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Synthesis, Photophysical, Photochemical and Biological Properties of Caged GABA, 4-[[(2H-1-Benzopyran-2-one-7-amino-4-methoxy) carbonyl] amino] Butanoic Acid¶

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ABSTRACT

The photorelease of a caged neurotransmitter can be used to investigate the function of neuronal circuits in tissues. We have designed and synthesized a stable, caged γ-aminobutyric acid (GABA) derivative, 4-[[(2H-1-benzopyran-2-one-7-amino-4-methoxy)carbonyl]amino] butanoic acid (BC204), that releases the neurotransmitter in physiological medium when irradiated with UV light at 300–400 nm in PBS at pH 7.4. The release of GABA occurs with the formation of the major photoproduct, 7-amino-4-(hydroxymethyl)-2H-1-benzopyran-2-one, via a solvolytic photodegradation mechanism of the coumarin moiety and was confirmed by electrospray mass spectrometry/mass spectrometry (ESI MS/MS). BC204 is chemically stable and shows no intrinsic activity after many hours under physiological dark conditions. These properties suggest that BC204 is an excellent form of caged GABA that is well suited for long-term biological studies.

INTRODUCTION

Caged neurotransmitters, which are photolabile, biologically inert prodrugs, have been increasingly used as a tool in cell biology, particularly in studying neuronal processes (1–4). In the past, many caged forms of the major fast excitatory neurotransmitter glutamate have been produced. Homologous forms of caged glutamate have different properties regarding their kinetics, quantum yield of photolysis, stability or release probability upon two-photon excitation. The differences between these molecules give researchers the opportunity to select the compound that best accommodates their specific experimental needs. In contrast with the wide variety of caged glutamate, only a few caged forms of the fast inhibitory neurotransmitters γ-aminobutyric acid (GABA) and glycine are currently available (5–7). These forms have several drawbacks, such as low stability under physiological dark condition (8), noninert photolysis intermediates or byproducts (7) and the need for high laser power (9–11), all of which limit a more widespread use of these compounds in neurobiological research. In order to develop an improved form of caged GABA, which would overcome some of these limitations, we predicted new structures based on molecular modeling studies.

Caging reagents based on the coumarin structure originated in 4-bromomethyl-7-methoxycoumarin that was used as a phosphate protecting group (12). These substances were reported to have good quantum yields and sufficient stability in aqueous medium (13). Brominated 7-hydroxycoumarin-4-ylmethyl esters and carbamates of glutamate where shown to release the neurotransmitter under one- and two-photon photolysis (14,15). Coumarins show a large variability in their absorption properties, dependent on the substitution pattern, and are readily synthesized by employing a modified Pechmann cyclization (16). Therefore, this substance class appeared to be a suitable candidate for further development to improve its quality as a photosensitive protecting group. In this article, we report a facile synthesis for an amino-substituted coumarin-caged GABA (BC204), the investigation of its photo physical and photochemical properties, and its biological activity.

MATERIALS AND METHODS

Anhydrous THF, DMF, DMSO (Aldrich Chem. Co., Milwaukee, WI) and all reagents were used as received. Other solvents were reagent grade and used without further purification if not stated otherwise. Tetrodotoxin (TTX) was purchased from Alomone Labs (Jerusalem, Israel), bicuculline methiodide from Tocris (Ballwin, MO), 2-OH-saclofen from RBI (Natick, MA). If not stated otherwise, all other chemicals used for electrophysiological recordings were from Sigma (St. Louis, MO).

Analytical high-performance liquid chromatography (HPLC) was performed on a Waters Delta 600 system with UV-VIS detection. A C_{18} column (Nova-Pak 4 μ, 3.9 × 300 mm, Waters) was used for HPLC analysis at a flow rate of 1 mL/min at 20°C with an injection volume of 10 μL. The solvent system comprises eluent A acetonitrile/water/TFA = 80/20/0.07%, and eluent B water/0.1% TFA. ^{1}H-NMR spectra were recorded at 300 MHz with the residue solvent peak as reference relative to TMS. ^{13}C-NMR spectra were recorded at 75.4 MHz and calibrated to the solvent peak. The spectra recorded in D_2O were calibrated using MeOH as an internal standard. Flash chromatography was performed on silica gel (J. T. Baker, 40 mm particle size). Melting points are not corrected. UV/VIS spectra were recorded on

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Abbreviations: ACSF, artificial cerebrospinal fluid; BC204, 4-[[(2H-1-benzopyran-2-one-7-amino-4-methoxy)carbonyl]amino] butanoic acid; ESI MS, electrospray ionization mass spectrometry; GABA, γ-aminobutyric acid; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS/MS, mass spectrometry/mass spectrometry; TTX, tetrodotoxin.

a Hewlett-Packard spectrophotometer. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA).

*Syntheses: (3-Hydroxyphenyl)-carbamic acid ethyl ester **2***. Commercially available 3-aminophenol 1 (10.0 g, 92 mmol) was dissolved in a mixture of 10 mL dry THF and 9.0 mL (113.4 mmol, 1.2 equivalents) pyridine under argon atmosphere. The reaction mixture was cooled to +3°C and 8.8 mL (10.0 g, 92 mmol) of ethyl chloroformate were added slowly, so that the temperature remained below +10°C. Stirring was continued at room temperature for 14 h. Then 50 mL of water were added and the mixture was extracted with 30 mL of CH_2Cl_2. The organic layer was separated, washed with 3 × 30 mL water, dried over Na_2SO_4, filtered and the solvent was evaporated. The greenish, oily residue was treated with 5 mL of toluene and cooled to +4°C for 1 h yielding 10.26 g (62%) of colorless crystals: mp 95°C; IR (KBr) 3301 (s, br, OH+ NH), 1693 (s, CO); ^{1}H-NMR (acetone-d_6) δ 8.39 (s, br, 1 H), 8.17 (s, 1 H), 7.20–6.93 (m, 3 H), 6.50–6.46 (m, 1 H), 4.12 (q, 2 H, ^{3}J = 7.1 Hz), 1.23 (t, 3 H, ^{3}J = 7.1 Hz); ^{13}C-NMR (acetone-d_6) δ 159.7, 155.3, 142.4, 131.1, 111.3, 111.2, 107.2, 61.9, 15.8; analytically calculated for $C_9H_{11}NO_3$: C, 59.66; H, 6.12; N, 7.73; found C, 59.69; H, 6.20; N, 7.76.

*[4-(Chloromethyl)-2-oxo-2H-1-benzopyran-7-yl]-carbamic acid ethyl ester **3***. Sulfuric acid (80%, 50 mL) was precooled with an ice bath. Then 1.81 g (10 mmol) of the protected aminophenol and 1.81 mL (2.20 g, 13 mmol) ethyl 4-chloroacetoacetate were added. The mixture was stirred at room temperature for 4 h, then poured into 50 mL ice water, and the grayish precipitate was filtered and dried in the air. The semisolid was treated with 50 mL MeOH at 40°C for 20 min. Filtration of this slurry yielded 1.89 g (67%) of a white solid: mp 247°C (decomp.); IR (KBr) 3284 (m, br, NH), 1702 (s, CO), ^{1}H-NMR (DMSO-d_6) δ 10.15 (s, 1 H), 7.77-7.74 (m, 1 H), 7.59-7.58 (m, 1 H), 7.45-7.40 (m, 1 H), 6.51 (s, 1 H), 4.97 (s, 2 H), 4.18 (q, 2 H, ^{3}J = 7.0 Hz), 1.27 (t, 3 H, ^{3}J = 7.35 Hz); ^{13}C-NMR (DMSO-d_6) δ 198.0, 168.5, 159.9, 153.3, 150.6, 143.1, 125.7, 114.3, 112.6, 104.6, 60.7, 41.2, 14.3; UV (MeOH) λ = 332 nm, ε = 13976; analytically calculated for $C_{13}H_{12}ClNO_4$: C, 55.43; H, 4.29; N, 4.97; found C, 55.81; H, 4.35; N, 5.22.

*7-Amino-4-(chloromethyl)-2H-1-benzopyran-2-one **4***. [4-(Chloromethyl)-2-oxo-2H-1-benzopyran-7-yl]-carbamic acid ethyl ester **3** (1.0 g, 3.55 mmol) was suspended in a mixture of 3 mL concentrated H_2SO_4 and 3 mL glacial acetic acid. The suspension was heated to 125°C for 2 h. After cooling to room temperature, the brown solution was poured on 100 mL crushed ice and brought to pH 9 with 1 N NaOH solution. The light-yellow solid was filtered and dried *in vacuo* (yield 63%). The product was used for synthesis without further purification. A sample amount was purified for analysis purposes as follows: The product was dissolved in THF and acidified with diluted HCl. The precipitate was extracted with water. The aqueous layer was separated and basified with concentrated NaOH solution until precipitation occurred. The purified product was filtered and dried *in vacuo,* yielding 64% of a yellowish solid, mp 187°C (decomp.); IR (KBr) 3450 (s, NH_2), 3351 (s, NH_2), 1689 (ss, CO), 1622 (s, NH_2); ^{1}H-NMR (DMSO-d_6) δ 7.53–7.49 (m, 1 H), 6.65–6.61 (m, 1 H), 6.49–6.48 (m, 1 H), 6.23–6.21 (s+s, nonres. 3 H), 4.90 (s, 2 H); ^{13}C-NMR (DMSO-d_6) δ 160.5, 1559, 153.3, 151.1, 126.0, 111.2, 107.8, 106.1, 98.6, 41.3; UV (MeOH) λ_{max} 366 nm, ε = 15 066; analytically calculated for $C_{10}H_8ClNO_2$: C, 57.30; H, 3.85; N, 6.68; found C, 57.31; H, 3.84; N, 6.79.

*4-[7-Amino-2H-1-benzopyran-2-one)-methyl acetate **5***. 7-Amino-4-(chloromethyl)-2H-1-benzopyran-2-one **4** (500 mg, 2.39 mmol) and KOAc (1.2 equivalents) were dissolved in 2 mL dry DMF and a small amount of tetrabutylammonium bromide was added. The mixture was warmed up to 50°C and stirred at room temperature for 5 h. The reaction mixture was poured into 15 mL water, the solid was suction filtered and dried. The raw product was crystallized from ether affording 445 mg of **5** as a yellowish solid (80%): mp 218 °C; IR (KBr): 3447 (s, NH), 3355 (s, NH), 1750 (s, C=O ester), 1688 (s, br, C=O, lactone); ^{1}H-NMR (DMSO-d_6) δ 7.42–7.39 (m, 1 H), 6.62–6.59 (m, 1 H), 6.48–6.47 (m, 1 H), 6.20 (s, br, 2 H), 6.00 (s, 1 H), 5.27 (d, 2 H, J = 1.5 Hz), 2.20 (s, 3 H); ^{13}C-NMR (DMSO-d_6) δ 170.0, 160.6, 155.7, 153.3, 150.8, 125.5, 111.4, 105.9, 104.9, 98.7, 61.1, 20.5; analytically calculated for $C_{12}H_{11}NO_4$: C, 61.80, H, 4.75, N, 6.01; found: C, 61.57, H, 4.85, N, 5.84.

*7-Amino-4-(hydroxymethyl)-2H-1-benzopyran-2-one **6***. 4-[7-Amino-2H-1-benzopyran-2-one)-methyl acetate **5** (100 mg, 0.43 mmol) and K_2CO_3 (60 mg, 1.1 equivalents) were suspended in 5 mL MeOH and stirred at room temperature for 18 h. Then 20 mL THF and Na_2SO_4 were added and the solution was filtered to remove all inorganic salts. The solvents were evaporated and the residue was treated with MeOH to afford the product **6** as a light-yellow solid (yield: 45 mg, 55%): mp 187°C (decay); IR (KBr) 3445 (s, br, NH_2), 3350 (ss, br, NH_2+ OH), 1674 (ss, br, CO-valence, lactone); ^{1}H-NMR (acetone-d_6 + 2 dr. DMSO-d_6) δ 7.35–7.32 (m 1 H), 6.63–6.59 (m, 1 H), 6.52–6.51 (m, 1 H), 6.16 (s, 1 H), 5.63 (s, br, 2 H), 5.10 (s, br, 1 H), 4.72 (s, br); ^{13}C-NMR (DMSO-d_6) δ 161.2 157.1, 155.5, 152.9, 125.0, 111.2, 106.4, 103.8, 98.7, 59.1; analytically calculated for $C_{10}H_9NO_3$: C, 62.82, H, 4.74, N, 7.25; found: C, 62.62, H, 4.78, N, 1.25.

*4-[[(2H-1-Benzopyran-2-one-7-amino-4-methoxy)carbonyl] imidazolide **7***. Crude 7-Amino-4-(hydroxymethyl)-2H-1-benzopyran-2-one **6** (191 mg, 1 mmol) and carbonyldiimidazole (194 mg, 1.2 equivalents) were dissolved in 2 mL dry DMF. The product **7** precipitates within 1 h. The yellow solid is filtered off, washed with 2 mL dry DMF, followed by 4 mL dry acetonitrile and dried under vacuum. The product can be stored at room temperature as a dry powder in a dark glass bottle in a desiccator over a prolonged period of time. ^{1}H-NMR (DMSO-d_6) δ 8.32 (s 1H), 7.64 (s, br 1H), 7.4 (d 1 H), 7.06 (s, br 1 H), 6.54 (dd, 1 H), 6.42 (d, 1 H), 6.15 (s, br, 2 H), 6.12 (s, 1H), 5.55 (s, 2H).

*4-[[(2H-1-Benzopyran-2-one-7-amino-4-methoxy)carbonyl]amino] butanoic acid **8***. 7-Amino-4-(hydroxymethyl)-2H-1-benzopyran-2-one **6** (100 mg, 0.52 mmol) and carbonyldiimidazole (104 mg, 1.2 equivalents) were dissolved in 1 mL dry DMSO. The solution was stirred at room temperature for 3 h, then GABA (65 mg, 1.2 equivalents) was added and the mixture was heated to 80°C for 1 h. After cooling to room temperature, 50 mL of water were added and the mixture was acidified to pH 3–4 with 1 M HCl. The precipitate was extracted with 3 × 40 mL ethyl acetate. The organic layers were combined, washed with 3 × 30 mL water, dried (Na_2SO_4) and the solvent evaporated. The residue was crystallized by treatment with a mixture of 1 mL acetone and 1 mL ether yielding 88 mg (53%) of **8** (yellowish solid), mp 194 °C; IR (KBr) 3464 (s, br, NH), 3363 (s, br, NH), 3093 (s, br, OH carboxylic acid), 1724 (ss, CO carboxylic acid), 1695 (ss, CO urethane + CO lactone); ^{1}H-NMR (DMSO-d_6) δ 7.53 (t, 1 H, ^{3}J = 6.0 Hz), 7.40–7.37 (m, 1 H), 6.62–6.58 (m, 1 H), 6.48–6.47 (m, 1 H), 5.97 (s 1 H), 5.22 (s, 2 H), 3.08 (m, 2 H), 2.28 (t, 2 H, ^{3}J = 7.4 Hz), 1.70 (m_c, 2 H); ^{13}C-NMR (DMSO-d_6) δ 174.1, 160.7, 155.6, 155.5, 153.2, 152.2, 125.3, 111.3, 105.8, 104.2, 98.6, 60.8, 39.8, 30.8, 24.7; analytically calculated for $C_{15}H_{16}N_2O_6$: C, 56.25, H, 5.03, N, 8.75; found: C, 56.02, H, 4.90, N, 8.59.

*4-[[(2H-1-Benzopyran-2-one-7-amino-4-methyl)amino] butanoic acid **11***. 7-Amino-4-(chloromethyl)-2H-*1-benzopyran-2-one* **4** (418 mg, 2 mmol) and the tetrabutylammonium salt of GABA (1.2 equivalents) were dissolved in 2 mL dry DMF and a small amount of potassium iodide was added. The mixture was warmed to 60°C and stirred for 5 h. The solvent was removed under high vacuum and the residue was dissolved in water. The pH was adjusted to 3–4 with 1 N HCl. The reaction mixture was concentrated and purified on RP-18 (eluent: water; water/25% methanol). Yield: 40 mg: ^{1}H-NMR (DMSO-d_6) δ 7.45 (t, 1 H, ^{3}J = 6.0 Hz); 6.64 (dd, 1H); 6.41 (d, 1H); 6.09 (s, 1 H); 3.89 (s, br, 2 H), 2.58 (t, 2 H), 2.28 (t, 2 H), 1.68 (m, 2 H). The molecular weight was determined by electrospray mass spectrometry/mass spectrometry (ESI MS) to be 275.9 Da (calc. 276.1Da) and the mass spectrometry/mass spectrometry (MS/MS) product ions at *m*/*z* 259.0 and 174.0 agree with a loss of water and $H_2NCH_2CH_2CH_2COOH$.

*Decay of 4-[[(2H-1-benzopyran-2-one-7-amino-4-methoxy)carbonyl]amino] butanoic acid **8** and quantification of GABA release upon irradiation with UV light*. Photolysis was carried out using a high-pressure mercury lamp (HBO 200, Oriel) with a filter allowing 240–400 nm light transmission. For the quantification of the GABA release, the irradiated solutions were postderivatized and analyzed by HPLC. Six hundred microliters of a 1.25×10^{-4} M solution of, in 0.1 M PBS, pH 7.4 were placed in a quartz corvette with a path length of 0.25 cm and irradiated for time periods ranging from 5 to 90 s. Time points were taken every 5 s. The amount of remaining caged compound **8** was determined by direct injection of 10 μL of the irradiated sample. For the determination of GABA, a 100 μL aliquot was removed and added to 100 μL of sodium bicarbonate buffer (0.2 M, pH 9) containing the valine standard (1×10^{-4} M). To this solution, 200 μL of dabsyl chloride (2 nmol/μL in acetone) were added, the mixture was vortexed and incubated at 60°C for 15 min. Under these dabsylation conditions, **8** is stable and does not undergo degradation. After cooling, samples of 10 μL were analyzed by HPLC. The gradient is 10–20% A from 0 to 4 min; 20–60% A from 4 to 8 min; 60–10% A 8–8.5min and 100%A from 8.5 to 16 min. The dabsylated derivatives were detected at λ = 436 nm. Coumarin derivatives were detected at λ = 345 nm.

Quantum yield of photodegradation. To provide an average quantum yield for the decaging reaction, we calibrated the irradiation system in the 300–400 nm range using ferrioxalate actinometry (17). The quantum yield of decaging was calculated for each Hg spectral line according to the equation $\varphi_{dec} = (dc/dt)_{obs} I_{abs}/(1 - V)$. The value of the reaction rate, dc/dt (mol L^{-1} s^{-1}), was calculated from the initial slope of the degradation curve of **8** (Fig. 2B). The value of I_{abs} (absorbed light intensity in moles of

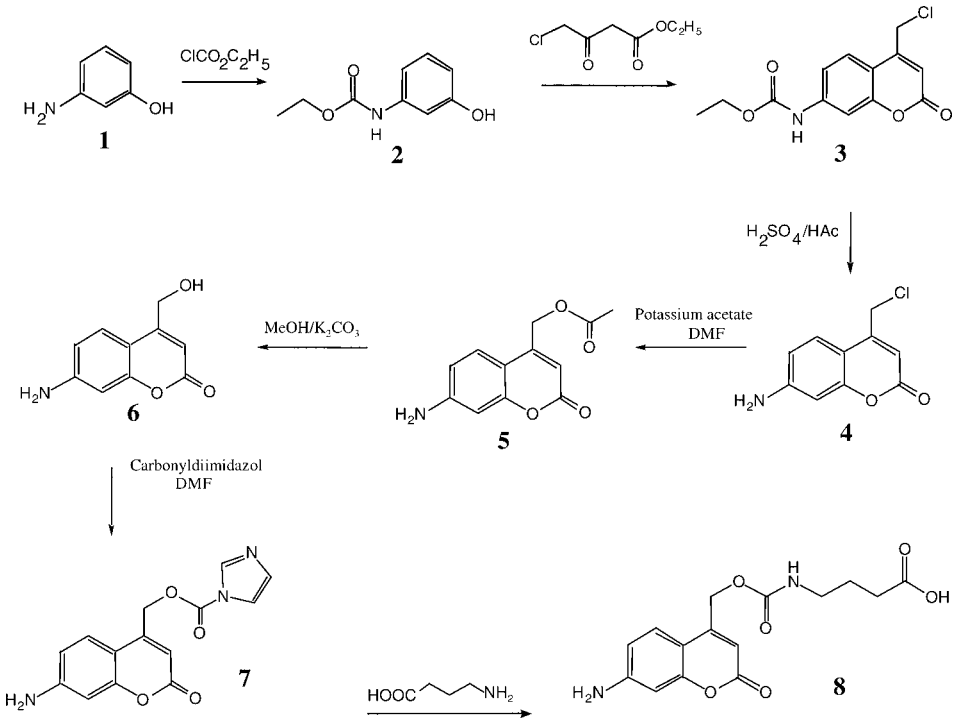


Figure 1. Synthesis of 4-[[(2H-1-benzopyran-2-one-7-amino-4-methoxy)carbonyl]amino] butanoic acid (BC204) prepared in seven steps from 3-aminophenol.

photons per second at $t = 0$ and $\lambda = 313$, 333 or 365 nm) was calculated by multiplying the absorption factor (taken from the UV spectrum of **8**) by the light intensity of the HBO 200 Oriel lamp (determined using the actinometer compound.). The solution volume was 3 mL.

Liquid chromatography/mass spectrometry/mass spectrometry. Liquid chromatography MS/MS analysis was done using a Magic 2002 liquid chromatograph (Michrom BioResources, Inc. Auburn, CA) operated with EZChrom software (Scientific Software, Inc.). The 1-mm column (Michrom Bioresources, Inc. Auburn, CA) was packed with C18 particles with 200 angstrom pores to a length of 15 cm. The mobile phase A was 99.9% water and 0.1% acetic acid (v/v) and mobile phase B was 99.9% acetonitrile and 0.1% acetic acid (v/v). The chromatographic runs were performed at a flow rate of 50 μL/min and used a gradient from 0% B to 100% B in 15 min. The column effluent was electrosprayed directly into the LCQ ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA) operated in positive ion mode for either full scan MS or MS/MS mode. The LCQ was operated with Xcalibur software version 1.2.

Animals and brain slice preparation. Experiments were performed in living brain slices prepared from 5–7 day old mice (C57Bl/6J, Jackson Laboratory, Bar Harbor, ME). Experimental procedures were in accordance with National Institutes of Health guidelines and were approved by the IACUC at the University of Pittsburgh. Preparation and maintenance of slices was performed as described in detail elsewhere (18). In short, animals were deeply anesthetized by hypothermia and decapitated. The brain was quickly removed and transferred to cold (4–8°C) artificial cerebrospinal fluid (ACSF; composition in mM: NaCl 124, $NaHCO_3$ 26, Glucose 10, KCl 5, KH_2PO_4 1.25, $MgSO_4$ 1.3, $CaCl_2$ 2, pH = 7.4, when aerated with 95% O_2/5% CO_2). ACSF used for preparation also contained 1 mM kynurenic acid. Coronal slices of the brainstem (250 μm thick) were cut on a vibrating microtome and kept at room temperature in an interface chamber until recordings.

Electrophysiological recordings. All recordings were made from neurons in the lateral superior olive, an auditory brainstem nucleus that receives GABAergic input (19). Slices were placed in a submerged-type recording chamber and continuously superfused with freshly oxygenated ACSF circulating at a rate of 2 mL/min (total ACSF volume 15 mL). Standard whole-cell patch clamp recordings were obtained with pipettes filled with solution containing (in mM) K-gluconate 84, KCl 38, $MgCl_2$ 2, HEPES 10, Na_2-phosphocreatine 5, Mg-ATP 3, Na_2-GTP 0.3, adjusted with KOH to pH 7.2. Whole-cell currents were recorded in voltage-clamp using an Axopatch 1D amplifier (Axon Instruments). All recordings were made in the presence of the sodium channel antagonist tetrodotoxin (1 μM, Alomone Labs, Jerusalem, Israel) to prevent action potentials and synaptic transmission. Membrane resistance was determined by applying 50 ms long, 5–10 mV voltage steps. Series resistance (less than 50 MΩ) was compensated 65–70%. Data were filtered at 3 KHz, digitized at a rate of 10 KHz and stored on hard disk for off-line analysis using custom-written software (P.H.M.K.) in the LabVIEW environment (National Instruments, Austin, TX).

Focal photolysis of BC204. For photolytic cleavage, BC204 stock solution (0.1 M in 0.1 M NaOH) was added to the ACSF to result in a final concentration of 200 μM. Focal photolysis was achieved by guiding the light from a 100 Watt mercury arc lamp (Oriel, Stratford, CT) to the recorded cell using small diameter fused silica optical fibers (core diameter of 50 μm; Polymicro Technologies, Phoenix, AZ). Duration of the light pulses was regulated by an electronically controlled shutter (Vincent Associates, Rochester, NY) located between the arc lamp and the optical fiber. For more details, see (20).

RESULTS AND DISCUSSION

Design approach and theoretical calculations

Our design criteria called for a caged GABA derivative that was stable under physiological dark conditions, but would rapidly release GABA when illuminated with a mercury arc lamp as used in a previously described one-photon uncaging setup (21). Thus we focused on a coumarin-caging reagent that was linked to GABA via a carbamate function, which combines a high chemical stability with useful properties for photodeprotection (as proven for nitrobenzyl systems). In order to tune the absorption properties of caging reagent to an optimal irradiation wavelength range, we investigated the influence of the substitution pattern on the 2H-1-benzopyran system on its long wavelength absorption maximum. In aqueous media under physiological pH, the substituent in the 7-position of the 2H-1-benzopyran-2-one derivatives had the most significant influence on the absorption. This effect was simulated by theoretical calculations using CAChe 3.0 (Oxford Molecular). Geometry optimization of the structures was carried out with Molecular Mechanics (MM2) (22), followed by a MOPAC (Molecular Orbital Package) calculation using the PM3 Hamiltonian (23). From

Table 1. UV and fluorescence data for coumarin caged GABA **8–10** (5×10^{-6} M solutions in PBS buffer solution, pH 7.4)

Substituent-R	$\lambda_{max,abs}$ nm (theoretical)	$\lambda_{max,abs}$ nm (experimental)	$\lambda_{max,em}$ nm (experimental)	ε 1/mol cm	ϕ_{fi}
9, -O-CH_3	308	324	418	13 000	0.41
10, 7-OH	310	326	483	10 000	0.21
8, 7-NH_2	320	348	477	14 000	0.40

the optimized structures, UV spectra were calculated with ZINDO3 (Professor M. C. Zerner's Intermediate Neglect of Differential Overlap program). We calculated 7-methoxy-, 7-hydroxy- and 7-amino-substituted coumarin-caged GABA derivatives and obtained theoretical UV absorption wavelengths that showed a bathochromic shift of 10 nm in the absorption maximum of the 7-amino-substituted coumarin derivatives *versus* the 7-hydroxy- and 7-methoxy-substituted compounds. These calculations encouraged our efforts to synthesize and study the photochemical and photophysical properties of 4-[[(2H-1-benzopyran-2-one-7-amino-4-methoxy)carbonyl]amino] butanoic acid **8.**

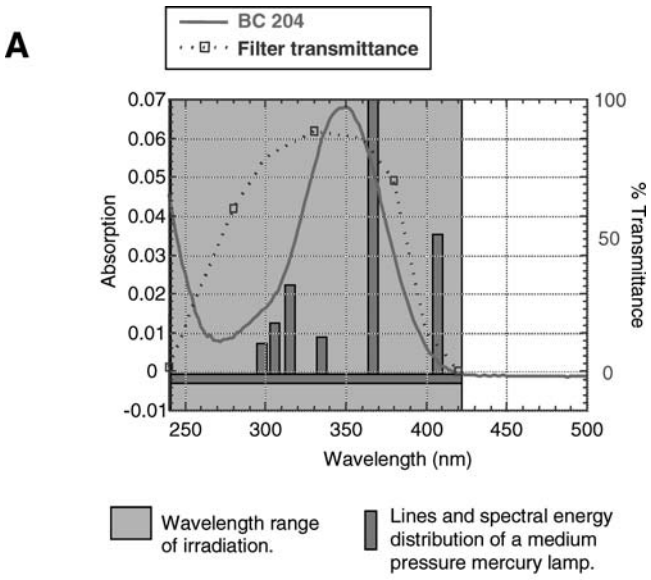
Synthesis and photophysical properties

To synthesize BC204, **8**, 7-Amino-4-(chloromethyl)-2H-1-benzopyran-2-one, **4** was prepared via Pechmann cyclization of (3-hydroxyphenyl)-carbamic acid ethyl ester **2** (24) followed by deprotection of the amino group (25). The protection of the amino group is required to avoid excessive oxidation during the cyclization reaction. To allow the introduction of a carbamate linker, 7-amino-4-(chloromethyl)-2H-1-benzopyran-2-one **4** had to be converted into the 4-hydroxymethyl derivative. Simple heating in water for 48 h, as reported for 6-bromo-4-chloromethyl-7-hydroxy coumarin (15), did not result in the desired product. Thus, 7-amino-4-(chloromethyl)-2H-1-benzopyran-2-one **4** was converted into 7-amino-4-(hydroxymethyl)-2H-1-benzopyran-2-one **6** as shown in Fig. 1 by a two-step reaction that involved the formation of 4-[7-amino-2H-1-benzopyran-2-one)-methyl acetate **5** under S_N2 conditions and its subsequent methanolysis. This reaction sequence could be combined in a two-step-one-pot synthesis. Coupling of **6** with GABA via a carbamate linker was achieved in another multistep-one-pot procedure. In the first step, 7-amino-4-(hydroxymethyl)-2H-1-benzopyran-2-one **6** was reacted with carbonyl-diimidazol to give the imidazolide **7** and without isolation directly reacted with GABA to afford 4-[[(2H-1-benzopyran-2-one-7-amino-4-methoxy)carbonyl]amino] butanoic acid **8** in good yield. For this reaction, unprotected GABA could be used due to the higher reactivity of the amino group.

When DMF is used as a solvent in the reaction of 7-amino-4-(hydroxymethyl)-2H-1-benzopyran-2-one **6** with carbonyl diimidazol, the imidazolide **7** precipitates from the reaction mixture and can be isolated by simple filtration. The solid can be washed and stored as a stable dry powder. This variant allows the use of crude 7-amino-4-(hydroxymethyl)-2H-1-benzopyran-2-one **6** and is particularly valuable for obtaining a clean active caging reagent **7** with out a sophisticated purification step. We have successfully used **7** to synthesize caged glycine and caged glutamate (results will be published elsewhere).

Spectral properties

As shown in Table 1, the absorption maximum of the 7-amino substituted coumarin **8** exhibits a large bathochromic shift of



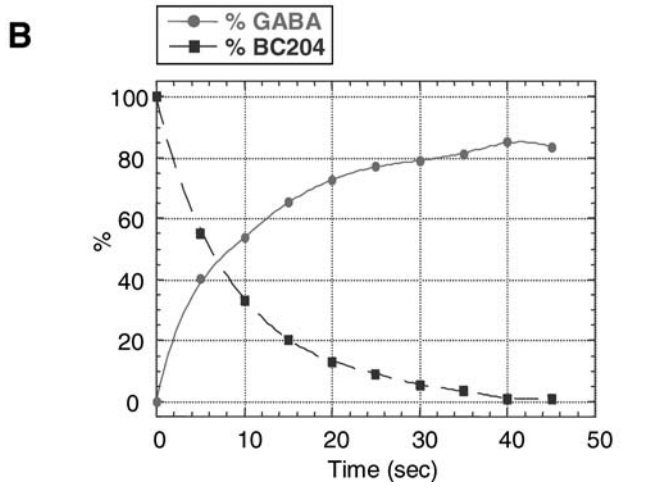


Figure 2. A: Absorption spectrum of BC204; wavelength range of irradiation; lines and spectral energy distribution of a medium-pressure mercury arc lamp. B: BC204 photodegradation where 50% of the GABA is release within the first 10 s.

$\Delta\lambda = 22$–24 nm as compared with the 7-hydroxy- and 7-methoxy-substituted compounds **9** and **10**, respectively (structures not shown). The experimental data confirmed the trend that we had seen in our theoretical calculations, although our absolute values for the theoretical maximum absorption wavelengths are low. The bathochromic shift in **8** can be attributed to the enhanced donor effect of the amino group. At pH 7.4, the amino group on the 7 position is not protonated and thus can display its donor effect in contrast with 7-hydroxy–substituted coumarin derivative **10**.

Photolysis

We studied the photochemical degradation under similar conditions that are employed in the biological decaging studies. Thus we used a 1.25×10^{-4}M solution of **8** in PBS and irradiated a 600 μL aliquot with UV light from a medium-pressure mercury arc lamp used with a bandpass filter between 240 and 400 nm. Figure 2A shows the

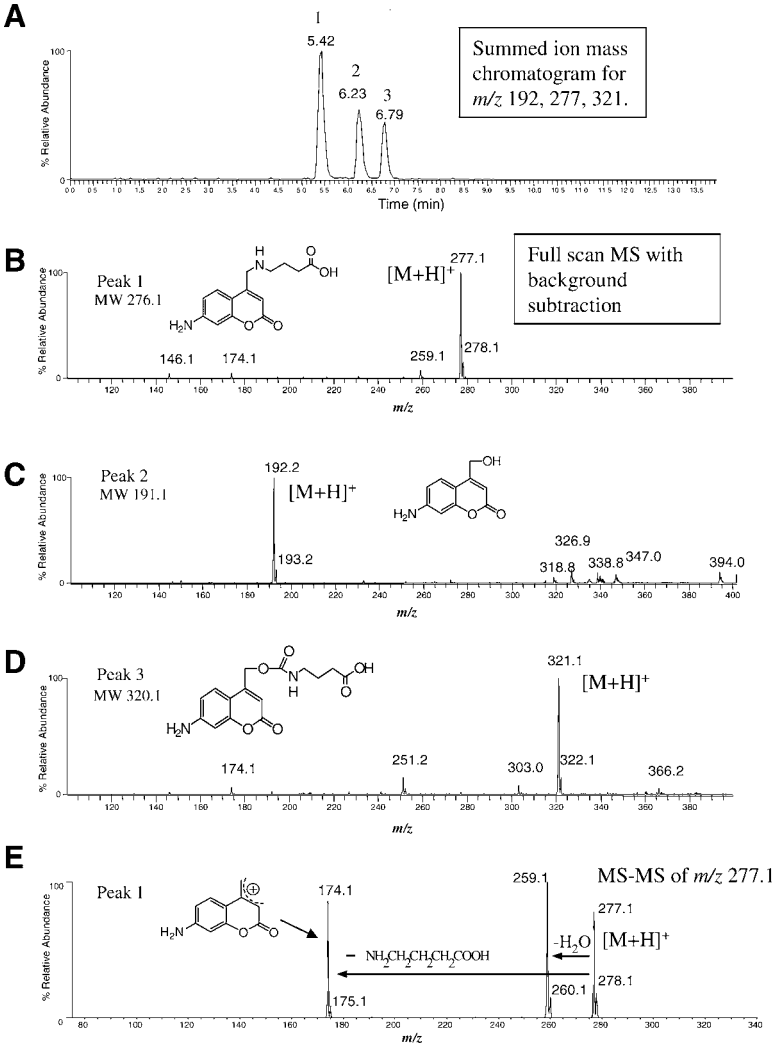


Figure 3. A: Summed selected mass chromatogram for each $[M+H]^+$ ion at *m/z* 192.1, 277.1 and 321.1 of peaks 1, 2 and 3, respectively. B: ESI mass spectrum of peak 1 with assigned structure. C: ESI mass spectrum of peak 2 with assigned structure. D: ESI mass spectrum of peak 3 with assigned structure. E: ESI MS-MS of peak 1, *m/z* 277.1, with a precursor isolation window of *ca* 4 amu, showing fragment ions from the loss of water and

absorption spectrum of **8**, the range of irradiation and the spectral lines of the mercury arc lamp. Under these irradiation conditions, 4-[[(2H-1-benzopyran-2-one-7-amino-4-methoxy)-carbonyl]amino] butanoic acid **8** degrades rapidly, as shown qualitatively by NMR-spectroscopy and quantitatively by HPLC. The fluorescamine assay (26) as a quantification method for the GABA release upon irradiation can not be applied for 7-amino-substituted coumarin derivatives because fluorescamine forms a chromophore with the 7-amino group that strongly interferes with the detection of the GABA-fluorescamine adduct. Thus, we postderivatized the released GABA with dabsyl chloride and quantified the dabsylated GABA via HPLC (27). Valine was added after the irradiation as an internal standard. Fifty percent of the GABA is released within the first 10 s from **8**, as shown in Fig. 2B. The quantum yield of photodegradation was determined to be 0.04 and is within the range of quantum yields reported for various (coumarin-4-yl)methyl esters of cAMP (28). After complete photodegradation of **8**, only 85% of free GABA was determined. To investigate the fate of the residual GABA, we separated the photoproducts by HPLC and analyzed the peaks by ESI MS.

Liquid chromatography electrospray mass spectrometry

Liquid chromatography (LC) electrospray ionization (ESI) mass spectrometry (MS) was used to ionize and determine the molecular weight of reaction products with little to no fragmentation due to the soft ionization process. The summed ion mass chromatogram from LC ESI MS for a reaction mixture that was irradiated for 20 s shows the parental compound **8** and two photoproducts in Fig. 3A. As expected, one of these photoproducts was identified as 7-amino-4-(hydroxymethyl)-2H-1-benzopyran-2-one **6** *m/z* 192.2 (Fig. 3C). In addition, a second photoproduct with a molecular mass $[M+H]^+$ of *m/z* 277.1 is formed (Fig. 3B) and the ESI mass spectrum for the parent compound **8** is shown in Fig. 3D. The ion signals in the mass chromatogram are not quantitative because of the differences in proton affinity among the photoproducts. ESI mass spectrometry/mass spectrometry (MS/MS) was performed on the precursor $[M+H]^+$ at *m/z* 277.1 as shown in Fig. 3E and the mass assignments support the structure of 4-[(2H-1-benzopyran-2-one-7-amino-4-methyl]- amino butanoic acid **11** showing loss of water and GABA. Isolation and characterization of **11** for NMR analysis proved to be too difficult because the compound forms only in small amounts. Thus, we synthesized the predicted compound 4-[(2H-1-benzopyran-2-one-7-amino-4-methyl]- amino butanoic acid, and performed LC, MS and MS/MS to further confirm its structure. The LC retention time, molecular weight and product ions formed from MS/MS agreed with the assigned structure **11**.

In order to understand the formation of **11** from **8**, we added valine as a competing nucleophile to the PBS solution prior to the

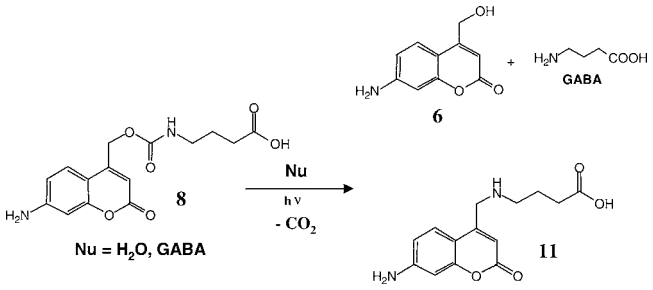


Figure 4. The formation of **6** and GABA is the result of photosolvolysis of **8**. For the formation of **11,** two mechanisms are proposed: an intramolecular 1–3 rearrangement under extrusion of carbon dioxide **C** or a nucleophilic competition reaction **B** of the photoreleased GABA with the water photosolvolysis step **A**.

irradiation (nucleophiles such as cysteine and isoleucine work equally well). Under dark conditions, the valine did not cause a release of GABA, however, the concentration of photoreleased GABA increased when valine competitively inhibited the formation of **11**. Thus, we conclude that **11** most likely forms through an ion-pair return of the photoreleased GABA after the loss of CO_2. This pathway is supported by the fact that the ratio of GABA to **11** remained fairly constant during photolysis. This process is relevant in a closed system where the released GABA stays present. In a biological open system, GABA will be immediately taken up by receptors or can diffuse away and thus minimize the formation of the by-product **11**.

Biological Activity of BC204

To determine the biological properties of BC204 and to estimate its potential as a tool in neurobiological research, BC204 was tested in auditory neurons in the lateral superior olive in brainstem slices of mice. In these experiments, we focused on those basic properties that are most important for the general use of caged neurotransmitters (3,29). First, the caged neurotransmitter should be biologically inactive and in particular should not act as an agonist or antagonist on the receptors under study. Second, under physiological darkness conditions, the compound should be stable so that biological specimens can be exposed to the caged compound over longer time periods. Third, the compound should be readily cleavable with light a wavelength >300 nm and with moderate intensity to minimize UV-induced physiological changes (30) or cell damage and to avoid the necessity of using expensive, high-power light sources.

The effects of photolyzed BC204 on membrane currents was tested using whole-cell patch clamp recordings from individual neurons. For uncaging, the output of a mercury arc lamp was aimed to a small area around the recorded cell using a 50 μm diameter quartz fiber as previously described (18). Action potentials and synaptic transmission were blocked with tetrodotoxin (1 μM) and metabotropic $GABA_B$ receptors were blocked by 2-OH-saclofen (10 μM). Under these conditions, photolysis of BC204 (200 μM) with short light pulses (5–40 ms) produced transient whole-cell inward currents. Increasing the duration of the pulses generated currents of larger amplitude and longer duration. For a given pulse length, response amplitudes and durations were remarkably reproducible (Fig. 5A), which indicated that hydrolysis products or the amount of the UV component delivered during repetitive illumination had no obvious detrimental effects on the recorded neuron.

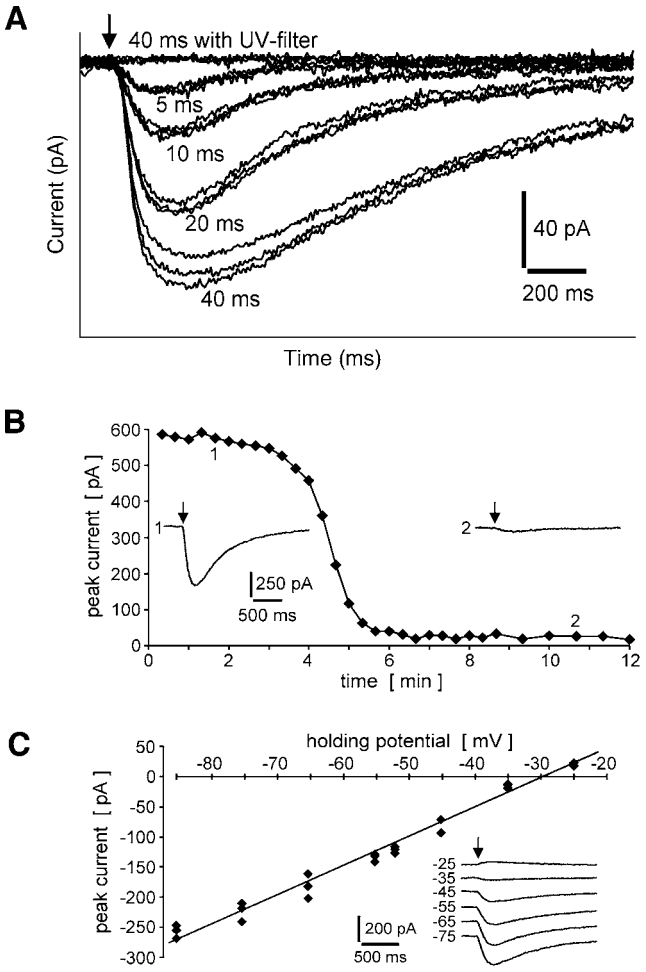


Figure 5. A: Photolysis of BC204 elicits whole-cell currents in auditory brainstem neurons. The slice was bathed in ACSF containing 200 μM BC204. The inward currents evoked by illuminating the area of the slice around the recorded neuron increase in amplitude and duration with increasing illumination times (opening of light shutter indicated by arrow). For a given flash duration, repeated photolysis of BC204 results in reproducible current responses. No responses are observed when the UV component of the illuminating light is removed by placing a filter in the light path. B: Currents elicited by photolysis of BC204 are due to activation of $GABA_A$ receptors. The specific $GABA_A$ receptor antagonist bicuculline (20 μM) eliminates the current response evoked by photolysis (15 ms flash duration) of BC204 (200 μM). Wash-in of bicuculline starts at time 0, reaching the slice after about 3 min. Current trace insets show single responses at the times indicated in the graph. C: The reversal potential of BC204-evoked currents is consistent with the activation of a ligand-gated chloride conductance. Currents elicited by photolysis of BC204 were measured while holding the cell membrane at various potentials. Currents reverse polarity at –29 mV in this cell, close to the theoretical chloride reversal potential of –30 mV. Current trace inset shows averaged responses at the holding potential indicated to the left (200 μM BC204, flash duration 3 ms). The arrows above the traces indicate the time of UV flash.

As expected from the absorption spectrum of BC204 (Fig. 2A), blockade of the UV light component (filter with center frequency 540 nm, bandpass 25 nm) completely prevented these responses.

Two lines of evidence indicate that photolysis-evoked membrane currents were mediated by specific activation of $GABA_A$ receptors, a ligand-gated chloride channel (31). First, responses were abolished when $GABA_A$ receptors were blocked by the specific antagonist bicuculline (20 μM) (Fig. 5B). Second, when responses were elicited

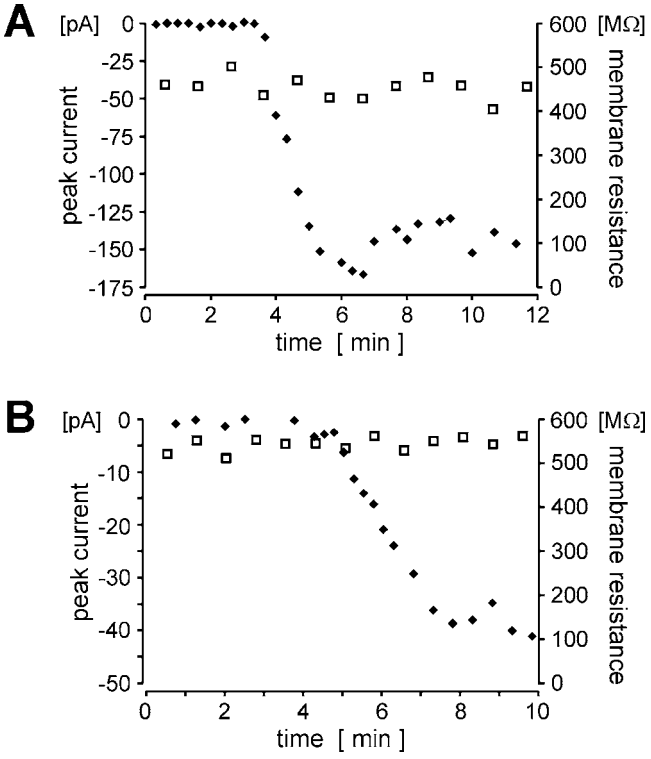


Figure 6. BC204 in solution is stable and can be kept for several days without showing signs of spontaneous degradation. A: Wash-in of BC204 taken from freshly prepared stock solution (0.1 M in 0.1 M NaOH) does not result in a change of neuronal membrane resistance (◆ peak current to 10 ms light pulse, □ membrane input resistance). B: For another neuron, wash-in of BC204 from a stock solution prepared 11 days before and kept at 4°C also has no effect on membrane resistance, indicating the absence of free GABA (both cells: 200 μM BC204; flash duration 10 ms).

at depolarized membrane potentials, current amplitudes decreased or reversed in polarity (Fig. 5C), with a reversal potential of 32.8 ± 4.3 mV (n = 8 cells). This reversal potential is close to the theoretical value of –30 mV as calculated with the Nernst equation for the chloride concentration in our pipette (42 mM) and bath solution (133 mM), indicating that photolysis of BC204 activated chloride membrane currents. Together, these results provide strong evidence that responses elicited by photolysis of BC204 are not caused by unspecific effects of hydrolysis byproducts, but rather are generated by free GABA acting on $GABA_A$.

To address the stability of BC204 in solution, prephotolytic activity and photolysis responses were tested in freshly prepared and stored stock solutions. Prephotolytic activity due to the presence of free GABA was assessed by monitoring the membrane input resistance with short voltage command steps during wash-in of diluted stock solutions (200 μM). Under these conditions, the presence of free GABA would become apparent as a reduction in the membrane resistance due to activation of $GABA_A$-activated chloride channels. As shown in Fig. 6, wash-in of freshly dissolved BC204 had no effect on the membrane resistance, indicating the absence of detectable free GABA. More importantly, wash-in of BC204 (200 μM) taken from stock solution, which was prepared 11 days before and stored in the dark at 4°C, also did not change the membrane resistance (n = 3 cells). The absence of any detectable membrane resistance change was not due to an unresponsiveness of the recorded cells to GABA, as all of the tested cells responded readily to photolyzed BC204. Furthermore, bath application of 20 μM GABA to these cells induced a steep drop in the membrane resistance to 25 ± 3.6% of its initial value (n = 2). This indicates that the absence of any noticeable membrane resistance changes upon BC204 application cannot be attributed to desensitization of $GABA_A$ receptors due to slow buildup of GABA in the slice.

GABA α-carboxy-2-nitrobenzyl ester [CNB-caged GABA] at more than 180 μM depresses presynaptic calcium influx at granule cell-Purkinje cell synapses (32), consistent with the original report of the synthesis of this substance by Gee *et al*. (33) (also: absorption maximum of CNB-caged GABA at 262 nm). Use of a 1 day old stock solution kept at room temperature also had no effect on the membrane resistance (n = 3 cells). Storage of stock solution did not seem to affect the ability to produce responses on uncaging, as we observed no obvious differences in response amplitudes between using fresh and stored stock solutions (fresh, 125–160 pA; 1 day old, 100–450 pA; 11 day old, 40–700 pA). Finally, our recordings also indicate that BC204 is also stable at physiological pH, a basic requirement for using caged compounds in longer experiments (34). In our recordings lasting up to 1 h, we found no indication for spontaneous degradation of BC204 in ACSF at pH 7.4, allowing us to reuse BC204-containing ACSF in another slice or to keep a slice in BC204-containing ACSF for 2 h before starting another recording. In summary, these results indicate that BC204 does not spontaneously degrade over the period tested at a rate detectable with our biological system while it still maintains its ability to undergo fast photolysis.

The 4-[[(2H-1-benzopyran-2-one-7-amino-4-methoxy)carbonyl] amino] butanoic acid (BC204) is a stable form of caged GABA that can be easily synthesized. This compound is chemically stable under physiological dark conditions and shows no intrinsic biological activity. ESI MS supports the stability of the structure as little to no fragmentation was observed. GABA is readily released from BC204 with an inexpensive one-photon irradiation system that uses light at 300–400 nm from a mercury arc lamp. The photo products were identified by LC ESI MS and the structure of the by-product 4-[(2H-1-benzopyran-2-one-7-amino-4-methyl]-amino butanoic acid was confirmed by LC ESI MS/MS and comparison to synthetically derived compound. These properties suggest that BC204 is an excellent form of caged GABA that is particularly suited for the use in long-term neurobiological studies.

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