

Two-phase dynamic combinatorial discovery of a spermine transporter†

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The discovery, in a two-phase dynamic combinatorial library, of an unexpected linear receptor and transporter for spermine is described.

Dynamic combinatorial chemistry has emerged as a powerful tool for the discovery of new molecular receptors. Dynamic combinatorial libraries (DCLs) rely on reversible chemical reactions that interchange under thermodynamic control.^{1,2} Introducing a guest that interacts with members of the library will shift the equilibrium composition, amplifying the strong binders at the expense of other library members.³ The amplified host can then be isolated and the host–guest system characterised. Most of the molecular receptors discovered in DCLs have drawn inspiration from previously known synthetic receptors,⁴ and the discovery of structurally unexpected receptors using DCLs is still relatively rare.⁵

We present here the discovery of an unexpected receptor for the polyamine spermine using our recently-described two-phase DCL approach and we also show that the receptor acts to carry spermine across a bulk liquid membrane.⁵ In the course of this work we developed a sensitive analytical method to selectively quantify small amounts (μM) of spermine in solution. Spermine is an attractive target for recognition as it has many biological roles in normal cell division and growth,⁶ it forms strong interactions with DNA and RNA,⁷ and it has an undefined geometry in solution.⁸

A DCL was set up with building blocks **1**,⁹ **2**,¹⁰ and **3** (Fig. 1). Recently we described the idea of two-phase dynamic combinatorial chemistry in which building blocks in one phase combine with building blocks in the other phase, increasing the available structural diversity.⁵ In particular we addressed the possibility of achieving molecular recognition of polar templates by non-polar receptors and *vice versa*. The discovery of receptors soluble in one solvent for compounds that are soluble in a different, immiscible, solvent is a challenge for design, synthesis and characterisation. Spermine appeared to be an ideal target for this approach.

Building blocks **1** and **2** are aromatic thiols equipped with carboxylate groups and are therefore soluble in aqueous solution at neutral pH, whereas the mono-thiol building block **3** bearing a methyl ester group is soluble in chloroform. The thiol functionality allows the building blocks to be oxidised to disulfides in the presence of air; thiolate anions then induce disulfide exchange under thermodynamic control.¹¹

When a mixture of building blocks **1**, **2** (dissolved in aqueous buffer solution) and **3** (dissolved in chloroform) was allowed to oxidise and equilibrate after addition of *N*-methyl morpholine (NMM) as a base and Bu_3N as a phase-transfer reagent, a DCL was formed (Fig. 2). Analysis of the composition of the library was by LC-MS, injecting each phase separately. The presence in the organic phase of building blocks that are usually only soluble in aqueous phases was encouraging. The experimental conditions established in previous work were used, ensuring equilibration under thermodynamic control, where it was shown that more of the water soluble materials could be forced into the organic phase using Bu_3N as a phase-transfer reagent.^{5a} None of the building block **3** was observed in the aqueous phase in any of the experiments performed, nor was any hydrolysis of the methyl ester **3** observed. Likewise, none of the dicarboxylic acid building block (**2**) was observed in the organic phase.

The top chromatogram in Fig. 2 shows that the aqueous phase exclusively contains the linear dimer of **2** (**2**₂), both in the templated and the untemplated library. In the organic layer, the untemplated library (Fig. 2, middle) contains the linear dimer of **3** (**3**₂), the cyclic trimer of **1** (**1**₃), the linear trimer of **1** and **3** (**3**-**1**-**3**), and small amounts of the linear tetramer (**3**-**1**-**1**-**3**). The transfer of the water soluble building block **1** and the library members containing **1** is enhanced by the Bu_3N . In the spermine templated library (Fig. 2, bottom) an amplification of the linear trimer (**3**-**1**-**3**) by 50% is observed by integration of the peaks in the HPLC chromatogram at the expense of the other library members present in the organic phase. This type of amplification in a DCL is an indication of a favourable interaction between the species and the template.³ The change in composition of the linear tetramer (**3**-**1**-**1**-**3**) is less than 2%.

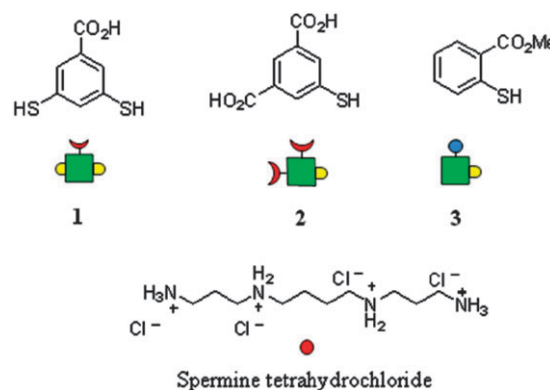


Fig. 1 Structures of the mono-thiol and di-thiol building blocks (**1**, **2** and **3**) and the polyamine template spermine tetrahydrochloride.

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† Electronic supplementary information (ESI) available: Calibration curves, LC-MS data and a description of how spermine concentrations were determined. See DOI: 10.1039/b902842k

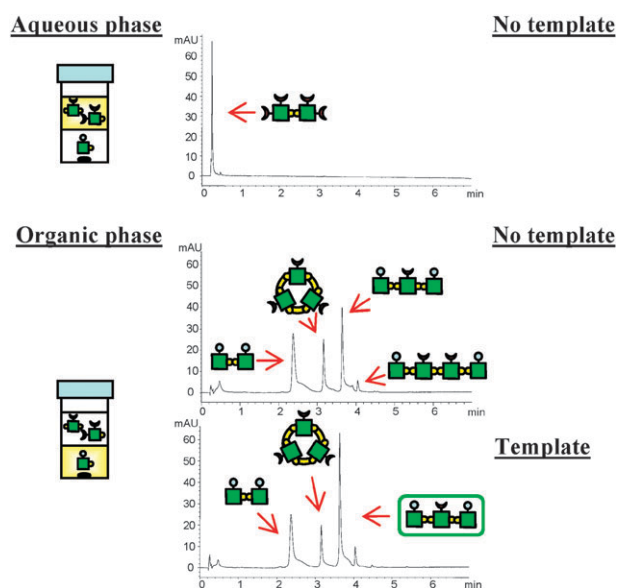


Fig. 2 Reverse phase HPLC chromatograms (260 nm) of libraries containing building blocks (**1**, **2** and **3**). Top: control library, aqueous phase (no template). Middle: control library, organic phase (no template). Bottom: organic phase after addition of spermine tetrahydrochloride as a template.

The amplified linear trimer (Fig. 3) was conveniently separated by chromatography on silica and isolated as the carboxylate salt; Bu_3NH^+ was identified as the counter ion by NMR analysis in CDCl_3 .

As spermine tetrahydrochloride is insoluble in CDCl_3 , and since a solution of the trimer in CDCl_3 did not bring the spermine salt into solution, complexation studies were attempted using free-base spermine. Upon addition of a solution of free-base spermine to a solution of the receptor in CDCl_3 , free-base Bu_3N appeared in the ^1H NMR spectrum, demonstrating that the Bu_3N had been replaced by spermine. Unfortunately, the linear bis-disulfide trimer was not stable for extended periods in the presence of excess free-base spermine.† Equilibration of the bis-disulfide generated a library of macrocycles from **1** (**1**₃ and **1**₄) which started to precipitate, and the dimer of **3** (**3**₂) which started to appear in the NMR spectrum. This prevented further two-dimensional NMR experiments to elucidate the structure of the complex in solution. Nevertheless, it was possible to construct a Job plot using free-base spermine (Fig. 4a), and this showed the stoichiometry of the complex to be two molecules of receptor to one molecule of spermine.

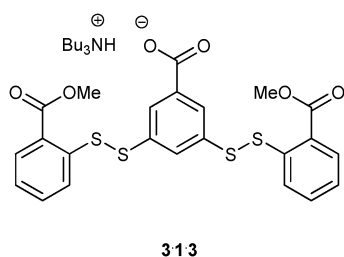


Fig. 3 Structure of amplified trimer (**3-1-3**).

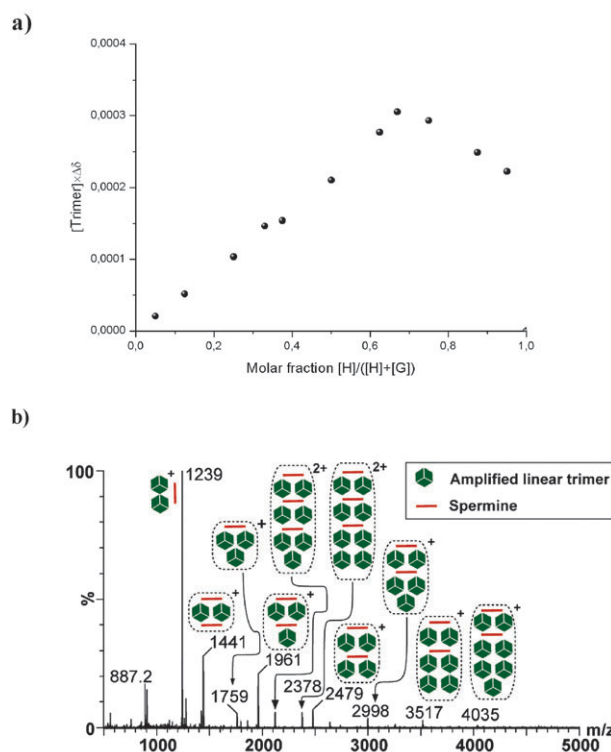


Fig. 4 (a) Job plot between the receptor (**3-1-3**) and free-base spermine giving 2 : 1 stoichiometry. (b) Nano-electrospray mass spectrum of a 1 : 1 mixture of receptor and spermine.

The 2 : 1 stoichiometry was further indicated by nano-electrospray ionisation (n-ESI) mass spectrometry (MS) (Fig. 4b). The most intense peak in the mass spectrum of a freshly prepared mixture of free-base spermine and the receptor is clearly the 2 : 1 complex. Additionally, aggregates larger than the 2 : 1 complex were also observed in the mass spectra. These species can be accounted for by the small size of the receptor–spermine complex compared to the fission droplets formed in the ESI process. The micromolar concentration of receptor and spermine allows for multiple occupancies of the complex and any free receptor or spermine within a single fission droplet and can result in ‘non-specific’ interactions.¹² Most crucially the absence of a complex with 1 : 1 stoichiometry allows us to conclude that the 2 : 1 complex is preferentially formed.

The fact that the receptor binds a guest template in a phase where the template is insoluble complicates the study of the solution phase behaviour of the system. However, one attractive application for such receptors is the transport of molecular species across bulk liquid membranes. One of the applications of artificial membrane carriers is as vehicles for drug delivery.¹³

Transport studies were performed in a U-tube cell (Fig. 5),¹⁴ consisting of a chloroform phase separating two neutral aqueous sources and receiving phases. When the U-tube was charged with a 5 mM solution of the receptor in CHCl_3 and the source phase was charged with a 600 mM solution of spermine tetrahydrochloride with NMM and Bu_3N , significant transport of spermine to the receiving phase took place.

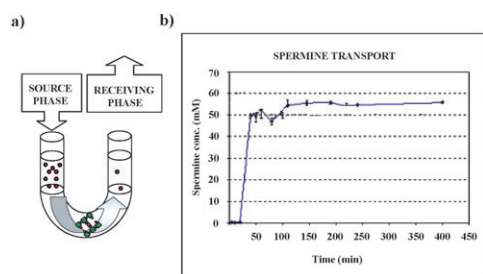


Fig. 5 (a) U-tube experiment showing transport of spermine across an organic phase using the receptor found in the DCL. (b) Transported spermine (average of 5 determinations) determined at different sample periods by FIA-MRM.

The system was able to transport approximately 60 mM of a 600 mM solution of spermine using 5 mM solution of the bis-disulfide receptor molecule. The transport takes place over approximately 30 minutes, after which it halts, apparently due to the instability of the bis-disulfide under the experimental conditions: the disulfides start to equilibrate to give a DCL, diminishing the amount of receptor in the organic phase and shutting down transport.[†] Preliminary transport studies using the organic phase from a pre-equilibrated DCL (as in Fig. 2) gave similar transport results. Control experiments showed no detectable transport of spermine into the aqueous receiving phase when carrier **3-1-3** was omitted.

The amount of spermine transported was measured using a FIA-MSMS method (flow injection analysis coupled to tandem mass spectrometry).¹⁵ Mass spectral quantification of spermine was performed by electrospray ionisation in positive ion mode, monitoring the mass transitions m/z 203 \rightarrow 129 by a multiple reaction monitoring (MRM) experiment. In MRM, the spermine (m/z 203) is isolated in the ion trap and then fragmented into two daughter ions; the more intense daughter ion (m/z 129) is isolated in the ion trap and measured with high sensitivity. Only molecules with a m/z of 129 fragmented from m/z of 203 are observed. A calibration curve is constructed by comparing the known concentrations of spermine in the calibration standard solutions (range 0.5 to 5 μ M) to the peak area of the daughter ion m/z 129 obtained with the FIA-MRM method. The concentration of spermine in the unknown sample could be calculated by comparing the peak area obtained with FIA-MRM method for the m/z 129 ions with the calibration curve. The transport samples were diluted with water before analysis to reach a concentration of spermine within the calibration range. The FIA-MRM method was optimised to obtain 5 replicate injections of sample and standard solutions in 2.5 minutes with good precision. The FIA-MRM method developed was validated in terms of linearity, precision, accuracy and stability in a solution of spermine (see ESI[†] for details).

In conclusion, we have demonstrated for the first time that unexpected receptors can be identified using two-phase dynamic combinatorial chemistry. We have identified a receptor for the biologically relevant polyamine spermine, showed that the stoichiometry of the complex is 2 : 1 and that the receptor works as a carrier of spermine across bulk liquid membranes. A conceptually similar set of observations using different

chemistry is reported by the Kiel group in an accompanying communication.¹⁶ In addition, this is the first example of the amplification of a linear species in a disulfide based DCL where there is a possibility of forming cyclic species.

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Notes and references

[†] Disulfide exchange is dependent on pH and other physical experimental changes, thus the bis-disulfide may re-equilibrate. The excess of spermine (template) and different solvent conditions can modify the 'library' composition as compared to the original two-phase conditions, thus allowing different oligomers to dominate the composition. This leads to the loss of the **3-1-3** trimer in this experiment.

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