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Selective detection of choline in pseudo-physiological medium with a fluorescent cage receptor

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A new fluorescent cage receptor for the detection of choline in pseudo-physiological medium is described. Not only does this capsule complex choline with an association constant greater than $9.9 \times 10^4 \text{ M}^{-1}$ but it is also highly selective towards acetylcholine.

Choline (Ch), known as vitamin B4 is an essential nutrient. It is also the precursor and the metabolite of acetylcholine (ACh), one of the major neurotransmitters. Both play an important role in various physiological activities, including learning and memory processes,¹ emotion or motor coordination.² In addition, abnormal levels of these compounds are suspected to be involved in mood disorders³ as well as in neurodegenerative pathologies such as Alzheimer's and Parkinson's diseases.^{4, 5} Consequently, a plethora of electrochemical sensors⁶⁻⁹ for ACh and Ch have emerged in the last two decades. Numerous optical sensors including chemiluminescent, electrochemiluminescent, colorimetric, fluorogenic and ratiometric sensors^{6, 10-14} have also been described. Nevertheless, an electrochemical oxidation or an enzymatic transformation of the analyte into hydrogen peroxide, betaine or hydroxyl radical is required with most of electrochemical and optical sensors which limits their use to the quantification of the analyte. To detect these species, fluorescent molecular probes^{15, 16} have been designed relying mostly on the fluorophore displacement concept.¹⁷⁻³¹ In this case, a non-emissive complex resulting from the interaction between a macrocyclic host and a fluorophore serves as a probe. If the affinity of the host for the target molecule is strong enough, the fluorophore is released in the presence of the target and an emission of fluorescence is observed. Although

sensitive, such systems allow for the detection and the quantification of ACh and Ch, but not for their imaging. In order to perform a real-time monitoring of the species, it is preferable to use molecular probes whose fluorescence emission is modified when the analyte is recognized.

Fluorescent cavitands able to recognize ACh in chloroform were reported by groups of Rebek³² and Dutasta.³³ Sarmentero and Ballester developed a cavitand for the selective recognition of Ch in methanol ($K_{\text{Ch/ACh}}=3.2$).³⁴ Moreover, our group, interested in the complexing properties of cyclotrimeratrylenes (CTV), described fluorescent CTV based probes for ACh and Ch in buffered aqueous media.³⁵⁻³⁷ It is worth noting that some of these fluorescent hosts interact more strongly with either ACh³⁶ or Ch.³⁷ Nevertheless, their association constants in these competitive media are too weak, especially for Ch ($K_{\text{Ch}} < 250 \text{ M}^{-1}$). So we turned our attention towards hemicyptophanes (HC), compounds known to complex, among other species, ammonium in solution.³⁸ They are capsules made of a CTV unit linked to another C_3 -symmetrical moiety. Martinez's group described the first water-soluble HC able to bind selectively Ch at basic pH.³⁹ This HC is characterized by a tris(2-aminoethyl)amine (TREN) group. These capsules can be made fluorescent by either introducing a fluorophore in the linker connecting both C_3 symmetrical moieties⁴⁰ or grafting a fluorescent entity onto the CTV unit. The main advantages of the second strategy are the possibility to introduce various fluorophores according to a methodology developed for the functionalization of CTV⁴¹ and modify the size and the structure of the capsule without being limited by the presence of the fluorophore. The first approach allowed us to access to fluorescent HC **1**, **2** and **3**; the second one to the HC **4** (Fig. 1).⁴²⁻⁴⁴ All these hosts bind ACh in organic solvent (containing 2% of water) with association constant superior to 10^4 M^{-1} . HC **4** was not able to discriminate between ACh and Ch in organic solvent but HC **1**, **2** and **3**, having a tripodal benzenic platform as second C_3 -symmetrical unit, were found to be selective for ACh ($K_{\text{ACh/Ch}}=4.1$, $K_{\text{ACh/Ch}}=2.8$ and $K_{\text{ACh/Ch}}=4.4$ respectively). Very recently, diastereoisomeric fluorescent cages with sucrose

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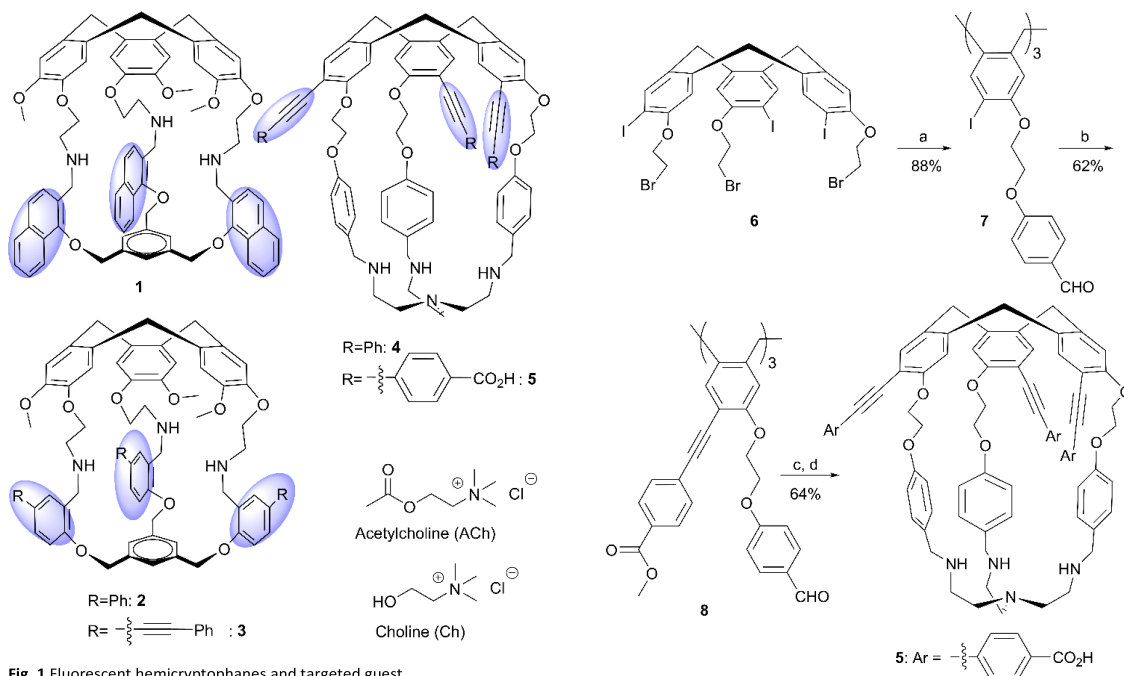


Fig. 1 Fluorescent hemicyptophanes and targeted guest

were reported by Szyszka *et al.* for the recognition of Ch and ACh in acetonitrile.⁴⁵ Despite one of these hosts is selective for ACh ($K_{ACh}/K_{Ch}=3.1$), the selectivity of the most efficient receptor for Ch is modest ($K_{Ch}/K_{ACh}=1.7$). Thus, although some hosts selective for ACh exist, those for Ch are very scarce and selective imaging of ACh or Ch with fluorescent supramolecular host remains very challenging, more particularly in aqueous medium. Here we report the synthesis of the water-soluble and fluorescent hemicyptophane **5**. Interestingly, this fluorescent cage recognizes Ch in pseudo-physiological medium with a better selectivity over ACh than hosts working in organic solvents.

The hemicyptophane **5** was synthesized from the CTV **6**, in four steps, following the synthetic route described in Scheme 1. In DMF, nucleophilic substitution of the bromine atoms by the phenoxides of 4-hydroxybenzaldehyde afforded **7** in 88% yield. This compound reacted with methyl-4-ethynylbenzoate under Sonogashira coupling conditions to afford **8** with 62% yield. Reductive amination in the presence of tris(2-aminoethyl)amine drove to a hemicyptophane intermediate that was immediately saponified in basic conditions. Removal of the solvent followed by acidification to pH 1 led to the precipitation of the desired water-soluble hemicyptophane **5**. This one was recovered in 64% yield from **8** after filtration and was fully characterized by NMR in deuterated methanol. As shown in Fig. 2, the proton NMR spectrum is consistent with the C_3 -symmetrical structure of the HC **5**. The two singlets at 7.6 and 7.3 ppm, attributed to the aromatic protons H_a and H_b , as well as the two signals around 4.9 and 3.7 ppm, corresponding to the diastereoisomeric protons H_c and $H_{c'}$, are characteristic of a

- a) p-hydroxybenzaldehyde (3.3 equiv.), Cs_2CO_3 (4.4 equiv.), DMF, 40°C, 18h
 b) Methyl-4-Ethynylbenzoate (9 equiv.), Et_3N (excess), CuI (0.6 equiv.), $PdCl_2(PPh_3)_2$ (0.6 equiv.), toluene, 40°C, N_2 , 2 days.
 c) 1) Tris(2-aminoethyl)amine (1.1 equiv.), DMF/MeOH, 45°C, N_2 , 3 days.
 2) $NaBH_4$ (excess), 0°C to 25°C, 4h.
 d) 1) 5M $NaOH_{aq}$, THF, 50°C, 24h. 2) 1M HCl_{aq} .

Scheme 1 Access to the water-soluble hemicyptophane **5**

symmetrical disubstituted CTV. The diastereoisomeric protons of the tris(2-aminoethyl)amine group H_u , H_v , H_w and $H_{u'}$ and the benzylic protons H_n and $H_{n'}$ appears as four multiplets between 2.4 and 3.2 ppm and two doublets at 3.7 and 3.9 ppm respectively. The signals between 4.3 and 4.9 ppm were assigned to the methylene protons H_d , $H_{d'}$, H_e and $H_{e'}$. Finally, the doublets around 6.8 and 7.2 ppm correspond to the aromatic protons H_k and H_l and those around 7.3 and 7.8 ppm to the protons H_r and H_s .

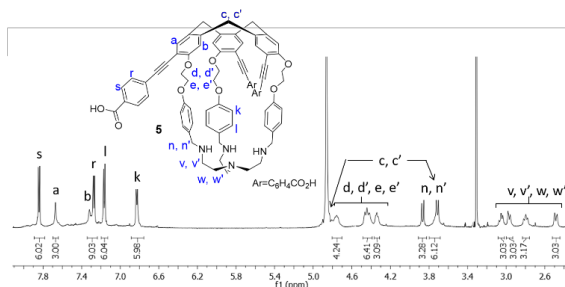


Fig. 2 1H NMR spectrum of hemicyptophane **5** in deuterated methanol (600 MHz, 25°C).

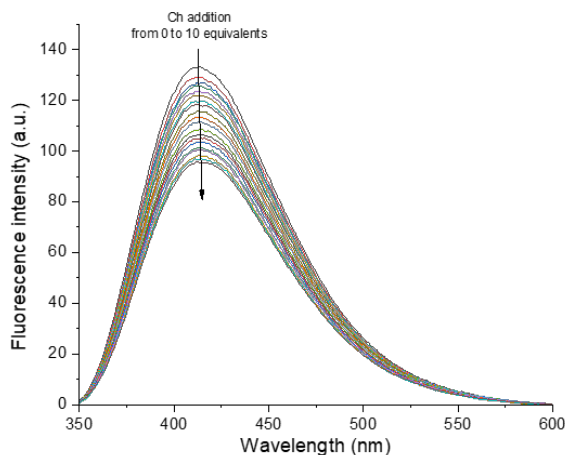


Fig. 3 Fluorescence emission of HC 5 at 2.5×10^{-5} M in a 140 mM PBS buffered solution (pH 7.4) upon addition of Ch. Chloride ions were used as ammonium counter ions. The temperature was set at 293 K.

The absorption and emission properties of hemicryptophane **5** were investigated in a 140 mM PBS buffer (pH 7.4) containing 2% of DMSO. Under these conditions, HC **5** shows three absorption peaks at 282 ($\epsilon = 3.5 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$), 302 ($\epsilon = 3.8 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and 330 ($\epsilon = 3.5 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) nm and exhibits a maximum emission wavelength at 413 nm when excited at 330 nm (Fig. S1 in ESI). DFT calculations were used to calculate the UV-visible electronic absorption spectrum involved in HC **5** (Fig. S4 and S6a in ESI) with Gaussian 09,⁴⁶ using CAM-B3LYP/6-31g(d,p) as quantum method. The theoretical absorption spectrum is consistent with the experimental one. Indeed, from the calculated electronic transitions in water, theoretical absorption bands at 278-282, 319 and 333 nm are obtained (Fig. S7 in ESI).

The fluorescent quantum yield Φ and the decay time τ were determined ($\Phi = 24\%$ using 9,10-diphenylanthracene as reference and $\tau = 4.7$ ns). Compared with the non-water-soluble fluorescent HC **4**, the spectroscopic characteristics are improved. Indeed, the excitation and emission wavelengths are red-shifted from 11 and 59 nm respectively. Moreover, both the quantum yield and the decay time are approximately doubled. This can be explained by the acceptor mesomeric effect of the carboxylate groups that increases the charge transfer from the oxygen atoms of the CTV skeleton.

The binding properties of HC **5** toward Ch and ACh were investigated in this pseudo-physiological medium at 20°C. Increasing amounts of guest were added to a 2.5×10^{-5} M solution of HC **5**; both absorption and emission spectra ($\lambda_{\text{ex}} = 330$ nm) were recorded. While the addition of guest does not change the absorption spectrum (see Fig S7 in ESI), a drop in fluorescence emission is observed for both guests as seen in Fig. 3 for Ch. The plot of the fluorescence emission ratio *versus* the addition of titrating reagent shows a slope break after addition of one equivalent of guest which suggests the formation of 1:1 stoichiometry complexes (Fig. 4). Moreover, the emission intensity is much more affected by Ch and slopes

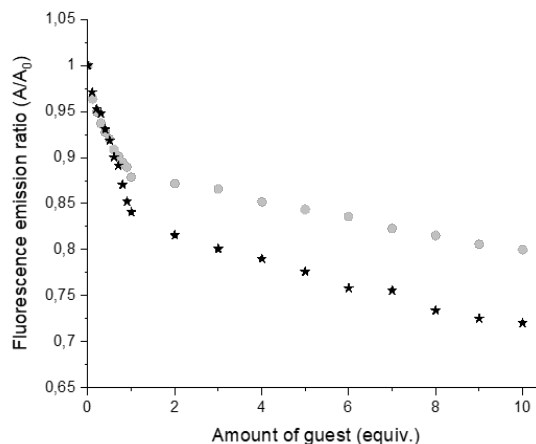


Fig. 4 Fluorescence emission of HC 5 at 2.5×10^{-5} M in a 140 mM PBS buffered solution (pH 7.4) upon addition of ACh (grey discs) or Ch (black stars). A_0 and A are the fluorescence emission area of the free and bound HC **5**, respectively. Chloride ions were used as ammonium counter ions. The temperature was set at 293 K.

slop is steeper in case of this guest, suggesting a higher affinity of HC **5** for Ch. ACh and Ch titration experiments were repeated, using, each time, fresh solutions of host and guest. Ch titration experiments were done by two experimenters. Results are reproducible (Fig. S3 in ESI).

Complexes of stoichiometry 1:1 were confirmed using Hypsep®. The binding constants K_{ACh} and K_{Ch} of such complexes ($K_{\text{ACh}} = 2.91 \times 10^4 \text{ M}^{-1}$, $K_{\text{Ch}} = 9.92 \times 10^4 \text{ M}^{-1}$) confirm the selectivity of the hemicryptophane **5** for Ch ($K_{\text{Ch}}/K_{\text{ACh}} = 3.4$). To our knowledge, this is the best selectivity for Ch over ACh in pseudo physiological environment observed to date. Such a selectivity may be surprising since no discrimination between ACh and Ch was observed with HC **4**. The response in emission is also different. In organic medium, an increase of fluorescence was observed with HC **4** while an extinction of fluorescence is observed with HC **5** in aqueous medium. To better understand the interactions involved in Ch@HC **5** and ACh@HC **5** complexes and rationalize the quench of the fluorescence emission observed, host-guest interactions were modelled via time dependent density functional theory (TD-CAM-B3LYP) calculations (see ESI for details). Stable (1:1) complexes were obtained with both guests (Fig. S6b and S6c in ESI). Various guest's orientations inside the HC were studied and their stabilization energy (SE) calculated from the electronic energies of complexes, guests and host ($\text{SE} = E_{\text{Guest@HC 5}} - (E_{\text{HC 5}} + E_{\text{Guest}})$, Table S2). For both guest, the most stable complex is always the one in which the quaternary ammonium of the guest points to the TREN moiety (namely BOTTOM in the ESI in opposition to the other forms named TOP). In particular, a difference in stabilization energy of 13.6 kcal/mol was found for both BOTTOM and TOP ACh@HC **5** complexes studied. For Ch@HC **5** complexes this difference in the stabilization energy is of the same order of magnitude i.e. 7.0 kcal/mol. Calculations finally show that the Ch@HC **5**_BOTTOM complex is globally the most stable conformation by about 4.5 kcal/mol in comparison to the BOTTOM form in the ACh@HC **5** situation. This energy

difference corresponds to the equivalent of a moderate hydrogen bond contribution. More specifically for the BOTTOM forms complexes, ionic interactions were found between the ammonium of the guest and the lone pair of one of the nitrogen atoms of the TREN as well as hydrogen bonds between the methyl groups of the guests and the carboxylates or ethylenedioxy chains of the cage (Fig. S6b and S6c of the ESI). Specific interactions into the ACh@HC **5** complex were also detected i.e. weak Me(RNMe₃⁺) – ethylenedioxy chain interactions and cation- π interactions between aromatics of alkynylaryl groups of the host and the trimethylammonium of ACh (Fig. S6b). The situation is different for the Ch@HC **5** complex where the hydroxyl group of Ch interacts with one benzoate via 1) π -hydrogen bond with the benzoate's aromatic ring and 2) weak hydrogen bonds with oxygen atoms of the carboxylate (Fig. 5). These interactions seem to stabilize the Ch@HC **5** complex (see ESI – paragraph 7.2) and may partly explain the high selectivity observed for Ch.

The analysis of the theoretical data reported in ESI (paragraph 7) allows to obtain another kind of information. The comparison of the distances between aromatic rings in the host and in the complexes shows that a swelling of the capsule occurred upon complexation and that some distances between benzoic acid and phenoxy groups are shorter in the complexes. These π - π interactions, more important in the Ch@HC **5** complex, may be responsible for the observed quench of fluorescence emission. An entry of water in the host, favoured by the most hydrophilic guest, may also contribute to this phenomenon. In the case of the free HC **4**, these π - π interactions were much stronger in organic solvent, compared to those observed for the free HC **5** in aqueous medium, and they decreased in the presence of guest. If the initial interactions within the cage are not the same depending on the solvent, it is likely that the solvent also affects the interactions in the presence of guest and thus the selectivity.

Finally, these calculations are consistent with the fact that complexation has virtually no impact on the absorption wavelengths (Fig. S7 in ESI).

Conclusions

In conclusion, the fluorescent hemicyptophane **5** was found to be an effective cage for strongly and selectively recognize Ch. To our knowledge, such selectivity for Ch over ACh has never been achieved before with supramolecular fluorescent host in pseudo-physiological environment. These results, supported by DFT calculations, confirm the crucial role of hydrogen bond to discriminate these species in water.

Conflicts of interest

There are no conflicts to declare.

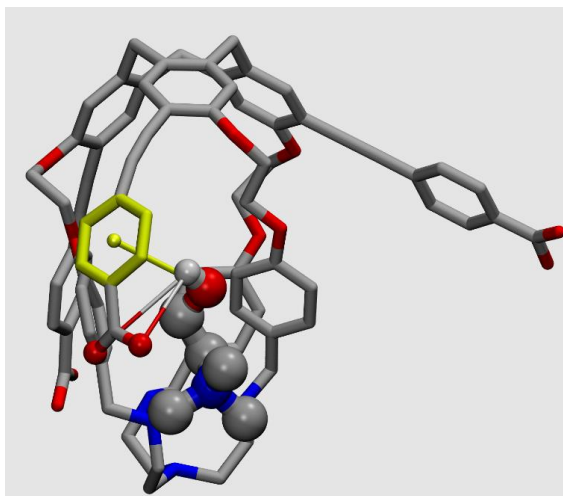


Fig. 5 DFT structure of the most stable Ch@HC **5** complex. For clarity purpose only the specific interactions implying the hydroxyl group of the guest are shown (one π -hydrogen bond (3.5 Å, yellow bond) and two hydrogen bonds with one carboxylate (3.4 and 3.9 Å))

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