Structure–activity studies on a library of potent calix[4]arene-based PDGF antagonists that inhibit PDGF-stimulated PDGFR tyrosine phosphorylation

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Platelet-derived growth factor (PDGF) and its receptor PDGFR are required for tumor growth and angiogenesis, so disruption of the PDGF–PDGFR interaction should lead to starvation of tumors and reduction of tumor growth. Potent PDGF antagonists have been discovered through the synthesis of a series of calix[4]arene-based compounds that are designed to bind to the three-loop region of PDGF. The effect of lower-rim alkylation, linker and number of interacting head groups on the calix[4]arene scaffold on PDGF affinity and cellular activity has been investigated.

Introduction

Platelet-derived growth factor (PDGF) is a mitogen that plays a key role in cell proliferation, angiogenesis, wound healing, chemotaxis, and inhibition of apoptosis.[1–3] In malignant diseases such as cancer, PDGF or its receptors are often found to be overexpressed and required for tumor growth.[4–7] PDGF has also been shown to induce high levels of vascular endothelial growth factor (VEGF), an angiogenesis inducer that is required for the initiation of the formation of new blood vessels.[8,9] To elicit its biological activity, PDGF binds to PDGFR, a receptor tyrosine kinase (RTK).[10] As a result of binding, the receptor dimerizes and undergoes autophosphorylation that triggers the recruitment of a series of signaling proteins and the activation of the corresponding signal transduction pathways.[11]

Based on the understanding of this biological process, disruption of the PDGF–PDGFR interaction should impede the critical angiogenesis process thus leading to starvation of tumors and reduction of tumor growth.[12–16] To this end, there have been several studies on antagonists against PDGFR or PDGF. Known PDGFR antagonists include antibodies against PDGFR,[17,18] peptides corresponding to the PDGF binding area[19,20] and small molecule inhibitors of receptor dimerization and tyrosine kinase activity.[12,13] Gleevec,[21] the Abl tyrosine kinase inhibitor, which also inhibits PDGFR tyrosine kinase, has been validated in clinical trials and approved recently by the FDA as a new cancer therapeutic. In contrast, only a few PDGF antagonists are known, such as anti-PDGF antibodies,[22] soluble extracellular domains of PDGFR[23] and PDGF-binding DNA aptamers,[24] and these have had only marginal success. We have been expanding our strategies of synthetic protein surface antagonists[25–27] to the design of PDGF antagonists. Synthetic PDGF antagonists have the potential advantages of stability toward protease and nuclease cleavage, smaller size and the convenience of systematic modifications. Over the past several years we have developed a calixarene-based system[28,29] that represents the only family of non-natural antagonists of PDGF.

The design of synthetic molecules that can bind to a protein surface and disrupt physiologically important protein–protein interactions remains a major challenge.[30–34] In contrast to the binding of substrates to well-defined enzyme active site cavities, protein–protein interfaces are much less defined.[35–37] They involve large and lightly featured domains that in many cases cover an area of 1200–2000 Å^2. The X-ray crystal structure[38] (Fig. 1) of the homodimer of PDGF-B (PDGF-BB) shows that it is a dimer of polypeptide chains that folds into two antiparallel pairs of β-strands and contains five intra- and inter-chain disulfide linkages. The three loop regions I–III are exposed to solvent, loop I being highly disordered and thus missing in the solved structure.

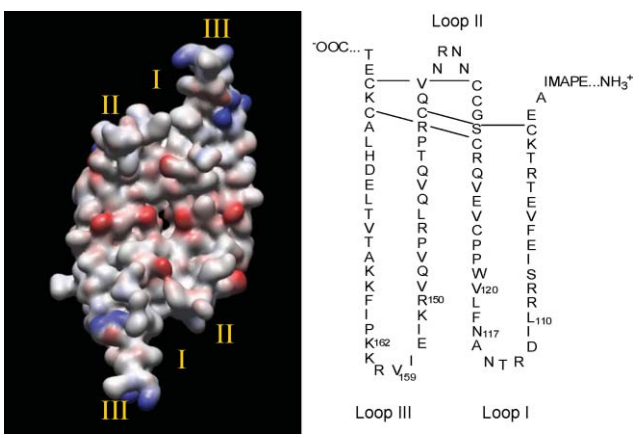

Fig. 1 X-Ray crystal structure of recombinant human PDGF-BB dimer and sequence of PDGF-B monomeric chain. Adapted from ref. 38.

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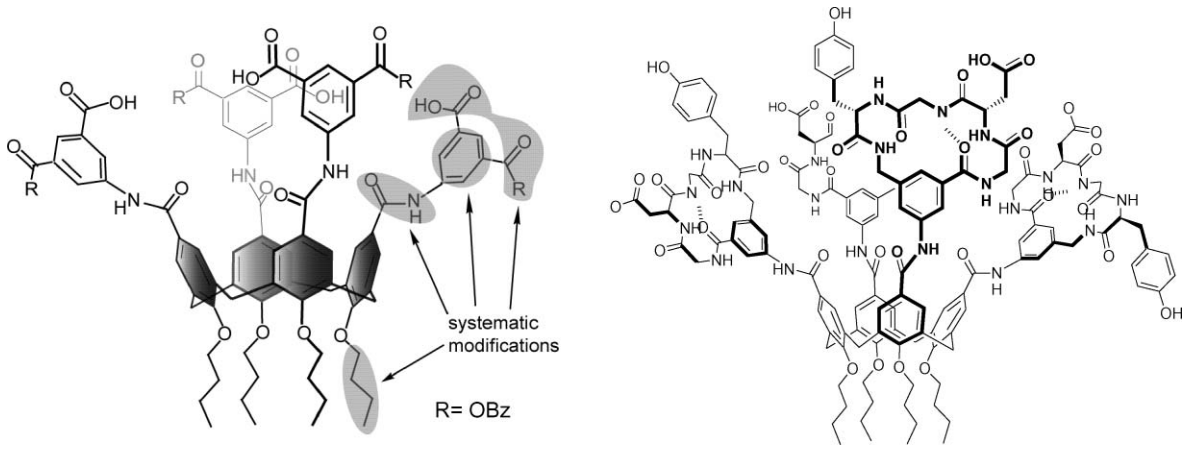

Fig. 2 SAR studies involved systematic modifications of the calix[4]arene scaffold, shown as the shaded domains on the structure of **GFB 204**. The structure of **GFB111** is also shown.

We have previously reported a PDGF antagonist that has 4 peptidic loops attached to calix[4]arene, **GFB111** (Fig. 2).[28,29] This molecule was shown to disrupt the PDGF–PDGFR interaction and have anti-angiogenic and anti-proliferative activity against human tumors. To overcome the drawbacks of **GFB111**, its large size, difficulty of synthesis and aggregation in water, we have recently discovered a second-generation PDGF antagonist **GFB204** (Fig. 2).[39] Simple acyclic isophthalate groups functionalized with an acidic and a hydrophobic group replaced the peptide loops, thus significantly reducing the molecular weight without diminishing activity. **GFB204** was found to block PDGF receptor phosphorylation and Erk1/2 activation with an IC_{50} of 0.19 μM. Further evaluation of its antitumor activity showed that it inhibited tumor growth in xenografted nude mice (Fig. 3). GFB204 was also shown to inhibit angiogenesis by a mechanism involving PDGF as well as VEGF.

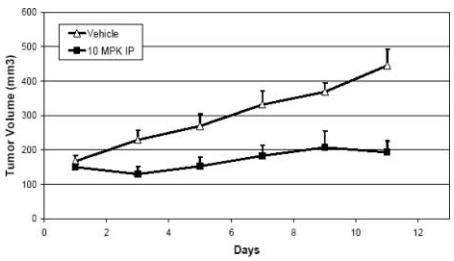

Fig. 3 Antitumor activity of **GFB204** in the nude mouse tumor xenograft model. ◆: control animals that received a vehicle. ■: treated animals that were injected with **GFB204** (10 mg kg^{-1}) daily.

Here, we report an investigation of the effect of a wide range of structure variations of **GFB204** on PDGF affinity and cellular activity including different numbers of isophthalate groups, variation of the linkage between isophthalate and calix[4]arene scaffold and substituents on the lower rim hydroxyls (Fig. 2). We have derived a structure–activity analysis based on the direct binding of PDGF to these antagonists as well as cell-based assays, providing valuable insights into PDGF recognition and directing us to a third-generation PDGF antagonist.

Results and discussion

Design and syntheses of PDGF antagonists

Our approach is based on the calix[4]arene scaffold that bears different functionalities complementary to the three binding loops of PDGF-BB. The three loops critically involved in PDGFR binding are composed of hydrophobic (loop I), hydrophobic and cationic (loop II) and cationic (loop III) residues. Epitope mapping and mutagenesis studies[40–45] have shown that of the three loops, the residues in loop I are most important in the binding to PDGFR. In our previous two generations of calixarene derivatives, the combination of hydrophobic and acidic groups[28] turned out to be essential to achieve optimal binding affinity. In this study, we report the synthesis of a third-generation library in order to evaluate more comprehensively the key structure–activity relationships.

Calix[4]arenes with different numbers of isophthalate groups were synthesized by reacting the acyl chloride form of the tetraacid derivative of calix[4]arene (**1**) with monobenzyl mono-*t*-butyl 5-aminoisophthalate (**2**) in the presence of methanol, followed by preparative thin layer chromatography to obtain the products with one to four isophthalate groups, with the remaining carboxylates blocked as methyl esters (**3b–6b**). The necessity of the aromatic isophthalate frame was assessed with compound **7b**, which contains in its place a dialkyl amide, and was prepared by coupling benzyl ((*t*-butoxycarbonylmethyl)amino)acetate to the tetraacid chloride of calix[4]arene, followed by either benzyl ester deprotection (H_2, Pd–C) or *t*-butyl ester removal (TFA) (Fig. 4).

To investigate the effect on affinity of different linker groups between the isophthalate and calix[4]arene components, compounds **8b–12b** were prepared. Compound **8b** came from the coupling of tetraacid chloride calix[4]arene to 1-benzyl 3-*t*-butyl 5-(2-aminoacetylamino)isophthalate. The preparation of **9b** involved the treatment of tetraamino calix[4]arene with triphosgene to generate the tetra(carbamoyl chloride) that subsequently reacted with aniline **2** to give the urea derivative **9b**. The tetra(diazo)calix[4]arene **10b** was obtained by reacting the parent

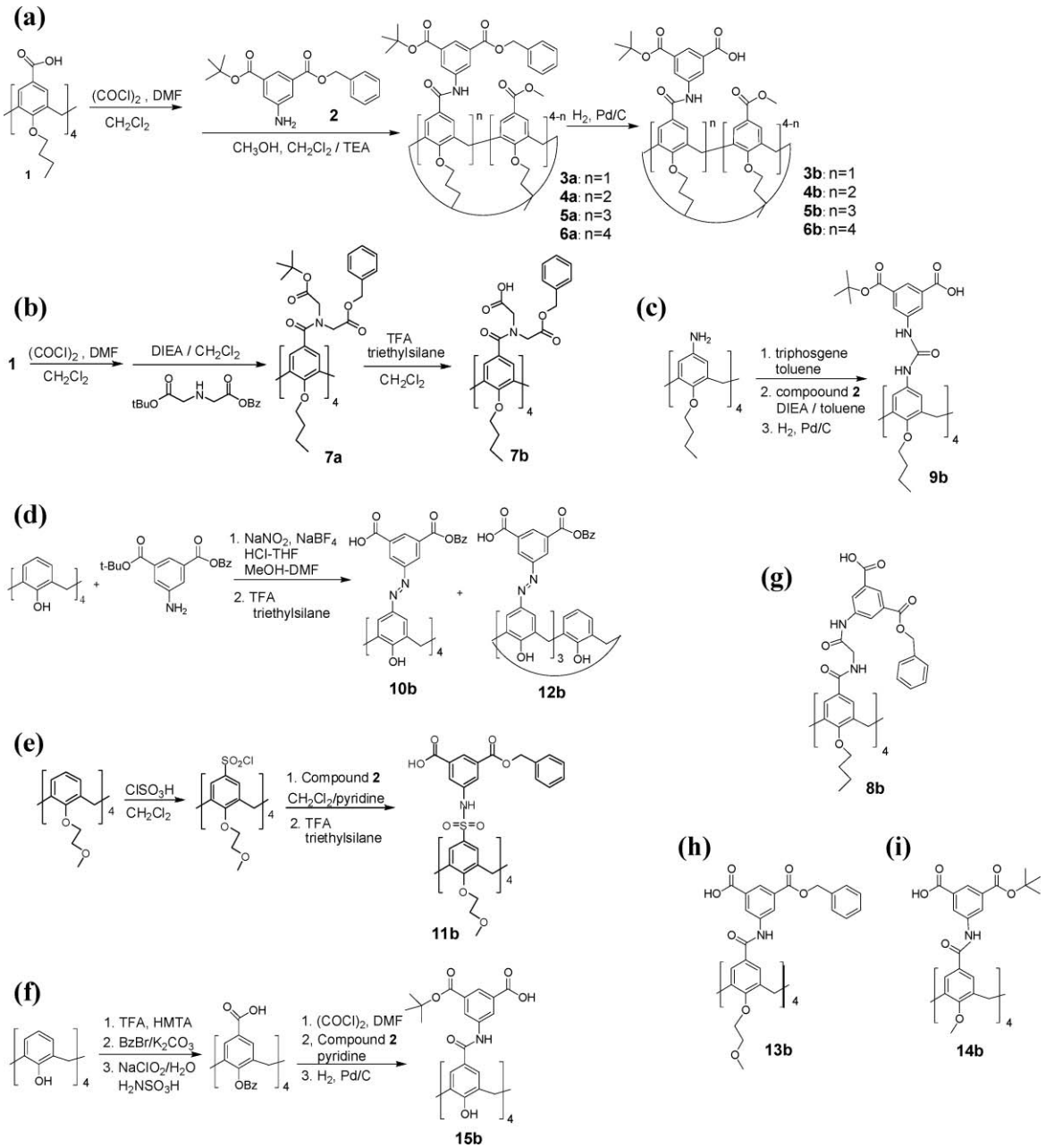

Fig. 4 (a)–(f): Synthetic routes for PDGF antagonists **3b–6b**, **7b**, **10b–12b** and **15b**. (g)–(i): Structures of PDGF antagonists **8b**, **13b** and **14b**.

calix[4]arene with the diazonium salt derived from **2** by treatment with sodium nitrite under acidic conditions. During this reaction, the tris(diazo)calix[4]arene **12b** was also obtained after recrystallization. As a close analog of the amide linker, a sulfonamide group was introduced into compound **11b** by coupling the corresponding tetrasulfonyl chloride calix[4]arene with aniline **2**.

The role of alkylation at the lower rim was exemplified by compounds **10b**, **13b**, **14b** and **15b**. Compounds **13b** and **14b** were synthesized similarly to **GFB204** except that the lower rim was alkylated with either 2-methoxyethyl bromide or methyl iodide. In the preparation of **15b**, the lower rim hydroxyls were first protected by benzyl groups that were later removed along with the isophthalate benzyl ester by hydrogenation.

Binding affinity of PDGF-BB antagonists

There is a single tryptophan residue in each PDGF-B monomer near binding loop I, which provides a fluorescent probe for detecting antagonist binding to this region. The dissociation constants were derived from the change of fluorescent intensity of tryptophan upon binding by the antagonists. The titration curves were fitted using Scientist software to a 1 : 1 binding equation, as described in the Experimental section.

First, an array of antagonists with one to four isophthalate groups was investigated to understand the optimal number of interacting moieties needed for efficient binding. A gradual decrease in binding affinity was observed when the number of isophthalate

groups was reduced from four to one, as in compounds **6b–3b**. The tetra(isophthalate) calix[4]arene **6b** has the highest affinity (K_d = 9.3 nM) in this series, while the tris(isophthalate) **5b** has a slightly weakened affinity (K_d = 21.1 nM). The affinity decreased further to K_d = 137 nM when, as in compound **3b**, there is only a single isophthalate arm remaining (Fig. 5). The affinity of tetra-substituted diazocalix[4]arene **10b** (K_d = 22.6 nM) toward PDGF-BB is only marginally larger than that of tri-substituted **12b** (K_d = 23.8 nM). These observations suggest that the three-armed calix[4]arenes may be comparably active *in vivo* since, despite their slightly weakened affinity, they may have the advantages of a significantly lower molecular weight compared to their four-armed analogs.

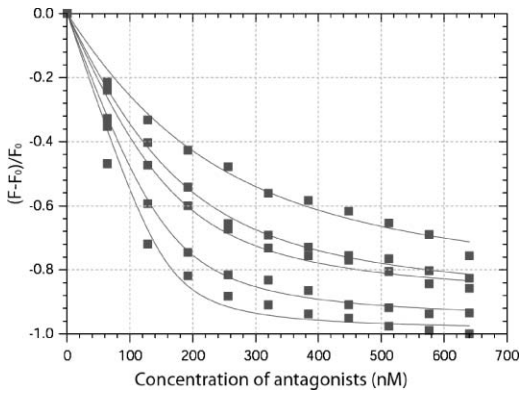

Fig. 5 Fluorescence titrations of PDGF-BB with compounds **3b–6b** and **1**. From top to bottom: K_d/nM: **3b** (137), **1** (68), **4b** (39.4), **5b** (21.1), **6b** (9.26).

Our binding target is the three loops of PDGF-BB that contain both hydrophobic and cationic groups and are critical for its binding to PDGFR. The most efficient binding is seen with those synthetic molecules containing several isophthalate residues bearing both hydrophobic and anionic groups. These binding domains provide a number of potential points of interaction, which should lead to stronger affinity. However, this effect would likely be saturated at a certain point due to limited surface contact and increased steric congestion. Based on our evaluation of K_d and IC_{50} values of the above compounds, the three-armed calix[4]arenes such as **5b** and **12b** appear to be comparably effective PDGF-BB antagonists (Table 1 and Fig. 6).

Second, the effect of the lower rim alkyl groups on the binding affinity was investigated. In our initial design, *n*-butyl groups at the lower rim were introduced to constrain the calix[4]arene scaffold into a cone conformation in which the phenyls are unable to invert through the cavity of the ring.[46] The butylated compound **GFB204** exists in a well-defined cone conformation, as indicated by the pair of doublets from the bridging methylene protons in the ^{1}H NMR spectrum. When the *n*-butyl group was replaced by a methoxyethyl group in compound **13b**, the cone conformation was retained while the binding affinity K_d decreased slightly from 17.3 nM (**GFB204**) to 46.9 nM (**13b**).

Extensive NMR and computational studies have shown that a calix[4]arene with four free hydroxyl groups at the lower rim is stabilized in its cone conformation by a circular array of four hydrogen bonds.[46] Although the cone is in equilibrium with the inverted cone conformation ($\Delta G^{\neq}$ = 15 kcal mol^{-1}) through a

Table 1 The dissociation constants of PDGF-BB with antagonists and the corresponding IC_{50} values for the inhibition of PDGFR auto-phosphorylation

Compound names	K_d/nM	IC_{50}/μM
GFB204	17.3	0.19 ± 0.06
1 (GFB248)	68.0	10
3b (GFB224)	137	>10
4b (GFB223)	39.4	>10
5b (GFB239)	21.1	0.43 ± 0.35
6b (GFB210)	9.26	0.35 ± 0.31
7b (GFB221)	—	2.32 ± 1.81
8b (GFB222)	—	0.6 ± 0.45
9b (GFB227)	—	>10
10b (GFB229)	22.6	0.11 ± 0.04
11b (GFB234)	13.7	>10
12b (GFB247)	23.8	0.48 ± 0.1
13b (GFB237)	46.9	0.58 ± 0.13
14b (GFB210)	31.3	0.35 ± 0.31
15b (GFB238)	10.0	>10

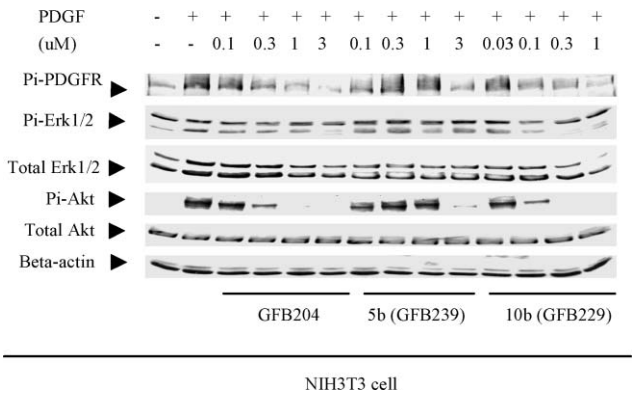

Fig. 6 **GFB204**, **5b** and **10b** inhibit PDGF-BB stimulation of PDGFR tyrosine phosphorylation, Erk1/Erk2 and Akt phosphorylation. NIH 3T3 cells were treated with increasing concentrations of **GFB204**, **5b** or **10b** for 5 min prior to stimulation with PDGF-BB (10 ng mL^{-1}) for 10 min. The cells were then lysed and processed for SDS/PAGE Western blotting with an antibody specific for phosphotyrosine for PDGFR tyrosine phosphorylation, phospho-Erk1/2 or phospho-Akt. Actin was blotted as a control.

partial cone intermediate, only the cone conformation is observed by NMR at room temperature. We rationalized that tetra-hydroxy calix[4]arene derivatives should also give satisfactory affinity to PDGF-BB since the prevalent conformation is cone. Indeed, the tetra-hydroxy calix[4]arene **15b** binds to PDGF-BB with a high affinity of K_d = 10 nM. Therefore, the alkylation of the lower rim is not required for the calix[4]arene to pre-organize into a favorable cone shape that is complementary to the protruding loop region of PDGF-BB. Removal of four lower rim *n*-butyl groups significantly reduced the molecular weight of the molecules, which is desired to improve solubility and bioavailability. The other tetra-hydroxy compound **10b** also showed a high affinity (K_d = 22.6 nM) as did the tetramethyl compound **14b** (K_d = 31.3 nM), further suggesting that a low barrier to conformational interconversion also gives an advantage for potency.

Third, the role of the linkage between isophthalate and calixarene subunits was studied. The affinity was retained when the initial amide linker was substituted by sulfonamide (**11b**) or diazo (**10b**). Compared to the partial double bond characteristics of

an amide, the sulfonamide linker should have more rotational freedom as a single bond, while the diazo bond should be more constrained. The comparable K_d values of the three compounds (**GFB204**: 17.3 nM; **11b**: 13.7 nM; **10b**: 22.6 nM) suggested that different degrees of rigidity in the linker can be tolerated without significant loss of affinity toward PDGF-BB. This is advantageous, as it provides a wide choice of linkers that could be further selected for optimal *in vivo* properties.

The difference of the chemical shift ($\Delta\delta$/ppm) of the pair of bridging methylene protons is a marker of the planarity of the cone conformation in calix[4]arenes.[46] The smaller the $\Delta\delta$ value the more planar is the cone, reaching zero ppm when it is completely planar. A survey of the PDGF-BB antagonists reported in this paper showed a wide range of $\Delta\delta$ values (0.70–1.32 ppm). This observation suggested that although the resting states of the calix[4]arene scaffolds change significantly due to their varying planarity, upon binding to PDGF-BB they may adjust to an optimal conformation without any major energy barrier due to the conformational flexibility of the calix[4]arene scaffold.

PDGF-BB antagonists block PDGF-BB induced PDGF receptor tyrosine phosphorylation and Erk1/2 activation

The calix[4]arene-based antagonists were shown to block the subsequent autophosphorylation of PDGFR and activation of downstream kinases Erk1 and Erk2 in NIH 3T3 cells, as described in the Experimental section. The IC_{50} values provided a direct evaluation of the cellular activity of our PDGF-BB antagonists (Table 1 and Fig. 6). Three of the compounds, **GFB204**, **10b** and **5b**, showed potent IC_{50} values: 0.19 μM, 0.11 μM and 0.43 μM respectively. These three compounds are also among those with the highest PDGF-BB affinity from the fluorescent binding experiments, with K_d values of 17.3 nM, 22.6 nM and 21.1 nM, respectively.

To our surprise, compound **15b** had a very low activity (IC_{50} >10 μM), although its affinity for PDGF-BB was high (K_d = 10.0 nM). This may be due to non-specific interaction of the calix[4]arene derivatives within the cellular assay. The cell-based assay provided a useful tool to screen the preliminary specificity and physiological stability of the initial leads. For example, compound **11b** showed excellent binding affinity (K_d = 13.7 nM) but only gave poor cellular activity (IC_{50} >10 μM) (Table 1).

The isophthalate group appears to be necessary for high cellular activity, since compound **7b**, which lacks the isophalate aromatic ring, showed much reduced activity. This is probably due to the decreased hydrophobicity when the four aromatic isophthalate groups were removed.

Conclusion

To understand the structure–activity relationship of calix[4]arene derivatives towards PDGF-BB binding, a library of compounds that address different aspects of the structural variation of the parent **GFB204** was prepared and their dissociation constants towards PDGF and their ability to inhibit PDGF-stimulated PDGFR tyrosine phosphorylation were obtained through fluorescent titrations and cell-based assays, respectively. Three isophthalate arms turned out to be sufficient to obtain good binding affinity and also to give competitive cellular activity. This observation has led us to design and synthesize new antagonists based on three-folded symmetry.[47]

The linker between the isophthalate and calix[4]arene scaffolds had an insignificant effect on the affinity, but notably influenced the cellular efficacy. Compounds **10b**, **GFB204** and **8b** (with diazo, amide and glycine linkers, respectively) all have satisfactory activity in cell culture. In contrast, compounds **11b** and **9b** with sulfonamide and urea linkers gave very low activity. The alkylation at the lower rim hydroxyls was found to be unnecessary because the four OH groups form an array of hydrogen bonds to stabilize the calix[4]arene scaffold into a cone conformation that is potentially well pre-organized to interact with the loop regions of PDGF-BB. To probe the structure of the complex, we have used the ligand–protein docking program GOLD which searches for the optimal docking of a flexible ligand to a rigid protein framework. Using the GoldScore fitness function, docking of the diazo-calixarene (**10b**) to PDGF-BB showed a cone-shaped calixarene binding to a large surface area of the loop region of PDGF-BB (Fig. 7).

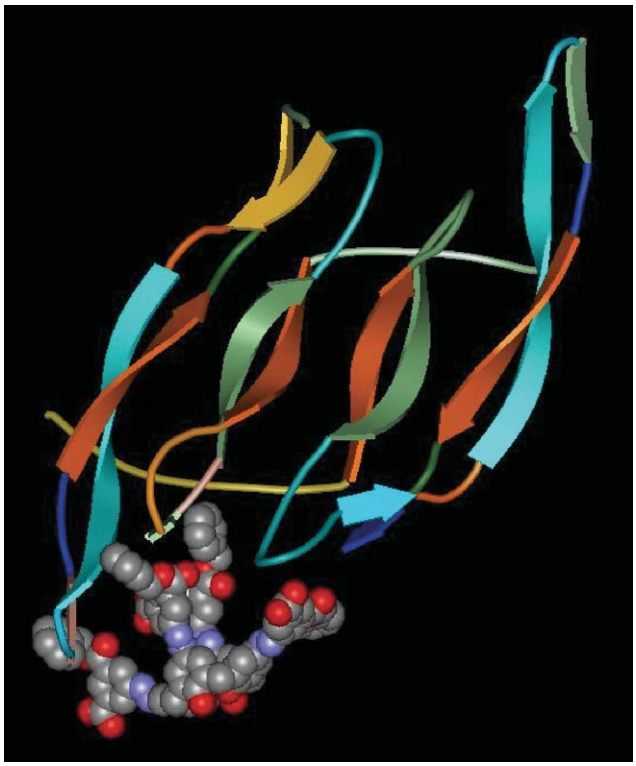

Fig. 7 The docking between PDGF-BB and diazo-calixarene (**10**). Docking was carried out using GOLD (GoldScore fitness function) and the graph was generated using WebLab Viewer Pro.

These observations are now guiding us in new strategies for the design of molecules with reduced molecular weight and elevated *in vivo* activity.

Experimental

Materials and instruments

PDGF-BB was purchased from Cell Sciences. All solvents were purchased from Mallinckrodt or Aldrich and all reagents were

purchased from Aldrich unless otherwise stated and used without further purification. All moisture-sensitive reactions were carried out under nitrogen atmosphere. Purification by column chromatography was carried out using silica gel (230–400 mesh size). Analytical thin layer chromatography (TLC) was conducted using Baker 0.25 mm silica gel pre-coated glass plates with fluorescent indicator active at UV245. Preparative TLC was conducted using Analtech 1000 mm silica gel pre-coated plates with fluorescent indicator active at UV245. ^{1}H NMR and ^{13}C NMR spectra were acquired on either Bruker DPX 400 or DPX 500 series spectrometers. Chemical shifts are expressed as parts per million using solvent as internal standard. Preparative HPLC was performed on a Waters 600E controller in conjunction with Waters 490E multi-wavelength UV detector. Analytical HPLC was performed on a Rainin HP controller with a Rainin UV detector, both attached to a Dell Optiplex PC running Varian Star Workstation software. Nominal mass spectrometry data was obtained by Dr Walter McMurray at the W. M. Keck Foundation Biotechnology Resource Laboratory of Yale University. High resolution mass spectrometry data was obtained by the Washington University Resource for Biomedical and Bio-organic Mass Spectrometry. Fluorescence titration experiments were carried out on a Photon Technology International spectrofluorometer equipped with a LPS-220B lamp power supply, a MD-5020 motor driver and an 814 photomultiplier detection system.

Fluorescence tritration

Lyophilized PDGF-BB (10 μg) was reconstituted by addition of deionized water (100 μL). This stock solution was diluted to 5 mM phosphate buffer pH 7.4 to reach a final concentration of 160 nM. To a quartz cuvette that contained 500 μL of 160 nM PDGF-BB solution, one of the antagonists was added in 32 μM × 1 μL aliquots and incubated at room temperature for 3 min before measurement. The excitation wavelength was 275 nm and the fluorescence of tryptophan at 340 nm was monitored after each addition. A blank buffer was titrated with the same antagonist in the same manner to get a background to be subtracted before curve fitting. The titration curve was fitted to an equimolar binding model using the Scientist program. The equations are $F = (F_0/(1.6 \times 10^{-7}))P + f_2KPL$, $P = 1.6 \times 10^{-7}/(1 + KL)$ and conc. $= (L + KPL)$. The initial values and constraints of parameters are $f_2 = 0$, $0 < L <$ conc., $0 < P < 1.6 \times 10^{-7}$ and $K = 1.0 \times 10^{6}$. F: fluorescence intensity at any titration point; F_0: fluorescence intensity at the beginning; P: unbound PDGF; f_2: unit fluorescence intensity of bound protein; K: association constant; L: unbound antagonist.

Antitumor activity in the nude mouse tumor xenograft model

Nude mice (Charles River, Wilmington, MA) were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) procedures and guidelines. A-549 cells (ATCC) were harvested and resuspended in PBS. A-549 cells were injected subcutaneously (s.c.) into the right and left flanks (10×10^6 cells per flank) of 8 week old female nude mice. When tumors reached about 200 mm^3, animals were dosed intraperitoneally (i.p.) with 0.1 mL once daily. Control animals received a vehicle, whereas treated animals were injected with **GFB204**. The tumor volumes were determined by measuring the length (l) and the width (w) and calculating the volume ($V = lw^2/2$). The statistical significance between control and treated animals was evaluated using Student's t-test.

Inhibition of growth factor-dependent receptor tyrosine phosphorylation by GFBs

Starved NIH 3T3 cells were pretreated with GFBs for 5 min before stimulation with PDGF-BB (10 ng mL^{-1}) for 10 min. The cells were then harvested and lysed, and proteins from the lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes then were blotted with anti-phospho-tyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY, USA) for activated PDGFR.[28] Phosphotyrosine PDGFRs were quantified using a Bio-Rad Model GS-700 Imaging Densitometer (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Inhibition of growth factor-mediated stimulation of phosphorylation of Erk1/2 and Akt by GFBs

Starved NIH 3T3 cells were pretreated with the indicated concentration of GFBs for 5 min, before 10 min stimulation with PDGF-BB (10 ng mL^{-1}). Cell lysates were run on SDS-PAGE gels, and then transferred to nitrocellulose and Western blotted with anti-phosphorylated Erk1/Erk2 (Cell Signaling Technologies) and anti-phosphorylated Akt as described previously by us.[28]

Synthesis

General procedure for removal of benzyl group. For example, the fully protected compound **5a** (5 mg, 0.0028 mmol) and palladium on carbon (10%, 0.5 mg) in methanol–ethyl acetate (2 mL, 1 : 1) was stirred under 1 atm H_2 for 8 h. The catalyst was filtered off and the filtrate was concentrated *in vacuo* to yield the deprotected product **5b** (3.5 mg, 83%) as a white solid. **5b**: ^{1}H NMR (400 MHz, CD_3OD): δ = 10.16 (s, 2H, NH), 9.77 (s, 1H, NH), 8.27–8.59 (m, 9H, Ar), 7.76 (s, 2H, Ar calix), 7.71 (s, 2H, Ar calix), 7.20 (s, 2H, Ar calix), 7.14 (s, 2H, Ar calix), 4.59 (t, J = 13.2 Hz, 4H, H_{ax} of $ArCH_2Ar$), 4.19 (t, J = 6.5 Hz, 4H, $OCH_2CH_2CH_2CH_3$), 3.93 (t, J = 6.5 Hz, 4H, $OCH_2CH_2CH_2CH_3$), 3.53 (s, 3H, $COCH_3$), 3.43 (t, J = 13.2 Hz, 4H, H_{eq} of $ArCH_2Ar$), 1.97 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.63 (s, 18H, $C(CH_3)_3$), 1.56 (s, 9H, $C(CH_3)_3$), 1.44 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.06 (m, 12H, $OCH_2CH_2CH_2CH_3$). HR ESI-MS m/z: calcd $[M + 2Na]^{2+}$ 770.8205, found 770.8196.

General procedure for removal of *t*-butyl group. For example, the fully protected compound **8a** (15 mg, 0.007 mmol), triethylsilane (5 μL, 0.035 mmol), and trifluoroacetic acid (150 μL, 2.1 mmol) in 3 mL of CH_2Cl_2 was stirred overnight. The deprotected product **8b** (11 mg, 80%) as a white solid was obtained with satisfactory purity after evaporation of the solvent followed by addition of diethyl ether and evaporation to dryness. **8b**: ^{1}H NMR (400 MHz, CD_3OD): δ = 8.32 (s, 8H, Ar), 8.25 (s, 4H, Ar), 7.38–7.24 (m, 28H, Ph and Ar calix), 5.28 (s, 8H, CH_2Ph), 4.50 (d, J = 13.3 Hz, 4H, H_{ax} of $ArCH_2Ar$), 3.98 (br s, 16H, $COCH_2$ and $OCH_2CH_2CH_2CH_3$), 3.30 (d, J = 13.3 Hz, 4H, H_{eq} of $ArCH_2Ar$), 1.93 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.51 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.03 (t, J = 7.3 Hz, 12H,

$OCH_2CH_2CH_2CH_3$). ^{13}C NMR (125 MHz, $CDCl_3$–CD_3OD, 1 : 1): δ = 168.3 (s, CO), 167.9 (s, CO), 167.1 (s, CO), 165.3 (s, CO), 159.3 (s, Ar), 138.4 (s, Ar), 135.3 (s, Ph), 134.6 (s, Ar), 131.3 (s, Ar), 130.5 (s, Ar), 128.1 (d, Ph), 127.8 (d, Ph), 127.7 (d, Ph), 127.5 (d, Ar), 126.9 (s, Ar), 125.7 (d, Ar), 124.7 (d, Ar), 124.3 (d, Ar), 74.8 (t, $OCH_2CH_2CH_2CH_3$), 66.7 (t, OCH_2Ph), 43.7 (t, $COCH_2$), 31.9 (t, $OCH_2CH_2CH_2CH_3$), 30.5 (t, $ArCH_2Ar$), 18.9 (t, $OCH_2CH_2CH_2CH_3$), 13.4 (q, $OCH_2CH_2CH_2CH_3$). HR ESI-MS *m*/*z*: calcd $[M - H]^-$ 1643.6650, found 1643.7494.

Monobenzyl mono-*t*-butyl 5-aminoisophthalate (2). To a solution of monomethyl 5-nitroisophthalate in 40 mL of dry methylene chloride (3 g, 13 mmol) was added oxalyl chloride (5.1 g, 40 mmol) and 100 μL of DMF, and then the solution was stirred for 3 h. The solution was evaporated *in vacuo* and added to *t*-BuOH (19 g, 260 mmol), DMAP (1.63 g, 13 mmol) and DIEA (17 g, 130 mmol) then stirred overnight. The solution was evaporated, and dissolved in 50 mL of methylene chloride and washed with 1 N NaOH (3 × 50 mL) and 1 N HCl (3 × 50 mL). The organic extracts were concentrated and dried *in vacuo* to afford monomethyl mono-*t*-butyl 5-nitroisophthalate (2.5 g, 68%) as a brown solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.97 (s, 1H), 8.93 (s, 1H), 8.89 (s, 1H), 4.00 (s, 3H), 1.65 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 164.1, 162.4, 148.1, 135.4, 134.1, 131.9, 127.7, 127.4, 83.0, 52.7, 27.8. FAB-MS *m*/*z*: calcd. $[M + H]^+$ 282.1, found 282.4.

To the above product (2.4 g, 8.5 mmol) was added 1 N LiOH (15 mL), followed immediately by THF (15 mL). After stirring vigorously for 2 h, the solution was poured into methylene chloride (60 mL), and 1 N HCl (60 mL) was added. The organic extracts were combined and evaporated *in vacuo* to afford mono-*t*-butyl 5-nitroisophthalate (2.2 g, 96%) as a light yellow powder. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.78 (s, 1H), 8.72–8.70 (m, 2H), 1.60 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 165.2, 163.0, 148.5, 135.0, 134.8, 133.5, 127.7, 127.0, 83.1, 28.0. FAB-MS *m*/*z*: calcd. $[M + H]^+$ 268.1 found 268.2.

The obtained powder (2.1 g, 7.8 mmol) was taken up in MeOH (200 mL) and added to a suspension of 10% Pd/C (200 mg) in H_2O (5 mL), and stirred for 3 h under 1 atm of H_2. The catalyst was filtered off and the filtrate evaporated *in vacuo* to provide mono-*t*-butyl 5-aminoisophthalate (1.74 g, 94%) as a white solid. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.88 (s, 1H), 7.53 (s, 1H), 7.48 (s, 1H), 1.61 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 167.18, 149.10, 131.72, 118.13, 117.61, 117.37, 116.97, 80.52, 27.7.

This product (2 g, 8.4 mmol) was taken up in DMF (50 mL) and cooled to 0 °C, with stirring. Then Cs_2CO_3 (2.7 g, 8.4 mmol) and benzyl bromide (1.44 g, 8.4 mmol) were added, and the solution stirred for 5 h at RT. After extraction with methylene chloride, the organic layer was washed with H_2O and the combined organic extract was evaporated *in vacuo.* The residue was chromatographed (hexanes–EtOAc, 3 : 1) to provide monobenzyl mono-*t*-butyl 5-aminoisophthalate (**2**) (1.8 g, 66%) as a white powder. **2**): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.10 (s, 1H), 7.59 (s, 1H), 7.55 (s, 1H), 7.44–7.32 (m, 5H), 5.35 (s, 2H), 1.58 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 166.1, 165.1, 145.3, 136.1, 133.7, 131.6, 128.8, 128.5, 128.4, 121.9, 120.9, 120.4, 81.8, 67.1, 28.4. FAB-MS *m*/*z*: calcd. $[M + H]^+$ 328.1, found 328.3.

Tetrakis(butoxy)calix[4]arene tetrakis(monobenzyl isophthalate) (GFB204). To a solution of tetrakis(butoxy)calix[4]arene tetracarboxylic acid (**1**) (100 mg, 0.12 mmol) in dry methylene chloride (5 mL) was added oxalyl chloride (0.65 mL, 7.2 mmol) and 1 drop of dry DMF. After stirring overnight, the reaction mixture was evaporated *in vacuo* to give the acyl chloride. A solution of **2** (235 mg, 0.72 mmol) and dry pyridine (64 μL, 0.72 mmol) in dry methylene chloride (7 mL) were added and stirred overnight. After evaporation *in vacuo*, the residue was purified by column chromatography (hexanes–acetone, 4 : 1) to afford the fully protected precursor of **GFB204** (168 mg, 67%). The *t*-butyl groups were removed as described previously to obtain **GFB204** as a white solid. **GFB204**: ^{1}H NMR (400 MHz, acetone-d_6): δ = 9.40 (s, 4H, NH), 8.49 (s, 4H, Ar), 8.47 (s, 4H, Ar), 8.26 (s, 4H, Ar), 7.56 (s, 8H, Ar calix), 7.47–7.28 (m, 20H, Ph), 5.32 (s, 8H, CH_2Ph), 4.63 (d, J = 13.6 Hz, 4H, H_{ax} of $ArCH_2Ar$), 4.07 (t, J = 7.2 Hz, 8H, $OCH_2CH_2CH_2CH_3$), 3.45 (d, J = 13.6 Hz, 4H, H_{eq} of $ArCH_2Ar$), 1.98 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.55 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.04 (t, J = 9.0 Hz, 12H, $OCH_2CH_2CH_2CH_3$). ^{13}C NMR (400 MHz, acetone-d_6): δ = 166.73 (CO), 165.83 (CO), 165.73 (CO), 160.74 (4°, Ar), 140.78 (4°, Ar), 137.11 (4°, Ar), 135.86 (4°, Ar), 132.01 (4°, Ar), 131.54 (4°, Ar), 129.34 (3°, Ar), 129.14 (3°, Ar), 129.09 (3°, Ar), 129.04 (4°, Ar), 128.97 (3°, Ar), 126.07 (3°, Ar), 125.93 (3°, Ar), 125.51 (3°, Ar), 75.95 ($PhCH_2$), 67.45 ($OCH_2CH_2CH_2CH_3$), 33.05 ($OCH_2CH_2CH_2CH_3$), 31.85 ($ArCH_2Ar$), 20.05 ($OCH_2CH_2CH_2CH_3$), 14.39 ($OCH_2CH_2CH_2CH_3$). FAB-MS *m*/*z*: calcd. $[M - H]^-$ 1835.7 found 1835.2.

To obtain tetrakis(butoxy)calix[4]arene tetra(mono-*t*-butyl isophthalate) (**6b**) from the same precursor as that of **GFB204**, the benzyl groups were removed according to the procedure described previously, to provide compound **6b** as a white solid. **6b**: ^{1}H NMR (400 MHz, acetone-d_6): δ = 9.38 (s, 4H, NH), 8.47 (s, 4H, Ar), 8.40 (s, 4H, Ar), 8.20 (s, 4H, Ar), 7.60 (s, 8H, calix Ar), 4.63 (d, J = 13.2 Hz, 4H, H_{ax} of $ArCH_2Ar$), 4.10 (br t, 8H, $OCH_2CH_2CH_2CH_3$), 3.47 (d, J = 13.2 Hz, 4H, H_{eq} of $ArCH_2Ar$), 2.01 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.57 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.53 (s, 9H, $C(CH_3)_3$), 1.06 (t, J = 7.2 Hz, 12H, $OCH_2CH_2CH_2CH_3$). ^{13}C NMR (400 MHz, acetone-d_6): δ = 167.39 (CO), 165.87 (CO), 165.07 (CO), 160.61 (4°, Ar), 140.38 (4°, Ar), 135.85 (4°, Ar), 133.32 (4°, Ar), 132.23 (4°, Ar), 129.40 (4°, Ar), 129.12 (3°, Ar), 125.93 (3°, Ar), 125.69 (3°, Ar), 125.36 (3°, Ar), 81.67 (4°, $C(CH_3)_3$), 76.03 (2°, $OCH_2CH_2CH_2CH_3$), 33.10 (2°, $OCH_2CH_2CH_2CH_3$), 31.83 (2°, $ArCH_2Ar$), 28.27 (1°, $C(CH_3)_3$), 20.07 (2°, $OCH_2CH_2CH_2CH_3$), 14.41 (1°, $OCH_2CH_2CH_2CH_3$). HR ESI-MS *m*/*z*: calcd $[M + 2Na]^{2+}$ 873.3569, found 873.3577.

Tetrakis(butoxy)calix[4]arene mono(mono-*t*-butyl isophthalate) tris(methoxy) (3b), tetrakis(butoxy)calix[4]arene bis(mono-*t*-butyl isophthalate) bis(methoxy) (4b), tetrakis(butoxy)calix[4]arene tris(mono-*t*-butyl isophthalate) mono(methoxy) (5b). To a stirred suspension of tetrakis(butoxy)calix[4]arene tetracarboxylic acid (**1**) (30 mg, 0.036 mmol) in dry CH_2Cl_2 (2 mL) was added oxalyl chloride (190 μL, 2.18 mmol) and 1 drop of dry DMF. After stirring overnight the solvent was evaporated, the resulting residue was dried *in vacuo* and then dissolved in dry CH_2Cl_2 (1 mL). A solution of **2** (35 mg, 0.108 mmol), methanol (5 μL, 0.108 mmol) and triethylamine (40 μL, 0.288 mmol) in dry CH_2Cl_2 (1 mL) was added dropwise and the reaction mixture was stirred for 12 h. The reaction mixture was diluted with CH_2Cl_2 (3 mL), washed with 0.5 N HCl (5 mL) and H_2O (2 × 3 mL), dried

over Na_2SO_4 and concentrated *in vacuo*. The crude product was separated by preparative TLC (CH_2Cl_2–methanol, 100 : 1) to obtain the fully protected compounds **4a** (10 mg, 19%), **6a** and a mixture of **3a** and **5a** as white solids. **4a** is the mixture of 1,2- and 1,3-substituted isomers, and no attempt was made to separate them. The mixture of **3a** and **5a** was further purified by preparative TLC (hexanes–acetone, 4 : 1) to obtain **3a** (8 mg, 19%) and **5a** (10 mg, 17%). **3a**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.52 (s, 1H, Ar), 8.41 (s, 1H, Ar), 8.38 (s, 1H, Ar), 7.87 (s, 1H, NH), 7.46–7.31 (m, 9H, Ph and Ar calix), 7.17 (s, 2H, Ar calix), 7.09 (s, 2H, Ar calix), 5.37 (s, 2H, CH_2Ph), 4.45 (d, J = 13.5 Hz, 2H, H_{ax} of $ArCH_2Ar$), 4.44 (d, J = 13.6 Hz, 2H, H_{ax} of $ArCH_2Ar$), 3.99 (t, J = 7.6 Hz, 4H, $OCH_2CH_2CH_2CH_3$), 3.89 (t, J = 7.3 Hz, 2H, $OCH_2CH_2CH_2CH_3$), 3.86 (t, J = 7.3 Hz, 2H, $OCH_2CH_2CH_2CH_3$), 3.82 (s, 6H, $COCH_3$), 3.54 (s, 3H, $COCH_3$), 3.27 (d, J = 13.5 Hz, 2H, H_{eq} of $ArCH_2Ar$), 3.26 (d, J = 13.6 Hz, 2H, H_{eq} of $ArCH_2Ar$), 1.90–1.81 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.58 (s, 9H, $C(CH_3)_3$), 1.47–1.39 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.01–0.96 (m, 12H, $OCH_2CH_2CH_2CH_3$). **4a**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.52 (s, 2H, Ar), 8.43 (s, 2H, Ar), 8.37 (s, 2H, Ar), 8.02 (s, 2H, NH), 7.42 (d, 4H, J = 6.9 Hz, Ph), 7.35–7.22 (m, 14H, Ph and Ar calix), 5.34 (s, 4H, CH_2Ph), 4.51–4.44 (m, 4H, H_{ax} of $ArCH_2Ar$), 3.98–3.92 (m, 8H, $OCH_2CH_2CH_2CH_3$), 3.60 (s, 6H, $COCH_3$), 3.32–3.26 (m, 4H, H_{eq} of $ArCH_2Ar$), 1.91–1.83 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.56 (s, 18H, $C(CH_3)_3$), 1.47–1.41 (m, 8H, $OCH_2CH_2CH_2CH_3$), 0.99 (t, J = 7.3 Hz, 12H, $OCH_2CH_2CH_2CH_3$). **5a**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.30–8.50 (m, 9H, Ar), 7.87 (s, 2H, NH), 7.70 (s, 1H, NH), 7.28–7.48 (m, 21H, Ph and Ar calix), 7.17 (s, 1H, Ar calix), 7.09 (s, 1H, Ar calix), 5.33 (s, 4H, CH_2Ph), 5.32 (s, 2H, CH_2Ph), 4.52 (dd, J_1 = 13.6 Hz, J_2 = 4.8 Hz, 4H, H_{ax} of $ArCH_2Ar$), 4.05 (t, J = 7.6, 4H, $OCH_2CH_2CH_2CH_3$), 3.91 (m, 4H, $OCH_2CH_2CH_2CH_3$), 3.52 (s, 3H, $COCH_3$), 3.34 (dd, J_1 = 13.6 Hz, J_2 = 4.8 Hz, 4H, H_{eq} of $ArCH_2Ar$), 1.90 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.56 (s, 18H, $C(CH_3)_3$), 1.54 (s, 9H, $C(CH_3)_3$), 1.50 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.02 (m, 12H, $OCH_2CH_2CH_2CH_3$). The deprotected **3b**, **4b** and **5b** were obtained after removal of the benzyl ester groups from **3a**, **4a** and **5a** respectively, as previously described. ESI-MS m/z: **3b**: calcd $[M + Na]^+$ 1108.5034, found 1108.5051; **4b**: calcd $[M - H]^-$ 1289.6, found 1289.7; **5b**: calcd $[M + 2Na]^{2+}$ 770.8205, found 770.8196.

Tetrakis(butoxy)calix[4]arene tetrakis(*N*-benzylacetate *N*-acetate) (7b). To a stirred suspension of **1** (26 mg, 0.03 mmol) in dry CH_2Cl_2 (2 mL) was added oxalyl chloride (167 μL, 1.92 mmol) and 1 drop of dry DMF. After 5 h the solvent was evaporated, the resulting residue dried *in vacuo* and then dissolved in dry CH_2Cl_2 (2 mL). A solution of benzyl ((*tert*-butoxycarbonylmethyl)amino)acetate (53 mg, 0.19 mmol) (obtained by coupling glycine *t*-butyl ester hydrochloride to benzyl bromoacetate in the presence of NEt_3 in dry THF) and diisopropylethylamine (33 μL, 0.19 mmol) in dry CH_2Cl_2 (1 mL) was added dropwise and the reaction mixture was stirred for 2 h. The reaction was then quenched by the addition of H_2O (2 mL) and the organic layer was separated, washed with H_2O (2 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by preparative TLC (CH_2Cl_2–methanol, 97 : 3) to obtain the fully protected **7a** as a colorless oil (32 mg, 53%). **7a**: ^{1}H NMR (400 MHz, DMSO-d_6, 90 °C): δ = 7.35 (m, 20H, Ph), 6.82 (s, 8H, Ar), 5.15 (s, 8H, CH_2Ph), 4.39 (d, J = 13.1 Hz, 4H, H_{ax} of $ArCH_2Ar$), 3.98 (br s, 8H, $OCH_2CH_2CH_2CH_3$), 3.89 (br s, 16H, NCH_2COOBz and NCH_2COO*t*-Bu), 3.13 (d, J = 13.1 Hz, 4H, H_{eq} of $ArCH_2Ar$), 1.86 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.41 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.38 (s, 36H, $OCCH_3$), 0.98 (t, J = 7.3 Hz, 12H, $OCH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6, 90 °C): δ = 169.9 (s, CO), 168.0 (s, CO), 167.1 (s, CO), 156.7 (s, Ar), 135.4 (d, Ar), 133.2 (s, Ph), 128.1 (s, Ar), 127.6 (d, Ph), 127.2 (2d, Ph), 126.7 (s, Ar), 80.6 (s, $C(CH_3)_3$), 80.4 (t, $OCH_2CH_2CH_2CH_3$), 73.9 (t, OCH_2Ph), 65.4 (2t, NCH_2), 30.9 (t, $OCH_2CH_2CH_2CH_3$), 30.0 (t, $ArCH_2Ar$), 27.1 (q, $C(CH_3)_3$), 18.1 (t, $OCH_2CH_2CH_2CH_3$), 12.9 (q, $OCH_2CH_2CH_2CH_3$). After removal of the *t*-butyl ester groups as previously described, compound **7b** was obtained as a white solid. HR ESI-MS m/z: **7a**: calcd $[M + Na]^+$ 1891.9129, found 1891.9429; **7b**: calcd $[M - H]^-$ 1643.6650, found 1643.7494.

Tetrakis(butoxy)calix[4]arene tetrakis(monobenzyl mono-*t*-butyl-isophthalate-glycine-linked) (8a). To a stirred suspension of **1** (24 mg, 0.029 mmol) in dry CH_2Cl_2 (2 mL) was added oxalyl chloride (150 μL, 1.74 mmol) and 1 drop of dry DMF. After 5 h the solvent was evaporated, the resulting residue was dried *in vacuo* and then dissolved in dry CH_2Cl_2 (2 mL). A solution of 1-benzyl 3-*t*-butyl 5-(2-aminoacetylamino)isophthalate (134 mg, 0.35 mmol) (obtained by coupling of **2** with *N*-Fmoc-glycine *via* the acyl chloride, followed by removal of Fmoc by diethylamine) in dry CH_2Cl_2 (1 mL) was added dropwise and the reaction mixture was stirred for 7 h. The solvent was evaporated and the residue was purified by crystallization from CH_2Cl_2–methanol (1 : 1) to yield the fully protected product **8a** as a white solid (45 mg, 68%). **8a**: ^{1}H NMR (400 MHz, $CDCl_3$–MeOD, 1 : 1): δ = 8.37 (s, 4H, Ar), 8.33 (s, 4H, Ar), 8.25 (s, 4H, Ar), 7.40–7.19 (m, 28H, Ph and Ar calix), 5.30 (s, 8H, CH_2Ph), 4.45 (d, J = 13.4 Hz, 4H, H_{ax} of $ArCH_2Ar$), 4.28 (s, 8H, $COCH_2$), 3.94 (br s, 8H, $OCH_2CH_2CH_2CH_3$), 3.26 (d, J = 13.4 Hz, 4H, H_{eq} of $ArCH_2Ar$), 1.88 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.52 (s, 36H, $C(CH_3)_3$), 1.46 (m, 8H, $OCH_2CH_2CH_2CH_3$), 0.99 (t, J = 7.3 Hz, 12H, $OCH_2CH_2CH_2CH_3$). ^{13}C NMR (125 MHz, $CDCl_3$–MeOD, 1 : 1): δ = 168.2 (s, CO), 167.8 (s, CO), 165.4 (s, CO), 164.6 (s, CO), 159.4 (s, Ar calix), 138.4 (s, Ar), 135.3 (s, Ph), 134.7 (s, Ar calix), 132.6 (s, Ar), 130.6 (s, Ar), 128.1 (d, Ph), 127.9 (d, Ph), 127.7 (d, Ph), 127.5 (d, Ar calix), 126.9 (s, Ar calix), 125.4 (d, Ar), 124.4 (d, Ar), 124.0 (d, Ar), 81.7 (s, $C(CH_3)_3$), 74.8 (t, $OCH_2CH_2CH_2CH_3$), 66.7 (t, OCH_2Ph), 43.7 (t, $COCH_2$), 31.9 (t, $OCH_2CH_2CH_2CH_3$), 30.6 (t, $ArCH_2Ar$), 27.5 (q, $C(CH_3)_3$), 18.9 (t, $OCH_2CH_2CH_2CH_3$), 13.5 (q, $OCH_2CH_2CH_2CH_3$). ESI-MS m/z: **8a**: calcd $[M + H]^+$ 2290.02, found 2289.96. The *t*-butyl ester groups were then removed to obtain the deprotected product **8b**.

Tetrakis(butoxy)calix[4]arene tetrakis(mono-*t*-butylisophthalyl ureido) (9b). To a solution of tetrakis(butoxy)calix[4]arene tetraamino (50 mg, 0.069 mmol) in toluene (2 mL) was added triphosgene (27 mg, 0.089 mmol), and the mixture was stirred at 110 °C for 4 h. After cooling the reaction to 30 °C, a solution of **2** (99 mg, 0.30 mmol) and diisopropylethyl amine (52 μL, 0.30 mmol) in toluene (1.5 mL) was added. The mixture was stirred overnight at room temperature and the solvent was evaporated. The residue was dissolved in CH_2Cl_2 (5 mL), washed with H_2O (2 × 3 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography

(CH_2Cl_2–methanol, 50 : 1) to yield the fully protected product **9a** as a white solid (58 mg, 40%). The benzyl groups were removed as described previously to obtain deprotected **9b** as a white solid (11 mg, 78%). **9b**: ^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD, 1 : 1): δ = 8.02 (s, 4H, Ar), 7.99 (s, 4H, Ar), 7.82 (s, 4H, Ar), 6.66 (br s, 8H, Ar calix), 4.28 (d, J = 13.6 Hz, 4H, H_{ax} of $ArCH_2Ar$), 3.72 (br s, 8H, $OCH_2CH_2CH_2CH_3$), 3.99 (d, J = 13.6 Hz, 4H, H_{eq} of $ArCH_2Ar$), 1.75 (m, 8H, $OCH_2CH_2CH_2CH_3$), 1.39 (s, 36H, $C(CH_3)_3$), 1.28 (m, 8H, $OCH_2CH_2CH_2CH_3$), 0.83 (t, J = 7.6 Hz, 12H, $OCH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$–CD_3OD, 1 : 1): δ = 167.6 (s, CO), 164.9 (s, CO), 160.3 (s, Ar calix), 153.3 (s, CO), 139.3 (s, Ar), 134.9 (s, Ar calix), 132.0 (s, Ar), 131.9 (s, Ar), 130.9 (s, Ar calix), 123.9 (d, Ar), 122.9 (s, Ar), 122.8 (d, Ar), 119.3 (d, Ar calix), 81.3 (s, $C(CH_3)_3$), 74.6 (t, $OCH_2CH_2CH_2CH_3$), 31.7 (t, $OCH_2CH_2CH_2CH_3$), 30.6 (t, $ArCH_2Ar$), 27.3 (q, $C(CH_3)_3$), 18.9 (t, $OCH_2CH_2CH_2CH_3$), 13.4 (q, $OCH_2CH_2CH_2CH_3$). ESI-MS m/z: **9a**: calcd $[M + H]^+$ 2121.97, found 2122.18; **9b**: calcd [M − H] 1759.77, found 1759.74.

Calix[4]arene tetrakis(monobenzyl isophthalyl diazo) (10b) and calix[4]arene tris(monobenzyl isophthalyl diazo) (12b). To a solution of **2** (263 mg, 0.80 mmol) in 1 N HCl (1.6 mL) and THF (1.6 mL), $NaNO_2$ (58 mg, 0.84 mmol) was added. The mixture was cooled to 0 °C and stirred for 20 min. $NaBF_4$ (171 mg, 1.56 mmol) was added and the solution was stirred for 10 min. This solution was then added to a solution of calix[4]arene (76 mg, 0.18 mmol) in methanol–DMF (1.6 mL, 1 : 1). After stirring for 2 h the reaction was quenched by the addition of H_2O (5 mL) and extracted with CH_2Cl_2. The organic layer was washed with H_2O (3 mL), dried over Na_2SO_4 and concentrated *in vacuo.* The residue was dissolved in CH_2Cl_2, and methanol was added to precipitate an orange solid. This solid was crystallized from CH_2Cl_2 + MeOH three times to obtain the fully protected product **10a** (100 mg, 32%). The three filtrate fractions were combined and evaporated to obtain **12a** (65 mg, 25%). The *t*-butyl groups were removed as described previously to obtain **10b** and **12b**. **10b**: ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.48 (s, 12H, Ar), 7.90 (s, 8H, Ar calix), 7.45 (d, J = 7.2 Hz, 8H, Ph), 7.35–7.27 (m, 12H, Ph), 5.37 (s, 8H, CH_2Ph), 4.38 (br s, 4H, H_{ax} of $ArCH_2Ar$), 3.67 (br s, 4H, H_{eq} of $ArCH_2Ar$). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 165.9 (s, CO), 164.4 (s, CO), 160.0 (s, Ar calix), 152.5 (s, Ar), 144.4 (s, Ar calix), 135.6 (s, Ph), 132.5 (s, Ar), 131.1 (d, Ar calix), 130.5 (s, Ar), 130.0 (d, Ar calix), 128.4 (d, Ph), 128.1 (2d, Ph), 126.5 (d, Ar), 126.0 (d, Ar), 124.3 (d, Ar), 66.7 (t, OCH_2Ph), 31.6 (t, $ArCH_2Ar$). **12a**: ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.30 (br, 3H, NH), 8.56–8.69 (m, 9H, Ar), 7.77–7.87 (m, 6H, Ar calix), 7.30–7.46 (m, 16H, Ph and Ar calix), 7.23 (s, 1H, Ar calix), 7.21 (s, 1H, Ar calix), 5.38–5.42 (m, 6H, CH_2Ph), 4.38 (br t, 4H, H_{ax} of $ArCH_2Ar$), 3.88 (br d, 2H, H_{eq} of $ArCH_2Ar$), 3.75 (br d, 2H, H_{eq} of $ArCH_2Ar$), 1.61 (m, 27H, $C(CH_3)_3$). **12b**: ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.55 (m, 9H, Ar), 7.87 (s, 2H, Ar calix), 7.84 (d, J = 1.6 Hz, 2H, Ar calix), 7.77 (d, J = 1.6 Hz, 2H, Ar calix), 7.29–7.46 (m, 16H, Ph and Ar calix), 7.09 (s, 1H, Ar calix), 7.07 (s, 1H, Ar calix), 5.34–5.39 (m, 6H, CH_2Ph), 4.40 (br, 4H, H_{ax} of $ArCH_2Ar$), 3.80 (br, 2H, H_{eq} of $ArCH_2Ar$). ESI-MS m/z: **10b**: calcd $[M - H]^-$ 1551.42, found 1551.48.

Tetrakis(methoxyethoxy)calix[4]arene tetrakis(monobenzyl isophthalate sulfonamide-linked) (11b). To a solution of tetrakis(methoxyethoxy)calix[4]arene (synthesized in a manner similar to **1**) (436.6 mg, 0.66 mmol) in dry CH_2Cl_2 (10 mL), chlorosulfonic acid (1.85 mL, 28.0 mmol) was added at 0–10 °C. After stirring at room temperature for 3.5 h, the reaction was poured onto ice, extracted with CH_2Cl_2, washed with brine, dried over Na_2SO_4 and dried *in vacuo* to obtain tetrakis(methoxyethoxy)calix[4]arene tetrakis(sulfonylchloride) (697 mg, 99.7%). To a solution of tetrakis(methoxyethoxy)calix[4]arene tetrakis(sulfonylchloride) (63 mg, 0.06 mmol) in dry CH_2Cl_2 (5 mL) was added **2** (122 mg, 0.37 mmol) in dry CH_2Cl_2 (1 mL) and dry pyridine (32 μL 0.37 mmol). After stirring overnight, the reaction was concentrated *in vacuo* and the residue was purified by column chromatography (hexanes–acetone, 7 : 3 to 6 : 4) to obtain fully protected **11a** as a white solid (72.4 mg, 73%). After removal of the *t*-butyl groups as described previously, **11b** was obtained as a white solid. **11b**: ^{1}H NMR (400 MHz, acetone-d_6): δ = 8.35 (t, J = 1.6 Hz, 4H, Ar), 8.09 (dd, J = 2.4, 1.6 Hz, 4H, Ar), 8.04 (dd, J = 2.4, 1.6 Hz, 4H, Ar), 7.53–7.32 (m, 20H, Ph), 7.26 (s, 8H, Ar calix), 5.41 (s, 8H, CH_2Ph), 4.50 (d, J = 13.6 Hz, 4H, H_{ax} of $ArCH_2Ar$), 4.13 (t, J = 4.4 Hz, 8H, $CH_2CH_2OCH_3$), 3.62 (t, J = 4.4 Hz, 8H, $CH_2CH_2OCH_3$), 3.17 (d, J = 13.6 Hz, 4H, H_{eq} of $ArCH_2Ar$), 3.10 (s, 12H, OCH_3). ^{13}C NMR (400 MHz, acetone-d_6): δ = 166.3 (CO), 165.5 (CO), 160.9 (Ar calix), 139.8 (Ar), 137.1 (Ph), 136.3 (Ar calix), 134.5 (Ar or Ar calix), 132.8 (Ar or Ar calix), 132.5 (Ar or Ar calix), 129.4 (Ph), 129.2 (Ph), 129.1 (Ph), 128.2 (Ar calix), 126.7 (Ar), 126.0 (Ar), 125.6 (Ar), 74.4 (CH_2Ph), 72.1 ($CH_2CH_2OCH_3$), 67.7 ($CH_2CH_2OCH_3$), 58.3 (OCH_3), 31.4 ($ArCH_2Ar$). ESI-MS m/z: **11a**: calcd $[M + Na]^+$ 2235.69, found 2235.71; **11b**: calcd $[M - H]^-$ 1987.45, found 1987.67.

Tetrakis(methoxyethoxy)calix[4]arene tetrakis(monobenzyl isophthalate) (13b). To a solution of tetrakis(methoxyethoxy)alix[4]arene tetracarboxylic acid (synthesized in a manner similar to **1**) (168 mg, 0.20 mmol) in dry CH_2Cl_2 was added oxalyl chloride (1.06 mL, 12.1 mmol) and 1 drop of dry DMF. After stirring overnight, the solvent was evaporated, and the residue was dried *in vacuo* and then dissolved in dry CH_2Cl_2 (4 mL). A solution of **2** (392 mg, 1.2 mmol) in dry CH_2Cl_2 (1 mL) was added, followed by diisopropylethylamine (278 μL, 1.6 mmol) and the reaction mixture was stirred for 12 h. It was poured into water, extracted with CH_2Cl_2, washed with water, dried over $MgSO_4$ and concentrated *in vacuo.* The residue was purified by column chromatography (ethyl acetate–hexanes–CH_2Cl_2, 7 : 3 : 1) to obtain the fully protected product **13a** as a white solid (142 mg, 40%). After removal of the *t*-butyl groups as previously described, compound **13b** was obtained as a white solid. **13b**: ^{1}H NMR (400 MHz, acetone-d_6): δ = 9.41 (s, 4H, NH), 8.49 (br, 8H, Ar), 8.26 (br, 4H, Ar), 7.57–7.27 (m, 28H, Ph and Ar calix), 5.31 (s, 8H, CH_2Ph), 4.74 (d, J = 13.6 Hz, 4H, H_{ax} of $ArCH_2Ar$), 4.26 (t, J = 4 Hz, 8H, $CH_2CH_2OCH_3$), 3.87 (t, J = 4 Hz, 8H, $CH_2CH_2OCH_3$), 3.42 (d, J = 13.6 Hz, 4H, H_{eq} of $ArCH_2Ar$), 3.40 (s, 12H, OCH_3). ^{13}C NMR (400 MHz, acetone-d_6): δ = 171.5 (CO), 171.0 (CO), 170.5 (CO), 161.8 (Ar calix), 141.7 (Ar), 138.2 (Ph), 137.0 (Ar, calix), 133.2(Ar, Ph or Ar calix), 132.7 (Ar, Ph or Ar calix), 130.4 (CH of Ph), 130.3 (Ar, Ph or Ar calix), 130.1 (CH of Ph), 130.0 (CH of Ph), 127.2 (CH of Ar), 127.0 (CH of Ar), 126.6 (CH of Ar), 75.6 (CH_2Ph), 73.7 ($CH_2CH_2OCH_3$), 68.5 ($CH_2CH_2OCH_3$), 59.8 (OCH_3), 32.7 ($ArCH_2Ar$). ESI-MS m/z: **13b**: calcd $[M - H]^-$ 1843.58, found 1843.63.

Tetrakis(methoxy)calix[4]arene tetrakis(mono-*t*-butyl isophthalate) (14b). To a stirred suspension of tetrakis(methoxy)-calix[4]arene tetracarboxylic acid (synthesized in a manner similar to **1**) (20 mg, 0.03 mmol) in dry CH_2Cl_2 (2 mL) was added oxalyl chloride (160 μL, 1.83 mmol) and 1 drop of dry DMF. After 5 h the solvent was evaporated, the resulting residue was dried *in vacuo* and dissolved in dry CH_2Cl_2 (1 mL). A solution of **2** (118 mg, 0.36 mmol) in dry CH_2Cl_2 (1 mL) was added dropwise and the reaction mixture was stirred for 12 h. The solvent was evaporated and the residue was purified by column chromatography using CH_2Cl_2–methanol (50 : 1) to yield the fully protected product **14a** as a white solid (47 mg, 82%). After removal of the benzyl groups as described previously, **14b** was obtained as a white solid. **14b**: ^{1}H NMR (400 MHz, CD_3OD): δ = 8.72 (s, 1H, Ar *paco* (*partial cone conformation*)), 8.68 (s, 1H, Ar *paco*), 8.65 (s, 1H, Ar *paco*), 8.59 (s, 1H, Ar *paco*), 8.47 (s, 4H, Ar *cone*), 8.37 (s, 4H, Ar *cone*), 8.35–8.34 (3s, 4H, Ar *paco*), 8.28 (s, 2H, Ar *paco*), 8.22 (s, 4H, Ar *cone*), 8.16 (s, 2H, Ar *paco*), 8.10 (s, 2H, Ar calix *paco*), 7.88 (s, 2H, Ar calix *paco*), 7.74 (s, 2H, Ar calix *paco*), 7.59 (s, 8H, Ar calix *cone*), 4.53 (d, J = 13.2 Hz, 4H, $ArCH_2Ar$ *cone*), 4.22 (d, 2H, J = 13.5 Hz, $ArCH_2Ar$ *paco*), 3.98 (s, 12H, OCH_3 *cone*), 3.95 (br s, 4H, $ArCH_2Ar$ *paco*), 3.82 (s, 9H, OCH_3 *paco*), 3.49 (d, J = 13.2 Hz, 4H, $ArCH_2Ar$ *cone*), 3.39 (d, 2H, J = 13.5 Hz, $ArCH_2Ar$ *paco*), 3.20 (s, 3H, OCH_3 *paco*), 1.65 (s, 9H, $C(CH_3)_3$ *paco*), 1.61 (s, 9H, $C(CH_3)_3$ *paco*), 1.52 (s, 18H, $C(CH_3)_3$ *paco*), 1.51 (s, 36H, $C(CH_3)_3$ *cone*). ^{1}H NMR (400 MHz, DMSO-d_6, 90 °C): δ = 10.09 (s, 4H, NH), 8.56 (s, 4H, Ar), 8.51 (s, 4H, Ar), 8.13 (s, 4H, Ar), 7.82 (br s, 8H, Ar calix), 3.95 (br s, 8H, $ArCH_2Ar$), 3.51 (br s, 9H, OCH_3), 1.55 (s, 36H, $C(CH_3)_3$). ^{13}C NMR (100 MHz, DMSO-d_6, 90 °C): δ = 165.0 (s, CO), 163.7 (s, CO), 163.0 (s, CO), 160.0 (s, Ar), 133.2 (s, Ar), 131.7 (s, Ar), 128.4 (s, Ar), 124.5 (d, Ar), 124.3 (d, Ar), 123.9 (d, Ar), 80.6 (s, $C(CH_3)_3$), 27.2 (q, $C(CH_3)_3$). ESI-MS m/z: **14a**: calcd $[M + H]^+$ 1893.74, found 1894.09; **14b**: calcd $[M - H]^-$ 1531.54, found 1531.53.

Calix[4]arene tetrakis(mono-*t*-butyl isophthalate) (15b). To a stirred suspension of tetrakis(benzyloxy)calix[4]arene tetracarboxylic acid (synthesized in a manner similar to **1**) (25 mg, 0.026 mmol) in dry CH_2Cl_2 (2 mL) was added oxalyl chloride (90 μL, 1.56 mmol) and 1 drop of dry DMF. After stirring overnight the solvent was evaporated, the resulting residue was dried *in vacuo* and then dissolved in dry CH_2Cl_2 (2 mL). A solution of **2** (51 mg, 0.156 mmol) in dry CH_2Cl_2 (1 mL) was added, followed by pyridine (15 μL, 0.156 mmol), and the reaction mixture was stirred for 12 h. The reaction was poured into water, then extracted with CH_2Cl_2, and washed with brine and dried over Na_2SO_4. The solvent was evaporated and the residue was purified by column chromatography (hexanes–acetone, 4 : 1) to yield **15a** as a white solid. (25 mg, 45%). After removal of the benzyl ester groups as described previously, **15b** was obtained as a white solid. **15b**: ^{1}H NMR (400 MHz, acetone-d_6 and drops of CD_3OD): δ = 8.61 (s, 4H, Ar), 8.53 (s, 4H, Ar), 8.23 (s, 4H, Ar), 7.84 (s, 8H, Ar calix), 4.50 (br, 4H, H_{ax} of $ArCH_2Ar$), 3.60 (br, 4H, H_{eq} of $ArCH_2Ar$), 1.52 (s, 36H, $C(CH_3)_3$). ^{13}C NMR (400 MHz, $CDCl_3$): δ = 165.9 (s, acetone-d_6 and drops of CD_3OD): δ = 168.9 (CO), 166.1 (CO), 158.7 (Ar), 140.7 (Ar), 133.8 (Ar), 132.8 (Ar), 131.3 (Ar), 129.7 (Ar), 126.9 (Ar), 126.6 (Ar), 126.3 (Ar), 82.7 ($C(CH_3)_3$), 33.3 ($ArCH_2Ar$), 28.3 ($C(CH_3)_3$). HR ESI-MS m/z: **15b**: calcd $[M + Na]^+$ 1499.4747, found 1499.4722.

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