Molecular recognition of nucleotides by a new bis(guanidinium)tetrakis(β-cyclodextrin) tetrapod†

Stephane Menuel, Raphaël E. Duval, Diana Cuc, Pierre Mutzenhardt and Alain Marsura*

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The facile synthesis of a novel bis(guanidinium)tetrakis(β-cyclodextrin) tetrapod, the first member of a new host family with a pre-defined number of guanidinium centres and cyclodextrins, is presented. Its molecular recognition towards nucleotide guest molecules was investigated. The formation of ditopic bimolecular complexes was observed, combining host–guest hydrophobic inclusion into cyclodextrins and electrostatic interactions between guanidinium and phosphate anions.

Introduction

Synthetic multivalent architectures using non-covalent bonding interactions as the supramolecular "glue" are well-defined systems for studying the concept of multivalency in nature.[1a] Multivalent interactions, which rely upon non-covalent bonds, are essential in the mediation of biological processes as well as in the construction of complex structures. A fundamental understanding of multivalency in supramolecular chemistry is necessary to synthesize model systems to provide insight into how biological processes work.[1b] In this field, the design of selective synthetic receptors for biological anion recognition remains a particularly daunting challenge. Among the biological anions, pentavalent phosphates and their polyphosphate nucleotides are thought to be the most important in living organisms.[1c] Moreover, they also represent the main pathway for energy supplies in cells.[2a,b] Elsewhere, considerable effort and time has been devoted to promoting sensitive and chemoselective fluorescent water-compatible sensors for detection and quantification of these nucleotides.[3a–f] One of the most typical supramolecular compound families that strongly and selectively bind nucleotides is represented by the macrocyclic polyamines and guanidinium-type receptors. When protonated, they strongly bind nucleotides *via* electrostatic interactions between the ammonium or guanidinium sites and polyphosphate anions.[4a–f] Elsewhere, examples of synthetic macrocyclic receptors, including chemically modified cyclodextrins (CyDs), have only very rarely been reported.[5a–f] We chose to investigate a new concept of non-polymeric CyD oligomer "bottom-up" synthesis (illustrated by Fig. 1), having cavities regularly distributed around a central skeleton and which possess a pre-defined number of cationic guanidinium centres. The structure was designed to perform three types of possible interaction between the host and the guest: electrostatic, hydrogen bonding, and hydrophobic inclusion in CyDs.

UMR 7565 SRSMC, Nancy-Université, CNRS Faculté de Pharmacie, 5 rue A. Lebrun, B.P. 80403, 54001 Nancy Cedex, France. E-mail: Alain.Marsura@pharma.uhp-nancy.fr; Fax: +33 (0) 3 83682345; Tel: +33 (0) 3 83682324

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Results and discussion

Synthesis

Here, we report a fast and easy route to a new bis(guanidinium)tetrakis(β-cyclodextrin) tetrapod **5** ($n = 0$), a peripherally functionalized cyclodextrin dendrimer that readily forms complexes with ATP, ADP and AMP nucleotides. The pure tetrapod **5** was obtained in fairly good yield (96%) and in two steps from a carbodiimide-β-cyclodextrin dimer **3**, which is afforded by the phosphine imide strategy from the monoazido-CyD[6a,b] (Scheme 1). Analyses of **4** and **5** by FTIR, NMR and ES-MS are in accordance with the proposed structures. The FTIR spectra of **4** and **5** show the presence of absorption bands at 1736–1663 cm^{-1} and 1737–1639 cm^{-1}, characteristic of the guanidine functions,[7] which were also confirmed by the quaternary carbon ^{13}C-NMR corresponding signals at 175 and 174 ppm.

The positive mode ESI mass spectrum of **4** showed the presence of the molecular ion at $[M + H]^+$ 2292 amu, while the spectrum of **5** afforded the tripodal molecular fragment $[M - (\beta\text{-CD-NH})^+]$ at 3662 amu by losing one 6-amino-β-CD moiety. Finally, the NMR spectra of **5** show the presence of methylene protons at 3.17 ppm and carbon signals at 22.3 ppm, corresponding to the linker ethyl chain.

Binding studies

Since both host and guest compounds are ionic, it is reasonable to expect the strongest binding to occur in the pH range where guanidine groups of **5** are protonated and positively charged and where at least one phosphate group of the nucleotides exists in anionic form.

Looking at the ^{1}H-NMR titration of **5** at pH 6.5 where both conditions are followed, shows that 5′-$ATP^{2-}2Na^+$, 5′-ADP^-Na^+ and 5′-AMP^-Na^+ species are readily complexed by the tetrapod **5** to give [2Nu:1TP] water soluble supramolecular complexes (see Fig. 2).

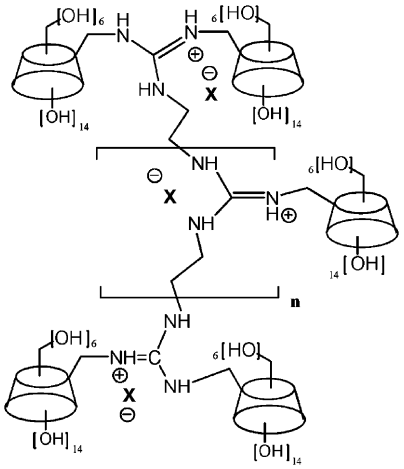
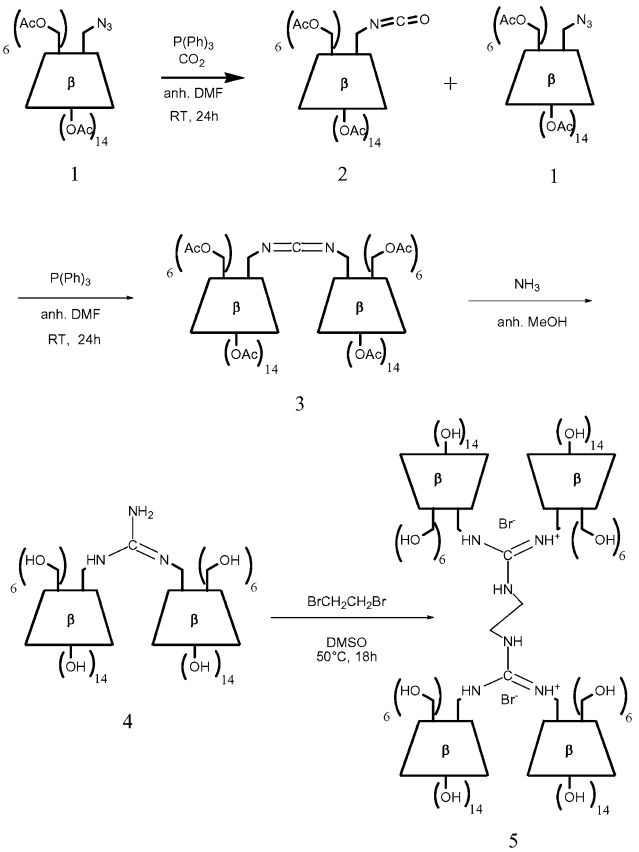


Fig. 1 Model for non-polymeric CyD foldamer development. In the following example $n = 0$. The extra cyclodextrin unit (n) is shown as future devices could possibly be extended.

Evidence of complexation was detected first from the chemical induced shifts (CIS) of some protons of the host and guest signals compared to those of the free compounds (Table 1). The CIS values of *e.g.* the H^8 nucleotide protons are indicative with respect to complexation (see Fig. 2). The stoichiometries of the complexes were confirmed by the Job plot continuous variation method, as illustrated in Fig. 3. A value of $R = 0.7$ was reached at the maximum which strengthens the evidence for the [2Nu:1TP] stoichiometry. Binding



Scheme 1 Synthetic pathway of tetrapod **5**.

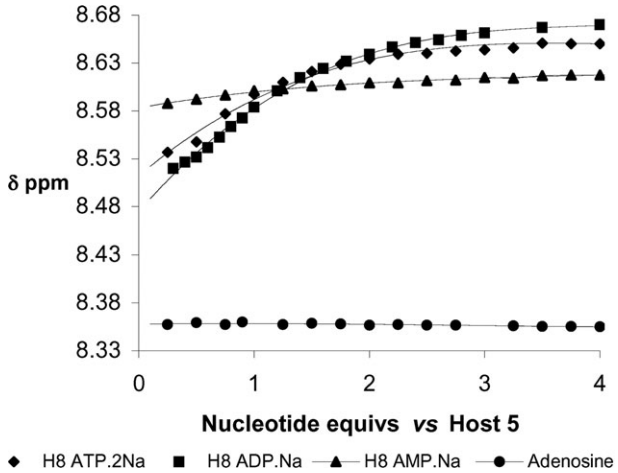
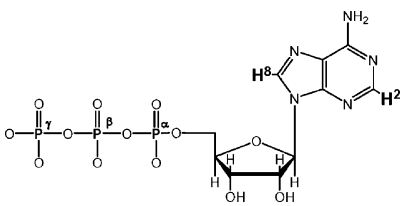


Fig. 2 Chemical induced shifts of the adenine nucleobase H^8 protons induced by complexation in CyD cavities plotted *vs.* the number of equivalents of 5′-ATP, 5′-ADP, 5′-AMP and adenosine. Tetrapod **5** concentration was 1.25 mg ml^{-1} (0.23×10^{-3} mol l^{-1}).

constants K (M^{-2}) have been determined by the COMPLEX approach.[8a,b] They are $K = 2.12 \times 10^6$ M^{-2}, 1.86×10^6 M^{-2} and 2.06×10^6 M^{-2} for [2Nu:1TP] complexes with 5′-$ATP^{2-}2Na^+$, 5′-ADP^-Na^+ and 5′-AMP^-Na^+, respectively, showing that **5** has similar strong binding constants with the three nucleotides. Considering the interaction between a ligand with multiple identical and independent binding sites and a substrate with a unique binding site, a series of stepwise equilibria can be written.[8c]

In tetrapod **5** the constant K (M^{-2}) value corresponds to the second equilibrium, which is of the same order as those found for the first equilibrium constant K (M^{-1}) which governs the first ATP molecule complexation and which is also of the same order as K (M^{-1}) found for the dipod **4**. Consequently, the K (M^{-2}) value found for tetrapod **5** is in perfect agreement with a non-cooperative or statistical binding effect in which the two complexation steps are independent of each other. Schematically, ATP complexation in tetrapod **5** could be compared to

Table 1 Values (ppm) of ^1H chemical induced shifts (CIS) of nucleotide guests and CyD hosts by complexation with **5** at pH 6.5 and 25 °C



Guest[a]	H^2 Ad	H^8 Ad	H^1 Rib	H^4 Rib	H^3 CD[b]	H^5 CD[b]
5′-$ATP^{2-}2Na^+$	0.16	0.12	0.05	0.02	0.02	0.01
5′-ADP^-Na^+	0.14	0.15	0.06	0.02	0.01	0.01
5′-AMP^-Na^+	0.02	0.04	0.00	0.00	0.03	0.02

[a] No complexation occurs with adenosine. [b] H^3 and H^5 are protons located inside the CyD cavity.

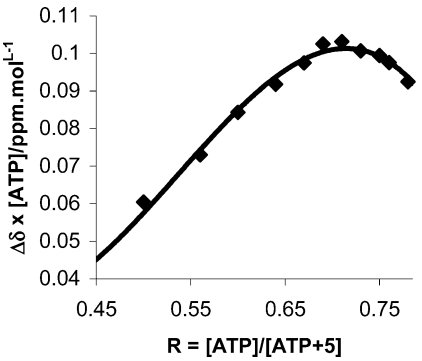


Fig. 3 Job plot corresponding to the chemical shift displacements of the H^8 protons of ATP for the [**5**:2(5′-$ATP^{2-}2Na^+$)] complex in D_2O.

the stepwise formation of a multiple pseudorotaxane in a pertinent example described by Stoddart *et al.*[1b] and should be looked on as a complexation arising with two independent covalently bound dipods.

This result was consistent with those found for a fully protonated heptakis(amino-CyD), with regards to the relative number of positive charges and electrostatic interactions, and *e.g.* AMP dianion.[8d] Results obtained by Suzuki *et al.*[8e] on a single β-CyD host having a cationic group on the primary hydroxy group side, confirm our observations, considering, on one side, the CIS values and, on the other side, the proposed host–guest interactions between ATP and the β-CyD host. The conformational situation depicted by the authors[8f] reproduces those found with both tetrapod **5** and dipod **4** with nucleotides. Notably, improvement in affinity from AMP to ATP and stabilization of the host–guest complex with β-CyD cavities, which are poor hosts for hydrophilic compounds such as nucleotides, are clearly favoured by ion pair formation between the guanidinium and phosphate groups. The signals of both ribose and nucleobase moieties are mainly shifted downfield and confirm the interaction of the whole nucleotide molecule with the CyDs of **5**, except with 5′-AMP in which smaller shifts for H^2 and H^8 protons are observed. This would indicate a smoother interaction of the 5′-AMP nucleobase with the CyD cavities. Also, it is interesting to note that no shifts were detected for protons of the ribose moiety of 5′-AMP, indicating clearly that the sugar unit remains, in this case, outside the CyD cavity. Furthermore, all the guest compounds exert slight shielding effects on H^3 and H^5 CyD protons, which are located inside the CyD cavity. This means and confirms that the guest molecules are well included inside the cavity. Elsewhere, one can see (Fig. 2) that adenosine was not complexed by **5**. This result was in accordance with previous results in the literature[9a–c] on the complexation scheme of nucleobase derivatives with CyDs, which revealed that the binding was very weak and restricted mainly to adenine derivatives. These features confirm the accepted idea that host–guest interactions with CyDs are essentially of a hydrophobic nature, whereas nucleotides, and more so their anionic forms, possess low hydrophobicity.

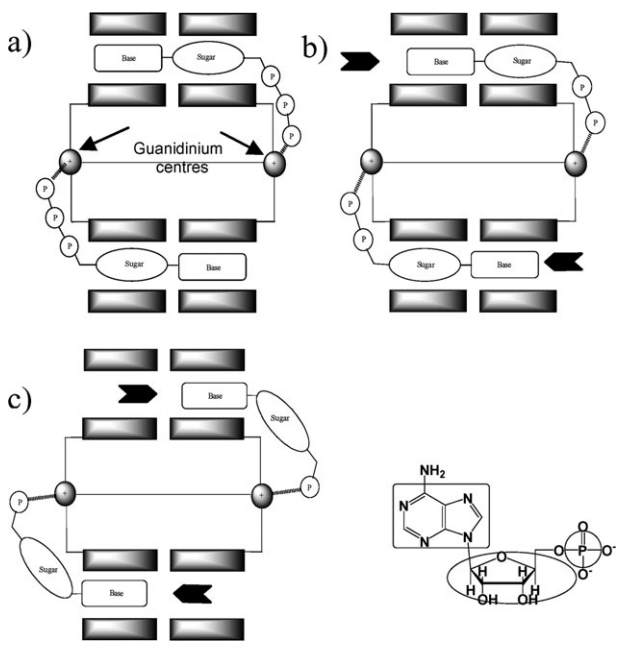
Electrostatic interactions between phosphate anions and guanidinium cationic centres were detected by the ^{31}P-NMR induced shifts of phosphorus signals of the complexed nucleotides compared to those of the free ones. As shown in Table 2, all phosphorus signals for the three nucleotides are shifted. Looking at Table 2, increasing shift values for 5′-$ATP^{2-}2Na^+$ from Pγ to Pα are detected, whereas the terminal phosphates of 5′-ADP^-Na^+ and 5′-AMP^-Na^+ species give the highest shift values. Knowing that in 5′-$ATP^{2-}2Na^+$ γ- and β-phosphate groups are ionized, it could be assumed that two salt bridges exist in this case with a very probably bis-protonated trisubstituted guanidinium group.[10] The registered Pα highest value of −0.22 ppm in 5′-$ATP^{2-}2Na^+$ was attributed in that case, more to strong interactions of the non-ionized phosphate hydroxyls by multiple hydrogen bonds with the primary hydroxyls of the small upper rim of the CyD that lies (Scheme 2a) in a close vicinity. The latter situation was imposed by the deeper inclusion of the 5′-$ATP^{2-}2Na^+$ nucleotide guest far inside the CyD cavities compared to the lesser inclusion of the two other, shorter, substrates.

Table 2 Values (ppm) of ^{31}P chemical induced shifts (CIS) of phosphate groups by complexation with **5** at pH 6.5 and 25 °C

Guest	Status	Pα[a]	Pβ[a]	Pγ[a]
5′-$ATP^{2-}2Na^+$	[2:1] complex	−0.22	−0.15	−0.10
5′-ADP^-Na^+	[2:1] complex	+0.01	+0.07	—
5′-AMP^-Na^+	[2:1] complex	−0.18	—	—

[a] See formula in Table 1.

The inclusion patterns postulated in Scheme 2 are consistent with ^{1}H- and ^{31}P-NMR shifts due to the complex formation but cannot be considered as the sole possibility.[5b] For instance, attempts to detail the geometries of these complexes more accurately (by *e.g.*, 2D-NMR ROESY experiments) unfortunately gave no useful supplementary information. To the best of our knowledge these results demonstrate for the



Scheme 2 Graphic representation of the complexation schemes for the [2:1] complexes of nucleotides with the tetrapod **5**: (a) with 5′-$ATP^{2-}2Na^+$; (b) with 5′-ADP^-Na^+; (c) with 5′-AMP^-Na^+.

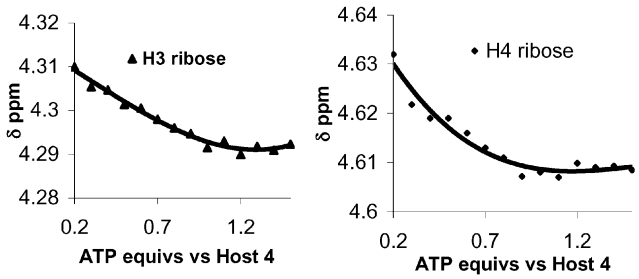


Fig. 4 Chemical induced shifts (CIS) of the ribose H^3 and H^4 protons induced by complexation in the CyD cavity, plotted *vs.* the number of equivalents of 5′-ATP. Dipod **4** concentration was 1.25 mg ml^{-1} ($0.64.10^{-3}$ mol l^{-1}).

second time[5b] the possible real inclusion of sugars inside the lipophilic CyD cavity.

Here, the ion-pair proximity with guanidiniums clearly provides strong electrostatic interactions, which could be considered as the driving and tuning forces for the selective inclusion of the [ribose unit-nucleobase] moieties in the CyD cavities.

A significant increase in the diffusion coefficient of tetrapod **5** is observed in the system [ATP:tetrapod **5**]. This can be explained only if the tetrapod **5** folds when it complexes the ATP. It must be recalled that the diffusion coefficient depends on $1/r_H$, where r_H is the hydrodynamic radius of the molecule; therefore, an increase in the diffusion coefficient (from $10^{-10.1}$ to $10^{-9.9}$ m^2 s^{-1}) demonstrates the packing of the system (see ESI†).

In order to determine which particular contribution was induced by using the multiple CyD cavities in the complexation scheme, we report results on the dimer **4** with $ATP^{2-}2Na^+$ guest molecules under the same conditions. Evidence of complexation was detected from CIS of the ribose H^3 and H^4 protons (Fig. 4) of the ATP unit, which were upfield shifted upon complexation as was found in the literature,[5b] whereas nucleobase aromatic protons remained unmodified. This feature confirms the localization of the sugar moiety inside the CyD cavity, whereas the nucleobase moiety stays outside. The stoichiometry of the complex was confirmed from the Job plot illustrated in Fig. 5. A value of $R = 0.5$ was reached at the maximum, which corresponds to a [1Nu:1dipod] stoichiometry. The binding constant K was 1.98×10^6 M^{-1}, in accordance with the results of the literature estimated for the 5′-ATP tetraanion.[5c]

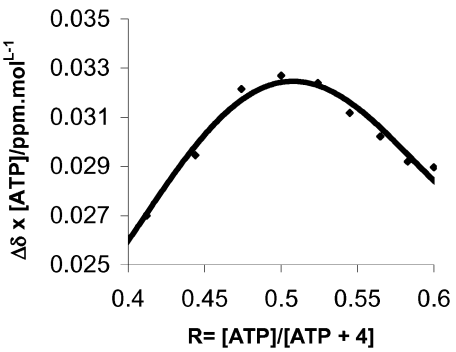
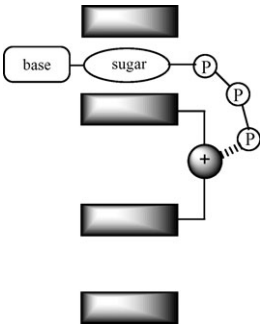


Fig. 5 Job plot corresponding to the chemical shift displacements of the ribose H^3 proton of ATP for [**4**:$ATP^{2-}2Na^+$] complex in D_2O.



Scheme 3 Graphic representation of the complexation scheme for the [1:1] complex of 5′-$ATP^{2-}2Na^+$ with the dipod **4**.

At this time, it is interesting to note that the ribose moiety was included in the CyD cavity as in the tetrapod **5**, that contrary to **5**, only one molecule of ATP could be complexed by the dipod **4** and that the nucleobase moiety remained free of inclusion as illustrated in Scheme 3.

These results clearly indicate that one of the two CyD cavities in the [ATP:dipod **4**] complex does not participate in the complexation. A small variation of the NMR diffusion coefficient is observed for ATP and no modification is seen for dipod **4**. This is consistent with the existence of an inclusion complex (see ESI†). This also undoubtedly established that a different complexation mode occurs for tetrapod **5** with the nucleotides and strengthens our above given interpretation illustrated by Scheme 2.

Toxicity studies

We decided to report here a toxicity study of the tetrapod **5** because this parameter is considered of primary importance in a first step to appreciate the validity of new drugs. The toxicity of the tetrapod **5** was estimated in an *in vitro* test by measuring cellular viability in the presence of increasing amounts of **5**. It was found that MRC-5 cell viability was weakly affected, as shown by the viability evolution during the time course of experiments of MRC-5 cells treated with tetrapod **5** (Fig. 6).

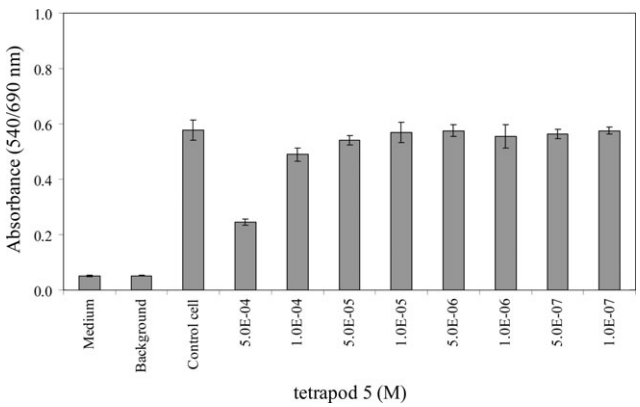


Fig. 6 MTT assay (see Experimental) performed with tetrapod **5** on MRC-5 at 168 h. Histograms were typical of three independent experiments. Medium: MEM alone. Background: MEM with drugs. Control cells: untreated MRC-5 cells.

After 24 h and 48 h of treatment, IC_{50} values were approximately of the same order of magnitude (6.9×10^{-4} mol $l^{-1}$and 6.7×10^{-4} mol l^{-1}, respectively); while at 168 h, **5** became slightly more toxic (3.9×10^{-4} mol l^{-1}). Thus, it appears that **5** was poorly cytotoxic to MRC-5 cells, with a rather more toxic effect on prolonged exposure times (*e.g.* 168 h).

Conclusion

In summary, we found that the proposed phosphine imide strategy is an efficient and easy way to obtain designed CyD water soluble foldamers, allowing full control of the number of cationic centres and CyD cavities introduced in the final molecular framework. It was also established that the tetrapod **5** is able to recognize nucleotidic material at a supramolecular level, combining host–guest hydrophobic inclusion of both ribose and nucleobase moieties into CyDs and electrostatic interactions between guanidinium sites and phosphate anions. Toxicity studies clearly demonstrate the cellular harmlessness of **5** at a high concentration. As initially planned for the aim of this research, new investigations into the self-assembly process of tetrapod **5** with oligonucleotides and DNA, along with their cellular transfection ability, are now under way.

Experimental

Syntheses

Compounds **1**, **2** and **3** were synthesized by known literature procedures.[6a,b]

Typical procedure for the synthesis of 6-monoguanidino[bis(β-cyclodextrin)] dimer 4. Carbodiimide **3** (1.40 g; 0.615 mmol)[6a] was dissolved in dichloromethane–methanol (5 : 1 by volume, 60 ml) and the solution was stirred at 25 °C under NH_3 for 18 h. After evaporation of the solvent, the residue was dissolved in methanol (5 ml) and precipitated with diethyl ether, filtered and evaporated to dryness to give pure **4** (92%). FTIR (KBr): ν = 3374.5 cm^{-1} (OH); 1736–1663 cm^{-1} (N═C–(NH_2)–NH); ES-MS: 2292 $[M + H]^+$; ^{1}H-NMR (400 MHz, D_2O, 25 °C, TMS): δ = 5.07 (m, 14H, H1), 3.95 (m, 14H, H3), 3.90–3.80 (m, 26H, H6a; H5), 3.71–3.62 (m, 16H, H6b; H2), 3.51 (m, 14H, H4); ^{13}C-NMR (100 MHz, D_2O, 25 °C, TMS): δ = 175.1 (N═C–(NH_2)–NH), 102.2 (C1), 81.5 (C4), 73.4 (C2), 72.3 (C3), 72.2 (C6), 60.6 (C5).

Typical procedure for the synthesis of the bis(guanidinium) tetrakis(β-cyclodextrin) tetrapod 5. 6-Monoguanidino[bis(β-cyclodextrin)] dimer **4** (0.622 g; 0.271 mmol) and 1,2-dibromoethane (0.025 g; 0.135 mmol; 0.5 equiv.) was dissolved in DMSO (25 ml) and the solution was stirred at 50 °C for 18 h under argon. The reaction was stopped by precipitation with diethyl ether (100 ml) at 25 °C and filtered. The residue was dissolved in methanol–water (1 : 2 by volume, 53 ml). This solution was concentrated to dryness, the residue was dissolved in water (100 ml). After lyophilization, this solution gave pure **5** (96%). (Found: C, 33.02; H, 6.53; N, 1.58; S, 5.31. $C_{127}H_{286}Br_2N_6O_{136} \cdot 10DMSO \cdot 19H_2O$ requires C, 32.96; H, 7.23; N, 1.57; S, 5.99%); FTIR (KBr): ν = 3359.7 cm^{-1} (OH); 1737–1639 cm^{-1} ($^+$NH═C–(NH_2)–NH, Br^-); ES-MS: 3662 $[M - (\beta\text{-CD-NH})^+]$; ^{1}H-NMR (400 MHz, D_2O, 25 °C, TMS): δ = 5.10 (m, 28H, H1), 3.99 (m, 28H, H3), 3.95–3.81 (m, 52H, H6a; H5), 3.74–3.65 (m, 32H, H6b; H2), 3.50 (m, 28H, H4), 3.17 (m, 4H, CH_2); ^{13}C-NMR (100 MHz, D_2O, 25 °C, TMS): δ = 174.0 (NHBr═C–(NH_2)–NH), 102.3 (C1), 81.6 (C4), 73.6 (C2), 72.4 (C3), 72.3 (C6), 60.7 (C5), 22.3 (CH_2).

Biological studies

Cell line, cell culture. MRC-5 cells (human embryonic lung fibroblasts) were kindly provided by Dr V. Venard (UMR CNRS-UHP 7565, GEVSM, Nancy, France). Freshly trypsinized cells were seeded at 1×10^5 cells cm^{-2} and grown in minimum essential medium (MEM, 41090, Invitrogen, France) supplemented with 10% decomplemented fetal calf serum (FCS, 10270, Lot 40Q5150 K, Invitrogen, France). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Cell viability was determined by the trypan blue dye exclusion method. For all experiments, cells were allowed to adhere and grow for 48 h in culture medium prior to exposure to tetrapod **5**.

MTT assay. To evaluate the effect of tetrapod **5** on MRC-5 cells, the MTT colorimetric assay was performed as described in the literature.[11] This test is based upon the selective ability of living cells to reduce the yellow salt MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] (MTT, 135038, Aldrich, France), to a purple-blue insoluble formazan precipitate. Experiments were performed in 100 µl of medium in flat-bottom 96-well plates (Sarstedt, France). After 48 h incubation of MRC-5 cells at an initial density of 1×10^4 cells per well, the medium was removed and replaced by 2% decomplemented FCS medium containing tetrapod **5** (5×10^{-4} to 1×10^{-12} M). After each period of incubation (24 h, 48 h and 168 h), stock MTT solution (5 mg ml^{-1} MTT in phosphate buffered saline (PBS)) was added (10 µl per 100 µl medium) and plates were incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 4 h. Then 100 µl sodium dodecyl sulfate (SDS, L-5750, Sigma, France) (0.1 g ml^{-1} SDS in PBS, with 445 µL HCl 0.01 M) were added to each well, in a humidified atmosphere with 5% CO_2 at 37 °C for 4 h more. The amount of formazan formed was obtained by scanning with an ELISA reader (Titertek Multiscan MCC/340 MK II apparatus, Labsystems, Helsinki, Finland) at a wavelength of 540 nm with reference at 690 nm. Height wells per dose and time point were counted in three different experiments.

NMR experiments. NMR diffusion experiments have been performed on free ATP, free tetrapod **5**, free dipod **4**, as well as on mixtures of [ATP:tetrapod **5**] and [ATP:dipod **4**]. DOSY maps and diffusion coefficients are reported in the ESI.† Results reveal that the systems [ATP:tetrapod **5**] and [ATP:-dipod **4**] are in fast exchange relative to the chemical shift timescale, which means that only a single "averaged" resonance is detected for all nuclei. All NMR experiments were carried out at 298 K on a Bruker AVANCE 600 MHz operating at 14.1 T fitted with a ^{1}H/^{13}C/^{15}N triple resonance cryoprobe equipped with a 56.3 G cm^{-1} gradient coil. Stimulated echo bipolar longitudinal eddy-current delay (LED) diffusion sequence was used for each diffusion experiment.[12]

The diffusion delay time (Δ) was 200 ms and the gradient pulse length (δ) was 1.5 ms. The durations (δ) of the gradient pulses (g) were optimized and the LED was kept equal to a low value (5 ms). The data for measuring the diffusion coefficient were acquired with 32 768 points in the direct dimension and 16 points in the diffusion dimension. Diffusion coefficients were extracted using the Bruker XWINNMR software (version 3.5).

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