A fluorescent cyclotriveratrylene: synthesis, emission properties and acetylcholine recognition in water†

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A fluorescent cyclotriveratrylene **1** was synthesized and characterized in methanol and water. Soluble in pure water and physiological media, compound **1** has binding properties towards acetylcholine. This detection is direct, contrary to most fluorescent systems which rely upon a competition principle between the guest and a fluorophore.

Introduction

Present in the mammalian brain, the neurotransmitter acetylcholine (ACh) is involved in nervous transmission.[1,2] Recent interest in this molecule is due to its implication in neuronal disorders such as chronic degenerative diseases (Alzheimer's), ischemia and neural trauma.[3] Currently, the role of this small molecule is still to be elucidated under pathological conditions. Considering its participation in different biological events, real-time and real-space monitoring of ACh would be highly valuable for a better understanding and diagnosis of these pathologies. Therefore there is a great interest in the design of fluorescent probes for acetylcholine in physiological media. As a consequence the topic of fluorescent chemosensors has been broadly developed and reviewed,[4,5] and some nice examples of fluorescent systems sensitive to ACh in polar solvents have been published. Their principle of ACh detection is based on the competitive recognition between ACh and a previously complexed fluorophore (F) (Fig. 1.1).[6,7] When ACh is complexed by a non-fluorescent host, the fluorophore is released and recovers its luminescence. Submicromolar concentrations of ACh are discernable through the fluorophore emission.

Nevertheless, with this strategy information about the space-resolved migration of ACh is not available. Thus, suitable probes for ACh imaging should be fluorescent and directly sensitive to the guest (Fig. 1.2). Cyclotriveratrylenes (CTV) are rigid bowl-shaped units with a size suitable for quaternary ammoniums.[8,9] Only few CTV derivatives have binding properties[10,11] and no fluorescence emission titrations were conducted. Nevertheless, CTV architectures can be functionalized to introduce fluorescent emission properties.

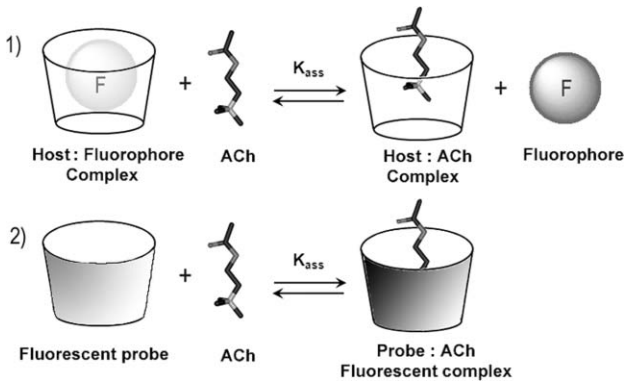
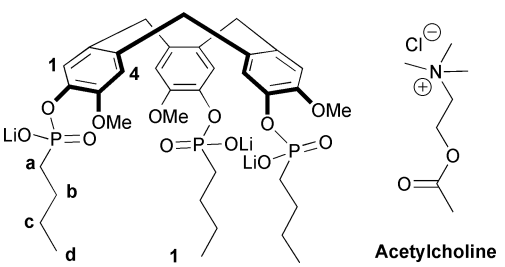


Fig. 1 Principle of ACh detection using fluorescence: (1) indirect strategy through the release of an encapsulated fluorophore, (2) direct host sensing.

A fluorescent cyclotriveratrylene

The new probe **1** has a hydrophobic architecture substituted with three electron-acceptor groups (phosphonate) and three electron-donor groups (methoxy) showing fluorescence emission properties (Scheme 1). At neutral pH, charged phosphonate groups will ensure hydrosolubility of **1** and ionic interactions with ammonium guests. Herein we report the synthesis of the first fluorescent cyclotriveratrylene **1** and its binding properties towards ACh in water and in a physiological medium using ^{1}H NMR and fluorescence emission spectrometries. To the best of our knowledge, this is the first example of a fluorescent CTV.



Scheme 1 Molecular structure of probe **1** and acetylcholine (ACh).

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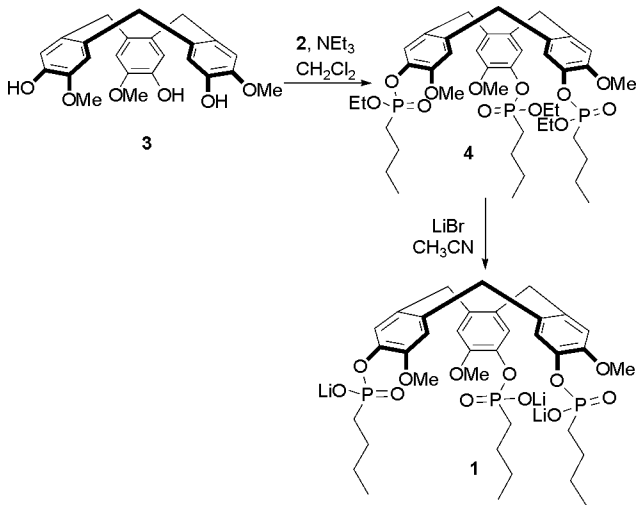
† Electronic supplementary information (ESI) available: Synthesis of compound **2**, UV-Vis and fluorescence spectra of **1**, NMR spectra of compounds **1**, **2**, **4** and **5** and NMR titration data of ACh. See DOI: 10.1039/b904590b

Synthesis and characterization

Synthesis of **1** was achieved in two steps from (±)-2,7,12-trihydroxy-3,8,13-trimethoxy-10,15-CTV **3**[11] (Scheme 2). Reaction of ethyl *n*-butylphosphonochloridoate **2**[12] with **3** gave compound **4** in 31% yield after column chromatography. The tris-phosphonate **4** was smoothly hydrolyzed using an excess of lithium bromide[13] in refluxing acetonitrile. After filtration and extensive washings with hot acetonitrile, host **1** was isolated in 80% yield. Its ^{1}H and ^{31}P NMR spectra confirmed a C_3 symmetry. In water, **1** exhibits a strong UV absorption band (λ_{max} = 289 nm, ε = 8015 M^{-1} cm^{-1}) and a fluorescent emission (λ_{ex} = 280 nm, λ_{em} = 314 nm, Φ = 0.14).



Scheme 2 Synthesis of probe **1**.

Acetylcholine recognition in water

NMR titrations were first undertaken to investigate host–guest associations. Probe **1** (10^{-3} M) was studied in the presence of ACh (0 to 0.2 M) in deuterated water, monitoring host or guest proton signals. The binding isotherm was fitted with a 1 : 1 model and the **1** : ACh binding constant was 76 ± 3 M^{-1}.[14]

Chemical shift variations were examined to determine the nature of this host–guest association.[15] ^{1}H NMR signals of ACh showed a small modification at its ammonium extremity ($\Delta\delta_{max} \approx -0.06$ ppm for CH_3N^+ and CH_2N^+) which is consistent with a weak cationic interaction with **1** (Table 1). Considering **1**, the *n*-butyl group protons H_{a-d} were shifted (Table 1, $\Delta\delta_{max}$ from +0.11 to +0.31 ppm respectively). It was noteworthy that the terminal methyl protons (H_d) were the most downfield shifted ones. Molecular modeling was undertaken to understand this unexpected observation, a perturbation of the *n*-butyl group while **1** binds ACh.

Geometry optimization in a vacuum using the MM^+ molecular mechanics calculation was carried out to represent a stable conformation of **1** : ACh structure as well as probe **1** (Fig. 2).[16] The **1** : ACh complex showed size complementarity between the bowl-shaped cavity and the quaternary ammonium. The ammonium group is not located in the middle of the cavity but is a little off-centre. Ionic interactions between the negative phosphonate groups and ammoniums exist at least between two of the phosphonate groups (distances of 2.88; 3.03; 5.50 Å have been measured). It was also noticed that the butyl chains of CTV **1** are then located outside the cavity. In contrast, the molecular model of **1** indicated that the *n*-butyl chain orientation is free and may interact with CTV aromatics *via* a favorable hydrophobic effect (Fig. 2, views 3 and 4). A change in the *n*-butylphosphonate group orientation in the presence or in the absence of a competitive ammonium may explain changes in the ^{1}H NMR spectra (H_{a-d}). This local modification of probe **1** is also promising for its fluorescence emission modification during ACh recognition.

Table 1 Maximum binding-induced chemical shift of probe **1** protons in D_2O

Probe **1**	H_1	H_4	OCH_3	H_a	H_b	H_c	H_d
$\Delta\delta_{max}$ (ppm)	0	0.05	0	0.11	0.16	0.22	0.31

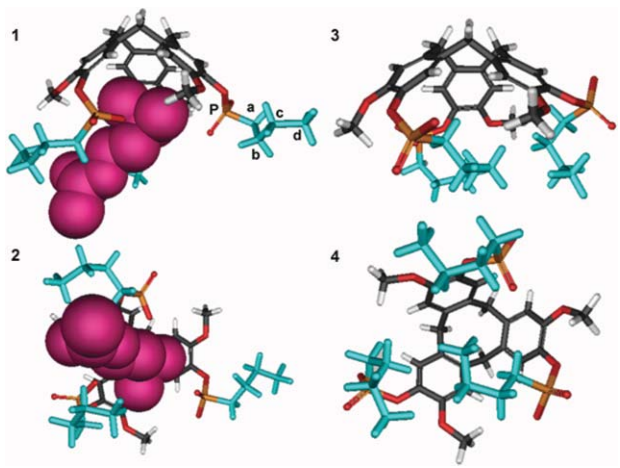


Fig. 2 Molecular mechanics of the **1** : ACh complex (lateral view 1 and bottom view 2) and probe **1** (lateral view 3 and bottom view 4).

In water, titration of probe **1** with ACh was achieved using fluorescence emission (Fig. 3). An enhancement of the fluorescence intensity was observed. The binding isotherm was fitted with a 1 : 1 model and an association constant of 63 ± 5 M^{-1} was measured which is consistent with the NMR titration data.

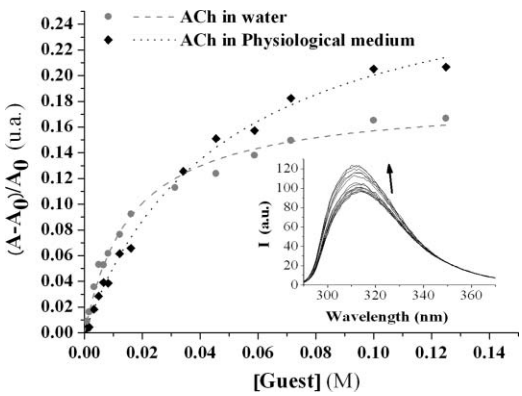


Fig. 3 Relative fluorescence area of **1** (10^{-5} M) with increasing ACh concentration (0 to 0.125 M) (●) in water and (◆) in a physiological medium, fitted with a 1 : 1 binding model. The inset shows fluorescence emission of **1** upon ACh addition in a physiological medium.

Acetylcholine recognition in a physiological medium

These promising results prompted us to investigate the properties of probe **1** in a physiological medium used for neuronal culture[17]: NaCl (113 mM), KCl (4.5 mM), $CaCl_2{\cdot}2H_2O$ (2 mM), $MgCl_2{\cdot}6H_2O$ (1 mM), $NaHCO_3$ (25 mM), $NaH_2PO_4{\cdot}H_2O$ (1 mM), D-glucose (11 mM), pH = 7.6. As this medium presents a high salt concentration, we had to check first that this solution had no dramatic effect on the fluorescence properties of the probe. In this solution, fluorescence emission was exactly the same as the one measured in water ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 314$ nm, $\Phi = 0.14$). In addition, the probe emission properties were not modified by increasing concentrations of ionic species (NaCl, 0 to 500 mM) or D-glucose (0 to 15 mM). The independence of the fluorescence properties of probe **1** with respect to the physiological medium was thus compatible with the titration experiments.

ACh was complexed by probe **1** with a 1 : 1 association constant of 23 ± 2 M^{-1}. This K_{ass} value is lower than the one measured in pure water. As solvation of ammonium is larger in highly ionic solvents, this result was expected. Despite a moderate K_{ass} value, it is noteworthy that the fluorescence intensity enhancement during titration is about +20% which is even bigger than the one observed in water. This observation might be due to the reorganization of the *n*-butyl groups when ACh is complexed.[18]

Conclusions

In conclusion, the first fluorescent CTV architecture was prepared and studied as a probe for acetylcholine. The *n*-butyl substituent in **1** appeared to be responsible for the sensitive response to ^{1}H NMR titrations and probably also to fluorescence titrations. The association constant of the probe is moderate in water and a physiological medium. Nevertheless, probe **1** allows direct fluorescent sensing of ACh in physiological media.

Experimental

Synthesis

General procedure. All reagents were purchased at the highest commercial grade and used as supplied. Anhydrous acetonitrile and dichloromethane were distilled from calcium hydride under a nitrogen atmosphere. Triethylamine was freshly dried and distilled from sodium hydride. Melting points were determined on a Kofler apparatus Thermophan from Reichert and are uncorrected. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 300 K. ^{1}H and ^{13}C chemical shifts (δ) are reported in ppm with the solvent reference relative to tetramethylsilane employed as the internal standard. ^{31}P chemical shifts (δ) are reported in ppm from an 85% H_3PO_4 reference. Infrared spectra were recorded on a Nicolet IR210 FT-IR spectrometer; peaks are reported in cm^{-1}. Low- and high-resolution electronic ionisation mass spectra (liquid phase secondary ion mass spectrometry) were carried out using AutoSpec equipment arranged in an EBE geometry (Micromass, Manchester, UK).

Synthesis of 2,7,12-tris(ethyl *n*-butylphosphonate)-3,8,13-trimethoxy-10,15-dihydro-5*H*-tribenzo-[*a,d,g*]cyclononene 4. A solution of (±)-CTV **3** (1.634 g, 4 mmol) in dry dichloromethane (50 mL) was cooled to 0 °C under argon. Dry triethylamine (2.79 mL, 19.9 mmol) and a solution of reagent **2** (3.323 g, 18 mmol) in dichloromethane (5 mL) were successively added dropwise. The mixture was stirred overnight under argon at 20 °C. Distilled water (100 mL) was added to the reaction mixture. After decantation, the aqueous phase was extracted with dichloromethane (2 × 50 mL). The combined organic phases were washed with distilled water (3 × 200 mL) and brine (150 mL), dried over magnesium sulphate, filtered and concentrated in a vacuum. Purification of the crude product by column chromatography over silica gel (dichloromethane–methanol 96 : 4) followed by precipitation in pentane led to CTV **4** (1.060 g, 31%) as a yellow oil; ν_{max} (NaCl) 3415, 2988, 2869, 1514, 1266, 1036, 912 cm^{-1}; δ_H(300.13 MHz; $CDCl_3$) 0.88 (9H, t, *J* 7.96, $CH_2CH_2CH_2CH_3$), 1.30 (9H, t, *J* 7.96, OCH_2CH_3), 1.37 (6H, m, *J* 7.93, $CH_2CH_2CH_2CH_3$), 1.64 (6H, m, $CH_2CH_2CH_2CH_3$), 1.85 (6H, m, $CH_2CH_2CH_2CH_3$), 3.57 (3H, d, *J* 15.07, CH_2), 3.83 (9H, s, OCH_3), 4.14 (6H, m, OCH_2CH_3), 4.68 (3H, d, *J* 15.07, CH_2), 6.88 (3H, s, H_{arom}), 7.30 (3H, s, H_{arom}); δ_C(75,5 MHz; $CDCl_3$) 13.52 (s, C_d), 16.10–16.37 (m, OCH_2CH_3), 23.43–23.77 (m, C_c), 24.31–24.38 (m, C_b), 26.28–26.43 (m, C_a), 36.11 (CH_2), 56.06 (OCH_3), 61.90–62.05 (m, OCH_2CH_3), 114.08–114.11 (m, C_{arom}), 122.93–123.00 (m, C_{arom}),131.29–131.49 (m, C_{arom}), 136.52 (C_{arom}), 138.23 (m, C_{arom}), 149.34 (m, C_{arom}); δ_P (160.1 MHz, $CDCl_3$) 30.76–30.89 (4 s); *m/z* (LRMS, LSIMS) 875 ([M + Na]$^+$, 100%), 853 ([M + H]$^+$, 91%); *m/z* (HRMS, LSIMS) calc. for $C_{42}H_{63}O_{12}P_3$ + H: 853.360840, found: 853.361068.

2,7,12-Tris(lithium *n*-butylphosphonate)-3,8,13-trimethoxy-10,15-dihydro-5*H*-tribenzo-[*a,d,g*]cyclononene 1. To a solution of **4** (0.425 g, 0.5 mmol) in dry acetonitrile (10 mL), lithium bromide (0.519 g, 6 mmol) was added under inert conditions. The mixture was refluxed for seven days. After cooling to room temperature, the reaction mixture was filtered and the precipitate was extensively washed with hot dry acetonitrile. The crude solid was solubilized in absolute ethanol (3 mL) and precipitated in acetonitrile. The solid was filtrated and dried under vacuum. Compound **1** (0.315 g, 80%) was isolated as a white solid; mp >265 °C; ν_{max} (NaCl) 2943, 2830, 1452, 1120 cm^{-1}; δ_H (300.13 MHz; CD_3OD) 0.85 (9H, t, *J* 8, $CH_2CH_2CH_2CH_3$), 1.32 (6H, m, $CH_2CH_2CH_2CH_3$), 1.61 (12H, m, $CH_2CH_2CH_2CH_3$), 3.56 (3H, d, *J* 15, CH_2), 3.82 (9H, s, OCH_3), 4.75 (3H, d, *J* 15, CH_2), 7.00 (3H, s, H_{arom}), 7.39 (3H, s, H_{arom}); δ_C(75.5 MHz; CD_3OD) 14.40 (C_d), 25.58 (d, *J* 13.2, C_c), 27.05 (d, *J* 3.3, C_b), 28.36 (d, *J* 124, C_a), 37.07 (s, CH_2), 57.02 (s, OCH_3), 115.61 (s, C_{arom}), 124.67 (s, C_{arom}), 133.45 (s, C_{arom}), 136.83 (s, C_{arom}), 141.86 (d, *J* 5.5, C_{arom}), 151.05 (d, *J* 3.3, C_{arom}); δ_P(120.1 MHz; CD_3OD) 24.58 (s); *m/z* (LRMS, LSIMS) 793 ([M + Li]$^+$, 68%), 808 ([M + Na]$^+$, 54%); *m/z* (HRMS, LSIMS) calc. for $C_{36}H_{48}Li_3O_{12}P_3$ + Na: 809.275247, found: 809.273651.

Titration experiments

General procedure. Composition of physiological medium: NaCl (113×10^{-3} M), KCl (4.5×10^{-3} M), $CaCl_2{\cdot}2H_2O$ (2×10^{-3} M), $MgCl_2{\cdot}6H_2O$ (1×10^{-3} M), $NaHCO_3$ (25×10^{-3} M), $NaH_2PO_4{\cdot}H_2O$ (1×10^{-3} M), D-glucose (11×10^{-3} M); pH = 7.6.

Binding constants (K_{ass}) were calculated at 293 K using ^{1}H NMR (400.13 MHz) and fluorescence emission (Varian Cary Eclipse Spectrofluorimeter) titrations and non-linear regression analysis.[19] Cells of 10 mm inner diameter were used for the fluorescence experiments and solutions were degassed under argon.

Standard procedure for fluorescence titration in aqueous media (293 K). Guest aliquots (guest stock solution 0.5 M) were added to a solution of receptor **1** (1.0×10^{-5} M, 3.0 mL) placed in the fluorimeter cell. After each guest addition the cell was carefully shaken and allowed to equilibrate for 2 min before recording the next spectrum. The excitation wavelength was set at 280 nm. Intensity changes in the emission spectra of the receptor **1** were monitored and analyzed using a specifically written non-linear least squares curve-fitting program implemented within Origin. Assuming a 1 : 1 stoichiometry, the binding constant (K_{ass}) was calculated.

Standard procedure for NMR titration (293 K). 5×10^{-2} M and 5×10^{-1} M guest stock solutions in the appropriate deuterated solvent were prepared. Fifteen solutions containing identical quantity of host **1** (10^{-3} M) and increasing aliquots of the guest stock solution were prepared. The total sample volume was kept constant (0.5 mL) by addition of deuterated solvent. For each NMR tube, guest ([G]) and host ([H]) concentrations were calculated and ^{1}H and ^{31}P spectra were recorded at 293 K. The host's chemical shift variations were monitored and analyzed using the same procedure as above.

Notes and references

1 J. R. Cooper, F. E. Bloom and R. H. Roth, *The Biochemical Basis of Neuropharmacology*, Oxford University Press, New York, 1991, p. 190.
2 E. R. Kandel, J. H. Schwartz and T. M. Jessell, *Principles of Neural Science*, MacGraw Hill, United States, 4th edn, 2000, p. 280.
3 J. Klein, S. S. Chatterjee and K. Loffelholz, *Brain Res.*, 1997, **755**, 347.
4 J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Kluwer Academic/Plenum Publishers, New York, 2nd edn, 1999.
5 J.-P. Desvergne and A. W. Czarnik, *Fluorescent Chemosensors of Ion and Molecule Recognition*, NATO-ASI Series, Kluwer Academic Publishers, Dordrecht, 1996; A. P. De Silva, H. Q. N. Gunaratne, T. Gunnlaugsson, J. M. Huxley, C. P. McCoy, J. T. Rademacher and T. E. Rice, *Chem. Rev.*, 1997, **97**, 1515; B. Valeur and I. Leray, *Coord. Chem. Rev.*, 2000, **205**, 3.
6 M. Inouye, K. Hashimoto and K. Isagawa, *J. Am. Chem. Soc.*, 1994, **116**, 5517.
7 K. N. Koh, K. Araki, A. Ikeda, H. Otsuka and S. Shinkai, *J. Am. Chem. Soc.*, 1996, **118**, 755; Y. J. Zhang, W. X. Ko and J. Xu, *Chin. J. Chem.*, 2002, **20**, 322; T. Jin, *J. Inclusion Phenom. Macrocyclic Chem.*, 2003, **45**, 195; S. D. Tan, W. H. Chen, A. Satake, B. Wang, Z. L. Xu and Y. Kobuke, *Org. Biomol. Chem.*, 2004, **2**, 2719; H. Bakirci and W. M. Nau, *Adv. Funct. Mater*, 2006, **16**, 237; N. Korbakov, P. Timmerman, N. Lidich, B. Urbach, A. Sa'ar and S. Yitzchaik, *Langmuir*, 2008, **24**, 2580.
8 A. Collet, in *Comprehensive Supramolecular Chemistry*, ed. J. L. Atwood, J. M. Lehn, J. E. D. Davies, D. D. MacNicol and F. Vögtle, Elsevier, Amsterdam, 1996, vol. 2, p. 325; T. Brotin and J.-P. Dutasta, *Chem. Rev.*, 2009, **109**, 80.
9 S. Zhang and L. Echegoyen, *C. R. Chim.*, 2006, **9**, 1031; O. Reynes, F. Maillard, J.-C. Moutet, G. Royal, E. Saint-Aman, G. Stanciu, J.-P. Dutasta, I. Gosse and J.-C. Mulatier, *J. Organomet. Chem.*, 2001, **637–639**, 356.
10 L. Garel, B. Lozach, J.-P. Dutasta and A. Collet, *J. Am. Chem. Soc.*, 1993, **115**(24), 11652.
11 J. Canceill, A. Collet and G. Gottarelli, *J. Am. Chem. Soc.*, 1984, **106**, 5997; I. Gosse, J.-P. Dutasta, M. Perrin and A. Thozet, *New J. Chem.*, 1999, **23**, 545.
12 Prepared from monochloration of diethyl *n*-butylphosphonate which was previously synthesized using a modified Michaelis–Arbuzov rearrangement (see ESI†).G. M. Kosolapoff, *J. Am. Chem. Soc.*, 1945, **67**, 1180; S. Gobec, I. Plantan, J. Mravljak, R. A. Wilson, G. S. Besra and D. Kikelj, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 3559.
13 R. Karaman, A. Goldblum, E. Breuer and H. Leader, *J. Chem. Soc., Perkin Trans. 1*, 1989, 765; E. Breuer, M. Safadi, M. Chorev and D. Gibson, *J. Org. Chem.*, 1990, **55**, 6147; M. Herm and T. Schrader, *Chem.–Eur. J.*, 2000, **6**, 47.
14 Titration curves and fitting data are available in the ESI†.
15 M. Herm and T. Schrader, *Chem.–Eur. J.*, 2000, **6**, 47.
16 HyperChem™, release 8.0, hypercube, Inc. (2002) Waterloo, Ontario N21 3X2, Canada.
17 E. Bracci and S. Panzeri, *J. Neurophysiol.*, 2006, **95**, 1285.
18 Fluorescence titrations were also conducted between probe **1** and choline (Ch), the biological precursor and metabolite of ACh (in water, $K_{ass} = 70 \pm 4$ M^{-1} and in a physiological medium, $K_{ass} = 23 \pm 2$ M^{-1}). In the literature and in our case, synthetic hosts complexed ACh and Ch *via* the ammonium group. This strategy cannot allow selective recognition of ACh against Ch.
19 K. A. Connors, *Binding Constants*, Wiley, New York, 1987.

