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Article:

Milanesi, L., Hunter, C.A., Sedelnikova, S.E. et al. (2006) Amplification of bifunctional ligands for calmodulin from a dynamic combinatorial library. *Chemistry – A European Journal*, 12 (4). pp. 1081-1087. ISSN: 0947-6539

<https://doi.org/10.1002/chem.200500357>

This is the peer reviewed version of the following article: Milanesi, L., Hunter, C.A., Sedelnikova, S.E. and Waltho, J.P. (2006), Amplification of Bifunctional Ligands for Calmodulin from a Dynamic Combinatorial Library. *Chemistry – A European Journal*, 12: 1081-1087, which has been published in final form at <https://doi.org/10.1002/chem.200500357>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions. This article may not be enhanced, enriched or otherwise transformed into a derivative work, without express permission from Wiley or by statutory rights under applicable legislation. Copyright notices must not be removed, obscured or modified. The article must be linked to Wiley's version of record on Wiley Online Library and any embedding, framing or otherwise making available the article or pages thereof by third parties from platforms, services and websites other than Wiley Online Library must be prohibited.

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**Amplification of Bifunctional Ligands for Calmodulin from a
Dynamic Combinatorial Library**

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Abstract

A well known strategy to prepare high affinity ligands for a biological receptor is to link together low affinity ligands. DCC (dynamic combinatorial chemistry) was used to select bifunctional protein ligands with high affinity relative to the corresponding monofunctional ligands. Thiol/disulfide generated a small dynamic library of bifunctional ligands in the presence of calmodulin, a protein with two independently mobile domains. The binding constant of the bifunctional ligand (disulfide) most amplified by the presence of calmodulin is nearly 2 orders of magnitude higher than that of the corresponding monofunctional ligand (thiol).

Keywords: calmodulin, chemical biology, combinatorial chemistry, multivalency, protein-ligand binding.

Introduction

Dynamic combinatorial chemistry (DCC) is a new approach successfully used in the discovery of high affinity ligands for biological and non biological receptors.^[1] This method uses reversible covalent chemistry to generate a mixture of compounds under thermodynamic control. The addition of a molecular target induces a change in the composition of the equilibrating mixture resulting in amplification of components with the highest binding affinity for the target.^[2] Using both dynamic and conventional combinatorial approaches, potent inhibitors of proteins have been obtained by linking two low affinity ligands together in a single bifunctional ligand.^[3] This strategy has been applied to the design of ligands that bind to one large pocket or to two pockets of a monomeric or multimeric protein.^[2a-b, 2d, 4-6] A common feature of this binding mode is that the binding sites are in a fixed positional orientation and thus formation of the complex does not involve large rearrangements of the protein receptor. Proteins used to screen dynamic or static combinatorial libraries are considered as *locks* to which one of the library components (the *key*) would fit.^[1, 2a-b] However for some proteins, binding to the natural substrate involves a hinge or shear motion of an entire domain with subsequent loss of flexibility in both the protein and the substrate.^[7] An example of such a protein is the calcium transducer calmodulin (CaM), which regulates a wide range of physiological processes by binding to numerous enzymes. The structures of the calcium loaded calmodulin (Ca²⁺-CaM) and its complexes with small antagonists and peptides have been elucidated.^[8] The peptide sequences are derived from the

natural protein targets of calmodulin, and thus the structures of the CaM-peptide complexes provide a good model of the binding mode under physiological conditions.^[9] In the Ca²⁺-CaM form, the two protein domains are connected by a flexible linker and are independently mobile.^[10] In Ca²⁺-CaM complexes with peptides, the peptides adopt an α -helical structure and the central linker bends resulting in a rearrangement of the two domains around the peptide. A similar structure is observed for complexes of CaM and small antagonists, although in this case binding involves more than one molecule of antagonist per molecule of Ca²⁺-CaM.^[8d, 11]

It has been suggested that it is the simultaneous involvement of both domains and the additional interactions with the central linker that determine the higher binding constant of peptides in comparison with small ligands ($K_d = 10^{-9}$ M and 10^{-6} M respectively).^[11b] Thus an improvement in the affinity of ligands for CaM may be achieved by preparing ligands that mimic the bidentate binding mode of the peptides. Indeed in previous work, we have used non-combinatorial methods to prepare bifunctional ligands that show higher affinity for CaM than the corresponding monofunctional ligands.^[7d] Our data show that the bifunctional ligands bind simultaneously to both protein domains as observed for peptides. This finding illustrates the validity of a multivalency strategy in the design of high affinity ligands for a receptor with motionally independent binding sites.^[7d, 10]

Here, we implement the DCC approach as a tool for the selection and amplification of high affinity dimeric ligands for Ca²⁺-CaM, a model for systems in which binding is

associated with considerable entropic loss due to a decrease in the flexibility of both the ligand and the protein upon complexation.

RESULTS AND DISCUSSION

Design of the library: The library components were designed based on the structures of CaM-peptide or CaM-antagonist complexes, incorporating functionality for the reversible covalent chemistry required to generate a dynamic mixture.^[1] A general structure for a bifunctional ligand for CaM is two hydrophobic groups connected by a linker, in this case a disulfide bond (Figure 1). Four of the library components contain hydrophobic groups that mimic the amino acid side chains residues found in the binding pockets of the Ca²⁺CaM-peptide complexes: leucine (compound LL), phenylalanine (compound PP), tyrosine (compound TT) and benzoyl (compound BB).^[9] All these library components were commercially available. Many CaM antagonists are naphthalene sulphonamides that bind with a 2:1 stoichiometry.^[11b] Thus a naphthalene sulphonamide component was included in the library: (NN) was synthesised by standard literature procedures.^[3d] The recent discovery that a naphthalene sulphonamide CaM antagonist inhibits the proliferation of prostate cancer cells, suggests that such ligands could be of interest as therapeutic agents.^[12] A cystine residue was used as linker between the two hydrophobic groups of the library components. Our previous work indicates that the length of this linker is sufficient for the two hydrophobic groups to bind simultaneously to the two protein domains.^[7d] The disulfide bond of the cystine

linker provides the reversible chemistry necessary in the generation of the library. The versatility and adaptability of thiol/disulfide exchange for the generation of dynamic library with biological receptors is well documented. [2b, 3d 13]

Generation and analysis of the dynamic libraries: A requirement for the generation of the dynamic library is that the experimental conditions are compatible with thiol/disulfide exchange chemistry and with the mode of binding of the targeted receptor. Thiol/disulfide exchange was initiated by the addition of an excess of dithiothreitol (DTT) (Figure 2). [2b, 3d The library was buffered at pH 7.5 to allow rapid thiol/disulfide exchange, ligand binding and complete dissolution of all the symmetric disulfide precursors. The stability of the DTT cyclic disulfide means that DTT should not be incorporated into oligomeric disulfides in the equilibrated mixture, and the number of disulfides present at equilibrium is determined only by scrambling of the original symmetrical disulfides. Air oxidation of the thiols formed after addition of DTT, eventually stops the equilibration and freezes the library. Starting with five disulfides gives a library of fifteen components after scrambling. [2b]

A control library (without CaM) was prepared and analysed every 24 hours by UV-HPLC and MS-HPLC. After 48 hours, 15 disulfides were found in the mixture, and their expected identities were confirmed by mass spectrometry (Figure 3). No thiols were detected. The composition of the library was unchanged for a further 76 hours indicating that the thiol/disulfide exchange was complete. The library composition was independent of the concentration of DTT

demonstrating that the library contains an equilibrium distribution of disulfides.

To analyse the library composition in the presence of protein, micro centrifuge filtration was used to separate the free and bound library components. The protein and any compounds bound to it are selectively concentrated in one compartment of the ultra filtration device, while unbound compounds are in the filtrate of the other compartment (Figure 2).

The filtrate solution (containing the free components) was analysed by UV-HPLC (Figure 4A). For all the chromatograms, the signal of oxidised DTT was used as an internal standard and thus it is the relative intensities of the peaks that are indicative of any effect induced by the protein. In principle, the chromatogram in Figure 4A could be compared with a reference library without CaM (Figure 3B). However, since it is not possible to rule out small variations in the filtrate composition due to the experimental procedure (e.g., the absorption of small quantities of library components on the membranes of the filtration unit due to non specific interactions), a control experiment was carried out for a library equilibrated without CaM and then subjected to micro centrifuge filtration. The HPLC trace of the filtrate solution of the control library is shown in Figure 4B. A comparison of the trace for the control experiment after filtration (Figure 4B) with the one before filtration (Figure 3B) shows subtle differences in the intensity distribution of the three signals with retention times (t_r) between 10 and 11 minutes, and the disappearance of the signal at $t_r = 13.8$ minutes in the control library after filtration. This suggests that the membrane of the

filtration unit does change the composition of the library to some extent.

This makes it difficult to draw conclusions from the differences in the HPLC traces of the filtrates with and without CaM (Figure 4A vs 4B): the compounds that show a lower peak intensity in the CaM equilibrated library ($t_r = 10-11$ minutes) compared to the control library may be adsorbed to the protein or to the membrane, which could lead to false positives in this experiment.

However a clearer picture emerged from analysis of the supernatant fractions (containing the bound library components), where an increase in the concentration of a library component in the presence of protein relative to the control can unambiguously be ascribed to protein binding. The supernatant solutions were treated with EDTA to remove calcium from CaM. In the *apo* form, the protein hydrophobic surfaces that are crucial for ligand binding are hidden, and so any bound ligands should be released from the protein.^[9] A second ultra filtration step was then used to separate *apo*CaM from the "bound" library components. There are clear differences in intensities of some of the peaks in the trace of the CaM containing solution compared with the control (Figure 5A vs 5B). The largest changes are in the NN ($t_r = 13.8$ min.) and NB ($t_r = 12.2$ min.) peaks that are significantly more intense in the presence of protein.

The NN peak is present in the filtrate of both CaM and the control library whereas in the first filtration step all the NN appears to be bound to the membrane of the filtration unit (Figure 4B vs 5B).

We attribute this to the use of excess EDTA in the second filtration step that will inhibit to some extent the absorption of NN to the membrane. Normalising the peak

intensities relative to DTT gives increases of 80% and 10% in the concentrations of NN and NB respectively in the presence of CaM, but these figures are probably underestimates due to losses on the membrane. Thus disulfides NN and NB are amplified by CaM, and there is a corresponding decrease in the intensity of the BB peak ($t_r = 10.1$ min.).

To eliminate the possibility that the amplification of NN and NB resulted from a non-specific interaction with CaM, a control experiment was carried out starting with the apo form of the protein under otherwise identical conditions. The results using apoCaM were identical to those obtained in the protein-free control experiment (see supplementary material), suggesting that the amplification observed in the presence of CaM is the result of a specific recognition process.

Binding studies. In order to determine the binding affinity of NN and NB for CaM, the mixed disulfide was synthesised on a preparative scale from the corresponding symmetric disulfide using a procedure similar to that used for the library preparation. A ^1H NMR titration was used to determine the dissociation constants and the values are reported in Table 1.

The symmetric disulfide NN showed the greatest affinity for CaM. This result is in agreement with the higher amplification observed for this disulfide in the selection experiment and reflects the homology of the two protein domains. There is a possibility that one of the other library members could be a better inhibitor than NN, but we have only investigated those systems that showed significant amplification. To test whether the disulfides act as

bifunctional ligands with improved affinity relative to the corresponding monofunctional ligands, the binding constants for the corresponding thiols were determined. The binding constant of NSH, the thiol obtained by reduction of NN with DTT, was nearly 2 orders of magnitude lower than the binding constant of NN (Table 1). The mixed disulfide NB also shows an increase in affinity relative to the monofunctional thiol, NSH.

The increased affinity for calmodulin observed for the bifunctional disulfides confirm that these compounds bind simultaneously to both protein domains as previously observed with structurally related compounds.^[7d]

Conclusions

A dynamic combinatorial library of disulfides capable of binding to calmodulin has been designed. A high affinity inhibitor has been found. For this inhibitor, the affinity for CaM is two orders of magnitude greater than that of the corresponding monofunctional ligand. Although the bifunctional approach has been used with disulfides based DCLs before, we chose CaM as a clearly defined model for the simplest form of multivalency (a divalent interaction with nearly identical binding sites).

Experimental section

Materials and instruments. All reagents were purchased and used without further purification. Solvents and N,N'-Dibenzoyl-L-cystine (BB) were purchased from Fluka. Naphthalene sulphonylchloride was purchased from Sigma-

Aldrich. L-Cystinyl-bis-L-phenylalanine (PP), L-Cystinyl-bis-L-Tyrosine (TT), L-Cystinyl-bis-L-leucine (LL), were purchased from Bachem. Reverse phase chromatography was performed with a HP 1100 equipped with a quaternary gradient pump, a UV/Vis detector and an auto sampler. For all HPLC analyses, a UV/Vis detector set at $\lambda = 220$ nm was used. For analysis of the library composition by RP-18 chromatography, a Vydac (300 Å, 5 μm) prepacked column size A (150x4.6 mm, flow rate: 1 mL min⁻¹) was used. For RP-18 preparative-chromatography, a Phenomenex-Luna (5 μm , 100 Å) prepacked column size A (flow rate: 1 mL min⁻¹) and a Phenomenex-Luna (100 Å, 5 μm) prepacked column size B (150x21 mm, flow rate 16 mL min⁻¹) were used. For ESI-MS analysis of the library composition, a Micromass LC spectrometer equipped with a Phenomenex-Jupiter C18 (300 Å, 5 μm) prepacked column of size C (250x4.6 mm) was used. Ultra filtration was carried out with Vivaspin concentrators (20 mL and 0.5 mL, M_w 10000) through a PES (Polyethylensulphone) membrane.

NMR spectra were recorded on Bruker AMX-250, AMX-400 or DRX-500 equipped with a cryoprobe. The following abbreviations are used for the multiplicities: s = singlet, d = doublet, t = triplet, q= quartet, m= multiplet, br = broad.

General Methods

Buffers composition: buffer A (50 mM TRIS, 10 mM CaCl₂·2H₂O, 50 mM KCl, 0.5 mM NaN₃, pH 7.5), buffer B (50 mM TRIS, 10 mM CaCl₂·2H₂O, 50 mM KCl, 0.5 mM NaN₃, pH 7.5), buffer C (1 mM TRIS, 10 mM CaCl₂·2H₂O, 50 mM KCl, 0.5 mM NaN₃ in D₂O, pH 7.5), buffer D (1 mM TRIS, 10 mM CaCl₂·2H₂O, 50 mM KCl, 0.5 mM NaN₃, 50 mM DTT in D₂O, pH 7.5). All buffers were filtered through a 0.2 µm cellulose membrane (Amicon) and the pH values given are not corrected for the deuterated solutions.

The following abbreviations are used: EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; DTT, 1,4- dithio-D,L-threitol; TRIS, tris(hydroxymethyl)aminomethane; TSP, 3-(trimethylsilyl)propionic-2,2,3,3,-d₄ acid, sodium salt.

CaM preparation. Recombinant bovine CaM was prepared as described previously.^[14] Protein samples were prepared from lyophilised, apoCaM. ^[14] Protein concentration was estimated by dry weight on a balance accurate to 1 mg and by using a molar extinction coefficient ϵ_{280} of 3300 M⁻¹ cm⁻¹.^[15]

Synthesis of NN. A solution of naphthalene sulphonyl chloride (0.14 g, 6.4 mmol) in CH₃CN (30 mL) was added

dropwise to a solution of L-cystine (0.5 g, 2.1 mmol) in 40 ml of 0.3 M Na₂CO₃ heated to 50°C. The reaction mixture was then stirred for 5 hours, and the solvent was removed under reduced pressure. The solid was redissolved in water (pH 10.0) and acidified to pH 2.0. The precipitate formed was filtered and washed with water, followed by cold DCM. The solid was dissolved in DCM, dried over Na₂SO₄ and concentrated under reduced pressure to give NN (0.8g, yield: 61%).

¹H NMR (250 MHz, CDCl₃): δ = 8.58 (d, *J* = 8.2, 2H), 8.21 (dd, *J* = 7.3, 0.9, 2H), 8.05 (d, *J* = 8.0, 2H), 7.92 (dd, *J* = 7.6, 1.8, 2H), 7.68–7.56 (m, 4H), 7.51 (t, *J* = 8, 2H), 5.98 (d, *J* = 7.5, 2H) 4.18–4.14 (m, 2H), 3.18 (dd, *J* = 14, 4, 2H), 2.80 (dd, *J* = 15, 4.5, 2H); ¹³C NMR (75 MHz, CD₃OD): δ = 173.0, 136.8, 135.7, 135.4, 130.3, 130.0; 129.5, 128.9, 127.9, 126.22, 125.22, 56.23, 41.27. HRMS (ESI): calcd for C₂₆H₂₅N₂O₈S₄ [M+H]⁺: 621.0494, found: 621.0483.

Synthesis of NB. 0.05g (0.64 mmol) of DTT were added to a solution of NN (0.2 g, 0.32 mmol) and BB (0.14 g, 0.32 mmol) dissolved in 10 mL of MeOH and 2 mL of 200 mM TRIS (pH 8). The solution was stirred exposed to air overnight. To follow the reaction, 4 μL of the reaction mixture were diluted in

100 μL of water and transferred to a 100 μL vial for HPLC analysis (Column: Vydac). 10 μL were injected via an autosampler eluting with 0.1% TFA-MeCN and 0.1% TFA - H_2O with the following gradient: 0-12 min, 5 \rightarrow 55% MeCN, 12-22 min, 55 \rightarrow 100% MeCN, 22-30 min 100 \rightarrow 5% MeCN. No thiols were detected in the chromatogram, and the reaction mixture was concentrated under vacuum at room temperature. The mixture was dissolved in 3 mL of methanol, and aliquots of 500 μL were purified by HPLC eluting with 0.1% TFA-MeCN/0.1% TFA- H_2O with the following gradient: 0-13 min, 33% MeCN, 13-20 min, 33 \rightarrow 100% MeCN, 20-30 min 100 \rightarrow 33% MeCN. Fractions corresponding to NB were collected and concentrated under reduced pressure at room temperature. Residual water was removed with a freeze drier to give pure NB (0.07 g, yield = 20%).

^1H NMR (400 MHz, buffer C): δ = 8.54 (dd, J = 8.8, 0.7, 1H), 8.18 (dd, J = 7.3, 1.2, 1H), 8.07 (d, J = 8.2, 1H), 7.85 (d, J = 8.0, 1H), 7.77-7.75 (m, 2H), 7.72-7.59 (m, 4H), 7.53-7.45 (m, 2H), 4.43 (dd, J = 10.1, 4.0, 1H), 3.80 (dd, J = 9.9, 3.8, 1H), 2.99 (dd, J = 13.8, 3.8, 1H), 2.92 (dd, J = 14.1, 3.8, 1H), 2.66 (dd, J = 13.8, 10.3, 1H), 2.56 (dd, J = 14.1, 10.0, 1H); ^{13}C -NMR (75 MHz, CD_3OD): δ = 135.3, 134.3, 133.8, 131.4, 128.8, 128.7, 128.5, 128.4, 128.1, 128.0, 127.5,

127.4, 127.0, 126.4, 126.3, 124.8, 124.6, 123.6, 66.5, 40.6,
38.8, 26.5; HRMS (ESI): calcd for C₂₃H₂₃N₂O₇S₃ [M+H]⁺:
535.0667, found: 535.0667.

Synthesis and analysis of combinatorial libraries

Stock solutions of the symmetric disulfides in buffer A or B were prepared at the following concentrations: 490 μ M NN, 650 μ M PP, 900 μ M LL, 1.8 mM of TT, 780 μ M BB. Aliquots of the stock solutions were diluted in 14 mL of buffer to give a final concentration of 140 μ M for each disulfide. 5 mL of this solution were used to dissolve 7.3 mg of apoCaM (70 μ M). To each solution (6 mL), 330 μ L of 0.1 M DTT (6.6 mM) was added, and the solutions were left at RT for 4 days under gentle stirring in a plastic vial. The vials were closed with a rubber septum and exposed to air via a needle. For analysis of the library without CaM (2 hours after addition of DTT and every subsequent 24 hours), 100 μ L of the library was transferred to an HPLC vial and 10 μ L were injected onto the column via an autosampler. Chromatographic conditions were the same as described for the analysis of NP (Column: Vydac). Control samples of buffer A, buffer B, each disulfide, DTT, oxidised DTT, and mixtures of disulfides

before addition of DTT were analysed in parallel. LC/MS (ES+) was carried out for the library in buffer A, as described for the HPLC analysis using column size D and formic acid instead of TFA in the eluent mixture.

For analysis of the equilibrated libraries (after 4 days at room temperature), 3.5 mL of each library solution (with and without CaM) were transferred to 6 Vivaspin concentrators previously washed with H₂O (2 x 20 mL) and buffer A or B (1 x 20 mL). The solutions were centrifuged at 3000g for 25 min to give 400 µL of CaM-libraries and 350 µL of buffers and libraries in one compartment of the centrifugal device. The filtrate was kept aside for RP-HPLC, and the solutions concentrated to 350 µL were diluted to 400 µL with buffer A or B. To the concentrated solutions 7 mg of EDTA (100 mM) and 30 µL of 0.1 M TRIS (pH 8) were added. The solutions were stirred for 1h at room temperature and then transferred to 4 Vivaspin concentrators previously washed with water (2 x 400 µL) and buffers A or B (1 x 400 µL in each case). The solutions were centrifuged at 12000 rpm for 12 minutes. 100 µL of the filtrate obtained from the Vivaspin concentrators were analysed by RP-HPLC (Vydac) using the

same conditions described for the analysis of the equilibrating disulfide library.

Binding studies

NMR spectroscopy. All titrations were carried out at 310 K at least twice. For ^1D and ^1H experiments, spectral widths were 13500 Hz and relaxation delays were 0.7 s. Solvent suppression was achieved using presaturation. To test the stability of the ligand a ^1H NMR spectrum of the ligand solution was recorded before and after leaving the solution at 310 K for 16 h.

CaM and NN, NB. ^1H NMR titrations were carried out as follows: stock solutions of the ligand at known concentration (400-600 μM) were prepared in buffer C (final pH 7.4). An aliquot of this solution was diluted with buffer C and TSP 10mM TSP in D_2O was added to give a solution of 40 μM ligand and 40 μM TSP. 500 μL of this ligand solution was used to dissolve 6 mg of apoCaM accurately weighted (equivalent to 550 μM CaM, pH = 5). 10-15 μL of 0.1 M NaOD were added to the CaM/ligand solution to give a final pH of 7.4. The solutions of ligand and CaM containing ligand were incubated at RT under gentle stirring for 20 h to allow complete H/D exchange for the amide protons of CaM. ^1H NMR

spectra were recorded of the ligand solution (0.6 mL) and for each successive addition (10-50 μ L aliquots) of the protein solution containing ligand up to a molar ratio of 3:1 protein:ligand.

CaM and NSH: ^1H NMR titrations were carried as described for NN and BN with the following differences: buffer D was used, and addition of protein solution containing ligand continued to a molar ratio of 6:1 protein:ligand. Prior to titration, a control experiment was carried out to verify that formation of NSH from a 48 μM solution of NN in buffer D was complete in 16 hours at 310 K.

Variations in ligand chemical shift greater than 0.06 ppm were fitted to a 1:1 or a 2:1 binding isotherm for the bifunctional (NN, NB) and monofunctional ligands (NSH) respectively. The data were analysed using non-linear curve fitting (*NMRTit_HG* for 1:1 binding isotherm and *NMRTit_HHG* for 2:1 binding isotherm with independent binding sites).^[16] This procedure optimises the binding constant and the limiting and bound chemical shifts. Representative data sets are illustrated in Figure 4 of the supplementary material.

Acknowledgments

We thank Dr. Arthur Moir for technical assistance and we acknowledge the University of Sheffield and BBSRC (LM) for financial support.

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Figures

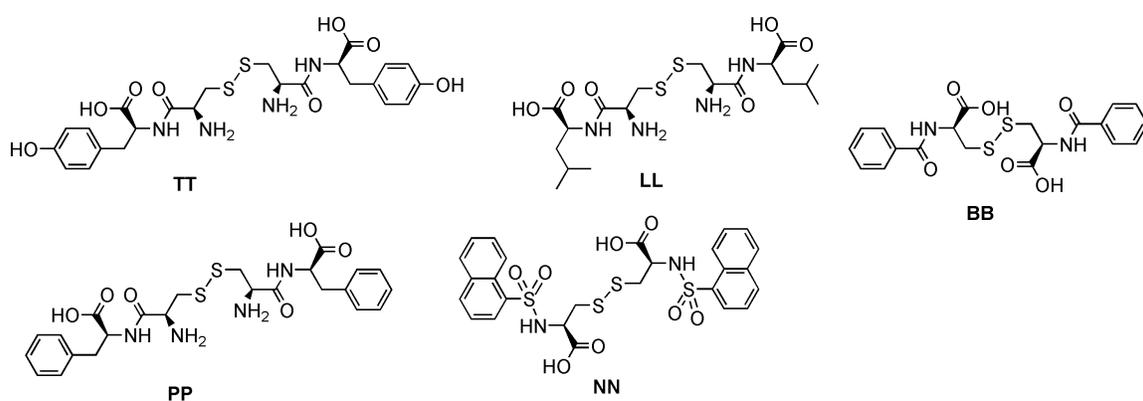


Figure 1. Structure of the disulfides used as precursors in the DCL.

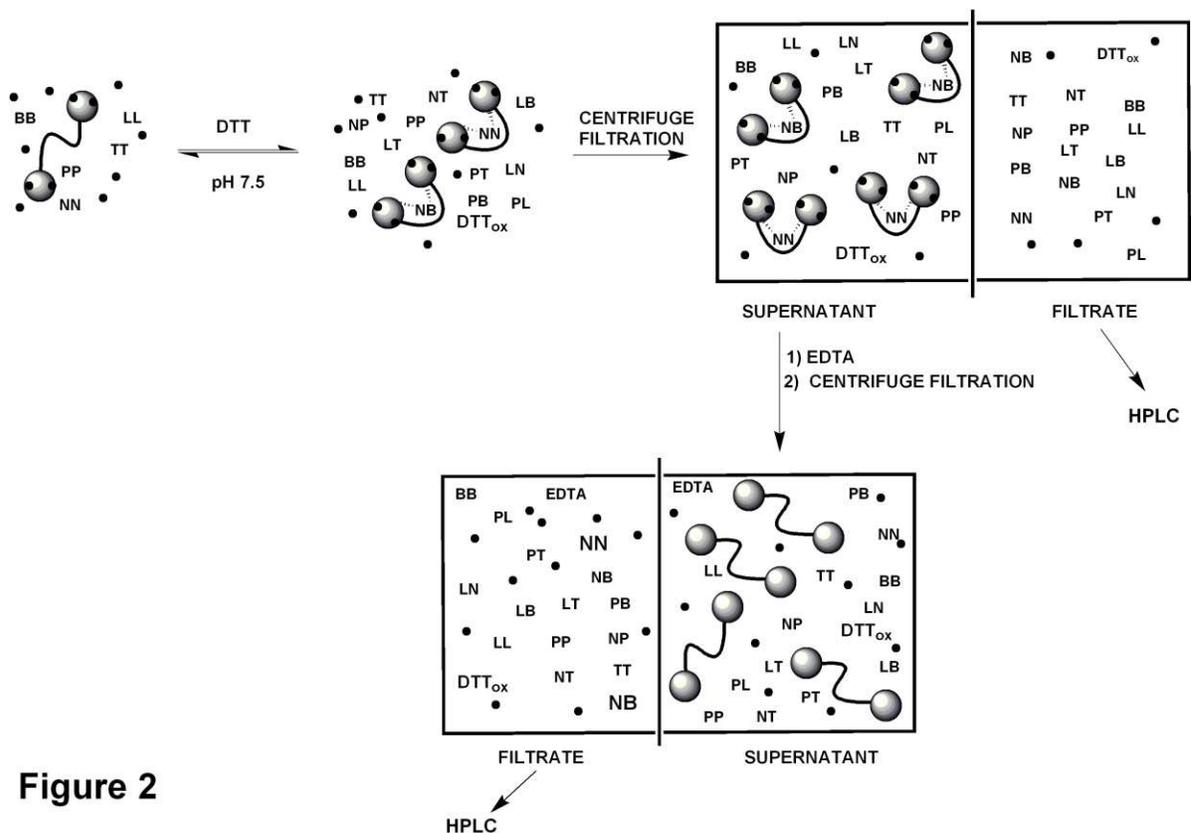


Figure 2

Figure 2. Schematic illustration of the conditions used for the synthesis and analysis of the library in the presence of CaM. In the first step five symmetric disulfides are equilibrated in the presence of DTT at basic pH to give a mixture of 15 disulfides and oxidised DTT (DTT_{ox}). This solution is then subjected to centrifuge filtration against a membrane non permeable to protein. The two solutions generated on the two compartments of the centrifugal device, one containing disulfides (filtrate) and the other containing protein and disulfides (surnatant) are depicted. In the final step the solution of concentrated protein and disulfides is treated with EDTA to give apoCaM and release bound disulfides and Ca²⁺ ions (depicted as black spheres). A second centrifuge filtration allows to separate the protein from the disulfides. The filtrate obtained in each step is subjected to HPLC analysis.

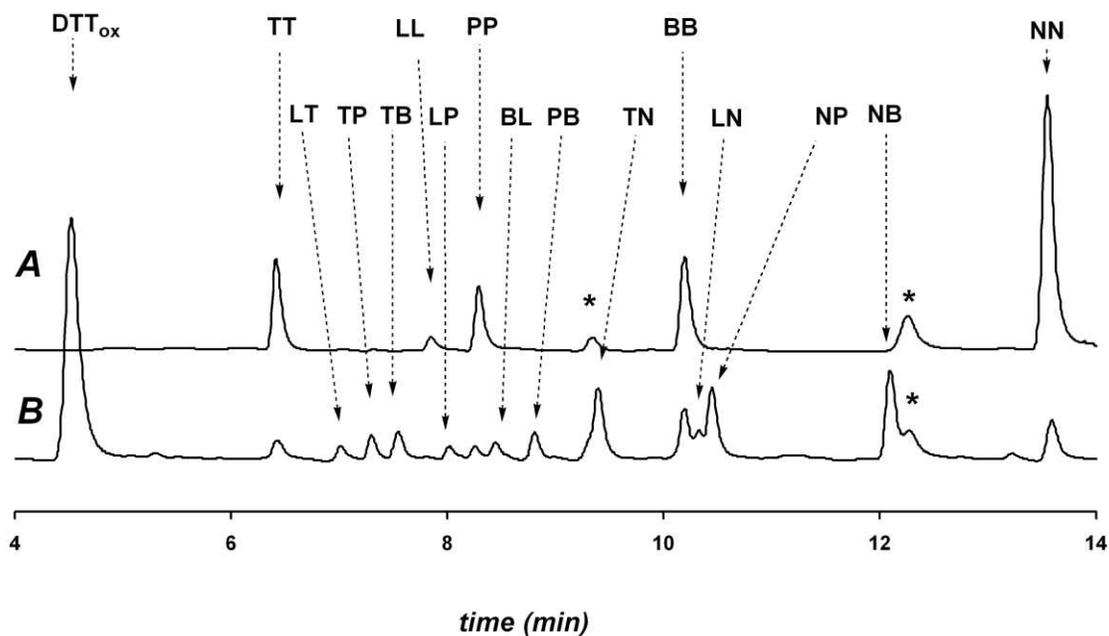


Figure 3. HPLC traces of the disulfide mixtures in 50 Mm TRIS, 10mM CaCl₂, 50 mM KCl, 0.5 mM NaN₃, pH 7.5. A) Chromatogram of the symmetric disulfides precursors. B) Chromatogram of the final reaction mixture containing the expected 15 disulfides and oxidised DTT (DTT_{ox}). Asterisk marks indicate unidentified impurities. Differences in signal intensities arise from differences in the extinction coefficients of the symmetric disulfides.

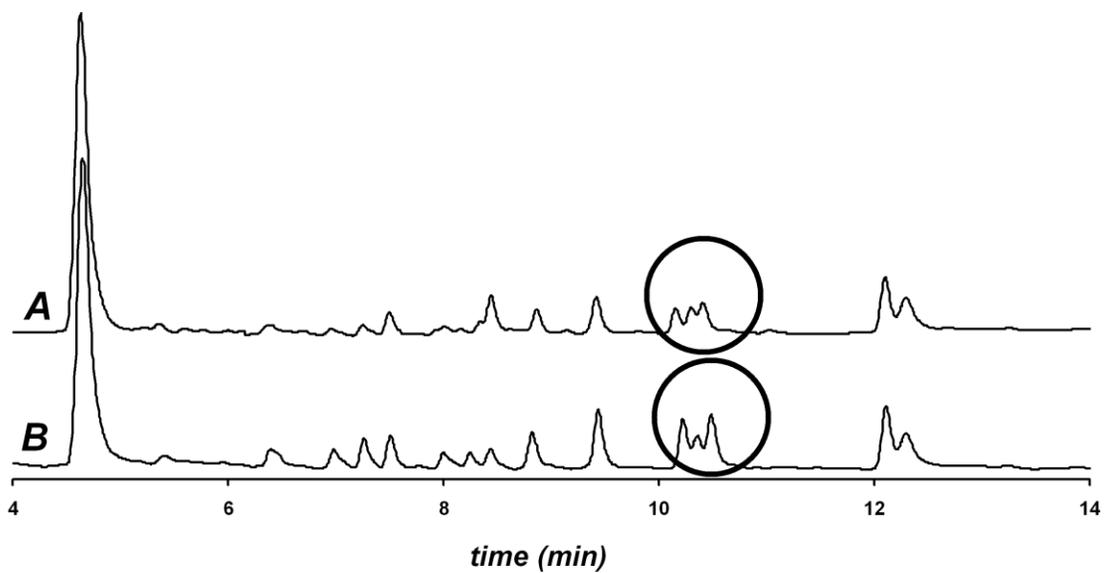


Figure 4. HPLC traces of the filtrate solutions obtained after centrifuge filtration of the disulfides mixtures. A) Trace corresponding to the library equilibrated in the presence of CaM. B) Trace corresponding to the library equilibrated without CaM. A black circle indicate the peaks that show the largest difference in relative intensities (see text for details).

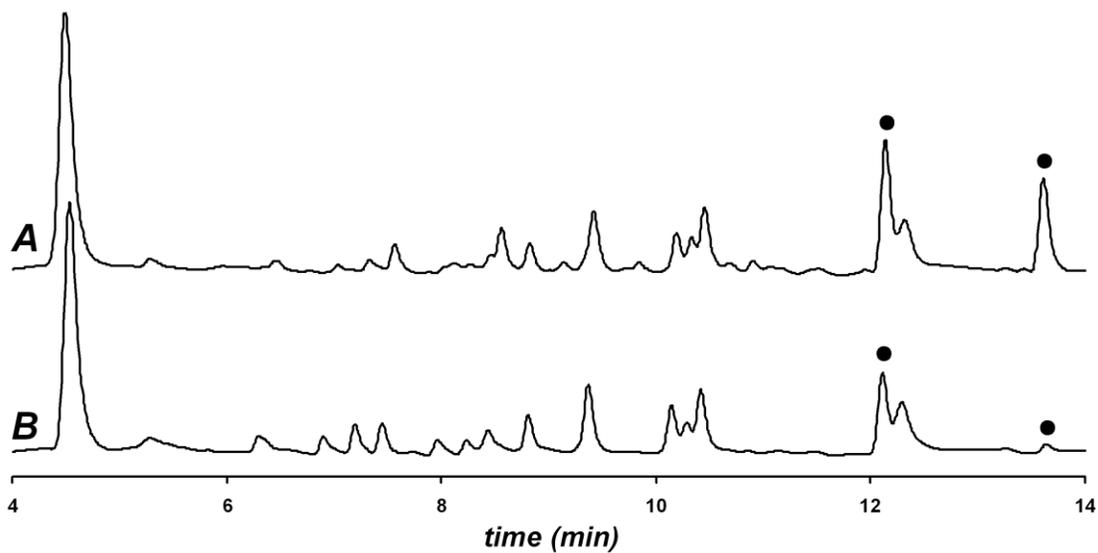


Figure 5. HPLC traces of the filtrate solutions obtained after centrifuge filtration of the disulfides mixtures treated with EDTA A) Trace corresponding to the library equilibrated in the presence of CaM. B) Trace corresponding to the library equilibrated without CaM. Dot marks indicate the peaks corresponding to compounds amplified by CaM.

Table 1

Dissociation constants and stoichiometries for CaM complexed with disulfides NN, NB (bifunctional) and thiol NSH (monofunctional)

Ligand	K_d (μM)	Stoichiometry (ligand:CaM)
NN	10±1.4	1
NB	210±10	1
NSH	814±89	2

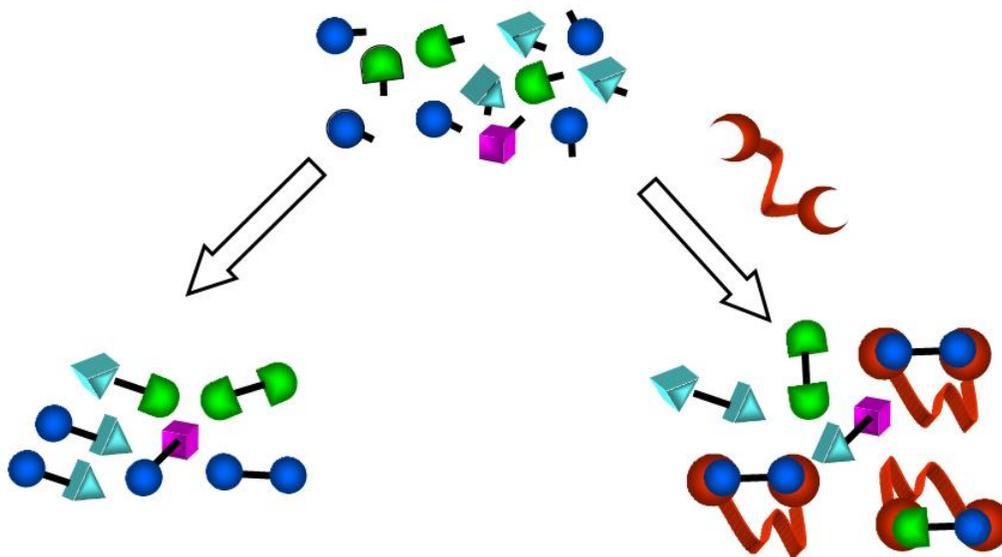


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Dynamic combinatorial chemistry is used to select high affinity bifunctional ligands for a receptor with independently mobile binding sites.