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Selective recognition of the di/trimethylammonium motif by an artificial carboxycalixarene receptor

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Chemical tools that recognise post-translational modifications have promising applications in biochemistry and in therapy. We report a simple carboxycalixarene that selectively binds molecules containing di/trimethylammonium moieties in isolation, in cell lysates and when incorporated in histone peptides. Our findings reveal the potential of using carboxycalixarene-based receptors to study epigenetic regulation.

Post-translational modifications (PTMs) are chemical alterations to proteins that occur after their biosynthesis in ribosomes.[1] PTMs often play important biological roles, including in protein folding, signalling and epigenetic regulation of transcription. However, despite their functional importance, the underlying mechanisms by which PTMs induce their biological functions are largely undefined, thus hindering biomedical efforts. This is particularly pertinent in the field of epigenetics, where transcriptional control is likely induced by combinations of PTMs, predominantly on the *N*-terminal tail of histone H3, in a highly context-dependent manner.[2]

Deciphering the functional roles of PTMs relies on bio-chemical/biological methods that enable the recognition and discrimination of specific PTMs. In this regard, the majority of reported studies use antibodies;[3] however, antibodies are often sub-optimal PTM binders in these experiments due to (often) incomplete characterisation of their binding profiles/selectivity, their propensity to undergo unfolding/ degradation, their relatively high cost and batch-to-batch variations in their binding affinities to their targets. Chemical tools that selectively recognise PTMs represent a promising alternative to antibodies and have found applications in biochemical and cellular activity and binding assays, and in biomedical applications including disease diagnostics.[4]

We are interested in developing molecules, *i.e.* artificial receptors, that selectively recognise and bind PTMs, with a particular focus on developing binders of methylated lysines. Lysine N^{ε}-methylation, which exists in mono-, di- and tri-methylated forms, occurs at multiple positions on histones and is often reported to either induce or repress gene expression by mechanisms that are dependent on the locations and extent of methylation.[5] However, the precise mechanisms by which these effects are induced and propagated remain largely uncharacterised. Furthermore, the enzymes that deposit and remove histone lysine methyl groups (lysine methyltransferases and lysine demethylases respectively), as well as proteins that bind to methylated histone lysines (*e.g.* plant homeobox domain- and chromodomain-containing proteins), are important therapeutic targets, particularly in cancer.[6] However, medicinal chemistry efforts in this field are hampered by a lack of suitably robust activity and binding assays that can report the methylation states of histone lysines.

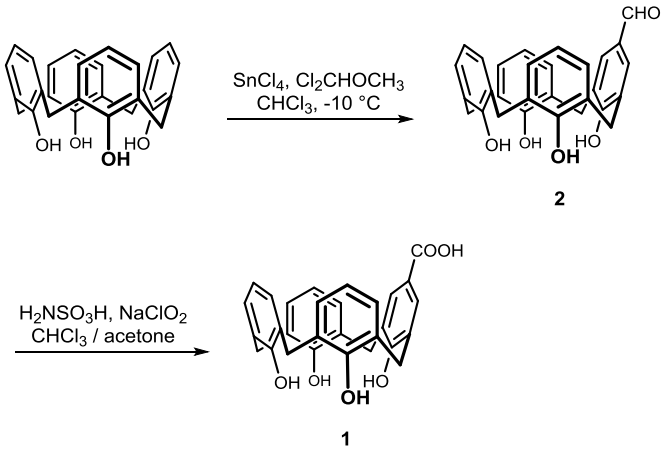

Fig. 1 Synthesis of carboxycalixarene **1**. Initial treatment of the commercially available calix[4]arene with 1,1-dichlorodimethylether and tin(IV)chloride afforded the aldehyde intermediate **2**. This is oxidized using sulphamic acid and sodium chlorite to yield monoacid **1**.

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In general, the recognition and binding of amino acids, oligopeptides and proteins by artificial receptors under physiological conditions, *viz.* in aqueous solution, has always been a challenging task in supramolecular chemistry. As many peptides and proteins contain amino acids with basic side chains, which are positively charged under neutral conditions, their recognition is in most cases ascribable to the complexation of alkylammonium ions.[7] Besides steric and molecular complementarity, three types of interactions between host and guest are typical: a) Hydrogen bonds, b) Cation···π-interactions, c) Ion pairs and salt bridges.[8] Using this concept, a large variety of artificial ammonium ion receptors have been introduced, ranging from crown ethers, cyclopeptides and calixarenes to molecular tweezers, cucurbiturils and cyclodextrins.[9] Early work on this topic was limited to recognition of ammonium ions in organic solvents, allowing strong hydrogen bonding between host and guest. In recent years, some progress has been made concerning the complexation of amino acids and peptides in more polar solvents, such as methanol and especially in water.[10] In this more challenging medium, hydrogen bonding between host and guest will provide less impetus for binding.[11] As introduction of methyl groups to basic amino acid residues results not in a change of the overall charge but in more lipophilic side chains, C-H···π interactions and the hydrophobic effect are having a substantial reinforcing effect on the complexation.

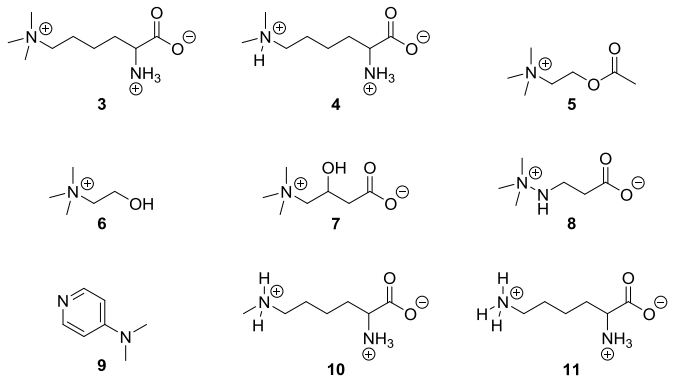

Fig. 2 Small molecules tested in this study. These include trimethyllysine (**3**), dimethyllysine (**4**), acetylcholine (**5**), choline (**6**), carnitine (**7**), meldonium (mildronate) (**8**), 4-dimethylaminopyridine (**9**), methyllysine (**10**) and lysine (**11**). Unless otherwise specified, racemic compounds are used in this study.

When dealing with the recognition of biologically active species, like amino acids and peptides featuring post-translational modifications, the artificial host provides a ''minimized'' representation of the binding sites of natural receptors. Focusing on methylated amino acid residues here structural studies on methyllysine effectors in cognate peptide–bound or apo forms suggest general mechanisms that are used by binding pockets to achieve specificity toward distinct methylation states; for recognizing di- and trimethylated lysine residues, "aromatic cages" are typical.[12] Such cavities can be mimicked by aromatic macrocycles, which already delivered promising results for a great variety of guest compounds such as lipids, nucleotides and (unsubstituted) amino acids and peptides.[13] One particular class are so-called calix[4]arenes *i.e. m*-cyclophanes with four phenolic groups, featuring electron-rich aryl moieties.[14] The inclusion properties of differently substituted calix[4]arenes towards amines and ammonium ions are well-established. Thereby, very often ionisable groups, such as carboxylic or sulfonic acids, have been introduced to the macrocylce.[15] Due to the rigid multiaromatic cavity and charge complementarity this artificial hosts can be also applied to imitate the natural aromatic cages found in lysine modifying enzymes. The inclusion behavior of calixarenes depends profoundly on their conformation, their substitution pattern and the solvents employed for inclusion formation, with some examples discussed in the following for carboxylated calix[4]arenes. Respective cycles with free OH groups[16] and double alkylated[17] species feature very often a so-called 'cone' conformation with an open cavity facilitating C-H···π interactions between host and guest. Tetralkylation of the phenolic moieties mostly leads to a closed cavity making guest inclusion into the cavity difficult;[18] an exception are tetra-methoxy and tetraethoxy calix[4]arenes as they show a much higher conformational flexibility.[19]

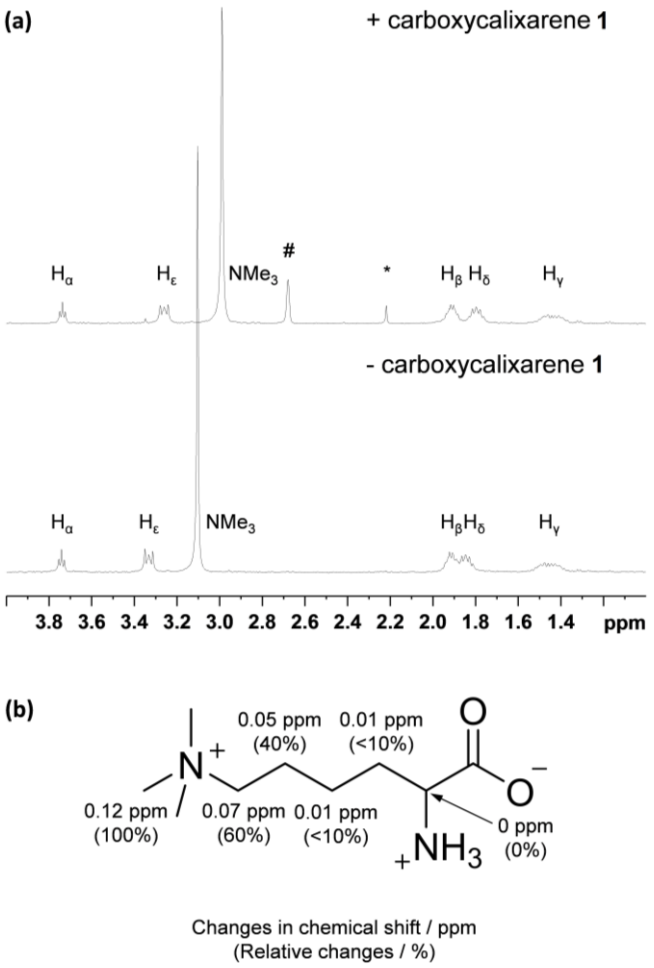

Fig. 3 NMR analysis of the binding of trimethyllysine **3** to carboxycalixarene **1**. (a) Relative changes of chemical shifts of trimethyllysine **3** in the presence and absence of carboxycalixarene **1**. The hash symbol (#) indicates resonance of residue DMSO-d_6 and the asterisk (*) indicates an impurity in **1**; (b) Binding epitope mapping of trimethyllysine **3** showing changes in chemical shifts (and relative percentage) upon binding to carboxycalixarene **1**.

Herein we present a simple, easily synthesised monocarboxy-calix[4]arene (**1**) in the cone conformation that binds methylated lysines. This compound is related to, but is distinct

from, previously reported methyllysine binders.[20] Carboxycalixarene **1** was synthesised from aldehyde **2** by applying a Gross formylation[21] followed by oxidation with sodium chlorite (Fig. 1 and Supplementary Information).[22,23]

The binding of **1** to a library of methylammonium-containing compounds (Fig. 2) was then studied using ^{1}H NMR. Initial screening was conducted with 250 µM of carboxycalixarene **1** and a 2-fold excess of potential ligand (500 µM) to ensure the detection of intermolecular binding interactions. Out of the nine compounds screened, carboxycalixarene **1** was found to selectively bind compounds that contain the dimethylammonium and trimethylammonium moieties (compounds **3**–**9**), as evidenced by significant changes in the *N*-methyl chemical shift, and in some cases, changes in peak width. No or very little spectral changes (≤0.01 ppm) were observed for compounds containing only amine or mono-methylammonium moieties (compounds **10** and **11**; Supplementary Fig. S3–S11), indicating no or very weak binding to **1**. The relative chemical shift changes of the di- and trimethylammonium-containing compounds were then mapped onto their structures (Fig. 3); by comparing relative perturbations in chemical shifts, binding epitopes of the small molecules to carboxycalixarene **1** were determined. With trimethyllysine **3**, the most significant chemical shift perturbations were observed in resonances corresponding to the N^{ε}-methyl groups (0.12 ppm). The perturbation observed for the ε-CH_2 resonance was 0.07 ppm, which is 60% relative to the Me_3 signal perturbation, and no change was observed for the α-proton, which is far removed from the trimethylammonium group, suggesting that **1** selectively recognises and interacts with the trimethylammonium group of trimethyllysine (Fig. 3b). Epitope mapping of compounds **4**–**9** shows the same selective recognition of **1** to the dimethylammonium and trimethylammonium motifs (Supplementary Fig. S12–S17).

Table 1 Dissociation constants, K_D, of the small molecule library screened in this study with carboxycalixarene **1** and tetrasulfonatocalixarene.[24]

Guest	K_D – Host: Carboxycalixarene **1**	K_D – Host: Tetrasulfonatocalixarene
3	60 ± 10 µM	27 µM [c]
4	70 ± 5 µM	62 µM [c]
5	n.d.[a]	-
6	65 ± 5 µM	-
7	60 ± 15 µM	-
8	50 ± 1 µM	-
9	95 ± 10 µM	-
10	> 500 µM [b]	250 µM [c]
11	> 500 µM [b]	1900 µM [c]

[a] K_D cannot be determined as **5** was unstable under assay condition. [b] No or little (<0.01 ppm) chemical shift changes observed under experimental conditions. K_D cannot be estimated precisely due to limited solubility of carboxycalixarene **1**. [c] Ref. 24

Titration experiments were then conducted to determine the binding constants (K_D) for carboxycalixarene **1** and the small molecule binders identified by NMR. Apart from **9**, which is aromatic in nature, all K_D values obtained were around 60 µM with no discernible preference between dimethylammonium and trimethylammonium moieties (Table 1, Supplementary Fig. S18–S23). Due to the limited solubility of **1**, it was not possible to obtain binding constants for compounds **10** and **11**, which showed no or very little chemical shift changes (≤0.01 ppm) upon addition of **1** in the initial NMR experiments. Interestingly, in contrast to other reported methyllysine-binding macrocycles like tetrasulfonatocalixarene,[24] carboxycalixarene **1** has a similar affinity to Kme3 and Kme2 and is selective over Kme1 and Kme0, which is different to the selectivity profile of the reported tetrasulfonatocalixarene.

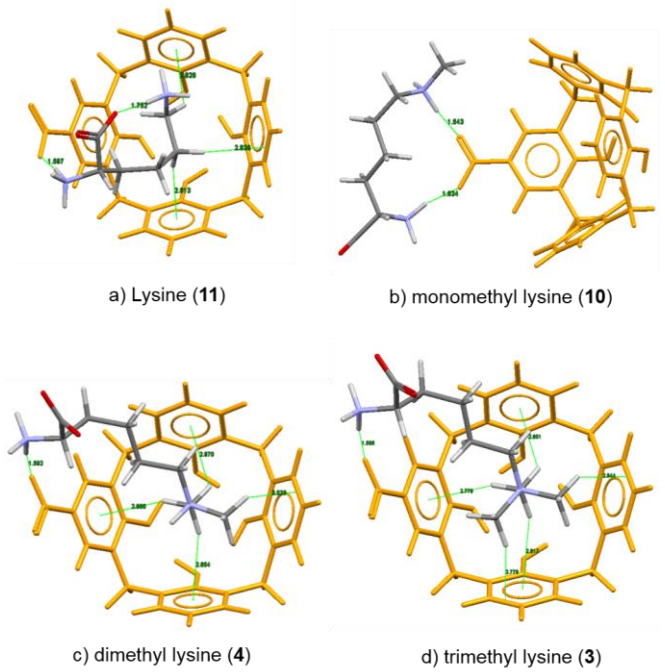

Fig. 4 Energy-minimised complexes of carboxycalixarene **1** with lysines of different methylated states (a-d). In the lysine and monomethyllysine complex, the positively charged nitrogen is pointing away from the cavity; the ε-ammonium moieties of dimethyllysine and trimethyllysine were found to be accommodated in the calixarene cavity. (The host was introduced as an anion, the guests as zwitterions with the N^{ε} as an ammonium ion. For the C-H···π-interactions the centroids of the aromatic rings or the centroids of two respective carbo atoms have been used. The energy minimization has performed with the programme MacroModel V.9.8 using the following parameter: OPLS_2001 force field, MCMM, solvent: water, 20,000 steps.)

In order to gain insights into the molecular interactions of **1** with dimethylammonium and trimethylammonium moieties, molecular modelling studies were conducted. Energy-minimised complexes of **1** with the four lysine binders (compounds **3**, **4**, **10** and **11**) were obtained using Monte Carlo simulations employing the OPLS_2001 force field (MacroModel V.9.8, solvent: water, 20,000 steps, Fig. 4 and Supplementary Table 1).[25] Interestingly, the side chains of dimethyllysine **4** and trimethyllysine **3** were found to be accommodated within the host, with the ε-ammonium moieties pointing into the calixarene cavity; for lysine and monomethyllysine, it is pointing away from the cavity. This suggests that cation···π-interactions[26] contribute to the recognition between **1** and dimethyllysine or trimethyllysine. Furthermore, four [d(H···π) = 2.654–3.023 Å] and five [d(H···π) = 2.778–2.944 Å] C-H···π-interactions were observed between **1** and dimethyllysine **4** and trimethyllysine **3** respectively, suggesting all *N*-methyl groups interact with **1**. By way of interest the X-ray structure of a tetrasulfonato-calix[4]arene/lysine complex[27] shows a very similar orientation of the lysine in the cavity of the host as the respective complex of lysine with **1**. Crystalline inclusion

compounds of higher alkylated ammonium ions with calix[4]arenes are much rarer. Very often the tetraalkylation of the phenolic groups leads to closed cavities preventing the ammoniumion entering the molecular cavity.[28] Though, tetrasulfonato-calixarenes with free phenol moieties were found to form respective complexes with the opened chalice facilitating C-H···π and cation···π interactions between host and guest together with the ammonium ion/sulfonate ion pair.[29] By way of interest, also one of the phenolic OH groups of undecorated calix[4]arenes can get ionized to form complexes with tetra- and trialkylammonium ions. Thereby, acetonitrile as a solvent was found to compete with the alkyl groups in the formation cavity inclusions and respective C-H···π interactions.[30]

Table 2 Dissociation constants, K_D, of H3 peptide fragments with carboxycalixarene **1**

H3 peptide fragments	K_D with **1**
PATGGV(Kme1)KPHRY	> 500 µM [a]
PATGGV(Kme2)KPHRY	60 ± 1 µM
PATGGV(Kme3)KPHRY	65 ± 5 µM
AR(Kme1)STGGK	> 500 µM [a]
AR(Kme2)STGGK	50 ± 5 µM
AR(Kme3)STGGK	50 ± 2 µM

[a] No or little (<0.01 ppm) chemical shift changes observed under experimental conditions. K_D could be estimated due to limited solubility of carboxycalixarene **1**.

Having identified binding between **1** and methylammonium-containing small molecules, experiments were conducted to determine whether **1** can recognise di/trimethyllysine residues within biologically relevant fragment peptides. Two histone H3 peptide fragments corresponding to residues 7-14 and 30-41 (sequence = AR(K9)STGGK and PATGGV(K36)KPHRY respectively) and containing trimethyllysine, dimethyllysine and monomethyllysine at residues 9 and 36 respectively, were synthesised and purified before binding analyses using ^{1}H NMR. H3K9 methylation are generally associated with repressive genes, whereas H3K36 methylation is thought to correlate with transcriptional activation. In agreement with the small molecule studies, **1** was found to selectively recognise peptides containing dimethyllysine or trimethyllysine residues over those containing monomethyllysine residues (Supplementary Fig. S24–S29). K_D values between **1** and the dimethyllysine and trimethyllysine-containing peptides, obtained by ^{1}H NMR titration experiments, were comparable to the K_D values for **1** and dimethyllysine and trimethyllysine (Table 2 and Supplementary Fig. S30–S33).

Finally, we tested the binding of **1** to small molecules that contain the di/trimethylammonium moiety in *Escherichia coli* cell lysates representing a complex analytical matrix. Bacterial cells were grown at 37 °C overnight before harvesting and lysing using sonication. After centrifugation and filtration, the cell lysate was supplemented with 450 µM of dimethylammonium and trimethylammonium-containing small molecules. Using ^{1}H NMR, the binding of the spiked compounds to **1** was clearly observed, revealing the potential for **1** to identify methylated species in complex mixtures (Fig. 5 and Supplementary Fig. S34–S37).

Overall, a simple, readily produced monocarboxycalix[4]arene (**1**) that selectively binds to di/trimethylammonium groups is presented. **1** was observed to discriminate between di-/tri- and mono/non-methylated lysines as free amino acids and when incorporated into histone fragment peptides. Our results indicate **1** to be a useful chemical tool to analyse differentially methylated lysines in biochemical and potentially cellular assays. In addition, it is also potentially useful in, for example, the monitoring and study of agricultural waste, which usually contains methylammonium ion moiety-containing compounds. Furthermore, molecular modelling studies suggest that **1** binds methylammonium groups via π-cation interactions, revealing potential avenues for synthetic optimisation. The research presented here will help in the development of libraries of synthetic methyllysine receptors useful in biochemical, cellular and potentially therapeutic applications.

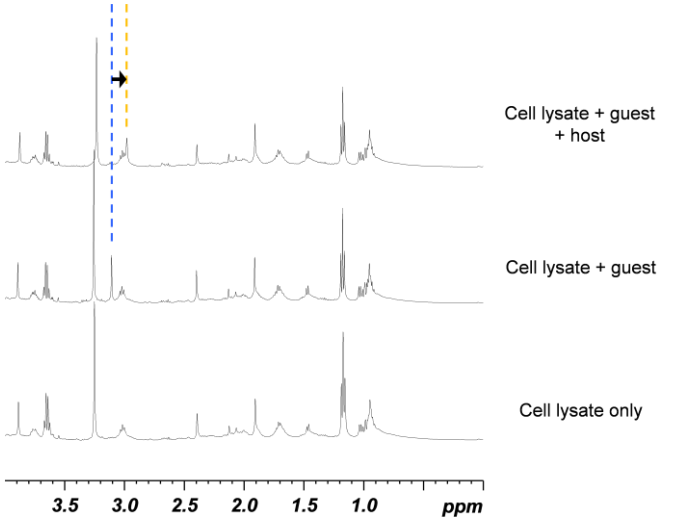

Fig. 5 Carboxycalixarene **1** recognised and bound **3** in cell lysates, as indicated by changes in the *N*-Me resonance chemical shift of **3** (blue to orange). The other signals in the spectrum are metabolites and molecules from the cell lysate.

Notes and references

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