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Synthesis and characterization of cell-permeant 6-nitrodibenzofuranyl-caged IP₃[†]

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Abstract

We have synthesized in a 6-nitrodibenzofuranyl (NDBF) derivative of inositol-1,4,5-trisphosphate (IP₃) for efficient two-photon uncaging in living cells. As its hexakis acetoxymethyl ester, this caged compound may be applied at low concentration to the extracellular milieu to load the intact astrocytes in acutely isolated brain slices from the mouse cortex. Two-photon irradiation of single astrocytes evoked intracellular calcium signals that required 10% of the energy dosage compared to nitroveratyl (NV)-IP₃. Since NDBF-IP₃ has a 5-fold higher quantum yield than NV-IP₃, these data imply that photolysis of the new NDBF caged compound mobilized intracellular calcium about twice as efficiently as the NV cage.

Ionized calcium (Ca²⁺) is the single most important second messenger inside cells.¹ Fluctuations in the intracellular concentration of Ca²⁺ are involved in the birth, life and death of every cell type.² For example, at key moments in the cell cycle, Ca²⁺ dictates the timing of cell division. Important cell functions, such as excitation–secretion coupling, are regulated by Ca²⁺, with the exocytosis^{3,4} of molecules, such as insulin, glutamate and neurotrophins (to name a few), all being stimulated by increases in Ca²⁺. Ca²⁺ is also involved in the apoptotic events that end the cell's life. Thus, Ca²⁺ truly is multiple-functional, a “birth, life and death messenger”.² Because of its preeminence, the ability to monitor and control the concentration of intracellular calcium ([Ca²⁺]_i) has been a major focus of chemical biology for three decades.^{3,5}

Techniques to monitor [Ca²⁺]_i were developed in the 1980s.⁵ Roger Tsien and co-workers synthesized a series of fluorescent dyes that responded selectively to increases in [Ca²⁺]_i in three fundamentally different ways: (1) fura-2 showed changes in its absorption spectrum upon Ca²⁺ binding but not its fluorescence quantum yield;⁶ (2) indo-1 showed changes in its fluorescence emission maximum but not its absorption spectrum;⁶ and (3) fluo-3 showed changes in its fluorescence quantum yield.⁷ Many others have subsequently made variations on the themes defined by these small organic molecular probes.⁸ In 1997 a conceptually different approach to the measurement of [Ca²⁺]_i was developed by Tsien's lab, using GFP-based probes.⁹ The first such genetically encoded calcium indicator (GECI) involved Ca²⁺

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binding to calmodulin to change the fluorescence energy transfer (FRET) from a cyan fluorescent protein (CFP)-yellow (Y)FP pair. Again since the seminal contribution, a very wide range of GECI have been made by many laboratories using this FRET technique that allow measurement of Ca^{2+} in ways that are analogous to fura-2 and indo-1. GECI analogous to fluo- 3 have also been developed.¹⁰ Thus chemists and cell biologists have provided immensely powerful methods to monitor Ca^{2+} inside living cells using synthetic organic or protein-based probes.

The ability to control $[\text{Ca}^{2+}]_i$ photochemically has not received as much attention as the ability to monitor it, however such technology¹¹⁻¹³ is conceptually no less important.^{3,14} *A priori*, there are two ways for photocontrol of $[\text{Ca}^{2+}]_i$, either directly through “caged Ca^{2+} ” probes³ or through modulation of Ca^{2+} stores.^{15,16} It is the latter that is the focus of this report. We have previously developed a new caging chromophore, NDBF, which had a significantly higher two-photon cross section for photolysis than other widely used nitrobenzyl (*e.g.* the basic *o*-nitrobenzyl or its veratyl (NV) derivative) caging chromophores.¹⁷ In our initial application of the NDBF chromophore to caged Ca^{2+} (*i.e.* NDBF-EGTA) we found that the two-photon uncaging cross section of NDBF-caged ethers was 0.6 GM¹⁷ (NV-caged compounds are 0.01 GM^{18,19}). These data suggest that a 6-NDBF-derivative of IP_3 should also be more effective for two-photon uncaging since C–O bond scission is the uncaging reaction for NDBF-EGTA and compound **1**. It should be noted that the NDBF chromophore has been recently applied to the caging of nucleotides to be incorporated into DNA fragments^{20,21} and to uncaging iron *via* modulation of crown chelator affinity.²² Also, other caged inositols, apart from IP_3 , have been synthesized in the past: 3-NV- IP_4 ²³ and 6-(*ortho*-nitrobenzyl)- IP_6 .²⁴ However, no biological applications of these nitrobenzyl or NDBF compounds have been described. In this report we extend the utility of NDBF to caging inositol-1,4,5-trisphosphate (IP_3), the ubiquitous molecule used by cells for mobilizing Ca^{2+} from intracellular stores.^{1,2,25} We show that a derivative of NDBF caged- IP_3 may be applied at low concentration to brain slices acutely isolated from mice such that, when compared to a simpler nitrobenzyl-caged IP_3 compound previously synthesized,²⁶ significantly less photochemical energy is required to evoke increases in $[\text{Ca}^{2+}]_i$.

Synthesis of NDBF- IP_3 /AM

The synthesis of the NDBF-caged IP_3 (**1**) analog starts with dibenzofuran (**2**), see Fig. 1, which is known to have a distinct reactivity for acylation/bromination and nitration.²⁷ The former occurs at the 2-position, whereas the latter occurs at the 3-position. Initially, we coupled the known¹⁷ secondary bromide **3** to diol **4** to give alcohol **5** with high regioselectivity but low yield (5%). Since we have shown that photolysis of compound **5** proceeds cleanly,¹⁷ we attempted phosphorylation of this alcohol. However all attempts to effect this transformation failed, most probably due to the steric hindrance at the 1-position brought about by the presence of the methyl group. Therefore, we synthesized 2-bromomethyl-3-nitrodibenzofuran from the known 2-methyl derivative (**6**).²⁸ This material could be cleanly nitrated at the 3-position with NaNO_3 in TFA in 36–63% yield to compound **7**. Monobromination of **7** was achieved with *N*-bromosuccinimide under carefully controlled conditions to **8** in 45% yield (with 48% of starting material recovered).

Bromide **8** was coupled much more efficiently to diol **4**, *via* its tin ketal, than **3**, to give alcohol **9** in a yield of 34–46% ($n = 3$). This intermediate was phosphitylated with bisfluorenylmethyl-di-*iso*-propylphosphoramidate²⁹ to give the monophosphate **10** ($R_1 = \text{Fm}$) in a low yield of 14%. Selective deprotection¹⁸ of 4,5-cyclohexylidene moiety using Dowex- H^+ proceeded cleanly (crude yield 96%) to give diol **11**. However, unlike the equivalent NV analog¹⁸ of this material, **11** did not undergo efficient phosphitylation with bisfluorenylmethyl-di-*iso*-propylphosphoramidate, as the yield of the trisphosphate **12** was <1%. Thus we turned to the less bulky bis(2-cyanoethyl)-di-*iso*-propylphosphoramidate reagent to produce compound **10** ($R_1 = 2\text{-cyanoethyl}$) in 58–74% yield. All attempts at selective deprotection the 4,5-cyclohexylidene of **10** ($R_1 = 2\text{-cyanoethyl}$) failed, only tetrol **13** was isolated, suggesting that a more bulky substituent at the 1-position was required for such selectivity. The solution to this key synthetic transformation (deprotection of the 4,5-cyclohexylidene moiety) was found to be initial protection of alcohol **9** with camphoryl chloride^{16,26} to yield intermediate ester **14** in a yield of 80%. The ring conformation induced by the presence of the camphoryl group was such that brief treatment of **14** with acetyl chloride cleanly deprotected the 4,5-cyclohexylidene moiety to give diol **14** in 60–68% yield ($n = 3$). Treatment of **15** with NaOH produced the key synthetic intermediate triol **16** in high yield (90–100%, $n = 3$). A variety of phosphitylating reagents were used to attempt the conversion of **15** into a hexakisphosphate ester with a view to the production of trisphosphate of compound **1** (*i.e.* $R_3 = R_4 = \text{H}$). Only the bis(2-cyanoethyl)-di-*iso*-propylphosphoramidate reagent produced a trisphosphate derivative **17** in 42–44% yield ($n = 3$). Other reagents, such as bis(*tert*-butyl)-di-*iso*-propylphosphoramidate and bisfluorenylmethyl-di-*iso*-propylphosphoramidate, did not produce an equivalent clean transformation. Since the 2-cyanoethyl protecting group has been widely used in inositol phosphate syntheses,³⁰ we assumed it would be straightforward to remove all protecting groups of **17** to give 6-NDBF-IP₃. However, compound **17** could not be completely deprotected. At best, only “hemi” deprotection of **17** to **18** (NMR analysis of the reaction mixture showed three cyanoethyl groups) could be effected. Use of an extremely wide array of standard reagents for phosphate ester hydrolysis³¹ did not allow us to deprotect fully compound **17**. This intransigence was quite surprising to us, when one considered the analogous NV caged compounds have proved quite flexible in terms of the scope of conditions that can be used for the synthesis of IP₃ derivatives.^{16,18,23} We discovered that intermediate **17** could be cleanly transformed into compound **1** by treatment with an organic base for 24 h, followed by reprotection of the exposed phosphate anions with acetoxymethyl (AM)-bromide to give (presumably) intermediate **19**. Fortunately, the remaining cyanoethyl protecting groups of **19** could be exchanged for AM esters in subsequent deprotection–reprotection steps in the same reaction flask to give the target caged compound **1** in 35% from phosphate **17** (Fig. 1).

Photochemistry NDBF-inositol derivatives

We compared the photochemistry of NDBF- and NV-caged inositols and found that the quantum yield of photolysis of the former was five times higher than the latter. Solutions of triols in aqueous buffer were used for such measurements, since AM esters are not water soluble at high enough concentrations. Thus, compound **15** was photolyzed with near-U.V.

light in the presence of a photochemical inert standard. A solution of the equivalent NV-triol,¹⁸ which absorbed the same amount of light, was photolyzed under identical conditions. The initial rate of disappearance of the NDBF-triol was five-fold greater than the NV-triol ($n = 3$), implying the quantum yield of NDBF inositol photolysis was 0.5. This value is similar to that of NDBF-EGTA,¹⁷ this is to be expected, as it is an ether bond that is photocleaved in both uncaging reactions. Finally, it should be noted that the uncaging of NV-^{32,33} and NDBF-caged¹⁷ ethers is significantly faster than simple *ortho*-nitrobenzyl caged ethers.^{33,34} This enables relatively fast mobilisation of intracellular Ca^{2+} from the photolysis of NV-caged^{16,18} and NDBF-caged IP_3 derivatives (Fig. 2).

We also measured the efficacy of two-photon uncaging^{35,36} of NDBF- IP_3 in living cells. Solutions of compound **1** or 6-NV- IP_3/AM (50 μM) were applied to living brain slices acutely isolated from mouse cortex ($n = 3$ mice, ages were 10–12 days, one mouse were used just for NDBF and one just for NV, and one for both caged compounds, 10 brain slices in all). The caged compounds were co-loaded with a fluorescent calcium indicator as its AM ester (fluo-4). We compared the amount of energy from a mode-locked Ti:sapphire laser tuned to 720 nm, which was required to trigger robust intracellular calcium oscillations (efficacy is (power dose) $\times$ (power dose) $\times$ [cage]^{17,35}). The uncaging beam was directed into the cell body of an astrocyte in a 4×4 grid of 16 equally spaced, diffraction-limited points for 10 ms per point. In the case of the NDBF cage, two bursts of 25 mW irradiation elicited large increases in fluorescence (Fig. 2). The NV-loaded cells required four sets of uncaging pulses of 40 mW. The latter calcium transients were blocked by bath application of 2-APB (100 μM),³⁷ implying that IP_3 -receptors were involved in calcium mobilization. Note that we could measure the effects of 2-APB on the same cells but this cannot be accomplished for the two types of caged compounds. In contrast to extracellular caged probe application, when each probe can be applied to the same cell in succession,³⁸ the AM esters are de-esterified internally, so the caged compound is permanently trapped inside the cell. Thus direct comparison of NDBF- and NV-caged IP_3 inside the same cell is not technically possible. However we did load cells in different brain slices made from the same mouse with each caged compound under identical conditions. The results of these experiments are shown in Fig. 2. Since our comparative uncaging data was gathered from cells isolated from several mice, it can be considered to show significant differences in the relative efficacy of the NDBF- and NV-caged compounds and imply that NDBF caged- IP_3 requires about one tenth the photon dosage compared to the NV cage to produce robust release of Ca^{2+} from intracellular stores. This result is lower than that observed from comparative 2-photon uncaging of Ca^{2+} from NDBF-EGTA: Ca^{2+} and DM-nitrophen: Ca^{2+} complexes in a cuvette.¹⁷ The difference in these results probably reflects the differential membrane permeabilities of the NDBF and NV AM esters. In our previous studies we loaded water-soluble caged probes *via* the patch pipette,^{17,18} so the concentrations were defined to allow precise comparison of the correlation between photon flux and Ca^{2+} signal. In contrast, in the current work the use of cage AM esters precludes exact control of [cage]_i. Nevertheless, cells loaded with the NDBF-caged compound consistently required significantly less energy than their NV-loaded counterparts, implying that the NDBF chromophore undergoes more effective two-photon excitation in living cells in acutely isolated brain slices.

Experimental

Synthesis

3-Nitro-2-methyldibenzofuran (7)—To a solution of **6** (3.70 g, 20 mmol) in trifluoroacetic acid (40 mL) was cooled to 10 °C and NaNO₃ (1.70 g, mmol) was added. The reaction mixture was allowed to warm to RT, stirred for 1 h, then poured into crushed ice. The crude solid was filtered off and crystallized from ethanol to give **7** (2.85 g) in 63% yield. ¹H NMR δ (300 MHz, CDCl₃) 8.24 (s, 1H), 7.98 (ddd, J = 7.8, 1.3, 0.7 Hz, 1H), 7.87 (s, 1H), 7.62 (ddd, J = 8.4, 1.3, 0.7 Hz, 1H), 7.56 (ddd, J = 8.4, 6.9, 1.3 Hz, 1H), 7.40 (ddd, J = 7.8, 6.9, 1.3 Hz, 1H), 2.76 (s, 3H).

¹³C NMR δ (70 MHz, CDCl₃) 158.71, 153.95, 147.99, 129.76, 129.29, 128.99, 124.11, 124.08, 122.94, 122.11, 112.73, 109.26, 21.73.

HRMS C₁₃H₁₀NO₃ requires 227.0582, 228.0667 (M + H) found.

2-Bromomethyl-3-nitro-dibenzofuran (8)—To a solution of **7** (2.27 g, 10 mmol) in carbon tetrachloride (100 mL), *N*-bromosuccinimide was added (1.78 g, 10 mmol) and benzoyl peroxide (0.24 g, 1.0 mmol). The reaction mixture was heated at reflux temperature for 7 h, cooled then filtered and concentrated *in vacuo*. Flash chromatography (25% hexane in dichloromethane, *R*_f 0.45) gave **8** (1.08 g) in 35% yield.

¹H NMR δ (300 MHz, CDCl₃) 8.32 (s, 1H), 8.11 (s, 1H), 8.02 (ddd; J = 7.8, 1.4, 0.7 Hz; 1H), 7.65 (ddd; J = 8.3, 1.4, 0.7 Hz; 1H), 7.61 (ddd; J = 8.3, 6.8, 1.4 Hz; 1H), 7.54 (ddd; J = 7.8, 6.8, 1.4; 1H), 5.04 (s; 2H).

¹³C NMR δ (70 MHz, DMSO-*d*₆) 158.19, 153.61, 146.27, 134.28, 130.31, 128.83, 124.66, 123.10, 122.79, 121.07, 112.79, 109.16, 61.07.

Mass spectrum: **8** debrominated during ionization, so a parent molecular ion was not detected.

(±)-2,3-4,5-di-O-cyclohexyldene-6-O-(2-methylene-3-nitro-dibenzofuran)-myo-inositol (9)—A mixture of **4** (1.40 g, 4.11 mmol) and di-*n*-butyltin oxide (1.1 g, 4.2 mmol) in benzene (50 mL) was heated at reflux temperature for 7 h with azeotropic removal of water. The clear solution was concentrated *in vacuo*. This crude product was dissolved in DMF (20 mL) with **9** (1.29 g, 4.1 mmol) and CsF was added (0.76 g, 5.0 mmol). The reaction mixture was heated at 100 °C under nitrogen during which time it became almost clear light red solution. After 21 h the reaction mixture was concentrated *in vacuo*, then dissolved in dichloromethane and extracted with water, brine, dried over MgSO₄ and concentrated *in vacuo*. Flash chromatography (20% ethyl acetate in hexane, *R*_f 0.45) gave **9** (1.01 g) in 44% yield.

¹H NMR δ (300 MHz, CDCl₃) 8.32 (s, 1H), 8.11 (s, 1H), 8.02 (ddd; J = 7.8, 1.4, 0.7 Hz; 1H), 7.65 (ddd; J = 8.3, 1.4, 0.7 Hz; 1H), 7.61 (ddd; J = 8.3, 6.8, 1.4 Hz; 1H), 7.54 (ddd; J = 7.8, 6.8, 1.4; 1H), 5.04 (s; 2H).

^{13}C NMR δ (70 MHz, DMSO- d_6) 158.23, 153.97, 146.90, 130.45, 130.18, 128.74, 124.69, 122.97, 122.75, 122.07, 112.94, 112.20, 110.71, 109.49, 82.02, 78.14, 77.82, 76.74, 76.34, 71.81, 68.76, 40.11, 39.92, 35.13, 25.53, 25.36, 24.49, 24.27, 24.18.

HRMS $\text{C}_{31}\text{H}_{35}\text{NO}_9$ requires 565.2312, 588.2238 ($\text{M} + \text{Na}$) found.

($\pm$)-1-O-Camphanyl-2,3,4,5-di-O-cyclohexilydene-6-O-(2-methylene-3-nitro-dibenzofuran)-myo-inositol (14)—A solution of **9** (0.665 g, 1.17 mmol), triethylamine (12 mL), dimethylaminopyridine (0.15 g) and (1S)-(-)-camphanic chloride (0.26 g, 1.2 mmol) in dichloromethane (6 mL) was stirred at RT for 20 h. The reaction mixture was washed with water, dried over Na_2SO_4 , and concentrated *in vacuo*. Flash chromatography (1% ethyl ether in dichloromethane, R_f 0.45, 0.40) gave **14** (0.493 g) in 65% yield. The NMR spectra of the mixture of diastereomers are complex.

^1H NMR δ (300 MHz, CDCl_3) 8.38 & 8.36 (s, 1H), 8.35 & 8.34 (s, 1H), 8.12–8.06 (m, 1H), 7.64–7.56 (m, 2H), 7.48–7.41 (m, 1H), 5.42–5.21 (m, 3H), 4.68–4.61 (m, 1H), 4.37 (dd, $J = 8.9, 6.0$ Hz, 1H), 4.04–3.96 (m, 1H), 3.93–3.84 (m, 1H), 3.58 (dd, $J = 10.4, 9.1$ Hz, 1H), 2.74–2.32 (m, 1H), 2.02–1.85 (m, 2H), 1.78–1.35 (m, 20H), 1.09, 1.07, 1.01, 0.93, 0.89 five methyl singlets resolved.

^{13}C NMR δ (70 MHz, CDCl_3) 177.48, 166.50, 158.14, 153.75, 145.50, 129.45, 129.32, 129.27, 123.59, 122.37, 121.80, 120.30, 120.24, 113.26, 113.21, 112.00, 111.52, 111.47, 108.73, 90.69, 90.62, 79.25, 78.94, 78.10, 77.78, 77.45, 75.73, 74.27, 74.07, 73.29, 73.17, 69.55, 69.09, 54.71, 54.67, 54.38, 54.22, 37.20, 36.93, 36.39, 34.54, 34.27, 30.70, 30.55, 28.94, 28.88, 24.91, 23.36, 23.71, 23.68, 23.60, 23.47, 16.90, 16.71, 16.61, 16.58.

HRMS $\text{C}_{41}\text{H}_{48}\text{NO}_{12}$ requires 746.3177, 746.3208 (M^+) found.

($\pm$)-2,3-O-cyclohexilydene-6-O-(2-methylene-3-nitro-dibenzofuran)-myo-inositol (16)—A solution of **14** (0.156 g, 0.20 mmol) in methanol/dichloromethane (6 mL, 1 : 5) was cooled to 5–10 °C and acetyl chloride (0.050 mL) was added and the reaction mixture stirred for 15 min. The reaction was quenched with triethylamine (0.5 mL), diluted with dichloromethane (15 mL), washed with water, separated, dried over MgSO_4 and concentrated *in vacuo*. This crude product was dissolved in methanol/dichloromethane (5 mL, 1 : 1) and KOH (0.5 mL, 1 N) was added and the reaction mixture was stirred at RT for 4 h. The reaction was diluted with dichloromethane (20 mL), water (2 mL), separated, dried with MgSO_4 , and concentrated *in vacuo*. Flash chromatography (3% methanol in dichloromethane, R_f 0.3) gave **17** (0.066 g) in 68% yield.

^1H NMR δ (300 MHz, $\text{MeOH}-d_4$) 8.73 (s, 1H), 8.34 (s, 1H), 8.17 (ddd, $J = 7.9, 1.4, 0.7$, 1H), 7.65 (ddd, $J = 8.3, 1.4, 0.7$, 1H), 7.62 (ddd, $J = 8.3, 6.8, 1.4$ Hz, 1H), 7.46 (ddd, $J = 7.9, 6.8, 1.4$ Hz, 1H), 5.35 (s, 2H), 4.39 (dd, $J = 5.3, 4.2$ Hz, 1H), 4.001–3.95 (m, 2H), 3.72–3.63 (m, 2H), 3.41 (dd, $J = 9.8, 8.2$ Hz, 1H), 1.8–1.4 (m, 10H).

^{13}C NMR δ (70 MHz, $\text{MeOH}-d_4$) 146.09, 130.92, 129.34, 128.93, 123.73, 122.86, 121.90, 121.14, 111.84, 110.20, 108.06, 82.36, 78.92, 76.48, 75.72, 74.18, 71.10, 70.37, 37.94, 35.08, 25.18, 23.99, 23.66.

HRMS $C_{25}H_{27}NO_9$ requires 485.1686, 486.1753 ($M + H$) found.

(±)-2,3-O-Cyclohexyldene-6-O-(2-methylene-3-nitro-dibenzofuran)-myo-inositol 1,4,5-trisphosphate hexakis(2-cyanoethyl) ester (17)—To a solution of **16** (0.048 g, 0.10 mmol), *N,N*-diisopropyl-bis(2-cyanoethyl)-phosphoramidite (0.16 g, 0.5 mmol) in dichloromethane (4 mL) was added tetrazole (0.035 g, 0.5 mmol) in acetonitrile (1 mL) at RT. When the reaction mixture (RM) showed one spot on TLC (*ca.* 18 h), it was cooled to 5–10 °C, and *tert*-butyl hydrogenperoxide (0.25 mL of 30% solution in water) was added. The clear RM was allowed to warm to RT. After stirring for 3 h, the RM was diluted with dichloromethane (20 mL) and washed with water (10 mL), saturated $NaHCO_3$ (10 mL), and saturated NaCl solution (20 mL), dried with $MgSO_4$, and concentrated *in vacuo*. Flash chromatography (4% methanol in dichloromethane) gave **16** (0.045 g) in 46% yield.

1H NMR δ (300 MHz, $CDCl_3$) 8.42 (s, 1H), 8.33 (s, 1H), 8.14 (ddd, $J = 7.8, 1.3, 0.7$ Hz, 1H), 7.66 (ddd, $J = 8.3, 1.3, 0.7$ Hz, 1H), 7.60 (ddd, $J = 8.3, 6.9, 1.3$ Hz), 7.46 (ddd, $J = 7.8, 6.9, 1.4$ Hz, 1H), 5.31 (ABq, $J = 54.0, 13.3$ Hz, 2H), 4.94 (ddd, $J = 8.4, 6.6, 3.9$ Hz, 1H), 4.87 (dd, $J = 15.9, 8.2$ Hz, 1H), 4.73 (dd, $J = 8.8, 4.9$ Hz, 1H), 4.68 (ddd, $J = 7.8, 4.1, 1.2$ Hz, 1H), 4.44–4.22 (m, 14H), 2.87–2.67 (m, 12H), 1.81–1.45 (m, 10H).

^{13}C NMR δ (70 MHz, $CDCl_3$) 155.75, 149.56, 140.47, 131.12, 119.10, 119.01, 118.86, 118.68, 118.61, 113.24, 112.12, 109.54, 81.91, 79.88, 77.75, 76.51, 75.34, 72.46, 64.94, 64.84, 64.77, 64.66, 64.59, 64.53, 57.789, 57.43, 38.53, 36.18, 26.29, 25.70, 25.43, 20.91, 20.85, 20.81, 20.66, 20.55.

^{31}P NMR δ (121 MHz, $CDCl_3$) –1.7, –2.1, –2.2.

HRMS $C_{43}H_{49}N_7O_{18}P_3$ requires 1044.2347, 1044.2368 (M^+) found.

(±)-2,3-O-Cyclohexyldene-6-O-(2-methylene-3-nitro-dibenzofuran)-myo-inositol 1,4,5-trisphosphate hexakis(acetoxymethyl) ester (1)—To a solution of **17** (0.040 g, 0.038 mmol) in acetonitrile (2 mL) was added ethyldiisopropyl amine (0.5 mL), and the RM was stirred at RT. After 18 h, the RM was concentrated *in vacuo* and redissolved in dry acetonitrile (3 mL) to which was added ethyldiisopropyl amine (0.3 mL) and acetoxymethyl bromide (0.40 mL). The RM was stirred for 96 h at RT. Flash chromatography with ethyl acetate (100%) gave **1** (0.013 g) in 30% yield.

1H NMR δ (300 MHz, $MeOH-d_4$) 8.64 (s, 1H), 8.41 (s, 1H), 8.25 (brd, $J = 7.8$ Hz, 1H), 7.69 (brd, $J = 8.4$ Hz, 1H), 7.63 (ddd, $J = 8.4, 1.3, 0.7$ Hz, 1H), 7.47 (ddd, $J = 7.8, 6.9, 1.3$ Hz, 1H), 5.75–5.35 (m, 14H), 4.99 (td, $J = 8.6, 3.8$ Hz, 1H), 4.73–4.64 (m, 3H), 4.45 (t, $J = 7.0$ Hz, 1H), 4.28 (td, $J = 6.6, 2.6$ Hz, 1H), 2.14 (s, 3H), 2.13 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 1.95 (s, 3H), 1.93 (s, 3H), 1.88–1.40 (m, 10H).

^{31}P NMR δ (121 MHz, $MeOH-d_4$) –1.7, –2.1, –2.2.

HRMS $C_{43}H_{54}NO_{30}P_3$ requires 1157.1943, 1180.1792 ($M + Na$) found.

Quantum yield determination

The quantum yield of photolysis of NDBF-caged inositol was determined as previously described.¹⁸ A solution of compound **16** was dissolved in aqueous buffer (40 mM Hepes, 100 mM KCl, pH 7.2) at a concentration of 0.017 mM such that the OD in a cuvette of path length 1 mm was 0.25. Inosine (0.5 mM) was included in the solution as a photochemically inert standard. The solution was irradiated with the filtered output (350–400 nm) from a 500 W Hg arc lamp. The time course of photolysis was followed by RP HPLC using a linear gradient of acetonitrile (10–100%) over 30 min, with a flow rate of 1 mL min⁻¹ through an Alltech Altima C-18 column (4 × 250 mm). TFA (0.1%) was part of the eluents. The reaction mixture (20 µL) was injected thrice for each time point analyzed. The rate of photolysis of compound **16** was compared to that of its equivalent NV-triol (0.05 mM).

Two-photon uncaging in living cells

Brain slices were isolated from C57BL/6 mice as previously described.³⁸ All experiments were carried out under institutional IACUC guidelines. The caged compounds were applied to brain tissue by first dissolving 50 µg of cage and 25 µg of fluo-4/AM in 5 µL of 20% pluronic acid in DMSO. To this solution a small volume (0.1 mL) of artificial cerebral spine fluid (ACSF) was added. This solution was sonicated for 5 min to ensure the organic probes were completely solubilized. This solution was then carefully added to a chamber containing a brain slice covered with ACSF (0.9 mL) under an atmosphere of carbogen. The brain slice was loaded at RT for 60 min, and then placed in a large chamber of ACSF for a further 30 min. The brain slice was then transferred to a two-photon microscope and maintained under a flow of ACSF (2 mL min⁻¹), which was constantly bubbled with a stream of carbogen to ensure pH = 7.4 and sufficient oxygen for cell sustenance.³⁹

A Prairie Technologies Ultima dual galvanometer scan head on an Olympus BX61 microscope was used for two-photon uncaging microscopy. Cells were visually selected for photolysis by two-photon imaging at 820 nm with a Vision 2 laser (Coherent). The pixel dwell time was 4 µs, and each frame was 512 × 512 pixels. Uncaging was effected by directing a second laser (Mai Tai, Spectra-Physics) to 16 equally spaced points in a 4 × 4 lattice within a single cell body. Each point was irradiated for 10 ms at 720 nm with 25–40 mW through an Olympus 60x LUMPLFL60XW/IR2 objective lens (numerical aperture = 0.9). The time course of fluorescence was analyzed off-line using imageJ software as previously described.²⁶

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Fig. 1.

The synthesis of NDBF-caged IP₃. a) (Bu)₂SnO; b) TFA, NaNO₃; c) NBS; d) (iPr)₂P(OCH₂CH₂CN)₂ or (iPr)₂P(OFm)₂, tetrazole, then ^tBuOOH; e) (1S)-(-)-camphanic chloride, DMAP; f) acetyl chloride; h) NaOH; i) (iPr)₂NEt; j) AM-Br, (iPr)₂NEt.

Fig. 2.

Two-photon uncaging of IP₃ in astrocytes in living brain slices. The caged IP₃ probes (6-NDBF-IP₃, **1** or 6-NV-IP₃/AM) were co-loaded into cells with fluo-4/AM. (a,b) Fluorescent images of Ca²⁺-dye loaded astrocytes before and after photolysis of 6-NV-IP₃. (c) Time-course of fluorescence response in the absence (red) and presence (blue) of an IP₃-R inhibitor (2-APB) produced by photolysis of 6-NV-IP₃. (d,e) Fluorescent images of Ca²⁺-dye loaded astrocytes before and after photolysis of **1**. (f) Time-course of fluorescence response as a result of photolysis of **1**. Warmer colors in (a,b) and (d,e) imply higher concentrations of free Ca²⁺. Scale bar 10 microns.