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# Synthesis and Two-photon Photolysis of 6-(*ortho*-Nitroveratryl)-Caged $IP_3$ in Living Cells

Srinivas Kantevari,[a] Caroline J. Hoang,[b] Jakob Ogrodnik,[c] Marcel Egger,[c] Ernst Niggli,[c] and Graham C. R. Ellis-Davies*[a]

*The synthesis of a photolabile derivative of inositol-1,4,5-trisphosphate ($IP_3$) is described. This new caged second messenger (6-ortho-nitroveratryl)-$IP_3$ (6-NV-$IP_3$) has an extinction coefficient of 5000 $M^{-1}cm^{-1}$ at 350 nm, and a quantum yield of photolysis of 0.12. Therefore, 6-NV-$IP_3$ is photolyzed with UV light about three times more efficiently than the widely used $P^{4(5)}$-1-(2-nitrophenyl)ethyl-caged $IP_3$ (NPE-$IP_3$). 6-NV-$IP_3$ has a two-photon cross-section of about 0.035 GM at 730 nm. This absorbance is sufficiently large for effective two-photon excitation in living cells at modest power levels. Using near-IR light (5 mW, 710 nm, 80 MHz, pulse-width 70 fs), we produced focal bursts of $IP_3$ in HeLa cells, as revealed by laser-scanning confocal imaging of intracellular $Ca^{2+}$ concentrations. Therefore, 6-NV-$IP_3$ can be used for efficient, subcellular photorelease of $IP_3$, not only in cultured cells but also, potentially, in vivo. It is in the latter situation that two-photon photolysis should reveal its true forte.*

## Introduction

Inositol-1,4,5-trisphosphate ($IP_3$) is one of the most important intracellular second messengers.[1] It is fundamental to the regulation of biological processes as diverse as glycogenesis in hepatocytes, to fertilization in oocytes and modulation of excitability and synaptic plasticity in Purkinje neurons. $IP_3$ is generated by hydrolysis of phosphatidylinositol bisphosphate, which is present in the cell membrane. $IP_3$ then binds to a calcium channel on the endoplasmic reticulum, and thus mobilizes $Ca^{2+}$ from these intracellular stores.[1] $IP_3$ has been the subject of intense study, both from chemical and biochemical points of view.[2]

Optical microscopy is an essential tool for studying cells, and the photocontrol of cell chemistry with caged compounds has proved extremely useful since the initial deployment of this technique in 1977.[3] The term "caged" is used to designate that a cellular-signaling molecule has been rendered biologically inert by covalent modification of its active functionality with a photoremovable protecting group.[4] Irradiation with a brief pulse of light frees the caged substrate. In cellular experiments, uncaging provides a means for rapidly and specifically activating the biological process that is controlled by the caged substrate.

Trentham and co-workers were the first to synthesize a caged $IP_3$.[5,6] They made 4/5-(*ortho*-nitrophenethyl)-caged $IP_3$ (NPE-$IP_3$) by direct alkylation of the $IP_3$ phosphates by using 1-(2-nitrophenyl)diazoethane. These researchers found that attachment of the chromophore to either the 4- or 5-phosphate rendered $IP_3$ biologically inert.[6] This caged $IP_3$ has been commercially available for some time and has been used by many laboratories. Since the pioneering work of the Trentham group, three syntheses of caged inositols have been published. In 1998 Tsien and co-workers reported a 15-step synthesis of a cell-permeant version of a caged $IP_3$, called cm$IP_3$/PM. Photolysis of this caged compound releases an analogue of $IP_3$ that bears a propionyloxymethyl protecting group on the 2- and 3-hydroxyl groups.[7] Dinkel and Schultz synthesized 3-(*ortho*-nitroveratryl)-caged $IP_4$ by using a ten-step synthetic route, starting from inositol.[8] Prestwich and colleagues synthesized a caged $IP_6$,[9] and have recently used this probe to study intracellular signaling in plants.[10] In this report we describe a seven-step synthesis of 6-(*ortho*-nitroveratryl)-caged $IP_3$ (6-NV-$IP_3$, **1**) starting from inositol (Scheme 1). The biological efficacy of **1** was tested by measuring $IP_3$-induced $Ca^{2+}$ signals and $Ca^{2+}$-regulated potassium-channel currents by rapid uncaging of **1** in cerebellar Purkinje cells (Figure 1), and by UV- and two-photon uncaging in HeLa cells (Figure 2).

## Results and Discussion

### Synthesis

The synthesis of **1** is outlined in Scheme 1. Starting from 2,3:4,5-di-*O*-cyclohexylidene-inositol,[11] the *ortho*-nitroveratryl caging group was introduced at the 6-position by regioselective attack of the tin ketal[12] with *ortho*-nitroveratrylbromide,

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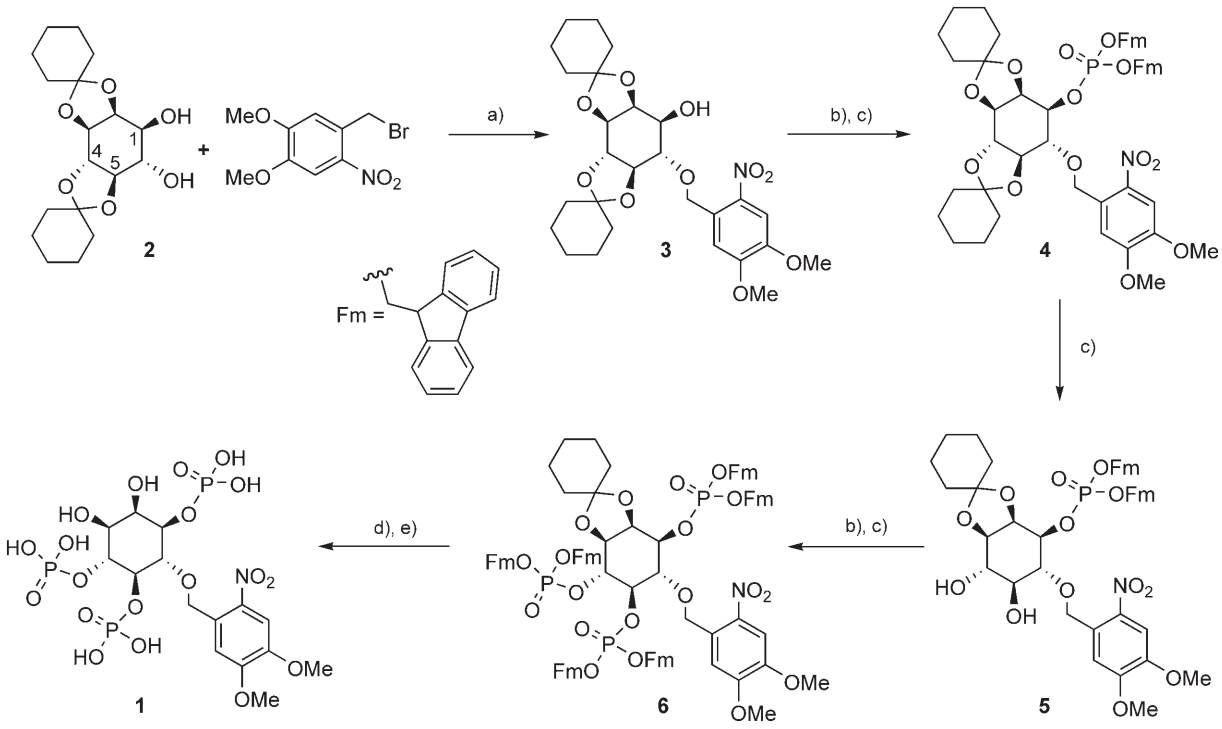


**Scheme 1.** Synthesis of 6-(*ortho*-nitroveratryl)-inositol-1,4,5-trisphosphate. a) $(n\text{Bu})_2\text{SnO}$ in toluene, then NV-Br with CsF in dimethyformamide, 24 h, RT; b) $(\text{Fm})_2\text{PN}(i\text{Pr})_2$ and tetrazole, 6 h, RT; c) *t*BuOOH, 30 min, 4 °C; d) Dowex $H^+$, 24 h, RT; e) piperidine, 4 h, RT.

to give alcohol **2** in a yield of 48%. The ratio of 6/1 isomers was about 4:1; Schultz and co-workers have used the same chemistry with a 3:1 selectivity.[13] This alcohol was then phosphytilated with difluorenylmethyl di-*iso*-propylphosphoramidate[14] at room temperature for 6 h, after which it was oxidized to the phosphate at ice temperature by using *tert*-butyl hydrogen peroxide to give monophosphate **4** in a yield of 70% (based on recovered starting material).

Selective deprotection of the 4,5-cyclohexylidene protecting group was accomplished by using Dowex $H^+$ resin, to give diol **5** in a yield of 81% (based on 56% recovered starting material). The diol was converted to trisphosphate **6** by using the same conditions as described for the conversion of **3** to **4**. Due to the steric congestion of product **6**, diphosphytilation gave only 65% yield from diol **5** (based on recovered starting material).

The fluorenylmethyl protecting groups were removed by treatment of **6** with piperidine at room temperature for 4 h. All solvents were removed from the reaction mixture, and the remaining cyclohexylidene-protecting group was removed by using Dowex $H^+$ resin. The target 6-(*ortho*-nitroveratryl)-*myo*-inositol-1,4,5-trisphosphate (**1**) was purified from the crude product mixture by RP-HPLC in a yield of 53% for two steps. The resultant caged $IP_3$ is in the racemic form, however, only the D-isomer mobilizes $Ca^{2+}$.[2] The L-isomer has a 2000-fold lower affinity for the $IP_3$ receptor,[2] and thus will act neither as agonist nor antagonist in biological experiments.

### Photochemical Properties

We measured the quantum yield of 6-NV-$IP_3$ photolysis and compared it with a known standard, 4-methoxy-7-nitroindolinyl–glutamate (MNI–glutamate). In order to determine the relative percent photolysis of the two caged compounds, a solution containing both caged compounds was photolyzed with near-UV light (300–380 nm). Under these conditions, both compounds experience the same flux density of photons; this makes the determination of the quantum yield for 6-NV-$IP_3$ more reliable. (We also photolyzed both cages separately in order to check that there was no interference from simultaneous photolysis.) Inosine was also included in the photolysis-reaction mixture as a photochemically inert standard. MNI–glutamate has a quantum yield of photolysis of 0.085.[4b] Analysis of the reaction mixture by HPLC showed that 6-NV-$IP_3$ was photolyzed about 1.4-times faster than MNI–glutamate. This implies a quantum yield ($\phi$) of photolysis of 0.12. 6-NV-$IP_3$ has an extinction coefficient ($\varepsilon$) of 5000 $\text{M}^{-1}\text{cm}^{-1}$ at 350 nm. This value is similar to other dimethoxynitrobenzyl caged compounds.[4a] Thus, 6-NV-$IP_3$ is uncaged about four times more efficiently that the original caged NPE-caged $IP_3$, as the product ($\varepsilon\cdot\phi$) is 600 for 6-NV-$IP_3$ and 240 for NPE-$IP_3$.[6] It should be noted that wavelengths below 340 nm are not practical for uncaging in living cells when using typical confocal microscopes for two reasons: 1) most objectives are opaque below this wavelength, and 2) confocal microscopes use lasers that emit light at 351–364 nm (i.e., Ar/Kr lasers).

There has been a great deal of interest over the past 25 years in the rate of uncaging of nitrobenzyl-caged compounds. This started with the pioneering studies of Trentham and co-

  

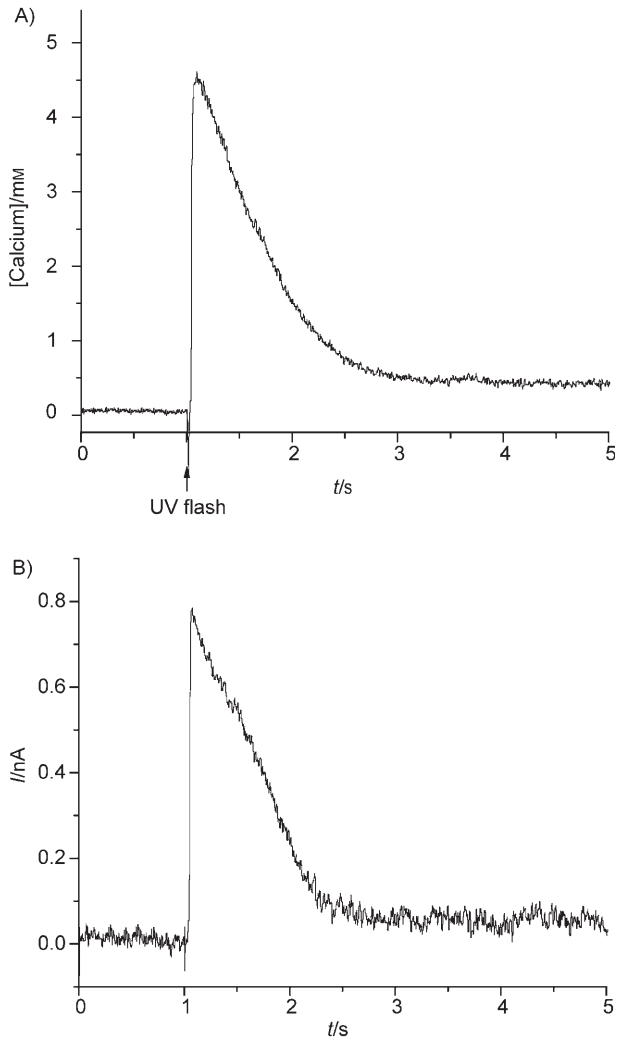


**Figure 1.** Responses evoked from cerebellar Purkinje neurons in acutely isolated brain slices. A) Changes in $[Ca^{2+}]_i$ as reported by fura-6F after photolysis (arrow) of 6-NV-$IP_3$ with a 1 ms pulse from a Xe flash lamp. B) Changes in $Ca^{2+}$-gated potassium channel currents during the same period.

workers in 1980.[15] Recently, detailed studies of *ortho*-nitrobenzylmethylether by Wirz and co-workers have shown definitively, that by using time-resolved IR spectroscopy simple ethers can be uncaged with a long-lived (many seconds) hemiacetal.[16] The uncaging of 6-NV-$IP_3$ inside cells evokes quite a fast appearance of intracellular calcium ($[Ca^{2+}]_i$) from intracellular stores (within 30 ms, Figure 1 A). This suggests that the presence of the electron-donating methoxy groups of the caging chromophore must speed up the rate of uncaging of *ortho*-nitrobenzylethers considerably. Furuta and co-workers have reported that *ortho*-nitrobenzyl-caged steroidal compounds (bile acids) uncage slowly in aqueous buffer (release required 5 min), whereas the analogous NV-caged compounds uncage with a rate that is faster by several orders of magnitude.[17]

We measured the two-photon uncaging cross section of 6-NV-$IP_3$ in a similar manner to the quantum-yield determination. The light source was a mode-locked Ti:sapphire laser (730 nm) that was focused through a low numerical-aperture (NA) lens (0.2, 10× magnification) by the scanhead of a two-photon microscope into a low-volume cuvette. By using multiple uncaging events (1024), sufficient photolysis of a volume large enough for accurate HPLC analysis could be achieved (5–20% uncaging). We found that 6-NV-$IP_3$ was photolyzed about 0.6-times more slowly than MNI–glutamate; again inosine was the photochemically inert internal standard. Since MNI-glutamate has a two-photon cross-section of 0.06 GM,[18] this implies that 6-NV-$IP_3$ had a two-photon cross section of 0.035 GM.

### Physiological experiments

Cerebellar Purkinje neurons have the highest density of $IP_3$ receptors in mammals.[19–21] Activation of metabotropic-glutamate receptors on Purkinje neurons produces an increase in intracellular $IP_3$ concentration.[1] This in turn binds to ligand-gated ion channels on the endoplasmic reticulum and causes an increase in $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ has been shown to be sufficient to produce long-term depression (LTD) of synaptic transmission in these neurons.[20] Such LTDs are thought to be one of the central processes in learning and memory.

The effectiveness of our new caged $IP_3$ was demonstrated by dialyzing single cerebellar-Purkinje neurons in acutely isolated brain slices with **1** and a fluorescent indicator of $Ca^{2+}$, fura-6F; such dyes can be used in microfluorimetry and they enable absolute $Ca^{2+}$ concentrations to be easily determined. Figure 1 A shows a representative example of the amount of $Ca^{2+}$ released by the binding of $IP_3$ to $IP_3$-regulated channels in the soma of Purkinje cells after photolysis of **1**. Peak increases in $[Ca^{2+}]_i$ of about 4 μM were recorded. The delay before the onset of $Ca^{2+}$ release was about 20 ms and the time to the peak was approximately 12 ms. These measurements are similar to values reported previously for these cells.[19] The whole-cell current recorded before and after uncaging is shown in Figure 1 B. This current must be mediated by a ligand-gated ion channel, that is, the $Ca^{2+}$-activated potassium channel, as we clamped all the voltage-gated channels at −60 mV. We believe that the release of $IP_3$ in such studies is preferable to the release of its analogues,[10] because $IP_3$ is the actual second messenger that is generated within the cell.

Comparison of the physico–chemical properties of 6-NV-$IP_3$ and NPE-$IP_3$ suggests that the former might mobilize $[Ca^{2+}]_i$ more effectively than the latter. This was indeed found to be the case. UV photolysis of the caged $IP_3$ compounds in HeLa cells produced a $Ca^{2+}$ signal that was about two- to three-times larger for 6-NV-$IP_3$ than NPE-$IP_3$ (compare Figures 2 A and 3 A). Laser-scanning confocal microscopy was used for these measurements with the $Ca^{2+}$ dye, fluo-3. This dye is not a ratiometric probe like fura-6F, but it has low fluorescence in quiescent cells and becomes much brighter when $[Ca^{2+}]_i$ increases. Even though we took care to match the concentrations of probes and light intensities in both experiments, this comparison can only be regarded as an approximation. This is because there is a very subtle, symbiotic relationship between $[Ca^{2+}]_i$ and intracellular $IP_3$ concentrations (which is bell-shaped[22]), and the number or density of $IP_3$ receptors can vary from cell to cell—the photolysis experiments must be performed in different cells. Nevertheless, there is a reasonably good agree-

  

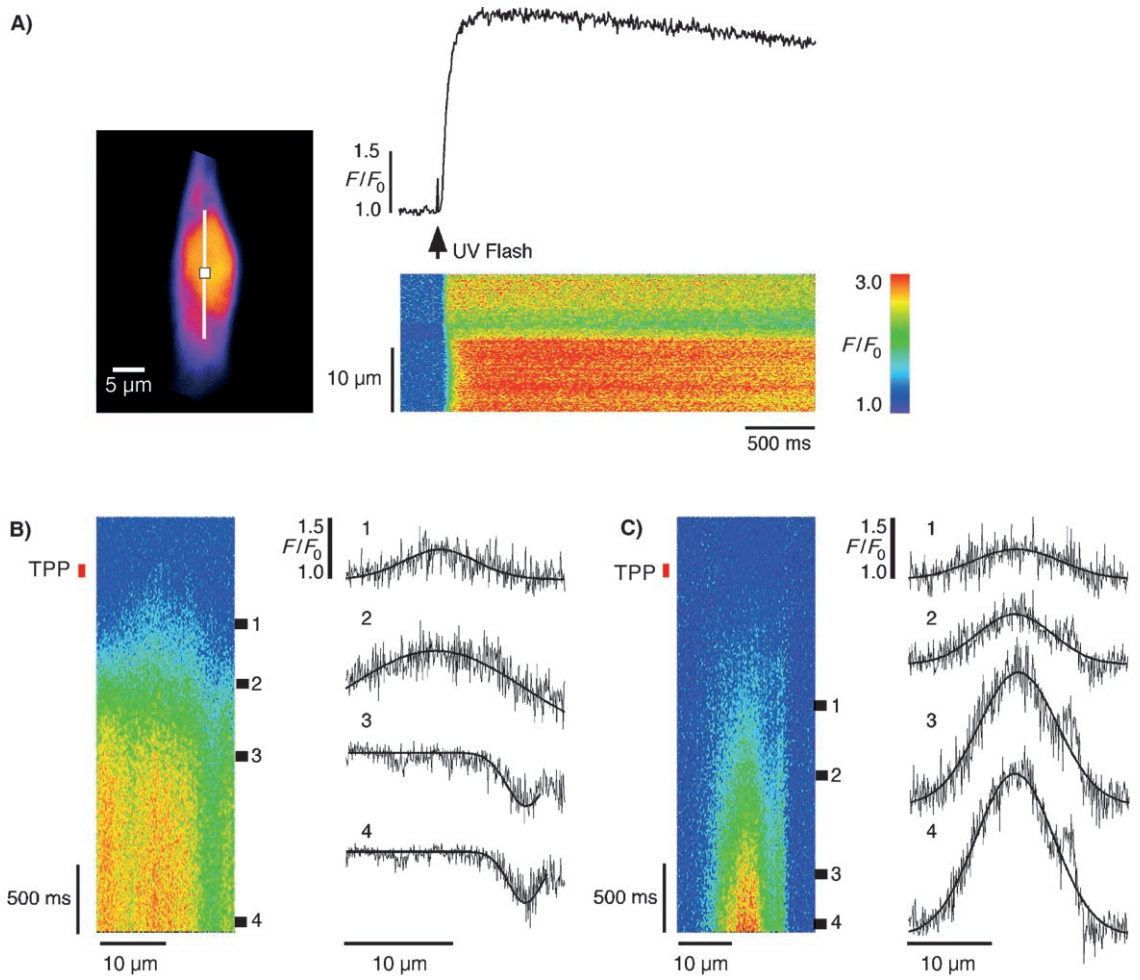


**Figure 2.** Local activation of $Ca^{2+}$ release in HeLa cells triggered by two-photon photolysis of 6-NV-$IP_3$. A) Left: HeLa cell after 1 day in culture. The position of the linescan is indicated by a white line. The spot for local uncaging of $IP_3$ is marked by a white square. Right: linescan image and the corresponding whole-cell $Ca^{2+}$ transient (global $Ca^{2+}$ response, as reported by fluo-3) induced by UV-flash photolysis. The pipette solution contained 6-NV-$IP_3$ (30 µM). B) Two-photon photolysis (TPP) of 6-NV-$IP_3$ (710 nm, 100 ms, 5 mW, red bar) triggered local $Ca^{2+}$-release events as reported by fluo-3. A local $Ca^{2+}$-release event evoked by local uncaging of $IP_3$, triggered a propagating $Ca^{2+}$ wave and global $Ca^{2+}$ transient; C) an elementary $Ca^{2+}$ signal in HeLa cells was evoked by diffraction-limited uncaging of $IP_3$. In contrast to the example shown in B), the $Ca^{2+}$ signal remained locally confined. Line-scan images in B) an C) show spatial spreading of the $Ca^{2+}$-release event at selected times. The continuous lines were obtained by a Gaussian fit to the points. The spatial spreading was determined to be 8.8 µm (full width at half maximum height). $F/F_0 = 2.1$.

ment between the relative size of the predicted [$Ca^{2+}$] signals and the product ($\varepsilon \cdot \phi$) for the two caged-$IP_3$ compounds.

The difference between the new and old caged-$IP_3$ molecules under two-photon excitation was also tested in HeLa cells. We found that NPE-$IP_3$ did not mobilize $Ca^{2+}$ under the conditions we chose (710 nm, 5 mW for 100 ms, Figure 3B), whereas 6-NV-$IP_3$ did (Figure 2B and C). Similar results have been reported for caged $Ca^{2+}$.[23] We could evoke different types of $Ca^{2+}$ signaling with two-photon photolysis of 6-NV-$IP_3$: calcium waves and calcium puffs. Figure 2B shows a local $Ca^{2+}$-release event that was evoked by local uncaging of $IP_3$. This triggered a propagating $Ca^{2+}$ wave (by $Ca^{2+}$-induced $Ca^{2+}$ release) that ended in a global $Ca^{2+}$ transient. Figure 2C, however, shows an example of a local $Ca^{2+}$-release event that corresponds to an elementary $Ca^{2+}$ signal in HeLa cells ($Ca^{2+}$ puffs[24]) and is evoked by diffraction-limited uncaging of $IP_3$. In contrast to the example shown in Figure 2B, the $Ca^{2+}$ signal remained locally confined. These data represent the first reports of two-photon uncaging of $IP_3$ with near-IR light in living cells and, as such, are a significant step forward in uncaging technology. This is because the physiological importance of subcellularly highly targeted signals, such as local increases of $Ca^{2+}$ concentration ($Ca^{2+}$ sparks and puffs) or second messengers, such as cAMP and $IP_3$, has only recently been recognized. Subcellular targeting of biological signals is a means by which cells are able to generate specific responses with an otherwise rather unspecific messenger.[25]

## Conclusion

The two-photon excitation technique has become essential for [$Ca^{2+}$] imaging in vivo (i.e., in living animals) over the past ten years. One reason for this is that the depth penetration of near-IR light is 500–600 µm for two-photon excitation, com-

  

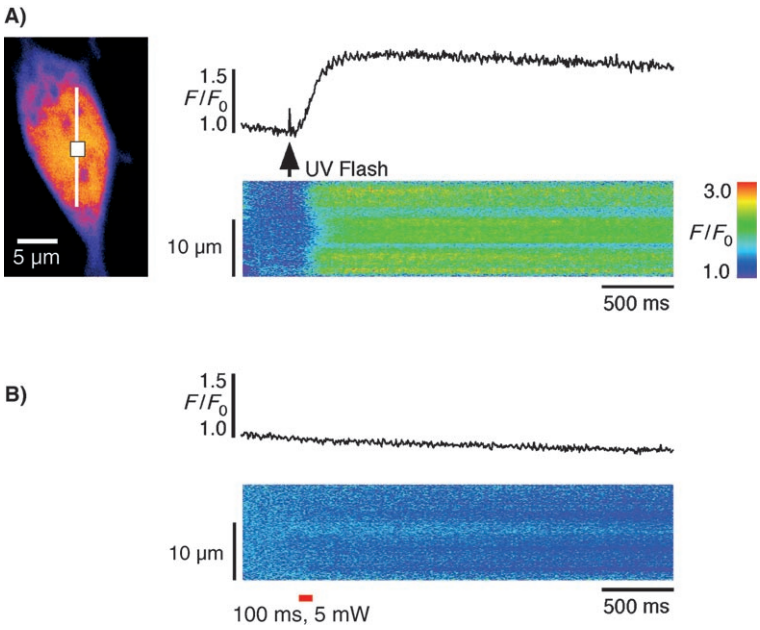


**Figure 3.** Whole-cell $Ca^{2+}$ transient induced by global UV-flash photolysis of NPE-$IP_3$ in HeLa cells. A) Left: HeLa cell after 1 day in culture. The position of the linescan is indicated by the white line and the spot for local uncaging of $IP_3$ is marked by a white square; right: a linescan image and the early phase of the corresponding whole-cell $Ca^{2+}$ transient (global response, as reported by fluo-3) induced by UV-flash photolysis. B) Local uncaging of NPE-$IP_3$ (30 µM) failed to trigger a $Ca^{2+}$ response on the local level. The output from a Ti:sapphire laser (710 nm, 100 ms, 5 mW, red bar) was applied to the cell to photolyze NPE-$IP_3$. $F/F_0 = 1.6$.

pared to only 30–50 µm for visible light.[26] With the introduction of "one-box" Ti:sapphire mode-locked lasers (the Chameleon by Coherent and Mai-Tai by Spectra-Physics are self-tuning, hermetically sealed lasers that are simple to operate), two-photon microscopy has become a practical proposition. However, only a few laboratories have been able to use two-photon excitation to uncage bioactive molecules in a way that mimics normal cellular signals. The major hindrance has been the lack of useful caged compounds. Thus, we think that the development of optical probes that undergo two-photon photolysis is the next frontier for uncaging technology. Therefore, we believe that the straightforward synthesis of 6-NV-$IP_3$ presented here, and its ready two-photon photolysis in living cells is a significant addition to the arsenal of tools for neurobiologists. Further, with the recent introduction of the dual two-photon microscope,[27] we are in a position to use two-photon excitation not only to observe cellular chemistry in real time, but also to control this chemistry simultaneously and independently at the single-cell level, not only in cultured cells as described in this report, but also in vivo. Such studies are now underway.[28]

## Experimental Section

**NMR spectroscopy**: NMR spectra were recorded on a 300 MHz Varian Mercury VX spectrometer. Chemical shifts are reported as $\delta$ values in ppm relative to TMS, TMSPA, or external phosphoric acid (85%, $\delta = 0.00$). The low-resolution mass spectra (*m/z*) were recorded on a Finnigan 4500 quadrupole spectrometer.

**Synthesis of 6-(*ortho*-nitroveratryl)-*myo*-inositol-1,4,5-trisphosphate**: Difluorenylmethyldiisopropyl phosphoramidate was prepared from dichlorodiisopropyl phosphoramidate and 9-fluorenylmethanol as described in the literature.[13]

**(±)-2,3:4,5-Di-*O*-cyclohexylidene-*myo*-inositol (2)**: This was synthesized from *myo*-inositol as described in the literature.[20]

**(±)-2,3:4,5-Di-*O*-cyclohexylidene-6-*O*-(*ortho*-nitroveratryl)-*myo*-inositol (3)**: A solution of **2** (340 mg, 1 mmol) and dibutyl tin oxide (249 mg, 1 mmol) in toluene (25 mL) was heated at reflux temperature for 24 h with azeotropic removal of water. The clear solution was concentrated under reduced pressure. The gummy residue obtained was stirred with CsF (162 mg, 1 mmol) and 3,4-dimethoxy-6-nitrobenzyl bromide (288 mg, 1 mmol) in DMF (5 mL) at room temperature for 24 h. The reaction mixture was poured into water and extracted with dichloromethane (3×20 mL), dried over $MgSO_4$, filtered, and evaporated by using a rotary evaporator. The crude product was purified by flash-column chromatography (hexane/ethyl acetate, 2:1) to give **3** as a gum (120 mg) in a yield of 48% (based on recovered starting material), along with the second isomer (32 mg, 12%). Elution with hexane/ethyl acetate (1:1) yielded 180 mg of starting material. $^1$H NMR (300 MHz, $CDCl_3$) $\delta = 7.75$ (s, 1H), 7.21 (s, 1H), 5.14 (dd, $J = 7.4$, 6.1, 2H), 4.48 (dd, $J = 2.6$, 1.3, 1H), 4.40 (t, $J = 2.3$, 1H), 4.1–4.2 (m, 2H), 4.08 (s, 3H), 3.94 (s, 3H), 3.87 (dd, $J = 3.0$, 0.9, 1H), 3.61 (dd, $J = 3.9$, 2.7, 1H), 2.6 (s, 1H), 1.4–1.8 (m, 20H). MS (ESI) *m/z*: 536 $[M+H]^+$; 558 $[M+Na]^+$.

**(±)-1-Bisfluorenylphospho-2,3:4,5-di-*O*-cyclohexylidene-6-*O*-(*ortho*-nitroveratryl)-*myo*-inositol (4)**: **3** (1.18 g, 2.2 mmol) was dissolved in acetonitrile (20 mL) and treated with difluorenylmethyldiisopropyl phosphoramidate (1.15 g, 2.2 mmol) and tetrazole (0.160 g, 2.25 mmol). The reaction mixture was stirred at room temperature for 6 h, cooled to 4 °C, and *tert*-butyl hydrogen peroxide (0.3 mL, 40% solution in water) was added. After stirring for 30 min at room temperature, dichloromethane (20 mL) and water (20 mL) were added and the organic phase was separated, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation. Flash chromatography (hexane/ethyl acetate, 3:1) gave product **4** as a gum (1.08 g) in a yield of 70% (based on 330 mg of recovered starting material). $^1$H NMR (300 MHz, $CDCl_3$) $\delta = 7.6$–7.8 (m, 8H), 7.20–7.45 (m, 10H), 5.06 (dd, $J = 25.5$, 5.6, 2H), 4.00–4.48 (m, 9H), 3.96 (m, 1H), 3.88 (s, 3H), 3.86 (s, 3H), 3.82 (dd, $J = 3.6$, 2.5, 1H), 3.39 (dd, $J = 4.1$, 3.1, 1H), 1.25–1.70 (m, 20H); $^{31}$P NMR ($CDCl_3$) $\delta = -0.88$. MS (ESI) *m/z*: 994 $[M+Na]^+$.

**(±)-1-Bisfluorenylmethylphospho-2,3-*O*-cyclohexylidene-6-*O*-(*ortho*-nitroveratryl)-*myo*-inositol (5)**: **4** (972 mg, 1 mmol) and Dowex $H^+$ resin (300 mg) in methanol/dichloromethane (1:1, 15 mL) was stirred for 24 h, at room temperature. The reaction mixture was filtered and concentrated by rotary evaporation. The residue was purified by flash-column chromatography (ethyl acetate) to give **5** as a gum (305 mg) in a yield of 81% (based on 560 mg recovered starting material). $^1$H NMR (300 MHz, $CDCl_3$, RT, TMS) $\delta = 7.58$–7.79 (m, 6H), 7.52 (s, 1H), 7.16–7.42 (m, 11H), 5.01 (dd, $J = 29.9$, 4.7, 2H), 4.47 (dt, $J = 3.3$, 1.9,1H), 4.24–4.38 (m, 2H), 4.04–4.18 (m, 3H), 3.92–3.97 (m, 2H), 3.86 (s, 3H), 3.84, (s, 3H), 3.82–3.88 (m,

  

1H), 3.74 (t, $J=3.3$, 1H), 3.62 (t, $J=2.8$, 1H), 3.33 (t, $J=3.3$, 1H), 3.00 (brs, 2H), 1.4–1.8 (m, 10H); $^{31}$P NMR (121 MHz, $CDCl_3$) $\delta=-1.06$. MS (ESI) $m/z$ : 914 $[M+Na]^+$, 892 $[M+H]^+$.

**(±)-1,4,5-Tris[bisfluorenylmethylphospho]-2,3-*O*-cyclohexylidene-6-*O*-(*ortho*-nitroveratryl)-*myo*-inositol (6)**: Difluorenylmethyldiisopropyl phosphoramidate (525 mg, 1 mmol) and tetrazole (70 mg, 1 mmol) were added to a solution of **5** (330 mg, 0.37 mmol) in dichloromethane (10 mL), and the mixture was stirred at room temperature for 20 h. The reaction mixture was cooled to 4 °C, and *tert*-butyl hydrogen peroxide (0.4 mL, 40% aqueous solution) was added. After being stirred for 30 min at room temperature, the solution was diluted with dichloromethane (10 mL) and water (10 mL). The organic phase was separated, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation. The crude product was purified by flash chromatography (hexane/ethyl acetate, 1:1) to give **6** as a gum (305 mg) in a yield of 65% (based on 92 mg of recovered starting material). $^1$H NMR (300 MHz, $CDCl_3$, RT, TMS) $\delta=6.9$–7.8 (m, 50H), 5.02 (s, 2H), 4.35–4.62 (m, 5H), 4.22–4.34 (m, 5H), 3.76–4.18 (m, 10H), 3.68 (s, 3H), 3.67 (s, 3H), 3.52–3.62 (m, 3H), 3.08 (t, $J=2.9$, 1H), 1.4–1.7 (m, 10H); $^{31}$P NMR (121 MHz, $CDCl_3$) $\delta=-1.48$, $-0.73$, $-0.11$. MS (ESI) $m/z$: 1786 $[M+Na]^+$.

**(±)-6-*O*-(*ortho*-Nitroveratryl)-*myo*-inositol-1,4,5-trisphosphate (1)**: Piperidine (0.06 mL, 0.75 mmol) was added to a solution of **6** (176 mg, 0.1 mmol) in dichloromethane (5 mL), and the mixture was stirred at room temperature for 4 h. The reaction was concentrated under high vacuum, the residue was dissolved in dichloromethane/methanol (9:1, 4 mL), Dowex H+ (300 mg) was added until pH 2.0 was reached, and the reaction mixture was stirred for 24 h at room temperature. The resin was removed by filtration, washed with methanol, and the filtrate concentrated. The crude product was dissolved in water (5 mL) washed with dichloromethane (3×5 mL), and lyophilized. The product was purified by RP-HPLC by using a Hamilton PRP-1 column (21.5×250 mm) with a linear gradient from 0 to 6% acetonitrile/0.1% TFA, to give 33 mg of**1** as a gum (53%). $^1$H NMR (300 MHz, $D_2O$, RT, TMSPA) $\delta=7.78$ (s, 1H), 7.51 (s, 1H), 5.20 (s, 2H), 4.44 (dd, $J=6.2$, 3.0, 1H), 4.22–4.36 (m, 3H), 4.04 (t, $J=3.2$, 1H), 3.95 (s, 3H), 3.90 (s, 3H), 3.76 (dd, $J=3.7$, 0.9, 1H); $^{13}$C NMR (75 MHz, $CD_3OD$, RT, TMS) $\delta=155.0$, 148.4, 139.6, 132.6, 111.9, 108.3, 80.3, 80.0, 79.7, 78.2, 72.8, 72.4, 71.5, 57.0, 56.7; $^{31}$P NMR (121 MHz, $D_2O$, RT, $PO_4H_3$) $\delta=0.20$, 0.85, 1.90. MS (ESI) $m/z$: 614.3 $[M-H]^{+1}$, 317.4 $[M-3H+Na]^{+2}$, 306.5 $[M-2H]^{+2}$.

**Preparation of slices**: Wistar rats (12 to 23 days old) were anesthetized with halothane and killed by decapitation. Sagittal slices (300 μm thick) were prepared from the vermis of the cerebellum with a vibratome (Campden Instruments, Loughborough, UK). Slices were maintained at room temperature in the recording solution until use (1–8 h). Animals were prepared according to a protocol approved by the IACUC at Albert Einstein College of Medicine.

**Recording and analysis**: Slices were mounted in a chamber on the stage of an upright Zeiss (Oberkochen, Germany) microscope and visualized by using a 0.8 NA, 63× water immersion objective with infrared optics. Slices were superfused continuously with recording solution (125 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM $NaH_2PO_4$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, pH 7.4 when gassed with 5% $CO_2$/95% $O_2$) at a rate of 1.5 mL min$^{-1}$. The intracellular solution contained K gluconate (125 mM), HEPES (8 mM), $MgSO_4$ (3 mM), NaATP (3 mM) at pH 7.2. The internal solution also contained fura-6F (500 μM; Molecular Probes, Eugene, OR, USA) and caged $IP_3$ (200 μM). All experiments were performed at room temperature.

Excitation light and flash photolysis were produced by the arc of a xenon lamp and flashlamp, respectively (Cairn Research Ltd., Faversham, UK). Series resistance was in the range of 5–10 MΩ. Experiments were terminated if the holding current dropped below −200 pA. Data were sampled at 2 kHz by using the Cairn Optopatch amplifier (Cairn Research Ltd., Faversham, UK) which was controlled through a National Instruments interface (Austin, TX, USA) with custom-written software. The area viewed was restricted to the soma of the Purkinje cell by an adjustable rectangular diaphragm, and emitted light was detected by a photon-counting photomultiplier. Fura-6F was excited at 400–440 nm with emission at >480 nm. The dye has been previously calibrated in neurons and we used the same method and $K_d$ value for $Ca^{2+}$.[29] Cells were dialyzed for 30 min with the cage/dye mixture after entering whole-cell configuration.

**Cell cultures**:[24] HeLa cells were grown in minimal essential medium that was supplemented with mixed serum (5%; 50% newborn calf/50% fetal calf), glutamine (2 mM), penicillin (60 units per mL), and streptomycin (50 μg mL$^{-1}$). The cultures were incubated in a humidified atmosphere (5% $CO_2$, 95% air) at 37 °C.

**Uncaging and imaging**: Cells were viewed with a 40× oil-immersion objective lens (Fluor, 1.3 NA; Nikon) and fluo-3 was excited with the 488 nm line of an argon-ion laser (model 5000, Ion Laser Technology, Salt Lake City, USA) at 150 μW intensity on the cell. The fluorescence was detected at 540±30 nm with a confocal laser-scanning microscope (MRC 1000, Bio-Rad, Glattbrugg, Switzerland) and operated in the linescan mode. The beam of a mode-locked Ti:sapphire laser (Mira 900, Coherent, Santa Clara, CA) was tuned to 710 nm, at 80 MHz repetition rate, 70 fs pulse width, and was guided through the camera port of the confocal microscope to produce a stationary diffraction-limited spot within the cell. Photolysis was elicited by the opening of a voltage-operated shutter, which was synchronized to the pixel clock of the scanner to coerce synchronization with image acquisition. The power of the two-photon laser was attenuated with neutral-density and linear-polarization filters that were placed in series. The extracellular bath solution contained NaCl (121 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgCl_2$ (0.8 mM), $NaHCO_3$ (6 mM), HEPES (25 mM), glucose (5.5 mM), at pH 7.3 (adjusted with NaOH). The pipette-filling solution contained KCl (140 mM), HEPES (10 mM), EGTA (0.1 mM), $MgCl_2$ (1.0 mM), fluo-3 (0.1 mM; Teflabs, Austin, USA), and NPE-$IP_3$, (30 μM), [D-*myo*-inositol-1,4,5-triphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester, 3Na], Calbiochem, San Diego, USA) or 6-NV-$IP_3$ (30 μM) at pH 7.2 (adjusted with KOH). Prior to the whole-cell experiments, cells were loaded with fluo-3 by incubation with fluo-3 AM (1 μM; Teflabs) for 30 min. Cells were voltage clamped in the whole-cell configuration and held at −70 mV. Global uncaging of $IP_3$ was performed by using a xenon short-arc flash lamp (350 nm, duration 400 μs, discharged energy up to 230 mJ). For the two-photon photolysis of NPE-$IP_3$ (30 μM) or 6-NV-$IP_3$ (30 μM) the output from a Ti:sapphire laser (710 nm, 100 ms, 5 mW) was applied in a diffraction-limited spot within the cell and was parfocal with the plane of fluorescence detection. The excitation-point spread function was determined so that it extended over 880 nm (full width at half-maximal amplitude) in the *x*–*y* direction and 1200 nm in the *z* direction. The spot for local uncaging of $IP_3$ is marked by a white square in Figures 2A and 3A.

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