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Photo-cleavable Analog of BAPTA for the Fast and Efficient Release of Ca^{2+}

Mariia V. Sutton, Matthew McKinley, Revathy Kulasekharan and Vladimir V. Popik[a*]

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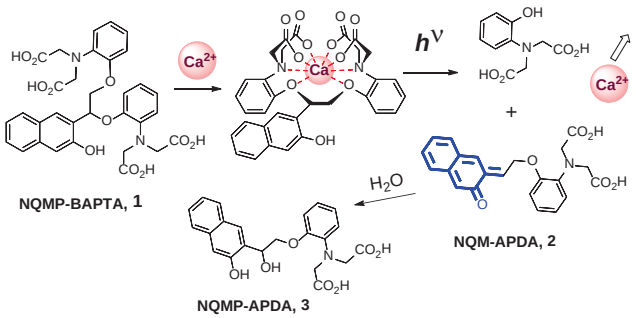
A new photocleavable analog of BAPTA chelating ligand has a high affinity towards Ca^{2+} ions (K= 2.5*10^6 M^{-1}). The use of photolabile 3-(hydroxymethyl)-2-naphthol core in the design of photo-BAPTA allows for the efficient (Φ=0. 63) and very fast (τ <12 μs) release of Ca^{2+} ions upon 300 or 350 nm irradiation.

Free calcium ions (Ca^{2+}) play an important role as a secondary messenger for a wide variety of regulatory functions in physiological and biological processes in cells.[1] The fluctuations of intracellular Ca^{2+} concentration regulate many cellular functions, such as neurotransmitter release,[2] operation of ion channels in the plasma membrane,[3] hormone secretion,[4] muscle contraction,[3] and many others. In recent years, the flash photolysis of photoresponsive calcium-ion chelators has become a common method in biochemical studies.[5] The light-triggered release of free Ca^{2+} allows for following the system response to a spike in calcium ion concentration.

The majority of photoresponsive calcium chelators are developed around polydentate ligands with high affinity to Ca^{2+}, such as ethylenediaminetetraacetic acid (EDTA),[6,7] ethylene glycol-*bis*(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA),[7,8] and 1,2-*bis*(*o*-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA).[9,10] For biomedical applications, BAPTA-based ligands are preferable since BAPTA offers the highest selectivity towards calcium ions (versus magnesium and other divalent cations).

Two general strategies are commonly employed in the design of photo-responsive calcium chelators. In the first approach, a photoreactive moiety conjugated to the amino group can change the availability of the nitrogen atom lone pair, thus modulating the calcium binding constant upon irradiation.[10] For example, the Tsien's design of the first photoresponsive calcium chelator was based on light-driven intramolecular oxidation of *p*-hydroxymethyl group of aniline moiety into a ketone.[9] Alternatively, a photolabile linker can be incorporated between two aminodicarboxylic acid fragments. The



Scheme 1.

calcium affinity of tridentate ligands, produced upon photochemical cleavage, is many orders of magnitude lower than that of the octadentate precursor.[8] The latter strategy for calcium photorelease is preferable to the former, as it permits a much higher overall change in Ca^{2+} affinity of a ligand. However, photo-cleavable analogues of calcium-selective BAPTA chelators have not been reported so far. Additional consideration in the design of photolabile chelator is the rate of ion release. The photochemical cleavage of *o*-nitrobenzyl or *o*-nitroveratryl ether linkers, that are commonly employed in photo-cleavable chelators,[8] is a relatively slow process. It involves several dark steps and might take tens of seconds to complete.[11]

Here, we report the synthesis, photochemical properties, and Ca^{2+} release kinetics of a novel photo-cleavable analogue of BAPTA (**1**, Scheme 1). 3-(Hydroxynaphth-2-yl)methyl ether servers as photolabile linker connecting two aminodicarboxyl "claws". Upon excitation, the former undergoes very fast (T~12 μs) scission of the benzylic ether bond to produce *o*-napthoquinone methide (o-**NQM**, structure highlighted in the intermediate **2**).[12] 3-(Hydroxynaphth-2-yl)methyl derivatives are, therefore, dubbed NQMPs for *o*-NaphthoQuinone Methide Precursors. In neutral aqueous solutions, o-**NQM**s undergo rapid (T~7 ms) hydration to produce 3-hydroxymethyl-2-haphthols (e.g., **3**, Scheme 1). NQMP derivatives show no dark- and photo-cytotoxicity below 100 μM.[13]

The synthesis of NQMP-BAPTA starts from commercially available methyl 3-hydroxy-2-naphthanoate (**4**, Scheme 2). The protection of

[a] *Department of Chemistry, University of Georgia, Athens, GA 30677, USA.*
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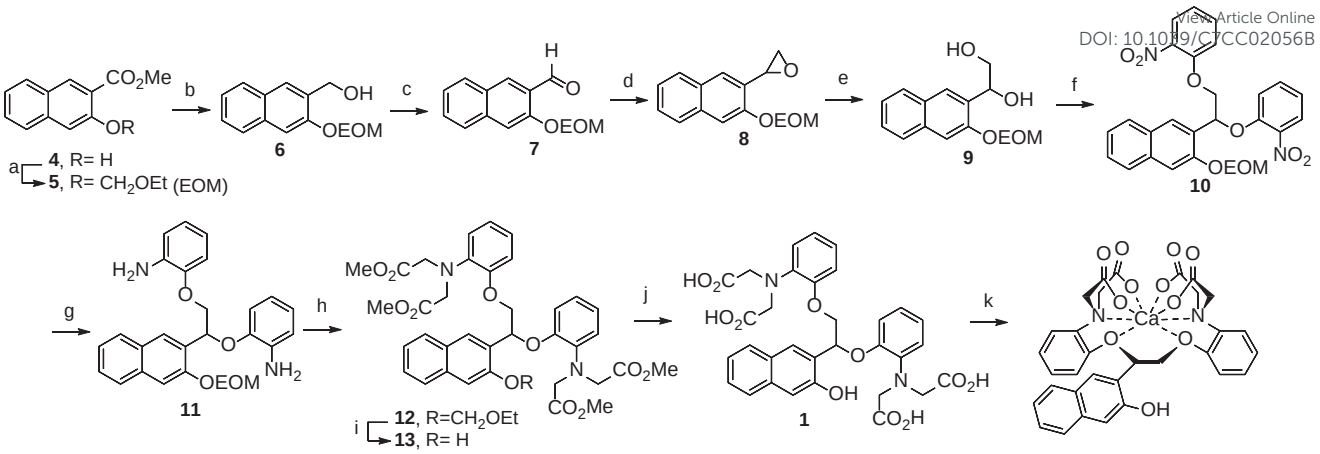


Scheme 2. Reagents and conditions: a) i) NaH, ii) chloromethyl ethyl ether, 99 %; b) $LiAlH_4$, 72 %; c) PCC, AcONa, molecular sieves, DCM, 89%; d) Trimethylsulfonium iodide, NaH, DMSO, THF, 99%; e) DMF-H_2O, 100 °C, 60%; f) 1-Fluoro-2-nitrobenzene , NaH, DMF, 120 °C, 78%; g) Zn, AcOH, MeOH, THF, 98%; h) Methyl bromoacetate, 1,8-bis(dimethylamino)naphthalene, NaI, 75%; i) Amberlyst-15(H), MeOH, 63%; k) KOH, MeOH, 1,4-dioxane, quant.; l) $CaCO_3$, HEPES/KCl.

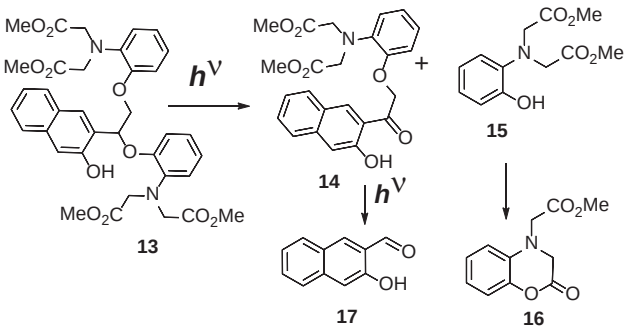
the phenolic hydroxyl with ethoxymethyl chloride (EOMCl), followed by LAH reduction of the ester produced 3-(hydroxymethyl)-2-naphthyl EOM ether (**6**).[14] The PCC oxidation of the latter gave naphthaldehyde **7**, which was converted to epoxide **8** under Corey-Chaykovsky conditions in an excellent yield.

Several attempts of the nucleophilic ring opening of epoxide **8** with 2-nitrophenolate produced only mediocre yields of *o*-nitrophenyl ether.[15] We, therefore, employed an alternative approach to the introduction of 2-nitrobenzyl moieties. First, the hydrolysis of **8** produced diol **9**, which, in turn, was treated with 2 equivalents of *o*-fluoronitrobenzene to give bis(*o*-nitrophenylether) **10**. The nitro groups of the latter were quantitatively reduced by zinc in acetic acid. The alkylation of amino groups in resulting *bis*-aniline **11** with methyl bromoacetate, followed by the deprotection of naphthol with Amberlyst-15(H), produced NQMP-BAPTA tetra-methyl ester (**13**). Finally, saponification of the latter gave a quantitative yield of the target NQMP-BAPTA chelator (**1**). Tetra-acid **1** in the neat form slowly decomposes on prolonged storage. Its potassium salt, however, is perfectly stable at room temperature.

The photochemical behavior of NQMP-BAPTA was first studied on the example of its tetra-ester **13**. Above 250 nm, the UV spectrum of **13** in 50% aqueous acetonitrile contains two pairs of close-lying bands of similar intensity: at 278 and 288 nm (log ε = 3.84 and 3.82), as well as at 320 and 332 nm (log ε = 3.25 and 3.33).[15] The irradiation of a solution of **13** in 50% aqueous acetonitrile with 300 nm fluorescent lamps resulted in the efficient cleavage of the starting material (Φ = 0.63±0.03). After 5 min of irradiation, no traces of tetra-ester **13** could be detected in the photolysate. HPLC analysis of the reaction mixtures at low conversion (90 s of irradiation), allowed us to identify two initial photoproducts: ketone **14** and *o*-dicarbmethoxymethylamino-phenol **15**. Phenol **15** is rather unstable and undergoes rapid acid-catalysed cyclization to lactone **16**. Exhaustive irradiation of the reaction mixture led to the formation of an additional photoproduct, 3-hydroxy-2-naphthaldehyde (**17**) (scheme 3).[15] The photoproducts **14**, **16**, and **17** were isolated in the preparative photolysis of **13** and fully characterized.[15] The identification of **16** required the independent preparation,[15] as spectroscopic data corresponding to the structure of the lactone was previously assigned to a lactam isomer of **16**.[16] Phenol **15** was also prepared independently, however, it always contains impurities of lactone **16**; and on silica gel, it forms lactone **16** quantitatively.

Based our previous experience with NQMP derivatives, we believe that the initial photo-cleavage of **13** occurs through the excited state scission of the benzylic C-O bond. This results in the formation of phenol **15** and *o*-aminophenoxymethene-N,N-diaacetic acid derivative of *o*-naphthoquinone methide (**NQM-APDA**, **2**, Scheme 1). The latter then adds water to form **NQMP-APDA** (**3**, Scheme 1). The isolated ketone **13** (Scheme 3), is an apparent product of the oxidation of the benzylic hydroxy group in **3** by atmospheric oxygen. Second primary product, phenol **15**, apparently undergoes intramolecular trans-esterification to produce lactone **16**. The detailed investigation of the mechanism of NQMP-BAPTA tetra-ester photo-transformations is underway in our laboratory.

The Ca^{2+} affinity of NQMP-BAPTA (**1**) was determined by spectrophotometric titration at pH=7.4. The UV spectrum of buffered solution (HEPES, pH= 7.4) of the tetra-potassium salt of **1** is



Scheme 3. Conditions: 300 nm, CH_3CN/water, 1:1.

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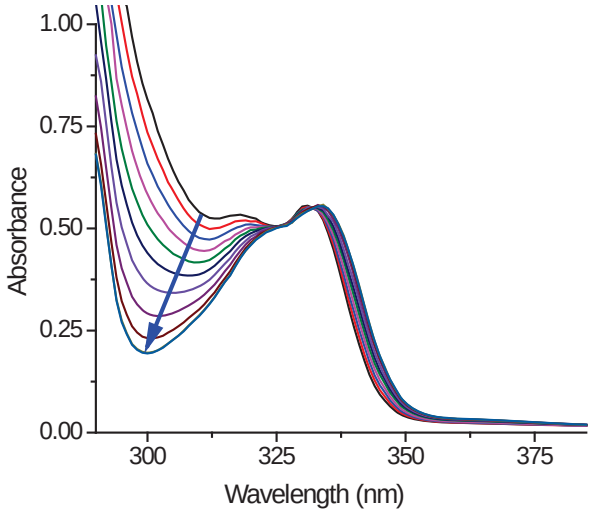

Figure 1. UV spectra of 0.2 mM aqueous solution of potassium salt of **1** at pH =7.4 at variable Ca^{2+} concentrations.

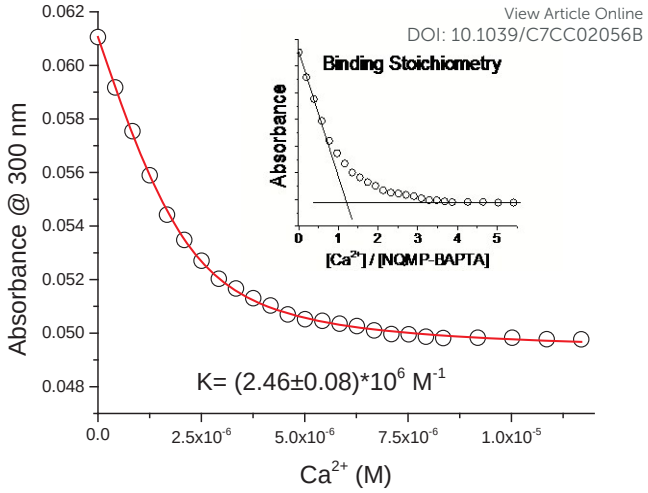

Figure 2. Spectrophotometric titration of 2.16 μM aqueous solution of NQMP-BAPTA with 0.25 mM of $CaCl_2$.

similar to that of ester **13**, only blue shifted by ~2 nm: bands at 276, 286, and 331 nm (nm (log ε = 3.86, 3.77, and 3.26) are observed.[15] The band with λ_{max}= 318 nm blends with broad absorbance around 300 nm. Upon the increase in Ca^{2+} ion concentration, absorbance around 300 nm is progressively reduced, and 331 nm band suffers red shift (Figure 1). These changes saturate at higher Ca^{2+} concentrations. The final UV spectrum Ca^{2+}:NQMP-BAPTA complex contains three absorbance bands above 250 nm: 269 (log ε = 3.80), 277 nm (log ε = 3.84), and 333 nm (log ε = 3.39, Figure 1.)[15]

Due to the high calcium affinity of NQMP-BAPTA, the titration of **1** was conducted at 2.16 μM concentration of the substrate. The buffered solution of potassium salt of **1** (HEPES, pH= 7.4) was titrated with 0.25 mM of $CaCl_2$ following the changes in absorbance at 300 nm (Fig. 2).

The absorbance of the NQMP-BAPTA solution at 300 nm kept decreasing with successive addition of $CaCl_2$ until it levels off above 10 μM Ca^{2+} concertation (Figure 2). The calcium ion binding constant of NQMP-BAPTA was calculated using eq. 1:

Eq. 1

$$[LM] = \frac{([L]_0 + [M]_0 + \frac{1}{K})}{2} + \sqrt{\frac{([L]_0 + [M]_0 + \frac{1}{K})^2}{4} - [L]_0[M]_0}$$

Where $[LM]$ is the equilibrium concentration of NQMP-BAPTA (**1**) – Ca^{2+} complex, $[L]_0$ and $[M]_0$ are stoichiometric concentrations of ligand **1** and Ca^{2+}, K is a binding constant. We assumed that the difference in absorbance of the solution of **1** at $[Ca^{2+}] = 0$ (A_0) and at $[Ca^{2+}] > 10$ μM (A_∞) is mostly due to the differences in the extinction coefficients between the free and fully complexed ligand. The changes of the absorbance at 300 nm in the course of the titration is, therefore, represented by the equation 2.

$$A = A_0 - \frac{[LM]}{[L]_0}(A_\infty - A_0) \qquad \textbf{Eq. 2}$$

Fitting of the experimental data to the equations 1 and 2 allowed us to calculate K_{bnd}= (2.46±0.08)x10^6 M^{-1} (at pH= 7.4 and ionic strength of 0.1 M). This value is similar to the parent BAPTA ligand.[10a] The midpoint of titration, determined from the absorbance values vs. $[Ca^{2+}]$/[**1**] ratio plot, indicates 1:1 binding stoichiometry (Insert in Figure 2).

The release of calcium ions from NQMP-BAPTA:Ca^{2+} complex under irradiation was explored with the help of Calcein indicator. The binding constant of the latter is 3-4 orders of magnitude lower than that of NQMP-BAPTA,[17] thus calcein only forms the complex with free Ca^{2+} ions in solution. Since the response of this indicator is known to depend on the media, we have built the calibration curve under the conditions of irradiation.[15] An aqueous solution (HEPES buffer, pH= 7.4, IS = 0.1 M) containing 1.13 mM of NQMP-BAPTA and 1.25 mM of $CaCl_2$ were irradiated with 300 nm fluorescent lamps. The 3.5 mL samples were exposed to UV light for various duration of time, from 30 s to 8 min, and a solution of Calcein was added to each sample to achieve 0.7 nm concentration of the indicator. The concentration of free calcium in solution was calculated from the observed fluorescence intensity at 513 nm. As Figure 3 illustrates, the photorelease of calcium from NQMP-BAPTA occurs in a dose-dependent manner and is essentially complete after 6 min of irradiation. The increase of calcium concentration

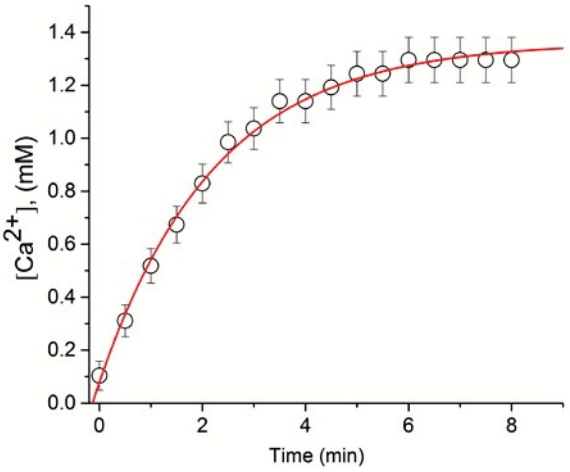

Figure 3. Photo-release of Ca^{2+} from the NQMP-BAPTA (**1**) - Ca^{2+} complex under 300 nm irradiation.

under given conditions can be approximated by a first-order rate law to give the apparent rate constant of $(7.4\pm0.3)*10^{-3}$ s^{-1}. It is important to note that Ca^{2+} is released quantitatively. This observation also indicates that the fragmentation of NQMP-BAPTA results in at least five orders of magnitude loss of calcium ion affinity (well below K_{bnd} of calcein).

In summary, we have developed the first photo-cleavable analog of BAPTA Ca^{2+}chelator, NQMP-BAPTA (**1**), by incorporating photo-labile 3-(hydroxymethyl)-2-naphthol linker in the structure. NQMP-BAPTA possess very high calcium affinity (K_{bnd}= $2.46*10^6$ M^{-1}), but upon irradiation, **1** looses one of its tridentate chelating arms resulting in the efficient calcium ion release. The high quantum yield (Φ = 0.63) of the NQMP-BAPTA cleavage makes it one of the most efficient of photosensitive calcium-ion chelators. [7,18] The NQMP linker photo-cleavage occurs on the microsecond time scale making NQMP-BAPTA suitable for time-resolved experiments.

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