Supporting Information:

Multipoint Recognition of Basic Proteins at a Membrane Model

R. Zadmard, T. Schrader*

General Remarks: DMSO-d_6 (Aldrich), deuterium oxide (Merck) and methanol-d_4 (Merck) were purchased each in ≥99.8% purity. Thin layer chromatography (TLC) analyses were performed on silica gel 60 F_{254} (Merck) with a 0.2 mm layer thickness. Preparative chromatography columns were packed with silica gel 60 from Aldrich. All solvents were dried and freshly distilled before use.

25,26,27,28-Tetrabutoxy-5,11,17,23-tetrakis(diethoxyphosphoryl)calix[4]arene. A solution of 25,26,27,28-tetrabutoxy-5,11,17,23-tetrabromocalix[4]arene (0.52 g, 0.5 mmol) and $NiCl_2$ (0.03 g, 0.25 mmol) in benzonitrile (3 mL) was treated dropwise with solution of $P(OEt)_3$ (0.7 g, 0.83 mmol) in benzonitrile (2 mL) under argon at ca. 180°C und stirring was continued for 1 h. The reaction mixture was poured into toluene (100 mL), washed 5 times with 5% aqueous NH_3, dried over Na_2SO_4 and evaporated. At 70-80°C and 0.01 mbar the excess of $P(OEt)_3$ and benzonitrile was removed completely; the remaining oil was chromatographed over silica gel eluting with ethyl acetate / methanol = 1:10 (R_f = 0.08). Yield 380 mg (0.32 mmol; 62 %); mp 154 - 156°C; **^{1}H NMR (300 MHz, DMSO):** δ 0.96 (t, 12 H, J = 7.29 Hz), 1.07 (t, 24 H, J = 6.96 Hz), 1.40 (m, 8 H, J = 7.63 Hz), 1.91 (m, 8 H, J = 7.63 Hz), 3.46 (d, 4 H, J = 12.93 Hz), 3.76 (m, 16 H, J = 7.3 Hz), 3.91 (t, 8 H, J = 7.62 Hz), 4.34 (d, 4 H, J = 12.95 Hz), 7.17 (d, 8 H, J_{P-H} = 12.93 Hz). **^{13}C NMR (75 MHz, DMSO):** δ 14.20, 16.34(d, J = 5.65 Hz), 19.11, 30.06, 32.10, 61.76 (d, J = 5.66 Hz), 73.59, 120.96, 123.48, 132.09 (d, J =10.74 Hz), 134.75 (d, J = 15.82 Hz), 159.58 (d, J = 3.96 Hz). **^{31}P NMR (81MHz, DMSO):** δ 23.80. **MS (FD):** *m/z* = 1215 ($M + Na^+$); calcd: 1192 (M^+); **elemental analysis cald**. for $C_{60}H_{16}O_{16}P_4$: C 60.39, H 7.77; found C 61.13, H 7.83.

25,26,27,28-Tetrabutoxy-5,11,17,23-tetrakis(hydroxyethylphosphoryl)calix[4]arene, tetralithiumsalt 1. 25,26,27,28-Tetrabutoxy-5,11,17,23-tetrakis(diethoxyphosphoryl)-calix[4]-arene (100 mg, 0.08 mmol) and LiBr (28.65 mg, 0.33 mmol) were heated in 2-hexanone (10 mL) under argon at 130°C. After 1.5 h the white precipitate was filtered off and washed ten times with diethyl ether and subsequently dried in vacuo. Yield 83 mg (0.075 mmol; 90 %); mp >255°C; **^{1}H NMR (300 MHz, DMSO):** δ 0.52 (t, 12 H, J = 6.97 Hz), 0.79 (t, 12 H, J = 6.96 Hz), 1.25 (m, 8 H, J = 7.29 Hz), 1.80 (m, 8 H, J = 7.63 Hz), 3.14 (d, 4 H, J = 13.27 Hz), 3.18 (m, 8 H, J = 7.63 Hz), 3.78 (t, 8 H, J = 7.62 Hz), 4.27 (d, 4 H, J = 12.60 Hz), 7.03 (d, 8 H, J_{P-H} = 12.60 Hz); **^{13}C NMR (75 MHz, DMSO):** δ 14.32, 16.04 (d, J = 5.65 Hz), 19.77, 32.29, 61.34 (d, J = 5.66 Hz), 75.53, 121.16, 125.32, 132.17 (d, J = 10.74 Hz), 134.87 (d, J = 15.82 Hz); **^{31}P NMR (81MHz, DMSO):** δ 18.22; TOF-MS (ESI-negative): *m/z* found 1079 ($M + 3H^+$); calcd: 1076 (M^+).

3,5-Bis(dimethoxyphosphorylmethyl)-1-hexadecanoic acid phenyl amide
30 mg 3,5-bis(dimethoxyphosphorylmethyl)-1-aminobenzene[1] (89 mmol, 1.0 eq) and 26 mg hexadecanoic acid (101 mmol; 1.1 eq) were dissolved in 30 mL dry dichloromethane. To this solution was added 77 µL T3P[2] (52% in ethylacetate, 134 mmol, 1.5 eq) and 20 µL N-methylmorpholine (178 mmol; 2.0 eq). The reaction was monitored by TLC. After 12 hours the solvent was evaporated and the remaining yellow oil separated by column chromatography on silica (dichloromethane/methanol 10:1). Yield: 47 mg (82 mmol; 92%).

^{1}H-NMR (200 MHz, $CDCl_3$): δ[ppm] = 7.41 (s, 2H, CH-arom.), 6.92 (s, 1H, CH-arom.), 3.66 (d, $^3J_{P,H}$ = 10.8 Hz, 12H, P-O-CH_3), 3.10 (d, $^2J_{P,H}$ = 21.7 Hz, 4H, P-CH_2), 2.69 (t, $^3J_{H,H}$ = 6.9 Hz, 2H, NOC-CH_2-), 2.03 (m, 2H, OC-CH_2-**CH_2**-) 1.42 (s, 25H, alkyl-chain), 1.12 (t, $^3J_{H,H}$ = 6.3 Hz, -CH_3). **^{31}P-{^{1}H}-NMR (81 MHz, $CDCl_3$):** δ[ppm] = 29.1.

[1] Y.-C. Kim, K. Jacobson *J. Med. Chem.* 43 (2000) 746-755.
[2] provided by Clariant GmbH.

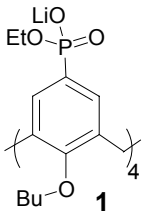
3,5-Bis(methoxyphosphorylmethyl)-1-hexadecanoicacid-phenylamid dilithiumsalt 7

To a solution of 20 mg of 3,5-Bis(dimethoxyphosphorylmethyl)-1-hexadecanoicacid-phenylamide (35 mmol, 1.0 eq) in 30 mL dry acetonitrile was added 3.0 mg dry lithium bromide under argon. The solution was heated to reflux for 24 hours. The white precipitate was filtered off and washed several times with acetonitrile. After drying under vacuum remains 16 mg of the dilithium salt (29 mmol, 82%).

^{1}H-NMR (300 MHz, methanol-d_4): δ[ppm] = 7.24 (s, 2H, CH-arom.), 6.92 (s, 1H, CH-arom.), 3.40 (d, $^3J_{P,H}$ = 10.1 Hz, 6H, P-O-CH_3), 2.83 (d, $^2J_{P,H}$ = 20.8 Hz, 4H, P-CH_2), 2.23 (t, $^3J_{H,H}$ = 7.3 Hz, 2H, NOC-CH_2-), 1.58 (m, 2H, OC-CH_2-**CH_2**-) 1.18 (s, 25H, alkyl-chain), 0.80 (t, $^3J_{H,H}$ = 6.6 Hz, -CH_3). **^{31}P-{^{1}H}-NMR (81 MHz, methanol-d_4)**: δ[ppm] = 26.2.

Lewis structures of all host molecules:

Bisphosphonate:



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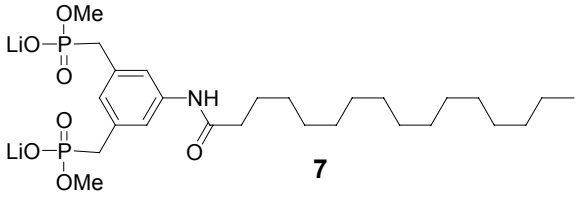

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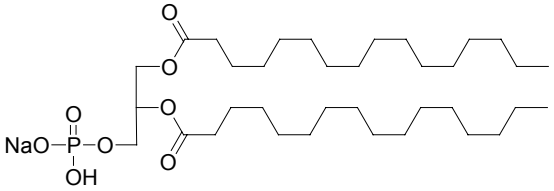
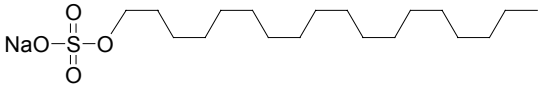
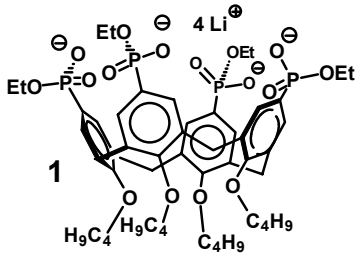
Monophosphate:



SDS:



Host 1



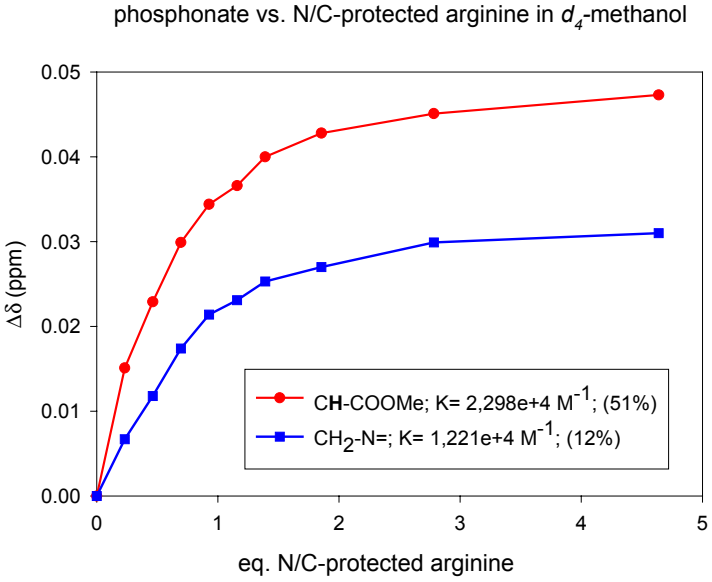
Mass Spectrometric Measurements: ESI mass spectra were recorded on a Finnigan MAT 95. Samples (20 µL) were introduced as 10^{-4} M solutions in HPLC-grade methanol at flow rates of 20 µL min^{-1}. Heated capillary temperature: 150 °C. Ion spray potential: 3.5 kV (positive ESI), 3.0 kV (negative ESI). About 20–30 scans were averaged to improve the signal-to-noise ratio.

^{1}H NMR Titrations: Ten NMR tubes were filled each with 0.80 mL of a solution of the guest compound (c_{guest} = 0.5–4 mM) in a deuterated solvent (DMSO, CD_3OD or D_2O). The host compound (1.525 total equivalents corresponding to the total guest amount) was dissolved in 0.61 mL of the same solvent, and the resulting solution was added, in amounts increasing from 0 to 5.0 equiv., to the ten guest solutions. Owing to their strong hygroscopicity, the phosphonate solutions contained approx. 0.3–0.6% of water. Volume and concentration changes were taken into account during analysis. The association constants were calculated by non-linear regression methods.

NMR titration: calix[4]arenetetraphosphonate **1** vs. N_α-tosyl-L-arginine methylester hydrochloride **2** in d_4-methanol.

Guest **2** solution: 1.19 mg ($3.14*10^{-3}$ mmol) N_α-tosyl-L-arginine methylester hydrochloride in d_4-methanol (6 mL).
Host **1** solution: 8.05 mg ($7.28*10^{3}$ mmol) calix[4]arenetetraphosphonate in d_4-methanol (1 mL).

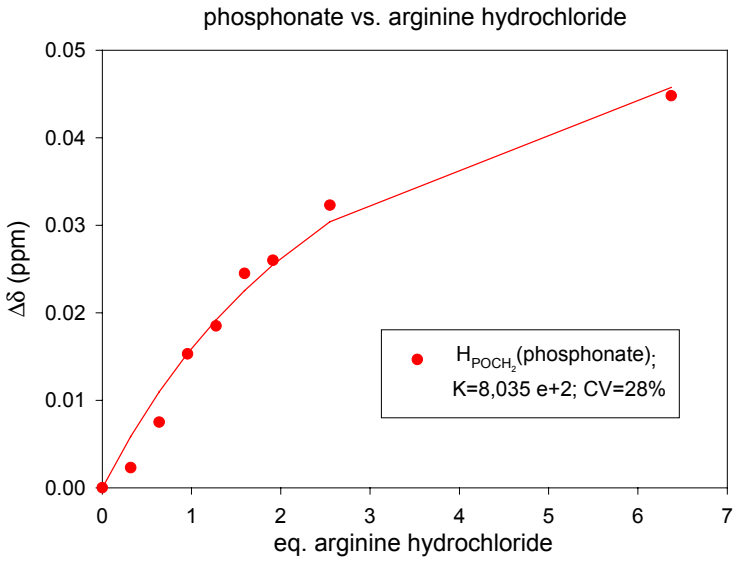
Sample	Guest soln. µL	Host soln. µL	Δδ(1) [ppm]	δ(1) [ppm]	Δδ(2) [ppm]	δ(2) [ppm]
1	600	0	0.000	3.892	0.000	3.178
2	600	10	0.015	3.877	0.006	3.185
3	600	20	0.022	3.869	0.011	3.190
4	600	30	0.029	3.862	0.017	3.196
5	600	40	0.034	3.858	0.021	3.200
6	600	50	0.036	3.856	0.023	3.201
7	600	60	0.040	3.852	0.025	3.203
8	600	80	0.042	3.849	0.027	3.205
9	600	120	0.045	3.847	0.029	3.208
10	600	200	0.047	3.845	0.031	3.209



NMR titration: calix[4]arenetetraphosphonate **1** vs. L-arginine hydrochloride **3** in d_4-methanol.

Guest **1** solution: 2.94 mg ($2.66*10^{-3}$ mmol) calix[4]arenetetraphosphonate in d_4-methanol (6 mL).
Host **3** solution: 1.09 mg ($5.17*10^{-3}$ mmol) arginine hydrochloride in d_4-methanol (0.61 mL).

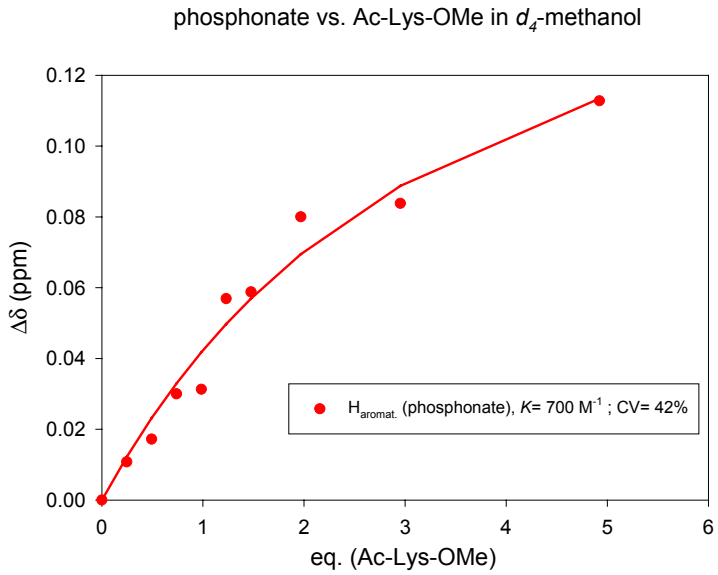
Sample	Guest soln. µL	Host soln. µL	Δδ(1) [ppm]	δ(1) [ppm]
1	600	0	0.000	3.721
2	600	10	0.002	3.719
3	600	20	0.007	3.713
4	600	30	0.015	3.706
5	600	40	0.018	3.702
6	600	50	0.024	3.696
7	600	60	0.026	3.695
8	600	80	0.032	3.689
9	600	120	0.027	3.693
10	600	200	0.044	3.676



NMR titration: calix[4]arenetetraphosphonate **1** vs. Ac-Lys-OMe*HCl **4** in d_4-methanol.

Guest **1** solution: 3.08 mg ($2.78*10^{-3}$ mmol) calix[4]arenetetraphosphonate in d_4-methanol (6 mL).
Host **4** solution: 1.00 mg ($4.18*10^{-3}$ mmol) Ac-Lys-OMe*HCl in d_4-methanol (0.61 mL).

Sample	Guest soln. µL	Host soln. µL	Δδ(1) [ppm]	δ(1) [ppm]
1	600	0	0.000	7.308
2	600	10	0.010	7.297
3	600	20	0.017	7.291
4	600	30	0.030	7.278
5	600	40	0.031	7.277
6	600	50	0.056	7.251
7	600	60	0.058	7.249
8	600	80	0.080	7.228
9	600	120	0.083	7.224
10	600	200	0.112	7.195



NMR titration: calix[4]arenetetraphosphonate **1** vs. Ac-Leu-OMe **5** in d_4-methanol.

Guest **1** solution: 3.08 mg ($2.78*10^{-3}$ mmol) calix[4]arenetetraphosphonate in d_4-methanol (6 mL).
Host **5** solution: 1.00 mg ($5.34*10^{-3}$ mmol) Ac-Leu-OMe **5** in d_4-methanol (0.61 mL).

Proton	K_a [M^{-1}]	δ_0 [ppm]	δ_9 [ppm]	$\Delta\delta_{sat}$ [ppm]
1b	0	7.3010	7.2844	0.016
1c	0	1.1051	1.1040	0.0011

NMR titration: calix[4]arenetetraphosphonate **1** vs. Boc-Ser-OMe **6** in d_4-methanol.

Guest **1** solution: 3.08 mg ($2.78*10^{-3}$ mmol) calix[4]arenetetraphosphonate in d_4-methanol (6 mL).
Host **6** solution: 1.00 mg ($4.18*10^{-3}$ mmol) Boc-Ser-OMe **6** in d_4-methanol (0.61 mL).

Proton	K_a [M^{-1}]	δ_0 [ppm]	δ_9 [ppm]	$\Delta\delta_{sat}$ [ppm]
1b	0	7.3010	7.2844	0.016
1c	0	1.1051	1.1040	0.0011

Job Plots: Equimolar solutions (10 mmol/10 mL, approx. 10 μM) of both the amino acids and the tetraphoshonate were prepared and mixed in various ratios. ^{1}H NMR spectra of the mixtures were recorded, and the chemical shifts were analysed by Job's method modified for NMR results.

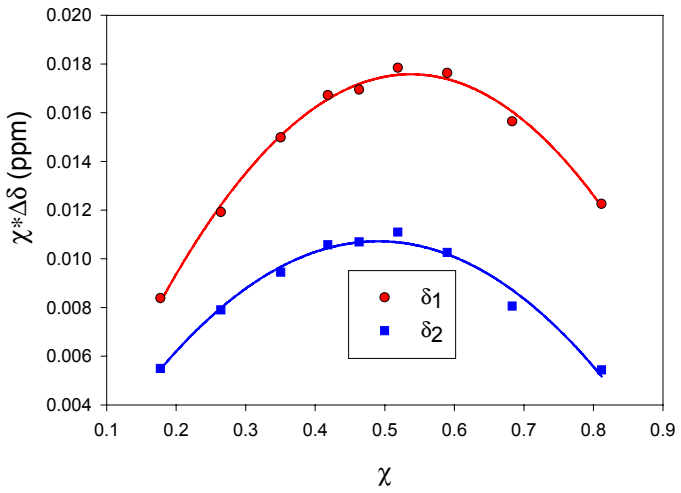
Job plot between calix[4]arenetetraphosphonate **1** and N_α-tosyl-L-arginine methylester hydrochloride **2** in d_4-methanol.

Amounts: Host **1** solution: 7.73 mg (7 μmol) in d_4-methanol (4 mL).
Guest **2** solution: 2.62 mg (7 μmol) in d_4-methanol (4 mL).

Sample	$V_{\text{host soln.}}$ [μL]	$V_{\text{guest soln.}}$ [μL]	χ [host]	$\chi_* \Delta\delta_1$ [ppm]	$\chi_* \Delta\delta_2$ [ppm]
0	800	0	-	-	-
1	720	80	0.811	0.012	0.005
2	640	160	0.683	0.015	0.008
3	560	240	0.589	0.017	0.010
4	480	320	0.518	0.017	0.011
5	400	400	0.462	0.016	0.010
6	320	480	0.418	0.016	0.010

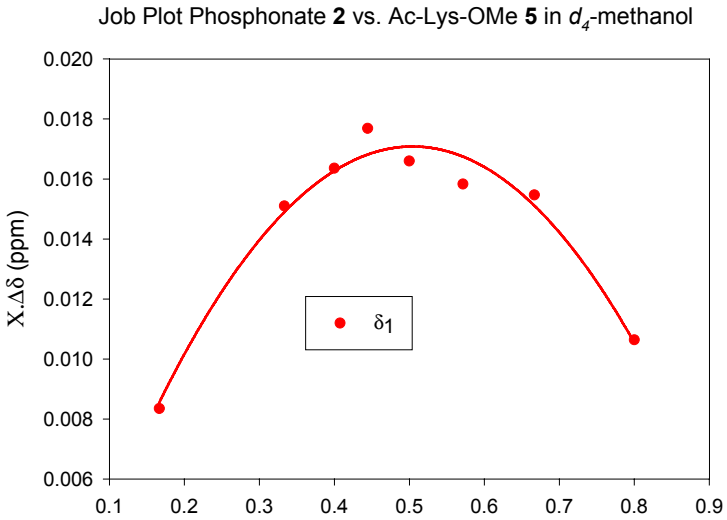
7	240	560	0.350	0.014	0.009
8	160	640	0.264	0.011	0.007
9	80	720	0.177	0.008	0.005

Job plot phosphonate **2** vs arginine **3** in d_4-methanol



Job plot between calix[4]arenetetraphosphonate **1** and Ac-Lys-OMe*HCl **4** in d_4-methanol.
Amounts: Host **1** solution: 7.73 mg (7 µmol) in d_4-methanol (4 mL).
Guest **4** solution: 2.62 mg (7 µmol) in d_4-methanol (4 mL).

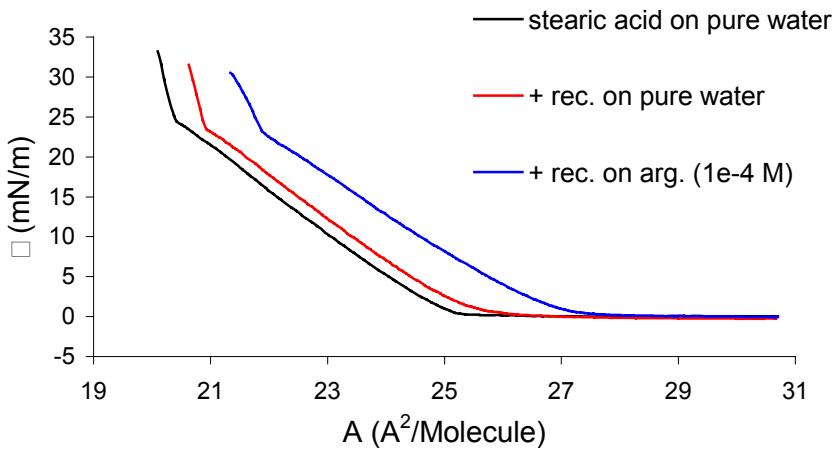
Sample	$V_{\text{host soln.}}$ [µL]	$V_{\text{guest soln.}}$ [µL]	χ [host]	$\chi_* \Delta\delta$ [ppm]
0	800	0	-	-
1	720	80	0.811	0.012
2	640	160	0.683	0.015
3	560	240	0.589	0.017
4	480	320	0.518	0.018
5	400	400	0.462	0.017
6	320	480	0.418	0.016
7	240	560	0.350	0.015
8	160	640	0.264	0.012
9	80	720	0.177	0.008



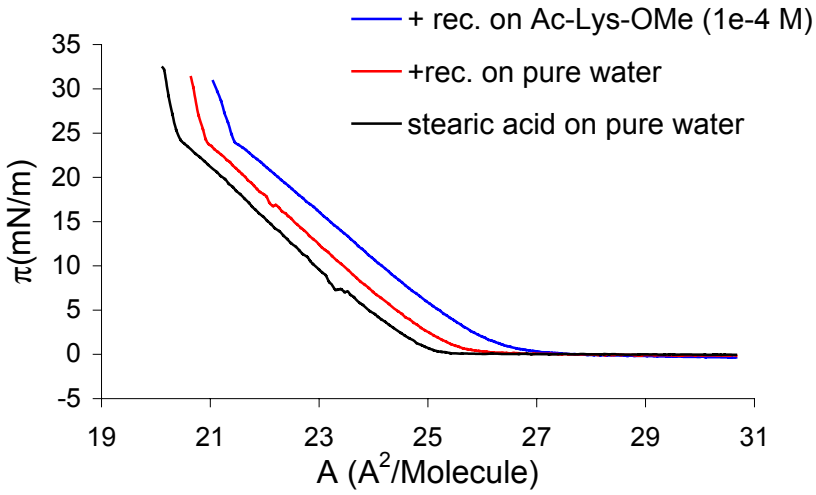
Film balance experiments: A NIMA 601BAM film balance (trough size 700 x 100 mm^2) equipped with a Wilhelmy plate was used for measuring the surface pressure as a function of molecular area at ambient temperature. Pure water (purifed by ELGA Purelab UHQ, > 18MΩ) and aqueous 100 µM amino acid solutions or 10 nM protein solutions were used as subphases, all of which did not give any appreciable values of surface pressure under compression in the examined area ranges. Lipid monlayers were obtained by spreading 50 µL of a 3.5 mM stearic acid solution in chloroform onto the subphases. The recorded π-A-isotherm cycles (barrier speed : 50 cm^2 / min) revealed no or only small isotherm changes of the stearic acid monolayer caused by dissolved guests (amino acids or proteins). Receptor **1** was incorporated into the lipid monolayer by dropping 5 µL of a 4.6 mM receptor solution in chloroform / methanol (1:1) onto the subphase at a surface pressure of 15 mN/m. Time-dependent π-A-isotherm cycles were recorded until no further effects could be observed.

1. Surface pressure / area isotherms of the stearic acid monolayer with embedded receptor 1 on cationic amino acids and peptides in the subphase.

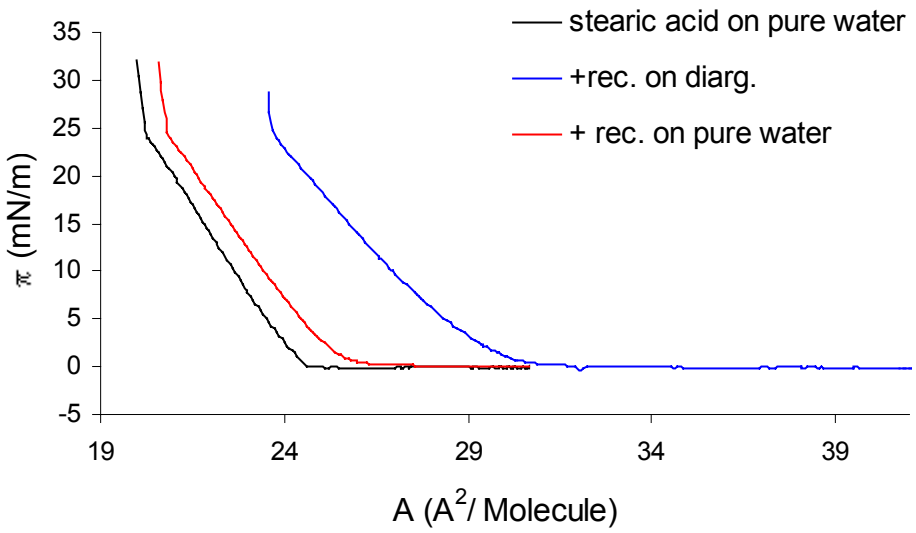
a: L-arginine hydrochloride 3 (100 µM) as subphase.



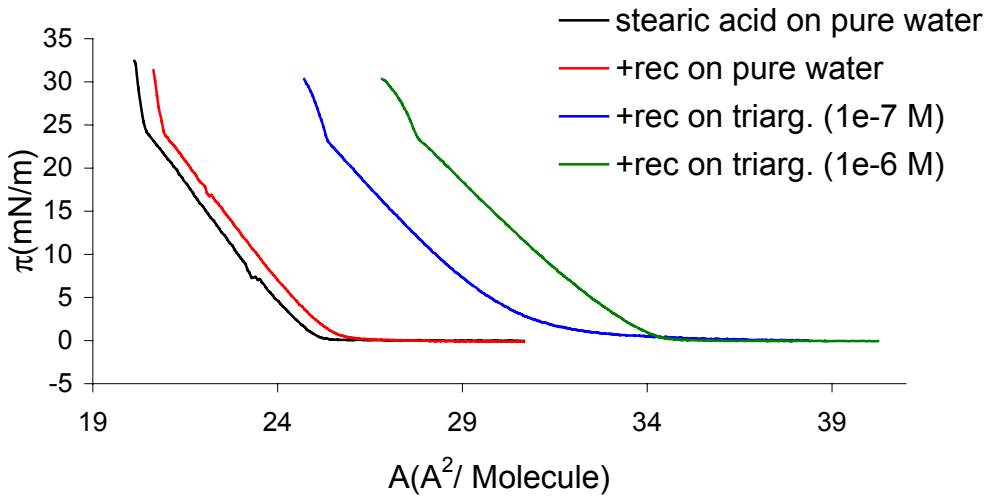
b: Ac-Lys-OMe*HCl 4 (100 µM) as subphase.



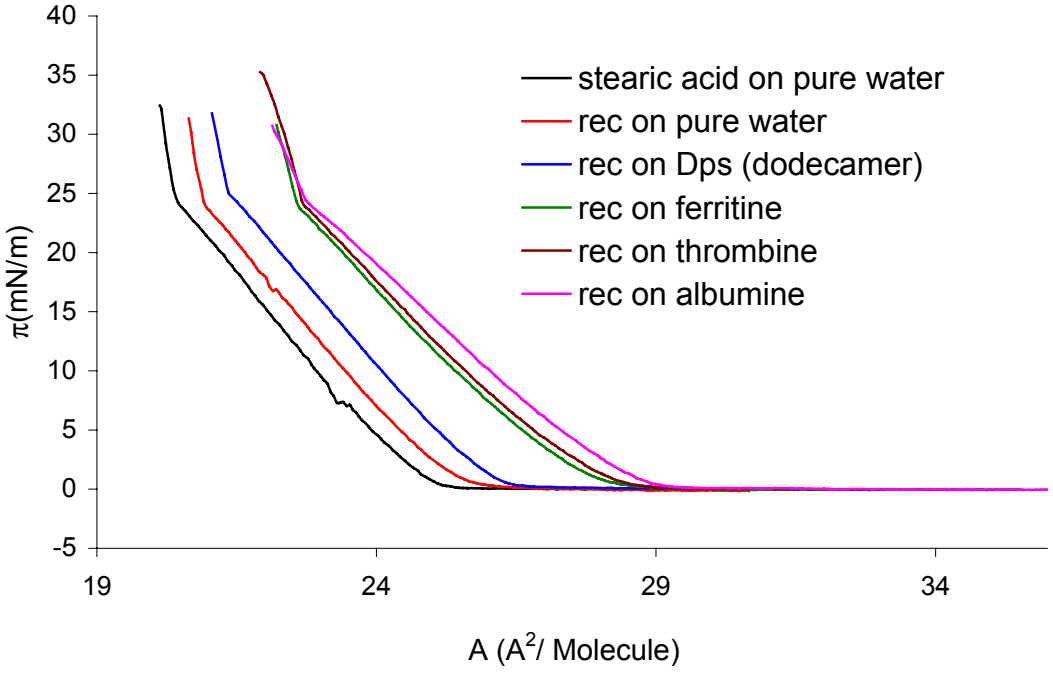
c: Diarginine acetate salt (10 μM) as subphase.



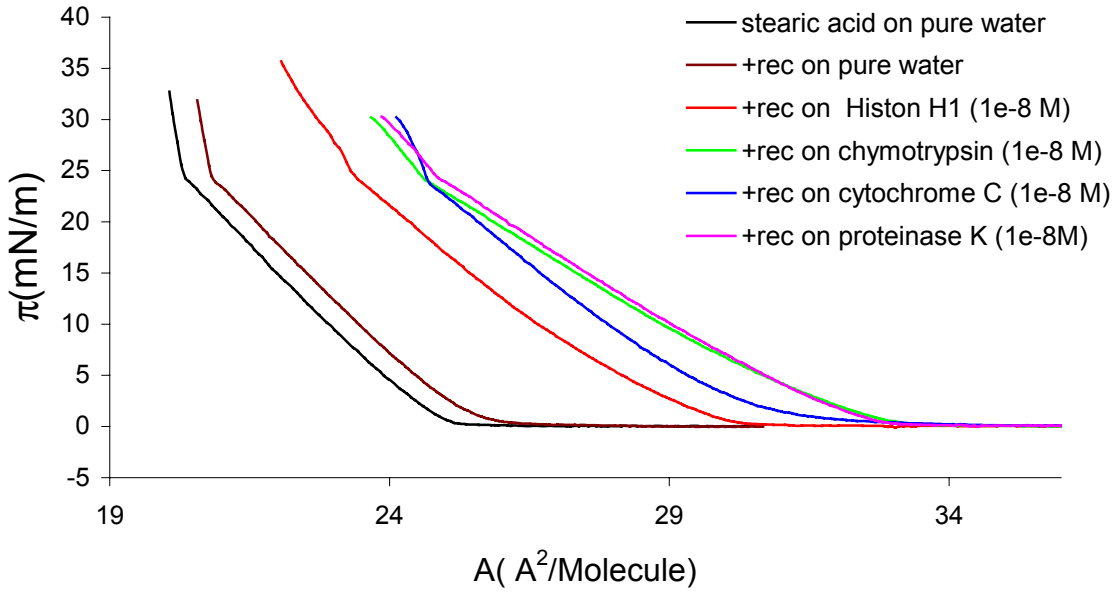
d: Triarginine acetate salt (1μM and 100nM) as subphase.



2. Surface pressure / area isotherms of the stearic acid monolayer with embedded receptor 1 on neutral and acidic proteins (10 nM) in the subphase.

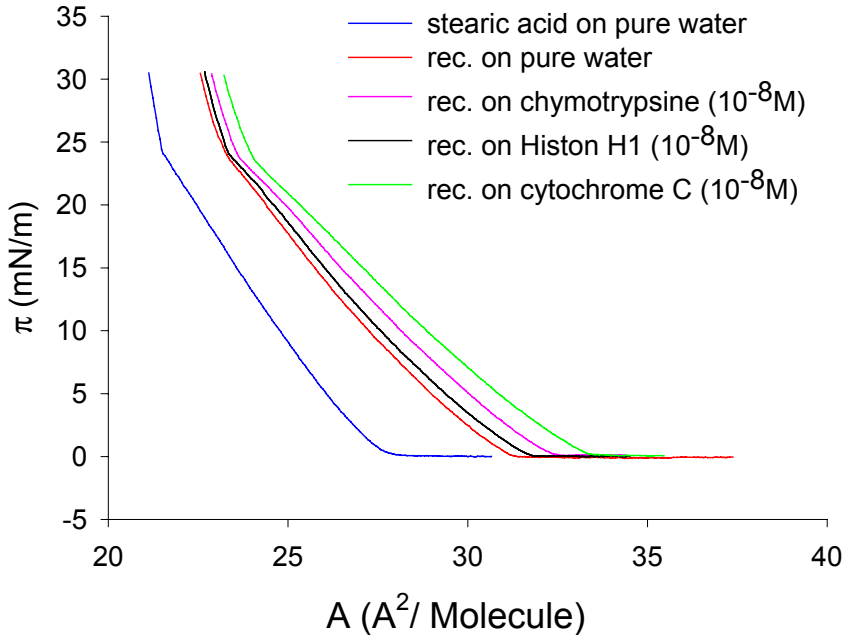


3. Surface pressure / area isotherms of the stearic acid monolayer with embedded receptor 1 on various basic proteins (10nM) in the subphase.



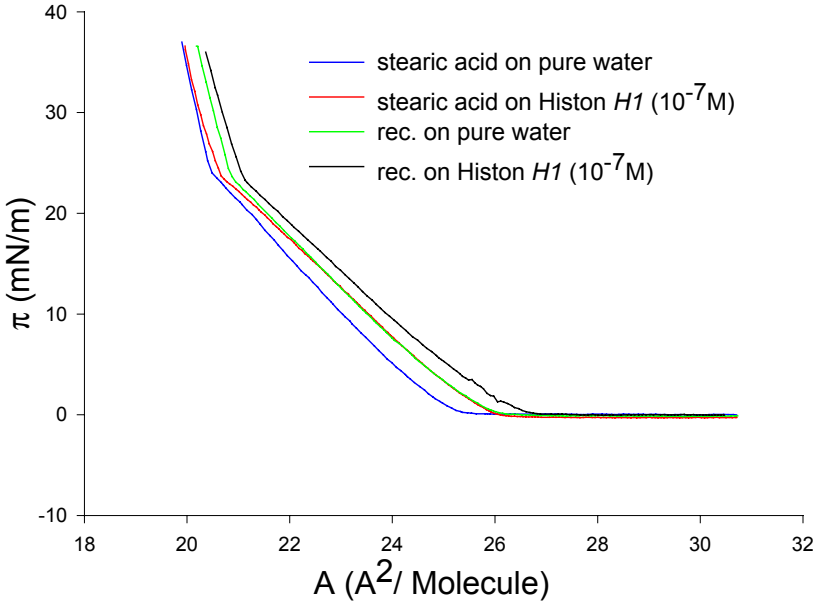
The binding isotherm of trypsin was identical to that of chymotrypsin.

4. Surface pressure / area isotherms of the stearic acid monolayer with embedded 1,2-Didodecanoyl-sn-glycero-3-phosphoric acid sodium salt on various basic proteins (10nM) in the subphase.



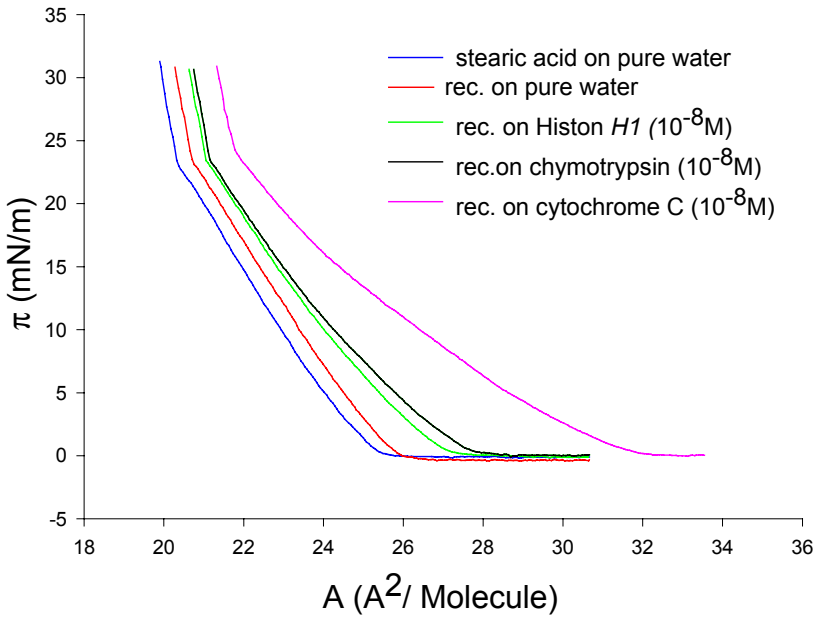
This simple phosphate anion shows only minute shift expansions of 0 - 0.5A^2 with basic proteins, as compared to ~ 5A^2 for calixarene **1**.

5. Surface pressure / area isotherms of the stearic acid monolayer with embedded 3,5-Bis(methoxyphosphorylmethyl)-1-hexadecanoicacid-phenylamid dilithiumsalt 7 on Histon *H1* (100nM) in the subphase.



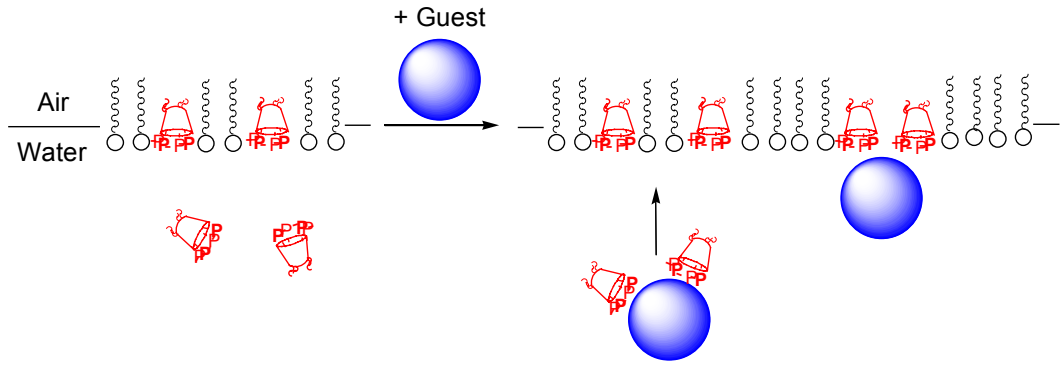
This moderately selective receptor molecule for basic amino acids shows only minute shift expansions of $0.2A^2$ with basic proteins, as compared to ~ $5A^2$ for calixarene **1**.

6. Surface pressure / area isotherms of the stearic acid monolayer with embedded Sodium Dodecylsulfate (SDS) on various basic proteins (10nM) in the subphase.



This simple sulfate anion shows only minute shift expansions of ~ $0.5A^2$ with basic proteins, as compared to ~ $5A^2$ for calixarene **1**. (Exception: cytochrome C).

Model rationale to explain the large π-A shifts in the filmbalance experiments:

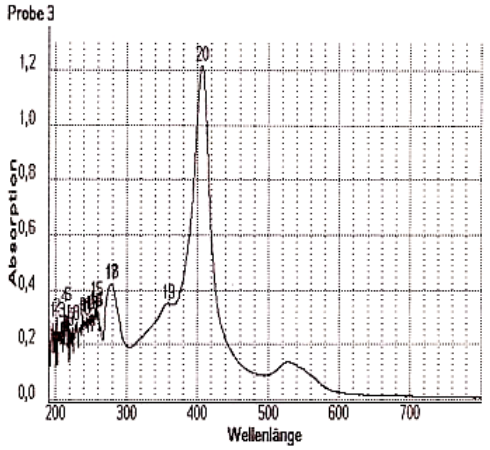


From all the above-mentioned collected experimental data we infer the following tentative mechanism of peptide and protein binding by the monolayer: As guest molecules are subinjected into the aqueous phase, they are bound by solvated receptor molecules close to the monolayer.

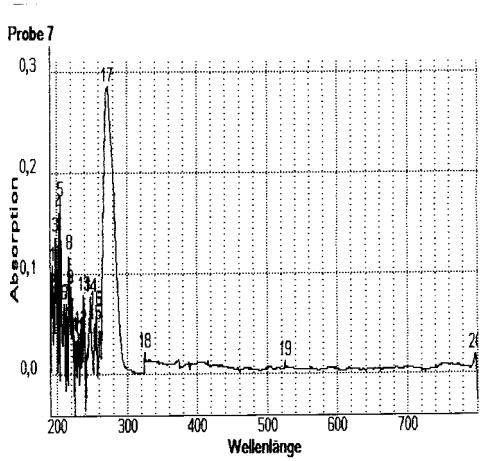
The host molecules' negative charges become in part neutralized and their lipophilicity increases. This in turn leads to reincorporation of the whole complex into the monolayer. Thus large amounts of receptor molecules which are formerly dissolved in the aqueous subphase, migrate back into the monolayer, as soon as they become complexed on the surface of a basic peptide or protein.

Langmuir-Blodgett experiments:

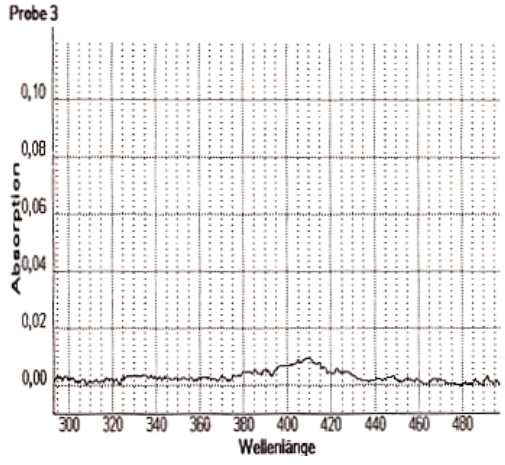
Monolayers at the air/water interface were prepared as described above with 0.1 eq or 0.4 eq of receptor **1**, embedded in the stearic acid phase. LB films were prepared on quarz plates (∅=1 cm, *d*=1 mm) by the vertical dipping method at a controlled surface pressure of 20 mN/m with a dipping rate of 10 mm/min. Thus, 160 layers were deposited on each side of the plate. UV-Vis spectra were recorded on a Pharmacia Biotech Ultrospec 3000 spectrometer at ambient temperature. The quarz plates were placed vertically in the optical path of the UV/Vis spectrometer and measured directly in the range of 200 to 600 nm at ambient temperature. The free receptor molecule **1** (150 µM) and free cytochrome C (25 µM) were prepared as aqueous solutions. Samples were measured in quartz cuvettes (d=1 cm) as solution in pure water (purifed by ELGA Purelab UHQ, > 18MΩ).



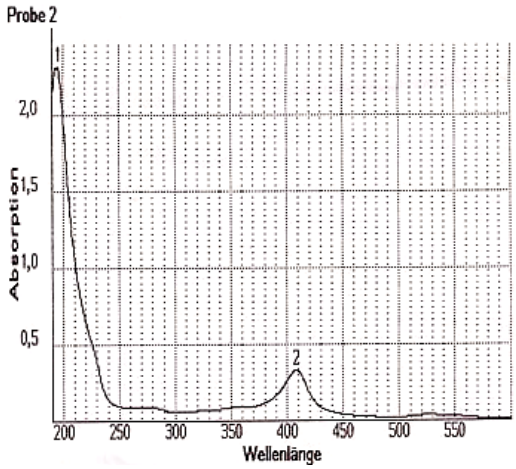
1.Cytochrome C in water (25 µM)



2. **1** in water (150 µM)

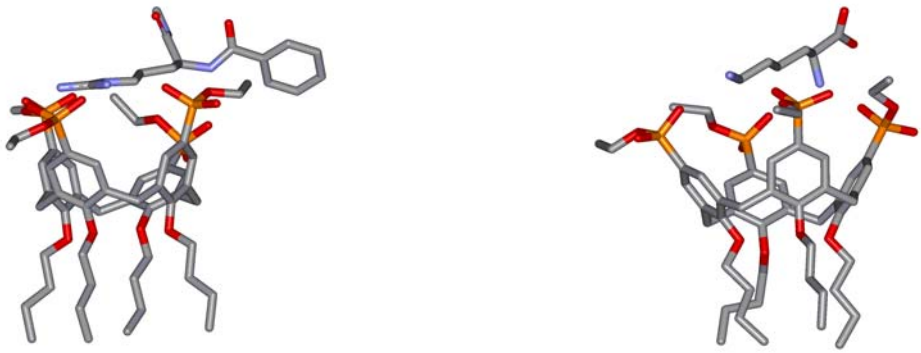


3. cytochrome C in the LB-film
(0.1 eq embedded receptor **1**)



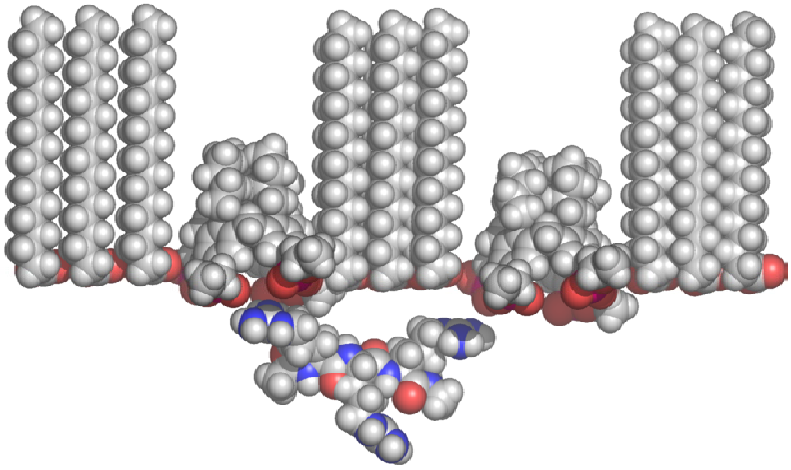
4. cytochrome C in the LB-film
(0.4 eq embedded receptor **1**)

Molecular Modeling: Force-field calculations were initially carried out as molecular mechanics calculations in water. To establish the minimum energy conformation of the free host and guest molecule as well as their 1:1 complex Monte-Carlo-simulations were subsequently carried out in water (MacroModel 7.0, Schrödinger Inc., 2000. Force-field: Amber*). A 3000-step Monte-Carlo-simulation was performed. All low energy structures were of very similar energy (ΔE ~5 kJ/mol) and conformation.



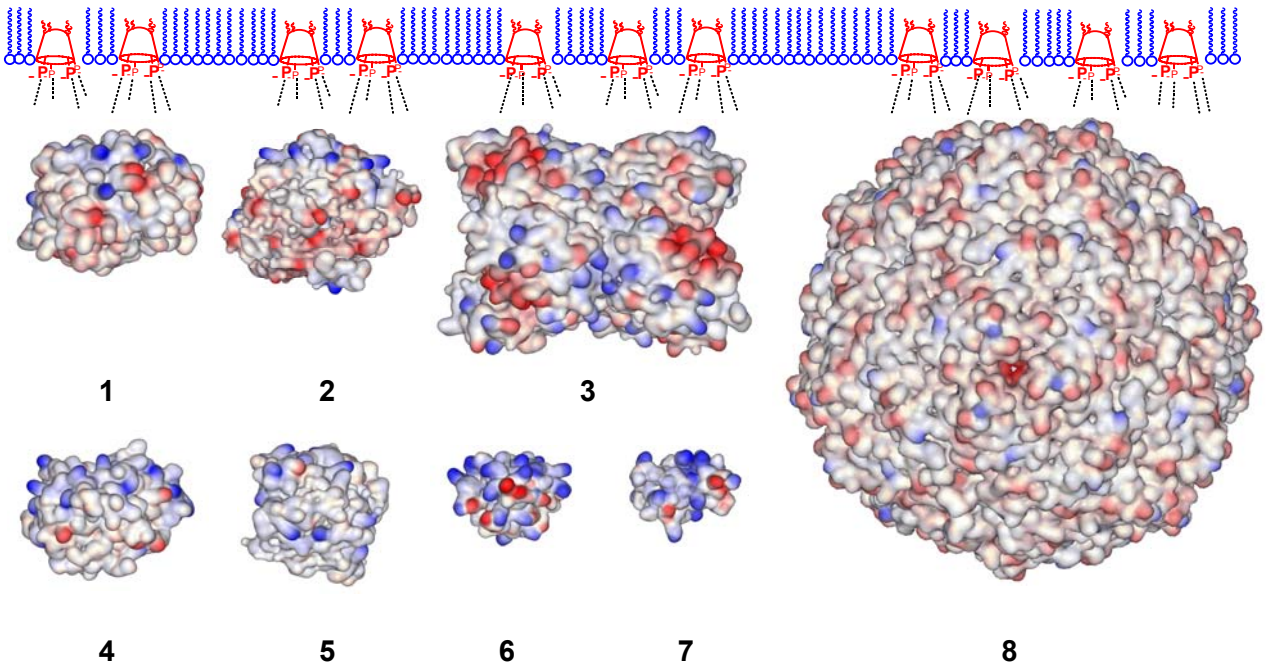
Energy-minimized structures of tetraphosphonate **1** in its complex with N_α-benzoylarginine methyl ester (left) and free lysine (right). Monte-Carlo simulations in water (MacroModel 7.0, Amber*, 3000 steps).

Graphic representation of the binding mode at the stearic acid monolayer: triarginine docking on to two calixarene tetraphosphonates.



Stearic acid molecules with embedded calixarenes, both oriented with their polar headgroups into the aqueous subphase. The diarginine molecules approaches the monolayer from beneath and draws the tetraphosphonates into the correct position to interact with both guanidinium functionalities in a double chelate arrangement (two-point binding mode). In the next step, the third guanidinium cation can pull a third calixarene from behind and form the third binding site.

Calculation of the protein binding domains: protein structures were drawn from the protein database (PDB), covered with their respective Connolly surfaces showing the electrostatic surface potential (ESP) and oriented towards the anionic binding sites of the receptor molecules in the monolayer. In the following illustration, they are depicted in their correct relative sizes.



Proteins which are bound by receptor **1** in the stearic acid monolayer, depicted in their correct relative sizes. The Connolly surface is patterned with the electrostatic surface potential (ESP), showing basic and acidic domains on the protein surfaces. The basic proteins are shown in their proposed orientation relative to the monolayer, presenting their positive domains (blue) upwards. 1 Proteinase K, 2 thrombin, 3 BSA, 4 chymotrypsin, 5 trypsin, 6 cytochrome C, 7 histone H1, 8 ferritine.