

Supporting Information for:

**Two-phase Dynamic Combinatorial Discovery of a Spermine
Transporter**

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Materials and Methods

All chemicals were purchased from commercial suppliers and used without further purification. All solvents were reagent grade quality (MeOH, CH₂Cl₂) or HPLC grade (water, chloroform, acetonitrile 190 far UV gradient, Romil). Formic acid was acquired from Romil.

Solvents used in the DCLs, the transport experiments and when using the LC-MS equipment were: Water, chloroform (stabilised with 1% EtOH) and acetonitrile 190 far UV gradient were HPLC quality from Rathburn. Formic acid used with the LC-MS equipment was acquired from Fluka.

The thiol derivative **3** was purchased from commercial suppliers.

The thiol derivatives **1**¹ and **2**² were prepared using slightly modified literature procedures.

¹ B. M. R. Liénard, N. Selevsek, N. J. Oldham and C. J. Schofield, *ChemMedChem*, 2007, **2**, 175-179.

² T. Tsuboi, Y. Takaguchi and S. Tsuboi, *Bull. Chem. Soc. Jpn.*, 2008, **81**, 361-368.

Dynamic Combinatorial Libraries

Aqueous phase: Building block **1** and **2** (5 mM overall) were dissolved in water and the pH was adjusted to pH 7-7.4 by addition of a base. In the case of tris-HCl (1M), 100 μ L was added to the aqueous stock solution.

Organic phase: Building block **3** (5 mM) was dissolved in chloroform. Then, 10 equivalents of base and of Bu₃N were added

Control experiments were performed in which the phase containing the building block was mixed with a pure solvent phase. The mixtures were allowed to oxidise and equilibrate during 4 weeks. The libraries generally reached equilibrium after 1 week.

Library Analysis

Analyses were performed using an Agilent 1100 series HPLC with a diode array UV/Vis detector and interfaced to an Agilent XCT ion-trap mass spectrometer. Analyses were performed using reversed phase HPLC silica based columns: Kovalsil MS-H (33 mm \times 4.6 mm, 1.5 μ m). Using an injection volume of 0.3 μ L, a flow rate of 1 mL/min and gradient elution for Kovalsil MS-H (30 to 40% over 1 min, 40 to 70% over 4 min and 70 to 90% over 1 min) of acetonitrile in water. Both acetonitrile and water contain 0.1% v/v formic acid. Analyses were monitored at 260 nm wavelength. The oven temperature was set up at 40 °C. Alternate positive and negative ion mass spectra were acquired in the ultrascan mode (26000 m/z \cdot sec⁻¹) using electrospray ionisation (drying gas temperature: 350 °C, nebulizer pressure: 55 psi, drying gas flow: 12 L/min, HV capillary: 4000 V; ICC target: 200 000) and atmospheric pressure chemical ionization (drying gas temperature: 400 °C, nebulizer pressure: 60 psi, drying gas flow: 7 L/min, HV capillary: 3500 V; corona current: 10000 nA, ICC target, 200 000).

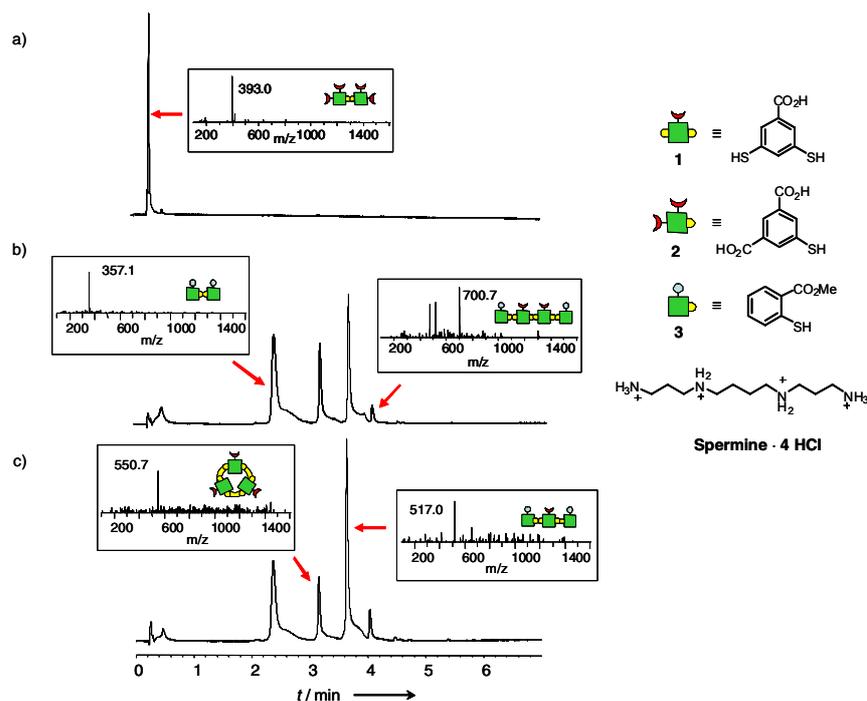


Figure S4. HPLC (monitored at 260 nm wavelength) and MS analysis of library set up with building blocks 1, 2 and 3 after equilibration, (a) aqueous phase injection (no template); (b) organic phase injection (no template); (c) organic phase after addition of spermine tetrahydrochloride as a template.

Transport experiment

The transport experiment was performed using the U-tube cell. The source phase consisted of 150 μL of a 600 mM solution of spermine tetrahydrochloride with 5 μL of NMM (solution at neutral pH); the membrane phase consisted of 400 μL of a receptor solution in chloroform (5 mM) with tributylamine (5 μL); the receiving phase was made of 150 μL of deionised water. Stirring in this experiment was kept constant. Samples from the aqueous phase (5 μL) were collected by a 25 μL Hamilton syringe and analysed by LC-MS under the following conditions.

The samples were introduced by FIA into the MS using an Agilent 1100 HPLC system with binary high pressure pump with a micro vacuum degasser and a 2 mL autosampler. The HPLC was controlled with ChemStation A10.02 software (Agilent). A 20 μm diameter, 1 m flexible stainless steel tubing was used to connect the outlet of the HPLC injector valve directly into the orthogonal ElectroSpray (ESI) ion source of an Agilent 1100 Series LC/MSD ion trap XCT. The software used for the ion trap was Bruker.

Method Development

Spermine, a biogenic amine occurring in food is commonly quantified in biological matrixes, requiring laborious samples preparation procedures and subsequently the need for internal standards.³ In the transport experiments there is no biological matrix and therefore there is no need for the use of internal standard for the quantification of spermine. Many methods have been developed to quantify various biogenic amines including spermine in the same analysis; most of these methods are chromatographic including capillary electrophoresis.⁴ In our transport experiments we are only interested in the quantification of spermine in the two aqueous phases so there is no need for the time consuming chromatography preferring a flow injection analysis (FIA) method. Spermine being an aliphatic amine cannot be analysed directly by UV our standard setup. Use of MS avoids the need for derivatisation.

Flow injection analysis (FIA)⁴ can deliver the sample directly into the Electrospray ion source transported by an appropriate mobile phase; the sample is injected into the HPLC flow path using a capillary stainless steel tubing to connect the HPLC injector to the Ion source. The ESI ion source is used in the positive mode to protonate the spermine.

The presence of large concentrations of strongly ionisable tributylamine (186.1 m/z) in the transport experiments gave a comparable weak (+)-ESP MS signal for the less well ionisable spermine ions (203.2 m/z) at the concentrations used.

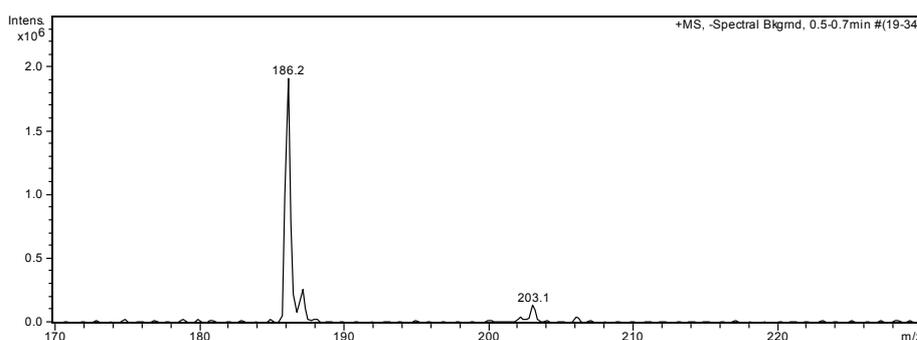


Figure S5. MS spectrum of spermine in the presence of tributylamine in (+)-ESP. The peak at 186 m/z is tributylamine and the peak at 203 m/z in spermine.

³ a) S. Millán, M. C. Sampedro, N. Unceta, M. A. Goicolea and R. J. Barrio, *Anal. Chim. Acta*, 2007, **584**, 145-152; b) A. Ōnal, *Food Chemistry*, 2007, **103**, 1475-148; c) C. Simó, M. V. Moreno-Arribas and A. Cifuentes, *J. Chromatogr. A*, 2008, **1195**, 150-156.

⁴ C. C. Sri, S. K. Shukla and P. N. Sarma, *J. Flow Injection Anal.*, 2008, **25**, 20-23.

By using Multiple Reaction Monitoring (MRM) experiments, the spermine ion (m/z 203.2) reaching the trap is isolated from tributylamine and all other positive ions by removing them from the trap. The isolated spermine (203.2 m/z) fragments into 2 daughter ions, an intense ion at 129.2 m/z and a less intense ion at m/z 112.2 under the MS-MS experimental conditions of the iontrap. The specific transition from the parent 203.2 to 129.2 gave excellent signal to noise levels and reproducible signals.

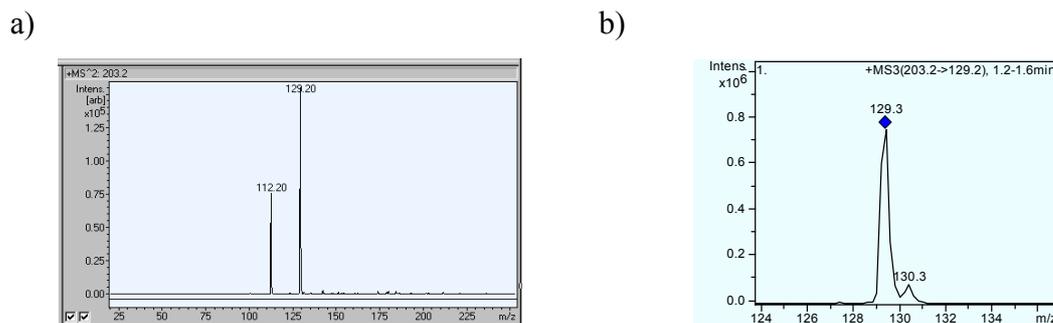


Figure S6. a) MS-MS fragmentation of spermine (m/z 203.2) giving two daughter ions (m/z 112.2 and 129.2); b) MRM peak for m/z 129.2 from parent m/z 203.2.

Optimisation of FIA-MRM parameters gave excellent limit of detection of 0.01 μM . However there was a drawback. While the range of concentration was linear up to the range of 1.5 μM with a correlation coefficient of > 0.90 , the concentration range up to 5 μM followed a pronounced polynomial curve. The parameters for the ionisation and the transport of the spermine to the trap were redeveloped to give acceptable signal intensity and peak shape while the concentration range of 0.1 to 5 μM was close to the initial linear region of the polynomial curve. The parameters used for the study were:

Capillary entry: -2300 V, Capillary exit +86.9 Vt, Skimmer + 30.3 Vs, Octopole -1 +7.95 V, Octopole-2 +1.57V with a Octopole Rf of 123 bpp, lens-2 at -3.4V and lens -48.4.

The trap parameters such as scanning range (120-140 m/z), maximum accumulation time, and number of replicates were optimised to give the best peak shape for the transition. Using ultrascan at 200.000 mz/sec gave enough points to accurately define the narrow peaks.

Method validation

Spermine was injected into the MS at a concentration of $< 5 \mu\text{M}$ to avoid contamination of the Trap. However, spermine at concentrations $< 10 \mu\text{M}$ was found to have very poor stability in solution. This was worse at higher dilution showing a 50% reduction in 30 minutes for a $0.5 \mu\text{M}$ concentration. Amines can react with CO_2 to form carbamic acid.⁵ The use of extensively degassed water and water purged with argon did not significantly improve the stability in solution of the low concentration of spermine. The use of plastic vials instead of glass vials and the dilution of spermine in a MeCN/water mixture did not improve the stability in solution either. The use of a concentration between 2-5 μM had good stability in solution within 30 minutes. Using 1 μL injection demonstrated good reproducibility while the injection of $< 5 \text{ pmol}$ into the MS did not result in a lasting contamination of the MS equipment. The LCMS method was proven to be robust to the presence of the expected levels of tributylamine.

By using 5 replicate injections of 1 μL of the sample and standard solutions into an HPLC flowing at 0.5 ml/min into the ion source using a 30:70 MeCN+0.1% HCOOH/MeCN+0.1% HCOOH gave good peak shapes and peak resolution. The 5 replicated peaks eluted within 2.5 minutes. The use of 5 replicates gave confidence in the data and the possibility of justifying the removal of obvious outliers.

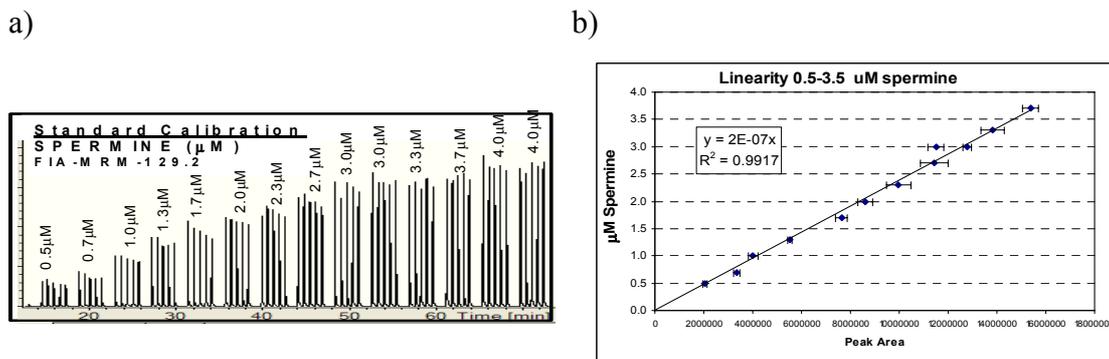


Figure S7. a) FIA-MRM peaks: 129.2 from 203.2 m/z transition (5 replicates injections per calibration standard) in the range 0.5 to 4.0 μM spermine, b) Corresponding calibration.

⁵ a) H. Fischer, O. Gyllenhaal, J. Vessman, and K. Albert, *Anal. Chem.*, 2003, **75**, 622-626; b) D. E. Penny and T. J. Ritter, *J. Chem. Soc., Faraday Trans. 1*, 1983, **79**, 2103-2109

Job plot

Stock solutions of the host trimer (8.0 mg, 1.14×10^{-5} mol in 3 mL CDCl_3 , 3.79 mM) and guest (free base spermine, 7.66 mg, 3.75×10^{-5} mol in 10 mL CDCl_3 , 3.79 mM) were prepared of the same concentration. The molar ratio were varied in the different $^1\text{H-NMR}$ experiments and all spectra were recorded within 5 minutes after the solutions were prepared ensuring that the mixtures had not started to equilibrate. The change in chemical shift of the protons from the central benzene ring of the receptor was monitored and the Job plot is shown in fig. 4a in the main paper.

Synthesis of receptor

Mono-thiol **2** (6.1 mg, 0.0307 mmol) was dissolved in H₂O (6 mL) and NMM (15 μ L) was added (0.00513 M solution in **2**). Di-thiol **1** (6 mg, 0.0322 mmol) was dissolved in H₂O (6 mL) and NMM (15 μ L) (0.00536 M solution in **1**). Mono-thiol **3** (6.96 mg, 0.0414 mmol) was dissolved in CHCl₃ (stabilized with 1% ethanol, 8 mL) and Bu₃N (15 μ L) was added (0.00517 M solution in **3**). Mix the organic solution (8 mL) with the solution of **1** (4 mL) and **2** (4 mL) in a vial and add one equivalent of spermine tetrahydrochloride to the mixture. The two-phase system was stirred vigorously (magnetic stirring) at room temperature for 2 weeks. The phases were separated and the aqueous phase extracted with CHCl₃ (2 \times 20 mL). The combined organic extracts were dried (Na₂SO₄), filtered through paper and concentrated *in vacuo*. The crude material was purified by dry column vacuum chromatography (CH₂Cl₂ to 15% MeOH in CH₂Cl₂ with 1% increments) to give a colorless oil that solidified upon standing. Yield: 10 mg, 65 %. ¹H-NMR (500 MHz, CDCl₃): 0.95 (t, 9H), 1.35-1.39 (m, 6H), 1.69-1.72 (m, 6H), 3.01 (t, 6H), 3.95 (s, 6H), 7.16 (t, 2H), 7.36 (t, 2H), 7.70 (t, 1H), 7.75 (d, 2H), 7.96-7.98 (m, 4H). ¹³C-NMR (125 MHz, CDCl₃): 13.6, 20.1, 25.1, 29.7, 52.4, 125.4, 125.6, 126.6, 126.8, 128.2, 131.4, 133.1, 133.1, 138.7, 140.2, 166.6, 168.8. MS (ESI): 517.0 *m/z*.

Nano-electrospray mass spectrometry

Nano-electrospray mass spectra were acquired on an LCT mass spectrometer (Waters, Manchester, UK) or a Qstar XL (MDS Sciex, Applied Biosystems, Concord, ON, Canada) using a previously described protocol optimized for the transmission of noncovalent complexes.⁶ The concentration of complexes, spermine, and linear trimer infused into the mass spectrometer was 0.565 mM. External calibration of the spectra and calibration of the Qstar mass spectrometer was achieved using a 100 mg mL⁻¹ solution of caesium iodide. Data were acquired and processed with Analyst QS (MDS Sciex, Applied Biosystems) and MassLynx 4.0 software (Waters, Manchester, U.K.). All spectra are shown with minimal smoothing and without background subtraction.

Typical instrument parameters, in positive ion mode, on the Qstar XL were: ion spray voltage 1.7 kV, declustering potential 50 V, focusing potential 50 V, declustering potential 15 V, quadrupole voltage (Q0) 15 – 80 V, collision gas (CAD) 3, ion release delay 6 and ion release width 5. Experiments were acquired at instrument base pressure of 6.5 mbar.

Typical instrument parameters, in positive ion mode, on the LCT were: capillary voltage 1.2 kV, sample cone 10 - 200 V, extraction cone 0 - 100 V, ion transfer stage pressure (monitored by a Pirani gauge), 2.6 – 3.3 mbar; TOF analyzer pressure (monitored by a Penning gauge), 1.1 – 1.7 × 10⁻⁶ mbar.

⁶ H. Hernández, C. V. Robinson, *Nature Protocols*, 2007, **2**, 3, 715-726.