# Synthesis and Characterization of Photolabile Compounds Releasing Noracetylcholine in the Microsecond Time Range**

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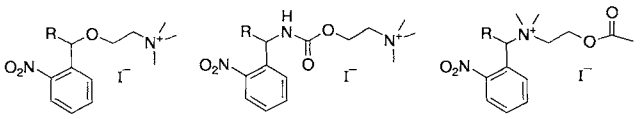
Temporally and spatially controlled release of biologically active molecules can be achieved with suitable photolabile precursors. Such compounds are important tools for the study of fast biological processes.[1] Hydrolysis of the neurotransmitter acetylcholine (ACh) by acetylcholinesterase (AChE[2]) is a particularly fast enzymatic process, with a turnover number approaching 20000 $s^{-1}$.[3] Knowledge of the 3-D structure of AChE[4] and several AChE–inhibitor complexes[5] has permitted a better understanding of structure–function relationships in the cholinesterases, but has also raised cogent new questions concerning the traffic of substrate and products to and from the active site[6, 7] in view of the high turnover rate. Time-resolved crystallography would present an ideal approach for investigating this issue at the atomic level and in real time, provided that suitable probes, which ensure efficient and synchronized initiation of the dynamic process, were available.[8] Photolabile precursors to the enzymatic product choline (**1a–3a**)[9] and the AChE substrate carbamoylcholine (**1b–3b**)[10] have been recently evaluated for their interaction with AChE and their potential use in time-resolved crystallographic studies.[11]



**1a**: R = Me
**2a**: R = H
**3a**: R = $CH_2NCS$

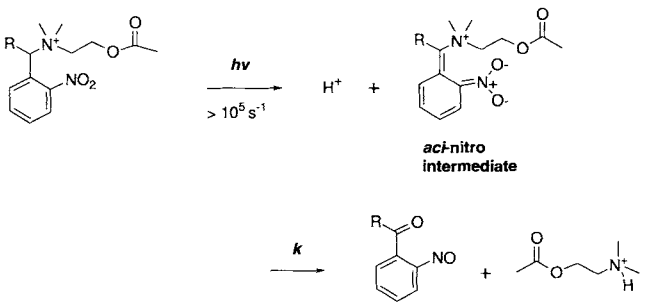
**1b**: R = Me
**2b**: R = H
**3b**: R = COOH

**1c**: R = Me
**2c**: R = H

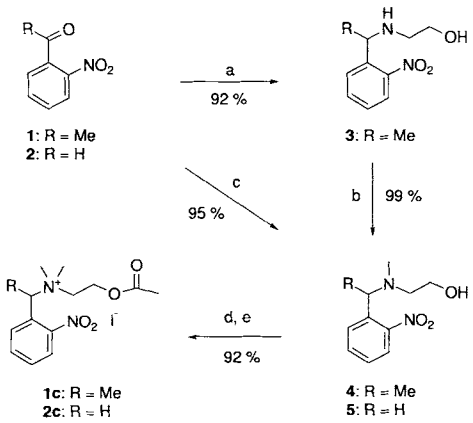
Here, we report the synthesis and characterization of a new type of photolabile probe (**1c** and **2c**) for time-resolved studies of AChE. These 2-nitrobenzyl derivatives of noracetylcholine (*N*,*N*-dimethylaminoethyl acetate) are close analogues of acetylcholine.[2] Hydrolysis of noracetylcholine by AChE follows an enzymatic reaction mechanism, which is similar to the hydrolysis of the natural substrate acetylcholine.[2, 12] Thus, hydrolysis of noracetylcholine, photogenerated from its precursor within the enzyme's active site, may closely mimic the hydrolysis of the neurotransmitter acetylcholine and serve as a paradigm for studying the catalytic mechanism of AChE under the conditions of time-resolved crystallography.

2-Nitrobenzyl groups have been used as photolabile protecting groups[13] to mask or "cage" biological molecules.[1, 14] The photochemical fragmentation reactions of 2-nitrobenzyl derivatives such as ethers, carbamates, carboxylic esters, and amines have been studied[1, 14] and parallels drawn to the analogous photochemical release of ATP from the 2-nitrobenzyl $P^3$-ester of ATP ("caged" ATP).[15] In this case, the decay of an "*aci*-nitro" intermediate (Scheme 1) was correlated to the formation of ATP. Compounds **1c** and **2c** are the first examples of 2-nitrobenzyl quaternary ammonium derivatives as "caged" effectors.

The syntheses of **1c** and **2c** are outlined in Scheme 2. The 2-nitrobenzyl group was introduced by reductive amination[16]




Scheme 1. Proposed mechanism for the photochemical fragmentation of 2-nitrobenzyl derivatives of noracetylcholine.




Scheme 2. Reagents and conditions: a) $HOCH_2CH_2NH_2$, $NaBH_3CN$, $ZnCl_2$, MeOH; b) $CH_2O$, $NaBH_3CN$, $ZnCl_2$, MeOH; c) $HOCH_2CH_2NHMe$, $NaBH_3CN$, $ZnCl_2$, MeOH; d) $CH_3COCl$, $NEt_3$, $CH_2Cl_2$; e) MeI, $CH_3CN$.

of either 2-nitroacetophenone (**1**) with ethanolamine or of 2-nitrobenzaldehyde (**2**) with *N*-methylethanolamine in the presence of sodium cyanoborohydride and zinc chloride. The tertiary amines **4** and **5** were subsequently acetylated and methylated, leading to the final products **1c** and **2c** in greater than 80% overall yields.

Photorelease of noracetylcholine from compounds **1c** was monitored by UV spectroscopy, HPLC, and an enzymatic assay (Figure 1). The observed isobestic points in the UV absorption spectra (Figure 1a) are consistent with a uniform photodecomposition process. This was confirmed by a quantitative HPLC analysis (Figure 1b) and a noracetylcholine assay (Figure 1c), which showed that the amount of starting material photolyzed matched the amount of nitroso product and noracetylcholine formed. These results also indicate a stoichiometric conversion of noracetylcholine from its precursor **1c**.

The quantum yields for compounds **1c** and **2c** (Table 1) were determined by comparing the extent of their photoconversion with that of 1-(2-nitrophenyl)ethyl carbamylcholine ($\Phi$ = 0.25).[9, 10] Dependence of the quantum yield of 2-nitrobenzyl derivatives on α-benzylic substituents has been previously documented,[1, 14] but the large difference between **1c** and **2c** is re-

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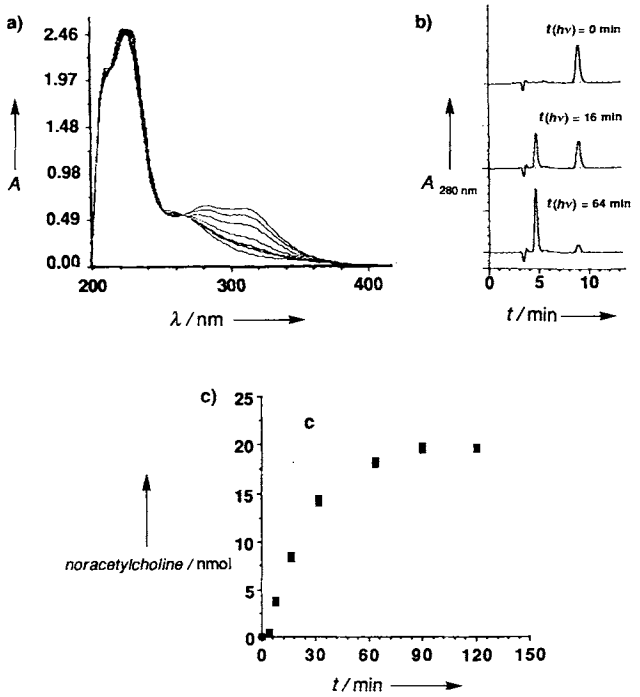


Figure 1. A solution of **1c** (0.2 mM) in 50 mM phosphate buffer at pH = 7.2 was exposed to irridiation (364.5 nm) from a Hg-Xe lamp. a) UV-spectra recorded during photolysis. The lowest trace at 230 and 300 nm and the highest trace at 260 nm correspond to the starting material **1c**. b) Aliquots (20 mL) of the irradiated sample were analyzed by HPLC. Compound **1c** had a retention time of 8.9 min. The peak at 4.7 min corresponds the photolysis by-product, 2-nitrosoacetophenone. c) The photoreleased noracetylcholine was quantified by an enzymatic assay with 100 mL aliquots of the irradiated sample.

Table 1. Spectroscopic properties, photofragmentation parameters, and inhibition constants for compounds **1c** and **2c**.

| Compd. | $\varepsilon$[M$^{-1}$cm$^{-1}$] [a] | $k$ [sec$^{-1}$] [b] | $\Phi$ [c] | $K_i$ [μM] [d] |
|---|---|---|---|---|
| **1c** | 3900 (254 nm) | $2.82\times10^4$ | 0.10 | $0.70\pm0.06$ |
| **2c** | 5100 (262 nm) | $2.90\times10^4$ | <0.01 | $4.52\pm0.35$ |

[a] Absorption spectra of 0.1 M phosphate buffer solutions at pH = 7.2 and 20 °C. [b] First-order rate constant of *aci*-nitro transient decay. [c] Quantum yield. [d] Inhibition constant on acetylcholinesterase.

markable. The low quantum yield of **2c** may limit its further application in time-resolved studies of AChE.

By the 351 nm laser flash photolysis of **1c** and **2c**, a transient absorption was detected around 400 nm (Figure 2, top), which is characteristic[1, 14, 15] of an *aci*-nitro intermediate. The transients were formed within the 20 ns duration of the laser pulse, and the subsequent decay accurately obeyed a first-order rate law (Figure 2, bottom). The decay rates (around $2.8\times10^4$ s$^{-1}$) were insensitive to pH at physiological values from 6.5 to 8.0. This observation may be explained by the mechanism proposed in Scheme 1. The release of noracetylcholine is therefore independent of pH since the benzylic nitrogen atom of the *aci*-nitro intermediate remains positively charged. Surprisingly, the substituent at the α-benzylic position does not influence the decay rate: the *aci*-nitro signals for compound **1c** and **2c** decayed at very similar rates (Table 1). Most importantly, the decay of the *aci*-nitro intermediates can be used as a kinetic measure for the release of the product.[15]

Both **1c** and **2c** showed inhibitory properties on purified *Torpedo* acetylcholinesterase. The inhibition constants are in the micromolar range (Table 1) and correspond to the values expected for aromatic molecules carrying a quaternary ammonium group. AChE remained fully stable when exposed to 351 nm laser flashes. The photolytic by-product from compound **1c**, 2-nitrosoacetophenone, had no toxic effects on the activity of acetylcholinesterase, even at 1 mM concentration (data not shown).

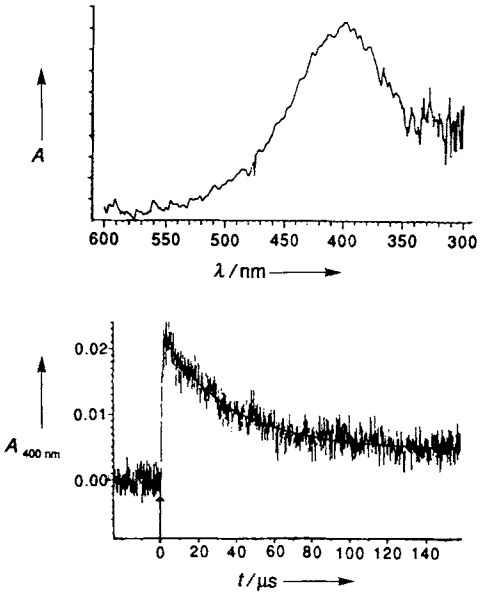


Figure 2. 351 nm laser flash photolysis of compound **1c** (1.5 mM **1c** in 0.1 M phosphate buffer, pH = 7.2, 20 °C). Top: transient absorption spectrum 1 ms after the laser pulse. Bottom: kinetic trace of the transient absorbance changes at 400 nm. The arrow indicates the begining of the laser flash.

In summary, our results demonstrate that photolysis of 2-nitrobenzyl quaternary ammonium derivatives **1c** and **2c** generates noracetylcholine over an *aci*-nitro intermediate. Probe **1c** shows excellent kinetic properties ($k = 2.82\times10^4$ s$^{-1}$, 20 °C) for the photolytic release of noracetylcholine and is comparable to the turnover rate of acetylcholinesterase ($k \approx 2\times10^4$ s$^{-1}$). Furthermore, the quantum yield for **1c** should ensure a sufficient photorelease of noracetylcholine. Thus, among all the probes synthesized up to now, compound **1c** is the most promising candidate for a time-resolved crystallographic study for investigating the molecular reaction mechanism of acetylcholinesterase.

## *Experimental Section*

All details for the photochemical reactions, activity assay of AChE, and inhibition of ligands on AChE can be found in ref. [9].

**1c:** Yellow crystals, m.p. 143–145 °C; $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 1.78 (d, 3H, $J$ = 4.5 Hz), 1.97 (s, 3H), 2.92 (s, 3H), 3.08 (s, 3H), 3.65–3.72 (m, 2H), 4.32–4.41 (m, 2H), 5.39 (q, 1H, $J$ = 4.5 Hz), 7.59–7.64 (m, 1H), 7.71–7.76 (m, 1H), 7.84–7.88 (m, 2H); MS (FAB positive): $m/z$(%): 281.1 (100) [$C_{14}H_{21}N_2O_4$]; correct elemental analysis.

**2c:** Yellow crystals, m.p. 123–125 °C. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 2.17 (s, 3H), 3.37 (s, 3H), 3.38 (s, 3H), 4.30–4.35 (m, 2H), 4.65–4.72 (m, 2H), 5.60 (s, 2H), 7.74–7.93 (m, 2H), 8.13–8.18 (m, 1H), 8.34–8.39 (m, 1H); MS (FAB positive): $m/z$(%): 267.1 (100) [$C_{13}H_{19}N_2O_4$]; correct elemental analysis

HPLC conditions: C18 reversed phase column (250 mm × 3.9 mm), isocratic elution with a mixture of 30% acetonitrile and 70% aqueous solution of 5 mM sodium dodecylsulfate and 5 mM sodium sulfate at pH = 2.00.

Enzymatic assay for noracetylcholine: 100 mL aliquots of photolysis samples were withdrawn and added to a 900 mL solution containing one unit of acetylcholinesterase, five units of choline oxidase, four 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 noracetylcholine was deduced from a standard reference.

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## The Trimesitylsilylium Cation**

Joseph B. Lambert* and Yan Zhao

No tricoordinated silylium ion, $R_3Si^+$, has been reported to date.[1] The most widely accepted criteria for determining silylium character are the $^{29}Si$ chemical shift and suitable structural parameters obtained from crystallography that indicate tricoordination and planarity. The closest approaches to such a species with alkyl groups have been our work[2] with the tetrakis(pentafluorophenyl)borate anion (TPFPB) and Reed's work[3] with various carborane anions. Chemical shifts in the range of $\delta(^{29}Si) = 90-115$ and C-Si-C angles of 114–117° were observed.[2, 3] The ideal angle for tricoordination is 120° and, according to calculations for the gas phase, chemical shifts of $\delta = 350-380$ are expected for these alkyl substituents.[3] Reed et al. noted that nonspecific solvation beyond the van der Waals radius of silicon may significantly lower the chemical shift. In their opinion, the optimal species observed to date have 36–52% silylium ion character.[3]

The high electrophilicity of tricoordinate silicon is responsible for its strong kinetic drive to coordinate. Calculations by Schleyer and co-workers[4] have shown that even rare gases, such as argon, and the C–H bonds of alkanes will coordinate with a tricoordinate silicon center. In order to avoid coordination by external nucleophiles, primarily solvent and anions, it is necessary to protect silicon sterically with appropriate substituents. Frenking[5] has suggested that the bis(pyrrolidino)-(2,5-di-*tert*-butylpyrrolidino)silylium ion would provide the appropriate steric protection. We have chosen to examine the trimesitylsilylium (mesityl = 2,4,6-trimethylphenyl = Mes) cation for similar reasons. Our molecular mechanics calculations suggest that the *ortho* methyl groups are very effective in sealing the silicon atom from large nucleophiles. To determine whether the *ortho* methyl groups are capable of interacting with the silicon atom, we carried out RHF/6-31G(d) calculations on the (2,6-dimethylphenyl)dihydrosilylium ion ($2,6\text{-}Me_2C_6H_3\text{-}SiH_2^+$). The methyl C–H bond lengths and other geometrical factors are not perturbed, which indicate little interaction. Evidently, the C–H bonds are sufficiently far from the silicon center.

Thus, the trimesitylsilylium ion appears to be an optimal choice since the *ortho* methyl groups shield the silicon center from attack by large nucleophiles but are prohibited by their geometry from interacting with the silicon. Indeed, the mesityl group proved successful in the preparation of the first monomeric, tricoordinate aluminum species, trimesitylaluminum.[6] The question then devolves on finding a method for generating the corresponding silylium ion in condensed phase. The most successful approach to date has been removal of hydride from a suitable silicon hydride ($R_3Si-H$) by reaction with the triphenylmethylium (trityl) cation.[2, 3] More than a decade ago, we tried to apply this approach to trimesitylsilane, but the reaction failed even upon prolonged heating. Apparently the *ortho* methyl groups prohibit the approach of trityl for reaction with the Si–H bond. We now report a novel leaving group that permits the preparation and NMR spectroscopic characterization of the trimesitylsilylium cation.

The strategy involves constructing a tetracoordinate silicon center with three mesityl substituents and a fourth group possessing a reactive site outside the steric sphere of influence of the *ortho* methyl groups. This fourth group must be capable of reaction with a suitable reagent that would result in its removal and generation of a silylium cation. The allyl group fulfills these specifications. We prepared allyltrimesitylsilane by reaction of chlorotrimesitylsilane with allyllithium. The X-ray crystal structure of this material (Figure 1) shows that the double bond extends beyond the *ortho* methyl groups and should be available for reaction with electrophiles.

A C–Si bond is weakened through hyperconjugation with a positive charge on a $\beta$-carbon atom. Uhlig[7] found that treatment of allylsilanes with triflic acid resulted in removal of the allyl group as an alkene. The proton attacks the double bond, which creates a positive charge at the position $\beta$ to the silicon atom. Triflate (or another nucleophile) then attacks silicon to generate a silyl triflate and an alkene. Similarly, Shade and Mayr[8] found that trityl tetrachloroborate reacts with allyltrimethylsilane to break the C–Si bond, expel the allyl group as allyltriphenylmethane, and produce chlorotrimethylsilane.

In the present case, the bulky mesityl groups should shield the silicon center from attack by large external nucleophiles, so that loss of the allyl group would produce the free silylium ion and an alkene (Scheme 1). The driving force would be provided by relief of steric strain around the original tetracoordinate silicon center. The same steric interactions then would prevent the silylium ion from reacting with the solvent, the anion accompanying the cationic electrophile, or the product alkene.

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