# Synthesis and Characterization of Photolabile Derivatives of Serotonin for Chemical Kinetic Investigations of the Serotonin 5-$HT_3$ Receptor†

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ABSTRACT: A series of photolabile *o*-nitrobenzyl derivatives of serotonin (caged serotonin) were synthesized: the amine-linked serotonin derivatives *N*-(2-nitrobenzyl) serotonin (Bz-5HT) and *N*-(α-carboxy-2-nitrobenzyl) serotonin (*N*-CNB-5HT), and *O*-α-carboxy-2-nitrobenzyl) serotonin (*O*-CNB-5HT), which has the caging group attached to the phenolic OH group. All the derivatives released free serotonin when excited by 308-nm or 337-nm laser pulses. The time constant of serotonin release from *N*-CNB-5HT was 1.2 ms, with a quantum yield of 0.08. This is too slow for rapid chemical kinetic measurements. *O*-CNB-5HT is suitable for transient kinetic investigations of the serotonin 5-$HT_3$ receptor. It released serotonin with a time constant of 16 μs and a quantum yield of 0.03. The biological properties of *O*-CNB-5HT were evaluated, and the applicability of the compound for kinetic studies of the 5-$HT_3$ receptor was demonstrated. *O*-CNB-5HT does not activate the 5-$HT_3$ receptor by itself, nor does it modulate the response of a cell when co-applied with serotonin. When irradiated with a 337-nm laser pulse, *O*-CNB-5HT released free serotonin that evoked 5-$HT_3$ receptor-mediated whole-cell currents in NIE−115 mouse neuroblastoma cells.

The rapid release of biologically active compounds from photolabile precursors ("caged compounds") has become an important technique in the study of fast biological processes (*1*−*5*). This is of particular importance in the investigation of neurotransmitter receptors which have to be studied on cell surfaces, where rapid mixing techniques do not have the time resolution required for the investigation of fast (microsecond to millisecond) processes of ion channel function (*6*, *7*). Caged derivatives of carbamoylcholine (*8*), glycine (*9*, *10*; see note added in proof), *N*-methyl-D-aspartic acid (NMDA) (*11*), kainate (*12*), γ-aminobutyric acid (GABA) (*13*, *14*), and glutamate (*15*) have been synthesized. Upon photolysis these compounds release the neurotransmitter in the microsecond time region and can be used in kinetic investigations of receptor-formed transmembrane channels (*4*, *5*, *16*). The release of free neurotransmitter, which occurs in the microsecond time region, is the rate-limiting step for such investigations. Neurotransmitter release by photolysis also affords excellent spatial resolution. The distribution of muscle-type acetylcholine receptors on $BC_3$-H1 cells has been studied using caged carbamoylcholine (*17*). Caged glutamate derivatives have been used to map neuronal connections in cells and tissue slices (*18*−*21*). Caged derivatives of glutamate, carbamoylcholine, and GABA were used to map neuronal connections in the pharynx of the nematode *Caenorhabditis elegans* (*22*). A caged serotonin derivative suitable for similar studies has not been available so far. The synthesis and chemical and biological characterization of a caged serotonin derivative that is biologically inert and is photolyzed to liberate serotonin in the microsecond time region is reported here.

The serotonin type-3 receptor (5-$HT_3$ receptor) is the only ligand-gated ion channel in the 5-HT receptor family (*23*−*26*). 5-$HT_3$ receptors are found mainly in the area postrema and the solitary tract nucleus of the central nervous system where they mediate fast synaptic transmission (*23*, *27*−*32*). They are also present in the peripheral nervous system and in the intestinal tract where they are involved in emesis, nociception, and gut motility (*33*−*35*). To date few studies of the 5-$HT_3$ receptor using chemical kinetic measurements have been published. It was reported that desensitization of the 5-$HT_3$ receptor is regulated by two or more complex processes with varying requirements for ATP hydrolysis (*36*). A double-exponential time course for desensitization was also reported for homomeric 5-$HT_3$ receptor channels expressed in *Xenopus* oocytes (*37*). Attempts to determine the rate of association of serotonin and 2-methyl serotonin with the 5-$HT_3$ receptor in NIE-115 cells have been published (*38*), but the time resolution was 60 ms, and events faster than this could not be detected. The laser-pulse photolysis method using caged neurotransmitter precursors provides a 100-μs time resolution for rapid chemical kinetic studies of receptor function, as demonstrated for the acetylcholine (*8*, *39*−*42*), glutamate (*15*), γ-aminobutyric acid (GABA) (*13*, *14*), and glycine (*9*, *10*) receptors.

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The mechanism of 5-$HT_3$ receptor activation and desensitization, and the effects of inhibitors on this receptor, have been studied (*43*) using a cell-flow technique with a time resolution of 10 ms (*6*). Studies of the nicotinic acetylcholine, GABA, glutamate, and glycine receptors showed that the rate constants for channel opening and closing are faster than the diffusion limit of the cell-flow method (*6*). The rate constants for channel opening and closing can be determined by equilibrating the receptors on cell surfaces with an inactive photolabile precursor of the neurotransmitter. Channel opening is then initiated by photolyzing the caged compound to generate free neurotransmitter in the microsecond time region, and the resulting current is recorded by using the whole-cell current-recording technique (*44*). This transient kinetic technique of laser-pulse photolysis allowed evaluation of the rate constants for channel opening and closing (*39*, reviewed in refs *4* and *45*). These rate constants together with the dissociation constant of the neurotransmitter determine the concentration of open receptor channels as a function of neurotransmitter concentration and time. This information is of interest because the concentration of open channels and their conductance determines the transmembrane voltage changes (*46*−*48*) that control signal transmission between cells (*49*). The laser-pulse photolysis technique, in which photolabile, biologically inert precursors of the neurotransmitter are used, also allows one to determine the effects of inhibitors on the elementary steps of channel opening (*40*, *41*, reviewed in ref *4*).

The synthesis and application of caged adrenergic receptor ligands has been reported and is pertinent to the synthesis of caged serotonin. Phenylephrine, like serotonin, contains a phenolic hydroxyl group and an aliphatic amino group. *N*-caged phenylephrine (*50*), as well as *O*-caged phenylephrine (*51*), was found to release the hormone, which triggered muscle contraction in tissue preparations, upon irradiation. Several caged derivatives of phenylephrine, epinephrine, and isoproterenol were synthesized and their chemical and biological properties evaluated (*52*). These compounds are photolyzed in the microsecond-to-millisecond time region, with quantum yields for loss of starting material in the 0.1−0.4 range. Here we report the synthesis and photochemical and biological characterization of photolabile precursors of serotonin for the study of the mechanism of the 5-$HT_3$ receptor using the laser-pulse photolysis technique.

## MATERIALS AND METHODS

*Synthesis.* Starting materials were obtained from Aldrich or synthesized according to published procedures. Solvents were from commercial sources, of analytical grade purity, dried with 4 Å molecular sieves, and used without further purification. Silica gel 60 and Sephadex LH-20 used for column chromatography were from Merck and Pharmacia Biochem, respectively. Serotonin hydrochloride was purchased from TCI America. 2-Oxo-2-nitrophenyl acetate was prepared by selenium dioxide oxidation of 2-nitroacetophenone in pyridine (*13*, *53*). Methyl 2-bromo-2-(2-nitrophenyl)-acetate was prepared according to a published procedure (*13*, *51*). Fluoroacetic acid was prepared by acidification of the potassium salt (Aldrich) with sulfuric acid, followed by distillation of the free fluoroacetic acid in vacuo. Melting points were determined in a Fischer-Johns melting point apparatus and are uncorrected. NMR data are given as the chemical shift in parts per million (multiplicity, integration, coupling constant, assignment). Elemental analyses were carried out by Galbraith Laboratories Inc., Knoxville, TN. Analytical mass spectroscopy was performed at the School of Chemical Sciences, University of Illinois, Urbana, IL.

*N-(2-Nitrobenzyl) Serotonin (Bz-5HT).* A mixture of 2-nitrobenzaldehyde (1.4 mmol), serotonin hydrochloride (1.0 mmol), and sodium acetate (1.2 mmol) in anhydrous ethanol was stirred for 4 h at room temperature. Sodium cyanoborohydride (0.8 mmol) was added in portions over 2 h, and stirring was continued overnight. The reaction mixture was filtered, rotary evaporated, and diluted with water and the pH adjusted to 2. The solution was then extracted with ethyl acetate (3×) and the aqueous layer lyophilized. The resulting solid was dissolved in water and purified by twice being passed through a Sephadex LH-20 column using water as eluent. Lyophilization gave the final product. Yield: 22%. Mp: 122−127 °C dec. $^1$H NMR ($D_2O$): $\delta$ 7.89 (d, 1H, $J$ = 8.4 Hz, $C_3'$H), 7.60 (t, 1H, $J$ = 8.2 Hz, $C_5'$H) 7.42 (m, 2H), 7.07 (m, 2H), 6.62 (m, 2H), 4.26 (s, 2H, $CH_2$), 3.30 (t, 2H, $J$ = 6.90, $CH_2CH_2N$), 2.98 (t, 2H, $J$ = 6.51, $CH_2CH_2N$). Anal. Calcd for $C_{17}H_{17}N_3O_3 \cdot H_2O$: C, 55.74; H, 5.46; N, 11.48. Found: C, 56.19; H, 5.21; N, 11.33.

*N-(α-Carboxy-2-nitrobenzyl) Serotonin (N-CNB-5HT).* This compound was prepared as described for Bz-5HT, except that sodium 2-oxo-(2-nitrophenyl) acetate was used instead of 2-nitrobenzaldehyde. Yield: 13%. Mp: 125−130 °C dec. $^1$H NMR ($D_2O$): $\delta$ 7.66 (d, 1H, $J$ = 8.18 Hz, $C_3'$H), 7.57 (d, 1H, $J$ = 7.69 Hz, $C_6'$H) 7.43 (m, 1H), 7.34 (m, 1H), 7.10 (m, 2H, $C_4$H and $C_7$H), 6.58 (dd, 1H, $J$ = 8.96 and 1.91, $C_6$H), 6.32 (d, 1H, $C_2$H), 7.34 (s, 1H, CH), 3.45 (m, 2H, $CH_2CH_2N$), 2.99 (m, 2H, $CH_2CH_2N$). Anal. Calcd for $C_{18}H_{17}N_3O_5 \cdot H_2O$: C, 57.91; H 5.09; N, 11.26. Found: C, 57.90; H 5.10; N, 11.21.

*N-(Butyloxycarbonyl) Serotonin (N-Boc-5-HT).* Serotonin hydrochloride (2.12 g, 10 mmol) was suspended in 20 mL of chloroform. Sodium bicarbonate (0.84 g, 10 mmol) in 15 mL of water, 2 g of sodium chloride, and di-*tert*-butyloxycarbonyl anhydride (2.18 g, 10 mmol) in 5 mL of chloroform were added in this order. The mixture was refluxed for 3 h with vigorous stirring. The aqueous phase was washed once with chloroform; the combined organic phases were washed with water (2×) and brine (1×) and dried over magnesium sulfate. Solvent was removed under reduced pressure to give the product as a solid foam. Yield: 97%. Mp: 53−55 °C. $^1$H NMR ($CDCl_3$): $\delta$ 7.93 (s (br), 1H), 7.22 (d, 1H, $J$ = 8.57 Hz, $C_4$H), 7.01 (d + s, 2H, $J$ = 2.54 Hz), 6.80 (dd, 1H, $J_1$ = 2.40 Hz, $J_2$ = 8.64 Hz, $C_6$H) 5.12 (s (br), 1H), 4.65 (s (br), 1H), 3.42 (t + m, 2H, $J$ = 6.10 Hz, $CH_2-CH_2-N$), 2.87 (t, 2H, $J$ = 6.70 Hz, $CH_2-CH_2-N$), 1.44 (s, 9H, $-C(CH_3)_3$).

*N-(Butyloxycarbonyl)-O-(α-carboxymethyl-2-nitrobenzyl) Serotonin (N-Boc-O-MeCNB-5-HT).* *N-Boc*-5HT (1.1 g, 4 mmol) was dissolved in 25 mL of dry THF. Under a nitrogen atmosphere and exclusion of light 0.56 g (5 mmol) of potassium *tert*-butylate and 2.4 g (12 mmol) of methyl 2-bromo-2-(2-nitrophenyl)acetate were added. The mixture was stirred at 60 °C for 12 h and neutralized with acetic acid and the solvent removed under reduced pressure. The resulting dark brown syrup was purified by column chromatography using silica 60 and chloroform as eluent. Yield: 15%. Mp: 86−89 °C. $^1$H NMR ($CDCl_3$): $\delta$ 9.82 (s (br), 1H),

8.14 (d, 1H, $J = 8.2$ Hz, C′$_3$H), 8.00 (d, 1H, $J = 8.4$ Hz, C′$_6$H), 7.82 (m, 1H), 7.67 (m, 1H), 7.25 (m, 2H, $C_4H + C_7H$), 7.16 (d (unresolved), 1H), 6.90 (dd, 1H, $J_1 = 2.2$ Hz, $J_2 = 8.6$ Hz, $C_6H$), 6.49 (s, 1H, benzyl-*H*), 5.95, (s (br), 1H), 3.87 (s, 3H, $-OCH_3$), 3.32 (m, $CH_2-CH_2-N$), 2.83 (m, $CH_2-CH_2-N$), 1.42 (s, 9H, $-C(CH_3)_3$). HRMS for $C_{24}H_{27}N_3O_7$ ($M^+$) Calcd 469.184 901. Found 469.185 100.

*N-(Butyloxycarbonyl)-O-(α-carboxy-2-nitrobenzyl) Serotonin (N-Boc-O-CNB-5-HT).* A 300 mg sample of *N-Boc*-O-MeCNB-5HT was dissolved in 15 mL of methanol. A 15 mL portion of a 7% aqueous solution of potassium carbonate was added and the mixture stirred under light exclusion for 10 min. The reaction was carefully neutralized with dilute hydrochloric acid and the solvent removed under reduced pressure. The temperature of the water bath was below 30 °C at all times, and the pH was adjusted to ca.5.5 several times during the evaporation. The product was passed over a silica 60 column using chloroform/acetone/acetic acid/water (40:40:20:5) as eluent. Yield: 85%. Mp: 67−72 °C. $^1$H NMR (acetone-$d_6$): $\delta$ 7.91 (m, 2H, C′$_6$H+C′$_3$H), 7.60 (m, 1H), 7.47 (m, 1H), 7.23 (m, 2H), 7.11 (s, 1H), 6.88 (dd (not fully resolved), 1H, J ≈ 9 Hz, $C_6H$), 6.37 (s, 1H, benzyl-*H*), 3.30 (t, 2H, $J = 7$ Hz, $CH_2-CH_2-N$), 2.81 (t, $J = 7$ Hz, 2H, $CH_2-CH_2-N$), 1.39 (s, 9H, $-C(CH_3)_3$). HRMS for $C_{23}H_{25}N_3O_7$ ($MH^+$) Calcd 456.177 076. Found 456.177 000.

*O-(α-Carboxy-2-nitrobenzyl) Serotonin (O-CNB-5-Boc-HT).* A 120 mg sample of *N-Boc-O*-CNB-5HT was dissolved in 25 mL of chloroform/acetone/water (10:10:1), and after addition of 1.5 g fluoroacetic acid the solution was stirred for 15 h at room temperature under nitrogen. A mL portion of toluene was added, and the solvents were removed with reduced pressure at temperatures below 30 °C. Toluene was evaporated twice from the residue to remove traces of fluoroacetic acid. The resulting solid was passed twice over a Sephadex LH-20 column using water as eluent. Yield: 90%. Mp: 141−142 °C dec. $^1$H NMR (acetone-$d_6$/$D_2O$): $\delta$ 7.97 (m, 2H, C′$_6$H + C′$_3$H), 7.75 (m, 1H), 7.61 (m, 1H), 7.26 (m, 3H), 6.86 (dd, 1H, $J_1 = 2$ Hz, $J_2 = 9$ Hz, $C_6H$), 6.37 (s, 1H, benzyl-*H*), 3.29 (m, $CH_2-CH_2-N$), 3.13 (m, $CH_2-CH_2-N$). Anal. Calcd for $C_{20}H_{20}N_3O_7 \cdot 3H_2O$: C, 49.28; H, 5.34; N 8.62. Found: C, 48.91; H, 5.11 N, 8.62. HRMS for $MH^+$ Calcd 356.124 646. Found 356.124 500.

*Cuvette Photolysis.* The instrumentation used for transient absorption spectroscopy has been described (*8*, *12*). A XeCl excimer laser (Lumonics TE 861 M) produced 10-ns, 15-mJ pulses of 308-nm light. Using a $N_2$ gas mixture, the same laser gave 4-ns, 2-mJ pulses of 337-nm radiation. Photolysis was carried out in a 2 × 10 mm cuvette using 70 μL of caged compound solution. The transient absorbance (10-mm path length) produced by the laser-induced photolysis was monitored perpendicular to the laser beam using a beam from a broad-spectrum tungsten halogen lamp (Newport 780) with a Corning WGS360 cutoff filter. Absorbance changes were observed at wavelengths between 350 and 500 nm with a 0.2-m McPherson model 275 single-pass monochromator and detected by a Thorn EMI 9635QB photomultiplier. The signal was amplified with a Thorn EMI model A1 preamplifier, digitized at rates of up to 0.1 MHz, and stored on a digital storage oscilloscope (LeCroy Scope Station 140). The transient decay signals were analyzed using Microcal Origin software on a PC. Quantum yields were determined by measuring the number of photons absorbed by a 70-μL solution of caged compound using a joule meter (ballistic thermopile, Gentec ED-200) and determining the amount of free serotonin released via analytical HPLC. The product quantum yield was calculated as the number of serotonin molecules liberated divided by the number of photons absorbed.

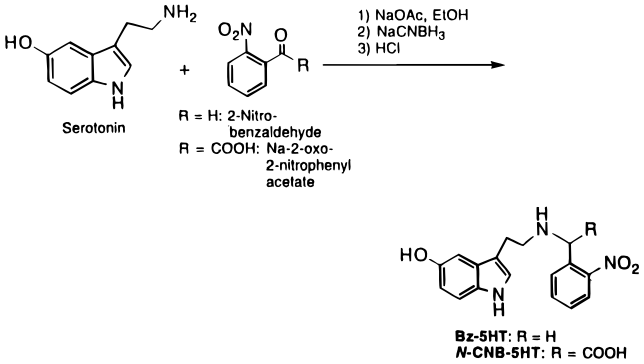
*UV Spectroscopy.* An Olis-14c spectrophotometer connected to a Dell 486 PC was used to record UV spectra of serotonin as well as of caged serotonin before and after photolysis. A 2 × 10 mm cuvette was used; the UV observation path length was 10 mm. When a solution was illuminated with laser light, the laser beam was focused to irradiate an area of approximately 1 × 1 × 8 mm inside the cuvette.

*Chromatography.* A Waters 600 E instrument with a 3.9 × 330 mm Waters Nova-Pak C-18 reversed-phase column was used for analytical HPLC. Isocratic elution was used to analyze the solution of caged compounds before and after photolysis with 1 mL/min of 50% methanol/50% 25 mM sodium phosphate buffer, pH 4.5. Free serotonin elutes at 2.4 min and *O*-CNB-5HT at 3.2 min under these conditions. Peaks were recorded with a Waters 486 UV detector at 254 nm and peak areas measured with a Hewlett-Packard 3392 integrator.

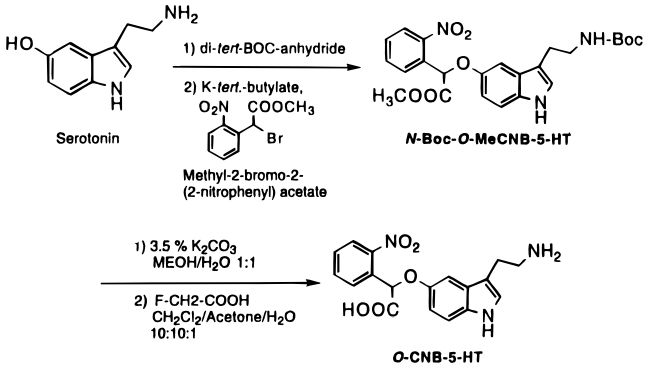
*Cell Culture and Whole-Cell Current Recording.* NIE-115 cells were grown in 80 cm$^2$, 260-mL Nunc culture flasks (Krackeler Scientific) at a temperature of 37 °C in a water-saturated atmosphere containing 5% $CO_2$. A 25 mL sample of culture medium made up of Dulbecco's modified Eagle's medium (DMEM; low glucose, Gibco) supplemented with 8% fetal bovine serum (FBS; Gibco) was used. Antibiotics (100 iu penicillin, 100 μg/L streptomycin, both from Sigma) were added to the medium only if contamination was observed. Cells were transferred weekly, seeded at $5 \times 10^5$ cells/flask, and fed twice during that period by replacing half the medium. On the day of transfer, cells were plated into 35-mm Falcon dishes (2 mL of cell suspension of $1 \times 10^4$ cells/mL per dish). These cells were then used for experiments within 2−5 days after plating. Recording glass electrodes were pulled from borosilicate glass (World Precision Instruments), using a two-stage puller (L/M 3 P-A, Adams & List, New York) and a Narishige flame polisher. The extracellular buffer consisted of (mM) NaCl (125), KCl (5.5), $CaCl_2$ (1.8), $MgCl_2$ (0.8), glucose (24.0), sucrose (37.0), and HEPES (20), pH adjusted to pH 7.2 with NaOH. The electrode solution contained (mM) KCl (150), NaCl (10), $MgCl_2$ (1), and HEPES (10.0), pH adjusted to 7.2 with KOH (both buffers according to H. P. M. Vijverberg, personal communication). An Axopatch 200A amplifier was used for recordings. Signal acquisition was carried out with pClamp software from Axon Instruments. Data were analyzed off-line on a PC using Microcal Origin software. All measurements were carried out at pH 7.2, 21−23 °C, and a transmembrane voltage of −60 mV.

*Laser-Pulse Photolysis.* A cell-flow device (*6*, *54*) was used to deliver solutions of caged serotonin or serotonin controls in extracellular buffer to cells suspended by a recording electrode in the whole-cell configuration (described in more detail in ref *43*). A Lumonics excimer laser produced single 4-ns laser pulses of ca. 200 μJ energy at 337 nm after coupling into an optical fiber (200-μm core diameter, SFS200/220N, FiberGuide Industries). The light was deliv-

Scheme 1




Scheme 2



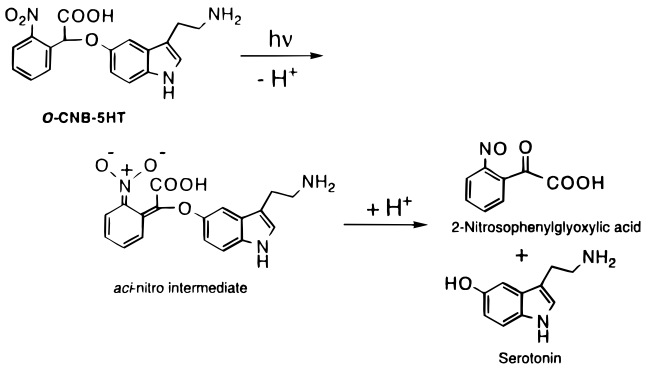

ered via this optical fiber to the cell attached to the recording electrode. Typically, the solution of caged neurotransmitter flows over the cells for only 200 ms before the laser is triggered. The traces in Figure 3 indicate that exposure of the receptor to caged serotonin for as long as 5 s does not have an experimentally significant effect on the current versus time traces. Data collection was synchronized to triggering of the laser by the acquisition system, which consisted of a Labmaster DMA digitizer board (Scientific Solutions), driven by the pClamp program (Axon). Electronic filtering of the signal from the current amplifier was set at 10 kHz (low pass) with digitization rates up to 50 kHz to accommodate the rapid current rise produced by receptor activation following photolysis of caged serotonin.

## RESULTS

The synthesis of the *N*-caged serotonin derivatives (Scheme 1), Bz-5HT and *N*-CNB-5HT, was accomplished in a single step from the α-oxo precursors by reductive amination with serotonin in the presence of sodium cyanoborohydride (*13*, *55*).

Synthesis of *O*-CNB-5HT, shown in Scheme 2, required protection of the primary amino group of serotonin (*56*). 2-Nitrophenylacetic acid was esterified with anhydrous methanol in the presence of sulfuric acid to yield methyl 2-nitrophenylacetate. Treatment with *N*-bromosuccinimide in carbon tetrachloride, using benzoyl peroxide as radical starter gave methyl α-(2-nitrophenyl)-α-bromoacetate in 23% yield (*13*, *51*). Reaction of the bromoester with *N*-Boc-5HT in the presence of potassium *tert*-butylate in anhydrous tetrahydrofuran afforded crude *O*-MeCNB-5HT (*51*). The compound could be purified by liquid chromatography. The methyl ester was cleaved rapidly with 3.5% potassium

Scheme 3




carbonate in 50% aqueous methanol. Care had to be taken in the removal of the *N*-protecting group since a labile benzylic bond was then present in the molecule. Fluoroacetic acid at room temperature selectively removed the *N*-group without attacking the nitrobenzyl function.

A rapid and efficient photoreaction releasing the desired products from their caged precursors is critical for the applicability of caged compounds in rapid kinetic studies (*57*). As shown in Scheme 3, the photolysis of 2-nitrobenzyl derivatives proceeds via an *aci*-nitro intermediate, which can be identified by its characteristic UV spectrum. The disappearance of the *aci*-nitro intermediate is coincident with product release (*58*−*65*).

Figure 1A shows the transient absorbance of the intermediate produced by irradiation of a solution of *O*-CNB-5HT in phosphate buffer of pH 7.0 with a single 4-ns pulse from a $N_2$ laser at 337 nm. The signal decays with a time constant of 16 ± 1.3 μs. The rate of *aci*-nitro decay is similar to that observed with caged carboxylates (*14*, *15*). *O*-Caged phenylephrine decays with a time constant of 500 μs (*51*). The spectral distribution of the transient intermediate is shown in Figure 1B. The absorbance maximum is around 420 nm. The observed wavelength dependence of the transient signal intensity is characteristic for the *aci*-nitro intermediate postulated for the uncaging reaction (*58*−*65*).

*N*-CNB-5HT was irradiated at pH 7.5 with 308-nm excimer laser pulses of 10-ns duration. As seen in Figure 1C, a transient is also observed; however, the time constant of the intermediate decay is in the millisecond region. The observed signal can be fit with two exponentials; the time constants are 1.2 and 6.2 ms, and the corresponding absorbance changes associated with these time constants are 0.15 and 0.06, respectively. When serotonin alone is irradiated with 308-nm laser pulses, a transient that decayed in the millisecond time domain is also observed (data not shown). Thus, the rapidly decaying fraction of the signal with a time constant of ca.1 ms might stem from the *aci*-nitro intermediate, but at an irradiation wavelength of 308 nm the serotonin ring system absorbs strongly (see Figure 2A) and the individual contributions of the CNB caging group and serotonin itself to the transient cannot be separated. In the best case, however, the uncaging reaction from *N*-CNB-5HT would have a rate constant of 1.2 ms, slower than serotonin release from *O*-CNB-5HT by a factor of ∼80. This slow release is in good agreement with previous results obtained with caged amines (*13*), which also photolyze slowly. The *N*-caged serotonin derivatives were, therefore, not further considered.

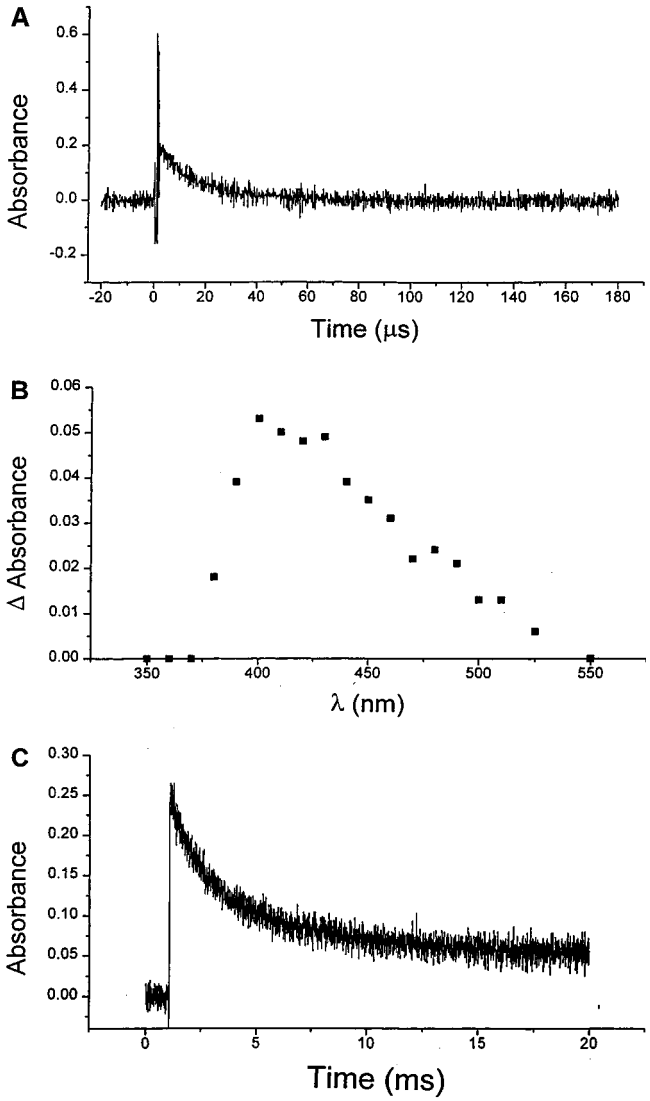



FIGURE 1: Transient absorbance spectra. (A) *aci*-Nitro intermediate absorbance decay of a 2.5 mM solution of *O*-CNB-5HT in 0.1 M phosphate buffer at pH 7.0 and room temperature. The transient absorbance was monitored at 420 nm in a 10 × 2 mm quartz cuvette, with a 10-mm path length for the monitoring light. A single pulse for excitation was produced by a $N_2$ laser at 337 nm, with an energy at the cuvette face of 2.2 mJ. The transient signal decay could be fit with a single exponential; the decay time constant is 16.0 ± 1.3 $\mu$s. (B) Spectral distribution of the maximum observed absorbance of the transient *aci*-nitro intermediate of *O*-CNB-5HT. Experimental conditions are the same as in panel A. (C) *aci*-Nitro intermediate absorbance of a 4 mM solution of *N*-CNB-5HT in 0.1 M phosphate buffer at pH 7.5 and room temperature. Excitation was produced with a single 10-ns, 30-mJ laser pulse at 308 nm from a XeCl excimer laser and was monitored at 425 nm. The faster component of the double exponential fit to the data has an amplitude of 0.15 absorbance unit and a time constant of 1.2 ms; the slow component has an amplitude of 0.06 absorbance unit and a time constant of 6.2 ms.

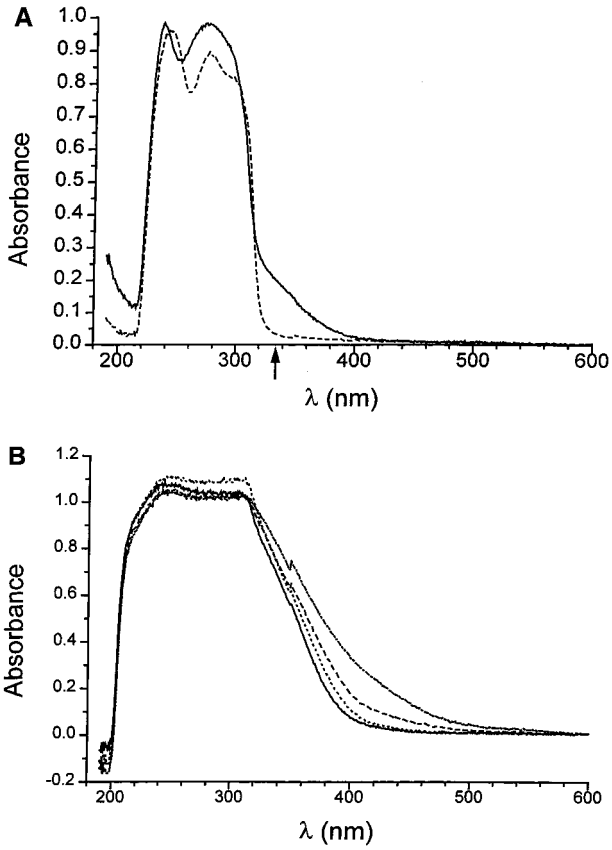



FIGURE 2: UV spectra of starting materials and photolysis products. (A) UV spectra of 0.25 mM solutions of serotonin (dashed line) and *O*-CNB-5HT (solid line) in 20 mM HEPES buffer, pH 7.2, at room temperature. Spectra were recorded on an Olis-14c spectrophotometer in a 10 × 2 mm quartz cuvette; the observation path length was 10 mm. Note that serotonin absorbs strongly below 325 nm. The arrow is at 337 nm, the wavelength of laser light produced by a $N_2$ laser. This wavelength is, therefore, suitable for uncaging of nitrobenzyl derivatives of serotonin. (B) Reaction spectra of a 1 mM solution of *O*-CNB-5HT in 0.1 M phosphate buffer at pH 7.0. All other experimental conditions were as described for panel A, except that the solution was purged with nitrogen and the cuvette sealed to prevent oxidation of liberated serotonin. The concentration of *O*-CNB-5HT was chosen to observe the spectral changes in the wavelength range from 330 to 500 nm; in this case the absorbance detector is saturated at wavelengths below 300 nm. The solution was irradiated with single 4-ns, 2.2-mJ laser pulses at 337 nm produced with a $N_2$ laser. The lower trace at 400 nm (solid line) corresponds to the spectrum of *O*-CNB-5HT before irradiation. The next spectra in succession were recorded after photolysis with 120, 350, and 800 laser pulses. The increase in absorbance at 400 nm and above is expected for the nitrosoketone that is being formed in the photolysis reaction together with free serotonin; the shorter wavelength region of the spectrum is dominated by the absorption of the serotonin system.

Serotonin is unique among the neurotransmitters that activate ligand-gated ion channels in that it has an aromatic indole system. Its intrinsic absorption must, therefore, be considered in photolysis experiments. Figure 2A shows UV spectra of serotonin (broken line) and *O*-CNB-5HT (solid line) recorded in a HEPES buffer at pH 7.2. Serotonin absorbs strongly at wavelengths of 325 nm and lower. Irradiation in this wavelength range would be expected to lead to the formation of photolysis side products, or, at least, to unproductive absorption. However, the absorption of the nitrobenzyl group tails out at around 400 nm. This window between 325 and 400 nm has to be used for the photorelease of serotonin from caged precursors. UV light from a $N_2$ laser at 337 nm (indicated by the arrow in Figure 2A) is suitable for these experiments.

Figure 2B shows UV−vis reaction spectra of a solution of *O*-CNB-5HT during photolysis with consecutive 337-nm excimer laser pulses. To prevent interference from air oxidation of the liberated serotonin, photolysis was carried out in a sealed cuvette under a nitrogen atmosphere. The concentration of the solution was 1 mM; at this concentration the detector was saturated at wavelengths below 320 nm.

However, spectral changes in the 330−500-nm range could be determined. The starting material is indicated by the solid line. The subsequent spectra were obtained after 120 (dotted line), 350 (broken line), and 800 (top line) laser pulses. A slight increase in absorbance at around 420 nm, tailing out at ca. 500 nm, is observed. This is in agreement with the formation of increasing amounts of 2-nitrosophenylglyoxylic acid during the photolysis reaction. This photoproduct of the α-carboxy-2-nitrobenzyl moiety has an extended $\pi$-system and, therefore, a higher absorbance at longer wavelengths than the starting material, *O*-α-carboxy-2-nitrobenzyl serotonin. Below 325 nm the absorption by serotonin is dominant. Since serotonin is both a product of the photoreaction and a component of the starting material *O*-CNB-5HT, only small absorbance changes occur at lower wavelengths after photolysis. This suggests that side reactions do not play a significant role under these conditions. It should be noted that the uppermost trace in Figure 2B was recorded after irradiation with 800 laser pulses; even at that point the reaction was not complete.

The low efficiency of conversion of *O*-CNB-5HT upon irradiation by multiple laser pulses, shown in Figure 2B, indicates a low quantum yield for the photolysis reaction. Product quantum yields were determined by irradiating a fixed volume of a solution of *O*-CNB-5HT with 1, 5, or 10 4-ns pulses of 337-nm laser light generated by a Lumonics $N_2$ laser. For each pulse, the energy absorbed by the solution was measured using a joule meter. The concentration of liberated serotonin was determined by using HPLC. The product quantum yield was calculated as the number of serotonin molecules produced divided by the number of photons absorbed by the solution. For *O*-CNB-5HT a quantum yield of 0.03 was found.

The time course of the photolysis reaction is essentially pH-independent. In the pH range from 2.5 to 10.5, no significant change in the rate of decay of the intermediate was observed (not shown). Likewise, the amount of intermediate formed during photolysis, as judged from the amplitude of its signal, seemed to be unaffected by changes in pH.

It should be noted that the 5-$HT_3$ receptor is activated by micromolar concentrations of serotonin. We determined that at a concentration of 2.8 $\mu$M serotonin half the maximum activation of the receptor is obtained; i.e., the EC-50 value is 2.8 uM. Therefore, a relatively low efficiency of serotonin release from its caged precursor can be tolerated in this case. Care has to be taken, however, to remove traces of free serotonin from the solution before the biological experiments. This rigorous purification of *O*-CNB-5HT was achieved by repeatedly passing the compound over a Sephadex LH-20 column using pure water as eluent. The eluate contained >99.5% pure *O*-CNB-5HT with no detectable trace of free serotonin, as judged by HPLC. Typically the concentration of *O*-CNB-5HT in the eluate was around 1 mM. After lyophilization and storage in a desiccator in the dark, however, a small but significant amount of free serotonin (<0.2%) was found in the samples. For elemental analysis a lyophilized sample was used without further drying. The analysis indicates the presence of some water with the compound. The stability of an aqueous solution was monitored over 3 days, by testing samples that were kept at room temperature and at 4 °C. In this case, no free serotonin was being formed at either temperature. This indicates that *O*-CNB-5HT does not undergo dark hydrolysis even after being kept in solution for longer times, which is of particular interest for locating receptors on particular cells (*17*−*22*, *23*, *24*), where the stability of the caged compounds is of the greatest importance. The compound was stored in aqueous solution at −20 °C and diluted into the appropriate buffers for experiments.

The applicability of *O*-CNB-5HT for biological studies is demonstrated in Figure 3, where the results of electrophysiological experiments with NIE-115 mouse neuroblastoma cells in the whole-cell recording configuration (*43*) are shown. These experiments were carried out at room temperature (21−23 °C), pH 7.2, and a transmembrane voltage of −60 mV. The solid traces 1 and 2 in Figure 3A are control measurements with 10 and 5 $\mu$M serotonin, respectively. At time 5000 ms (arrow) the laser was fired; it did not affect the whole-cell current response of the cell. This shows that the laser pulse does not produce receptor activation or cell leakage. Trace 3 is a measurement using 5 $\mu$M serotonin together with 450 $\mu$M *O*-CNB-5HT. In two consecutive applications of this solution to the same cell we observed maximum currents that were 78−88% of the control (5 mM serotonin) and desensitization rates of 100% and 140% of the control, respectively. Trace-to-trace variability of both the maximum current and the rate of desensitization was on the order of 20% with the NIE-115 cells used for our experiments. Therefore, within experimental error, the cell-flow experiments with 5 mM serotonin in the absence and presence of a 90-fold greater concentration of the caged compound give the same results. Trace 4 shows the whole-cell current response to the application of 900 $\mu$M *O*-CNB-5HT, without laser irradiation of the cell. A minimal rise in whole-cell current occurs and is most likely due to submicromolar concentrations of free serotonin present in the solution. Trace 5 is the response of the cell to a laser pulse alone; no serotonin or caged compound was present in this case. Again no receptor activation or cell leakage was observed. These experiments show that the application of neither *O*-CNB-5HT nor 337-nm laser pulses alone has a significant effect on the experimental error of the measurements that we estimate to be ±30%.

Figure 3B shows the current rise induced by photolysis of 900 $\mu$M *O*-CNB-5HT over another cell. The current rise can be described by a monoexponential function over at least 90% of its duration. The time constant for the whole-cell current rise due to photoliberated serotonin in experiments conducted with two different cells was 25.1 ± 0.2 and 22.2 ± 0.6 ms.

The whole-cell current responses to photoliberated serotonin were calibrated by applying 10 $\mu$M serotonin and determining the whole-cell current corrected for receptor desensitization (*6*). Concentrations of photoliberated serotonin were estimated from calibration curves of whole-cell currents versus serotonin concentration (*43*). The whole-cell current response to the application of a 900 $\mu$M solution of *O*-CNB-5HT (Figure 3A, trace 4) indicates that this solution contains approximately 0.3 $\mu$M free serotonin. The whole-cell current observed upon laser-pulse photolysis of 900 $\mu$M *O*-CNB-5HT, shown in Figure 3B, corresponds to a concentration of approximately 4 $\mu$M serotonin liberated over the cell. What effect does contamination with 0.3 mM free

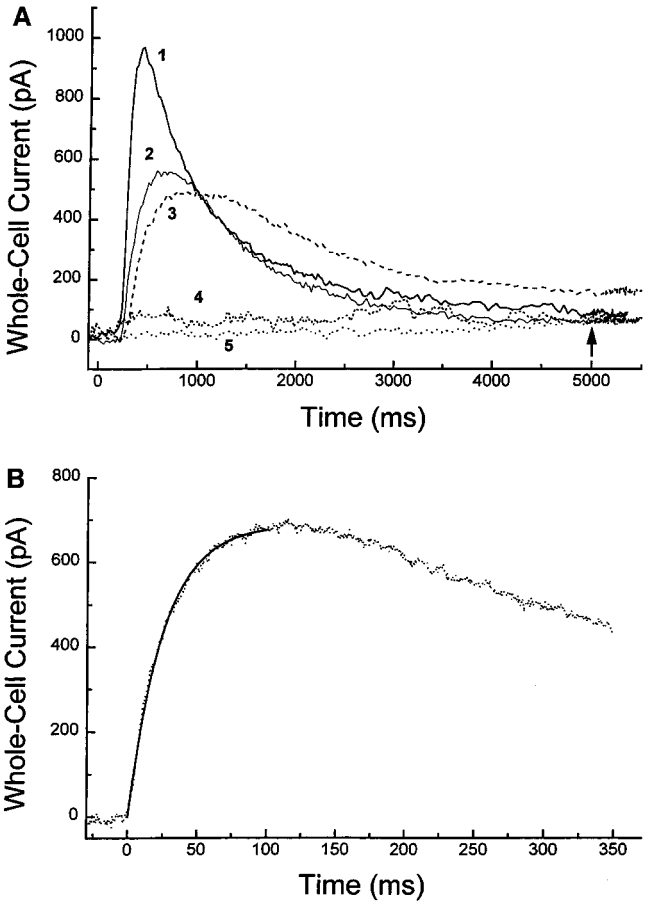



FIGURE 3: Whole-cell current responses of NIE-115 mouse neuroblastoma cells, pH 7.2, 22−24 °C, −60 mV. (A) Cell-flow experiments. Trace 1 corresponds to application of 10 $\mu$M serotonin; trace 2 is a 5 $\mu$M serotonin control experiment. A 4-ns, 150-mJ laser flash of 337-nm wavelength, produced by a Lumonics $N_2$ excimer laser, was fired after 5 s of neurotransmitter flow (arrow). It induced no additional receptor activation or cell leakage. Trace 3 was obtained by flow application of 5 $\mu$M serotonin together with 450 $\mu$M *O*-CNB-5HT. Note that the current response is not significantly different from that of the control (trace 2). Trace 4 represents the whole-cell response to application of 900 $\mu$M *O*-CNB-5HT. No laser was fired in trace 4, and there was only a minimal current response, considered to be due to some free serotonin in the solution of the caged compound (see the text). Trace 5 shows that there was no whole-cell current response to a laser flash (indicated by arrow at 5000 ms). (B) Laser-pulse photolysis experiment. A 900 $\mu$M *O*-CNB-5HT solution flowed over a cell for ca. 1.2 s, using a cell-flow device (*6*, *54*), before the reaction was initiated by a laser pulse (337 nm, ca. 150 $\mu$J) at time 0. The current rise of the whole-cell response (dotted line) could be fitted to a single-exponential function (solid line) with a time constant of 25.1 ± 0.2 ms. A cell-flow experiment with the same cell using 10 $\mu$M serotonin produced a maximum (corrected) current of 1086 pA. The laser-pulse photolysis-induced current of 689 pA corresponds to a concentration of ~4 $\mu$M liberated serotonin, as calculated from a standard curve of current amplitude versus serotonin concentration.

serotonin in a solution of 900 mM *O*-CNB-5HT have on the experiment in which 4 $\mu$M serotonin is photolytically released? In a typical experiment the cell is exposed to the solution of caged serotonin for 200 ms before the laser is fired. The rate of desensitization of 5-$HT_3$ receptors is concentration-dependent (*36*), and at the submicromolar concentrations present in the solution of *O*-CNB-5HT the extent of desensitization that occurs within the 200-ms time frame of a typical laser-pulse experiment can be neglected. From the EC-50 value of 2.8 $\mu$M serotonin and the slow desensitization rate ($t_{1/2}$ = 100 ms at a serotonin concentration 30 times higher than that of the contaminant) (see ref *43*) one can estimate that less than 2% of the receptors present would be affected by the free serotonin contaminant. To illustrate this, we have exposed the receptor to caged serotonin in the experiments in Figure 3 (see trace 4) considerably longer than the 200 ms usually used in these experiments.

The 5-$HT_3$ receptor desensitizes slowly; when rapid perfusion techniques are used, a double-exponential desensitization with rates of hundreds of milliseconds and several seconds can be observed (*30*, *43*). Under these circumstances. in laser-pulse photolysis experiments. the falling phase of the current reflects both receptor desensitization and diffusion of photoliberated serotonin away from the cell surface. The desensitization rate obtained in cell-flow experiments (*6*) obtained at 4 $\mu$M serotonin of 1.3 $s^{-1}$ cannot, therefore, be directly compared to that obtained in the laser-pulse photolysis experiment in Figure 3B of 1.9 $s^{-1}$.

## DISCUSSION

Photolabile precursors of serotonin were synthesized from readily available starting materials. Care had to be taken to protect the caged serotonin derivatives from light, particularly when aqueous solutions were handled. All compounds were sufficiently soluble in water or buffers (in excess of 2 mM) to allow study of biological processes. Upon irradiation with a pulse of laser light, serotonin was released from the caged precursors. Caged serotonin compounds can, therefore, be useful to introduce concentration jumps of serotonin at high spatial and temporal resolution.

The rate of release of a neurotransmitter from its *o*-nitrobenzyl-caged precursor can be followed by monitoring the decay of the characteristic *aci*-nitro intermediate signal, which can be identified by its similarity to the spectra obtained with other compounds (*58*, *59*, *62*−*65*). Generally, the decay of the *aci*-nitro signal is considered to reflect neurotransmitter release (*61*, *64*, *65*).

The rate of *aci*-nitro decay observed for *O*-CNB-5HT is similar to that observed with caged carboxylates (*5*, *14*, *15*). The spectral distribution of the transient intermediate, having its absorbance maximum around 420 nm, is consistent with the *aci*-nitro intermediate postulated for the uncaging reaction (*58*−*64*). Several examples of caged phenols have been reported in the literature. Caged dopamine, protected with the α-carboxynitrophenyl caging group, was found useful for the creation of rapid dopamine concentration jumps (*66*). The kinetics or quantum yield of the photolysis reaction was, however, not determined. Caged phenylephrine, derivatized on its phenolic hydroxyl with the α-carboxy-2-nitrobenzyl group (*8*), released the parent hormone with a time constant of 500 $\mu$s and a product quantum yield of 0.28 (*51*). The phenolic hydroxyls of isoproterenol and phenylephrine were caged using the 2-nitrobenzyl group. For this caged phenylephrine, an *aci*-nitro intermediate lifetime of ca. 10 ms and a quantum yield for the loss of starting material of 0.13 were reported (*52*). The increase in rate and quantum yield due to the α-carboxy substituent is well documented for carbamates (*8*), carboxylates (*5*, *14*, *15*), and phenols (*51*). Compared to α-carboxy-2-nitrobenzyl-caged phenylephrine (*51*), we find a significantly faster rate of photorelease of

serotonin from *O*-CNB-5HT (time constants of 500 $\mu$s for caged phenylephrine and 16 $\mu$s for serotonin), but a much less efficient release (the quantum yield for caged phenylephrine is 0.28 and for caged serotonin 0.03).

It should be noted that both the fast photolysis rate and the low observed quantum yield for this reaction are not in agreement with data from other caged phenolic groups. This, together with the apparent lack of an effect of the pH of the reaction medium on the rate of the photoreaction, also suggests that a different pathway of cleavage of the *aci*-nitro intermediate may be invoked in the case of *O*-CNB-5HT.

Opening of neurotransmitter-gated ion channels occurs in the microsecond-to-millisecond time region (*8*, *39*, *67*, reviewed in refs *4* and *45*). Liberation of free neurotransmitter from a caged precursor must, therefore, occur on a microsecond time scale to resolve kinetically channel-opening steps. For this reason, *N*-caged derivatives of serotonin were not considered in biological studies, since photorelease of serotonin occurred only in the millisecond time region. The *O*-caged serotonin, *O*-CNB-5HT, was found to photolyze rapidly (time constant 16 $\mu$s), thus releasing serotonin fast enough for rapid chemical kinetic studies of the function of the ion channel of the 5-$HT_3$ receptor.

Since micromolar concentrations of serotonin are sufficient to activate 5-$HT_3$ receptor channels (*43*), the rather low quantum yield observed for the liberation of serotonin by photolysis of *O*-CNB-5HT should not pose an insurmountable problem for kinetic studies of this receptor. By the same token, care has to be taken to remove traces of free serotonin from the solution. This can be accomplished by repeated chromatography over a Sephadex LH-20 column (*11*). An additional hindrance is the long wavelength of activation needed to avoid cell damage and side reactions of the serotonin system. If the photolysis is carried out with 308-nm laser light, the quantum yield (the number of serotonin molecules released per absorbed photon) is lower, as expected from the unproductive absorption by the serotonin ring system. The actual amounts of released serotonin, however, are higher, since the absorbance at this wavelength for serotonin, but also for the 2-nitrobenzyl moiety, is dramatically higher, and consequently many more photons per laser pulse are absorbed (see Figure 2A). When photolysis at a shorter wavelength is contemplated, however, cell damage may occur. The NIE-115 cells used in this study, for example, do not tolerate 308-nm irradiation.

Of the compounds synthesized, *O*-CNB-5HT, with the caging group attached to the phenolic hydroxyl function of serotonin, fulfilled the criteria for a caged neurotransmitter. The compound is stable toward hydrolysis in aqueous solution at pH 7, but liberates serotonin upon laser irradiation at 337 nm, a wavelength not damaging to the investigated cells or receptors. The rate of serotonin release, as judged from the *aci*-nitro decay rate, is 16 $\mu$s, sufficiently fast for rapid kinetic studies, and the quantum yield of 0.03, albeit low, is sufficient for activation of the 5-$HT_3$ receptor in NIE-115 mouse neuroblastoma cells. Judging from the experiments carried out so far, serotonin concentration jumps as high as 6−7 $\mu$M, corresponding to half-maximal activation of the receptor, should be attainable without undesired side effects. This should make it possible to cover a wide enough range of serotonin concentrations to determine the time constants of channel opening and closing. *O*-CNB-5HT by itself does not activate the 5-$HT_3$ receptor, and it does not modulate the whole-cell current response of a cell when it is co-applied with serotonin.

Particularly, its stability toward hydrolysis should make *O*-CNB-5HT well suited for locating specific receptors on neurons and in tissue slices (*17*−*21*), or within a known circuit of neurons controlling an observable response (*22*), where prolonged equilibration of the target tissue with the caged compound is required.

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## NOTE ADDED IN PROOF

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