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Identification and Recovery of an Asymmetric Calix[4]arene Tetranitrile Derivative using Liquid Chromatography and Mass Spectrometry

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A simple analytical liquid chromatography mass spectrometry (LC-MS) method and associated instrumentation has been adapted for use by the organic chemist to yield milligram quantities of target compound from a reaction mixture. Calix[4]arene 3 was identified as representing 51% of total peak area of a reaction mixture containing no less than 10 components, using LC-MS. This peak corresponded to a mass of 878.8, equivalent to a complex of 3 and an ammonium cation. Molecular models further rationalize this observation by showing that the asymmetric binding cavity of 3 is suitable for binding tetrahedral guests such as the ammonium ion. By scaling up the LC method, using analytical instrumentation, 55 mg of 98% pure 3 was isolated with a recovery yield of 90% in 1 h. The current method represents a powerful and easily adapted tool for monitoring a challenging synthesis that combines identification, efficient separation and partial characterization for reaction mixture components using readily available instrumentation and methods.

Keywords: Calixarene; HPLC; MS; Semi-preparative; Isolation

INTRODUCTION

Structures **1**–**6** (Fig. 1) belong to a large family of compounds called calixarenes. There are numerous compounds based on the calixarene molecular platform that all have certain features in common, namely a central aromatic cavity or annulus, an upper rim and a lower rim, substituted as required. Substitution often locks the calixarene into a rigid cup-like cone conformation, ideal for selective host recognition. In short, calixarenes make excellent platforms for the design of chemical sensor receptors for ions and neutral molecules. Several excellent publications are available describing the history, synthesis and characteristics of calixarenes [1–4].

To date, the most commercially successful sensor calixarenes are symmetrically substituted calix[4] arenes such as the tetraester **5**, which acts as a selective sodium host in chemical sensor applications [4].

Calix[4]arene **3** was synthesized by the method shown in Scheme 1. Structure **3** was envisaged as a precursor of a host for nonspherical-shaped cations and anions. This is because of the differing lengths in the alternate pendant groups represented in the spatial arrangement of functionality on the lower rim. One possible low-energy conformation is that shown in Fig. 2. Evidence for this cone conformation is provided by the two doublets observed at 4.33 and 3.28 ppm for the methylene protons in the calixarene's annulus, as seen in the proton NMR of **3** [1]. It must be acknowledged that the current model's lower rim pendant groups must have considerable flexibility due to the methyl and propyl spacers.

The mixture from the synthesis of **3** was analysed by LC-MS. The peak labelled **(3)** in Fig. 3 corresponds to a molecular ion $+m/e$ 878.8 ($[M + NH_4^+]$, calcd 878.6) with an area of 51% relative to the total peak area. Ammonium ions, presumably originating from the synthetic work-up, seemed to stabilize the molecule **3**. This observation suggests that **3** may be predisposed towards binding tetrahedrally shaped cations because of the spatial arrangement of its binding sites. Preliminary potentiometric screening of **3** revealed significant responses towards a number of cations, confirming that **3** is energetically capable of spontaneous complex formation.

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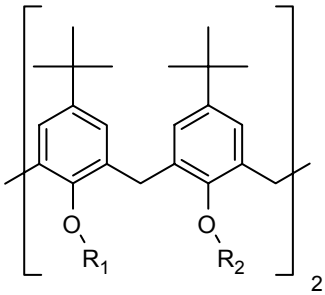


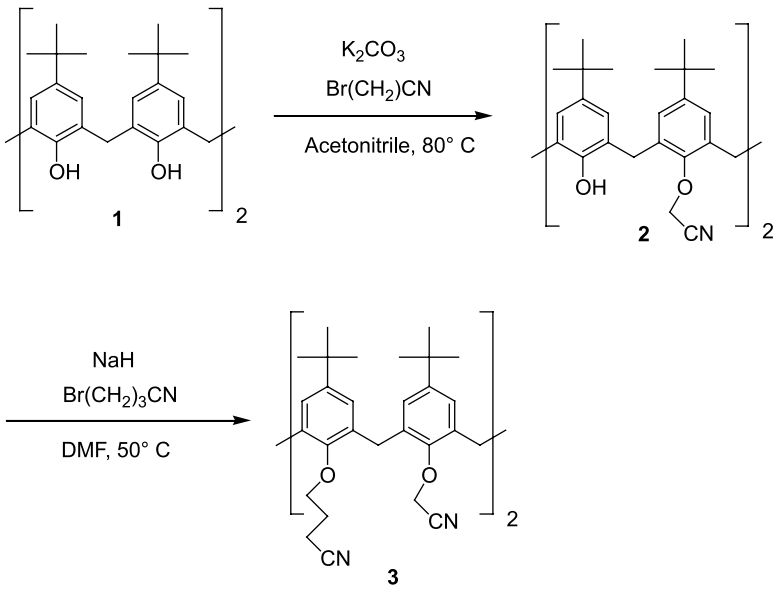
Calixarene	R_1	R_2
1	H	H
2	H	CH_2CN
3	$(CH_2)_3CN$	CH_2CN
4	$(CH_2)_3CN$	$(CH_2)_3CN$
5	$CH_2C(O)_2Et$	$CH_2C(O)_2Et$
6	$CH_2C(O)_2Et$	$C_2H_4C(O)_2Et$

FIGURE 1 Structural formulae of calix[4]arenes.

Potentiometric membranes were prepared by a well-established method [5]. Table I depicts the potential change in a PVC membrane containing **3** when in contact with a 10^{-1}M aqueous solution of the indicated cation compared to equivalent measurements in deionized water. This demonstrates that complex formation is indeed occurring with a particular order of selectivity, which favours ammonium and potassium over sodium. Further potentiometric work is in progress and will be the subject of a separate report.

Sodium selectivity often appears as the 'default' selectivity when cation-selective *t*-butyl calix[4] arenes are assessed experimentally [6]. This is because of the excellent fit of the sodium cation with the cavity of many calix[4]arenes, such as **5** for example [4,7]. The cavity of **5** is defined by localized electron density of four phenoxy and four carbonyl oxygen atoms. By contrast, for compounds such as **3** and **4**, cations interact with the nitrile functional groups, with less involvement of the phenoxy oxygens. Figure 4 shows the interaction of ammonium (for illustrative purposes) with **3** and **4**. This complexation arrangement seems to be similar for all common earth and alkali earth metals. The nitrile groups may offer an alternative binding pocket further removed from the lower rim defined, which involves the phenoxy oxygen atoms to a lesser extent, and this may also lead to radically different selectivity in ion-binding behaviour. Cation interaction further from the annulus is supported by preliminary results from potentiometric investigations of **3**, showing a selectivity for ammonium and potassium over sodium, and these larger cations are not optimally accommodated at the geometrically restricted region of the calix[4]arene lower rim. The ammonium cation is of particular interest because of the geometric complementarity with the spatial arrangement of the nitrile groups of **3**, and the potential for hydrogen bonding with these binding sites.

We have been interested in asymmetrically substituted compounds such as **3** because of the possibility of generating ligands with dramatically different ion-binding selectivities than observed previously. However, the synthetic route to these derivatives has proved to be more difficult than anticipated. For example, starting from **1**, we attempted to make the asymmetric derivative **6**, which has an additional methylene spacer in adjacent pendant ester groups. However, we found



SCHEME 1 Synthesis of **3**.

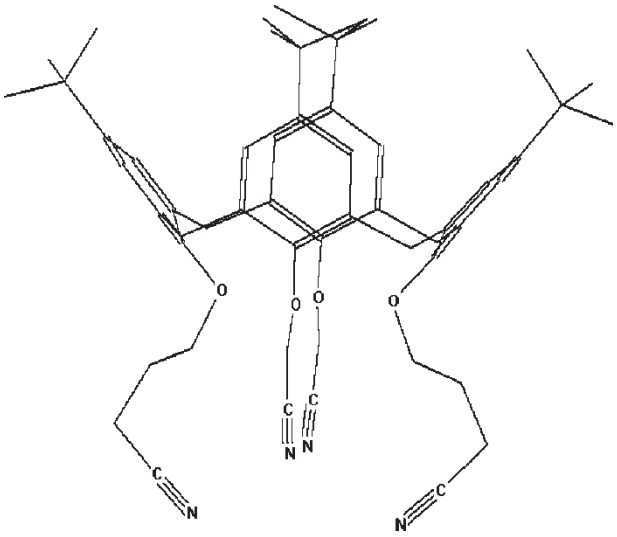


FIGURE 2 Energy-minimized model of **3** generated using MM2 in Chem 3-d Pro v.8.0 (Cambridgesoft Corporation).

that because of (a) the high degree of substitution, (b) increasing difficulty to deprotonate successive phenol hydrogens and (c) the sterically hindered nature of target compounds, yields obtained were very small or the reactions were unsuccessful. Analogously, the synthesis of **3**, as seen in Scheme 1, did not proceed as readily as that of **4** [8]. This prompted the use of more advanced work-up tools.

Normal-phase flash chromatography or open-column chromatography have been used for many years for separations [9]. To date, these techniques have been sufficient to separate and recover calixarene compounds from each other in the majority of cases. However, we found that LC-MS was necessary for the separation and identification of the more complex product profile obtained in these reactions. High-performance liquid chromatography (HPLC) is a common analytical technique that generally has much better separating efficiency than open-column or flash chromatography [10]. This improved efficiency can be crucial when dealing with a low yield of product in a complex mixture.

The use of HPLC for preparative purposes has largely been the preserve of industry as dedicated instrumentation can be expensive to acquire and run and, as such, preparative HPLC has been perceived as a specialist technique by most organic research chemists. Dedicated preparative instrumentation and materials have been used for asymmetrically substituted calix[4]arenes without MS in the past [11], and several useful analytical HPLC methods have been developed [12]. While scaling up of an existing analytical method for use in preparative HPLC must be done with care, we have found that good results can often be achieved in a relatively straightforward manner. It is our view that the ease of extending the use of both analytical-scale HPLC and MS for semi-preparative product characterization, isolation and collection has not been sufficiently highlighted in the literature. In this report, we highlight the importance of using advanced analytical characterization of reaction products coupled with semi-preparative methods for scaling up the process in order to obtain reasonable quantities of more elusive derivatives such as the asymmetric calix[4]arene **3**.

RESULTS AND DISCUSSION

The chromatogram in Fig. 3 reveals the complex nature of the reaction mixture obtained for the synthesis of **3**. Numerous peaks or bands are present with considerable co-elution. Calixarene **3** and some other components in the mix could be identified. Starting materials, including **2**, are seen at about

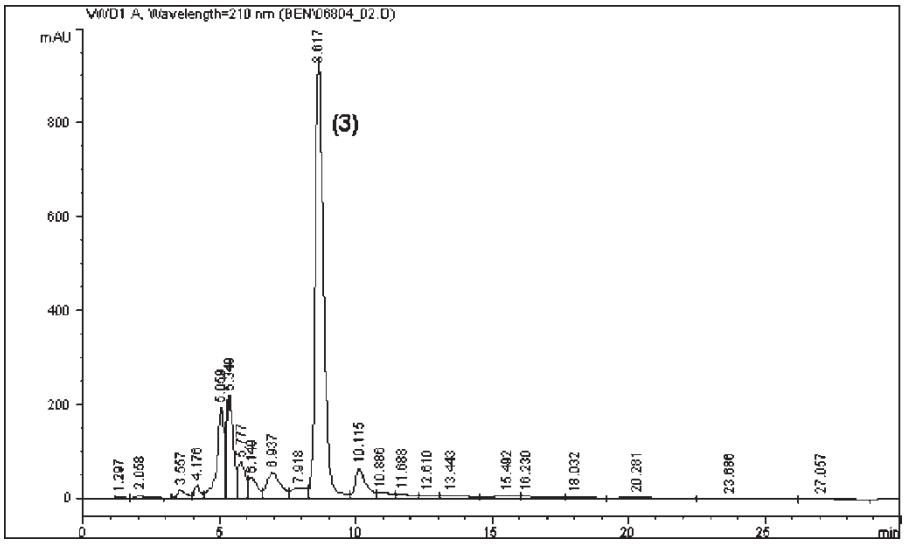


FIGURE 3 LC chromatogram of reaction mixture; **3** identified by MS.

TABLE I Potential changes in a potentiometric membrane containing **3** in contact with 10^{-1} M aqueous solutions of cation chlorides, compared to the equivalent potential in deionized water

Cation	Potential change (mV)
K^+	+216.0
NH_4^+	+206.9
Na^+	+154.0
Ca^{2+}	+79.4
Li^+	+71.4
Mg^{2+}	+62.4

5 min. Between 5 and 8.6 min (peak of **3**) various breakdown fragments of **3** appear. Molecular ions of **3** + K^+ were seen in this region too. An unidentified component of greater mass than **3** was seen at about 10.1 min. The reverse-phase nature of the column stationary phase ensures that components larger (and less polar) than **3** emerged after 8.6 min. By-products and unreacted or partially reacted starting materials were therefore largely confined to retention times below 8.6 min.

With a fairly complex reaction mixture containing **3** present, it was decided that the analytical HPLC method would be scaled up to isolate **3** as efficiently as possible, instead of resorting to open-column chromatography for separation. If normal-phase chromatography was used instead, the compound of interest would elute early and would be less likely to be resolved from related compounds. This scenario applies to the use of open-column chromatography where silica gel is often used as a stationary phase.

Using a simple scale-up factor as a guideline, supplied by most column manufacturers, HPLC parameters were altered for semi-preparative work. Column width went from 2.0 to 10.0 mm with a larger particle size of the same stationary phase. The stationary phase used for this work, Synergy Fusion-RP, has both reverse- and normal-phase characteristics, so that a mixture with a broad range of polarities can be effectively separated in the one chromatogram, thus saving time and consumables.

The flow rate was increased from 0.2 to 5 mL/min. These flow rates correspond to a flow rate of 1 mL/min on a more typical 4.6 mm diameter column. The injector, pump, detector cell and tubing of most analytical HPLC hardware is capable of running at these conditions. Indeed, even with a flow rate of 5 mL/min, the back pressure never rose above 33 bar, well within the instrument's maximum limit.

The 100 μL standard analytical injection loop was retained as it is more beneficial to increase injected sample concentration than volume for efficient separation [13]. Sample concentration was increased for preparative work from 0.5 to 300 mg/mL. As the UV detector cell was designed for analytical concentrations, the wavelength used for preparative work was changed to one showing less sensitivity towards the sample, thus avoiding detector saturation. Calixarene **3** absorbs about six times less UV radiation at 280 nm than at 210 nm. The analytical wavelength of 210 nm was therefore changed to the less sensitive 280 nm for preparative work. For sample collection, a Gilson 204 fraction collector was used. Injections were performed and the relevant peaks collected using an automation facility on the fraction collector, requiring minimal supervision. It was also possible to manually collect fractions without the use of a fraction collector. In a short time and with only small modifications, the analytical instrumentation was ready for semi-preparative work.

Figure 5 shows a typical semi-preparative chromatogram from the original synthesis mixture of **3**. As expected, all retention times are faster than in the analytical run in Fig. 3 and resolution is generally lower because of the higher sample concentrations injected [14]. Ultimately, the recovery yield and percentage purity of the target are the important parameters. In 1 h, unattended, 55 mg of 97.6% pure **3** was isolated from 120 mg of a mixture of no less

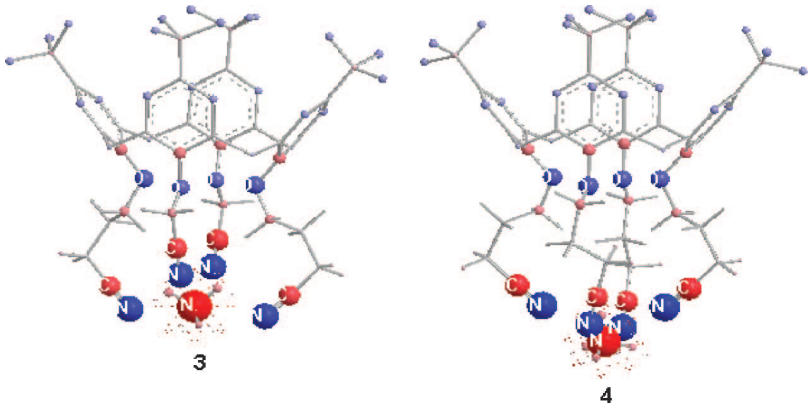


FIGURE 4 Complexes of **3** and **4** with an ammonium ion. Atoms are scaled by size according to Huckel partial charges. Red and blue are areas of positive and negative localized charge, respectively. The importance of the nitrile groups for complexation is revealed.

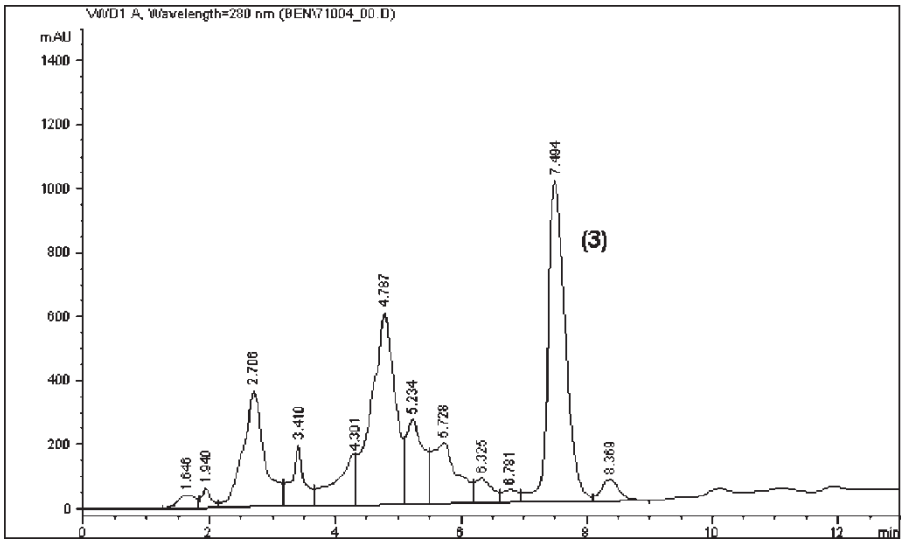


FIGURE 5 A semi-preparative-scale HPLC chromatogram obtained for a mixture containing **3**.

than 10 components (Fig. 6). This represents a recovery of about 90%. Given that the observed absorption coefficients for **2** and **3** are similar, this figure was deemed fairly accurate.

Theoretically, open-column or flash chromatography could not match this separation in terms of separation efficiency or time [10]. Practical considerations include the fact that open-column or flash chromatography is more prone to operator error, with increased possibility of product loss.

CONCLUSION

Following simple alterations to an analytical LC-MS instrumental set-up and synthetic method, quantities of calixarene **3** were isolated in 1 h that were sufficient for characterization, activity screening and further synthesis. MS also revealed that **3** may be predisposed towards forming complexes with ammonium ions rather than the more usual sodium ions. Energy-minimized structures suggest that this may be due to the spatial arrangement of the nitrile groups, which are out-of-plane compared to the well-known symmetrically substituted tetraester calix[4]arenes, and form an alternative binding site further away from the phenoxy oxygen atoms at the base of the calix[4]arene annulus. Hence **3** could provide a route to the generation of a range of new calix[4]arene derivatives with dramatically different ion-binding selectivity.

MATERIALS AND METHODS

HPLC was carried out using an HP1100 instrument with UV detection. For MS work, this was coupled

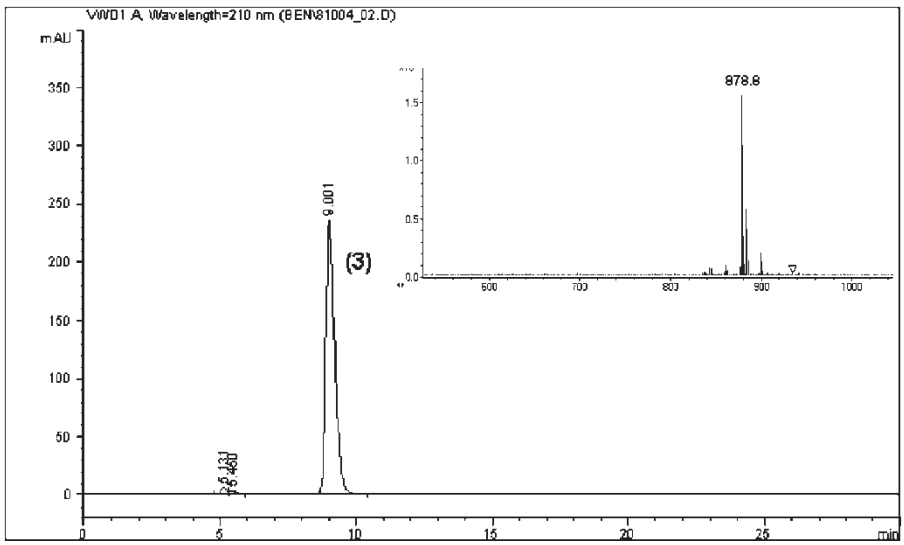


FIGURE 6 LC-MS chromatogram showing 97.6% pure **3** following semi-preparative HPLC separation. Inset shows MS identification of **3**.

to a Bruker/Hewlard-Packard Esquire system, using a positive ESI source and the software's default 'smart' settings. The mobile phase used was isocratic LC grade acetonitrile with 0.25% formic acid content. This also served as the sample solvent. For analytical LC-MS, a Synergy 150.0 × 2.0 mm, 4 μm Fusion-RP column was used. Flow rate was 0.2 mL/min. Detection wavelength was 210 nm. Injections were 5 μL of a 0.5 mg/mL sample. For semi-preparative HPLC, a Synergy 250.0 × 10.0 mm, 10 μm Fusion-RP column was used. Flow rate was 5.0 mL/min. Detection wavelength was 280 nm. Injections were 100 μL of a 300 mg/mL sample, filtered before use. Fraction collection was carried out manually or with a Gilson 204 fraction collector in automation mode. Recovery yield was based on percentage of total peak area.

NaH was used as a 60% dispersion in mineral oil. All reactions were carried out under argon. The name *p-tert*-butylcalix[4]arene was used for convenience instead of the IUPAC name, 5,11,17,23-tetra-*p-tert*-butyl-25,26,27,28-tetrahydroxycalix[4]arene.

Potentiometric membranes were prepared using 250 mg 2-nitrophenyl octyl ether, 125 mg PVC, 2.5 mg **3** and 0.5 mg potassium tetrakis(4-chlorophenyl) borate.

The electrochemical cell used consisted of a double junction reference electrode and a PVC membrane working electrode in the following arrangement: Ag AgCl 3 M NaCl 0.1 M LiOAc sample solution PVC membrane 0.1 M NH_4Cl AgCl Ag.

Membranes were conditioned in 0.1 M ammonium chloride for 3 h followed by deionized water for 30 min before analysing the 10^{-1} M cation chloride solution of interest. The potentiometric cell was interfaced to a PC using a National Instruments SCB-68 4-channel interface.

Energy-minimized molecular models were generated using Chem3D pro v.8.0 software, also used to calculate extended Huckel charges to display partial charge surfaces.

5,11,17,23-Tetra-*p-tert*-butyl-25,27-bis[(cyanomethyl)oxy]-26,28-dihydroxycalix[4]arene (2)

p-tert- Butylcalix[4]arene **1** (5.0 g, 7.72 mmol), K_2CO_3 (1.28 g, 9.26 mmol) and bromoacetonitrile (1.95 g, 16.20 mmol) were heated in CH_3CN (80 mL) at 50°C for 5 days. The reaction was monitored by LC-MS. The solvent was evaporated and the residue taken up in CH_2Cl_2 (300 mL), washed with 1 N HCl (100 mL), H_2O (50 mL) and brine (50 mL) and dried with Mg_2SO_4. CH_2Cl_2 was evaporated and the residue was recrystallized from $CHCl_3$/MeOH yielding a white solid: yield 73%; mp 285–290°C; UV–vis (ACN) 210 nm (ϵ/dm^3 cm^{-1} mol^{-1} 152 472), 280 nm (25 974); IR (KBr) 2250 cm^{-1} (CN), 3515 cm^{-1} (OH); ^{1}H NMR δ 7.12 (s, 4H), 6.73 (s, 4 H), 4.81 (s, 4H), 4.23 and 3.45 (ABq, 4H, $J = 13.6$), 1.33 (s, 18H), 0.87 (s, 18H); ^{13}C NMR δ 150.3 (s), 149.0 (d), 142.9 (d), 135.4 (s), 128.2 (s), 126.6 (s), 125.7 (s), 115.5 (s), 60.8 (s), 34.4 (t), 31.9 (t), 31.5 (s); ESI mass spectrum $+m/e$ 749.6 ([M + Na$^+$], calcd 749.4); HPLC purity: 96.8%. Anal. Calcd for $C_{52}H_{66}N_2O_4$(%): C, 79.30; H, 8.04; N, 3.85. Found: C, 78.94; H, 7.87; N, 4.00.

5,11,17,23-Tetra-*p-tert*-butyl-25,27-bis[(cyanopropyl)oxy]-26,28-bis[(cyanomethyl)oxy]calix[4]arene (3)

Calixarene **2** (4.0 g, 5.5 mmol) and NaH (0.44 g, 11.0 mmol) were stirred for 1 h at room temperature in anhydrous DMF (100 mL). 4-Bromobutyronitrile (1.63 g, 11.0 mmol) was added batchwise and the mixture was stirred at 80°C for 24 h. The reaction was monitored by HPLC-MS. The mixture was cooled and another equivalent of NaH and 4-bromobutyronitrile was added and heated as before. After a further 72 h the DMF was evaporated and the residue taken up in CH_2Cl_2 (250 mL), washed with 1 N HCl (100 mL), H_2O (50 mL), brine (50 mL) and saturated NH_4Cl (50 mL) and dried with Mg_2SO_4. After filtration the CH_2Cl_2 was removed to give 4.69 g of a beige solid. The solid was purified by semi-preparative HPLC to yield a white solid: recovery yield 90%; mp 234–236°C; UV–vis (ACN) 210 nm (ϵ/dm^3 cm^{-1} mol^{-1} 150 366), 280 nm (28 420); IR (KBr) 2248 cm^{-1} (CN); ^{1}H NMR δ 7.17 (s, 4H), 6.43 (s, 4 H), 4.95 (s, 4H), 4.33 and 3.28 (ABq, 4H, $J = 13.0$), 3.90 (t, 4H), 2.69 (t, 4H), 2.32 (m, 4H), 1.35 (s, 18H), 0.80 (s, 18H); ^{13}C NMR δ 152.3 (d), 148.3 (s), 145.8 (s), 135.7 (s), 131.5 (s), 126.7 (s), 125.3 (s), 119.7 (s), 118.0 (s), 74.2 (s), 58.5 (s), 34.7 (s), 34.3 (d), 31.7 (t), 26.3 (s), 15.0 (s); ESI mass spectrum $+m/e$ 878.8 ([M + NH_4^+], calcd 878.6); HPLC purity: 97.6%. Anal. Calcd for $C_{56}H_{68}N_4O_4$(%): C, 78.10; H, 7.96; N, 6.51. Found: C, 77.79; H, 8.13; N, 6.26.

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