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PAPER

C_3-triiodocyclotriveratrylene as a key intermediate to fluorescent probes: application to selective choline recognition†

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A new strategy to obtain fluorescent cyclotriveratrylene (CTV) probes is proposed. The key intermediate, a triiodo CTV, is prepared in 3 steps with 47% overall yield. The whole synthesis requires only one purification step. The potential of this triiodo CTV as an intermediate is illustrated through the synthesis of a fluorescent phosphorylated probe that is able to bind choline and acetylcholine in pseudo-physiological conditions, with selectivity towards choline. As a consequence, this intermediate should allow us to rapidly form a library of probes in order to highlight the most promising ones.

Introduction

Cyclotriveratrylenes (CTVs), cyclic trimers of veratrole, are supramolecular bowl-shaped structures known for their ability to complex with organic and organometallic guests.[1–3] Thus, there is a growing interest in the synthesis of modified CTVs from their first development by A. Collet *et al.* in the early 1980s.[4] In our group, we are particularly interested in developing CTVs as fluorescent probes, especially for acetylcholine (ACh) and choline (Ch) imaging (Fig. 1). In this area, we recently reported the first fluorescent CTV that is able to recognize similarly ACh and Ch in aqueous media.[5] Its fluorescence, due to an intramolecular charge transfer between electron-withdrawing and donating groups, located on the aromatic cores of the CTV, is altered in the presence of the guest. The recognition is mainly due to π–cation interactions between the CTV aryl groups and the ammonium part of the guest. Additional ionic interactions can also be induced by the substituents present on the aryl sub-units of the CTV.

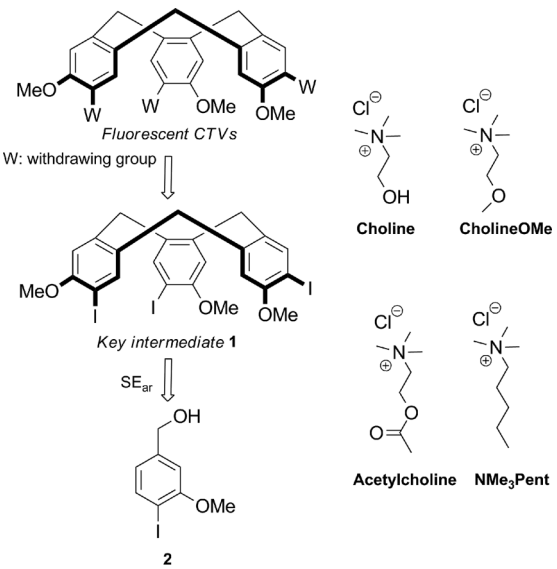

Fig. 1 Fluorescent CTVs for Ch and ACh detection: synthetic approach.

Other fluorescent probes are known to detect ACh and Ch, but their principle of detection is based on the competitive recognition between the guest and a previously complexed fluorophore.[6–8] When the guest is complexed by the non-fluorescent host, the fluorophore is released and recovers its luminescence. Recently, sub-micromolar concentrations of ACh have been detected using this strategy.[7] Nevertheless, with this method, information about the space-resolved migration of ACh is not available. As a consequence these systems do not allow the imaging of ACh or Ch. Hence, in order to highlight new molecular probes, the development of a library of CTVs functionalized by diverse groups

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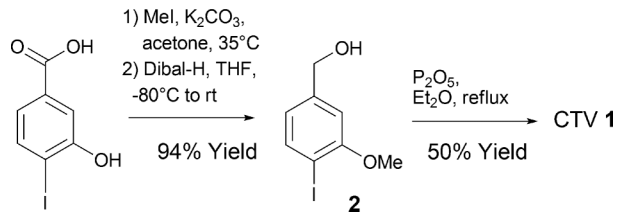
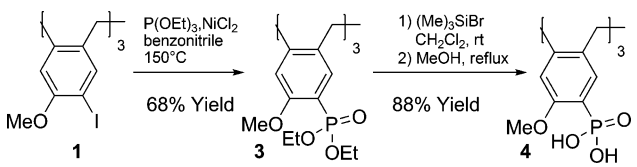
is of interest to evaluate both their fluorescence and recognition properties as well as their selectivity.

However, only few CTVs with electron-withdrawing groups have been synthesized to date.[1] Indeed, CTVs are obtained by aromatic electrophilic substitution (SE_{ar}) of 3,4-disubstituted benzyl alcohol derivatives. The trimerization reaction (SE_{ar}) is well-known to be efficient and regioselective when the substituents in positions 3 and 4 are both donating groups. In this case, the substituent in position 3 should be especially strongly activating towards SE_{ar}, most commonly a methoxy group. On the contrary, trimerization is less effective or even completely ineffective as soon as aromatic rings are functionalized with electron-withdrawing groups.[1,9] Therefore, with the aim of preparing a library of new fluorescent CTVs through a convergent and a minimum step synthesis, we focus our attention on the synthesis of the key CTV intermediate **1** (Fig. 1). This one presents in the *ortho*-position of the methoxy group, an iodine atom, designed to be replaced by electron-withdrawing groups *via* substitution reactions or more efficiently by organometallic coupling reactions.

The synthesis of **1** was reported a few years ago, in five steps and 43% overall yield.[10] The last step was a diazotization/iodination sequence of a triamino CTV intermediate. In this communication, we present an alternative route to access **1** in a minimum step synthesis *via* the trimerization of the 4-iodo-3-methoxybenzyl alcohol **2** (Fig. 1, Scheme 1). In addition, the potential of **1** as a key intermediate towards new fluorescent CTV probes is illustrated through the synthesis of the fluorescent phosphorylated CTV **4** which presents attractive recognition properties of ammonium guests in pseudo-physiological medium (100 mM buffer solution at physiological pH (Scheme 2)).



Scheme 1 Synthesis of CTV **1**.



Scheme 2 Functionalization of CTV **1**.

Results and discussion

As mentioned above, our strategy to prepare CTV **1** is based on the trimerization *via* SE_{ar} of the 4-iodo-3-methoxybenzyl alcohol **2** (Fig. 1). The trimerization of the 4-bromo analogue has been previously reported in the literature[11,12] with acceptable yields in the range of 40%, showing that a weak electron-withdrawing group does not deactivate the aromatic ring too much. The lower electron-withdrawing effect of an iodo substituent compared to a bromo one prompted us to attempt this strategy. With the iodine atom we expect to enhance the trimerization yield. As a result, we focused our attention on an efficient synthesis of compound **2**. Several methods exist to introduce an iodine atom on 3-hydroxy or 3-methoxy benzoic acid derivatives. Some of them are based on ortholithiation[13] or aromatic electrophilic substitution[14–17] reactions in the *ortho*-position of the hydroxy or methoxy groups. Others pass through the obtaining of a diazonium salt.[18–20] Nevertheless, as the 3-hydroxy-4-iodobenzoic acid is commercially available, it was decided that it would be the material of choice to prepare compound **2** (Scheme 1).

Thus, starting from 3-hydroxy-4-iodobenzoic acid, **2** was obtained without purification in two steps in 94% overall yield. After methylation with iodomethane, the methyl ester intermediate was efficiently reduced using diisobutylaluminium hydride. Not only do these conditions prevent the deiodination[21] observed when $LiAlH_4$ is used as the reductive agent but it also allows simultaneous attainment of the benzyl alcohol in 97% yield. Indeed, in a previously reported synthesis of **2**, the reduction of the methyl ester through the corresponding acid chloride, requires three steps, reducing the yield to 72% of the alcohol.[14]

The last step was the trimerization of **2** in the presence of phosphorus pentoxide in diethyl ether; the CTV **1** was obtained with 50% yield. At this step, we had to carry out the only and easy purification step of this synthesis. After filtration of the CTV **1** on a silica gel plug and elution with dichloromethane, the iodinate key intermediate was digested in a diethyl ether/CH_2Cl_2 (95/5) mixture.

Thus, CTV **1** was efficiently obtained in only three steps with a global yield of 47%. If this yield is similar to those previously reported by A. Collet *et al.*,[10] the main advances of the synthesis developed here lie in the minimum number of reaction and purification steps.

Introduction of electron-withdrawing groups from the triiodo CTV **1** was then investigated through the synthesis of the CTV **4** bearing phosphorylated groups (Scheme 2). Phosphonate groups have been chosen, in a first attempt, for their binding properties towards cations and their ability to improve the water solubility of hydrophobic molecules. Indeed, in the fluorescent CTV probe that we have previously described, phosphonate groups have shown a good capability to bind ammonium parts of choline and acetylcholine.[5] In the CTV probe **4** prepared in the present study and contrary to the former, phophorylated groups are directly conjugated to the aromatic cores that might be advantageous for the fluorescent properties in terms of charge transfer.

The synthesis of CTV **4** *via* the substitution of the iodine atoms is described in Scheme 2. The phosphorylation of **1** using triethyl phosphite[22] in the presence of nickel chloride as the catalyst was shown to be efficient, considering that three iodine atoms have to be substituted. The tri(diethyl phosphonate) CTV **3** was obtained with 68% yield. This intermediate was then dealkylated using bromotrimethylsilane as the silylating reagent[23] leading to CTV **4** with an 88% isolated yield.

The absorption and fluorescence properties of CTV **4** were investigated in pseudo-physiological conditions (HEPES buffer 100 mM, pH = 7.5) (Fig. 2a). In such conditions, the new water-soluble phosphorylated CTV **4** has an emission wavelength at 316 nm when excited at 295 nm. The fluorescent quantum yield ϕ and the time decay τ of **4** were determined ($\phi = 0.11$ and $\tau = 2.4$ ns).

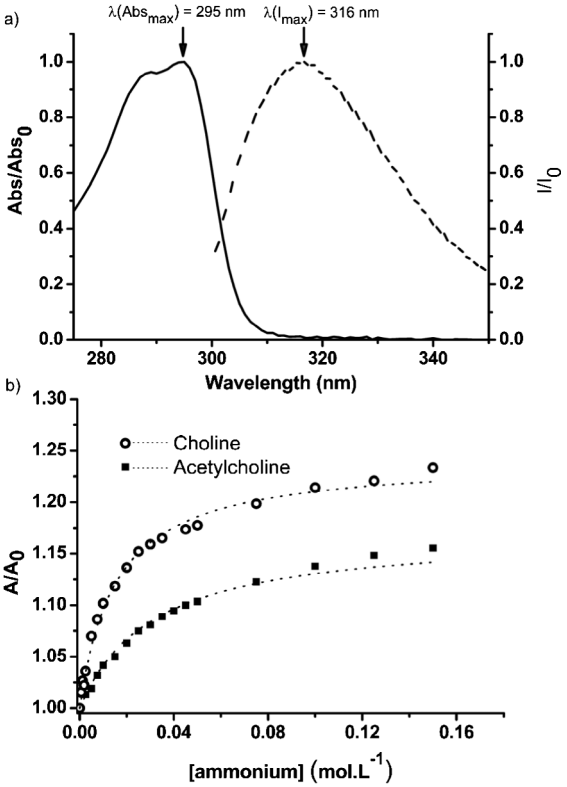


Fig. 2 a) UV-Vis (solid line) and fluorescence (dashed line) spectra of 10^{-5} M CTV **4** in 100 mM HEPES buffer pH 7.5; b) relative fluorescence area of **4** (10^{-5} M) with increasing ammonium concentrations (0 to 0.16 M) (○) choline and (■) acetylcholine fitted with a 1 : 1 binding model.

Binding properties of **4**, in the conditions described above, were studied. Titrations of CTV **4** with quaternary ammonium species of biological interest such as ACh and Ch, present in the brain, were achieved using fluorescence emission (Fig. 2b). Each titration was done three times and led to reproducible results. An increase of the fluorescence intensity of 15 and 23% for ACh and Ch respectively was observed. In contrast, the fluorescence intensity was unchanged after addition of salts such as sodium chloride, or tetrapentylammonium in the same concentration range (results not shown).

Assuming a 1 : 1 stoichiometry, the binding constants (K_{ass}) were calculated.[24] Binding constants of 23.5 ± 1 M^{-1} (R^2 = 0.998) for ACh and 66 ± 4 M^{-1} (R^2 = 0.993) for Ch were found. The affinity of CTV **4** for Ch or ACh may seem weak compared to those described in the literature,[6,7] however, the binding constants of the previously described systems are based on indirect detection methods and most of them are not determined in highly concentrated salt conditions.

Moreover, we confirmed by NMR experiments the formation of a complex between CTV **4** and both Ch and ACh. Indeed, on NOESY spectra, correlation spots were observed between the methyl groups of the guest (Ch or ACh) and the aromatic protons of CTV **4** (Fig. 3). Additional NMR diffusion experiments (DOSY), which have been particularly used in supramolecular chemistry to investigate issues of structure in molecular assembly,[29] were also consistent with the binding of Ch and ACh by the CTV **4**. Besides the free CTV **4** and guest populations, we observed a new population that enhances with the amount of the guest, corresponding and confirming the formation of CTV**4**:guest complexes (see ESI†).

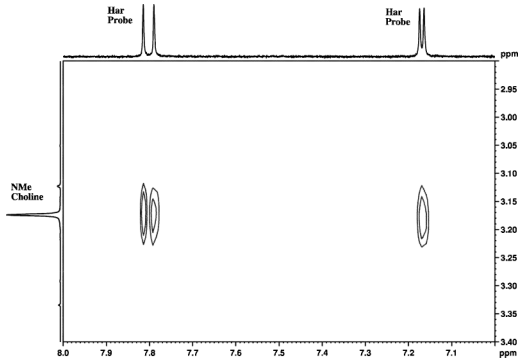


Fig. 3 Selected region of the NOESY spectrum in D_2O of CTV **4** in the presence of an excess of Ch (mixing time 2 s).

As for the selectivity, we observed by fluorescence experiments, a preference of CTV **4** for choline. It is noteworthy that only a few examples of ammonium probes show some selectivity between Ch and ACh.[25–28] With a $K_{Ch} : K_{ACh}$ ratio of 2.8, our system has a selectivity comparable to the best non-fluorescent selective probe previously published in DMSO.[25]

The structure/selectivity relationship is difficult to forecast although some papers[8,27,30] highlight hydrophobicity and/or hydrogen bond interactions as critical elements. To understand, in our case, the origin of the selectivity observed for choline, we performed additional titration experiments (see ESI†).

Using tetramethylammonium (NMe_4), trimethylpentylammonium (NMe_3Pent) and methoxycholine (CholineOMe) (Fig. 1), we expected to evaluate the influence of the guest chain on the selectivity. Complexation constants of 48 ± 2 M^{-1} (R^2 = 0.994), 16.5 ± 2 M^{-1} (R^2 = 0.996), 18.5 ± 1 M^{-1} (R^2 = 0.997) were respectively obtained. NMe_4 is better recognized than all the tested tetraalkylammonia except for Ch. This result indicates that if charge–charge and/or π–cation interactions are mostly responsible for the binding properties of the probe, additional interactions have to be considered to explain the selectivity observed for Ch. Polarity of the chain seems significant but is not the only factor since NMe_3Pent, ACh and CholineOMe have similar K_{ass}. In addition, the fact that CholineOMe is much less recognized than Ch allows to envisage the existence of a hydrogen bond between the hydroxyl group of the choline and one of the phosphonate groups of the probe.

In order to confirm the presence of this hydrogen bond in the CTV **4**:Ch complex, molecular modelling was undertaken. Geometry optimization in a periodic box containing water molecules using the MM^+ molecular mechanics calculation was carried out. A stable conformation of the **4**:Ch structure (Fig. 4)[31] showed size complementarities 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-center. Ionic interactions between the negative phosphonate groups and the ammonium part of Ch can occur (distances of 4.39, 4.46 and 4.94 Å have been measured). It is also noteworthy that one hydrogen bond is possible between one phosphonate of **4** and the OH group of the choline (a distance H . . . O of 3.8 Å and an angle of 110° are observed).[32] This interaction is probably responsible for the selectivity of CTV **4** for choline.

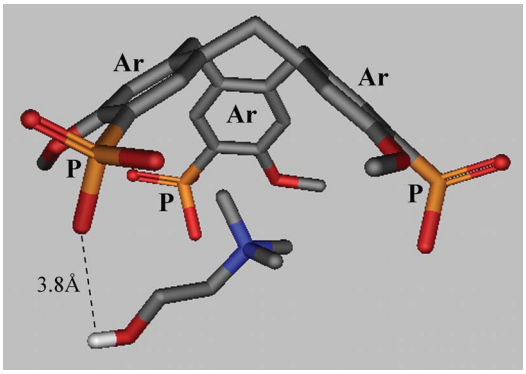


Fig. 4 Stable conformation of the **4**:Ch complex obtained by MM^+ molecular mechanics (cubic box of 56 Å containing 5833 water molecules). Water molecules and protons have been omitted for clarity.

Conclusion

To conclude, in this study we have developed a new route to prepare the C_3-triiodocyclotriveratrylene compound **1** in three steps with an overall yield of 47%. One of the major advantages of our synthesis compared to those previously reported[10] is that only a simple filtration as step of purification is required. Through the preparation of the phosphorylated CTV **4**, this study also proved the synthetic interest of **1** to access to fluorescent cyclotriveratrylenes bearing electron-withdrawing groups which are difficult or not possible to obtain by electrophilic aromatic substitution of corresponding benzyl alcohol derivatives. These CTVs are of interest as fluorescent molecular probes especially to bind biological ammonium species such as choline and acetylcholine. In particular, as the excitation wavelength of CTV **4** is too low for *in vivo* applications, it could be interesting to extend the conjugation between acceptor and donor groups to increase its excitation wavelength. Moreover it could also increase the hydrophobicity of the probes and modulate the selectivity. We plan to synthesize such more conjugated CTVs *via* carbon–carbon coupling reactions from the intermediate **1** and thus rapidly obtaining a library of probes to highlight the most promising ones.

Experimental

General methods and materials

All commercially available reagents were used as received. Dichloromethane was freshly distilled from CaH_2 before use. Tetrahydrofuran was freshly distilled from sodium and benzophenone before use. NMe_3Pent and CholineOMe as the chloride salts have been synthesized as previously reported.[33] Acetone was dried over $MgSO_4$ before use. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker Ultrashield Avance 400 spectrometer. Chemical shifts are reported using tetramethylsilane or the residual solvent peak as internal reference for ^1H or for ^{13}C. Infrared were determined on a Nicolet iS10 FT-IR spectrometer. Melting points were measured using a Kofler Heizbank melting point bench (model 7841). High resolution mass spectra were performed by the CESAMO (Bordeaux, France) on a QStar Elite mass spectrometer (Applied Biosystems). UV-Vis spectra were recorded on a CARY 100 scan spectrophotometer and fluorescence spectra on a CARY Eclipse spectrophotometer. Time-resolved fluorescence experiments were performed using a frequency-tripled Nd-YAG laser (355 nm, 8 ns pulse) for excitation. Right-angle detection was used to collect the luminescence spectra using a gated CCD camera and spectrograph at variable delays.

Synthesis

4-Iodo-3-methoxybenzyl alcohol (2). The 3-hydro-4-iodobenzoic acid (1 g, 3.8 mmol) was dissolved in 50 mL of dried acetone under a nitrogen atmosphere and potassium carbonate (2.1 g, 15.2 mmol) was added. The reaction mixture became white and cloudy. The iodomethane (1.42 mL, 22.8 mmol) was added slowly and the reaction mixture was warmed to 35 °C under stirring overnight. Then, acetone was evaporated under vacuum. The crude product was dissolved in 80 mL of ethyl acetate and washed with distilled water until neutrality of the aqueous phase was achieved. The organic phase was dried with anhydrous magnesium sulfate and concentrated under vacuum. The 4-iodo-3-methoxybenzoic acid methyl ester was obtained as a bright-yellow oil (1.1 g, 98% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, J = 8 Hz, 1H, Ar–H), 7.40 (d, J = 2 Hz, 1H, Ar–H), 7.35 (dd, J = 8 Hz, 2 Hz, 1H, Ar–H), 3.93 (s, 3H, $COOCH_3$), 3.90 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.2 ($COOCH_3$), 157.9 (C_{Ar}), 139.3 (C_{Ar}), 131.4 (C_{Ar}) 123.1 (C_{Ar}), 110.9 (C_{Ar}), 92.5 (C_{Ar}), 56.3 ($COOCH_3$), 52.2 (OCH_3). IR (ν cm^{-1}): 3021, 2971, 2857, 1728, 1586, 1485, 1442, 1228, 1186, 1100, 1042, 1013. HRMS (ESI): calc. $C_9H_9O_3I$, Na adduct 314.9488, found 314.9500.

The 4-iodo-3-methoxybenzoic acid methyl ester (2.4 g, 8.2 mmol) was dissolved in 40 mL of dried tetrahydrofuran under an argon atmosphere. The mixture was cooled to −80 °C before the dropwise addition of diisobutylaluminium hydride (Dibal-H) (41.1 mL of 1 M solution in hexane, 41.1 mmol). The mixture was stirred at room temperature overnight and then quenched at 0 °C by addition of 150 mL of 1 M HCl solution. The product was extracted with dichloromethane (150 mL × 3). All the combined organic phases were washed with distilled water until neutrality of the aqueous phase was achieved and then dried over anhydrous magnesium sulfate. After concentration under vacuum, the 4-iodo-3-methoxybenzyl alcohol **2** was obtained as a bright-yellow oil (2.07 g, 96% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 7.68 (d, J = 8 Hz, 1H, Ar–H), 6.81 (s, 1H, Ar–H), 6.64 (d, J = 8 Hz, 1H, Ar–H), 4.59 (s, 2H, CH_2OH), 3.84 (s, 3H, OCH_3), 2.44 (br, 1H, OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.2 (C_{Ar}), 143.0 (C_{Ar}), 139.4 (C_{Ar}), 120.8 (C_{Ar}), 109.5 (C_{Ar}), 84.6 (C_{Ar}), 64.7 (CH_2OH), 56.4 (OCH_3). IR (ν cm^{-1}): 3329, 2950, 2857, 1593, 1464, 1393, 1257, 1043, 1014. HRMS (ESI): calc. $C_8H_9IO_2$, Na adduct 286.9539, found 286.9539.

2,7,12-Triiodo-3,8,13-trimethoxy-10,15-dihydro-5*H*-tribenzo[*a*,*d*,*g*]cyclononene (1). A suspension of phosphorus pentoxide P_2O_5 (5.75 g, 40.4 mmol) in 70 mL of dried diethyl ether was added under nitrogen to a vigorously stirred mixture of 4-iodo-3-methoxybenzyl alcohol **2** (2.3 g, 9 mmol) in 2 mL of anhydrous diethyl ether. The reaction mixture was warmed to reflux and the stirring stopped, but the nitrogen conditions were maintained. After 3 days the diethyl ether was evaporated. The crude product was triturated with dichloromethane (100 mL) and filtered through silica gel in a large, coarse-fritted glass funnel. The silica gel was rinsed with dichloromethane (150 mL × 4). The filtrate

was concentrated under vacuum. The obtained solid was finally digested with diethyl ether : dichloromethane (95 : 5) (10 mL × 3) to give the CTV **1** as a white solid (524 mg, 50%). mp > 285 °C (decomposition). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (s, 3H, Ar–H), 6.74 (s, 3H, Ar–H), 4.65 (d, J = 13.6 Hz, 3H, Ar–CH_2), 3.86 (s, 9H, OCH_3), 3.58 (d, J = 13.6 Hz, 3H, Ar–CH_2). ^{13}C NMR (100 MHz, THF-d_8) δ 158.4 (C_{Ar}), 142.0 (C_{Ar}), 141.5 (C_{Ar}), 134.5 (C_{Ar}), 113.3 (C_{Ar}), 84.4 (C_{Ar}), 56.9 (Ar–CH_2), 36.2 (COCH_3). IR (ν cm^{-1}): 3010, 2914, 2839, 1585, 1448, 1470, 1431, 1249, 1039. HRMS (ESI): calc. $C_{24}H_{21}O_3I_3$, Na adduct 760.8517, found 760.8535.

Diethyl 12 - [bis(hydroxymethoxy)(methylene)phosphoranyl] - 7-(diethoxyphosphoryl)-3,8,13-trimethoxy-10,15-dihydro-5*H*-tribenzo[*a*,*d*,*g*]cyclononen-2-ylphosphonate (3). The CTV **1** (150 mg, 0.2 mmol) was diluted in 6 mL of benzonitrile under argon atmosphere. Nickel chloride (31.6 mg, 0.4 mmol) and triethylphosphite (660 µL, 3 mmol) were added, and the solution was allowed to react at 150 °C overnight. The reaction mixture was cooled down to room temperature. The crude solution was diluted in 50 mL of toluene and washed three times with a 5% aqueous ammonia solution. Then the organic phase was washed with water until neutrality of the aqueous phase, dried over anhydrous magnesium sulfate and concentrated under vacuum. The residue was finally purified by chromatography on silica gel, eluted with ethyl acetate : methanol 100 : 0 up to ethyl acetate : methanol, 90 : 10. CTV **3** was obtained as a white solid (106 mg, 68%). mp 245 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (d, J_{P-H} = 15 Hz, 3H, Ar–H), 6.96 (d, J_{P-H} = 7 Hz, 3H, Ar–H), 4.78 (d, J = 13.6 Hz, 3H, Ar–CH_2), 4.07 (br, 12H, OCH_2CH_3) 3.86 (s, 9H, OCH_3), 3.75 (d, J = 13.6 Hz, 3H, Ar–CH_2), 1.30 (t, J = 7.2 Hz, 9H, OCH_2CH_3), 1.26 (t, J = 7.2 Hz, 9H, OCH_2CH_3). ^{31}P NMR (162 MHz, $CDCl_3$) δ 16.8 (s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.2 (s, C_{Ar}), 146.2 (s, C_{Ar}), 137.5 (s, J_{P-C} = 8 Hz, C_{Ar}), 130.4 (d, J_{P-C} = 15 Hz, C_{Ar}), 115.3 (d, J_{P-C} = 187 Hz,C_{Ar}), 112.8 (d, J_{P-C} = 10 Hz, C_{Ar}), 62.2 (OCH_2CH_3), 56.1(OCH_3), 36.7 (Ar–CH_2), 16.5 (OCH_2CH_3). IR (ν cm^{-1}): 2980, 2908, 1599, 1488, 1469, 1242, 1064, 1024. HRMS (ESI): calc. $C_{36}H_{51}O_{12}P_3$, Na adduct 791.2485, found 791.2485.

3,8,13-Trimethoxy-7,12-diphosphono-10,15-dihydro-5H-tribenzo[a,d,g]cyclononen-2-ylphosphonic acid (4). CTV **3** (50 mg, 0.065 mmol) was diluted in 3 mL of dried dichloromethane, under argon atmosphere. Bromotrimethylsilane (155 µL, 1.17 mmol) was added, and the reaction mixture was left at room temperature overnight. Then the dichloromethane was evaporated and 8 mL of methanol were added to the crude mixture. The reaction mixture was warmed to reflux. After 6 h, the solution was cooled to room temperature and filtered. The crude solid was rinsed with methanol and dried overnight under vacuum at 40 °C to give the CTV **4** (34 mg, 88%). mp > 265 °C (decomposition). ^{1}H NMR (400 MHz, D_2O) δ 7.78 (d, J = 15.2 Hz, 3H, Ar–H), 7.14 (d, J = 6.4 Hz, 3H, Ar–H), 4.83 (d, J = 13.6 Hz, 3H, Ar–CH_2), 3.85 (s, 9H, OCH_3), 3.78 (d, J = 13.6 Hz, 3H, Ar–CH_2). ^{31}P NMR (162 MHz, D_2O) δ 12.1 (s). ^{13}C NMR (100 MHz, D_2O) δ 159.6 (s, C_{Ar}), 145.2 (s, C_{Ar}), 135.6 (d, J_{P-C} = 7 Hz, C_{Ar}), 130.8 (d, J_{P-C} = 13 Hz, C_{Ar}), 120.3 (d, J_{P-C} = 175 Hz, C_{Ar}), 112.8 (d, J_{P-C} = 9 Hz, C_{Ar}), 55.7 (OCH_3), 35.8 (Ar–CH_2). IR (ν cm^{-1}): 3372, 2940, 1595, 1491, 1463, 1260, 1063, 989, 907. MALDI-MS: m/z for $C_{24}H_{27}O_{12}P_3$, Na adduct, calc. 623.4, found 622.9.

Spectroscopic characterisations of CTV 4. All the measurements were performed in HEPES 100 mM, pH = 7.5 solution, at 293 K. The molar extinction coefficient $\varepsilon_{(295\,nm,\,HEPES\,100\,mM,\,pH=7.5)}$ = 7995 ± 95 (R^2 = 0.9991) M^{-1}.cm^{-1} was obtained in the range of 10^{-4}–10^{-7} M. The data curve absorbance *versus* concentration was fitted with a linear curve-fitting equation (with interception at 0 for x = 0) implemented within Origin®. Emission spectra were recorded with λ_{ex} = 295 nm ± 5 nm. The fluorescence quantum yield ϕ = 0.11 was obtained by using Rhodamine 101 in ethanol as a reference (ϕ_{ref} = 0.92 ± 0.02).

Fluorescence titration experiments

Guest aliquots (0.5 M guest stock solution of Ch or ACh) were added to a 10^{-5} M solution of receptor CTV **4** in HEPES 100 mM, pH = 7.5 (2.0 mL) placed in the fluorimeter cell at 293 K. After each guest addition, the cell was carefully shaken and allowed to equilibrate for 2 min before recording the emission spectrum. The excitation wavelength was set at 295 nm ± 5 nm. Intensity changes in the emission spectra of the CTV **4** were monitored. The integration of the signal is done between 300 and 500 nm in order to obtain the area of the curve as a function of the guest concentration. This curve can be analyzed using a specifically written nonlinear least square curve-fitting program implemented within Origin®. The volume change due to the guest addition is taken into account in the calculation. Assuming a 1 : 1 stoichiometry, the binding constants (K_{ass}) were calculated.

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