Synthesis and Characterization of Photolabile Choline Precursors as Reversible Inhibitors of Cholinesterases: Release of Choline in the Microsecond Time Range

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Three *o*-nitrobenzyl ether derivatives of choline (compounds **A**−**C**) were synthesized as photolabile cholinesterase inhibitors in order to study the mechanism of choline release from the enzyme active site. The key step of the synthesis was a simple and efficient Lewis acid-catalyzed opening of dioxolane rings derived from *o*-nitrobenzaldehyde and *o*-nitroacetophenone. Laser flash photolyses of compounds **A**−**C** were analyzed by UV spectroscopy, HPLC, and an enzymatic assay for choline. The quantum yields of photoconversion were determined, and the kinetics of choline release were analyzed by studying the decay of the transient *aci*-nitro intermediate at 405 nm. The observed rates varied considerably in function of both pH and the substituent at the α-benzylic position. Furthermore, we demonstrated that all three compounds possessed reversible inhibitory properties on both purified *Torpedo* acetylcholinesterase and purified human plasma butyrylcholinesterase. Compound **A**, *O*-[1-(2-nitrophenyl)ethyl]choline iodide, which displayed the highest quantum yield (0.27) and the most rapid photolysis rate (6.8×10^4 s^{-1} at pH 6.5), represents therefore an interesting tool for the study of the fast process of choline release from cholinesterases by time-resolved Laue crystallography.

Introduction

Photolabile compounds can provide control of temporal and spatial release of biologically interesting molecules by rapid photolysis and are thus important tools for the time-resolved study of fast biological processes.[1] Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are fast-acting enzymes which catalyze the hydrolysis of the neurotransmitter acetylcholine.[2] AChE operates at a nearly diffusion-limited rate[3] and possesses a turnover of 20 000 per second.[4] Although the 3-D structures of both AChE[5] and AChE−inhibitor complexes[6] as well as the deduced model structure of BuChE[7] were described, it is still unclear how choline is rapidly released from the active site of the enzyme in order to maintain its high turnover. To address this problem, we conceived photolabile precursors of choline, as inhibitors of cholinesterases, to investigate the process of rapid choline release from cholinesterases by future time-resolved crystallographic studies.[8]

o-Nitrobenzyl groups have been used as photolabile protecting groups[9] to mask or "cage" biological molecules.[1] The photochemical release of ATP from the *o*-nitrobenzyl P^3-ester of ATP ("caged" ATP)[10] was the first example of a thorough photochemical study, demonstrating that the decay of an "*aci*-nitro" intermediate could be correlated to the formation of ATP. Similar photochemical fragmentation mechanisms have also been proposed for the photolysis of *o*-nitrobenzyl derivatives, such as ethers,[11] carbamates,[12] carboxylic esters,[13] and amines.[14] These photolabile compounds have become interesting tools for the investigation of several fast biological processes.[1]

In the present paper, we describe the synthesis and characterization of three *o*-nitrobenzyl ethers of choline (Scheme 1, compounds **A**−**C**) as photolabile choline precursors which are reversible inhibitors of cholinesterases. These molecules differ from the classical photolabile "caged" compounds in their capability to interact with the enzymes, prior to photoactivation. In particular, probe **A** seems best adapted for further photoregulatory investigation of choline release from cholinesterases.

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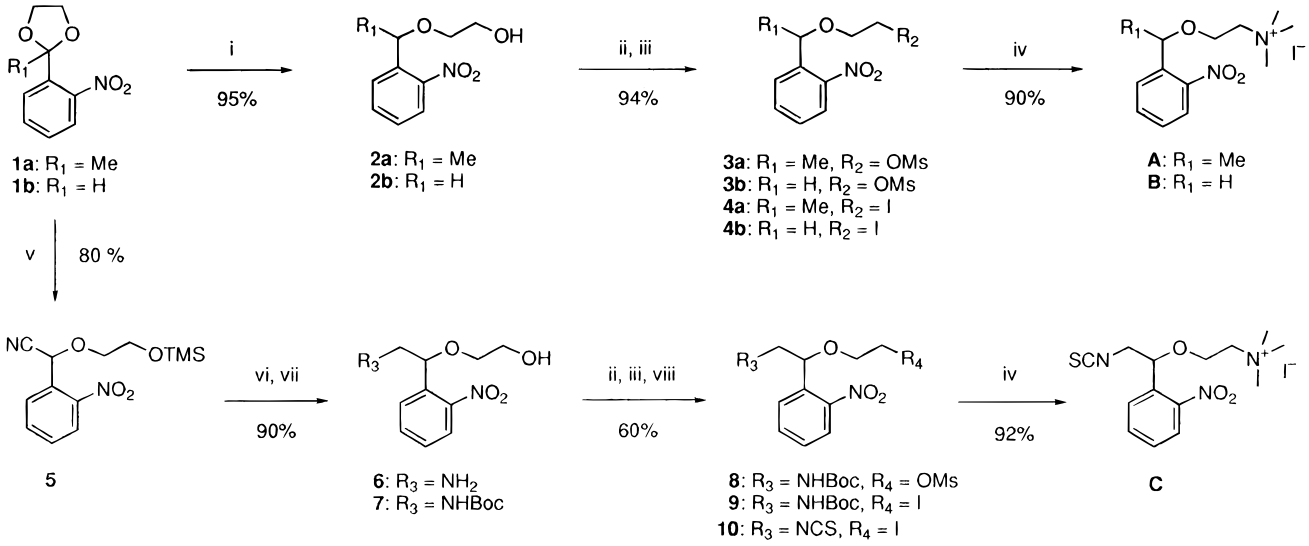
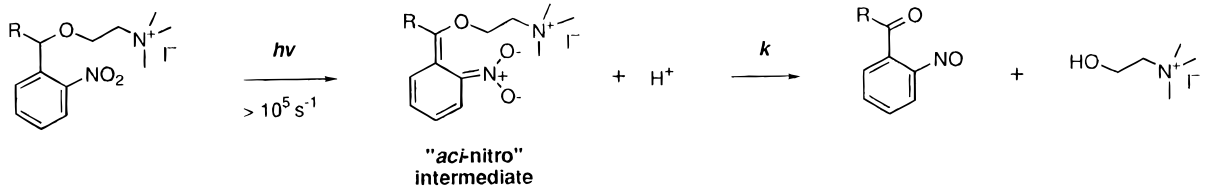
Scheme 1. Synthesis of 2-Nitrobenzyl Ethers of Choline, Compounds A–C[a]



[a] Reagents and conditions: (i) $NaBH_3CN$, $TiCl_4$, CH_3CN, rt, 2 h; (ii) MsCl, TEA, ether, rt, 1 h; (iii) NaI, acetone, reflux, 2 h; (iv) NMe_3, toluene, rt, 24 h; (v) TMSCN, ZnI_2, rt, 15 min; (vi) (a) BH_3·THF, rt, 4 h, (b) 6 N HCl, (c) 4 N NaOH; (vii) di-*tert*-butyl dicarbonate, TEA, THF, rt, 45 min; (viii) (a) CF_3COOH, CH_2Cl_2, rt, 10 min, (b) $CSCl_2$, $NaHCO_3$, CH_2Cl_2/H_2O, rt, 10 min.

Scheme 2. Proposed Mechanism of the Photochemical Reaction of 2-Nitrobenzyl Ethers of Choline



Results

Synthesis of 2-Nitrobenzyl Choline Derivatives A–C. The synthetic routes to the three photolabile choline derivatives (compounds **A**–**C**) are outlined in Scheme 1. The introduction of the choline moiety used a common strategy for all three compounds, namely the opening of the cyclic acetal **1a** and ketal **1b**.

Treatment of ketal **1a** with sodium cyanoborohydride, in the presence of $TiCl_4$,[15] led to the opening of the dioxolane ring and yielded ether **2a** which was subsequently mesylated (**3a**) and iodinated to give **4a**. Reaction of **4a** with trimethylamine in dry toluene led to the final product **A**. The use of dry toluene as solvent facilitated the final isolation and purification of quaternary ammonium compounds as they precipitated from the reaction medium. Starting from acetal **1a**, compound **B** was prepared in the same way.

Acetal **1b** was treated with trimethylsilyl cyanide in the presence of ZnI_2[16] to give nitrile **5**, which was further reduced with BH_3·THF to the corresponding amine **6** and protected as the *tert*-butyl carbamate **7**. This compound was subsequently mesylated (**8**) and transformed to the corresponding iodide **9**. The removal of the Boc protecting group in **9** by trifluoroacetic acid, followed by treatment with thiophosgene, gave the isothiocyanate derivative **10**. The final compound **C** was obtained by a S_N2-substitution of the iodine in **10** with trimethylamine in toluene.

Photochemical Release of Choline from Compounds A–C. The UV–vis absorption spectra of compounds **A**–**C** showed a maximum around 260 nm (Table 1), which is characteristic of the 2-nitrobenzyl moiety. During laser flash photolysis of **A**, a decrease in absorption at 260 nm was observed concomitant with an increase at 225 nm and the appearance of a new peak at 310 nm (Figure 1a), corresponding to the formation of the aromatic nitroso byproduct (Scheme 2). The observed isobestic points at 275, 248, and 210 nm are consistent with a unique photodecomposition process. This was further confirmed by HPLC analysis using an ion-pair partitioning technique on a reversed phase column (Figure 1b). To avoid the positively charged molecules **A**–**C** being immediately eluted in the void volume, we used the negatively charged dodecyl sulfate as a counterion which conferred to these probes a reasonable retention time (Table 1). Figure 1b shows an HPLC analysis during the photochemical decomposition of compound **A**. The photolytic byproduct, 2-nitrosoacetophenone, eluted earlier (retention time of 6.1 min) than the parent compound (retention time of 9.0 min). The decay of compound **A** was concomitant with the

Table 1. Spectral Properties, HPLC Characterization, and Quantum Yields of compounds A–C

	λ_{max},[a] nm	ϵ,[a] $M^{-1}cm^{-1}$	t_R,[b] min	Φ[c]
A	261	5300	11.1	0.27[d]
B	261	5000	9.3	0.19[e]
C	260	4300	16.3	0.26[d]

[a] Absorption properties were determined in 0.1 M phosphate buffer, pH 7.2, at 20 °C. [b] The HPLC retention time (t_R) of each compound on a reversed phase column using acetonitrile (40%) and a solution of 5 mM sodium dodecylsulfate and 5 mM sodium sulfate at pH 2.00 (60%). [c] Quantum yields (Φ). [d] Determined relative to 1-(2-nitrophenyl)ethyl carbamylcholine. [e] Determined relative to compound **C** as a reference.

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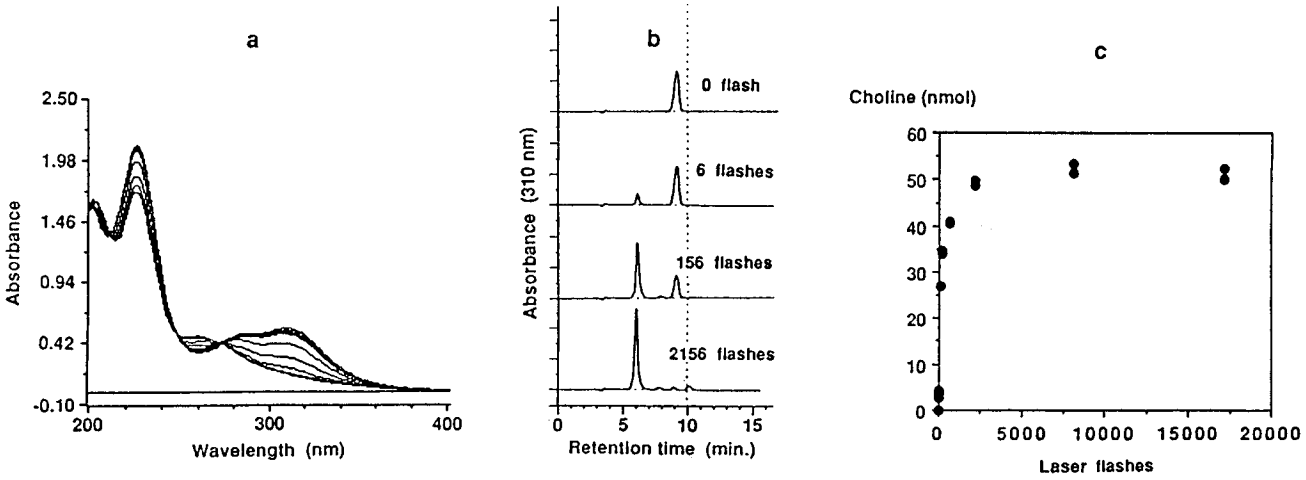

Figure 1. Laser flash photolysis of compound **A** was analyzed by (a) UV spectroscopy, (b) HPLC analysis, and (c) an enzymatic assay specific for choline. A solution of 1 mM compound **A** in 100 mM phosphate buffer at pH 6.5 was exposed to 351 nm laser flashes. (a) UV spectral recording of the photolysis. The lowest trace at 310 and 225 nm and the highest trace at 260 nm correspond to the starting material. b) Aliquots of irradiated sample (100 μL) were analyzed by HPLC with acetonitrile (45%) and a solution containing 5 mM sodium dodecylsulfate, 5 mM sodium sulfate at pH 2.00 (55%). Compound **A** has an HPLC retention time of 9.0 min, and the appearing peak at 6.1 min corresponds to the photolysis byproduct, 2-nitrosoacetophenone. (c) The photoreleased choline was quantified by an enzymatic assay[17] using 50 μL aliquots of samples (see Experimental Section).

formation of the corresponding aromatic nitroso product, implying a stoichiometric photoconversion with no other UV-absorbing byproducts being observed during this photolytic reaction.

In this study, the photoproduct of primary interest was choline. After photolysis of compounds **A**−**C**, the released choline was detected and quantified with a specific enzymatic assay[17] (Figure 1c). The amount of choline formed increased with increasing exposure to light and coincided with the disappearance of the parent compound **A** (Figure 1). This result also indicated a stoichiometric conversion of choline from its precursor. Similar results were also obtained for compounds **B** and **C** (data not shown).

Quantum Yields for Photoconversion. The quantum yields for the photoconversion of compounds **A**−**C** were determined by comparison with the photolysis of 1-(2-nitrophenyl)ethyl carbamylcholine ($\Phi = 0.25$).[12] The extent of the photolytic conversion in equimolar mixtures of choline derivatives and the reference compound was analyzed on HPLC (see Experimental Section). Compounds **A** and **C** photolyzed as efficiently as 1-(2-nitrophenyl)ethyl carbamylcholine and possessed quantum yields of 0.27 and 0.26, respectively (Table 1). However, 1-(2-nitrophenyl)ethyl carbamylcholine could not be used for the determination of the quantum yield of compound **B** because of their similar HPLC retention time, and we therefore used compound **C** as an internal reference. As compound **B** photolyzed 1.25-fold less efficiently than compound **C**, a quantum yield of 0.19 was deduced for compound **B**.

Laser Flash Kinetic Studies. The kinetics of the photolysis of compounds **A**−**C** was analyzed by monitoring the formation and decay of the presumed *aci*-nitro intermediate. Laser flash photolysis of **A**−**C** induced a rapid increase in absorbance around 400 nm, followed by an exponential decay to the initial level (Figure 2). The short-lived transient was detected with a delay of 0.2 μs after laser flash photolysis of **A** and showed a spectral maximum around 405 nm (Figure 2a). Similar transients have been observed with a variety of 2-nitrobenzyl compounds and were attributed to the *aci*-nitro species[1] (Scheme 2). Most importantly, the decay of this species can be used as a kinetic measure of release of the corresponding product.[10] For compound **A**, the decay of the *aci*-nitro transient is a first-order reaction (Figure 2b), while a two-component decay was observed for compounds **B** and **C** (data not shown). Furthermore, the decay rate of the transient was first order in $[H^+]$ (Figure 2c and Table 2) and was sensitive to substitution at the α-benzylic position (Table 2). The *aci*-nitro signal for compound **A** decayed about 50 times faster than compound **C** and approximately 800 times faster than compounds **B** at pH 6.5, 20 °C (Table 2). The fastest photolysis rate (6.8×10^4 s^{-1}) was observed with compound **A** at pH 6.5, a rate which is comparable with the turnover rate of acetylcholinesterase.

Biological Properties of Compounds A−C. Both AChE and BuChE display high affinity for quaternary ammonium ligands through specific interaction with aromatic residues at the active sites of the enzymes.[4,6,18] All three compounds **A**−**C** showed reversible inhibitory properties on both purified *Torpedo* acetylcholinesterase (mixed type of inhibition) and purified human plasma butyrylcholinesterase (competitive inhibition) with inhibition constants in the 10^{-5}−10^{-6} M range (Table 3). AChE and BuChE remained fully stable when exposed to 351-nm laser flashes. The photolytic byproduct from compound **A**, 2-nitrosoacetophenone, which could be scavenged by dithiothreitol or glutathione as previously described,[1] had no toxic effects on the activity of cholinesterases, even at 1 mM concentration (data not shown).

Discussion

The goal of this work was to synthesize and characterize photolabile precursors of choline to be used as cholinesterases inhibitors allowing a fast and efficient photorelease of choline. Such photolabile inhibitors should fulfill a number of criteria: they should show

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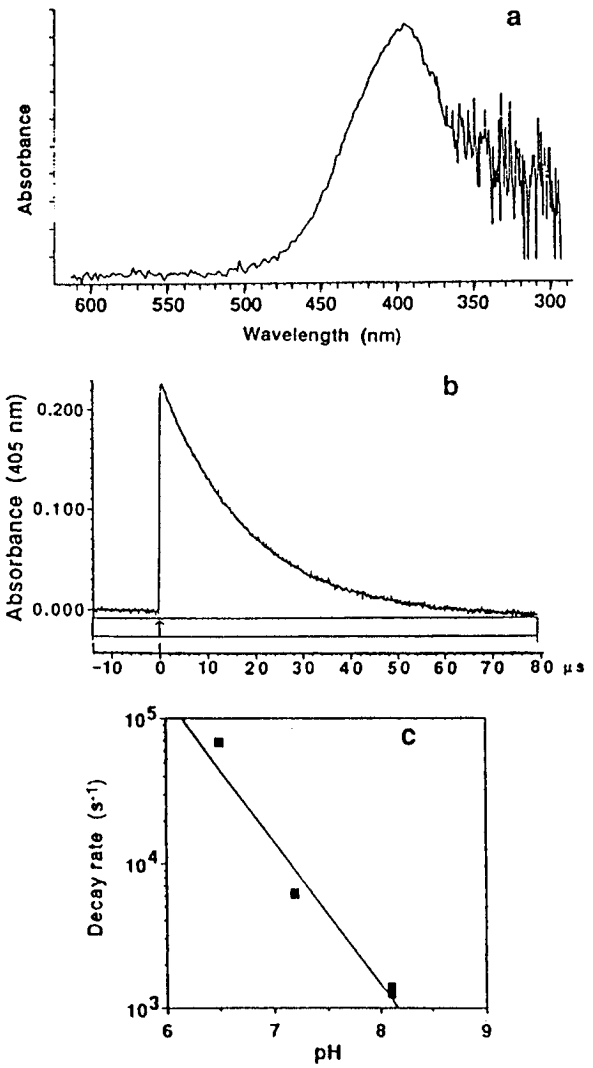

Figure 2. Kinetic analysis on the laser flash photolysis of compound **A**. A solution of 1 mM compound **A** in 0.1 M phosphate buffer was exposed to a single 351 nm laser pulse at 20 °C. (a) The UV spectrum of a transient was observed by recording the spectral change before and after (0.2 μs delay) laser flash photolysis of compound **A**. (b) Kinetic record at 405 nm after a single laser flash photolysis of compound **A** at pH 6.5. Arrow indicates the begining of the laser flash. The transient was formed immediately and followed by an exponential decay. (c) Rate constants for the decay of the transient intermediate in the photolysis of compound **A** as a function of pH.

Table 2. Half-Life ($t_{1/2}$) of the *aci*-Nitro Transients from Compounds A−C as a Function of pH

	$t_{1/2}$, pH 6.5[a]	$t_{1/2}$, pH 7.2[a]	$t_{1/2}$, pH 8.1[a]
A	10.2 μs	110 μs	525 μs
B	82.3 ms[b]	521 ms[b]	2.42 s[b]
C	0.56 ms[b]	1.11 ms[b]	1.84 ms[b]

[a] Spectral transients observed in flash photolysis were analyzed in 0.1 M phosphate buffer at 20 °C. [b] Value corresponding to the slower phase of the decay rate were presented.

inhibitory effect on the enzymes prior to photolysis; they should photorelease choline efficiently and rapidly; and the byproducts of photolysis should be chemically inert, biologically inactive, and nontoxic for the enzymes.

Three photolabile precursors of choline, **A**−**C**, were synthesized. Each contains a 2-nitrobenzyl moiety attached via an ether linkage to the oxygen of choline. Although several methods had previously been used for the synthesis of 2-nitrobenzyl ether derivatives,[11] none of them seemed suitable for the synthesis of the compounds **A**−**C**. The basic reaction conditions of Williamson ether synthesis gave mainly tarry mixtures, while the silver(I) oxide catalyzed condensations[19] proved unsuccessful for the 2-nitrobenzyl ether series (data not shown). The syntheses of compounds **A**−**C** (Scheme 1) in the present work took advantage of the possibility of using cyclic ketals and acetals as precursors of the choline moiety. Noticeably, the reductive and nucleophilic openings of the dioxolane rings were achieved in near-quantitative yield in the presence of Lewis acids (ZnI_2 and $TiCl_4$).

Table 3. Inhibition Constants of Compounds A−C on AChE and BuChE

	AChE (M)	BuChE (M)
A	1.30×10^{-5}	1.11×10^{-5}
B	1.00×10^{-5}	1.94×10^{-5}
C	3.73×10^{-6}	3.13×10^{-6}

Previous work[1,9,11] has shown that the 2-nitrobenzyl ethers can be readily and efficiently photodecomposed. Photorelease of choline from compounds **A**−**C** was investigated and analyzed by UV spectra, HPLC, and an enzymatic assay for choline (Figure 1). The kinetics of choline release was analyzed by studying the decay of the transient *aci*-nitro intermediate (Scheme 2). The observed decay rates were very different for the three compounds **A**−**C** (Table 2), showing a fast exponential decay for compound **A** but a slower biphasic process[20] for both compounds **B** and **C**. The strong dependence of the decay rate on the substituent at the α-benzylic position, although well-documented in analogous series,[11−13] is in our case remarkable by its extent; i.e., 4 orders of magnitude difference were found between the decay rates observed when R = CH_3 (**A**) and R = H (**B**). Furthermore, the observed first order pH-dependence of the decay rates (compound **A**, Figure 2c) is in agreement with the proposed mechanism (Scheme 2). Compound **A** shows excellent kinetic properties ($k = 6.8 \times 10^4$ s^{-1} at pH 6.5, 20 °C) for photolytic release of choline, one of the fastest photolysis reactions described with *o*-nitrobenzyl derivatives. This photolytic release of choline from probe **A** in the microsecond time range ($t_{1/2} \approx 10$ μs) is even faster than the enzymatic choline release from acetylcholinesterase (≈50 μs).[4] Finally, the observed quantum yields for the three probes **A**, **B** and **C**, respectively, 0.27, 0.19 and 0.26 (Table 1), are sufficient to ensure an efficient photorelease of choline.

All three compounds are reversible inhibitors of both AChE and BuChE. The inhibition constants are in the μM range (Table 3) and are expected reasonable values for aromatic molecules carrying a quaternary ammonium group.[4] Clearly, probe **C** did not react covalently with the catalytic serine as anticipated, suggesting an unfavorable positioning of the isothiocyanato moiety within the active site. Furthermore, the photolytic byproduct of compound **A**, 2-nitrosoacetophenone, has no toxic effect on the enzymes even at concentrations up to 1 mM.

AChE is a fast-acting serine protease which terminates signals at cholinergic synapses.[21] Despite the report on

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(20) Biphasic decays have already been reported for *ortho*-nitrobenzyl ethers (ref 11b).

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the 3-D structure of AChE,[5] the mechanism of the rapid clearance of choline from the active site of the enzyme remains unclear.[22,23] This problem could be addressed by time-resolved Laue crystallography at the atomic level[8] with suitable photolabile molecules that can release choline rapidly and efficiently. Among the synthesized photolabile inhibitors of cholinesterases, compound **A** is the most promising candidate for such studies because of its ability of rapid photorelease of choline with high quantum yield.

Experimental Section

Melting points are uncorrected. Elemental analyses and mass spectral data were obtained at the Faculté de Chimie, Université Louis Pasteur, Strasbourg. ^{1}H NMR and ^{13}C NMR were run at 200 and 50 MHz, respectively. Chemical shifts are given in parts per million (ppm) using the residue solvent peaks as reference relative to TMS. A C_{18} column (3.9 × 300 mm) was used for HPLC analysis.

Acetylcholinesterase and butyrylcholinesterase were purified as described.[24,25] 2-Methyl-2-(2-nitrophenyl)-1,3-dioxolane (**1**) and 2-(2-nitrophenyl)-1,3-dioxolane (**2**) were prepared from 2-nitroacetophenone and 2-nitrobenzylaldehyde, respectively. Isothiocyanate derivative was purified on neutral aluminum oxide 90 (70−230 mesh) by column chromatography; all other compounds were purified by flash column chromatography on silica gel (230−400 mesh).

Syntheses. General Procedure I for the Reductive Opening of Ketal or Acetal. To a solution of the ketal **1a** or acetal **1b** (1 equiv) in CH_3CN at 0 °C were added titanium tetrachloride (1.3 equiv) and sodium cyanoborohydride (1.2 equiv). The resulting yellow suspension was stirred at room temperature for 2 h and then neutralized with saturated $NaHCO_3$ solution before extraction of the product into CH_2Cl_2. The organic layer was dried over $MgSO_4$, the solvent was evaporated with a rotary evaporator, and the crude product was purified by flash chromatography.

General Procedure II for the Preparation of the Mesylate Derivatives 3a,b and 8. To a solution of the corresponding alcohol (**2a**,**b**, **7**) in ether were added triethylamine (1.3 equiv) and methanesulfonyl chloride (1.1 equiv). After 1 h at 25 °C, the organic solvent was removed and the residue was purified by flash chromatography.

General Procedure III for the Preparation of the Iodide Derivatives 4a,b and 9. Sodium iodide (5 equiv) was added to a solution of the mesylate derivative (**3a,b** and **8**) in acetone. The reaction mixture was refluxed for 2 h, and then the organic solvent was removed and the residue was purified by flash chromatography.

General Procedure IV for the S_N2 Substitution of 4a,b and 9 with Trimethylamine. The iodide derivative (**4a, b** and **9**) was treated with a solution of toluene saturated with trimethylamine. The reaction mixture was stirred at 25 °C for 24 h. The precipitate was collected by centrifugation, washed five times with toluene, and dried *in vacuo*.

2-[1-(2-Nitrophenyl)ethoxy]ethanol (2a). According to the general procedure **I**, ketal **1a** (0.59 g, 2.8 mmol) in CH_3CN (14 mL) at 0 °C was treated with titanium tetrachloride (0.40 mL, 3.7 mmol) and sodium cyanoborohydride (0.21 g, 3.4 mmol). Purification on silica gel with hexane/EtOAc 4:1 yielded product **2a** (0.57 g, 95%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.52 (d, 3H, J = 6.3 Hz), 2.20 (br s, 1H), 3.31−3.50 (m, 2H), 3.71 (br m, 2H), 5.05 (q, 1H, J = 6.3 Hz), 7.38−7.47 (m, 1H), 7.62−7.69 (m, 1H), 7.76 (dd, 1H, J = 7.9, 1.4 Hz), 7.89 (dd, 1H, J = 8.1, 1.1 Hz).

2-[(2-Nitrobenzyl)oxy]ethanol (2b). According to the general procedure **I**, acetal **1b** (39 mg, 0.20 mmol) in CH_3CN (1 mL) was treated with titanium tetrachloride (0.25 μL, 0.22 mmol) and sodium cyanoborhydride (13.2 mg, 0.21 mmol). Purification on silica gel with EtOAc/hexane 1:4 gave **2b** (37 mg, 95%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 2.02 (br s, 1H), 3.70−3.74 (m, 2H), 3.84 (br m, 2H), 4.96 (s, 2H), 7.43−7.50 (m, 1H), 7.63−7.71 (m, 1H), 7.78−8.00 (m, 1H), 8.07 (dd, 1H, J = 8.1, 1.2 Hz).

Methanesulfonic Acid 2-[(1-Nitrophenyl)ethoxy]ethyl Ester (3a). According to the general procedure **II**, alcohol **2a** (0.46 g, 2.2 mmol) in ether (10 mL) was treated with triethylamine (0.40 mL, 2.9 mmol) and methanesulfonyl chloride (0.19 mL, 2.4 mmol). Purification on silica gel with hexane/EtOAc 1:1 gave **3a** (0.61 g, 97%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.54 (d, 3H, J = 6.5 Hz), 3.10 (s, 3H), 3.47−3.67 (m, 2H), 4.28−4.39 (m, 2H), 5.07 (q, 1H, J = 6.4 Hz), 7.40−7.48 (m, 1H), 7.63−7.71 (m, 1H), 7.76−7.80 (m, 1H), 8.06 (dd, 1H, J = 8.1, 1.1 Hz).

Methanesulfonic Acid 2-[(2-Nitrobenzyloxy)ethyl Ester (3b). According to the general procedure **II**, alcohol **2b** (35 mg, 0.18 mmol) in ether (0.5 mL) was treated with triethylamine (32 μL, 0.23 mmol) and methanesulfonyl chloride (15 μL, 0.19 mmol). Purification on silica gel with hexane/EtOAc 1:1 gave **3b** (0.48 mg, 98%) as a colorless oil: ^{1}H NMR ($CDCl_3$) 3.10 (s, 3H), 3.84−3.89 (m, 2H), 4.43−4.47 (m, 2H), 4.96 (s, 2H), 7.42−7.51 (m, 1H), 7.63−7.71 (m, 1H), 7.76−7.80 (m, 1H), 8.06 (dd, 1H, J = 8.1, 1.2 Hz).

1-[1-(2-Iodoethoxy)ethyl]-2-nitrobenzene (4a). According to the general procedure **III**, **3a** (0.71 g, 2.5 mmol) in acetone (20 mL) was treated with sodium iodide (1.9 g, 12.5 mmol). Purification on silica gel with hexane/ether 4:1 gave **4a** (0.78 g, 97%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.55 (d, 3H, J = 6.3 Hz), δ 3.24 (t, 2H, J = 6.50 Hz), 3.47−3.67 (m, 2H), 5.09 (q, 1H, J = 6.3 Hz), 7.39−7.48 (m, 1H), 7.64−7.71 (m, 1H), 7.84 (dd, 1H, J = 7.9, J = 1.4), 7.92 (dd, 1H, J = 8.2, 1.2 Hz).

1-[(2-Iodoethoxy)methyl]-2-nitrobenzene (4b). According to the general procedure **III**, **3b** (45 mg, 0.16 mmol) in acetone (0.5 mL) was treated with sodium iodide (120 g, 0.8 mmol). Purification on silica gel with hexane/ether 4:1 gave **4b** (50 mg, 96%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 3.36 (t, 2H, J = 6.50 Hz), 3.87 (t, 2H, J = 6.50 Hz), 4.97 (s, 2H), 7.42−7.50 (m, 1H), 7.64−7.71 (m, 1H), 7.87 (d, 1H, J = 7.8 Hz), 8.06 (dd, 1H, J = 8.1, 1.2 Hz).

***O*-[1-(2-Nitrophenyl)ethyl]choline Iodide (A).** According to the general procedure **IV**, the iodide **4a** (128 mg, 0.37 mmol) was treated with toluene (20 mL) saturated with trimethylamine. Purification by washing the precipitate with toluene gave compound **A** (135 mg, 90%) as a light yellow solid: mp 138−139 °C; ^{1}H NMR (CD_3CN) δ 1.58 (d, 3H, J = 6.3 Hz), 3.25 (s, 9H), 3.53−3.85 (m, 4H), 5.11 (q, 1H, J = 6.3 Hz), 7.53−7.63 (m, 1H), 7.74−7.81 (m, 2H), 7.98 (ddd, 1H, J = 8.0, 0.7, 0.7 Hz); ^{13}C NMR (CD_3CN) δ 22.7, 54.3, 62.9, 66.0, 74.2, 124.6, 128.0, 129.0, 134.2, 138.2; MS ($C_{13}H_{21}N_2O_3$, FAB positive) 253.2 g/mol. Anal. Calcd for $C_{13}H_{21}N_2O_3I$: C, 41.07; H, 5.58; N, 7.37. Found: C, 41.26; H, 5.57; N, 7.40.

***O*-(2-Nitrobenzyl)choline Iodide (B).** According to the general procedure **IV**, the iodide **4b** (128 mg, 0.37 mmol) was treated with toluene (20 mL) saturated with trimethylamine. Purification by washing the precipitate with toluene gave compound **B** (135 mg, 90%) as a light yellow solid: mp 148−150 °C; ^{1}H NMR (CD_3CN) δ 3.18 (s, 9H), 3.62−3.66 (m, 2H), 3.97−4.05 (m, 2H), 4.94 (s, 1H), 7.55−7.62 (m, 1H), 7.75−7.79 (m, 2H), 8.07 (d, 1H, J = 8.0 Hz); MS ($C_{12}H_{19}N_2O_3$, FAB positive) 239.2 g/mol. Anal. Calcd for $C_{12}H_{19}N_2O_3I$: C, 39.36; H, 5.23; N, 7.65. Found: C, 39.56; H, 5.17; N, 7.40.

(2-Nitrophenyl)[2-[(trimethylsilyl)oxy]ethoxy]acetonitrile (5). Solid zinc iodide (20 mg) and trimethylsilyl chloride (0.18 mL, 1.4 mmol) were successively added to **1b** (0.25 g, 1.3 mmol). After 5 min of stirring at room temperature, the reaction mixture was purified by chromatography with hexane/ether 1:1 to afford product **5** (0.30 g, 80%) as a colorles oil: ^{1}H NMR ($CDCl_3$) δ 0.15 (s, 9H), 3.82−3.95 (m,

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4H), 6.12 (s, 1H), 7.58−7.66 (m, 1H), 7.72−7.80 (m, 1H), 8.00 (d, 1H, J = 8.3 Hz), 8.14 (d, 1H, J = 8.1 Hz); IR ($CHCl_3$) 2338 cm^{-1} ($\nu_{C\equiv N}$).

2-[2-Amino-1-(2-nitrophenyl)ethoxy]ethanol (6). To the nitrile **5** (0.96 g, 3.3 mmol) was added dropwise a 1 M BH_3·THF solution (40 mL) at 0 °C. After 4 h of stirring at 25 °C, 6 N HCl (40 mL) was added to the reaction mixture, giving a white precipitate. After evaporation of the organic solvent *in vacuo*, the aqueous phase was basified with 4 N NaOH to pH 10. The product was extracted with EtOAc, and the organic phase was dried over $MgSO_4$ and reduced *in vacuo*. The residue (0.68 g, 92%) was purified by flash chromatography with dichloromethane/methanol/triethylamine 8:2:1 to give the amine **6** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 2.55 (br s, 3H), 2.83 (dd, 1H, J = 13.4, 8.3 Hz), 3.15 (dd, 1H, J = 13.4, 2.8 Hz), 3.39−3.62 (m, 2H), 3.68−3.78 (m, 2H), 5.00 (dd, 1H, J = 8.3, 2.7 Hz), 7.40−7.49 (m, 1H), 7.62−7.70 (m, 1H), 7.78 (dd, 1H, J = 7.9, 1.2 Hz), 7.97 (dd, 1H, J = 8.1, 0.9 Hz).

[2-(2-Hydroxyethoxy)-2-(2-nitrophenyl)ethyl]carbamic Acid *tert*-Butyl Ester (7). To a solution of the amine **6** (0.43 g, 1.9 mmol) in THF (8 mL) were added triethylamine (0.27 mL, 1.9 mmol) and di-*tert*-butyl dicarbonate (0.41 g, 1.9 mmol). The reaction mixture was stirred at room temperature for 45 min and purified by flash chromatography. Elution with hexane/EtOAc 1:2 afforded 0.63 g (98%) of the product **7**: ^{1}H NMR ($CDCl_3$) δ 1.37 (s, 9H), 2.41 (br s, 1H), 3.38−3.53 (m, 4H), 3.72 (br s, 2H), 5.03 (dd, 1H, J = 5.2, 5.0 Hz), 5.13 (br s, 1H), 7.41−7.49 (m, 1H), 7.60−7.69 (m, 1H), 7.76 (dd, 1H, J = 8.0, 1.5 Hz), 8.00 (dd, 1H, J = 8.2, 1.0 Hz).

Methanesulfonic Acid 2-[2-[(*tert*-Butoxycarbonyl)-amino]-1-(2-nitrophenyl)ethoxy]ethyl Ester (8). According to the general procedure **II**, alcohol **7** (0.59 g, 1.8 mmol) in ether (8 mL) was treated with triethylamine (0.30 mL, 2.2 mmol) and methanesulfonyl chloride (0.17 mL, 2.0 mmol). Purification on silica gel with hexane/EtOAc 1:1 gave **8** (0.69 g, 95%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.37 (s, 9H), 3.10 (s, 3H), 3.51−3.64 (m, 4H), 4.33−4.37 (m, 2H), 4.98 (br s, 1H), 5.01 (dd, 1H, J = 6.0, 4.3 Hz), 7.43−7.52 (m, 1H), 7.63−7.75 (m, 2H), 7.98 (d, 1H, J = 8.1 Hz).

[2-(2-Iodoethoxy)-2-(2-nitrophenyl)ethyl]carbamic Acid *tert*-Butyl Ester (9). According to the general procedure **III**, mesylate **8** (0.59 g, 1.45 mmol) in acetone (8 mL) was treated with sodium iodide (1.1 g, 7.25 mmol). Purification on silica gel with hexane/ether 1:1 gave **4b** (0.62 g, 98%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.35 (s, 9H), 3.22 (t, 2H, J = 6.1 Hz), 3.38−3.66 (m, 4H), 5.02 (br s, 1H), 5.06 (dd, 1H, J = 10.2, 3.9 Hz), 7.44−7.52 (m, 1H), 7.63−7.70 (m, 1H), 7.76 (d, 1H, J = 7.9 Hz), 7.94 (d, 1H, J = 8.1 Hz).

1-[1-(2-Iodoethoxy)-2-isothiocyanatoethyl]-2-nitrobenzene (10). To a solution of the iodide **9** (100 mg, 0.23 mmol) in dichloromethane (1 mL) was added trifluoroacetic acid (0.25 mL). After 10 min of stirring at room temperature, the reaction solution was concentrated *in vacuo*. The obtained residue was dissolved in CH_2Cl_2/H_2O 1:1 (2 mL) and treated with sodium bicarbonate (0.15 g, 1.8 mmol) and thiophosgene[26] (33 μL, 0.42 mmol). After extraction of the product with CH_2Cl_2, the combined organic phase was dried over $MgSO_4$ and evaporated *in vacuo*. Purification by flash chromatography on Al_2O_3 with hexane/ether 4:1 gave the isothiocyanate product **10** (60 mg, 69%) as a light yellow oil:[27] ^{1}H NMR ($CDCl_3$) δ 3.26−3.33 (m, 2H), 3.36−3.79 (m, 2H), 3.80 (dd, 1H, J = 14.6, 6.5 Hz), 3.95 (dd, 1H, J = 14.5, 3.3 Hz), 5.24 (dd, 1H, J = 6.4, 3.3 Hz), 7.50−7.59 (m, 1H), 7.72−7.80 (m, 1H), 7.92 (dd, 1H, J = 7.8, 1.4 Hz), 8.06 (dd, 1H, J = 8.2, 1.2 Hz); IR ($CHCl_3$) 2112, 2212cm^{-1} ($\nu_{S=C=N}$).

***O*-[α-(Isothiocyanatomethylene)-2-nitrobenzyl]-choline Iodide (C).** According to the general procedure **IV**, the iodide **10** (80 mg, 0.21 mmol) was treated with toluene (15 mL) saturated with trimethylamine. Purification by washing the precipitate with toluene gave compound **B** (82 mg, 92%) as a light yellow solid: mp 121−123 °C; ^{1}H NMR (CD_3CN) δ 3.38 (s, 9H), 3.75−3.95 (m, 3H), 4.11 (dd, 1H, J = 15.9, 5.9 Hz), 4.10−4.16 (m, 1H), 4.33 (dd, 1H, J = 15.9, 3.4 Hz), 5.54 (dd, 1H, J = 6.0, 3.4 Hz), 7.68−8.06 (m, 3H), 8.29 (d, 1H, J = 8.1 Hz,); ^{13}C NMR (CD_3CN) δ 50.2, 54.6, 63.7, 66.2, 76.4, 125.6, 129.2, 130.4, 133.0, 134.8, 148.2, 153.1; IR (KBr) 2061, 2139 cm^{-1} ($\nu_{S=C=N}$); MS ($C_{14}H_{20}N_3O_3S$, FAB positive) 310.1 g/mol. Anal. Calcd for $C_{14}H_{20}N_3O_3SI$: C, 38.45; H, 4.61; N, 9.61. Found: C, 38.64; H, 4.83; N, 9.60.

Laser Flash Photolysis. An excimer laser was operated at 351 nm (XeF) with a pulse energy of 100−150 mJ and a pulse width of about 20 ns. A pulsed Xe high-pressure arc was used as the monitoring light. A shutter disposed between the lamp and the sample cell (pathlength = 4 cm) was opened shortly before the laser flash to avoid photolysis of the starting compound by the Xe arc. The laser flash was perpendicular to the probing light from the pulsed Xe lamp.

Transient Spectrum Detection. The detection system allowed the capture of the transient spectrum (300−600 nm) at a given time delay after excitation (time window ≥5 ns). The analyzing light was split with a prism and was focused with a lens on the entrance slit of polychromator of OMA (optical multichannel analyzer). The transient spectrum was captured by a diode array camera viewing a gateable microchannel plate image intensifier. The absorption spectrum was observed after a laser shot by using the spectral distribution of the monitoring light prior to the laser pulse as the reference light intensity at each wavelength.

Kinetics Records. The detection system allowed the simultaneous capture of the kinetics at a given wavelength by using a transient digitizer. The analyzing light through the cuvette was gathered with a lens and focused on the entrance slit of a monochromator. The signal was recorded by a photomultiplier and fed into the transient digitizer. Rate constants were calculated by least-squares fitting of decay curves with single or dual exponential function, as appropriate.

HPLC Analysis of the Photolysis of Compounds A−C. A solution (4 mL) of 1 mM **A** in 100 mM phosphate buffer, pH 6.5 was exposed to 351-nm laser flashes. Aliquots of samples (100 μL) were injected into a reversed phase column, which was equilibrated with acetonitrile (45%) and a solution containing 5 mM sodium dodecylsulfate, 5 mM sodium sulfate at pH 2.00 (55%), at a flow rate of 1.0 mL/min. The compounds were detected by UV absorption at 310 nm. Compound **A** has a retention time of 9.0 min and the corresponding photolytic byproduct, 2-nitrosoacetophenone, a retention time of 6.1 min. Experiments with **B** and **C** were performed similarly.

Extent of Photolysis of Compounds A−C. Solutions of equimolar mixtures of **A**−**C** and 1-(2-nitrophenyl)ethyl carbamylcholine (each 0.26 mM) in 20 mM sodium phosphate pH 7.0 were exposed for varying times (10 to 60 s) to 365-nm line from a 1000 W xenon−mercury lamp. Aliquots of the irradiated samples (40 μl) were analyzed by reversed phase HPLC (40% acetonitrile, 60% of a solution containing 5 mM sodium dodecylsulfate, 5 mM sodium sulfate, pH 2.00). The HPLC retention time for compounds **A**−**C** and 1-(2-nitrophenyl)ethyl carbamylcholine were 11.1, 9.3, 16.3, and 9.1 min, respectively.

Enzymatic Assay[17] for Choline. A solution (4 mL) of 1 mM **A** in 100 mM phosphate buffer, pH 6.5 was subjected to 351-nm laser flashes. Aliquots of samples (50 μL) were taken and added to a 950 μL solution containing 1.6 units of choline oxidase, 4.3 units of peroxidase, 0.74 mM 4-aminoantipyrene, 0.34 mM $CaCl_2$·H_2O, and 5.3 mM phenol in 50 mM Tris buffer, pH 7.8. After 20 min at 25 °C, the solution turned to red and its absorbance was measured at 505 nm. The corresponding amount of choline (Figure 1c) was deduced from a standard reference. Experiments with **B** and **C** were performed similarly.

Activity Assay of AChE and BuChE. AChE and BuChE activities were measured in 50 mM phosphate buffer, pH 7.2, at 20 °C by Ellman's test[28] using acetylthiocholine and butyrylthiocholine as the respective substrates.

Inhibition of A−C on AChE and BuChE. Kinetic measurements of enzyme were performed at variable substrate concentrations in the absence and presence of different

(26) Thiophosgene was handled in a well-ventilated cupboard.

(27) Purification of compound **10** was achieved by rapid filtration on an Al_2O_3 column.

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concentrations of inhibitor to constitute the Lineweaver−Burk double reciprocal plots. The apparent competitive inhibition constants were calculated by the increase in slope of (1 + [I]/Ki) in the presence of inhibitor.

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