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Tetrahedron 63 (2007) 10764–10767
Tetrahedron

Calix[4]arene-based ligands as endotoxin receptors

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Received 4 May 2007; revised 31 May 2007; accepted 4 June 2007
Available online 24 July 2007

Abstract—Three new calix[4]arene-based receptors as potential antimicrobial agents have been synthesized. Their recognition ability towards lipopolysaccharides of Gram-negative bacteria has been studied by NMR and UV titrations.

1. Introduction

The design and synthesis of high affinity protein or carbohydrate ligands represent an important frontier in bioorganic chemistry.[1] A promising strategy to obtain such ligands is the creation of multivalent compounds that bind to a multivalent target.[2]

Generally, the synthesis of multivalent compounds involves the coupling of a molecular scaffold bearing several functional groups to a number of monovalent ligands, attached with or without a spacer. Examples of scaffolds include partially substituted benzene derivatives, carbohydrate residues, azamacrocycles, calixarenes, cyclodextrins, gold nanoparticles, polymers, membranes and dendrimers.

Calixarene macrocycles[3] are a useful basis for the design and synthesis of multivalent receptors[4] due to their well understood chemistry, highly variable functionalization and particular conformational features for the different oligomers, from the conformationally rigid calix[4]arenes to the mobile calix[6]- and calix[8]arenes.

In our previous work, we have reported calix[8]arene derivatives functionalized with eight basic amino acid residues, which are complementary to the surface of the enzyme tryptase near its active site and work as tryptase inhibitors in the nanomolar range.[5] The same derivatives, owing to their cluster of positive charges, display an exceptional neutralization ability towards heparin, a sulfated polysaccharide known as one of the most powerful anticoagulant drugs.[6]

As part of a programme for the development of new synthetic receptors, significant in the recognition of biological structures, we have synthesized three amino acid derivatives of calix[4]arene that have shown powerful activity as lipopolysaccharide receptors.

Lipopolysaccharides (LPS) are the principal constituent of the outer cellular membrane of Gram-(−) bacteria,[7] better known as bacterial endotoxins, identified as the causative agent of septic shock syndrome.[8] LPS are complex amphiphilic molecule with average molecular weight of about 10 kDa consisting of a hydrophilic polysaccharide portion and a lipophilic lipid moiety called lipid A. The polysaccharide region is composed of three separate domains: an inner core, an outer core, and an O-specific chain, the composition of which is highly variable among Gram-(−) bacteria. The lipid A moiety, the toxic component of LPS,[9] consists of diphosphorylated glucosamine disaccharide carrying a number of higher fatty acid chains.[10] The oligosaccharide core and lipid A portion are structurally highly conserved across Gram-(−) genera (Fig. 1).

Lipid A has an anionic amphiphilic nature that enables it to interact with a variety of cationic hydrophobic ligands. Polymixin B (PMB),[11] a cationic amphiphilic cyclic decapeptide, and squalamine,[12] a cationic steroid isolated from shark, are two natural antibiotics able to bind lipid A and neutralize its toxicity. A property of these natural antibiotics

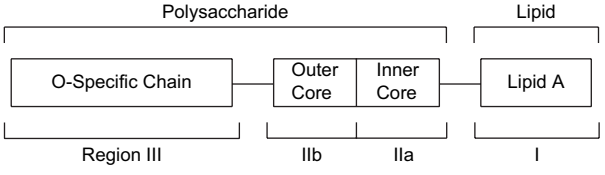

Figure 1. General architecture of lipopolysaccharides.

Keywords: Synthetic receptors; Molecular recognition; Calixarenes; Lipopolysaccharides.
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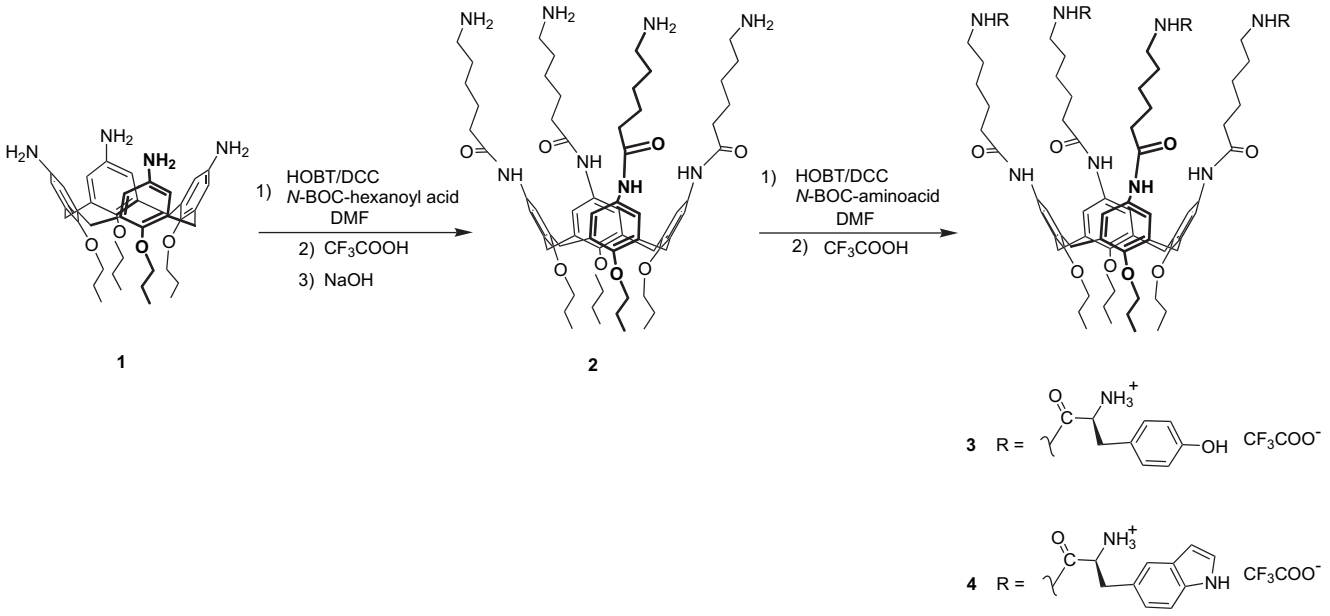
doi:10.1016/j.tet.2007.06.125



Scheme 1.

is their facially amphiphilic conformation.[13] One face of the molecule presents cationic groups and the other hydrophobic groups. Many designed cationic peptides or steroid antibiotics have been developed based upon the structure of naturally occurring molecules.[13] To the best of our knowledge, only two, very different, receptors for lipid A have been synthesized containing a cycloalkane core.[14]

While cationic, facially amphiphilic antimicrobial agents could be prepared from a number of building blocks, we chose as scaffold a tetrapropoxy-tetraamino-calix[4]arene **1** in the cone conformation[15] to ensure the facially amphiphilic nature of the receptor. To enhance the hydrophobic and basic properties of the receptors, we decided to conjugate the calixarene scaffold with both tryptophan and tyrosine amino acid residues. Moreover, we used a 6-aminohexanoyl arm as spacer between the calixarene scaffold and the acyl active residue in order to provide the receptors with the conformational mobility essential for an efficient induced fit recognition mechanism (Scheme 1).

2. Results and discussion

The first evidence of the recognition between derivatives **2–4** and LPS came from NMR titration experiments. These experiments were carried out in D_2O adding LPS into a solution of the calixarene derivative at a known concentration. Peaks in the NMR spectra of compounds **2** and **3** were sharp, consistent with the presence of monomeric species in solution. In contrast, compound **4** showed broad and unresolved signals, indicating the presence of aggregate forms. In each titration, consecutive additions of LPS caused a decrease in signal intensities, culminating in substantial broadening and subsequent disappearance of all resonances ascribable to the calixarene derivatives when about 0.3 molar equivalents of LPS was added, as shown in Figure 2 for titration of **3** with LPS.

These results, although providing evidence of the interaction between LPS and compounds **2–4**, were not of sufficient precision to allow a value of the stability constant for the LPS–calixarene complex (K_d) to be obtained. They are, however, consistent with those obtained by others in the examination of LPS-binding compounds.[14a,16]

While LPS show weak UV absorptions, those of calixarenes are strong and thus we attempted to measure the affinities of derivatives **2–4** for LPS by UV titrations. Compounds **2–4** are sufficiently soluble in buffered aqueous solution (Hepes 2 mM, pH 7) to permit binding analysis by this method. Addition of LPS solution to a buffered solution of the calixarene derivatives gave different results for each calixarene derivative. Titration of compound **2** with LPS showed no significant modification of the absorption of **2** even after the addition of a large excess. Probably, as the aromatic region of the calixarene scaffold responsible for the UV absorption of **2** is far from the interacting amino groups, the UV profile

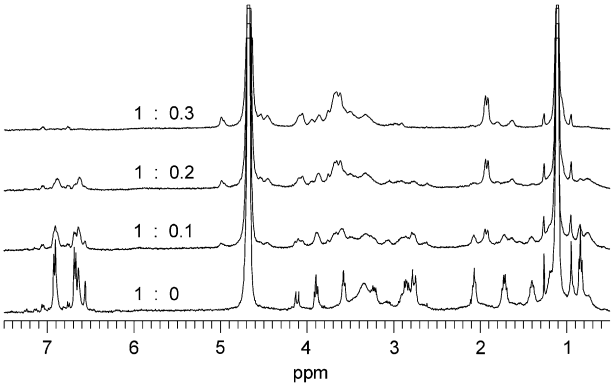


Figure 2. Selected spectra of ^{1}H NMR titration of **3** with LPS. Small aliquots (5 μL) of LPS solution (2.0 mM in D_2O) were added to the NMR sample of **3** in D_2O (500 μL, 0.5 mM); *tert*-butanol (0.6 μL) was added as a standard for NMR integration.

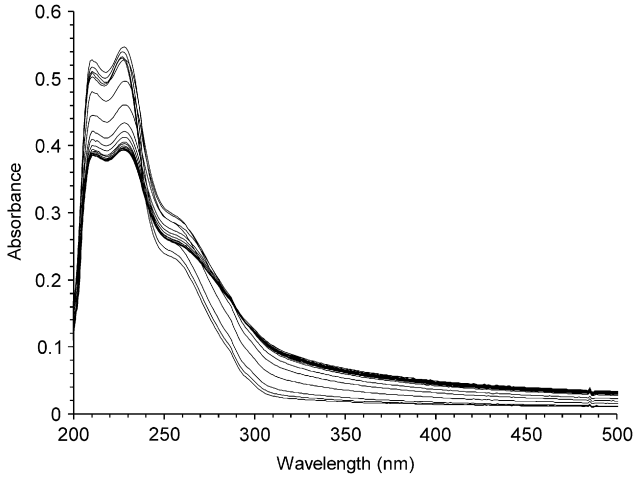


Figure 3. UV titration of **3** with LPS. A 3 mL solution of compound **3** (5.4 μM, 2 mM HEPES, pH 7) was titrated by addition of 4 μL aliquots of a LPS solution (35 μM, HEPES 2 mM, pH 7).

of **2** do not change in a significant way during titration experiments. In contrast, titration of compound **3**, due to the proximity of the interacting amino groups to the UV-active tyrosine residues, showed an evident but non-uniform variation of the absorbance measured at different wavelengths (Figs. 3 and 4).

Applying the mole ratio method[17] to the UV data, the stoichiometry deduced for the complex between **3** and LPS was >10:1 (Fig. 4). This finding, compared to 3:1 stoichiometric ratio deduced from NMR titrations, suggested that LPS at the concentration used to perform NMR experiments, are in aggregated forms.[18] To explain such high stoichiometric ratios, it appears necessary to conclude that the complexation process between **3** and LPS is nonspecific. Compound **3** probably recognizes not just a single lipophilic region of LPS but also binds to the polysaccharide chains of which they are composed. The complexity of the interaction profile prohibited any stability constant estimations. The interaction of **4** with LPS seemed very similar to that of **3**, confirming a high stoichiometric ratio for the complexation process.

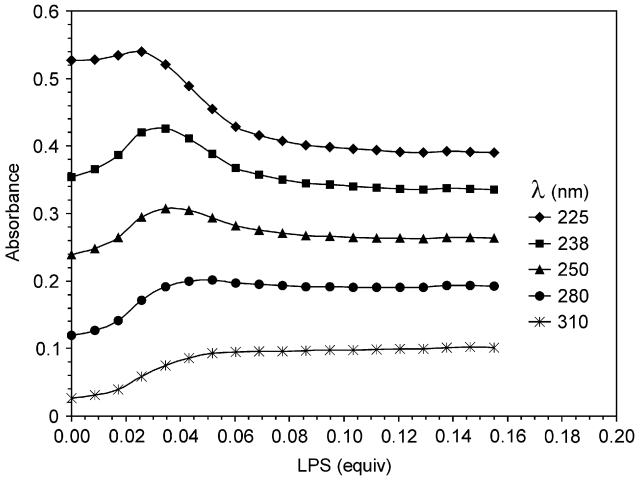


Figure 4. UV titration profiles at selected wavelengths obtained for the addition of aliquots of LPS to **3**.

To avoid the problem of multiple equilibria when working with LPS, lipid A, the conserved LPS headgroup, might be an easier target for molecular recognition studies. However, although this might allow the determination of association constants, it could not provide a reliable measure of the ability to neutralize bacterial endotoxins. Given the clinical importance of lipopolysaccharides as the causative agent of bacterial sepsis, the alternative of biological tests with different Gram-negative bacteria, to really assess the antimicrobial activity of **2**–**4**, is being pursued.

3. Conclusion

In this work we have described three new calix[4]arene-based synthetic receptors that have shown recognition ability towards LPS, the primary constituent of the outer cellular membrane of Gram-(−) bacteria. By exploiting a calix[4]-arene scaffold in the cone conformation, we have obtained molecules with facial amphiphilicity, a necessary requirement for disrupting bacterial membranes and generating active compounds against a broad spectrum of bacteria. Spectroscopic (UV and NMR) measurements have underlined the antimicrobial potentiality of these derivatives. Our findings could help in the search and development of new antibiotics, the most important strategy to overcome the constant evolution and spread of multidrug-resistant pathogens.

4. Experimental

4.1. General comments

All chemicals were reagent grade and used without further purification. Tetraamino-tetrapropoxy-calix[4]arene **1** was prepared following literature procedure.[15] Lipopolysaccharides, lyophilized powder from *Escherichia coli* Serotype 055:B5, were purchased from Sigma. ^{1}H and ^{13}C NMR spectra were acquired at 400.13 and 100.61 MHz, respectively, on a Bruker Avance™ 400 instrument. ESIMS spectra were acquired under positive ionization conditions on a Waters-Micromass ZQ2000 mass spectrometer. UV spectra were acquired on a Agilent 8453 UV–vis spectrophotometer.

4.1.1. General procedure for tetra-aminoacid-calix[4]-arene synthesis. *N*-Boc-aminoacid (1.2 mmol) and HOBT (1.4 mmol) in 5 mL of dry DMF were stirred in a round-bottomed flask at room temperature. DCC (1.3 mmol) and, after 15 min, derivative **1** or **2** (0.1 mmol) in 2 mL of dry DMF were added. The reaction was stirred for 3 h. The solvent was removed and reaction mixture was purified by preparative TLC on silica-gel using CH_2Cl_2/EtOH 94:6. Yield of the coupling reactions was ~80% with all the amino acids used. Further treatment with TFA at room temperature for 1 h, followed by several washings with ethanol and drying under vacuum, gave the protonated forms of **2**–**4**. Compound **2** was obtained as the free base by precipitation with 0.5 M NaOH.

4.1.1.1. 5,11,17,23-Tetraamino(*N*-6-aminohexanoyl)-25,26,27,28-tetra-*n*-propoxycalix[4]arene (2). ^{1}H NMR:

(400.13 MHz, MeOD) δ 1.02 (t, J=7.4 Hz, CH_3, 12H), 1.41 (m, CH_2, 8H), 1.51 (m, CH_2, 8H), 1.65 (m, CH_2, 8H), 1.96 (q, J=7.4 Hz, CH_2, 8H), 2.25 (t, J=7.3 Hz, CH_2, 8H), 2.62 (t, J=7.2 Hz, CH_2, 8H), 3.11–4.46 (AB, J=13.0 Hz, $ArCH_2Ar$, 8H), 3.85 (t, J=7.4 Hz, OCH_2, 8H), 6.88 (s, ArH, 8H). ^{13}C NMR: (100.61 MHz, MeOD) δ 9.2 (q), 22.8, 25.1, 26.1, 30.5, 32.1, 36.2, 40.8 (t) 120.3 (d), 132.1, 134.6, 152.8, 172.4 (s). ESMS calcd for $C_{64}H_{96}O_8N_8$ 1106.5288 (M+H$^+$), found 1105.9.

4.1.1.2. 5,11,17,23-Tetraamino[*N*-6-aminohexanoyl-(*N′*-tyrosinyl)]-25,26,27,28-tetra-*n*-propoxycalix[4]arene (3). ^{1}H NMR: (400.13 MHz, MeOD) δ 1.03 (t, J=7.5 Hz, CH_3, 12H), 1.24 (m, CH_2, 8H), 1.43 (m, CH_2, 8H), 1.61 (m, CH_2, 8H), 1.95 (q, J=7.4 Hz, CH_2, 8H), 2.24 (t, J=7.1Hz, CH_2, 8H), 2.95–4.43 (AB, J=13.0 Hz, $ArCH_2Ar$, 8H), 3.05 (m, CH_2, 8H), 3.83 (t, J=7.5 Hz, OCH_2, 8H), 3.89 (t, J=7.4 Hz, $CHNH_3^+$, 4H), 6.76 (d, J=8.4 Hz, ArH, 8H), 6.86–6.91 (AB, J=2.3 Hz, ArH, 8H), 7.06 (d, J=8.4 Hz, ArH, 8H). ^{13}C NMR: (100.61 MHz, MeOD) δ 9.2 (q), 22.6, 24.8, 25.9, 28.2, 30.5, 36.0, 36.6, 38.8 (t), 54.6 (d), 76.5 (t), 115.2, 120.2 (d), 124.7 (s), 129.9 (d) 132.0, 134.6, 152.9, 156.6, 168.2, 172.3 (s). ESMS calcd for $C_{100}H_{132}O_{16}N_{12}$ 1759.2348 (M+H$^+$), found 1760.1.

4.1.1.3. 5,11,17,23-Tetraamino[*N*-6-aminohexanoyl-(*N′*-tryptophanyl)]-25,26,27,28-tetra-*n*-propoxycalix[4]-arene (4). ^{1}H NMR: (400.13 MHz, MeOD) δ 0.99 (t, J=7.4 Hz, CH_3, 12H), 1.14 (m, CH_2, 8H), 1.33 (m, CH_2, 8H), 1.52 (m, CH_2, 8H), 1.91 (q, J=7.4 Hz, CH_2, 8H), 2.18 (t, J=7.1 Hz, CH_2, 8H), 2.99–4.36 (AB, J=12.9 Hz, $ArCH_2Ar$, 8H), 3.21 (m, CH_2, 8H), 3.76 (t, J=7.4 Hz, OCH_2, 8H), 4.01 (t, J=7.4 Hz, $CHNH_3^+$, 4H), 6.84–6.87 (AB, J=2.2 Hz, ArH, 8H), 7.03 (t, J=7.9 Hz, ArH, 4H), 7.11 (t, J=7.9 Hz, ArH, 4H), 7.16 (s, ArH, 4H), 7.35 (d, J=8.1 Hz, ArH, 4H), 7.59 (d, J=8.1 Hz, ArH, 4H). ^{13}C NMR: (100.61 MHz, MeOD) δ 9.2 (q), 22.8, 24.8, 25.6, 27.3, 28.0, 30.5, 36.0, 38.9 (t), 53.6 (d), 77.0 (t), 106.6 (s), 111.1, 117.6, 118.7, 120.4, 121.4, 124.0 (d), 127.3, 132.1, 134.6, 136.6, 152.8, 168.4, 172.8 (s). ESMS calcd for $C_{108}H_{136}O_{12}N_{16}$ 1851.3842 (M+H$^+$), found 1850.7.

4.1.2. General procedure for NMR titrations. Small aliquots (5 μL) of LPS solution (2.0 mM in D_2O) were added to an NMR tube containing 500 μL solution of compounds **2**, **3** or **4** (0.5 mM) in D_2O and *tert*-butanol (0.6 μL) as a standard for integration.

4.1.3. General procedure for UV–vis titration. A 3 mL solution of compounds **2**, **3** or **4** (5.4 μM) in 2 mM HEPES, pH 7 was titrated by addition of 4 μL aliquots of a LPS stock solution (35 μM, HEPES 2 mM, pH 7). After each addition, the solution was allowed to equilibrate for 2 min, then scanned for absorbance from 500 to 200 nm. LPS stock solution was sonicated for 2 h after preparation and then stored at 4 °C.

References and notes

1. (a) Yin, H.; Hamilton, A. D. *Angew. Chem., Int. Ed.* **2005**, *44*, 4130–4163; (b) Davis, A. P.; James, T. D. *Functional Synthetic Receptors*; Schrader, T., Hamilton, A. D., Eds.; Wiley-WCH: Weinheim, 2005; pp 45–109; (c) Davis, A. P.; Wareham, R. S. *Angew. Chem., Int. Ed.* **1999**, *38*, 2978–2996.
2. Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754–2794.
3. *Calixarenes 2001*; Asfari, Z., Böhmer, V., Harrowfield, J. M., Vicens, J., Eds.; Kluwer Academic: Dordrecht, 2001.
4. Baldini, L.; Casnati, A.; Sansone, F.; Ungaro, R. *Chem. Soc. Rev.* **2007**, *36*, 254–266.
5. Mecca, T.; Consoli, G. M. L.; Geraci, C.; Cunsolo, F. *Bioorg. Med. Chem.* **2004**, *12*, 5057–5062.
6. Mecca, T.; Consoli, G. M. L.; Geraci, C.; La Spina, R.; Cunsolo, F. *Org. Biomol. Chem.* **2006**, *4*, 3763–3768.
7. Young, L. S.; Martin, W. J.; Meyer, R. D.; Weinstein, R. J.; Anderson, E. T. *Ann. Intern. Med.* **1977**, *86*, 456–471.
8. (a) Raetz, C. R. H. *Annu. Rev. Biochem.* **1990**, *59*, 129–170; (b) Ulevitch, R. J.; Tobias, P. S. *Curr. Opin. Immunol.* **1994**, *6*, 125–130.
9. (a) Takahashi, I.; Kotani, S.; Takada, H.; Tsujimoto, M.; Ogawa, T.; Shiba, T.; Kusumoto, S.; Yamamoto, M.; Hasegawa, A.; Kiso, M.; Nishijima, M.; Amano, F.; Akamatsu, Y.; Harada, K.; Tanaka, S.; Okamura, H.; Tamura, T. *Infect. Immun.* **1987**, *65*, 57–69; (b) Rietschel, E. T.; Brade, H.; Brade, L.; Brandenburg, K.; Shade, U. F.; Seydel, U.; Zähringer, U.; Galanos, C.; Lüderitz, O.; Westphal, O.; Labishinski, H.; Kusumoto, S.; Shiba, T. *Prog. Clin. Biol. Res.* **1987**, *231*, 25–53.
10. (a) Rietschel, E. T.; Kirikae, T.; Shade, U. F.; Ulmer, A. J.; Holst, O.; Brade, H.; Schmidt, G.; Mamat, U.; Grimmecke, H.-D.; Kusumoto, S.; Zähringer, U. *Immunobiology* **1993**, *187*, 169–190; (b) Zähringer, U.; Lindner, B.; Rietschel, E. T. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 211–276.
11. Storm, D. R.; Rosenthal, K. S.; Swanson, P. E. *Annu. Rev. Biochem.* **1977**, *46*, 723–763.
12. Moore, K. S.; Wehrli, S.; Roder, H.; Rogers, M.; Forrest, J. N., Jr.; Mc Crimmon, D.; Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1354–1358.
13. Savage, P. B. *Eur. J. Org. Chem.* **2002**, *5*, 759–768 and references therein.
14. (a) Hubbard, R. D.; Horner, S. R.; Miller, B. L. *J. Am. Chem. Soc.* **2001**, *123*, 5810–5811; (b) Bucholtz, K. M.; Gareiss, P. C.; Tajc, S. G.; Miller, B. L. *Org. Biomol. Chem.* **2006**, *4*, 3973–3979.
15. Verboom, W.; Durie, A.; Egberink, R. J. M.; Asfari, Z.; Reinhoudt, D. N. *J. Org. Chem.* **1992**, *57*, 1313–1316.
16. David, S. A.; Bechtel, B.; Annaiah, C.; Mathan, V. I.; Balaram, P. *Biochim. Biophys. Acta* **1994**, *1212*, 167–175.
17. Tsukube, H.; Furuta, H.; Odani, A.; Takeda, Y.; Kudo, Y.; Inoue, Y.; Liu, Yu.; Sakamoto, H.; Kimura, K. *Comprehensive Supramolecular Chemistry*; Atwood, J. L., Davis, J. E. D., Macnicol, D. D., Vogtle, F., Eds.; Elsevier Science: New York, NY, 1996; pp 428–430.
18. Santos, N. C.; Silva, A. C.; Castanho, M. A. R. B.; Martins-Silva, J.; Saldanha, C. *ChemBioChem* **2003**, *4*, 96–100.