Template-Directed Synthesis of Bivalent, Broad-Spectrum Hosts for Neuromuscular Blocking Agents

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Abstract: We report on the synthesis of bivalent water soluble calix[4]arene and calix[5]arene hosts, **Super-sCx4** and **Super-sCx5** as new broad-spectrum supramolecular binders of neuromuscular blocking agents (NMBAs). Synthesis was achieved using the target bisquaternary amine NMBAs as a template to link two highly anionic *p*-sulfonatocalixarene building blocks in aqueous solution. Bivalent anionic hosts **Super-sCx4** and **Super-sCx5** bind by engaging both quaternary amines present on a variety of NMBAs. We report low μM binding to structurally diverse alkyl, steroidal, curarine and benzyisoquinoline NMBAs with high selectivity over the neurotransmitter acetylcholine.

Neuromuscular blocking agents (NMBAs) are frequently administered in clinical practice during anesthesia. NMBAs block the action of acetylcholine (ACh) by binding nicotinic acetylcholine receptors (nAChR) at the post synaptic endplate of the neuromuscular junction.^[1] A variety of structurally diverse NMBAs are used in clinical practice to accommodate different pharmacological needs.^[2] Although NMBAs vary in structure, they adhere to a pharmacophore model having two alkyl ammonium ions separated by ~ 14 Å in their lowest energy conformation.^[3] NMBAs can thus span the inner diameter of the nAChR and simultaneously block both ACh binding pockets (Figure 1a). After surgery, NMBA reversal facilitates patient recovery.

While NMBAs are bivalent, all reported supramolecular reversal agents contain a single macrocyclic binding motif.^[4] Supramolecular reversal agents administered by intravenous injection bind free NMBAs in the blood, lowering the concentration of NMBAs at the neuromuscular junction and reversing effects.^[5] Several NMBA reversal agents have been developed from macrocyclic hosts, including cyclodextrins, cucurbiturils and pillararenes (Figure 1b). These hosts operate via an inclusion binding mode that is inherently limited by the size of the host cavity and the fit of the NMBA. Sugammadex, a γ -cyclodextrin host that is approved for clinical use in over 50 countries, reverses steroidal NMBAs vecuronium and rocuronium.^[6] However, its cavity is too small to achieve reversal of wider curarine and benzyisoquinoline NMBAs and it is similarly inactive towards the thinner NMBA suxamethonium.^[7] Likewise, pillar[5]arene is too small to form a threaded complex with steroidal NMBAs, but thioether carboxylate decafunctionalized pillar[5]arene can form a nesting complex with rocuronium.^[8] Water-soluble carboxylatopillar[6]arene strongly binds the thinner NMBA suxamethonium,^[9] and sulfated Pillar[6]maxQ strongly binds steroidal NMBA's.^[10] Calabadiion 2, an acyclic cucurbituril, has broader binding preferences, reversing steroidal and benzyisoquinoline NMBAs, vecuronium, rocuronium and cisatracurium but binding to suxamethonium has not been reported.^[11]

Figure 1. a) Cross section of nAChR inhibited by an NMBA binding both ACh sites. b) Supramolecular reversal agents reported to date contain a single binding site. c) We propose a reversal agent with dual binding sites separated by a flexible linker.

To complement a family of molecules with diverse biscationic structures but a common, geometrically defined pharmacophore, we chose a conceptually new approach: building bivalent hosts using two promiscuous, but carefully spaced binding pockets (Figure 1c and Figure 2). We selected *p*-sulfonatocalixarenes (sCx4 and sCx5), as their anionic upper rims and dynamic bowl like cavities are known to bind many kinds of cationic moieties.^[12]

Figure 2. Chemical structures of bivalent calixarene hosts, NMBAs and choline.

Figure 3. NMBA templated synthesis forms bivalent calixarene hosts with a flexible hydrazine linker. **a)** Synthetic scheme of **Super-sCx4** templated by decamethonium in $(\text{NH}_4)_2\text{CO}_3$ (50 mM, pH 6.4) in D_2O . **b)** ^1H NMR spectrum of i) decamethonium ii) addition of 2 eq **sCx4-CHO** (1) (aldehyde highlighted by green diamond) iii) final crude after completed reductive amination (hydrazine linkage highlighted by blue star). Red dotted line shows encapsulation of methyl peaks throughout the reaction. **c)** UPLC-MS of final crude shows major product formation of bivalent **Super-sCx4** and minor formation of monovalent **sCx4-CH₂NHNH₂**. **d)** Synthetic scheme of **Super-sCx5** templated by pancuronium in $(\text{NH}_4)_2\text{CO}_3$ (50 mM, pH 6.4) in D_2O .

Templated synthesis with the NMBA decamethonium was effective at creating a new bivalent host (Figure 3a). Simple models predicted that a very short linker would be needed between calixarenes, and we repeatedly found that late-stage covalent coupling was hampered by the mutual repulsion of two highly anionic calixarene building blocks. To overcome this barrier, monofunctionalized sCx4-CHO (**1**) and decamethonium were mixed in a 2:1 ratio in aqueous solution. *In situ* ^1H NMR showed upfield shifts of bound decamethonium methyl groups (Figure 3bii). 1D DOSY NMR on the uncomplexed calixarene sCx4-CHO (**1**) provided a hydrodynamic radius of 7.4 ± 0.3 Å,^[13] which increased to 11.7 ± 0.3 Å upon complexation with decamethonium, supporting the formation of a templated 2:1 complex under the reaction conditions (Table 1, Figure S58). Addition of hydrazine creates a complex system with two reversible imine bonds and calixarenes engaged on either end of the templating NMBA. Reduction of imine bonds locks the structure, forming the bivalent host **Super-sCx4**. Continuous NMBA complexation throughout the reaction is observed by further upfield shifted methyl peaks in the final crude reaction mix (Figure 3biii). UPLC-MS of the crude reaction identified the bivalent host **Super-sCx4** as the major product (50% isolated yield) with minor formation of monovalent **sCx4-CH₂NHNH₂** (Figure 3c).

We also developed a bivalent calix[5]arene analogue (Figure 3d). Novel building block sCx5-CHO (**5**) was synthesized in four steps by site-specific functionalization of the calix[5]arene scaffold (See SI). Initial templation attempts with sCx5-CHO (**5**) and decamethonium showed the guest adopting a folded conformation inside the larger sCx5-CHO (**5**) cavity, forming a 1:1 complex (Figure S25) and preventing the success of the templated synthesis. The steroidal NMBA pancuronium was selected as a more rigid template, as it has been shown to form a 2:1 complex with similarly sized cucurbit[7]uril.^[14] Monofunctionalized sCx5-CHO (**5**) and pancuronium were equilibrated in a 2:1 ratio in aqueous solution. Upfield shifts of the quaternary amine methyl groups were observed by *in situ* ^1H NMR reaction monitoring (Figure S26). 1D DOSY NMR of the complexed calixarene supports the formation of a 2:1 complex (Table 1, Figure S63). Bivalent **Super-sCx5** was isolated in a 25% yield after HPLC purification, and a slightly larger hydrodynamic radius of 12.6 ± 0.2 Å for **Super-sCx5** was determined (Table 1, Figure S65).

Templation by NMBA is essential to the formation of each bivalent host. Control reactions without NMBA templates produced little or no detectable **Super-sCx** products (UPLC-MS), instead resulting in the formation of monovalent **sCx4-CH₂NHNH₂** and **sCx5-CH₂NHNH₂**, and other reduced monovalent calixarenes (Figure S24 and S27).

A wide variety of NMBA's form 1:1 complexes with the new bivalent hosts. Suxamethonium was selected for detailed 1D DOSY investigation as it displayed distinguishable sharp peaks in ^1H NMR when complexed with either **Super-sCx4** or **Super-sCx5**. The hydrodynamic radii of suxamethonium complexes with hosts **Super-sCx4** (11.2 ± 0.6 Å) and **Super-sCx5** (12.4 ± 0.6 Å) were similar to the values for the respective isolated Super-sCx host (Table 1, Figure S61 and S66). The 1:1 complexation demonstrated by DOSY is further supported by molecular modeling (see SI). Conformational searches were carried out on complexes of each Super-sCx host with a panel of structurally diverse NMBA's (decamethonium, suxamethonium, gallamine, pancuronium, tubocurarine and cisatracurium). In all cases, the most stable energy-minimized structures involved two quaternary amines from the NMBA's being engaged within the two pockets of the bivalent hosts (See SI).

Table 1. Diffusion coefficients and hydrodynamic radii determined by 1D DOSY.

Compound	D (10^{-10} m ² /s)	r_H (Å)
sCx4-CHO (1) ^[13]	3.29 ± 0.02	7.4 ± 0.3
sCx4-CHO (1)•decamethonium ^[a]	2.16 ± 0.05	11.7 ± 0.3
sCx4-CH₂NHNH₂ ^[b]	2.77 ± 0.05	9.1 ± 0.2
Super-sCx4 ^[b]	2.22 ± 0.02	11.4 ± 0.1
Super-sCx4 •suxamethonium ^[b]	2.3 ± 0.1	11.2 ± 0.6
sCx5-CHO (5) ^[b]	2.79 ± 0.03	9.0 ± 0.1
sCx5-CHO (5)•pancuronium ^[a]	2.2 ± 0.2	11 ± 1
sCx5-CH₂NHNH₂ ^[b]	2.7 ± 0.1	9.5 ± 0.5
Super-sCx5 ^[b]	2.00 ± 0.03	12.6 ± 0.2
Super-sCx5 •suxamethonium ^[b]	2.04 ± 0.09	12.4 ± 0.6

[a] (NH₄)₂CO₃ (50 mM, pD 6.4) in D₂O (500 MHz, 297 K) [b] NaH₂PO₄/Na₂HPO₄ (50 mM, pD 7.4) in D₂O (500 MHz, 297 K)

Six-point ^1H NMR titrations of NMBA's demonstrate qualitatively that matching of bivalent guests with bivalent hosts is required for strong 1:1 binding. The upfield shifts of NMBA methyl groups were monitored upon addition of hosts into NMBA's, with all titrations exhibiting fast exchange on the NMR time scale (See SI). Titrations of bivalent hosts **Super-sCx4** and **Super-sCx5** into NMBA's displayed strong binding, resulting in saturation of chemical shift perturbation at 1.0–1.5 equivalents. Titrations with monovalent control hosts **sCx4-CH₂NHNH₂** and **sCx5-CH₂NHNH₂** did not display saturation at ≤ 3 equivalents, demonstrating that the monovalent control compounds have weaker complexes of higher stoichiometries. Similarly, titrations of the bivalent **Super-sCx4** and **Super-sCx5** hosts into choline and acetylcholine showed weaker, unsaturated binding curves.

The new hosts exhibit pan-selective NMBA binding with selectivity over acetylcholine. We used an indicator-displacement assay (IDA) to determine equilibrium dissociation constant (K_d) values. Competitive titrations of NMBA's into the pre-formed Super-sCx•LCG complexes were conducted, with displacement of LCG resulting in increased fluorescence (Figure 4).^[15] **Super-sCx4** exhibited stronger binding to the thinner NMBA's, suxamethonium (71 ± 8 μM) and decamethonium (63 ± 9 μM), with selectivity over acetylcholine (Table 2). **Super-sCx5** exhibited strong broad-spectrum binding to all NMBA's tested (2–18 μM) and selectivity over acetylcholine (Table 2). Notably the larger calix[5]arene cavity offered strong binding to both the thinner NMBA suxamethonium (14 ± 3 μM) and the bulkier NMBA cisatracurium (6 ± 3 μM).

This work provides a new approach to developing reversal agents, using the target as a template to build synthetically challenging hosts in aqueous solution. While several examples of upper-rim-linked bivalent calixarenes exist,^[16] making covalent connections between highly anionic building blocks is especially challenging. The large magnitude of repulsive anionic charge of *p*-sulfonatocalixarenes poses a challenge to forming a short flexible linkage between the upper rims. Templation has been widely used, from small ion templation of macrocycles,^[17] to larger complex architectures like nanorings,^[18] nanorods,^[19] molecular knots,^[20] and polymers.^[21] More recently, templation has been applied to dynamic combinatorial chemistry, by forming mixtures of synthetic building blocks that favor linkage around a desired template.^[22]

Figure 4. Indicator displacement assay determines binding interactions of bivalent hosts to NMBAs **a)** Cartoon illustration of lucigenin displacement upon NMBA binding of Super-sCx host **b)** Competitive titration of suxamethonium (up to 640 μ M) into **Super-sCx4** (5 μ M) and lucigenin (0.25 μ M). Solutions in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (10 mM, pD 7.4), $\lambda_{\text{ex}} = 369$ nm $\lambda_{\text{em}} = 485$ nm.

Specific ternary complexes are required to drive the successful synthesis of new bivalent hosts. Reactions in the absence of template consistently failed to provide bivalent hosts. The transient 2:1 complex of **sCx4-CHO** and decamethonium was directly observed in the reaction mixture by using *in situ* DOSY NMR (Figure S58), while the mix of **sCx5-CHO** with decamethonium failed to generate a 2:1 complex (Figure S25) and also failed to produce the desired bivalent host. This situation was improved

by using pancuronium as the template for **Super-sCx5** synthesis. The success of a very short hydrazine linker and the NMR evidence for bis-hydrazone formation during the templation reaction means that upper-rim sulfonates are as close as 8 Å in the transient ternary complexes.

In some sense, a pharmacophore model is a useful message of promiscuity. It teaches us that many drugs presenting related functional groups in a similar 3D arrangement can be active against the same target. When paired with a promiscuous host macrocycle, the use of a single template during synthesis can provide bivalent hosts that have broad-spectrum activity and yet retain useful selectivity (e.g. over monovalent competitors). While it seems that only certain NMBAs are suitable for a given templation reaction, the bivalent hosts in this work, once synthesized, have good affinity for NMBAs and weak affinity for neurotransmitters. We are careful not to claim any pharmacological advantage for this combination of properties, but we point out that it is complementary to and distinct from the properties of other NMBA reversal agents.

Sulfonated calixarenes have demonstrated *in vivo* activity in several models of intoxication and have favorable toxicity profiles. sCx4 itself is biocompatible with low toxicity (100 mg/kg in mice) making it an excellent candidate for the development of novel supramolecular drug reversal agents.^[23] Sulfonatocalixarenes, however, are also known for forming intrinsically weak (~mM) complexes with a large variety of cationic guests in salty water.^[12] Until now, this has limited their biological applications to those that involve targeted analytes that exist at relatively high *in vivo* concentrations.^[24] We are exploring the application of this templation approach to other host classes, as well as the potential for Super-sCx hosts in a variety of *in vivo* applications.

Table 2. Equilibrium dissociation constants of Super-sCx hosts and their selectivity for NMBAs vs. acetylcholine.

Guest	Super-sCx4 (K_d , M) ^[a]	Selectivity over ACh (fold)	Super-sCx5 (K_d , M) ^[a]	Selectivity over ACh (fold)
Lucigenin	$6 \pm 2 \times 10^{-7}$	-	$3 \pm 1 \times 10^{-7}$	-
Choline	$2 \pm 1 \times 10^{-2}$	-	$1.1 \pm 0.7 \times 10^{-3}$	-
Acetylcholine	$9 \pm 5 \times 10^{-3}$	-	$1.4 \pm 0.8 \times 10^{-3}$	-
Decamethonium	$6.3 \pm 0.9 \times 10^{-5}$	140	$1.8 \pm 0.4 \times 10^{-5}$	78
Suxamethonium	$7.1 \pm 0.8 \times 10^{-5}$	130	$1.4 \pm 0.3 \times 10^{-5}$	100
Gallamine	$8 \pm 6 \times 10^{-6[b]}$	1100 ^[b]	$2 \pm 2 \times 10^{-6[b]}$	700 ^[b]
Pancuronium	$1.0 \pm 0.2 \times 10^{-5}$	900	$1.4 \pm 0.3 \times 10^{-5}$	100
Tubocurarine	$2 \pm 1 \times 10^{-4}$	45	$1.8 \pm 0.8 \times 10^{-5}$	78
Cisatracurium	$2 \pm 0.4 \times 10^{-4}$	45	$6 \pm 3 \times 10^{-6[b]}$	230 ^[b]

[a] All studies solutions in phosphate buffer (10 mM, pH 7.4). Dissociation constants were determined from fluorescence IDA titrations assuming 1:1 stoichiometry. 1:1 binding was supported by molecular modeling of NMBAs with Super-sCx hosts. For binding curves and residuals see SI. [b] Certain structurally complex NMBAs showed complex titration profiles that prove a non-1:1 stoichiometry. The apparent K_d values arising from 1:1 fits are provided, but must be considered only as rough approximations of potency for binding.

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Keywords: calixarenes • host-guest systems • reversal agents • supramolecular chemistry • template synthesis

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Entry for the Table of Contents

Broad-spectrum binding: New bivalent supramolecular reversal agents are formed by target templated synthesis in aqueous solution. Bivalent anionic Super-sCx calixarene hosts form strong complexes to a variety of structurally diverse target neuromuscular blocking agents.

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1. General methods and materials

1.1. General NMR spectroscopy methods

Deuterated solvents were purchased from Sigma Aldrich. Reactions monitored *in situ* were prepared using ammonium carbonate (50 mM, pD 6.4) in D₂O, pD was adjusted using 1 M DCl. Ammonium carbonate was selected due to its lack of interference in ¹H NMR and compatibility with UPLC-MS. Adjusting the pD to 6.4 favored the formation of hydrazone in the initial condensation reaction. ¹H NMR titrations were performed in phosphate buffer (50 mM, pD 7.4), prepared using sodium phosphate monobasic and sodium phosphate dibasic in D₂O, the pD was adjusted with 1 M NaOD/DCl.

¹H, ¹³C, and 1D DOSY were recorded on a Bruker Avance Neo 500 MHz spectrometer or 300 MHz spectrometer as specified. ¹H, ¹³C NMR spectra were processed using MestReNova by Mestrelab Research S.L. 1D DOSY experiments were pre-processed using TopSpin and diffusion coefficients were analyzed using Bruker Dynamics Center.

1.2. General UPLC-MS and HPLC methods

Reactions were monitored and final host purity was verified using a Waters UPLC-MS equipped with UV/vis and QDa detector. All samples were filtered through a 13 mm syringe filter with a 0.45 µm PTFE membrane then injected onto an Aquity UPLC BEH C18 1.7 µm (21 x 50 mm) column. A gradient of 90% H₂O (0.4% FA)/10% CH₃CN (0.4% FA) to 30% H₂O (0.4% FA)/70% CH₃CN (0.4% FA) over 5 minutes at 0.5 ml/min flow was used for all traces. Reaction mixtures were directly purified on a Teledyne ACCQPrep HP150 HPLC system with UV detection set at 280 nm. A Phenomenex Luna C18, 250 mm x 22 mm, 5 µm preparative column or a Teledyne Redi Sep C18, 150 mm x 20 mm, 5 µm were used for purification. The column and gradient methods are as specified for each synthetic protocol.

1.3. General HR-MS, MP and IR characterization methods

Accurate mass spectra of pure novel compounds were obtained by electrospray ionization using a Thermo Scientific™ Exactive™ Plus Orbitrap Ultimate 3000 LC-MS system. The eluent used was a 50:50 mix of Mili-Q™ water and Optima™ Acetonitrile. Melting points were determined using a Gallenkamp Melting Point apparatus. Infrared spectra were collected using a Perkin Elmer 1000 FT-IR spectrometer. IR data was reported as frequency of absorption (cm⁻¹) intensity; s = strong, m = medium, w = weak, br = broad.

1.4. Materials

Suxamethonium chloride (>98%) and decamethonium bromide (>98%) were purchased from Tokyo Chemical Industry. Choline chloride (>98%) was purchased from Alfa Aesar. Tubocurarine chloride (98%), cisatracurium besylate (88%), pancuronium bromide (97%) and gallamine triethiodide (95%) were purchased from Toronto Research Chemicals. Acetylcholine chloride (>98%), hydrazine hydrochloride, sodium cyanoborohydride and lucigenin (LCG) were purchased from Sigma Aldrich.

2. Synthesis

Starting material sCx4-CHO (**1**) was synthesized following the reported protocols.^[1]

*See main text for schematic version of this figure

Super-sCx4 – templated reaction. sCx4-CHO (**1**) (30 mg, 0.043 mmol, final concentration = 10 mM) and decamethonium bromide (9 mg, 0.022 mmol, final concentration = 5 mM) were dissolved in ammonium carbonate in D₂O (4.34 mL, 50 mM, pD 6.4). The starting material was equilibrated for 15 min at room temperature with stirring. The dissolved starting material formed a clear colourless solution. ¹H NMR of the starting material was taken to verify decamethonium complexation. Hydrazine hydrochloride (0.024 mmol, 24 μ L of 1 M solution in D₂O) was added and stirred at room temperature for 30 min. Upon hydrazine hydrochloride addition the reaction formed an orange precipitate. Sodium cyanoborohydride (0.43 mmol, 27 mg) was added, and stirred at room temperature for 2 h. Upon sodium cyanoborohydride addition the reaction formed a clear colourless solution. UPLC-MS verified the formation of bivalent **Super-sCx4** as the major product (see Figure S23). The reaction mixture was directly purified by HPLC using a gradient of 90% H₂O (0.1% TFA)/10% CH₃CN (0.1% TFA) to 68% H₂O (0.1% TFA)/32% CH₃CN (0.1% TFA) over 17 minutes on a Phenomenex Luna C18, 250 mm x 22 mm, 5 μ m preparative column. Fractions were collected, immediately flash frozen and lyophilized to yield a white fluffy solid (15 mg, 50%). m.p. decomposed >250°C; ¹H NMR (300 MHz, D₂O): δ 7.63 (s, 4H), 7.61 (s, 4H), 7.51 (s, 4H), 7.00 (s, 4H), 4.02 (d, *J* = 10.9 Hz, 16H), 3.75 (s, 4H); ¹³C NMR (126 MHz, *d*₆-DMSO): δ 151.6, 150.2, 149.4, 139.5, 139.1, 130.1, 128.1, 127.5, 127.2, 127.0, 126.6, 126.5, 126.4, 126.0, 51.0, 30.9, 30.6; FT-IR: 3243 (br), 1594 (w), 1454 (w), 1136 (m), 1111 (m), 1037 (s), 625 (s), 551 (s) cm⁻¹; HRMS (ESI): *m/z* calcd for C₅₈H₅₂N₂O₂₆S₆⁵⁻ 275.82265 [*M*-5H]⁵⁻; found 275.81569.

Super-sCx4 – control untemplated reaction. The control reaction was carried out exactly as above but omitting decamethonium bromide. The dissolved starting material formed a clear colourless solution. Upon hydrazine hydrochloride addition the reaction formed a clear light-yellow solution. Upon sodium cyanoborohydride addition the reaction formed a clear colourless solution. UPLC-MS verified that bivalent **Super-sCx4** was not synthesized in the absence of the decamethonium template. A mixture of the monovalent hydrazone and reduced starting material sCx4-CH₂NHNH₂ (**6**) were formed (see Figure S24).

sCx4-CH₂NHNH₂. sCx4-CHO (**1**) (50 mg, 0.072 mmol, final concentration = 30 mM) was dissolved in ammonium carbonate in D₂O (2.41 mL, 50 mM, pD 6.4), forming a colourless solution. Hydrazine hydrochloride (0.22 mmol, 217 μ L of 1 M solution in D₂O) was added. Upon addition the solution turned yellow. The reaction was stirred at room temperature for 30 min, forming a yellow precipitate. Sodium cyanoborohydride (0.72 mmol, 45 mg) was added, and stirred at room temperature for 2 h, forming a white cloudy solution. A minimal amount of water was added to dissolve the precipitate (~2 mL). The crude reaction mixture was directly purified by HPLC using a gradient of 90% H₂O (0.1% TFA)/10% CH₃CN (0.1% TFA) to 65% H₂O (0.1% TFA)/35% CH₃CN (0.1% TFA) over 17 minutes on a Phenomenex Luna C18, 250 mm x 22 mm, 5 μ m preparative column. The fractions were collected, immediately flash frozen and lyophilized, yielding a fluffy white product (35 mg, 69%). m.p. decomposed >250°C; ¹H NMR (500 MHz, D₂O): δ 7.81 (s, 2H), 7.68 (d, *J* = 2.2 Hz, 2H), 7.63 (s, 2H), 6.88 (s, 2H), 4.24-3.88 (m, 10H); ¹³C NMR (126 MHz, *d*₆-DMSO): δ 151.6, 150.2, 149.4, 139.5, 139.1, 130.1, 128.1, 127.5, 127.2, 127.0, 126.6, 126.5, 126.4, 126.0, 51.0, 30.9, 30.6; FT-IR: 3200 (br), 1609 (w), 1460 (w), 1156 (s), 1040 (s), 626 (s), 557 (m) cm⁻¹; HRMS (ESI): *m/z* calcd for C₂₉H₂₈N₂O₁₃S₃³⁻ 235.02512 [*M*-3H]³⁻; found 235.01876.

Calix[5]arene starting material was provided by Prof. Dong-Sheng Guo and Juan-Juan Li, Nankai University Tianjin. 4Bz-Cx5 (**2**) was synthesized following the reported protocol.^[2]

4Bz-Cx5-CHO (3). 4Bz-Cx5 (**2**) (500 mg, 0.52 mmol) was dissolved in trifluoroacetic acid (5 mL) and hexamethylenetetramine (665 mg, 4.75 mmol) was added. The solution was heated for 4 h at 70°C forming a clear pink solution. Water was added to the reaction forming a white precipitate. The precipitate was then dissolved upon addition of dichloromethane and the layers were separated. The water layer was back extracted twice with fresh dichloromethane. The combined organic layers were washed twice with water, then twice with brine and dried over Na₂SO₄. The filtered organic layer was concentrated to dryness on a rotary evaporator resulting in light yellow crystals (500 mg, 98% yield). This product was carried forward to debenzoylation after partial characterization, due to poor ionization in HRMS and multiple conformations resulting in intractable NMR spectra. ¹H NMR (500 MHz, CDCl₃): δ 9.91 (s, 1H), 8.39–5.99 (m, 34H), 4.23–3.11 (m, 10H).

Cx5-CHO (4). 4Bz-Cx5-CHO (**3**) (500 mg, 0.51 mmol) and sodium hydroxide pellets (410 mg, 10.2 mmol) were dissolved in methanol (20 mL). The reaction was heated at reflux overnight forming a clear orange-brown solution. The mixture was quenched with cold 1 M HCl, forming a white precipitate. The precipitate was collected by vacuum filtration then purified using a silica plug (~10 g). The crude was dissolved in minimal CH₂Cl₂ and wet loaded. The plug was run with a starting eluent of CH₂Cl₂ with a gradient to CH₂Cl₂ + 5% MeOH, affording a solid white product. (275 mg, yield 96%). m.p. decomposed >250°C; ¹H NMR (300 MHz, CDCl₃): δ 9.35 (s, 1H), 9.17 (s, 1H), 8.37 (s, 2H), 8.23 (s, 2H), 6.77–6.99 (m, 10H), 6.37 (t, *J* = 7.7 Hz, 2H), 6.35 (t, *J* = 7.7 Hz, 2H), 3.38 (br, 10H); ¹³C NMR (126 MHz, CDCl₃): δ 191.0, 156.0, 150.0, 149.9, 131.4, 130.6, 129.7, 129.4 (2x), 129.4, 127.8, 127.0, 126.8, 126.6, 125.8, 122.1, 121.8, 31.4, 31.3, 31.3; FT-IR: 3249 (br), 1686 (m), 1596 (m), 1446 (m), 753 (s) cm⁻¹; HRMS (ESI): *m/z* calcd for C₃₆H₃₀O₆⁻ 557.20424 [*M-H*]⁻; found 557.19679.

sCx5-CHO (5). CHO-Cx5 (**4**) (100 mg, 0.18 mmol) was dissolved in minimal dichloromethane (2 mL). Concentrated sulfuric acid (192 μL, 3.6 mmol) was added and the reaction was heated at 50°C for 3 h. An insoluble pink-purple residue formed on the sides of the reaction vial. Dichloromethane was decanted from the reaction. The residue was rinsed with fresh dichloromethane and decanted again. The solid residue was then suspended in ethyl acetate (~4 mL) by sonication and transferred into a 50 mL falcon tube. Suspension in ethyl acetate was repeated two more times to completely remove all residue from the reaction vial (~12 mL total). The suspension was diluted with cold diethyl ether (0°C, ~30 mL) forming a grey precipitate. The suspension was centrifuged into a pellet (10 min, 4°C, 3400 rpm) and the supernatant was decanted. The pellet was resuspended in cold diethyl ether (0°C, ~30 mL) with sonication, repeating the centrifugation and decanting process. The pellet was left to air dry overnight then purified by HPLC. A gradient of 90% H₂O (0.1% TFA)/10% CH₃CN (0.1% TFA) to 77% H₂O (0.1% TFA)/23% CH₃CN (0.1% TFA) over 12 minutes was used on a Teledyne Redi Sep C18, 150 mm x 20 mm, 5 μm preparative column. Fractions were concentrated by rotary evaporator then lyophilized, affording a white fluffy powder (122 mg, 78%). m.p. decomposed >250°C; ¹H NMR (300 MHz, D₂O): δ 9.62 (s, 1H), 7.70 (s, 2H), 7.64 (d, *J* = 2.4 Hz, 2H), 7.60–7.57 (m, 2H), 7.56 (d, *J* = 2.4 Hz, 1H), 3.92 (br, 10H); ¹³C NMR (126 MHz, D₂O): δ 195.5, 158.2, 153.8, 153.7, 135.7 (2x), 132.1, 129.7, 129.7, 129.6, 129.6, 129.5, 129.3, 127.1, 127.1, 127.0, 126.9, 31.4, 31.4, 31.1; FT-IR: 3282 (br), 1664 (w), 1111 (s), 1035 (s), 620 (m), 554 (m) cm⁻¹; HRMS (ESI): *m/z* calcd for C₃₆H₃₀O₁₈S₄³⁻ 291.67717 [*M-3H*]³⁻; found 291.66999.

*See main text for schematic version of this figure

Super-sCx5 – templated reaction. sCx5-CHO (**5**) (30 mg, 0.034 mmol, final concentration = 10 mM) and pancuronium bromide (12.6 mg, 0.017 mmol, final concentration = 5 mM) were dissolved in ammonium carbonate in D₂O (3.43 mL, 50 mM, pD 6.4). The starting material was equilibrated for 15 min at room temperature with stirring. The dissolved starting material formed a clear colourless solution. ¹H NMR of the starting material was taken to verify pancuronium complexation. Hydrazine hydrochloride (0.019 mmol, 19 μ L of 1 M solution in D₂O) was added and stirred at room temperature for 30 min. Upon hydrazine hydrochloride addition the reaction formed an orange solution. Sodium cyanoborohydride (0.34 mmol, 22 mg) was added, and stirred at room temperature for 2 h. Upon sodium cyanoborohydride addition the reaction formed a clear colourless solution. UPLC-MS verified the formation of bivalent **Super-sCx5** as the major product (see Figure S26). The reaction mixture was directly purified by HPLC using a gradient of 90% H₂O (0.1% TFA)/10% CH₃CN (0.1% TFA) to 68% H₂O (0.1% TFA)/32% CH₃CN (0.1% TFA) over 17 minutes on a Phenomenex Luna C18, 250 mm x 22 mm, 5 μ m preparative column. The fractions were collected, immediately flash frozen and lyophilized to yield a white fluffy solid (6 mg, 25%). m.p. decomposed >250°C; ¹H NMR (500 MHz, D₂O): δ 7.64–7.56 (m, 16H), 7.10 (s, 4H), 3.97 (s, 4H), 3.90 (s, 12H), 3.85 (s, 8H); ¹³C NMR (126 MHz, *d*₆-DMSO): δ 152.2, 152.1, 151.4, 138.7, 138.4, 130.1, 128.4, 127.6, 127.4, 127.2, 126.6 (x2), 126.5, 126.3, 124.8, 51.4, 31.5, 31.0, 30.8; FT-IR: 3344 (br), 1594 (w), 1474 (w), 1139 (m), 1113 (s), 1038 (s), 622 (m), 558 (m) cm⁻¹; HRMS (ESI): *m/z* calcd for C₇₂H₆₄N₂O₃₄S₈⁶⁻ 291.68510 [*M*-6H]⁶⁻; found 291.67723.

Super-sCx5 – control untemplated reaction. The control reaction was carried out exactly as above but omitting pancuronium bromide. The dissolved starting material formed a clear colourless solution. Upon hydrazine hydrochloride addition the reaction formed a clear light-yellow solution. Upon sodium cyanoborohydride addition the reaction formed a clear colourless solution. UPLC-MS verified that bivalent **Super-sCx5** was not synthesized in the absence of the template pancuronium. A mixture of the monovalent hydrazone and reduced starting material sCx5-CH₃ (**7**) were formed (see Figure S27).

sCx5-CH₂NHNH₂. sCx5-CHO (**5**) (50 mg, 0.057 mmol, final concentration = 30 mM) was dissolved in ammonium carbonate in D₂O (1.91 mL, 50 mM, pD 6.4), forming a colourless solution. Hydrazine hydrochloride (0.17 mmol, 171 μ L of 1 M solution in D₂O) was added. Upon addition the solution turned yellow. The reaction was stirred at room temperature for 30 min. Sodium cyanoborohydride (0.57 mmol, 36 mg) was added, and stirred at room temperature for 2 h, forming a colourless solution. The crude reaction mixture was directly purified by HPLC using a gradient of 90% H₂O (0.1% TFA)/10% CH₃CN (0.1% TFA) to 65% H₂O (0.1% TFA)/35% CH₃CN (0.1% TFA) over 17 minutes on a Phenomenex Luna C18, 250 mm x 22 mm, 5 μ m preparative column. The fractions were collected, immediately flash frozen and lyophilized, yielding a fluffy white product (28 mg, 69%). m.p. decomposed >250°C; ¹H NMR (500 MHz, D₂O): δ 7.73 (d, *J* = 2.3 Hz, 2H), 7.63 (d, *J* = 2.3 Hz, 2H), 7.59 (d, *J* = 2.3 Hz, 2H), 7.53 (d, *J* = 2.3 Hz, 2H), 7.01 (s, 2H), 3.99 (s, 2H), 3.96 (s, 6H), 3.90 (s, 2H). ¹³C NMR (126 MHz, *d*₆-DMSO): δ 152.2, 151.9, 151.3, 139.0, 138.7, 128.2, 127.5, 127.3, 127.3, 127.0, 126.9, 126.8, 126.4, 126.3, 54.0, 31.1, 30.9, 30.5; FT-IR: 3320 (br), 1593 (w), 1473 (w), 1139 (m), 1112 (s), 1037 (s), 623 (m), 558 (m) cm⁻¹. HRMS (ESI): *m/z* calcd for C₃₆H₃₄N₂O₁₇S₄³⁻ 297.02468 [*M*-3H]³⁻; found 297.01746.

2.1. Characterization data – ^1H , ^{13}C , DEPT-135 NMR and UPLC-MS

Figure S1. ^1H NMR spectrum of bivalent **Super-sCx4** (300 MHz, 300 K, in D_2O).

Figure S2. ^{13}C NMR spectrum of bivalent **Super-sCx4** (125 MHz, 298 K, in d_6 -DMSO).

Figure S3. DEPT-135 NMR spectrum of bivalent **Super-sCx4** (125 MHz, 298 K, in d_6 -DMSO).

Figure S4. UPLC-MS trace of bivalent **Super-sCx4** after preparative HPLC purification.

Figure S5. ¹H NMR spectrum of monovalent **sCx4-CH₂NHNH₂** (500 MHz, 298 K, in D₂O).

Figure S6. ¹³C NMR spectrum of monovalent **sCx4-CH₂NHNH₂** (125 MHz, 298 K, in *d*₆-DMSO).

Figure S7. DEPT-135 NMR spectrum of monovalent **sCx4-CH₂NHNH₂** (125 MHz, 298 K, in *d*₆-DMSO).

Figure S8. UPLC-MS trace of monovalent **sCx4-CH₂NHNH₂** after preparative HPLC purification.

Figure S9. ¹H NMR spectrum of 4Bz-Cx5-CHO (**3**) (500 MHz, 298 K, in CDCl₃). This compound was carried forward without full characterization due to the intractable NMR spectra.

Figure S10. ^1H NMR spectrum of Cx5-CHO (**4**) (300 MHz, 300 K, in CDCl_3).

Figure S11. ^{13}C NMR spectrum of Cx5-CHO (**4**) (125 MHz, 298 K, in CDCl_3).

Figure S12. ¹H NMR spectrum of sCx5-CHO (**5**) (300 MHz, 300 K, in D₂O).

Figure S13. ¹³C NMR spectrum of sCx5-CHO (**5**) (125 MHz, 298 K, in D₂O) calibrated with an external reference (5% MeOH in D₂O, 49.5 ppm).

Figure S14. UPLC-MS trace of sCx5-CHO (**5**) after preparative HPLC purification.

Figure S18. UPLC-MS trace of bivalent **Super-sCx5** after preparative HPLC purification.

Figure S19. ¹H NMR spectrum of monovalent **sCx5-CH₂NHNH₂** (500 MHz, 298 K, in D₂O).

Figure S20. ¹³C NMR spectrum of monovalent **sCx5-CH₂NHNH₂** (125 MHz, 298 K, in d₆-DMSO).

Figure S21. DEPT-135 NMR spectrum of monovalent **sCx5-CH₂NHNH₂** (125 MHz, 298 K, in d₆-DMSO).

Figure S22. UPLC-MS trace of monovalent **sCx5-CH₂NHNH₂** after preparative HPLC purification.

2.2. *In situ* reaction monitoring by ^1H NMR and UPLC-MS

Figure S23. Bivalent calix[4]arene host synthesis is templated by the NMBA decamethonium. **a)** ^1H NMR of i) decamethonium bromide (5 mM) ii) addition of sCx4-CHO (1) (10 mM) iii) crude reaction mixture after condensation and reductive amination. Red dashed line illustrates upfield shift of decamethonium methyl groups, indicating complexation of methyl groups inside the Cx4 cavity. Reaction in ammonium carbonate (50 mM, pD 6.4) in D_2O (300 MHz, 298 K). **b)** UPLC-MS of crude templated reaction shows minor formation of monovalent sCx4- CH_2NHNH_2 (1.96 min peak, left MS trace) and major product formation of bivalent **Super-sCx4** (2.12 min peak, right MS trace).

Figure S24. The calix[4]arene untemplated control reaction does not form a bivalent host. **a)** ^1H NMR of i) sCx4-CHO (**1**) (10 mM) ii) crude reaction mixture after condensation and reductive amination. Reaction in ammonium carbonate (50 mM, pD 6.4) in D_2O (300 MHz, 298 K). **b)** UPLC-MS of crude untemplated control reaction shows formation of monovalent **sCx4-CH₂NHNH₂** (1.84 min peak, left MS trace) and reduced starting material **sCx4-CH₃** (**6**) (2.00 min peak, right MS trace).

Figure S25. Decamethonium is not suitable for bivalent calix[5]arene host templation. **a)** ¹H NMR of i) decamethonium bromide (5 mM) and ii) addition of 2 eq **sCx5-CHO** (**5**) (10 mM). Red dashed lines illustrate upfield chemical shifts of decamethonium methylene peaks. **b)** COSY **c)** Change in chemical shifts ($\Delta\delta$) of decamethonium methylene peaks indicates decamethonium assumes a folded conformation inside the binding pocket of **sCx5-CHO**. Spectra collected in ammonium carbonate (50 mM, pD 6.4) in D₂O (300 MHz, 298 K).

Figure S26. Bivalent calix[5]arene host synthesis is templated by the rigid NMBA pancuronium. **a)** ^1H NMR of i) pancuronium bromide (5 mM) ii) addition of sCx5-CHO (**5**) (10 mM) iii) crude reaction mixture after condensation and reductive amination. Red dashed line illustrates upfield shifts of the quaternary amine methyl groups on pancuronium, indicating complexation throughout the reaction. Reaction in ammonium carbonate (50 mM, pD 6.4) in D_2O (300 MHz, 298 K). **b)** UPLC-MS of crude templated reaction shows formation of bivalent **Super-sCx5** (1.57 min peak, right MS trace) as the major product.

3. Indicator Displacement Assay

Direct and competitive titrations were done in NUNC black-walled, optical bottom 96-well plates. Lucigenin was used as the indicator in all titrations and stock concentrations were determined by $\epsilon_{410\text{nm}} = 8900 \text{ M}^{-1}\text{cm}^{-1}$. Direct titration solutions were composed of 10 mM phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$) at pH 7.4, 0.25 μM of lucigenin and varying concentrations of host (0–40 μM) made in Milli-Q® ultrapure water. Competitive titration solutions were composed of 10 mM phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$) at pH 7.4, 0.25 μM of lucigenin, 5 μM host and varying concentrations of guest (0–10 mM) made in Milli-Q® ultrapure water. Each well had a final volume of 100 μL . All fluorescence readings were collected on a BioTek Cytation-5, λ_{ex} 369 nm and λ_{em} 485 nm. Three sets of duplicates were performed for each guest on different days with new stock solutions.

A blank containing only the dye and no host (FI_0) was subtracted from each duplicate experiment. The data for each of the duplicates was plotted producing a K_d with standard error. The average K_d values of the three sets of duplicates was reported along with the propagated standard error for each host•guest interaction.

3.1. Determining direct and competitive binding affinities

Direct host•lucigenin affinities were determined by plotting the fluorescence ($\text{FI}-\text{FI}_0$, RFU) as a function of host concentration. The data was fitted in GraphPad Prism using a direct one site binding equation:

$$Y = A \times \frac{(D + x + K_{d \text{ LCG}}) - \sqrt{(D + x + K_{d \text{ LCG}})^2 - (4Dx)}}{2D}$$

Where, A = amplitude of change in fluorescence ($\text{FI}-\text{FI}_0$), D = concentration of lucigenin, x = amount titrated and $K_{d \text{ LCG}}$ = dissociation constant of lucigenin

Direct host•lucigenin fitting was constrained by the concentration of dye (0.25 μM). The graphs were plotted with standard deviation and a second plot of x residuals was determined. All graphs met a criterion of $R^2 > 0.95$. The IC_{90} points from the direct titrations were selected as the host concentrations used in competitive titrations, determined to be 5 μM for both **Super-sCx4** and **Super-sCx5**.

Competitive host•guest affinities were determined by plotting the logarithm of guest concentration and the fluorescence response from lucigenin displacement ($\text{FI}-\text{FI}_0$, RFU). The data was analyzed in GraphPad Prism using the competitive one site binding equation:

$$\log \text{EC}_{50} = \log \left(10^{\log K_{d \text{ guest}} * \left(1 + \frac{D}{K_{d \text{ LCG}}} \right)} \right)$$

Where, $K_{d \text{ LCG}}$ = dissociation constant of lucigenin, D = concentration of lucigenin, $K_{d \text{ guest}}$ = dissociation constant of guest of interest

Competitive host•guest fitting was constrained by the direct host•lucigenin dissociation constant ($K_{d \text{ LCG}}$) and the concentration of lucigenin (0.25 μM). This model fits $K_{d \text{ guest}}$ directly and does not report the EC_{50} . This analysis also assumes 1:1 host•guest interaction. Graphs were plotted with standard deviation and a second plot of x residuals were determined. All graphs met a criterion of $R^2 > 0.95$.

Figure S28. Exemplary duplicate data from direct titrations of **Super-sCx4** into lucigenin, and competitive titrations of guest into **Super-sCx4** + lucigenin. Error bars were plotted for each duplicate but are not visible in cases where the error is smaller than the plotted point.

Figure S29. Exemplary duplicate data from direct titrations of **Super-sCx5** into lucigenin, and competitive titrations of guest into **Super-sCx5** + lucigenin. Error bars were plotted for each duplicate but are not visible in cases where the error is smaller than the plotted point.

4. ^1H NMR titrations

Figure S30. ^1H NMR titrations of bivalent **Super-sCx4** (4 mM) into choline (800 μM). Red dashed line indicates binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S31. ^1H NMR titrations of monovalent **sCx4-CH₂NHNH₂** (4 mM) into choline (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S32. ^1H NMR titrations of bivalent **Super-sCx4** (4 mM) into acetylcholine (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S33. ^1H NMR titrations of monovalent **sCx4-CH₂NH₂NH₂** (4 mM) into acetylcholine (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S34. ^1H NMR titrations of bivalent **Super-sCx4** (4 mM) into decamethonium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S35. ^1H NMR titrations of monovalent **sCx4-CH₂NHNH₂** (4 mM) into decamethonium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S36. ^1H NMR titrations of bivalent **Super-sCx4** (4 mM) into suxamethonium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S37. ^1H NMR titrations of monovalent **sCx4-CH₂NH₂NH₂** (4 mM) into suxamethonium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S38. ^1H NMR titrations of bivalent **Super-sCx4** (4 mM) into gallamine (800 μM). Red dashed line indicates the binding of ethyl and methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S39. ^1H NMR titrations of monovalent **sCx4-CH₂NHNH₂** (4 mM) into gallamine (800 μM). Red dashed line indicates the binding of ethyl and methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S40. ^1H NMR titrations of bivalent **Super-sCx4** (4 mM) into pancuronium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S41. ^1H NMR titrations of monovalent **sCx4-CH₂NHNH₂** (4 mM) into pancuronium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S42. ^1H NMR titrations of bivalent **Super-sCx4** (4 mM) into cisatracurium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S43. ^1H NMR titrations of monovalent **sCx4-CH₂NHNH₂** (4 mM) into cisatracurium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S44. ^1H NMR titrations of bivalent **Super-sCx5** (4 mM) into choline (800 μM). Red dashed line indicates binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S45. ^1H NMR titrations of monovalent **sCx5-CH₂NHNH₂** (4 mM) into choline (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S46. ^1H NMR titrations of bivalent **Super-sCx5** (4 mM) into acetylcholine (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S47. ^1H NMR titrations of monovalent **sCx5-CH₂NHNH₂** (4 mM) into acetylcholine (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S48. ^1H NMR titrations of bivalent **Super-sCx5** (4 mM) into decamethonium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S49. ^1H NMR titrations of monovalent **sCx5-CH₂NHNH₂** (4 mM) into decamethonium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure 50. ^1H NMR titrations of bivalent **Super-sCx5** (4 mM) into suxamethonium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S51. ^1H NMR titrations of monovalent **sCx5-CH₂NHNH₂** (4 mM) into suxamethonium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S52. ^1H NMR titrations of bivalent **Super-sCx5** (4 mM) into gallamine (800 μM). Red dashed line indicates the binding of ethyl and methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S53. ^1H NMR titrations of monovalent **sCx5-CH₂NHNH₂** (4 mM) into gallamine (800 μM). Red dashed line indicates the binding of ethyl and methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S54. ^1H NMR titrations of bivalent **Super-sCx5** (4 mM) into pancuronium (800 μM). Migration of quaternary amine methyl groups were not distinguishable. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S55. ^1H NMR titrations of monovalent **sCx5-CH₂NHNH₂** (4 mM) into pancuronium (800 μM). Red dashed line indicates the binding of methyl groups. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S56. ^1H NMR titrations of bivalent **Super-sCx5** (4 mM) into cisatracurium (800 μM). Migration of quaternary amine methyl groups were not distinguishable. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Figure S57. ^1H NMR titrations of monovalent **sCx5-CH₂NHNH₂** (4 mM) into cisatracurium (800 μM). Migration of quaternary amine methyl groups were not distinguishable. All solutions were in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

*Note: ^1H NMR titrations were not completed for tubocurarine due to solubility issues at the concentrations required for NMR.

5. DOSY

For each DOSY experiment the 90° pulse was determined, T1 was estimated, Δ and δ were determined. The 90° pulse was determined by measuring the null pulse length at 360° and dividing by four using a zg pulse sequence. The T1 relaxation was estimated through an inversion recovery pulse sequence, t1ir1d. The relaxation time for each experiment was set to be 5x the estimated T1. For each experiment, the Δ and δ were determined by finding a 90-95% intensity difference between the first and last spectra in the power array. The δ was set to 2500 μs and Δ was initially set to 0.1 ms, then increased if needed. A stebppg1s1d pulse sequence was used in all DOSY experiments with an FID size of 32. The data was pre-processed and phased in TopSpin. Then the diffusion coefficients were calculated with Bruker Dynamic Center. Peak integrals were user defined. The data was fit using the following diffusion fit function with a variable gradient strength and 95% confidence level:

$$f(x) = I_0 \exp(-\gamma^2 G^2 \delta^2 (\Delta - \frac{\delta}{3}) D)$$

The data was plotted as a function of the field gradient strength (G) with a residuals plot. For each DOSY experiment the average diffusion coefficient was calculated with standard error. Then the Stokes-Einstein equation was used to calculate the hydrodynamic radius (r_H) assuming the molecules were spherical in shape.

$$r_H = \frac{k_B T}{6\pi\eta D}$$

Where, k_B = boltzmann constant, T = 297 ± 1 K and η = viscosity of water, 8.94 x 10⁻⁴ ± 2.78 x 10⁻⁵ Pa·s^[3]

Table S1. Parameters used in diffusion analysis of templated starting material sCx4-CHO (1)•decamethonium (2:1).

Used γ	26752 rad/(s*Gauss)
Used δ	0.0025000 s
Used Δ	0.13990 s

Table S2. Dynamics Center calculated diffusion coefficients output from user defined integrals for template starting material sCx4-CHO (1) •decamethonium (2:1).

Peak name	F2 [ppm]	D [m ² /s]	error
1	9.66	2.21×10^{-10}	7.230×10^{-13}
2	7.56	2.20×10^{-10}	2.301×10^{-13}
3	3.97	2.18×10^{-10}	1.149×10^{-12}
4	1.97	2.12×10^{-10}	1.248×10^{-13}
5	0.81	2.11×10^{-10}	1.493×10^{-13}

Figure S58. a) ¹H NMR of templated starting material sCx4-CHO (1)•decamethonium (2:1), the peak area integrals highlighted in grey were used to calculate diffusion coefficients b) 1D DOSY plots for each integral and c) the corresponding 1D residuals plot. Solution was in ammonium carbonate (50 mM, pD 6.4) in D₂O (500 MHz, 297 K).

Table S3. Parameters used in diffusion analysis of bivalent **Super-sCx4**.

Used γ	26752 rad/(s*Gauss)
Used δ	0.0025000 s
Used Δ	0.13990 s

Table S4. Dynamics Center calculated diffusion coefficients output from defined integrals for bivalent **Super-sCx4**.

Peak name	F2 [ppm]	D [m ² /s]	error
1	7.47	2.24×10^{-10}	3.563×10^{-13}
2	6.99	2.20×10^{-10}	3.790×10^{-13}
3	3.78	2.21×10^{-10}	6.865×10^{-13}

Figure S59. **a)** ¹H NMR of bivalent SuperPSC-sCx4, the peak area integrals highlighted in grey were used to calculate diffusion coefficients **b)** 1D DOSY plots for each integral and **c)** the corresponding 1D residuals plot. Solution was in phosphate buffer (50 mM, pD 7.4) in D₂O (500 MHz, 297 K).

Table S5. Parameters used in diffusion analysis of monovalent **sCx4-CH₂NHNH₂**.

Used γ	26752 rad/(s*Gauss)
Used δ	0.0025000 s
Used Δ	0.099900 s

Table S6. Dynamics Center calculated diffusion coefficients output from user defined integrals for monovalent **sCx4-CH₂NHNH₂**.

Peak name	F2 [ppm]	D [m ² /s]	error
1	7.73	2.82×10^{-10}	2.072×10^{-12}
2	7.55	2.76×10^{-10}	5.263×10^{-13}
3	6.64	2.73×10^{-10}	3.568×10^{-12}

Figure S60. a) ¹H NMR of monovalent **sCx4-CH₂NHNH₂**, the peak area integrals highlighted in grey were used to calculate diffusion coefficients b) 1D DOSY plots for each integral and c) the corresponding 1D residuals plot. Solution was in phosphate buffer (50 mM, pD 7.4) in D₂O (500 MHz, 297 K).

Table S7. Parameters used in diffusion analysis of **Super-sCx4•Suxamethonium (1:1)**.

Used γ	26752 rad/(s*Gauss)
Used δ	0.0025000 s
Used Δ	0.13990 s

Table S8. Dynamics Center calculated diffusion coefficients output from user defined integrals for **Super-sCx4•Suxamethonium (1:1)**.

Peak name	F2 [ppm]	D [m ² /s]	error
1	7.52	2.20×10^{-10}	1.703×10^{-13}
2	7.25	2.15×10^{-10}	5.150×10^{-13}
3	3.85	2.33×10^{-10}	2.292×10^{-12}
4	3.54	2.15×10^{-10}	1.544×10^{-12}
5	2.65	2.39×10^{-10}	4.716×10^{-13}
6	1.45	2.38×10^{-10}	1.961×10^{-13}

Figure S61. a) ¹H NMR of **Super-sCx4•Suxamethonium (1:1)**, the peak area integrals highlighted in grey were used to calculate diffusion coefficients b) 1D DOSY plots for each integral and c) the corresponding 1D residuals plot. Solution was in phosphate buffer (50 mM, pD 7.4) in D₂O (500 MHz, 297 K).

Table S9. Parameters used in diffusion analysis of sCx5-CHO (**5**).

Used γ	26752 rad/(s*Gauss)
Used δ	0.0025000 s
Used Δ	0.10990 s

Table S10. Dynamics Center calculated diffusion coefficients output from user defined integrals for sCx5-CHO (**5**).

Peak name	F2 [ppm]	D [m ² /s]	error
1	9.43	2.80×10^{-10}	1.350×10^{-12}
2	7.45	2.75×10^{-10}	1.427×10^{-12}
3	7.49	2.77×10^{-10}	7.515×10^{-13}
4	7.56	2.80×10^{-10}	3.358×10^{-13}
5	7.61	2.84×10^{-10}	1.183×10^{-12}
6	3.81	2.78×10^{-10}	7.702×10^{-13}

Figure S62. a) ¹H NMR of sCx5-CHO (**5**), the peak area integrals highlighted in grey were used to calculate diffusion coefficients b) 1D DOSY plots for each integral and c) the corresponding 1D residuals plot. Solution was in phosphate buffer (50 mM, pD 7.4) in D₂O (500 MHz, 297 K).

Table S11. Parameters used in diffusion analysis of templated starting material sCx5-CHO (1)•pancuronium (2:1).

Used γ	26752 rad/(s*Gauss)
Used δ	0.0025000 s
Used Δ	0.12990 s

Table S12. Dynamics Center calculated diffusion coefficients output from user defined integrals for templated starting material sCx5-CHO (1)•pancuronium (2:1).

Peak name	F2 [ppm]	D [m ² /s]	error
1	9.61	2.41×10^{-10}	1.172×10^{-12}
2	7.67	2.43×10^{-10}	2.274×10^{-13}
3	3.89	2.41×10^{-10}	1.172×10^{-12}
4	2.05	2.05×10^{-10}	4.022×10^{-13}
5	1.88	2.06×10^{-10}	7.070×10^{-13}
6	0.36	2.02×10^{-10}	7.619×10^{-13}

Figure S63. a) ¹H NMR of templated starting material sCx5-CHO (1)•pancuronium (2:1), the peak area integrals highlighted in grey were used to calculate diffusion coefficients b) 1D DOSY plots for each integral and c) the corresponding 1D residuals plot. Solution was in ammonium carbonate (50 mM, pD 6.4) in D₂O (500 MHz, 297 K).

Table S13. Parameters used in diffusion analysis of monovalent **sCx5-CH₂NHNH₂**.

Used γ	26752 rad/(s*Gauss)
Used δ	0.0025000 s
Used Δ	0.10990 s

Table S14. Dynamics Center calculated diffusion coefficients output from user defined integrals for monovalent **sCx5-CH₂NHNH₂**.

Peak name	F2 [ppm]	D [m ² /s]	error
1	7.52	2.61×10^{-10}	4.097×10^{-13}
2	6.96	2.74×10^{-10}	8.141×10^{-13}
3	3.92	2.82×10^{-10}	4.717×10^{-13}
4	3.79	2.50×10^{-10}	8.531×10^{-13}

Figure S64. a) ¹H NMR of monovalent **sCx5-CH₂NHNH₂**, the peak area integrals highlighted in grey were used to calculate diffusion coefficients b) 1D DOSY plots for each integral and c) the corresponding 1D residuals plot. Solution was in phosphate buffer (50 mM, pD 7.4) in D₂O (500 MHz, 297 K).

Table S15. Parameters used in diffusion analysis of bivalent **Super-sCx5**.

Used γ	26752 rad/(s*Gauss)
Used δ	0.0025000 s
Used Δ	0.14490 s

Table S16. Dynamics Center calculated diffusion coefficients output from user defined integrals for bivalent **Super-sCx5**.

Peak name	F2 [ppm]	D [m^2/s]	error
1	7.45	2.03×10^{-10}	2.739×10^{-13}
2	6.92	1.98×10^{-10}	8.215×10^{-13}
3	3.77	1.99×10^{-10}	2.339×10^{-13}

Figure S65. a) ^1H NMR of bivalent **Super-sCx5**, the peak area integrals highlighted in grey were used to calculate diffusion coefficients b) 1D DOSY plots for each integral and c) the corresponding 1D residuals plot. Solution was in phosphate buffer (50 mM, pD 7.4) in D_2O (500 MHz, 297 K).

Table S17. Parameters used in diffusion analysis of bivalent **Super-sCx5**•suxamethonium (1:1).

Used γ	26752 rad/(s*Gauss)
Used δ	0.0025000 s
Used Δ	0.14990 s

Table S18. Dynamics Center calculated diffusion coefficients output from user defined integrals for bivalent **Super-sCx5**•suxamethonium (1:1).

Peak name	F2 [ppm]	D [m ² /s]	error
1	7.58	2.05×10^{-10}	3.390×10^{-13}
2	7.33	1.88×10^{-10}	1.522×10^{-12}
3	3.88	2.08×10^{-10}	1.151×10^{-12}
4	3.32	2.04×10^{-10}	3.372×10^{-12}
5	2.24	2.14×10^{-10}	1.218×10^{-12}
6	1.59	2.04×10^{-10}	1.002×10^{-12}

Figure S66. a) ¹H NMR of bivalent **Super-sCx5**•suxamethonium, the peak area integrals highlighted in grey were used to calculate diffusion coefficients b) 1D DOSY plots for each integral and c) the corresponding 1D residuals plot. Solution was in phosphate buffer (50 mM, pD 7.4) in D₂O (500 MHz, 297 K).

6. Molecular modelling

Host-guest complexes were modeled in Avogadro. Each individual host and guest compound was geometry optimized using the molecular mechanics forcefield MMFF94. A systematic rotor search was used to find the lowest energy conformers of each compound. Then geometry optimization of the lowest energy conformer was done using a 5000 step with a steepest descent algorithm and $10\text{e-}7$ convergence. The pre-optimized structures were then combined to form a host-guest complex, where another systematic rotor search was used to find the lowest energy conformers for each host-guest pair. Optimization of the lowest energy host-guest conformers was completed using a 5000 step with a steepest descent algorithm and $10\text{e-}7$ convergence.

Figure S67. Molecular modelling of **Super-sCx4** shows 1:1 complexation with the dye lucigenin and the NMBA's decamethonium, suxamethonium and gallamine. In all cases the bisquaternary amines on the guest bind both cavity sites present on bivalent **Super-sCx4**.

Figure S68. Molecular modelling of **Super-sCx4** shows 1:1 complexation with the NMBA's pancuronium, tubocurarine and cisatracurium. In all cases the bisquaternary amines on the guest bind both cavity sites present on bivalent **Super-sCx4**.

Figure S69. Molecular modelling of **Super-sCx5** shows 1:1 complexation with the dye lucigenin and the NMBA's decamethonium, suxamethonium and gallamine. In all cases the bisquaternary amines on the guest bind both cavity sites present on bivalent **Super-sCx5**.

Figure S70. Molecular modelling of **Super-sCx5** shows 1:1 complexation with the NMBA's pancuronium, tubocurarine and cisatracurium. In all cases the bisquaternary amines of the guest bind both cavity sites present on the bivalent **Super-sCx5** host.

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Author Contributions

A.J.S.: investigation, formal analysis, supervision, writing, visualization. N.A.C.: investigation, formal analysis. F.H.: conceptualization, writing, review, and editing.