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DOI: 10.1002/cplu.201100081

Synthesis and Toxicology of *p*-Phosphonic Acid Calixarenes and O-Alkylated Analogues as Potential Calixarene-Based Phospholipids

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A series of *p*-phosphonic acid calix[*n*]arenes **1a–g** bearing hydroxy groups, $n=4$ and 5, or alkyl chains of varying lengths at their lower rim, $n=4$, were synthesised, and in vitro assessments of their effect on cell viability in rat PC12 cells and primary cultures of mixed retinal cells were conducted. Compounds **1a** and **1g**, bearing hydroxy groups at their lower rim, displayed lower toxicity towards PC12 cells than their alkylated counterparts; concentrations of up to 1 mg mL^{-1} did not affect the number of viable cells. Mixed retinal cultures showed a higher susceptibility towards toxic effects for all calixarenes tested. Compound **1b** self-assembles into nanofibres from a toluene solution, and **1c** assembles into micelles from an aqueous solution, with these nanoscale architectures showing potential applications for various biological roles, such as solubilising *p*-nitrophenol and curcumin.

Introduction

Calix[n]arenes are versatile macrocyclic compounds comprised of phenolic units linked by methylene bridges,[1] where '*n*' can vary from 4 to 20.[2] These compounds have been used in a wide variety of applications including sensor technology,[3] stabilisation of nanoparticles,[4] phase-transfer catalysts,[5] and guest encapsulation.[6,7] The widespread use of calixarenes relates in part to the ease at which their upper and lower rims can be modified with a wide range of different functionalities.[8,9]

Calix[n]arenes have also recently featured in a variety of examples in the field of biotechnology owing to their apparent low toxicity. For instance, parent *tert*-butylcalix[4]arenes attached to human antibodies do not provoke an immune response in test subjects.[10] Solid-lipid nanoparticles (SLNs) fabricated using amphiphilic calix[4]arenes bearing long alkyl chains incorporating either hydroxyl or phosphonate groups have no haemolytic properties.[11] This is thought to be due to the small cavity size of the calix[4]arenes, given that *p*-sulfonato calix[8]arenes with their significantly larger cavity display a much higher haemolytic effect than the calix[4 and 6]arene analogues.[12] Cellular uptake of fluorescent, water soluble calix[4]arene-based probes has also been demonstrated using a nonspecific uptake mechanism not linked to either of the main endocytic pathways, resulting in accumulation of the probe within the cell cytoplasm.[13] Calixarenes have also proved to be effective in the encapsulation of various biologically relevant molecules, including the encapsulation and subsequent extraction of cytochrome c by carboxylic functionalised *p*-*tert*-octylcalix[6] and [8]arenes,[14] encapsulation of the model photosensitiser chlorine e6 by micelles of PEGylated calix[4]arene,[15] and the formation of stable complexes between dipeptides and bis(hydrxoymethyl)phosphonic acid calix[4]arenes.[16] The ability of calixarenes to effectively encapsulate a diverse range of molecules, coupled with their documented low toxicity, makes them attractive candidates for a wide range of drug delivery systems. The biochemistry of various calix[*n*]arenes, including their complexation towards biologically active molecules, toxicity, and use in biosensors has already been covered by an in-depth Review on sulfonated calixarenes,[17] and a more general Review concerning anionic calixarenes.[18,19]

Recently a high yielding synthesis of *p*-phosphonic acid calix[*n*]arenes was established, as a six-step procedure.[20,21] This new class of calixarene has tuneable properties; examples include the binding of calcium ions in the solid state[22] and the

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cplu.201100081.

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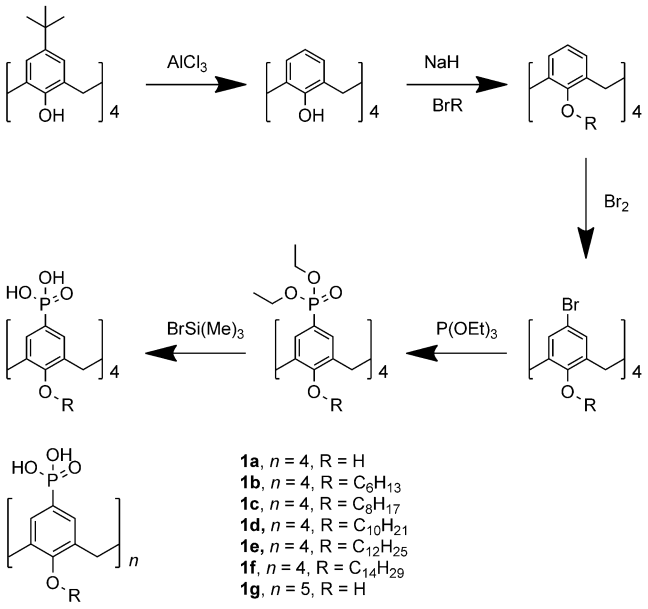
encapsulation of the potent anticancer agent carboplatin.[23] *p*-Phosphonic acid calixarenes bearing long C_{18} alkyl chains at their lower rim have also been synthesised and shown to self-assemble into uniform nanofibres.[24] The phosphonate ester of calix[4]arene O-alkylated with C_{18} chains is of particular interest as it shows a bilayer arrangement close to the size of cellular phospholipid bilayers.[25] As such, these phosphonated calixarenes may be useful for a range of biological applications, including the delivery of therapeutic agents to an injury site. In an effort to understand the biological role of the molecules and to gain insight into any potential toxicity, we prepared a series of *p*-phosphonic acid calix[*n*]arenes (where $n=4$, 5), bearing hydroxyl groups or alkyl chains at their lower rim, and have investigated their self-assembly prowess. The effects that these compounds have on cell viability in vitro on both an immortalised cell line and freshly isolated primary cultures have also been assessed.

Results and Discussion

Synthesis of *p*-phosphonic acid calixarenes

p-Phosphonic acid calix[*n*]arenes **1a** and **1g**, Scheme 1, were prepared according to a six-step published procedure from readily available starting materials.[20] The new lower rim O-alkyl analogues **1b–f**, Scheme 1, were prepared by a variation



Scheme 1. *p*-Phosphonic acid calix[*n*]arenes used in the toxicology studies, along with their associated synthetic procedure.

of the protocol for other O-alkyl compounds, where $R=CH_3$, *n*-C_4H_9, *n*-$C_{18}H_{37}$.[22] This variation shortens the synthetic protocol to five steps, replacing the acetylation step with an alkylation, thereby eliminating the deacetylation step. The presence of alkyl chains also slightly lowers the overall yields relative to the pristine calixarenes, R = H, with all compounds being obtained in overall yields of 50–60%.[20,25] Compounds **1a–g** are soluble in water at high pH (>10) and in DMSO. Only compounds **1a–c** and **1g** are readily soluble at neutral pH. Compounds **1a–f** were prepared with the molecules locked in the symmetrical C_{4v} cone conformation, with the conditions used for the alkylation step predetermining the formation of the cone conformer.

Effects of *p*-phosphonic acid calix[*n*]arenes on cell viabilities

To determine whether or not the calix[*n*]arenes are in any way toxic, the viability (using a LIVE/DEAD cell kit) of PC12 and mixed retinal cultures was assessed at a range of calix[*n*]arene concentrations: 0.001, 0.01, 0.1, 0.3, and 3 mg mL^{-1}. Both the concentrations at which viability was significantly reduced and the approximate concentrations at which viability was reduced by 50% (IC_{50} values) are displayed in Table 1, with representative data in Figure 1. We have established that both the length of the alkyl chain and the number of phenolic units in the macrocycle play an important role in the toxicity profiles of these compounds towards rat pheochromocytoma neural progenitor (PC12) cells. Relatively high concentrations (1 mg mL^{-1}) of both **1a** and **1g** were tolerated by PC12 cells, whereas **1b** and **1f** displayed an increased toxicity (toxic at 0.1 mg mL^{-1}). Compounds **1c**, **1d**, and **1e**, with intermediate alkyl chain lengths, were less toxic towards PC12 cells than for the shorter and longer chain lengths. Concentrations of up to 1.0 mg mL^{-1} were needed to cause a significant reduction in the number of viable cells, as summarised in Figure 1 and Table 1. Another trend observed was the decrease in the number of live cells as the calixarene concentration increased, 3 mg mL^{-1} being toxic for all calixarenes tested.

Table 1. Concentrations (mg mL^{-1}) where a significant decrease in cell viability is observed and (in parentheses) where viability is inhibited by approximately 50%, for calixarenes **1a–g** in both PC12 and mixed retinal cells.

Calixarene	PC12 cells		Mixed retinal cells	
	24 h	72 h	24 h	72 h
1a	>3.0 (>3.0)	3.0 (1.0–3.0)	1.0 (1.0)	0.01 (0.01)
1b	0.3 (1.0–3.0)	0.1 (0.3)	0.1 (0.3)	0.3 (0.3)
1c	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)	0.1 (0.3)
1d	0.3 (1.0)	1.0 (1.0)	0.3 (0.3)	1.0 (0.3–1.0)
1e	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)	0.01 (0.1)
1f	1.0 (1.0)	0.1 (0.1)	3.0 (1.0)	0.01 (0.01)
1g	3.0 (>3.0)	3.0 (1.0–3.0)	0.1 (0.1)	1.0 (0.3–1.0)

Mixed retinal cells were generally more susceptible than PC12 cells to the toxic effects associated with **1a–g**. Compounds **1a** and **1g**, which showed the least toxicity towards PC12 cells, nevertheless resulted in a significant decrease in the number of viable cells in the mixed retinal cultures at concentrations as low as 0.01 and 0.1 mg mL^{-1} for **1a** and **1g**, respectively. The addition of alkyl chains to the calixarenes had little effect on their toxicity towards the mixed retinal cells,

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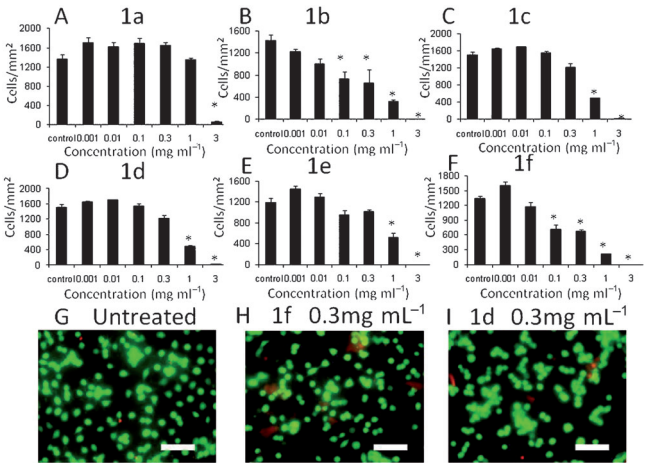

Figure 1. Effects of compounds **1a**--**f** on PC12 cell viabilities (mean ± SEM) after 72 h in culture and representative images of untreated and treated PC12 cells. Statistically significant reductions in viability of cultures treated with calixarene preparations compared to untreated cells are indicated by * ($p \leq 0.05$), scale bar = 100 μm.

with toxic effects generally observed following treatment between 0.1 and 0.01 mg mL^{-1} after 72 hours (Table 1). An exception to this was **1d**, where no decrease in the number of viable cells was recorded at a concentration of 0.1 mg mL^{-1} after 24 hours and at a concentration of 0.3 mg mL^{-1} after 72 hours (Table 1). Although some preparations showed a decrease in the concentration required for reduced viability as time passed, there was no consistent effect to indicate a time-dependent toxicity. However, longer term studies are important to address the possibility of chronic toxicity.

It is important to note that mixed retinal cultures are comprised of several cell types including retinal ganglion cells (neurons) and astrocytes. Although retinal ganglion cells will not replicate in culture, astrocytes may undergo several population doublings over a 72 hours culture period. As such, the decreased number of viable retinal cells may be a composite of increased proliferation of astrocytes and loss of retinal ganglion cells and perhaps other neurons. Accordingly, the variation between the different calix[*n*]arenes in terms of greater or less toxicity after 24 or 72 hours in culture may be due to differential effects on the balance of astrocyte proliferation and neuronal loss. Nevertheless, the increased toxicity of some of the calix[*n*]arene preparations towards mixed retinal cultures indicates that the preparations cannot all necessarily be deemed safe for in vivo use.

Self-assembly of *p*-phosphonic acid calix[*n*]arenes

Two different classes of *p*-phosphonated calixarenes were prepared, the amphiphilic calix[4]arenes with *n*-alkane chains, **1b–f**, and calix[4,5]arenes devoid of such chains, **1a** and **1g**, Scheme 1. Accordingly, different self-assembly outcomes are inevitable. In this context, we have previously shown that the pristine calix[4,5]arenes themselves, **1a** and **1g**, assemble into distinctly different structures. Compound **1a** forms nanorafts in solution with a bilayer arrangement of calixarenes, with similar bilayer arrangements present in the solid state, whereas compound **1g** forms molecular capsules in solution and nanofibres in the solid state, with a controlled encapsulation and release of carboplatin drug molecules.[20,23] The amphiphilic calixarenes, **1b–f**, were designed as phospholipid mimics. They have potential for forming micelles and/or vesicles with the phosphonate groups pointing either towards the solvent or towards themselves, depending on the polarity of the solvent and concentration effects.

Atomic force microscopy (AFM) established that compound **1b** assembles into nanofibres from toluene, their height varying between 5–8 nm and 45–55 nm, Figure 2. The smaller nanofibres, Figure 2b, are bridged by circular structures, with a residual solvent layer visible around the smaller fibres. Nanofibre formation is not observed in areas devoid of

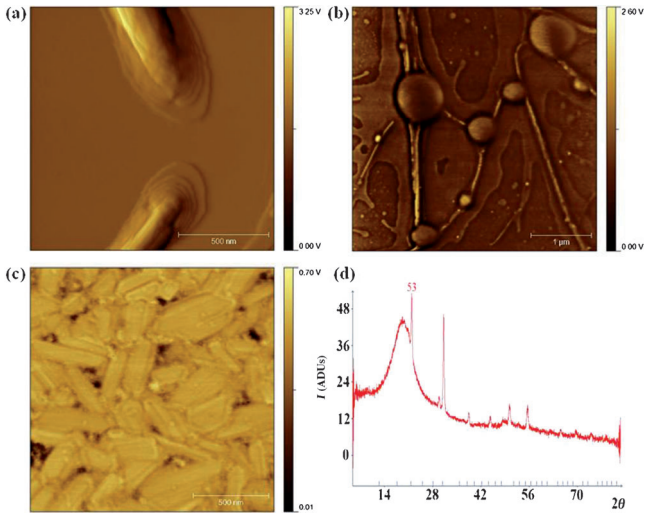

Figure 2. AFM micrographs of nanofibres of **1b** formed from a toluene solution, (a) and (b), and nanocrystals formed from an aqueous solution (c), along with their powder
X-ray diffraction pattern for the compound in (c) and (d). AFM scale bars are 500 nm, 1 μm, and 500 nm for (a), (b) and (c), respectively.

this layer. The appearance of fibre ends on the larger fibres, Figure 2a, suggests that the larger nanofibres have a stepwise growth mechanism similar to crystal growth, with each "step" corresponding to a height of 2–3 nm. This step-wise growth was also seen in the formation of nanocrystals of **1b**, Figure 2c, from an aqueous solution of the compound deposited onto a mica substrate. Repeated attempts to form large crystals of **1b** were unsuccessful, however, the powder X-ray diffraction pattern of **1b**, Figure 2d, shows some crystalline material offset by the amorphous material presumably associated with inefficient packing of alkyl chains, as disordered species. This is one line of evidence indicating that the alkane chains are in the fluid state at room temperature and is supported by the ease at which **1c** forms micelles at room temperature. Micelles of **1c** readily form by dissolving the compound in an aqueous solution at pH > 9. Decreasing the pH to 7 or lower produces micelles that have a stability of greater than weeks at concentrations as high as 30 mg mL^{-1} and as low as

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0.3 mg mL^{-1}. Micelles and vesicles generally require the alkane chains of the amphiphiles to be fluid or "above main lamellar chain-melting phase transition temperature" in order for the amphiphiles to form a close packed nonpolar system to the exclusion of water,[26]

Micelle formation

As mentioned in the previous section, it appears that **1c** spontaneously forms micelles at a pH > 9. Producing micelles from amphiphilic calix[4]arenes by controlling the pH value is a known technique and there are a number of examples where this class of compound has been shown to form micelles.[27] Further, the compounds in this paper were designed as phospholipid mimics and thus the formation of the micelles was expected. Herein, we give evidence that **1c** forms micelles without encapsulating the high-pH and hence toxic solvent.

The size of the micelles was independent of both the adjusted final pH value and concentration and had mean diameter between 4.2 and 5.6 nm, as measured by dynamic light scattering (DLS). This correlates with slightly larger than twice the length between the phosphonate group and the terminal methyl group of a straightened alkane chain of **1c** (1.9 nm as estimated by using Chem3D). Thus, the DLS data give a size which only allows the superstructures formed by **1c** to be micelles.

The colorimetric test gives evidence that there is no solvent stored in a pocket within the superstructures of **1c**. As shown by Figure 3a, *p*-nitrophenol forms a bright yellow solution at basic pH but is colourless at acidic pH. Figure 3b shows the solutions of *p*-nitrophenol alone, *p*-nitrophenol plus **1c** in micellular form (dissolved in a basic solution of water and *p*-nitrophenol), and **1c** in micellular form alone at a pH < 3.

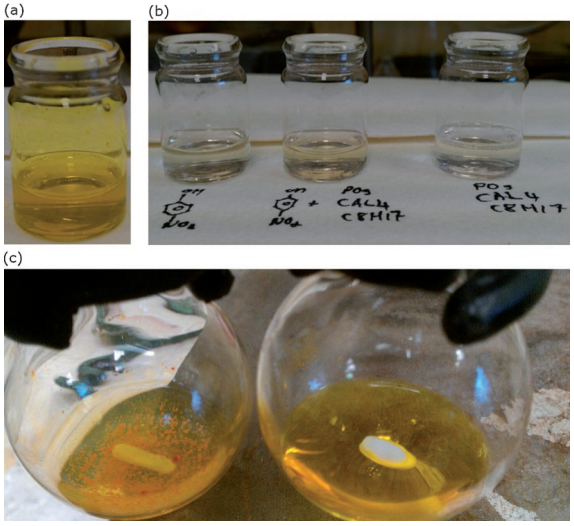

Figure 3. *p*-Nitrophenol (12 mg mL^{-1}) at pH 14 (a) and at a pH 3 after micelle-formation (b), from left to right solutions of *p*-nitrophenol (12 mg mL^{-1}) alone, *p*-nitrophenol (12 mg mL^{-1}) plus **1c** (7 mg mL^{-1}) and **1c** (7 mg mL^{-1}) alone and at pH 6.5 after micelle formation (c), left curcumin (1 mg mL^{-1}) alone and right curcumin (1 mg mL^{-1}) plus **1c** (7 mg mL^{-1}).

The micelles of **1c** formed at basic pH in the presence of *p*-nitrophenol result in a close to colourless solution. This observation indicates that **1c** does not encapsulate the solution it was formed in, as this would be indicated by the acidified solution maintaining the bright yellow colour of the original basic solution, owing to the *p*-nitrophenolate anion. Additional evidence for the formation of micelles without encapsulating any solvent comes from the toxicology data shown below.

Treatment of PC12 cells with 1 mg mL^{-1} of **1c** in micelle form resulted in only an approximately 25% decrease in cell viability compared to an approximately 75% decrease following treatment with 1 mg mL^{-1} of **1c** in monomeric form (Figure 1 and Figure 4). This finding indicates that the toxicity of the preformed micelles is lower than for **1c** in monomeric form, as seen in Figure 1. If **1c** encapsulated some of the basic pH solvent it was formed in, then an increase in toxicity would be expected, not a decrease. This apparent decrease in toxicity may also be a result of the preformed micelles of **1c** having a lower probability of integrating themselves into and subsequently disrupting the bilayer arrangement of the cells than monomeric **1c**.

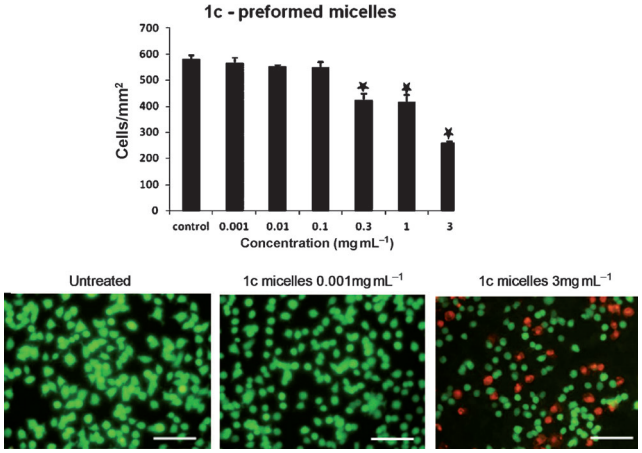

Figure 4. Effects of preformed micelles of **1c** on PC12 cell viabilities (mean ± SEM) after 72 h in culture and representative images of untreated and treated PC12 cells (objective 40×). Statistically significant reductions in viability of cultures treated with calixarene preparations compared to untreated cells are indicated by * ($p \leq 0.05$). Scale bar = 100 μm.

The novelty of the micelles of **1c** is their stability at low pH. Compound **1c** is only sparingly soluble in water at a pH < 7. However, once the micelles are formed they maintain their stability in water to pH < 1. The advantage of this is the similarity in the solubility profile to known antioxidants such as curcumin. This compound is known to be soluble under basic conditions but precipitates at low pH. Therefore, if a basic solution of **1c** and curcumin is prepared and the pH subsequently decreased to pH 7, the antioxidant is forced to intercalate into the micelle, as illustrated in Figure 3c. This solution of curcumin and **1c** displays remarkable stability. Both the curcumin and **1c** remained soluble for several months whereas solutions of curcumin alone were only sparingly soluble at pH < 9. The stability of this system has many possible advantages, such as

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remaining viable at a pH value lower than that of the stomach and higher pH value than the intestines, with implications in oral delivery methods. However, the activity of the antioxidant carried within these micelles and the location where **1c** accumulates has yet to be tested but is the subject of ongoing research, along with other polyphenol antioxidants such as resveratrol.[28]

Conclusion

We have synthesised a series of new amphiphilic calix[4]arenes bearing *p*-phosphonic acid groups at their upper rim and alkyl chains at their lower rim. The toxicology of these phospholipid mimics has been investigated, with mixed retinal cells proving to be more susceptible to the toxic effects of **1a**--**g** than PC12 cells. In the presence of hydroxy groups at the calixarene lower rim, toxicity is decreased and concentrations of at least 1.0 mg mL^{-1} can be tolerated in PC12 cells for **1a** or **1g**. Furthermore, the self-assembly of these phospholipid mimics was studied, with **1c** forming micelles of 4–5 nm diameter which are stable over a large range of pH values and are also able to intercalate curcumin, as a model antioxidant. These micelles also show an apparent decrease in toxicity towards PC12 cells compared to their monomeric analogues, thus making them possible candidates for targeted drug delivery.

Experimental Section

Synthesis of *p*-phosphonic acid calixarenes

Phosphonic acid calixarenes **1b**–**f** were synthesised according to a modified literature procedure.[17,18] Starting materials were obtained from Sigma–Aldrich and were used without purification. Calixarenes **1a** and **1g** have been previously reported.[22]

5, 11, 17, 23-tetra(dihydroxyphosphoryl) 25, 26, 27, 28-tetrahexoxycalix[4]arene (1b): m.p. > 300 °C (decomp); IR (KBr) 3437 (s), 2922 (s), 1594 (m), 1460 (m), 1271 (m), 1125 (m), 998 (m) cm^{-1}; ^{1}H NMR ([D$_6$]DMSO, 25 °C, 400 MHz) δ = 6.97 (s, 8H, ArH), 6.04 (br. s, 8H, OH), 4.35 (d, J = 13.5 Hz, 4H, ArCH$_2$Ar), 3.85 (t, J = 7.2 Hz, 8H, OCH$_2$), 3.27 (d, J = 13.5 Hz, 4H, ArCH$_2$Ar), 1.83 (m, 8H, CH2), 1.24–1.35 (br. s, 24H, CH$_2$), 0.87 ppm (t, J = 7.5 Hz, 12H, CH$_3$); ^{13}C NMR ([D$_6$]DMSO, 25 °C, 400 MHz) δ = 158.56, 134.21, 130.77, 74.89, 31.57, 31.49, 30.18, 29.80, 25.94, 25.50, 22.35, 13.86 ppm; ^{31}P NMR ([D$_6$]DMSO, 25 °C, 300 MHz) δ = 16.17 ppm; HRMS (FAB): *m/z* calcd for $C_{52}H_{76}O_{16}P_4$ [M + H$^+$]: 1081.4106; found: 1081.4184.

5, 11, 17, 23-tetra(dihydroxyphosphoryl) 25, 26, 27, 28-tetraoctoxycalix[4]arene (1c): m.p. > 300 °C (decomp); IR (KBr) 3413 (s), 2922 (s), 1618 (m), 1460 (m), 1384 (w), 1271 (m), 1125 (m), 1007 (m) cm^{-1}; ^{1}H NMR ([D$_6$]DMSO, 25 °C, 400 MHz) δ = 6.96 (s, 8H, ArH), 6.32 (br. s, 8H, OH), 4.31 (d, J = 13.3 Hz, 4H, ArCH$_2$Ar), 3.84 (t, J = 7.6 Hz, 8H, OCH$_2$), 3.26 (d, J = 13.3 Hz, 4H, ArCH$_2$Ar), 1.82 (m, 8H, CH$_2$), 1.22–1.38 (br. s, 40H, CH$_2$), 0.83 ppm (t, J = 7.5 Hz, 12H, CH$_3$); ^{13}C NMR ([D$_6$]DMSO, 25 °C, 400 MHz) δ = 158.59, 134.19, 130.74, 74.87, 31.52, 30.22, 29.96, 29.56, 29.22, 29.02, 26.02, 22.21, 13.78 ppm; ^{31}P NMR ([D$_6$]DMSO, 25 °C, 300 MHz) δ = 16.21 ppm; HRMS (FAB): *m/z* calcd for $C_{60}H_{92}O_{16}P_4$ [M + H$^+$]: 1193.5414; found: 1193.5445.

5, 11, 17, 23-tetra(dihydroxyphosphoryl) 25, 26, 27, 28-tetradecoxycalix[4]arene (1d): m.p. > 300 °C (decomp); IR (KBr) 3437 (s), 2922 (s), 1594 (m), 1464 (m), 1404 (w), 1273 (m), 1128 (m), 983 (m) cm^{-1}; ^{1}H NMR ([D$_6$]DMSO, 25 °C, 400 MHz) δ = 6.96 (s, 8H, ArH), 6.27 (br. s, 8H, OH), 4.29 (d, J = 13.5 Hz, 4H, ArCH$_2$Ar), 3.77 (t, J = 7.5 Hz, 8H, OCH$_2$), 3.22 (d, J = 13.5 Hz, 4H, ArCH$_2$Ar), 1.77 (m, 8H, CH$_2$), 1.26–1.40 (br. s, 56H, CH$_2$), 0.78 ppm (t, J = 7.6 Hz, 12H, CH$_3$); ^{13}C NMR ([D$_6$]DMSO, 25 °C, 400 MHz) δ = 158.56, 134.19, 130.75, 74.89, 31.54, 30.56, 29.79, 29.52, 29.11, 29.08, 26.17, 22.19, 13.55 ppm; ^{31}P NMR ([D$_6$]DMSO, 25 °C, 300 MHz) δ = 16.85 ppm; HRMS (FAB): *m/z* calcd for $C_{68}H_{108}O_{16}P_4$ [M + H$^+$]: 1275.4521; found: 1275.4537.

5, 11, 17, 23-tetra(dihydroxyphosphoryl) 25, 26, 27, 28-tetradodecoxycalix[4]arene (1e): m.p. > 300 °C (decomp); IR (KBr) 3411 (s), 2922 (s), 1605 (m), 1466 (m), 1405 (w), 1273 (m), 1127 (m), 995 (m) cm^{-1}; ^{1}H NMR ([D$_6$]DMSO, 25 °C, 400 MHz) δ = 6.95 (s, 8H, ArH), 6.19 (br. s, 8H, OH), 4.30 (d, J = 13.4 Hz, 4H, ArCH$_2$Ar), 3.87 (t, J = 7.2 Hz, 8H, OCH$_2$), 3.24 (d, J = 13.4 Hz, 4H, ArCH$_2$Ar), 1.80 (m, 8H, CH$_2$), 1.20–1.31 (br. s, 72H, CH$_2$), 0.79 ppm (t, J = 7.5 Hz, 12H, CH3); ^{13}C NMR ([D$_6$]DMSO, 25 °C, 400 MHz) δ = 158.54, 134.21, 130.76, 74.89, 31.55, 30.47, 29.80, 29.48, 29.12, 29.07, 26.15, 22.21, 13.65 ppm; ^{31}P NMR ([D$_6$]DMSO, 25 °C, 300 MHz) δ = 17.02 ppm; HRMS (FAB): *m/z* calcd for $C_{76}H_{124}O_{16}P_4$ [M + H$^+$]: 1417.7918, found: 1417.7990.

5, 11, 17, 23-tetra(dihydroxyphosphoryl) 25, 26, 27, 28-tetratetradecoxy-calix[4]arene (1f): m.p. > 300 °C (decomp); IR (KBr) 3412 (s), 2922 (s), 1617 (m), 1466 (m), 1405 (w), 1273 (m), 1127 (m), 999 (m) cm^{-1}; ^{1}H NMR (CDCl$_3$, 25 °C, 400 MHz) δ = 7.57 (s, 8H, ArH), 6.30 (br. s, 8H, OH), 4.28 (d, J = 13.6 Hz, 4H, ArCH$_2$Ar), 3.76 (t, J = 7.4 Hz, 8H, OCH$_2$), 3.09 (d, J = 13.6 Hz, 4H, ArCH$_2$Ar), 1.69 (m, 8H, CH$_2$), 1.12–1.38 (br. s, 88H, CH$_2$), 0.74 ppm (t, J = 7.3 Hz, 12H, CH$_3$); ^{13}C NMR (CDCl$_3$, 25 °C, 400 MHz) δ = 158.56, 134.23, 130.77, 74.91, 31.75, 30.49, 29.72, 29.66, 29.62, 29.54, 29.22, 22.49, 13.94 ppm; ^{31}P NMR (CDCl$_3$, 25 °C, 300 MHz) δ = 18.93 ppm; HRMS (FAB): *m/z* calcd for $C_{92}H_{156}O_{16}P_4$ [M + H$^+$]: 1642.0422; found: 1642.0380.

Cell culture

Rat pheochromocytoma cells (PC12) were obtained from the Mississippi Medical Centre (Jackson, Mississippi, USA) and grown in RPMI medium supplemented with 10% horse serum (HS), 5% foetal bovine serum (FBS), 2 mM L-glutamine, 2 mM penicillin-streptomycin, 1 mM MEM sodium pyruvate, and 0.1 mM MEM non-essential amino acids (GIBCO, Invitrogen, Carlsbad, California, USA). Cells were grown on flasks coated with poly-L-lysine (PLL; 10 μg mL^{-1}; Sigma, St Louis, Missouri, USA) and housed in an incubator at 37 °C, 5% CO_2. PC12 cells were seeded at 2×10^5 cells mL^{-1} for experiments assessed at 24 h and 1×10^5 cells mL^{-1} for experiments assessed at 72 h, in 96-well plates coated with PLL as above.

Mixed retinal cultures were prepared as follows. Rats were maintained and treated in accordance with National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and as approved by the University of Western Australia Animal Ethics Committee. Piebald Virol Glaxo (PVG) hooded rat pups age P0-P3 (Animal Resources Centre, Murdoch, Western Australia) were terminally euthanized (by overdose with Euthal). Retinae were isolated and incubated with papain (165 units mL^{-1}; Worthington Biochemical Corporation, Lakewood, New Jersey, USA), L-cysteine (5 mg; Sigma, St Louis, Missouri, USA), and DNase (10,000U mL^{-1}; Sigma, St Louis, Missouri, USA) in HBSS (10 mL) for 30 min at 37 °C, gently triturated in Neurobasal-A cell media, supplemented with 10% FBS and 2 mM L-glutamine (GIBCO, Invitrogen, Carlsbad, California, USA) using a sterile Pasteur pipette and filtered through sterile nylon gauze. Mixed reti-

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nal cultures were seeded at 8×10^5 cells mL^{-1} for experiments assessed at 24 h and 5×10^5 cells mL^{-1} for experiments assessed at 72 h, in 96-well plates precoated with PLL (10 μg mL^{-1}) and laminin (10 μg mL^{-1}; Invitrogen, Carlsbad, California, USA).

Treatment of cells with *p*-phosphonic acid calix[*n*]arenes

Compounds **1a–g** were assessed by dissolving each calixarene preparation (3 mg mL^{-1}) in DMSO (final maximum concentration of DMSO in solution 1%) by heating in a thermomixer (Eppendorf, Crown Scientific Pty Ltd. Minto, NSW, Australia) at 70 °C, 750 rpm and serially diluted to give final concentrations of 1, 0.3, 0.1, 0.01, and 0.001 mg mL^{-1}. The effects of each concentration of calixarene on cell viabilities were assessed in triplicate. Control cultures were incubated in complete media + 1% DMSO.

Assessment of cell viabilities

The effects of *p*-phosphonic acid calixarenes on PC12 and mixed retinal cell viability were assessed following 24 or 72 h treatment, using the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Molecular Probes Inc. Eugene, Oregon, USA). 1 μM calcein-AM and 2 μM Ethidium homodimer-1 (Ethd-1) diluted in PBS was added to cells and incubated at 37 °C with 5% CO_2 for 30 min. Cells were photographed at four predetermined locations in each well with a 40× objective for PC12 cells and 20× objective for mixed retinal cultures, by using a fluorescent inverted microscope (Olympus 1× 51, Japan) at excitation wavelength of 494 nm (calcein-AM). The number of live cells were counted in each field of view for the PC12 cells and in two of eight predetermined squares of a grid for mixed retinal cells, with the aid of Image J software.[29] The numbers of dead cells were not assessed as the calixarenes were autofluorescent in the red channel, which interfered with dead cell assessment. Viabilities were represented as the total number of live cells counted in each well. Results were represented as the mean total number of viable cells/mm^2 ± SEM of triplicate samples. Results were statistically analysed using ANOVA and Bonferroni/Dunn post hoc tests, with a significance value of $p \leq 0.05$.

General micelle formation procedure

Compound **1c** was added to a solution at pH 10 made up from MilliQ water and 1 M NaOH or from MilliQ water, curcumin (obtained from Sigma–Aldrich and used without purification) and 1 M NaOH or a solution at pH 14 made up from MilliQ water, *p*-nitrophenol and 1 M NaOH. The mixture was readjusted to pH 10 with 1 M NaOH where applicable, stirred until **1c** was completely dissolved and then the pH was adjusted to the necessary pH value with 1 M HCl.

Acknowledgements

Support of this study by the Australian Research Council is acknowledged, along with the facilities, scientific, and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, which is funded by The University of Western Australia, State and Commonwealth Governments. M.F. is supported by the Neurotrauma Research Program of Western Australia and S.A.D. by the NHMRC (APP1002347).

Keywords: curcumins · micelles · phospholipids · phosphonated calixarenes · toxicology

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Received: October 24, 2011
Revised: February 6, 2012
Published online on March 1, 2012

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