Synthesis and Photochemistry of a New Photolabile Derivative of GABA. Neurotransmitter Release and Receptor Activation in the Microsecond Time Region

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Rapid release of biologically active molecules from photolabile, or "caged", precursors is an important technique in the study of fast biological processes.[1] Photolysis of caged compounds provides temporal and spatial control of the distribution of substrate concentration in systems where diffusional mixing delays are a barrier to observation of rapid events. The most widely used caging group is the *o*-nitrobenzyl group. *o*-Nitrobenzyl ester derivatives of amino acids, including γ-aminobutyric acid (GABA), have been prepared previously, although slow photoactivation kinetics precluded use of the derivatives in receptor activation studies.[2] Introduction of a carboxyl substituent in the α-position of the caging group tends to significantly improve photolysis kinetics and quantum yield, as illustrated by the photolysis of a caged carbamoylcholine derivative, which upon photolysis gave carbamoylcholine with a quantum yield of 0.8 and a $t_{1/2}$ value of 45 μs.[3] This caged compound has been used in kinetic investigations of the acetylcholine receptor in BC_3H1 muscle cells.[4] More recently, the α-carboxy-*o*-nitrobenzyl group has been used to protect the amino group of glycine[5] and GABA.[6] These compounds are biologically inert until photolysis, which generates free amino acid, with quantum yields of 0.02 and photolysis half-times of about 1.5 ms. Herein we report the synthesis, physical, and biological characterization of a vastly improved caged version of GABA, in which the carboxylate group is protected as an α-carboxy-*o*-nitrobenzyl ester. This compound is of interest because the GABA receptor is the important inhibitory amino acid neurotransmitter in the central nervous system. The receptor is also important because it is modulated by clinically important compounds such as benzodiazepines and barbiturates which have application in the treatment of epilepsy and anxiety disorders.[7] The caged compound **4** is biologically inert before photolysis when tested with isolated $GABA_A$ receptor-containing[8] embryonic mouse cortical cells.

Alkylation of the carboxylate in the protected amino acid **1** with the bromide **2** was accomplished smoothly in refluxing benzene in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). Subsequent trifluoroacetic acid deprotection gave hydrolytically stable salt **4**, which was purified by Sephadex LH-20 chromatography and gave satisfactory spectral and combustion analysis data.[9] The analogous hydrochloride was also prepared, using anhydrous hydrogen chloride in dioxane in the deprotection step; this compound behaved identically to the trifluoroacetate **4** in both photolysis and receptor activation experiments.

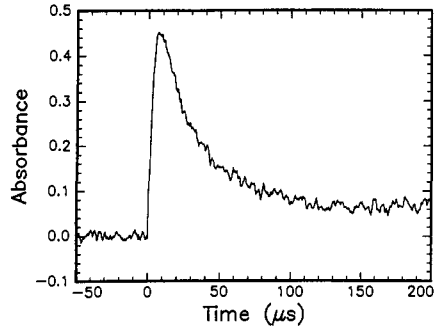
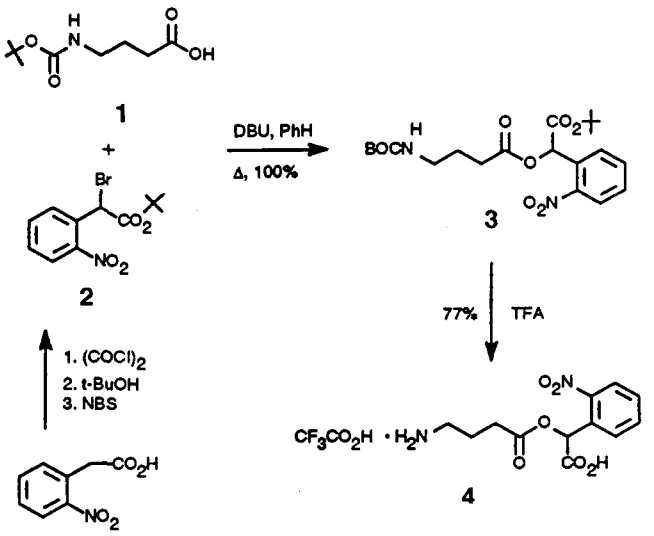


Figure 1. Absorbance transient produced by 308-nm XeCl excimer laser flash in a 5 mM solution of **4** in 100 mM phosphate buffer at pH 7.4 at 22 °C. A single-exponential approximation to the data yields a best fit decay time constant of 28 μs.



An absorbance transient produced by a laser flash at 308 nm[10] on a 5 mM solution of **4** in 100 mM phosphate buffer at pH 7.4 at 22 °C is shown in Figure 1. A single-exponential approximation to the data yields a best fit decay time constant of 28 μs. The maximum amplitude of the fast transient is plotted as a function of the wavelength between 370 and 500 nm. The resulting spectrum shown in Figure 2 has an absorbance peak centered at 425 nm and is characteristic of an *aci*-nitro intermediate[11] **5**. A

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(9) For **4**: mp 139–142 °C dec; ^1H NMR (D_2O) 8.13 (d, J = 8.1 Hz, 1H), 7.81 (t, J = 7.4 Hz, 1H), 7.70 (m, 2H), 6.69 (s, 1H), 3.07 (t, J = 6.4 Hz, 2H), 2.63 (m, 2H), 2.01 (t, J = 7.0 Hz, 2H). Anal. Calcd for $C_{14}H_{15}N_2O_8F_3$: C, 42.43; H, 3.82; N, 7.07. Found: C, 42.45; H, 3.97; N, 6.92.

(10) Details of the general photolysis method can be found elsewhere.[6] Briefly, photolysis of solutions of **4** was accomplished using a 308-nm Lumonics TE861M XeCl excimer laser, focused to irradiate a volume of 8 mm^3 of a 2 × 2 mm quartz cuvette. The pulse energy was between 10 and 50 mJ delivered in 10–20-ns pulses; flux densities of 1–8 mJ/mm^2 were produced at the front face of the cuvette. Transient absorbance of intermediates produced during photolysis was observed at right angles to the laser irradiation. For detection of the *aci*-nitro intermediate (**5**), the image of the irradiated cuvette volume closest to the laser was magnified and focused onto a monochromator slit, and the spectral distribution of the transmitted light was measured at selected wavelengths over the range 350–500 nm. The data collection time was determined by the decay rate of the signal for each compound and was at least 6 times the value of the time constant of the slowest component observed in the signal to ensure adequate representation of the decay for subsequent fitting operations. A nonlinear least-squares analysis program was used to fit single-exponential functions with constant base line offset to the transient absorbance signals.

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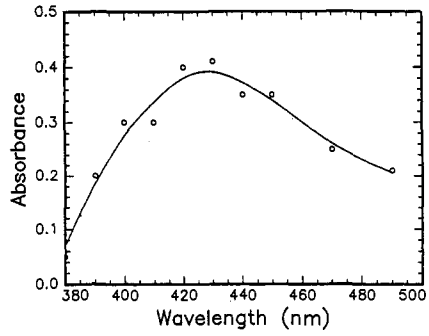
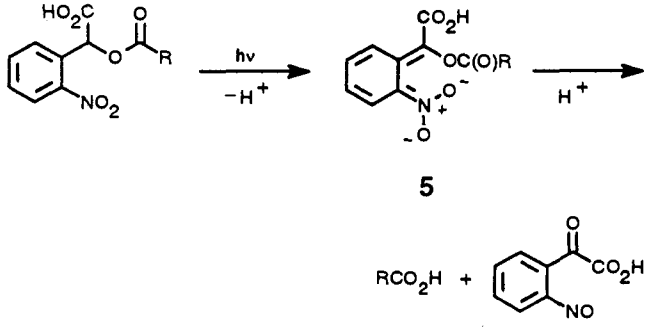


Figure 2. The maximum amplitude of the fast transient is plotted as a function of wavelength between 370 and 500 nm with a maximum at 425 nm and corresponds to the spectrum of the *aci*-nitro intermediate **5**.[11]

similar decay rate, spectrum, and quantum yield were seeen by Zhu et al.[12] for photolysis of α-phenyl-*o*-nitrobenzoate. This decay is thought to lead to the production of free amino acid in a concerted fashion and can thus be used as a kinetic measure of uncaged neurotransmitter production.[13]



High quantum yield of photolysis is a desirable property for the practical application of caged compounds in biological systems. The quantum yield calculated from six trials[14] with **4** was 0.16 ± 0.02. Single determinations performed using lower (6 and 12 mJ) and higher (26 mJ) energies gave similar quantum yields.

In order to study hydrolytic stability, an important feature, samples of **4** were stored for 12–48 h at 26 °C in 100 mM phosphate buffer at pH 7.4 in the dark. Aliquots were removed periodically and analyzed for free GABA by HPLC. A small but detectable conversion was seen which was estimated to be less than 1% in 24 h, which is considerably improved over other systems that release free neurotransmitter upon photolysis in the microsecond time region.[15]

Photolysis[16] of a neuron incubated in 500 μM **4** initiated a rapid transmembrane ion current through the GABA receptor

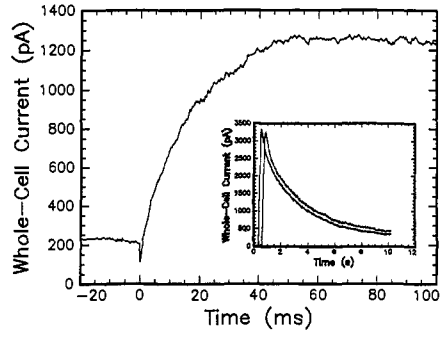


Figure 3. Current trace from fetal mouse cortical neurons.[19] A neuron was incubated for 0.5 s in a 500 μM solution of **4** in physiological saline, and membrane currents were observed by whole-cell recording. At 0 ms, the cell was exposed to a 0.5-mJ flash of 343-nm light generated by a pulsed dye laser and delivered through a fiber optic.[4b] Photolysis produced a concentration jump in this case of 40 μM free GABA as estimated by comparison to the current amplitude generated by application of control solutions of free GABA to the same cell. Inset: Cell flow experiments[18b] showed no inhibition of the current response when free 50 μM GABA was applied to a cell by rapid flow in the presence and absence of 500 μM **4** at pH 7.4.

(Figure 3) monitored by whole cell current recording.[17] The response is equivalent to the response elicited by application of 40 μM free GABA using a rapid mixing technique.[18] The amount of GABA released to the neuron could be adjusted by changing the concentration of **4** in the solution used to preincubate the cell before photolysis. The current response could be completely and reversibly inhibited in the presence of 100 μM bicuculline, a known $GABA_A$ receptor inhibitor. Also, no current response was produced by solutions of **4** when applied by a flow technique[18b] at concentrations ≤2 mM. Hydrolysis of **4** at 26 °C and pH 7.4 is slow (see above), and any background currents attributed to free GABA were eliminated by use of freshly prepared solutions of **4**. Applied free 50 μM GABA in the presence of 500 μM **4** at pH 7.4 produced a current response identical to that of 50 μM GABA alone (Figure 3 inset). Under these conditions **4** does not inhibit the current response produced by free GABA. The HCl salt of **4** was also used in parallel trials and produced identical results.

The new caged version **4** of GABA reported here possesses many desirable features for study of rapid biological events: (1) relatively high quantum yield of photolysis (uncaging); (2) very fast uncaging; (3) inertness, in terms of both receptor activation and inhibition before photolysis; (4) hydrolytic stability. Carboxylate caging with the α-carboxy-2-nitrobenzyl caging group, as described herein, should prove to be a useful and general method for the study of fast biological process involving carboxylate-containing activators.

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(14) Quantum yield was determined by photolysis of a 40-μL aliquot of 5 mM **4** (100 mM phosphate buffer, pH 7.4) in a 0.2 mm path length quartz cuvette with single shots of 308-nm light from the XeCl laser (Lumonics). Absorbed light was measured with a ballistic thermopile energy meter (Gentec), which measured the difference in transmission of light through the cuvette with sample and with buffer alone. Total absorbed energy was set at 22 ± 3 mJ (corrected for cuvette and buffer absorbance). Liberated GABA was measured by HPLC, performed by derivatizing aliquots with *o*-phthalaldehyde (Sigma protocol for amino acid OPA derivatization). Quantitation of the fluorescent derivative was determined on a Waters 300 × 4.5 mm Novapak C-18 reverse phase column; isocratic elution at 1 mL/min with 55% 50mM phosphate buffer at pH 6.5, 45% methanol eluted derivatized GABA at $k' = 5.1$. The peaks were recorded with an ISCO FL-2 fluorescence detector (350-nm excitation and 430-nm detection band filters) and quantified with a chromatographic integrator.

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(16) Neurons from fetal mouse cortical tissue were grown in culture and used in whole cell recording experiments[19] for photolysis studies on **4**. The extracellular recording buffer consisted of 145 mM NaCl, 1 mM calcium chloride, 10 mM glucose, and 10 mM HEPES (pH 7.4). The intracellular electrode buffer was 140 mM CsCl, 1 mM calcium chloride, 10 mM EGTA, and 10 mM HEPES (pH 7.2). The cell suspended by the recording electrode could be positioned in front of both a u-tube flow device[18] used for rapid exchange of GABA and caged GABA solutions and an optical fiber which delivered UV light from the laser to the cell.[4] For cell photolysis experiments with **4**, 300–500 μJ of 343 nm UV light generated in 500-ns pulses was provided by a flash lamp pumped dye laser (SLL 500, Candella) using oxazine 720 perchlorate in ethanol as the lasing medium.[4b]

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