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2-Nitrobenzyl Quaternary Ammonium Derivatives Photoreleasing Nor-butyrylcholine in the Microsecond Time Range

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Abstract: 2-Nitrobenzyl derivatives of nor-butyrylcholine (N,N-dimethylaminoethyl butyrate) were synthesized and characterized as photolabile inhibitors of butyrylcholinesterase, displaying the required photofragmentation kinetics for rapid release of the enzyme substrate, nor-butyrylcholine.
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Photolabile precursors of biologically interesting molecules, or "caged", compounds can provide control of temporal and spatial release of enzyme substrates or receptor ligands by rapid photolysis, and are thus important tools in the study of fast biological processes.[1] The hydrolysis of neurotransmitter acetylcholine by cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), is an extremely fast enzymatic process.[2] Different types of "caged" compounds have been synthesized and tested for their potential use in exploration of the catalytic mechanism of AChE.[3-5] Among them, the "caged" nor-acetylcholine, which was synthesized and developed in our laboratory, is of particular interest, because hydrolysis of nor-acetylcholine is chemically identical to that of acetycholine and may serve as a paradigm for the study of the catalytic mechanism of AChE.[5] However, no suitable caged compounds are available for the studies on BuChE. Understanding the catalytic mechanism of BuChE is very important because BuChE breaks down the muscle relaxant succinylcholine, although the exact biological role of this enzyme is unknown. Since BuChE hydrolyses butyrylcholine faster than the endogenous substrate acetylcholine, we synthesized and characterized a series of 2-nitrobenzyl derivatives of N,N-dimethylaminoethyl butyrate (nor-butyrylcholine), of N-methylaminoethyl butyrate and of aminoethyl butyrate as "caged" compounds for BuChE.

The synthesis (Scheme 1) started with a reductive amination[6] of 2-nitro-benzaldehyde **1** or 2-nitro-acetophenone **2** with ethanolamine. The amino function in **3** was selectively protected before the hydroxyl group was acylated for the synthesis of product **5**. The conversion of **3** and **4** to the corresponding product **8** and **9** was directly achieved by successive methylation and acylation. A further methylation of **8** and **9** gave the quaternary amines **10** and **11**,[7] respectively.

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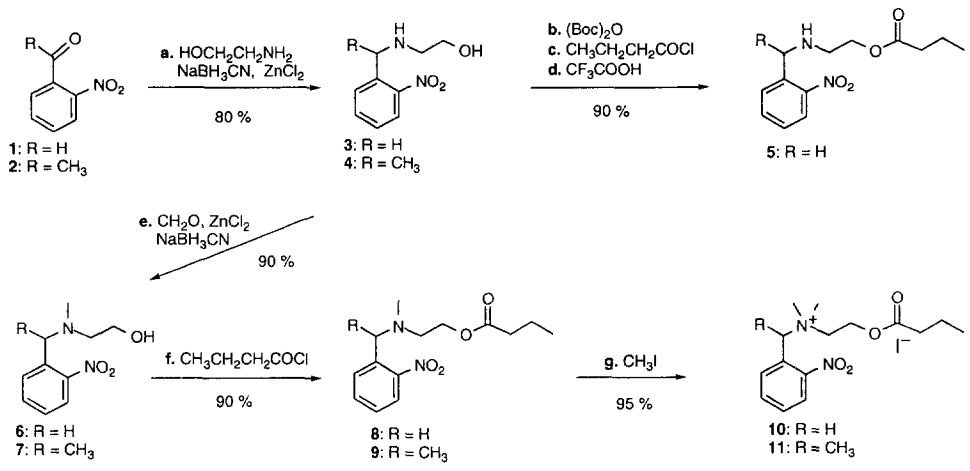
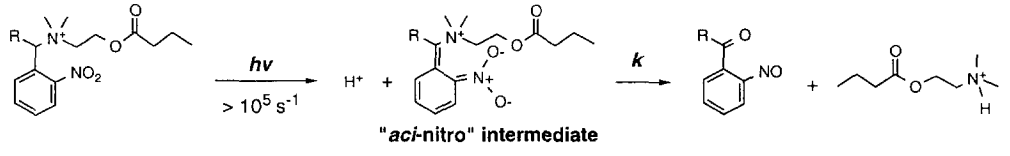


Scheme 1. Synthesis of 2-nitrobenzyl derivatives of N,N-dimethylaminoethyl butyrate (**10** and **11**), of N-methylaminoethyl butyrate (**8** and **9**) and of aminoethyl butyrate (**5**).

The application of caged compounds for the investigation of rapid kinetic processes depends critically on the ability of the photolysis reaction to give the desired products rapidly and with good yield. The kinetics of the photochemical fragmentation of 2-nitrobenzyl derivatives **5**, **8**, **9**, **10** and **11** were investigated by monitoring the presumed *aci*-nitro intermediate (Scheme 2), whose decay was show to be synchronous with the liberation of the corresponding photoproducts.[8]



Scheme 2. Proposed mechanism for the photofragmentation of 2-nitrobenzyl derivatives of N,N-dimethylaminoethyl butyrate.

Although photolysis of 2-nitrobenzyl derivatives of amine has been described as similar to that of the "caged" ATP,[8] no *aci*-nitro intermediate signals could be observed during the photolysis of either secondary amine **5** or tertiary amines **8** and **9** in aqueous buffer solution. Alternatively, the photofragmentation of quarternary ammonium derivatives **10** and **11** showed the expected *aci*-nitro intermediate (Figure 1a) allowing the pertaining kinetic studies (Figure 1b).

Similarly to what has been observed for caged nor-acetylcholine,[5] the substituent at the α-benzylic position in compounds **10** and **11** does not influence the decay rate: the half-time of their photolysis is around 25 μs (Figure 1b and Table 1), which is compatible with the turnover rate of BuChE.[2] Furthermore, the decay rate of the transient was not sensitive to pH around physiological values from 6.5 to 8.0 (data not show). This observation may be explained by the proposed mechanism in Scheme 2, where the release of nor-butyrylcholine is not pH-dependent, since the benzylic nitrogen atom of the *aci*-nitro intermediate remains positively charged.

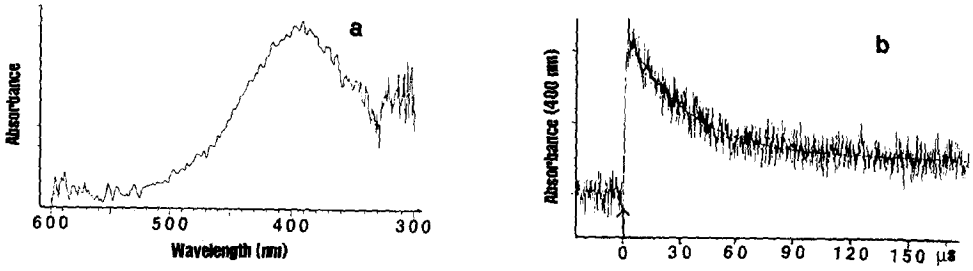


Figure 1. Kinetic analysis on the laser flash photolysis at 351 nm of compound **11** in 0.1 M phosphate buffer pH 7.2 at 20°C. a) The UV spectrum of a transient was observed by recording the spectral change before and after (0.2 µs delay) laser flash photolysis of compound **11**. b) Kinetic record at 400 nm after a single laser flash photolysis of compound **11**. Arrow indicates the beginning of the laser flash.

Table 1. Spectral Properties, Photofragmentation Parameters and Inhibition Constants of **10** and **11**.

Compound	λ_{max}[a] (nm)	ε_{max}[a] ($M^{-1}cm^{-1}$)	$t_{1/2}$[b] (µs)	Φ[c]	K_i[d], BuChE (µM)
10	260	4600	23	0.01	34.4 ± 1.7
11	256	3600	24	0.10	33.0 ± 1.1

[a] Absorption properties were determined in 0.1 M phosphate buffer, pH 7.2, at 20°C. [b] Half-time of photolysis. [c] Quantum yield. [d] Inhibition constant on BuChE.

The photochemical reaction of **10** or **11** was analyzed by UV spectroscopy, HPLC and an enzymatic assay for nor-butyrylcholine. The observed isobestic points in the UV absorption spectra (Figure 2a) are consistent with a uniform photodecomposition process. Further quantitative HPLC analysis[9] (Figure 2b) and enzymatic assay for nor-butyrylcholine[10] (Figure 2c) demonstrated that the amount of decomposed starting material **11** matched the amount of formed 2-nitroso-acetophenone and nor-butyrylcholine (Scheme 2). These results establish a stoichiometric conversion of nor-butyrylcholine from its precursor **11**.

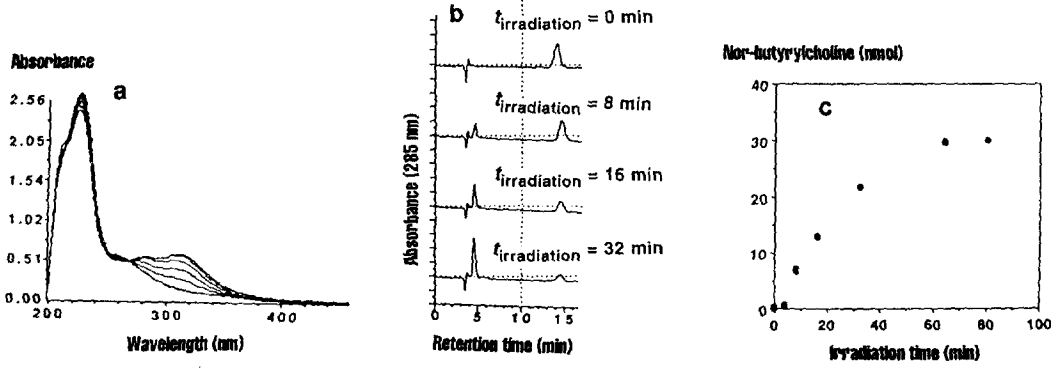


Figure 2. A solution of **11** (0.3 mM **11** in 50 mM phosphate buffer pH 7.2, 4°C) was exposed to 364.5 line of Hg-Xe lamp. a) UV-spectral recording of the photolysis. The lowest trace at 310 nm and 230 nm and the highest trace at 256 nm correspond to the starting material. b) In the HPLC,[9] compound **11** has a retention time of 14.3 min and the appearing peak at 4.7 min corresponds to the photolysis by-product, 2-nitrosoacetophenone. c) The amount of formed nor-butyrylcholine during the photolysis was quantified by an enzymatic assay.[10]

The quantum yields for the photoconversion of compounds **10** and **11** (0.10 and 0.01, respectively) (Table 1) were determined by comparison with the photolysis of 1-(2-nitrophenyl)ethyl carbamylcholine (Φ = 0.25).[11] The substituent at the α-benzylic position has a remarkable influence on the quantum yield of **10** and **11**, and the low quantum yield of **10** may limit its further application in the studies of BuChE.

Both compounds **10** and **11** showed competitive inhibition on purified human serum BuChE with inhibition constants around 30 μM (Table 1). This is not unusual since many simple quarternary ammonium compounds show inhibition in this range. Furthermore, laser irradiation at 351 nm has no observable damage on BuChE. The photolytic by-product from compound **11**, 2-nitroso-acetophenone, had no toxic effects, under the experimental conditions employed, on the activity of BuChE either.

In summary, our studies demonstrate that 2-nitrobenzyl quaternary ammonium derivatives **10** and **11** photodecompose, via an *aci*-nitro intermediate, with excellent kinetic properties ($t_{1/2}$ = 24 μs, 20 °C) allowing a rapid release of nor-butyrylcholine. In addition, the observed quantum yield for **11**, is sufficient to ensure an efficient photorelease of nor-butyrylcholine. Thus, compound **11** is a promising probe to photoregulate the BuChE activity for potential time-resolved studies on the catalytic mechanism of this enzyme.

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7. **10**: ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.96 (t, 3H, *J* = 7.31 Hz), 1.58 - 1.76 (m, 2H), 2.40 (t, 2H, *J* = 7.49 Hz), 3.36 (s, 6H), 4.29 - 4.34 (m, 2H), 4.64 - 4.72 (m, 2H), 5.65 (s, 2H), 7.78 (ddd, 1H, *J* = 1.46, 7.66, 8.04 Hz), 7.90 (ddd, 1H, *J* = 1.46, 7.66, 7.68 Hz), 8.16 (dd, 1H, *J* = 1.46, 8.04 Hz), 8.42 (dd, 1H, *J* = 1.46, 7.68 Hz). MS ($C_{15}H_{23}N_2O_4$, FAB positive) 295.1 g/mol. Anal. Calcd for $C_{15}H_{23}N_2O_4I$: C, 42.67; H, 5.49; N, 6.63. Found: C, 42.81; H, 5.59; N, 6.48.

   11: ^{1}H NMR (300 MHz, CD_3CN) δ 0.96 (t, 3H, *J* = 7.35 Hz), 1.60 - 1.70 (m, 2H), 1.89 (d, 3H, *J* = 6.78 Hz), 2.37(t, 2H, *J* = 7.34 Hz), 3.02 (s, 3H), 3.18 (s, 3H), 3.78 - 3.85 (m, 2H), 4.49 - 4.55 (m, 2H), 5.51 (q, 1H, *J* = 6.78 Hz), 7.74 - 7.84 (m, 1H), 7.88 - 7.93 (m, 1H), 8.04 - 8.05 (m, 1H), 8.07 - 8.08 (m, 1H). MS ($C_{16}H_{25}N_2O_4$, FAB positive) 309.1 g/mol. Anal. Calcd for $C_{16}H_{25}N_2O_4I$: C, 44.05; H, 5.78; N, 6.42. Found: C, 43.97; H, 5.83; N, 6.31.
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9. HPLC conditions: C18 reversed phase column (250 mm x 3.9 mm), isocratic elution using a mixture of 30 % acetonitrile and 70 % aqueous solution of 5 mM sodium dodecylsulfate and 5 mM sodium sulfate at pH 2.00.
10. Enzymatic assay for nor-butyrylcholine: 100 μL aliquots of samples of photolysis were withdrawn and added to 900 μL solution containing 1 unit of butyrylcholinesterase, 5 units of choline oxidase, 4 units of peroxidase, 0.74 mM 4-aminoantipyrine, 0.34 mM $CaCl_2 \cdot H_2O$, and 5.3 mM phenol in 50 mM Tris buffer, pH 7.8. After 30 min at 25 °C, the developed red dye was measured at 505 nm. The corresponding amount of nor-butyrylcholine was deduced from a standard reference.
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