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ORIGINAL ARTICLE

Complexing photolabile cholinergic ligands with synthetic and biological receptors: a dynamic survey

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Abstract Molecular recognition processes between synthetic or biological receptors and small photolabile cholinergic ligands are described for adaptive host–guest complexes with calixarene derivatives and for the photoregulation of cholinergic enzymes, respectively.

Keywords Bioorganic chemistry · Photochemistry · Ligand–receptor interactions · Calixarenes

Introduction

Photolabile precursors of biologically relevant molecules are molecules whose activity or function is masked by chemical modification using a photolabile protecting group [1–3]. Light excitation of these compounds result in rapid and specific removal of the protecting group and concomitant release of the corresponding biomolecules to restore their biological functions [4–7]. Consequently, we can make use of these photolabile compounds in combination with rapid laser photolysis to provide temporally and spatially controlled release of enzyme substrates or receptor ligands for studying fast biological processes, which are otherwise difficult to be investigated [8].

Hydrolysis of the neurotransmitter acetylcholine (ACh) by cholinesterases, namely acetycholinesterase (AChE) and butyrylcholinesterase (BuChE), is a particularly fast enzymatic process [9]. AChE operates at a rate which is almost diffusion-limited, with a turnover number approaching 20,000 s^{-1}. The description of the 3-D structure of AChE and BuChE as well as of several enzyme-inhibitor complexes has permitted a better understanding of structure-function relationships in the cholinesterases [10–12]. It has, however, also raised cogent new questions concerning the traffic of substrate and products, to and from the active site located near the bottom of a deep and narrow gorge. In order to decipher the rapid catalytic processes of cholinesterases, we have proposed to use photolabile cholinergic ligands in combination with time-resolved X-ray crystallography [13].

Furthermore, using macrocycles as artificial receptors or enzymes, host–guest inclusion chemistry could provide us valuable information about the nature and the mechanism of biological processes of molecular recognition. The calixarene family plays an important role in host–guest chemistry and is of special interest to mimic cholinergic receptors. Various calixarene-based synthetic receptors have been developed [14–18], including the water soluble tetra-, hexa-, and octasulfonated calix[4]arene, calix[6]arene, and calix[8]arenas, respectively [19–21]. They are able to host the neurotransmitter acetylcholine and related cholinergic ligands [22], and can, therefore, be used as synthetic cholinergic receptors. Our interest in studying

Dedicated to Prof. Jack Harrowfield and Dr. Jacques Vicens on the celebration of their 65th birthday.

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cholinesterases using photolabile cholinergic ligands led us to investigate their interactions with calixarenes as model cholinergic receptors to better understand molecular recognition processes *via* a supramolecular approach.

Here, we present a brief overview on the design, synthesis and characterization of various photolabile cholinergic ligands as well as their molecular recognition with synthetic cholinergic receptors and their use in the functional regulation of cholinesterases.

Photolabile cholinergic ligands

Scheme 1 shows the different types of photolabile cholinergic ligands that have been designed, synthesized and characterized for their photochemical and biological properties with respect to the above mentioned studies. They are *ortho*-nitrobenzyl derivatives of choline (**A** and **B**)[23–25], arsenocholine (**C**)[26], carbamylcholine (**D**)[27] and carbamylarsenocholine (**E**)[26], respectively.

We have chosen *ortho*-nitrobenzyl moiety as photolabile group because of its small size, excellent photochemical properties as well as easy and convenient syntheses. During our study, we found that the CF_3- group at the α-benzylic position could improve considerably the photochemical properties leading to a rapid photocleavage with extremely high quantum yield [25].

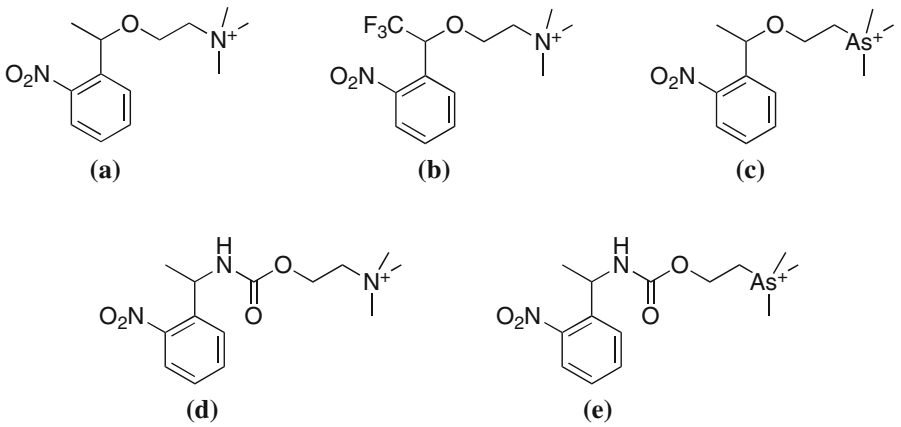
Choline is the enzymatic product of cholinesterases, and photolabile choline ligands **A** and **B** are expected to bind at the active site of the enzymes and to generate choline by subsequent photoactivation. **A** and **B** can offer us the possibility to study the rapid clearance of choline from the active site. It is further known that carbamylcholine acts as a slow substrate for cholinesterases: rapid carbamylation with release of choline is followed by a much slower decarbamylation process. Thus, photolabile carbamylcholine **D** can be used to investigate the mechanism of carbamylation as well as the subsequent release of choline after enzymatic reaction. Both compound **C** and **E** contain a heavy atom, arsenic in place of nitrogen [26], providing the unique advantage to contour the phase problem when we study the fast enzymatic reaction by time-resolved crystallography.

The photolabile cholinergic ligands **A**–**E** were synthesized either by us [23–26] or by other groups [27]. They are effective inhibitors of both AChE and BuChE, with Micheal binding constant ranging from 11 to 78 μM (Table 1). In addition, all the compounds, upon photoactivation, release rapidly the corresponding product within 10^{-5} seconds and in high yields, with quantum yield of **C** being as high as 70%. The fast photofragmentation and high quantum yield of these compounds are important prerequisites for their successful implementation in studying the fast catalytic process of cholinesterases by time-resolved crystallography.

Calixarenes as synthetic cholinergic receptors

We next used calixarenes as artificial receptors to study host–guest complexes formation with the photolabile cholinergic ligand **A**. Calixarenes are macrocylic molecules consisting of phenolic units connected at the *ortho*-position by methylene bridges. The existence of many conformational isomers of calixarenes generate a large number of cavities of different sizes and shapes, which can be used for molecular recognition processes. The water soluble tetra-, hexa-, and octasulfonated calix[4]arene, calix[6]arene, and calix[8]arenas **1**–**3** (Scheme 2), respectively, have been previously reported to be able to host the neurotransmitter acetylcholine and related cholinergic ligands [22]. The binding of cholinergic ligands to calixarenes is a complex process in which electrostatic forces, hydrophobic effects, and cation–π interactions [19–21] intervene, all playing a crucial role.

In our study on the host–guest complexes of calixarenes with the photolabile cholinergic ligand **A**, all the three calixarenes **1**–**3** formed stable 1:1 complexes with **A**.

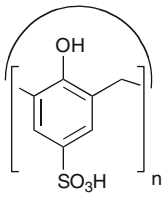


Scheme 1 Photolabile cholinergic ligands described in this work [23–27]

Table 1 Inhibition constants (K_I) for AChE and BuChE and photochemical properties (half time of photolysis $t_{1/2}$ and quantum yields Φ) of photolabile cholinergic ligands **A-E**

Ligand	K_I, AChE (μM)	K_I, BuChE (μM)	Photolysis	
			$t_{1/2}$ (μsec)	Φ
A	13	11	10	0.27
B	16	n.d.	2 & 45	0.70
C	17	18	7.6	0.25
D	44	78	24	0.25
E	63	29	13	0.33

Scheme 2 *p*-Sulfonated calix[4]arene (**1**), calix[6]arene (**2**), and calix[8]arene (**3**)



1 : n=4
2 : n=6
3 : n=8

Dissociation constants of 68, 77 and 29 μM were determined for calix[4]arene, calix[6]arene and calix[8]arene, respectively. The strongest interactions were observed with calix[8]arene (**3**), with dissociation constants similar to those observed with acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). The high affinities of these calixarenes for **A** are comparable to those of the biological recognition sites in cholinesterases, suggesting that the *para*-sulfonated calixarenes mimic the binding sites of cholinesterases [28].

Due to their small cavity size, both calix[4]arene (**1**) and calix[6]arene (**2**) molecules formed monotopic complexes

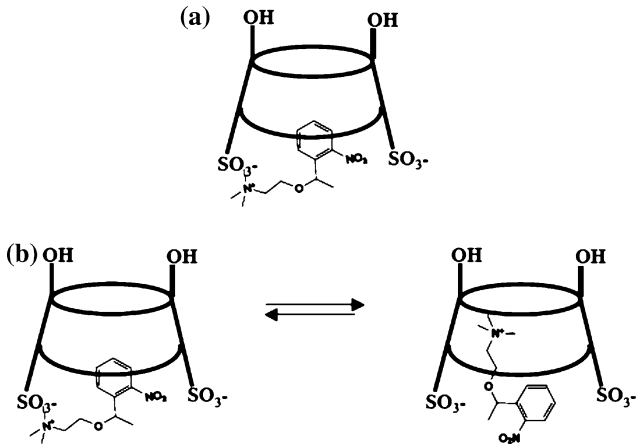


Fig. 1 Monotopic binding complexation of **1** with **A** at **a** pD 7.3 and **b** pD 0.4 [28]

with **A** (Fig. 1) [28]. Furthermore, the monotopic complexes between the calix[4]arene (**1**) and **A** may be regulated by the pH: at neutral pH, **1** specifically binds the cationic choline moiety; whereas at acidic pH, **1** non-selectively hosted either the cationic choline moiety or the aromatic group of **A** in its cavity [28]. Remarkably, the kinetics of photorelease of choline from the calixarene complex is similar to that obtained with **A** alone at neutral pH [29]. From these results, it is expected that the photofragmentation of our photolabile cholinergic ligands may occur similarly when they are complexed at the active site of the cholinesterases.

To our great interest, the larger calix[8]arene (**3**) formed ditopic binding complexes by concomitantly hosting the choline moiety and the photolabile group of **A** (Fig. 2) [30]. The ditopic complex was formed *via* a 'mutually induced fitting' process between the structurally flexible host and guest with calix[8]arene (**3**) and the guest of

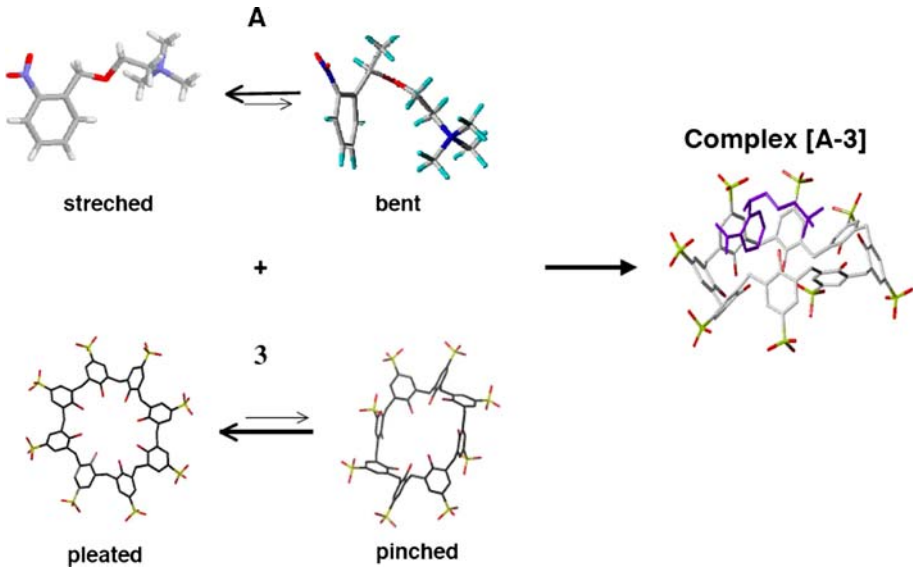


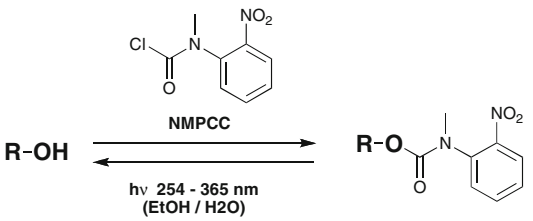
Fig. 2 The complex [**A**-**3**] is formed via mutually induced fitting with the conformationally flexible guest **A** and host **3** [30]. When **A** and **3** are not complexed, **A** prefers the streched lower-energy conformation (obtained from crystal structure [26]) to the bent, higher-energy conformation (from the NMR data [30]), while **3** prefers the lower-energy pleated form to the higher-energy pinched ones [30]. In the complex [**A**-**3**], **A** adopts the bent conformation and **3** the pinched conformation

A. Both **A** and **3** underwent conformational change, transforming their corresponding lower-energy conformations into the higher-energy ones in order to fit to each other and host **A** favorably into the cavity of **3** (Fig. 2). This is an original example of adaptive host-guest systems *via* a 'mutually induced fitting' process [30]. Mutually induced fit has been frequently found in biological macromolecules interactions, such as protein/DNA, protein/RNA, protein/protein complexes. Our study on the various mode of complexation between calixarenes and photolabile cholinergic ligands pinpoints the power of supramolecular chemistry in mimicking and studying biological systems.

Cholinesterases

The 3D structure of acetylcholinesterase revealed a remarkable structural feature with the catalytic triad located at the bottom of a deep and narrow gorge of the enzyme [9]. This raised the question of the fast catalytic reaction and the traffic of substrate and product through this gorge. The existence of possible alternative pathways occurring through rapid conformational change of the enzyme has been proposed and referred to the "back door" hypothesis [31]. To study in more details the functioning of cholinesterases (ChEs) and in particular to identify the traffic of substrate and product during the catalytic reaction, we intended to develop time-resolved macromolecular crystallographic studies on these enzymes. Such dynamic studies recommend the photoregulation of the activity of the ChEs by making use of the fast and efficient photochemical triggering to control spatially and temporally the enzyme activity, which can be achieved with either photolabile cholinergic ligands to mask the enzyme product and substrate (**A**-**E** in Scheme 1) or by caging directly the enzymes to mask the enzyme activity (see below).

Alternatively, it might be more advantageous to mask directly the enzyme activity because a stoichiometric irreversible modification of the protein leads to a stable inactivated enzyme which requires a minimum amount of photons for its reactivation. Such an enzyme caging can be envisaged either by steric blockade of the catalytic site or by chemical modification of an amino acid which is critical for its activity. We demonstrated that N-methyl-N-(*o*-nitrophenyl) carbamates were very convenient photoremovable alcohol protecting groups [32] (Scheme 3). Using N-methyl-N-(*o*-nitrophenyl) chlorocarbamate (MNPCC) as carbamylating reagent, we were able to demonstrate the carbamylation of BuChE by covalent modification of the catalytic serine residue, and the photoregulation of BuChE by uncaging process [33].



Scheme 3 N-Methyl-N-(o-nitrophenyl) carbamates as photoremovable alcohol protecting groups [19]

Treatment of AChE and BuChE with MNPCC led to a time- and concentration-dependent inactivation of both enzymes. The inactivation kinetics (Fig. 3) show that the process was very efficient, particularly for AChE, where sub-micromolar concentrations of MNPCC were sufficient for a rapid and complete inactivation [33]. The pseudo-irreversible inactivation of BuChE induced by MNPCC is in line with a carbamylation reaction of the catalytic serine as demonstrated by X-ray crystallography (Fig. 4) [33], showing electron density in agreement with a covalent bond between the serine-oxygen atom of the catalytic serine (Ser 198) and the carbonyl group of the MNPCC reagent. The carbamylation of the active serine is responsible, at least for BuChE, for the enzyme blockade.

We next examined the possibility of a photochemical regeneration of the ChEs activities by irradiation at 365 nm using a Xe–Hg light source. MNPCC-inactivated AChE and BuChE responded very differently to this photolytic treatment (Fig. 5a, b), showing very high recovery (100%) of the activity for BuChE in most conditions, while the activity of AChE was only marginally restored (≤20%). The complete recovery of the enzyme activity after photolysis of the MNPCC-inactivated BuChE, describes a remarkable example of a light-activated enzyme [33].

The photoregulation of AChE or BuChE, and especially the synchronization of the enzyme activity provided by this technique, allows us to investigate the mechanism of this class of protein by using time-resolved biophysical techniques. In this context, the combination of the concept of uncaging of "enzyme regulators" with real-time resolved crystallographic techniques should provide an insight into

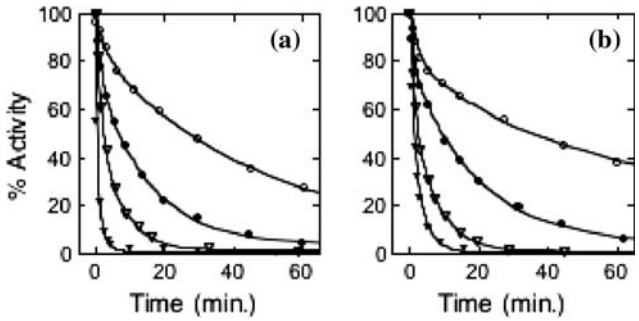


Fig. 3 MNPCC-induced inactivation kinetics **a** BuChE: MNPCC 5–50μM, **b** AChE : MNPCC 0.2–2μM

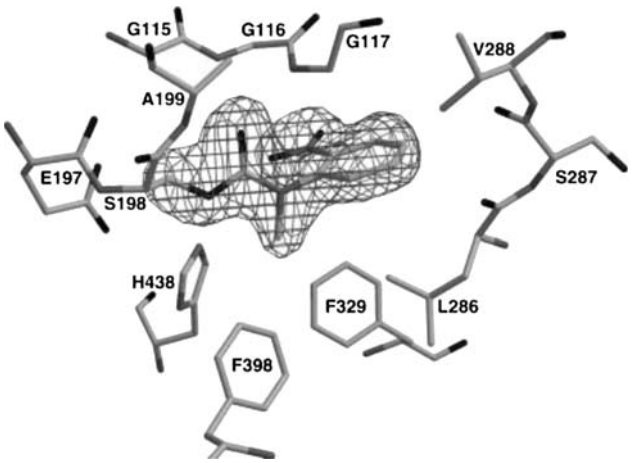


Fig. 4 X-ray structure (2.1 Å) of active site recombinant BuChE carbamylated by MNPCC[20]

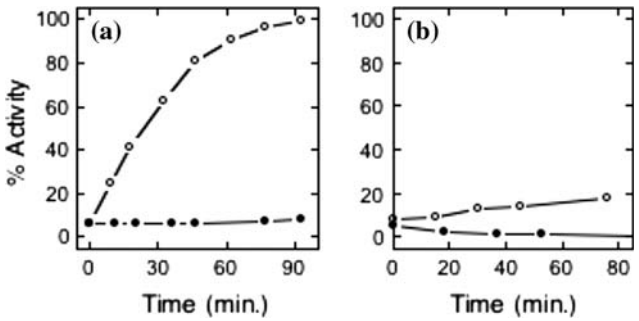


Fig. 5 Photochemical recovery of enzymatic activity. **a** BuChE: inhibition 20 μM MNPCC, photolysis in the presence of 0.1 M NH_2OH, control without light; **b** AChE: inhibition by 1 μM MNPCC, control without light

the structural analysis of the enzyme mechanism in three dimensions and at the atomic resolution within a crystal. The Laue crystallography (a technique which uses a white beam X-ray probe) is the only method used for such studies, and provides the unique ability to collect extremely rapidly a full X-Ray data collection on a non-rotating static crystal [34]. But even if this technique allows X-rays exposure times as short as 100 ps, the time resolution for such studies would be as high as permitted by the photoactivation process. Therefore, the biggest limitation for real-time resolved crystallography studies on Cholinesterases, employing caging groups, is to build up a very efficient and fast phototrigger to be able to collect a real movie on the catalytic process.

A nice alternative for time-resolved crystallography is the use of physical techniques to control the rates of reactions within crystals. Therefore, the biological reaction can be stopped (usually by lowering the temperature) and monochromatic X-ray can be used to collect the diffraction data [35]. The ability of photoremoval groups to be photolyzed at cryo-temperature [36, 37] (and below the dynamical transition temperature of the studied protein) represents an attractive way to accumulate protein intermediates which can then be trapped by cooling the crystal to 100 K. Such a 'kinetic' crystallography study was developed to structurally address the product traffic in acetylcholinesterase using a 'caged' arsenocholine derivative [38] (probe **C**) taking advantage of the *more intense* electron density due to the arsenic atom. The UV–laser-induced cleavage of this enzymatic product analogue (arsenocholine), was performed in a temperature-controlled X-ray crystallography regime, to allow the structural monitoring of the traffic of the enzymatic product from the enzyme active site. We were able to monitor the protein conformationals changes induced by arsonocholine release [24] and those results were consistent with the idea that choline, the enzymatic product, exits the enzyme catalytic side either via the gorge or via an alternative 'backdoor' trajectory.

Concluding remarks

In this review dedicated to "Jacques & Jack", we have recalled our initial purpose in developing photolabile cholinergic ligands to investigate the fast-acting catalytic process of cholinesterases. Various types of cholinergic ligands have been consequently designed, synthesized and characterized. They are the photolabile procursors of either choline, the enzyme product, or carbamylcholine, a slow substrate of cholinesterases, containing or not arsenic as a heavy atom to replace the nitrogen in the choline moiety. All of these compounds show a fast and efficient release of the corresponding cholinergic ligand upon photoactivation. They bind at the enzyme active site and photoregulate the enzyme activity either via the release of the photoproduct choline or through carbamylation of the cholinesterases. Combination with a temperature-controlled X-ray crystallographic regime, we were able to monitor the conformational change of acetylcholinesterase induced by arsenocholine relase, as well as to analyse the traffic of choline within the enzyme after catalysis. Alternatively, caging the cholinesterases directly with the photolabile group MNPCC to mask the serine residue at the enzyme active site proved to be an effective and advantagous approach to achieve photoregulation of enzyme activity for further time-resolved studies on the catalytic process of cholinesterases.

In parallel, we used *p*-sulphonated calixarenes as artificial cholinergic receptors to investigate their interactions with the photolabile cholinergic ligands to better understand the fundamental phenomenon of molecular recognition in biology by means of a supramolecular host-guest chemistry approach. The calixarenes can mimck the binding site of cholinesterase and form formed stable complexes with the photolabile cholinergic ligands, with binding constants

being similar to those of cholinesterases. Most interestingly, the larger calix[8]arene (**3**) formed ditopic binding complexes by concomitantly binding to both the cholinergic moiety and the photolabile group, whereas the smaller calix[4]arene (**1**) and calix[6]arene (**2**) molecules formed monotopic complexes due to their small cavity size. The ditopic complex was formed between the conformationally flexible calix[8]arene (**3**) and the structurally flexible ligand **A** *via* a 'mutually induced fitting' process, whereas the monotopic complexes between the calix[4]arene (**1**) and **A** may be regulated by the pH. Various modes of complexation are involved in the binding of the bifunctional photolabile cholinergic ligands in *para*-sulfonated calixarenes, depending on their size, geometry, and state of protonation. In these complexes, the cooperative effects of electrostatic forces, hydrophobic effects, cation–π interactions, and the size and geometry of the molecules are all decisive binding factors, which reflect many resemblances with the biological cholinergic receptors and enzymes.

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