

# An Antenna-Sensitized Nitroindoline Precursor to Enable Photorelease of L-Glutamate in High Concentrations

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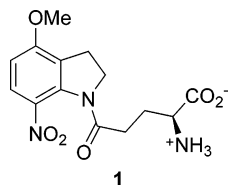
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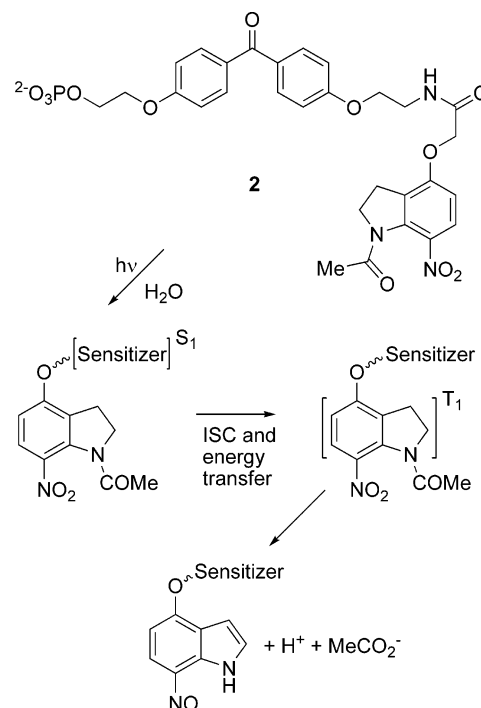
1-Acyl-7-nitroindolines are useful reagents for rapid release of carboxylates upon flash photolysis in aqueous solution and have been particularly effective for rapid (submicrosecond) release of neuroactive amino acids such as L-glutamate in biological experiments. In model systems the efficiency of photorelease has been shown to be greatly improved by attachment of a benzophenone triplet-sensitizing antenna. The present work describes synthesis and initial biological evaluation of the L-glutamate precursor **3**. A significant improvement in the overall synthesis uses double Boc protection of the glutamate amino group to avoid side reactions during introduction of the nitro group. To accommodate the multiple functionalities in **3**, linkage of the nitroindoline and benzophenone moieties is carried out late in the synthesis. Photolysis of **3** occurs with near-quantitative stoichiometry and the released L-glutamate efficiently activates neuronal glutamate ion channels.

## Introduction

We recently described photocleavage of 1-acyl-7-nitroindolines in oxygenated aqueous solution with intramolecular triplet sensitization<sup>1</sup> that significantly enhanced the photocleavage efficiency compared to that of nonsensitized analogues such as **1**. An outline of the photochemical process that results in photorelease of acetate ion from the model triplet-sensitized acetamide **2** in the above work is given in Figure 1. The enhanced photosensitivity is believed principally to arise from the antenna effect of the benzophenone sensitizer, which achieves more effective light absorption than by the nitroindoline alone. Triplet lifetime measurements confirmed intramolecular energy transfer within **2** that competed effectively with intermolecular triplet quenching of the benzophenone by oxygen.<sup>1</sup> Our interest in this chemistry originates from our previous work on 1-acyl-7-nitroindolines<sup>2</sup> such as **1** as tools for rapid delivery of



neuroactive amino acids, particularly L-glutamate, within experimental preparations from mammalian brain.<sup>3</sup> The specific inspiration for the intramolecular sensitization

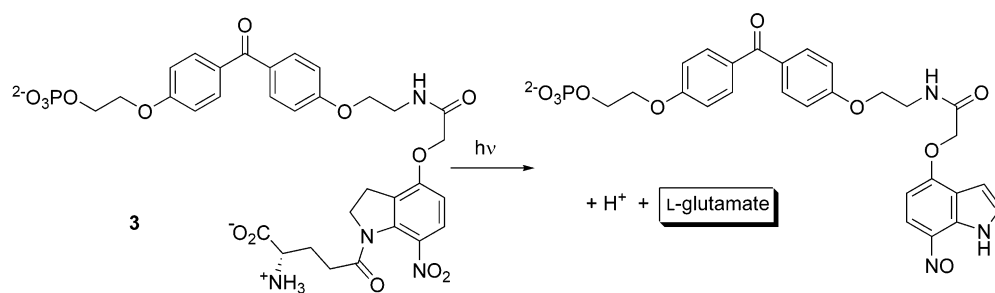


**FIGURE 1.** Outline of the photolysis pathway for photocleavage of sensitized 1-acyl-7-nitroindolines such as **2**. Details of the mechanistic steps between the nitroindoline triplet and the photoproducts are described by Morrison et al.<sup>4</sup>

came from our study of the photolysis mechanism of these compounds, which showed inter alia that the half-time for release of the acyl group as its corresponding carboxylate anion upon nanosecond laser flash photolysis was ~150 ns, and that photolysis proceeded via the triplet state of the nitroindoline.<sup>4</sup>

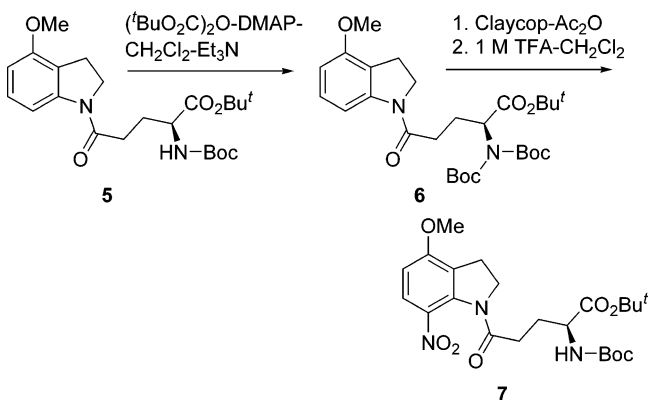
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**FIGURE 2.** Expected photolytic reaction pathway for **3**, showing release of L-glutamate.

#### SCHEME 1

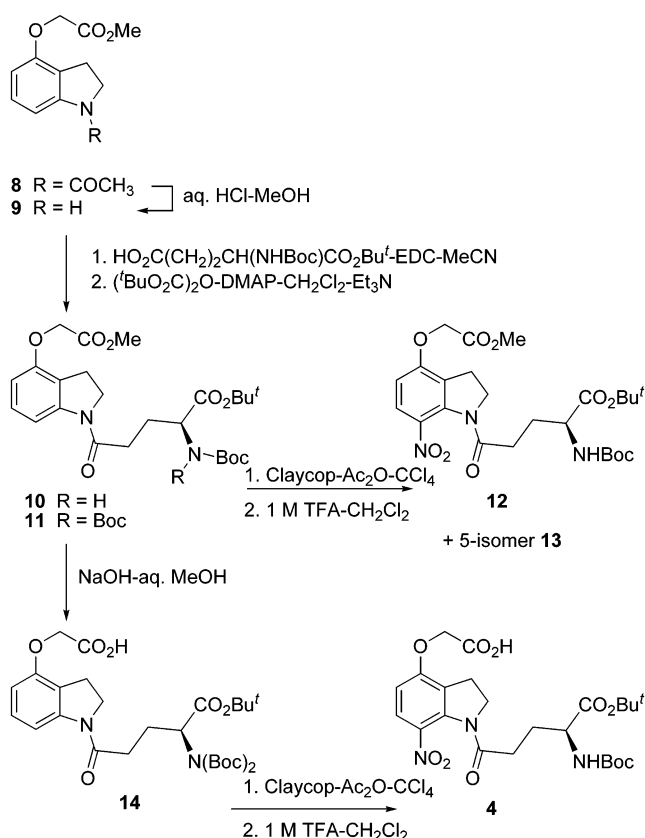


#### Results and Discussion

To extend the model demonstration of enhanced photosensitivity to release of a biologically useful compound, we required the L-glutamate derivative **3**. On the basis of our published results,<sup>1</sup> this compound was expected to photolyze according to the overall reaction shown in Figure 2. Evidence to support clean formation of the nitrosoindole byproduct from the sensitized conjugates was presented previously.<sup>1</sup>

A key intermediate for synthesis of **3** was the carboxylic acid **4** (see Scheme 2), which contains a protected glutamate residue. However, we had previously encountered problems from unwanted partial nitration/nitrosation of the Boc-protected amino function in the side chain during introduction of the 7-nitro group in synthesis of related compounds.<sup>2c</sup> We therefore began the present work by seeking to avoid this complication, as shown in Scheme 1. Conversion of **5** to its di-Boc derivative **6** was easily achieved in 86% yield with di-*tert*-butyl pyrocarbonate-DMAP,<sup>5</sup> and claycop nitration then gave a mixture of 5- and 7-nitrated products in a ratio of ~1:10. Although nitration was accompanied by partial loss of the newly introduced Boc group, there was now no evidence of nitration or nitrosation at the side-chain nitrogen. Evi-

#### SCHEME 2



dently, the second protecting group survives for a sufficient time to block reaction at this site. For simplicity, the crude product mixture was treated with 1 M TFA in dichloromethane to complete the removal of the second Boc group,<sup>6</sup> whereupon the 7-nitro compound **7** was obtained after flash chromatography and crystallization (to remove the 5-nitro isomer) in 81% yield (70% overall from **5**), a substantial improvement over the previous 43% yield of this compound by direct claycop nitration of **5**.<sup>2c</sup>

With this problem of the side reaction resolved, we proceeded to assemble the carboxylic acid **4**, as outlined in Scheme 2. The previously described<sup>1</sup> acetamide ester **8** was methanolized to give the amino ester **9**, which was coupled with the  $\alpha$ -*tert*-butyl ester of *N*-Boc-L-glutamate

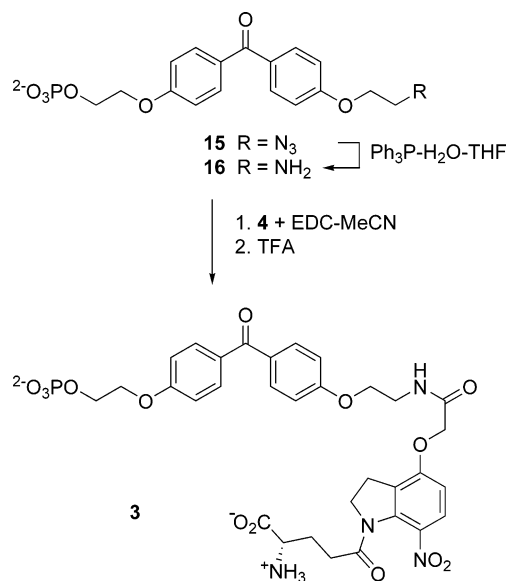
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## SCHEME 3



to yield amide **10**. A second Boc group was added as described above, giving **11**. Claycop nitration<sup>2c</sup> and subsequent treatment with dilute TFA in CH<sub>2</sub>Cl<sub>2</sub> then proceeded smoothly to give the 7-nitro compound **12**. A small proportion of the 5-nitro isomer **13** was removed by fractional crystallization. However, mild basic treatment of **12** to hydrolyze the methyl ester was accompanied by substantial loss of the protected glutamate moiety, probably because participation by the nitrogen in the side chain assists cleavage of the amide bond between the amino acid residue and the nitroindoline. Cleaner results were obtained by reversing the order of these two steps, i.e., initial base hydrolysis of **11** gave the acid **14** and claycop nitration then yielded the target nitro acid **4**, again after treatment with dilute TFA to effect complete removal of the second Boc group. In this case, the noncrystalline product (that contained a small proportion of the 5-nitro isomer) was used without separation of the two isomers.

To complete the synthesis of **3**, it was necessary to link **4** with the sensitizer unit, as shown in Scheme 3. In our previous preparation of the model conjugate **2**,<sup>1</sup> we had reduced the azide **15** by catalytic hydrogenation but this was frequently accompanied by partial reduction of the carbonyl group. Here we found that Staudinger reduction in moist THF<sup>7</sup> was more satisfactory and the crude amine **16**, without separation from the triphenylphosphine oxide byproduct, was suitable for carbodiimide-mediated coupling with the crude acid **4**. Finally, treatment with neat TFA removed all *tert*-butyl protecting groups to leave the water-soluble conjugate **3**. Reverse-phase HPLC purification removed the small proportion of the corresponding 5-nitro isomer that eluted after the major product and was identified by its distinctive UV-visible absorption spectrum (see the Supporting Information, Figures S1 and S2).

As previously described,<sup>1</sup> quantification of solutions of these sensitized conjugates was based on UV-visible spectroscopy, using data from computed absorption spec-

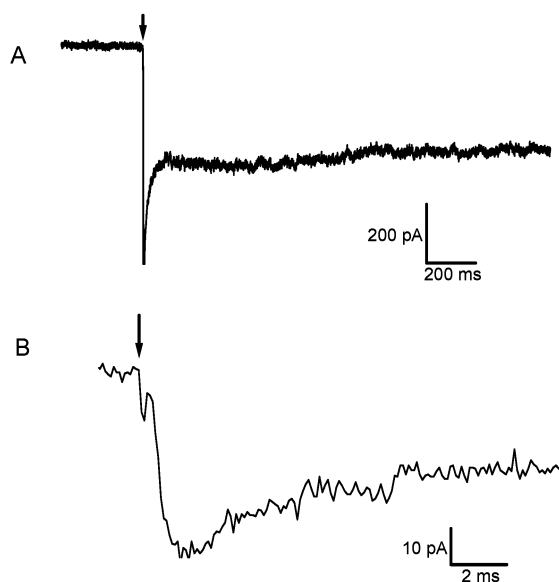
tra. These were obtained by adding the individual molar absorption spectra of the benzophenone and nitroindoline components and gave an estimated molar absorptance coefficient of 27 900 M<sup>-1</sup> cm<sup>-1</sup> at 300 nm for conjugates such as **3**.<sup>1</sup> In our previous work with these sensitized conjugates, we showed qualitatively that acetate was released upon photolysis of **2** (by appearance of an appropriate signal in the <sup>1</sup>H NMR spectrum of a photolyzed solution), but synthesis of the glutamate derivative **3** enabled us to make a quantitative measurement of the stoichiometry of product release. Thus, solutions of a known concentration of **3** were irradiated (Rayonet photochemical reactor, 300-nm lamps) for different periods of time to give extents of photolysis in the range 27–44%, which was estimated by the decrease in height of the reverse-phase HPLC peak compared to unphotolyzed control. The same samples were analyzed for free glutamate as described previously<sup>2a,b</sup> and were found to contain ~90% of the concentration of glutamate that was expected from the extent of photolysis. Free glutamate contamination in the unphotolyzed sample was measured at 0.2%. We note that UV-visible spectra of **3** recorded after incremental periods of irradiation (data not shown) revealed an identical clean spectral evolution over the same time intervals as reported for the acetyl compound **2** (see Figure 2 of ref 1). Given this fact and inevitable cumulative errors in the computed absorption spectrum and in the analytical measurements, it is reasonable to assume that release of glutamate from **3** proceeds with essentially 1:1 stoichiometry, as previously found for nonsensitized 1-acyl-7-nitroindolines such as **1**.<sup>2a,b</sup>

The effect of L-glutamate photoreleased from **3** by 1-ms flash photolysis in the external solution was tested on hippocampal neurons in primary culture and on cerebellar granule cells in an acute brain slice preparation. Recordings in a whole cell voltage clamp at -70 mV are shown in Figure 3 following photorelease of 50 μM L-glutamate on a small hippocampal neuron (A) or 500 μM L-glutamate on a cerebellar granule cell (B). In both cases an initial rapid rise and fall of current, mediated by rapid activation and subsequent desensitization of glutamate receptors, is followed by a sustained inward current due to persistence of L-glutamate in the bathing solution. The initial response of the granule cell at high glutamate concentration rises to a peak within the 1-ms flash lamp pulse, consistent with the rapid photorelease described previously for 1-acyl-7-nitroindolines.<sup>4</sup> These preliminary results indicate that neither intact **3** nor the intermediates or byproducts of the photolysis reaction interfere substantially with the action of L-glutamate. However, a more extensive study will be required to establish the concentration range for **3** that can be applied to neuronal preparations<sup>3b</sup> and to investigate (and if necessary minimize) toxicity that could arise from the triplet states generated, notably from singlet oxygen produced by oxygen quenching that competes with intermolecular energy transfer from the benzophenone triplet.

## Conclusion

A principal aim in developing the sensitized nitroindoline photochemistry was to facilitate release of high glutamate concentrations. From previous data for **2**,<sup>1</sup> the conjugate **3** is approximately 6-fold more photosensitive

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**FIGURE 3.** (A) Current recording from a hippocampal neuron in primary culture. Photorelease, at the time indicated by the arrow, of 50  $\mu\text{M}$  L-glutamate from 500  $\mu\text{M}$  **3** present in the bath. Whole-cell patch clamp, membrane potential  $-70$  mV, 1  $\mu\text{M}$  tetrodotoxin present, 1-ms xenon flash lamp pulse, UG5 filter. (B) Current recording from a cerebellar granule cell in an acute transverse slice, 20-day rat. Photorelease, at time indicated by the arrow, of a high concentration (approximately 500  $\mu\text{M}$ ) of L-glutamate from 500  $\mu\text{M}$  **3**. Conditions as in part A. The perturbation at the start of the inward current is an artifact arising from the flash lamp trigger.

(with 300-nm lamps) than its nonsensitized analogue **1**. Comparable data determined here with 350-nm lamps showed that the effect of the antenna was substantially reduced (approximately 2-fold more sensitive than **1**). We have previously argued that the enhanced photosensitivity of these conjugates is principally an effect of enhanced light absorption by the antenna, the absorption of which peaks at 300 nm, rather than a change in quantum yield.<sup>1</sup> We have not measured absolute quantum yields, since the polychromatic light source used here makes it difficult to assign the relative fractions of light absorbed by the antenna or directly by the nitroindoline. Similarly the relative photosensitivity of the sensitized and nonsensitized compounds is only indicative of trends with respect to wavelength. Measurements with monochromatic light sources would more usefully address these issues, although our principal future focus is to evaluate the two-photon photolysis efficiency (see below).

Data from initial biological tests reported above indicate that essentially complete photorelease of glutamate from 500  $\mu\text{M}$  **3** can be achieved with light intensities that would release only 120  $\mu\text{M}$  from a 1 mM solution of **1** in the same experimental conditions.<sup>3b</sup> A reviewer requested that we comment upon the use of 300-nm light, which is more damaging to biological tissues than longer wavelengths. However, many studies with photolabile precursors have used 308-nm light from XeCl excimer lasers without encountering significant problems. The absorbance coefficient of **3** at 308 nm is only 9% lower than that at the 300-nm maximum, so photolysis at this commonly used wavelength will retain the advantage of the antenna. It seems probable that the pharmacological

and physiological benefits of being able to transform a greater proportion of the compound will outweigh any disadvantages of the shorter wavelength irradiation. Furthermore, the high absorbance coefficient of the sensitized conjugate in the near-UV offers a prospect of highly localized release of glutamate by two-photon photolysis<sup>8</sup> if the two-photon cross-section is similarly increased. This technique achieves high 3-dimensional spatial localization since the light intensity needed to achieve simultaneous two-photon absorption is only attained in a very tightly focused laser spot. However, few examples in biological systems have yet been described, principally because most of the chromophores used to date, including that of **1**,<sup>3c,d</sup> have cross-sections such that light intensities approaching the limits imposed by phototoxicity have been required to effect useful extents of photolysis.<sup>9</sup> At least two examples of photocleavable compounds with larger two-photon cross-sections have been described<sup>10,11</sup> and these studies signaled the possibilities of further improvement. We plan to measure the two-photon cross-section of the sensitized nitroindoline system and will report results when they become available. As noted above, further pharmacological characterization of **3** will also be required to establish its full potential, but to date the combination of high light absorption, excellent resistance to hydrolysis, and sub-microsecond photorelease suggests that the sensitized nitroindolines may significantly extend the utility of nitroindoline photochemistry.

Finally we note a different type of antenna-enhanced photocleavage that appeared contemporaneously with our previous paper.<sup>1</sup> In this work, a 7-hydroxycoumarin derivative, derivatized on the phenolic group as its 1-(2-nitrophenyl)ethyl ether, underwent photocleavage of the nitrophenylethyl group with greatly enhanced efficiency compared to other 1-(2-nitrophenyl)ethyl-derivatized fluorophores.<sup>12</sup> The detailed mechanism operative in this system was not characterized but probably involves an energy transfer process analogous to that in a related study by Castellano and colleagues,<sup>13</sup> where strongly distance-dependent energy transfer mechanisms (dipole-dipole or electron exchange) have been proposed. Prospects for further innovation in this area of antenna-amplified photolysis are enticing.

## Experimental Section

**1-[4S-[(4-*tert*-Butoxycarbonyl)-4-(di-*tert*-butoxycarbonylamino)]butanoyl]-4-methoxyindoline (6).** 1-[4S-[(4-*tert*-Butoxycarbonyl)-4-(*tert*-butoxycarbonylamino)]butanoyl]-4-methoxyindoline (**5**) (2.17 g, 5 mmol), prepared as previously described,<sup>2b</sup> in a solution of dry  $\text{CH}_2\text{Cl}_2$  (20 mL) and  $\text{Et}_3\text{N}$  (30 mL) was treated with di-*tert*-butyl dicarbonate (2.73 g, 12.5 mmol) and DMAP (75 mg, 0.6 mmol) and the solution was refluxed under nitrogen for 5 h. The solvents were evap-

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orated and the residue was diluted with EtOAc (100 mL) and washed with 1 M KHSO<sub>4</sub>, saturated aq NaHCO<sub>3</sub>, and brine, dried, and evaporated. Flash chromatography [1:4 EtOAc–hexanes] followed by trituration with Et<sub>2</sub>O–hexanes gave **6** as fluffy white crystals (2.33 g, 86%), mp 80–81 °C (from Et<sub>2</sub>O–hexanes): <sup>1</sup>H NMR (500 MHz) δ 7.86 (d, *J* = 8.1 Hz, 1H), 7.15 (t, *J* = 8.1 Hz, 1H), 6.56 (d, *J* = 8.1 Hz, 1H), 4.86 (dd, *J* = 9.0, 5.7 Hz, 1H), 4.02–4.09 (m, 2H), 3.83 (s, 3H), 3.09 (t, *J* = 8.5 Hz, 2H), 2.50–2.60 (m, 2H), 2.41–2.47 (m, 1H), 2.16–2.25 (m, 1H), 1.48 (s, 18H), 1.45 (s, 9H). Anal. Calcd for C<sub>28</sub>H<sub>42</sub>N<sub>2</sub>O<sub>8</sub>: C, 62.90; H, 7.92; N, 5.24. Found: C, 63.02; H, 7.98; N, 5.23.

**1-[4S-(4-*tert*-Butoxycarbonyl)-4-(*tert*-butoxycarbonylamino)butanoyl-4-methoxy-7-nitroindoline (7).** A solution of **6** (2.14 g, 4 mmol) in CCl<sub>4</sub> (16 mL) and acetic anhydride (8 mL) was stirred at room temperature for 22 h with a suspension of claycop (2.56 g, prepared as described in ref 14). The solid was filtered off and washed with CCl<sub>4</sub>, and the combined filtrates were evaporated. The residue was dissolved in EtOAc and washed with saturated NaHCO<sub>3</sub> and brine, dried, and evaporated to a brown viscous oil that was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (80 mL), treated with TFA (1 M solution in CH<sub>2</sub>Cl<sub>2</sub>; 6 mL), and stirred at room temperature for 20 h. The solvent was evaporated and the residue was dissolved in EtOAc (100 mL), washed with saturated NaHCO<sub>3</sub> and brine, dried, and evaporated. Flash chromatography (1:1 EtOAc–hexanes) followed by crystallization (CHCl<sub>3</sub>–hexanes) gave **7** (1.56 g, 81%) as yellow crystals, mp 145–147 °C, identical with the material previously described (mp, <sup>1</sup>H NMR).<sup>2c</sup>

**Methyl {1-[4S-(4-*tert*-Butoxycarbonyl)-4-(*tert*-butoxycarbonylamino)butanoyl]indolin-4-yloxy}acetate (10).** A solution of methyl (1-acetylindolin-4-yloxy)acetate<sup>1</sup> (**8**) (2.74 g, 11 mmol) in a mixture of MeOH (230 mL), water (36 mL), and concentrated HCl (18 mL) was refluxed for 4 h. The solution was diluted with water (100 mL), concentrated to ~200 mL, basified with solid NaHCO<sub>3</sub>, and extracted with EtOAc (3 × 100 mL). The combined organic phases were washed with brine, dried, and evaporated to give methyl (indolin-4-yloxy)acetate (**9**) (1.77 g, 77%) as pale crystals: <sup>1</sup>H NMR (90 MHz) δ 6.92 (t, *J* = 7.5 Hz, 1H), 6.31 (d, *J* = 7.5 Hz, 1H), 6.12 (d, *J* = 7.5 Hz, 1H), 4.62 (s, 2H), 3.76 (s, 3H), 3.56 (t, *J* = 8 Hz, 2H), 3.05 (t, *J* = 8 Hz, 2H). The crude indoline (1.77 g, 8.5 mmol) was dissolved in dry MeCN (80 mL) and treated with *N*-*tert*-BOC-L-glutamic acid *γ*-*tert*-butyl ester (2.88 g, 9.5 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide·HCl (2.30 g, 12 mmol). The mixture was stirred at room temperature for 18 h, then evaporated. The residue was dissolved in EtOAc and washed with 0.5 M aq HCl, saturated aq NaHCO<sub>3</sub>, and brine, dried, and evaporated to give a white foam that, after trituration with ether, gave **10** as white crystals (3.73 g, 89%), mp 120–122 °C (EtOAc–hexanes): <sup>1</sup>H NMR (500 MHz) δ 7.88 (d, *J* = 8.1 Hz, 1H), 7.13 (t, *J* = 8.1 Hz, 1H), 6.44 (d, *J* = 8.1 Hz, 1H), 5.22 (d, *J* = 7 Hz, 1H), 4.66 (s, 2H), 4.12–4.26 (m, 1H), 4.03–4.09 (m, 2H), 3.78 (s, 3H), 3.20 (t, *J* = 8.5 Hz, 2H), 2.44–2.58 (m, 2H), 2.22–2.30 (m, 1H), 1.99–2.08 (m, 1H), 1.47 (s, 9H), 1.42 (s, 9H). Anal. Calcd for C<sub>25</sub>H<sub>36</sub>N<sub>2</sub>O<sub>8</sub>: C, 60.96; H, 7.37; N, 5.68. Found: C, 60.88; H, 7.47; N, 5.59.

**Methyl {1-[4S-(4-*tert*-Butoxycarbonyl)-4-(di-*tert*-butoxycarbonylamino)butanoyl]indolin-4-yloxy}acetate (11).** A solution of **10** (3.94 g, 8 mmol) in a mixture of dry CH<sub>2</sub>Cl<sub>2</sub> (32 mL) and Et<sub>3</sub>N (48 mL) was treated with di-*tert*-butyl dicarbonate (4.36 g, 20 mmol) and DMAP (98 mg, 0.8 mmol) and the mixture was refluxed under nitrogen for 6 h. The solvents were evaporated and the residue was dissolved in Et<sub>2</sub>O (100 mL) and washed with 1 M aq KHSO<sub>4</sub>, saturated aq NaHCO<sub>3</sub> and brine, dried, and evaporated to give a viscous oil. Flash chromatography (2:3 EtOAc–hexanes) gave **11** as a pale foam (4.61 g, 97%) that was used in the next step without further purification: <sup>1</sup>H NMR (500 MHz) δ 7.90 (d, *J* = 8.1

Hz, 1H), 7.12 (t, *J* = 8.1 Hz, 1H), 6.42 (d, *J* = 8.1 Hz, 1H), 4.86 (dd, *J* = 5.2, 9.3 Hz, 1H), 4.66 (s, 2H), 4.04–4.10 (m, 2H), 3.79 (s, 3H), 3.18 (t, *J* = 8.6 Hz, 2H), 2.50–2.60 (m, 2H), 2.42–2.48 (m, 1H), 2.18–2.25 (m, 1H), 1.48 (s, 18H), 1.46 (s, 9H). Low resolution MS (ES<sup>+</sup>): *m/z* 593.4 (M + H)<sup>+</sup>, calcd for (C<sub>30</sub>H<sub>44</sub>N<sub>2</sub>O<sub>10</sub> + H)<sup>+</sup> 593.3.

**{1-[4S-(4-*tert*-Butoxycarbonyl)-4-(di-*tert*-butoxycarbonylamino)butanoyl]indolin-4-yloxy}acetic Acid (14).** Aqueous NaOH (1 M, 11 mL, 11 mmol) was added to a solution of **11** (4.35 g, 7.3 mmol) in MeOH (200 mL). After 2.5 h, when consumption of the starting material was confirmed by TLC (90:10:1 EtOAc–hexanes–AcOH), the solution was neutralized with 1 M aq citric acid (11 mL) and concentrated. The residue was diluted with water, acidified to pH 2 with 1 M aq citric acid, and washed with EtOAc. The combined organic phases were washed with brine, dried, and evaporated to a pale foam which, after trituration with EtOH, gave **14** as white crystals (3.07 g, 72%), mp 65–67 °C (Et<sub>2</sub>O–hexanes): <sup>1</sup>H NMR (500 MHz) δ 7.91 (d, *J* = 8.2 Hz, 1H), 7.13 (t, *J* = 8.2 Hz, 1H), 6.46 (d, *J* = 8.2 Hz, 1H), 4.86 (dd, *J* = 4.9, 9.1 Hz, 1H), 4.68 (s, 2H), 4.02–4.11 (m, 2H), 3.16 (t, *J* = 8.6 Hz, 2H), 2.51–2.60 (m, 2H), 2.43–2.49 (m, 1H), 2.16–2.24 (m, 1H), 1.48 (s, 18H), 1.46 (s, 9H). Anal. Calcd for C<sub>29</sub>H<sub>42</sub>N<sub>2</sub>O<sub>10</sub> + H<sub>2</sub>O: C, 58.38; H, 7.40; N, 4.69. Found: C, 58.72; H, 7.40; N, 4.69.

**1-[4S-(4-*tert*-Butoxycarbonyl)-4-(*tert*-butoxycarbonylamino)butanoyl]-7-nitroindolin-4-yloxyacetic Acid (4).** Claycop (3.2 g) was added to a solution of **14** (1.15 g, 2 mmol) in a solution of CCl<sub>4</sub> (50 mL) and acetic anhydride (25 mL) and the mixture was stirred at room temperature overnight, when the consumption of the starting material was confirmed by TLC [100:2 EtOAc–AcOH]. The solid was filtered off and washed thoroughly with EtOAc (200 mL) and the combined filtrate was washed with brine, dried, and evaporated, then re-evaporated from toluene (2 × 50 mL). The residue was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (50 mL), treated with 1 M TFA in CH<sub>2</sub>Cl<sub>2</sub> (3 mL), and stirred at room temperature overnight. The solution was diluted with CH<sub>2</sub>Cl<sub>2</sub> (100 mL) and washed with brine, dried, and evaporated to a brown viscous oil. Flash chromatography (100:2 EtOAc–AcOH) gave **4** as a pale yellow foam (0.90 g, 86%), contaminated with a small proportion of the 5-isomer. This material was used in the next step without further purification.

**4-[2-[1-[4S-(4-Amino-4-carboxybutanoyl)]-7-(nitroindolin-4-yloxy)acetamido]ethoxy]-4'-[2-(dihydroxyphosphoryloxy)ethoxy]benzophenone (3).** A solution of the azide **15** (779 mg, 1.5 mmol, prepared as described<sup>1</sup>) in THF (10.25 mL) containing water (40 μL, 2.25 mmol) was treated with triphenylphosphine (590 mg, 2.25 mmol) and the mixture was stirred at room temperature under nitrogen for 20 h. TLC [4:1 EtOAc–hexanes] confirmed that all the azide was reduced to the amine **16**. The solvent was evaporated and the residue was dissolved in CHCl<sub>3</sub> (30 mL), dried, and evaporated to a viscous oil (1.41 g) that contained material identical with **16** as previously described<sup>1</sup> (assessed by <sup>1</sup>H NMR), together with triphenylphosphine oxide and unreacted triphenylphosphine. The mixture was dissolved in dry MeCN (50 mL) and treated with the crude acid **4** (785 mg, 1.5 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide·HCl (401 mg, 2.1 mmol). The mixture was stirred at room temperature under nitrogen for 18 h, then evaporated. The residue was dissolved in EtOAc and washed with 0.5 M aq HCl, saturated aq NaHCO<sub>3</sub>, and brine, dried, and evaporated. Flash chromatography (95:5 CHCl<sub>3</sub>–MeOH) gave a pale yellow foam (700 mg, 52%), which was used in the next step without further characterization. This material (645 mg, 0.71 mmol) was dissolved in TFA (30 mL), stirred at room temperature for 1 h, and concentrated in vacuo. The residue was dissolved in water (180 mL) and adjusted to pH 7.1 with 1 M aq NaOH. The solution was washed with ether and analyzed by reverse-phase HPLC [mobile phase 25 mM Na phosphate, pH 6.0–MeCN (10:3 v/v)], *t*<sub>R</sub> 3.9 min, with a small additional peak at 6.3 min. The solution was lyophilized, dissolved in 25 mM Na phosphate,

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pH 6.0 (130 mL), and pumped onto the preparative HPLC column. The column was washed with 25 mM Na phosphate, pH 6.0 for 1.5 h, then eluted with 25 mM Na phosphate, pH 6.0–MeCN (10:2 v/v). Fractions containing the first peak were analyzed as above, combined, and quantified by UV spectroscopy [ $\lambda_{\text{max}}$  (H<sub>2</sub>O)/nm 300 ( $\epsilon/\text{M}^{-1}$  cm<sup>-1</sup> 27 900)] to give **3** (230  $\mu\text{mol}$ ). Later fractions contained a compound that corresponded to the  $t_{\text{R}}$  6.3 min peak above, which was identified as the 5-nitro isomer of **3** from its UV–visible absorption spectrum (see the Supporting Information) but was not further characterized. The solution of **3** was concentrated and desalted by reapplication to the preparative HPLC column in 25 mM Na phosphate, pH 6.0. The column was first eluted with water for 2 h, then with water–MeOH (100:25 v/v). Fractions containing the product were analyzed, combined, and quantified (UV–visible spectroscopy) to give **3** (171  $\mu\text{mol}$ ). The solution was concentrated and the residue was dissolved in water, adjusted to pH 8, and stored frozen: <sup>1</sup>H NMR (Na<sup>+</sup> salt)  $\delta$  (500 MHz, D<sub>2</sub>O, acetone reference) 7.65 (d,  $J$  = 8.9 Hz, 2H), 7.63 (d,  $J$  = 8.9 Hz, 2H), 7.36 (d,  $J$  = 9 Hz, 1H), 7.08 (d,  $J$  = 8.9 Hz, 2H), 6.88 (d,  $J$  = 8.9 Hz, 2H), 6.54 (d,  $J$  = 9 Hz, 1H), 4.62 (s, 2H), 4.31 (t,  $J$  = 4.7 Hz, 2H), 4.17–4.20 (m, 4H), 4.12–4.15 (m, 2H), 3.75 (t,  $J$  = 6.3 Hz, 1H), 3.70 (t,  $J$  = 4.7 Hz, 2H), 3.05 (t,  $J$  = 7.9 Hz, 2H), 2.69 (t,  $J$  = 7.9 Hz, 2H), 2.08–2.15 (m, 2H). HRMS (ES<sup>+</sup>):  $m/z$  731.1951 (M + 3H)<sup>+</sup>; calcd for (C<sub>32</sub>H<sub>33</sub>N<sub>4</sub>O<sub>14</sub>P + 3H)<sup>+</sup>, 731.1960.

**Quantitative Product Analysis from Photolysis of 3.** Separate solutions of **3** (0.319 mM in 25 mM Na phosphate, pH 7.0 containing 5 mM dithiothreitol) were irradiated for varying times (3 or 5 s) in 1-mm path length cells in a Rayonet photochemical reactor fitted with 16  $\times$  300 nm lamps. The solutions were analyzed by reverse-phase HPLC (as above) and the extent of photolysis of each solution was determined by comparison of peak heights with those of nonirradiated controls. Aliquots of the photolyzed solutions were also subjected to quantitative amino acid analysis (Pharmacia AlphaPlus-Analyzer with ninhydrin detection). The extents of photolysis were 26.9% and 43.8% for the 3- and 5-s irradiations, respectively, and measured glutamate concentrations were 88–90% of the values expected from the calculated decreases in concentration of the starting conjugate **3**.

#### Relative Photolysis Efficiencies of 1 and 2 at 350 nm.

Separate solutions of **1** and **2** (each 0.31 mM in 25 mM Na phosphate, pH 7.0 with 5 mM dithiothreitol) were simultaneously irradiated for 7 s in 1-mm path length cells (Rayonet Reactor, 16  $\times$  350 nm lamps). Solutions were analyzed by reverse-phase HPLC as previously described<sup>1</sup> and the extent of photolysis of each solution was determined by comparison of peak heights with those of nonirradiated controls. Conversions for **1** and **2** were 18% and 35%, respectively, indicating that **2** photolyzed  $\sim$ 2-fold more efficiently than **1** under these conditions.

**Flash Photolysis of 3 During Whole Cell Recording from Neurons.** General details of the photolysis and patch clamp methodology have been described previously.<sup>3a,b,e</sup> Specific details for photolysis of **3** are given in the legend to Figure 3. Calibration of photolytic conversion in the experimental microscope in physiological experiments was as described previously<sup>3b</sup> by separate fluorometric determination of the proton released stoichiometrically with L-glutamate during photolysis, using 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) excited at 450 nm as the proton-sensitive fluorophore.

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**Supporting Information Available:** General experimental details, <sup>1</sup>H NMR spectra for compounds **3**, **6**, and **10–14**, computed UV–visible spectrum for the 5-nitro isomer of compound **3**, and experimental details for compounds **12** and **13**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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