenzaldehyde dimethoxyacetal, followed by hydrazinolysis of the methyl ester to generate the necessary hydrazide functionality (Scheme 1).

Cyclization of **3** in chloroform/TFA leads initially to a series of macrocyclic *N*-acyl hydrazones. Six hours after the reaction is initiated, **3** has been consumed completely and macrocycles from dimer to 15mer are detected by electrospray mass spectrometry (ESI-MS). At this time, HPLC analysis shows that the cyclic trimer constitutes the major product of the mixture (Fig. 3a). However, after a period of three days, the initial distribution of macrocycles drifts almost exclusively in favour of the cyclic dimer, which



Scheme 1. (a) TEA, CH_2Cl_2, EDC, DMAP; (b) $NH_2NH_2{\cdot}H_2O$, MeOH.

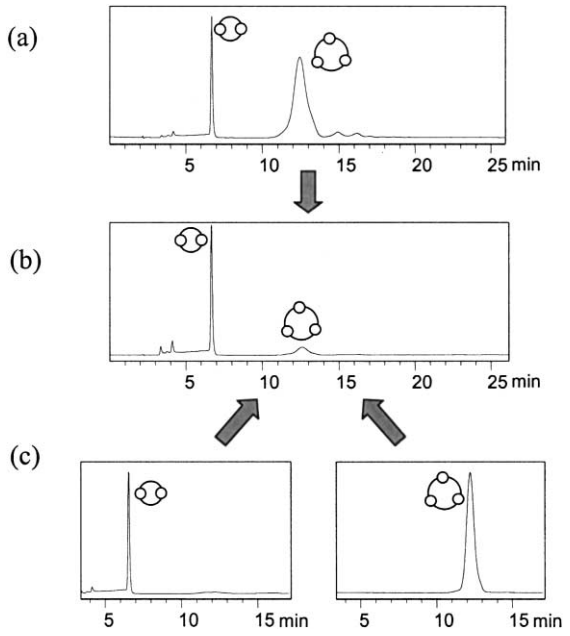


Figure 3. (a) HPLC trace of the reaction mixture 6 h after the reaction was initiated from building block **3**. (b) HPLC trace of the reaction mixture after 3 days. (c) HPLC trace of cyclic dimer and cyclic trimer.

then represents around 88% of the peptide material of the mixture (Fig. 3b).[9]

To ascertain that the reaction mixture has reached the equilibrium, the library was also generated from pure cyclic trimer, and from pure cyclic dimer. In both experiments, the final distribution of products was the same as in the library prepared from **3** (Fig. 3b and c).

Having a dynamic system in which the cyclic trimer is kinetically and thermodynamically accessible, we tested a series of tetra alkyl ammonium salts for their templating ability (Fig. 4). Four equivalents of each salt were

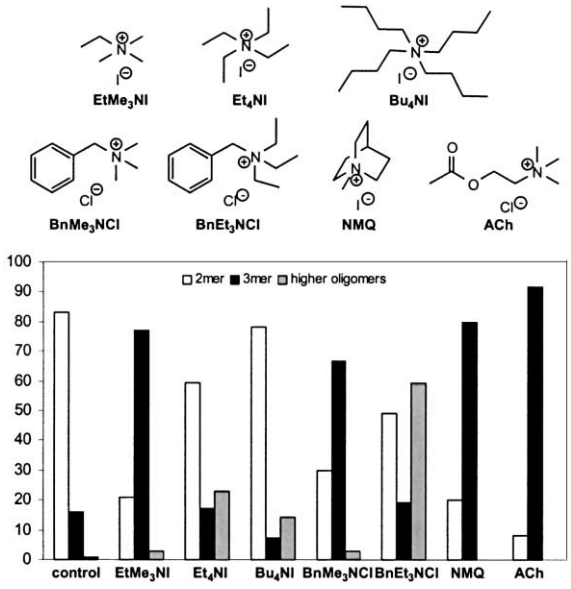


Figure 4. Relative amounts of cyclic products in absence (control) and in presence of different templates.

introduced into the system and the reaction was monitored by HPLC, observing different responses from the library. Salts containing three butyl or ethyl groups on the nitrogen produced weak non-specific amplification of higher oligomers. However, when trimethyl ammonium salts or *N*-methyl quinuclidinium iodide were used as templates, the cyclic dimer was consumed to generate more cyclic trimer. The final concentration of trimer depends on the template used and varied between 10 and 90% of the peptide material in the mixture.

If the observed amplification is due to the stabilization of the cyclic trimer via complexation with the ammonium salts, any change in the extent of binding should result in a corresponding change in the library composition. When the amount of added NMQ was varied in the range 1–7 equiv. the proportion of peptide material present as **2** similarly ranged from 12–81%.

Electrospray mass spectrometry (ESI-MS) has proved to be a powerful technique for the identification of supramolecular complexes.[10] ESI-MS analysis of the reacting mixtures in presence of the templates allowed the detection of complexes between the cyclic trimer and the corresponding effective templates. Cyclization reactions were carried out in presence of 5 equiv. of the different effective templates, and ESI mass spectra were recorded while the system was on its way towards the equilibrium. In all the experiments, peaks corresponding to the complex 3mer-template were observed at 817.5 (for 3mer: $EtMe_3N^+$), 879.5 (for 3mer: $BnMe_3N^+$), 855.5 (for 3mer: NMQ^+), and 875.6 (for 3mer: ACh^+). No such adducts were observed for the cations with any of the other library members (Fig. 5).

When working with dynamic systems, it is important to show that the final product distribution in presence of a template represents the equilibrium and is not the result of the introduction of a kinetic trap. It should be noted that the ammonium salts added into the DCL are able to undergo anion exchange with the TFA used as catalyst. This exchange could render the reaction conditions insufficiently acidic to permit hydrazone exchange.

If the macrocycles are interconverting, an equal number of linear hydrazinium intermediates generated during the ring opening/closure process must be present in the mixture. We have previously reported that the hydrazinium terminus of these transient species can be complexed by [18]crown-6.[11] This complexation leads to amplification of the linear species, which can therefore be detected by ESI-MS or HPLC demonstrating that the system is still dynamic. Solutions of **3** were hence established under thermodynamic cyclization conditions with 4 equiv. of *N*-methyl quinuclidinium and variable quantities of TFA, such that the series of experiments progresses from an excess of template to an excess of TFA. The equilibria were maintained for three days before [18]crown-6 was added to the solutions. For all the experiments, after the addition of the crown ether, a new signal was observed in the HPLC at 2.5 min. The UV spectrum recorded using the diode array of the HPLC facility shows the expected band at 257 nm characteristic of aromatic aldehydes. ESI mass spectra of the solutions are dominated by a major peak at 526.4 corresponding to the complex between [18]crown-6 and the protonated hydrazide monomer accompanied by small peaks for the protonated cyclic dimer (487.3), and trimer (730.4), as well as for the complex of the protonated linear dimer with [18]crown-6 (769.4) and for the complex of the cyclic trimer with NMQ (855.5) (Fig. 6).

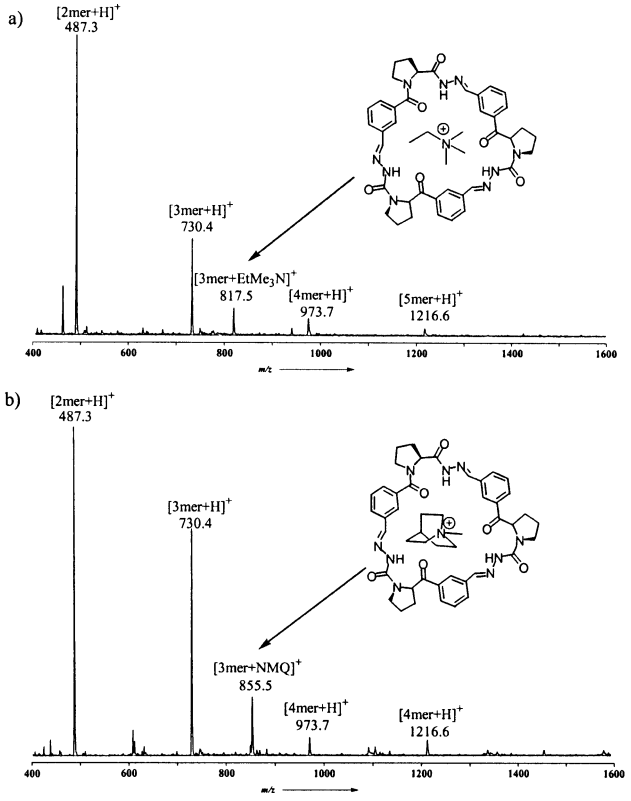


Figure 5. ESI mass spectra of the reaction mixture in presence of: (a) 5 equiv. of $EtMe_3NI$, and (b) 5 equiv. of NMQ.

In these experiments, the product distribution of the dynamic system is controlled by two simultaneous supramolecular effects: (1) towards linear monomer and linear dimer by virtue of crown ether recognition, and (2) towards cyclic trimer by ammonium cation binding; both effects act against the intrinsic thermodynamic preference towards the cyclic dimer. This intricate interplay of supramolecular

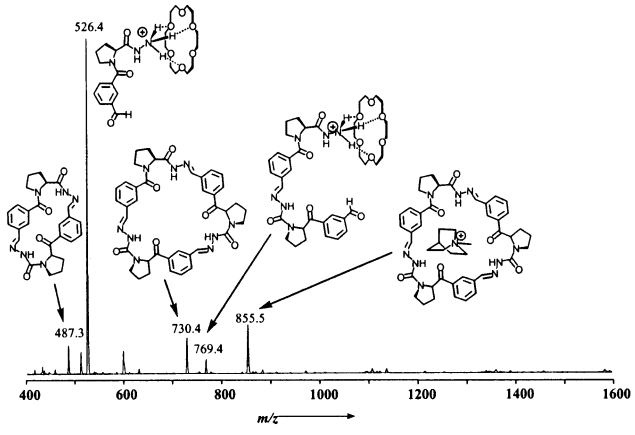


Figure 6. ESI mass spectrum of the dynamic system in presence of two competing templates: NMQ and [18]crown-6.

recognition and stabilization, through a more or less complex network of equilibria highlights the capabilities of DCLs.

The ^{1}H NMR spectrum of pure trimer **2** in a 95:5 chloroform/methanol mixture—to ensure complete solubility—shows the presence of different conformational isomers in slow exchange on the NMR chemical shift time scale (Fig. 7a). Inspection of the α-proton region of the spectrum reveals a range of α-proton signals between 4.5 and 6.0 ppm. That all of these conformers for the trimer are interconverting is clear from the NOESY spectrum, which shows exchange peaks between all the α-proton signals. This mixture of conformers and *cis*/*trans* isomers can be envisaged as a dynamic conformational or configurational library in itself.[12]

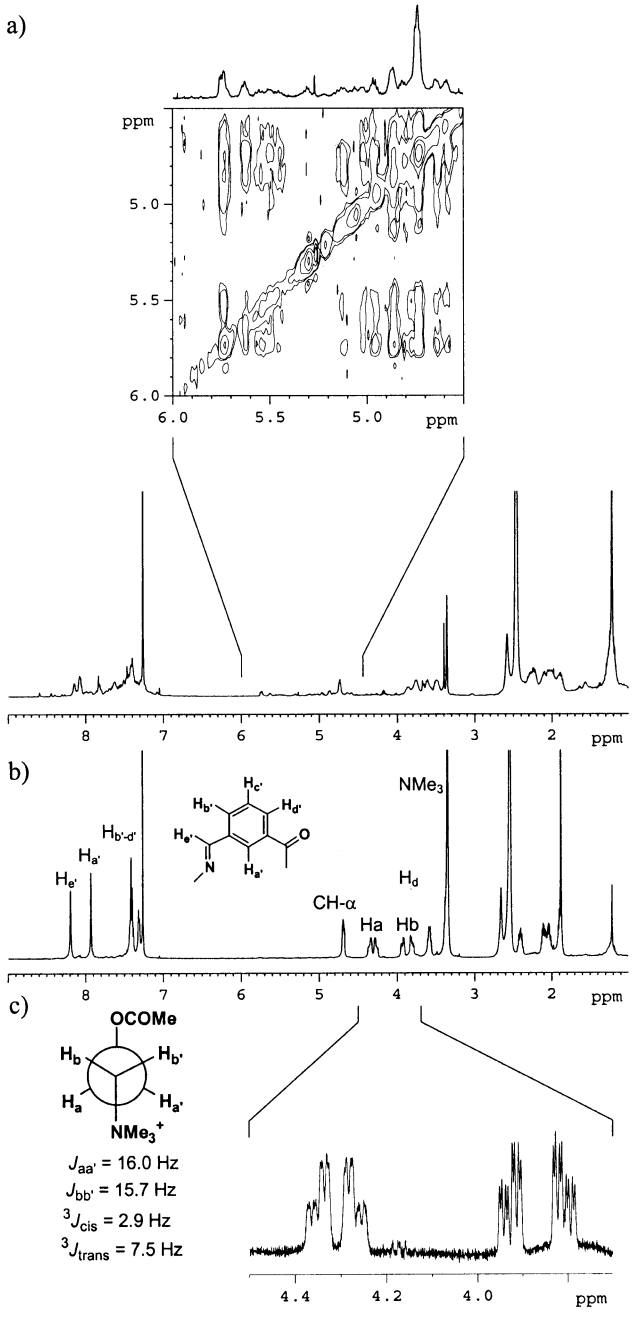


Figure 7. (a) ^{1}H NMR spectrum of trimer **2** and expansion of the ^{1}H NOESY spectrum showing the exchange of α proton signals for different conformers. (b) ^{1}H NMR spectrum of trimer **2** in presence of 1 equiv. of ACh. (c) Expansion of ^{1}H NMR spectrum of acetylcholine in presence of 1 equiv. of **2** showing CH_2a and CH_2b resonances. Conformation derived from the observed coupling constants.

Addition of different templates to the mixture of interconverting conformers of **2** dramatically changes its ^{1}H NMR spectrum. The presence of 1 equiv. of acetylcholine leads to the simplification of the whole spectrum of **2**, which is now dominated by a single C_3 symmetric conformer (Fig. 7b).[13] All the signals previously observed in the α proton region collapse into one multiplet at 4.74 ppm. Similarly two sharp singlets corresponding to the imine He′ and the aromatic Ha′ are observed at 7.97 and 8.23 ppm where several broad signals were previously present.

The binding to the chiral receptor **2** also affects the resonances of all the guest protons. The *N*-methyl proton signal is shifted downfield by 0.15 ppm while the signal corresponding to the acetyl group is shifted upfield by 0.23 ppm as compared to the unbound guest. In addition the $-CH_2$a– and $-CH_2$b– protons of acetylcholine become inequivalent and split into two pairs of signals. The appearance of the signals for $-CH_2$a– and $-CH_2$b– and the observed gem, *cis* and *trans* coupling constants for $-CH_2$b– (15.7, 2.9 and 7.5 Hz, respectively), led to the interpretation that acetylcholine is bound in the *trans* conformation (Fig. 7c).[14]

Further information on the host–guest complex between the cyclic trimer and acetylcholine was provided by ^{1}H ROESY studies. Analysis of a 1:1 mixture of acetylcholine and **2** in $CDCl_3/CH_3OD$ (95:5) revealed nOes between the *N*-methyl protons of the guest and the aromatic Ha′ and the C-α proton of proline in the receptor (Fig. 8a). NOes were also observed between Ha′ and the $-CH_2$a– and $-CH_2$b– protons of acetylcholine. These intermolecular nOes suggest that when it is bound to the guest, receptor **2** adopts a conformation in which Ha′ and the C-α proton of proline are exposed into the cavity. This could be achieved if the peptide bond is in the *trans* conformation and the carbonyl is anti to Ha′ and slightly out of the plane of the aromatic ring. This conformation places one Hd of proline in close proximity to Ha′ on the aromatic ring and is supported by the intramolecular nOe observed between these two protons (Fig. 8b).

The 1:1 complex stoichiometry for the complex between **2** and acetylcholine was confirmed by using Job's method of continuous variations.[15]

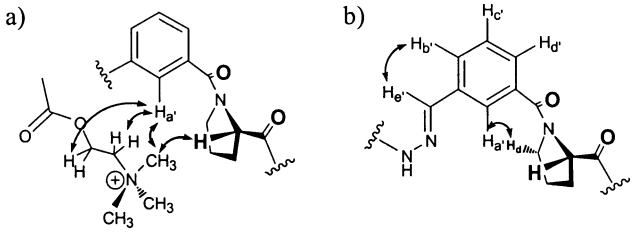


Figure 8. (a) Intermolecular nOes observed in a 1:1 mixture of ACh and receptor **2**. (b) Intramolecular nOes observed for bound receptor.

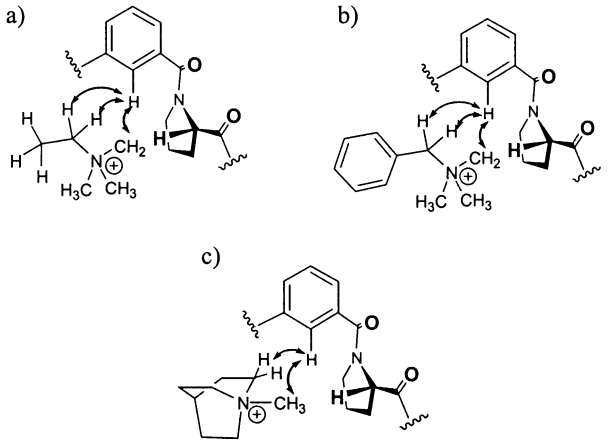


Figure 9. Observed intermolecular nOes between receptor **2** and (a) $EtMe_3NI$, (b) $BnMe_3NI$, and (c) NMQ.

Control experiments of 1:1 mixtures of the acetylcholine with cyclic dimer afforded ^1H NMR spectra, which show none of the changes in chemical shifts or splitting of CH_2 signals observed with cyclic trimer.

The simplification of the ^1H NMR spectrum of **2** observed in presence of acetylcholine was also produced by addition of $EtMe_3NI$, $BnMe_3NI$, and NMQ. The similarity between the spectra of receptor **2** in presence of the different cations suggests that the same conformer is selected and fixed by all the guests. The effect of complexation on the proton resonances of the different guests follows a similar trend: (a) small downfield shift in the $-NMe_3$ resonances, and (b) splitting of the signals corresponding to protons on the CH_2 α to the ammonium centre.

^1H ROESY experiments of 1:1 mixtures of **2** with NMQ, $EtMe_3NI$, and $BnMe_3NI$ showed similar intermolecular nOes between Ha′ in the receptor and the *N*-methyl protons of the guests as observed with ACh (Fig. 9).

The stability of the complexes between **2** and the four effective templates were estimated by means of NMR titrations. Increasing amounts of the receptor were added to solutions of the different salts in $CDCl_3/CH_3OD$ (95:5) and the shifts of the resonances of the guests' protons were followed in the ^1H NMR spectra. Stability constants were calculated using a non-linear least squares fitting method for 1:1 complexes.[16] The values obtained were: $230\ M^{-1}$ for acetylcholine, $150\ M^{-1}$ for *N*-methyl quinuclidinium, $140\ M^{-1}$ for ethyl-trimethyl ammonium, and $80\ M^{-1}$ for benzyl-trimethyl ammonium. These relative binding constants correlate well with the degree of amplification of **2** that each template produces. It should be noted that the conditions under which titrations were performed do not entirely reflect those of the cyclization reaction and it is reasonable to believe that the presence of 5% methanol could give values for the binding constants lower than would be operating in 100% chloroform.

3. Discussion and outlook

Hydrazone chemistry has been demonstrated as a suitable reaction for DCL generation. The libraries that result from hydrazone exchange have been proven to be under thermodynamic control, which is of huge significance if templating is to be realized in DCLs. This characteristic, in combination with the possibility of switching on and off of the exchange by acid catalysis and neutralization, has allowed the guest induced amplification and isolation of receptor **2**.

In the dynamic system studied, a significant increase in the cyclic trimer concentration was observed when four of the ammonium cations tested were used as templates. The first evidence that these observations are the result of a specific template effect is ESI mass spectrometry analysis, which reveals the in situ formation of complexes only between the templates and the amplified cyclic trimer.

The interaction between each effective template and the amplified receptor was proven by the observation of intermolecular nOes between the receptor and protons of the templates in the ^1H ROESY experiments. The binding constants for the complexes are in accordance to the response they exert on the system: Acetylcholine ($K=230\ M^{-1}$) is the best template, NMQ ($K=150\ M^{-1}$) amplifies the trimer to a similar extent as $EtMe_3NI$ ($K=140\ M^{-1}$), and $BnMe_3NI$ possesses the smallest binding constant ($K=80\ M^{-1}$) and produces the weakest response from the system.

Molecular recognition with [18]crown-6 was used to check that hydrazone exchange is not switched off by the excess of template necessary to shift the equilibrium towards cyclic trimer **2**. This simple experiment represents a useful test to confirm that hydrazone exchange based mixtures are dynamic at any moment.

The objective of this work was to generate a dynamic system which is simple enough to permit the use of conventional analytical techniques for its characterization, and still contains one interesting member for recognition based in a known receptor. The simplicity of the system facilitates carrying out the necessary control experiments in a proof of principle step; however, its low diversity decreases the chances of discovering new interesting receptors.

The next obvious step is to expand the diversity and screen with a range of templates. The main challenge is analytical in nature; how can active species be identified from such diverse mixtures? Although changes in product distribution should be indicative of lead compounds, many building blocks will not be components of these lead compounds and thus not all the library members will be consumed to generate the amplified species. The existence of a wide range of untemplated species could cloud traditional analysis such as HPLC.

Fourier transform ion cyclotron electrospray mass spectrometry (FT-ICR-MS) has been demonstrated as a powerful technique for the analysis of complex DCLs[4d,17] and conventional combinatorial libraries.[18] Taking advantage of the high resolution and sensitivity of FT-ICR-MS, small increases in the concentration of the best binders in the library may be detected. Once the best binders have been identified, the library composition can be

tuned by mixing only the right relative amounts of those building blocks which feature in the species that bind to the template.

Alternatively, templates supported on polymer beads can become a very useful tool.[4e,19,20] Since the template is attached to a solid support, the untemplated species will eventually become washed from the solid support by filtration leaving only 'strong' binders appended to the bead. The species attached to the solid phase may be washed off under different solvent conditions that disrupt the template–receptor interaction and subsequently identified. The development of selection techniques using guests appended to polymer beads is pivotal to the management and success of highly diverse DCLs.

What is clear is that dynamic combinatorial chemistry is a highly powerful technique, which by using reversible chemistry can generate and screen highly diverse libraries of candidate molecules for the identification of host and guest molecules with potential as catalysts or drugs. Thermodynamic control invites the template to dictate its preferred receptor thus amplifying the concentration of the receptor. This reversal of thinking, away from design to choreographed molecular amplification, may unlock the doors towards the discovery of unexpected novel receptors.

4. Experimental

4.1. General

HPLC analysis was performed on a Hewlett-Packard 1050 instrument using reversed phase conditions of H_2O/MeCN gradients with a 15 cm×4.6 mm i.d. 3 μm particle size, Supelco ABZ$^+$plus C16 alkylamide column. Data were analysed using HP ChemStation. All NMR spectroscopy was performed on Bruker DRX 400 or DPX 500 instruments and chemical shifts are quoted in parts per million with respect to TMS. Electrospray mass spectra were recorded on a Micromass Quattro-LC triple quadrupole apparatus fitted with a *z*-spray source. The electrospray source was heated to 100°C and the sampling cone voltage (V_c) was 30 V. Samples were introduced into the mass spectrometer source without work-up with an LC pump (Shimadzu LC-9A) at a rate of 4 μl min^{-1} of MeCN/H_2O (1:1). Calibration was performed using protonated horse myoglobin. Scanning was performed from *m*/*z* 200 to 2200 in 6 s and several scans were summed to obtain the final spectrum, which was processed using MassLynx V3.0 software. High-resolution mass spectra were recorded on a Micromass Q-tof instrument, incorporating time-of-flight analysis with electrospray ionization through a standard *z*-spray source. Several scans were summed to obtain the final spectrum which was processed using MassLynx V3.0 software. Calibration was performed using erythromycin as the standard.

4.2. Materials

All chemicals were purchased from Aldrich, Lancaster or Fluka and were used without further purification. All solvents were distilled prior to use and dry solvents freshly distilled from CaH_2 under Ar(g). *N*-methylquinuclidinium iodide was prepared according to a literature procedure.[21] Column chromatography was carried out using silica gel 60 F (Merck).

4.2.1. 3-Carboxybenzaldehyde dimethoxyacetal (4). 3-Carboxybenzaldehyde (5.00 g, 0.033 mol) and ammonium chloride (10 g, 0.20 mol) were refluxed in dry methanol (110 ml) during 72 h under an atmosphere of argon. The reaction mixture was filtered while warm and the solvent removed under reduced pressure to give a white solid. The product was purified by recrystallization from hexane to afford compound **4** as a white solid (6.1 g, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ=8.22 (1H, s, Ar-H), 8.08 (1H, d, *J*=7.8 Hz, Ar-H), 7.71 (1H, d, *J*=7.8 Hz, Ar-H), 7.49, (1H, t, *J*=7.8 Hz, Ar-H, *J*=7.8 Hz, Ar-H), 5.47 (1H, s, CH), 3.35 (6H, s, OMe); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ=171.94 (C=O), 138.73, 132.08, 130.28, 129.50, 128.49 (Ar), 102.33 (CH), 52.67 (OMe).

4.2.2. (*S*) *N*-(3-Dimethoxymethyl-benzoyl)-proline methyl ester (5). L-Proline methyl ester hydrochloride (0.59 g, 3.56 mmol) and 3-carboxybenzaldehyde dimethoxyacetal (0.70 g, 3.57 mmol) were dissolved in dry CH_2Cl_2 (20 ml) containing dry Et_3N (1.0 ml, 7.17 mmol) under Ar(g) and the solution was cooled to 0°C on an ice bath. To this solution were added EDC (0.68 g, 3.57 mmol) and DMAP (25 mg, 0.20 mmol) and the solution stirred at 0°C for 1 h before being allowed to warm to room temperature and stirred overnight. CH_2Cl_2 (120 ml) was added and the solution washed with H_2O (3×100 ml). The organic phase was dried ($MgSO_4$) and the solvent removed under reduced pressure to give a yellow oil. Column chromatography (SiO_2) [EtOAc/Hex, 8:2] yielded the titled compound **5** as a colourless oil (0.76 g, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) δ=7.65 (1H, s, Ar-H), 7.53–7.33 (3H, band, Ar-H), 5.39 (1H, s, C*H*(OMe)$_2$), 4.67–4.63 (1H, m, α-H), 3.76 (3H, s, O*Me*), 3.66–3.50 (2H, band, Pro-C*H*$_2$), 3.31 (6H, s, CH(O*Me*)$_2$), 2.35–2.24 (1H, m, Pro-C*H*$_a$CH$_b$), 2.03–1.99 (2H, band, Pro-C*H*$_2$), 1.91–1.82 (1H, m, Pro-C*H*$_a$H$_b$); ^{13}C NMR (100 MHz, $CDCl_3$) δ=174.1, 170.9 (C=O), 139.7, 137.5 (Ar*quat*), 129.9, 129.6, 128.8, 127.1 (Ar-H), 104.0 (*C*H(OMe)$_2$), 61.7 (α-H), 60.5 (COO*Me*), 54.1, 53.6 (CH(O*Me*)$_2$), 51.3, 30.8, 26.8 (CH$_2$); ESI-MS (+) *m*/*z*=308 [M+H]$^+$, 276 [M−OMe]$^+$.

4.2.3. (*S*) *N*-(3-Dimethoxymethyl-benzoyl)-proline carboxylic acid hydrazide (3). Proline ester **5** (0.74 g, 2.41 mmol) was treated with hydrazine monohydrate (1.2 ml, 24.7 mmol) in methanol (25 ml). The reaction was left overnight before removal of the solvent under vacuum to give a yellow oil which was purified by flash chromatography (SiO_2) [CH_2Cl_2/MeOH, 90:10] to afford the monomer **3** (0.57 g, 77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ=8.31 (1H, s, N*H*NH$_2$), 7.62 (1H, s, Ar-H), 7.53–7.4H (2H, band, Ar-H), 7.40 (1H, m, Ar-H), 5.39 (1H, s, C*H*(OMe)$_2$), 4.67–4.66 (1H, m, α-H), 3.94 (2H, bs, NHN*H*$_2$), 3.59–3.56 (1H, band, Pro-C*H*$_a$CH$_b$), 3.47–3.45 (1H, band, Pro-CH$_a$C*H*$_b$), 3.31 (6H, s, CH(O*Me*)$_2$), 2.37 (1H, m, Pro-C*H*$_a$CH$_b$), 2.11–2.04 (2H, band, Pro-C*H*$_2$), 1.82 (1H, m, Pro-C*H*$_a$H$_b$); ^{13}C NMR (100 MHz, $CDCl_3$) δ=172.0, 170.7 (C=O), 138.5, 135.9 (Ar*quat*), 128.7, 128.3, 127.3, 125.6 (Ar-H), 102.4 (*C*H(OMe)$_2$),

58.6 (α-H), 52.7 ($CH(OMe)_2$), 50.4, 27.8, 25.5 (CH_2); ESI-MS (+) m/z=308 $[M+H]^+$, 276 $[M-OMe]^+$, 244 $[M-2OMe]^+$; HRMS (QTOF) $[M+Na]^+$ $C_{15}H_{21}N_3O_4Na$ requires 330.1430, found 330.1446.

4.3. General procedure for cyclization and templating experiments

5 mM solutions of (*S*) *N*-(3-dimethoxymethyl-benzoyl)-proline carboxylic acid hydrazide (**3**) in freshly distilled chloroform containing TFA (25 mM) were stirred at room temperature during 5 days. The equilibrium is reached within 3 days. The amplification observed is achieved by addition of the ammonium salts either when the reaction is started or after it has reached equilibrium. All templates are solids and thus simply weighed out and added to the reaction. Comparative cyclization experiments were carried out in a 2 ml scale. HPLC or ESI-MS analysis was performed by the removal of 100 μl aliquots for analysis from the reaction mixture.

4.4. Preparation of the dynamic system from the cyclic dimer

2 ml of a 25 mM solution of TFA in freshly distilled chloroform were added to cyclic dimer (2.4 mg, 5×10^{-3} mmol) and the resulting solution stirred for 3 days. The reaction was followed by HPLC and ESI-MS.

4.5. Preparation of the dynamic system from the cyclic trimer (2)

2 ml of a 25 mM solution of TFA in freshly distilled chloroform were added to cyclic trimer (2.4 mg, 3.3×10^{-3} mmol) and the resulting solution stirred for 3 days. The reaction was followed by HPLC and ESI-MS.

4.6. Use of [18]crown-6 to check that the templated system is dynamic

Cyclization reactions were carried out at 5 mM concentration of building block **3** containing 4 equiv. of NMQ (with respect to the amount of **3**) and increasing amounts of TFA (from 1 to 10 equiv. with respect to the amount of **3**). The reaction were stirred for 3 days before [18]crown-6 was added (same number of equivalents as TFA). The amplified monomer was detected by HPLC analysis (260 nm, R_t=2.5 min) and by ESI-MS (m/z=526.4) in all the experiments.

4.7. Purification of cyclic dimer

(*S*) *N*-(3-Dimethoxymethyl-benzoyl)-proline carboxylic acid hydrazide (**3**) (0.28 g, 0.90 mmol) was dissolved in freshly distilled chloroform (180 ml) to afford a 5 mM solution. To this solution was added TFA (0.35 ml, 5 mmol) and the reaction stirred at room temperature for 3 days. After this time, the solution was treated with Amberlyst A-21® beads to remove the TFA, filtered and the solvent removed under reduced pressure to give a white solid. This solid was subjected to silica gel column chromatography [$CHCl_3$/MeOH, 90:10] to give the cyclic dimer as a white solid (0.12 g, 54%). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ=7.96 (2H, s, CH=NR), 7.52 (2H, d, J=6.1 Hz, Ar-H), 7.37 (2H, t, J=6.7 Hz, Ar-H), 7.33 (2H, d, J=6.7 Hz, Ar-H), 5.77 (2H, m, α-H), 3.90–3.80 (4H, m, Pro-CH_2), 2.51–2.49 (2H, m, ProCH_2), 2.16–2.01 (6H, band, Pro-CH_2); ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$) δ=173.3, 169.6 (C=O), 143.6 (Ar*quat*), 136.9 (Ar*quat*), 132.4, 130.7, 129.3 (Ar-H), 121.2 (CH=NR), 57.7 (α-C), 32.1, 28.8, 24.9 (Pro-CH_2); ESI-MS (+) m/z=487.3 $[M+H]^+$; HRMS (QTOF) $[M+Na]^+$ $C_{26}H_{26}N_6O_4Na$ requires 509.1913, found 509.1891.

4.8. Purification of cyclic trimer (2)

(*S*) *N*-(3-dimethoxymethyl-benzoyl)-proline carboxylic acid hydrazide (**3**) (400 mg, 1.30 mmol) was dissolved in chloroform (130 ml) to give a 10 mM solution. To this solution was added *N*-methyl quinuclidinium iodide (1.3 g, 5.21 mmol) and TFA (0.1 ml, 1.30 mmol)—as a solution in chloroform. The reaction was left stirring at room temperature for 2 days before washing with H_2O (100 ml) and subsequent washing of the aqueous layer with three portions of chloroform (100 ml). The organic extracts were combined, dried ($MgSO_4$) and the solvent removed under reduced pressure to give a white solid. This solid was subjected to silica gel column chromatography using gradient elution [$CHCl_3$/MeOH, 98:2→90:10] to give the cyclic trimer as a white solid (158 mg, 50%). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ=8.49–7.14 (15H, band, Ar-H and CH=NR), 5.82–4.67 (3H, band, α-H), 3.93–3.49 (6H, band, Pro-CH_2), 2.43–2.05 (12H, band, Pro-CH_2); ESI-MS (+) m/z=730.4 $[M+H]^+$; HRMS (QTOF) $[M+Na]^+$ $C_{39}H_{39}N_9O_6Na$ requires 752.2921, found 752.2913.

4.9. Job plot

Equimolar solutions (5 mM) of trimer **2** and acetylcholine chloride in 5% $CD_3OD/CDCl_3$ were prepared and mixed in various ratios. ^{1}H NMR spectra of the resulting solutions were recorded, and the change in chemical shift of the *N*-methyl proton signals of the guest was analysed.[15]

4.10. Host–guest titrations

Solutions of cyclic trimer **2** (25 mM, $CDCl_3/CD_3OD$, 95:5) were titrated into 5 mM solutions of the different guests ($CDCl_3/CD_3OD$, 95:5). The chemical shifts of the guest protons were plotted against the host concentration. The binding constants were calculated by non-linear least-squares fitting method for 1:1 complexes using EQNMR program.[16] The values were estimated to have errors within 20%.

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