Photoactivatable Compounds

(Coumarin-4-yl)methyl Esters as Highly Efficient, Ultrafast Phototriggers for Protons and Their Application to Acidifying Membrane Surfaces**

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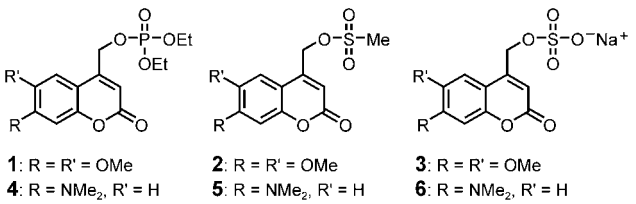
Protons play a crucial role in cellular signal transduction. They trigger protein conformational transitions and are coupling intermediates in electron transport phosphorylation, and their transmembrane gradients may serve as energy sources or stores. Kinetic studies of all these processes may be aided by photoactivatable proton precursors for the generation of rapid pH jumps. With these proton sources ("caged protons"), the spatial and temporal pH distribution can be controlled without diffusional mixing delays.[1,2] Most of the precursors are nitrobenzyl derivatives, namely 2-nitrobenzaldehydes,[3–5] as well as 2-nitrobenzyl and/or 1-(2-nitrophenyl)ethyl acetates,[1] phosphates,[6–8] tosylates,[9,10] and sulfates.[7,11] Their application is limited by the low efficiency of the activation in the long-wavelength UV/Vis range and the generation of reactive *o*-nitrosocarbonyl photoproducts.

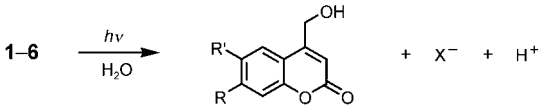
Here we introduce (6,7-dimethoxycoumarin-4-yl)methyl (DMCM) diethyl phosphate (**1**), DMCM methanesulfonate (**2**), and sodium DMCM sulfate (**3**), as well as [7-(dimethylamino)coumarin-4-yl]methyl (DMACM) diethyl phosphate (**4**), DMACM methanesulfonate (**5**), and sodium DMACM sulfate (**6**) as a new class of phototriggers for protons that facilitates the study of proton-dependent biological processes (Scheme 1). Compound **1**, for example, has already been used to study H^+ migration along lipid bilayers.[12]



1: R = R' = OMe **2**: R = R' = OMe **3**: R = R' = OMe
4: R = NMe_2, R' = H **5**: R = NMe_2, R' = H **6**: R = NMe_2, R' = H

Scheme 1. Structures of the phototriggers **1**–**6**.

Photocleavage of **1**–**6** is efficient and clean. It yields H^+, the respective anion, and the strongly fluorescent **7** or **8** (Scheme 2). The esters themselves are only very weakly



7: R = R' = OMe
8: R = NMe_2, R' = H

X^-: $OPO(OEt)_2^-$, OSO_2Me^-, OSO_3^{2-}

Scheme 2. Photolysis of **1**–**6**.

fluorescent as indicated by their particularly small fluorescence quantum yields (Table 1). Both the DMCM and the DMACM moieties have been introduced earlier for the photoreversible inactivation of cyclic nucleotide monophosphates.[13,14] The analogues (7-methoxycoumarin-4-yl)methyl (MCM) diethyl phosphate and methanesulfonate have been described previously,[15,16] but they were not classified or used as phototriggers for protons.

Table 1: Properties of **1**–**6**.[a]

Phototrigger	λ_{abs}^{max} [nm]	ε^{max} [M^{-1} cm^{-1}]	φ	λ_f^{max} [nm]	φ_f	c_S [μM]
1[b]	346	11400	0.08	447	0.005	>10000
2[c]	348	10700	0.23	437	0.002	40[b]
3[d]	345	11000	0.09	445	0.007	>10000
4[b]	386	16100	0.36	497	0.006	3700
5[c]	388	17100	0.79	487	0.002	100[b]
6[d]	382	14700	0.46	506	0.008	>10000

[a] Long-wavelength absorption maxima λ_{abs}^{max}, extinction coefficient ε^{max}, photochemical quantum yield φ, fluorescence maxima λ_f^{max}, fluorescence quantum yield φ_f, and concentration at saturation c_S. [b] In acetonitrile/HEPES (5:95), pH 7.2. [c] In acetonitrile/HEPES (20:80), pH 7.2. [d] In HEPES, pH 7.2.

Compounds **1**, **2**, **4**, and **5** were synthesized by esterification of the alcohols **7** and **8** with diethyl phosphoric acid chloride and methanesulfonic acid chloride, respectively, in CH_2Cl_2 in the presence of pyridine or triethylamine at 0°C with yields of 53–68%. The photoactivatable sulfates **3** and **6** were prepared by treatment of **7** and **8** with the triethylamine/SO_3 complex in dimethylformamide using the general method of Tserng and Klein.[17,11] The yields were 77% for **3** and only 16% for **6** (see the Supporting Information for preparative details and analytical characterization).

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The absorption maxima of the DMACM esters **4**–**6** are more intense and red-shifted by 40 nm relative to those of the DMCM derivatives **1**–**3** at about 346 nm. Thus, efficient photolysis of the DMCM esters occurs at 330–365 nm and of the DMACM esters at 365–420 nm. Conceivably, the photocleavage proceeds in analogy to the conversion of MCM esters,[16] by means of an S_N1 mechanism, including singlet-state activation, ion-pair formation, ion-pair separation, and subsequent hydroxylation. Because the reaction quantum yields should correlate with both the electron-donor strength of the substituent in position 7 of the coumarin chromophore (stabilization of the intermediate coumarinylmethyl carbocation) and the leaving-group ability, the reaction quantum yields were expected to be higher for the DMACM esters than for the DMCM esters and to increase in the order diethyl phosphates ≈ sulfates < methanesulfonates. The measured photochemical quantum yields (φ) for proton photorelease confirmed these predictions (Table 1).

The high quantum yield of **5** is unique for photoactivatable compounds with coumarinylmethyl moieties. The high quantum yield combined with the high extinction coefficient results in an unparalleled photosensitivity. By using φ multiplied by the molar absorptivity (ε) as a criterion for the overall sensitivity of photocleavage,[1] the DMACM compounds **4**–**6** were confirmed to be the most sensitive phototriggers described so far. The $\varphi\varepsilon^{max}$ values of **1**–**3** (900–2400 $M^{-1}cm^{-1}$) and of **4**–**6** (5800–13500 $M^{-1}cm^{-1}$) are very high. To illustrate the excellent photochemical properties of the coumarinylmethyl esters, H^+ photorelease from **6** was compared with that from the commercially available 2-hydroxyphenyl 1-(2-nitrophenyl)ethyl phosphate (NPE-HPP) (Figure 1).

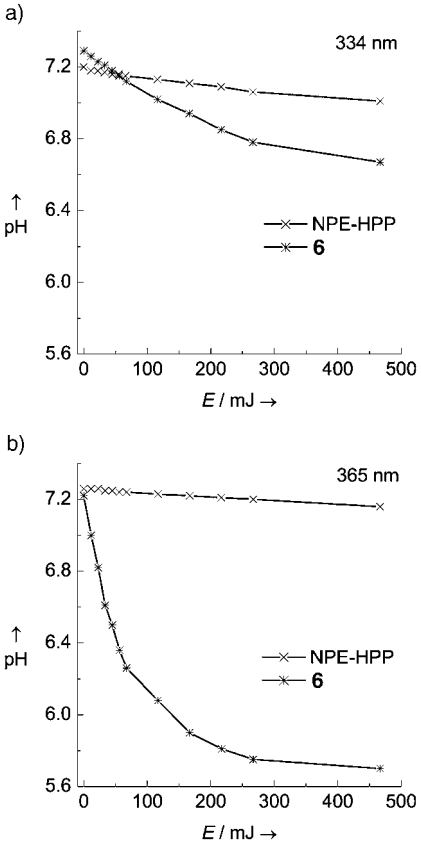

Figure 1. Comparison of the photosensitivity of H^+ release from 25 μM **6** and 25 μM NPE-HPP in aqueous solution (pH 7.2; reaction volume: 2.8 mL) a) at 334 nm, b) at 365 nm. E = energy of irradiation.

Exposure of **1** and **6** to femtosecond pulses of a mode-locked Ti:sapphire laser at 728 and 750 nm, respectively, caused a significant decrease of the fluorescence intensity of fluorescein isothiocyanate (FITC) dextran, indicating that photocleavage was possible with two-photon excitation as well (Figure 2). Sensitivity to two-photon photolysis has been described recently for photoactivatable (6-bromo-7-hydroxycoumarin-4-yl)methyl compounds.[18]

Proton sources relying on the 2-nitrobenzyl rearrangement provide H^+ photorelease with rate constants of $2\times10^7\ s^{-1}$ and $>4\times10^7\ s^{-1}$ for unbuffered and buffered solutions, respectively.[19] The photolysis pathway of the coumarinylmethyl ester cleavage suggests reaction rates that are significantly higher. To check the hypothesis, we conducted time-resolved energy-dependent fluorescence measurements. Deconvolution of the experimentally determined decay curves of high-intensity single-pulse excitations (0.5 ns half-width) of **1**, **3**, **4**, and **6** revealed fluorescence contributions from the corresponding coumarinylmethyl alcohols **7** or **8.** Since both **7** and **8** were released within the duration of the excitation pulse, photolytic H^+ formation occurred with a rate constant of at least $5\times10^8\ s^{-1}$, that is, H^+ was liberated within two nanoseconds or even faster.

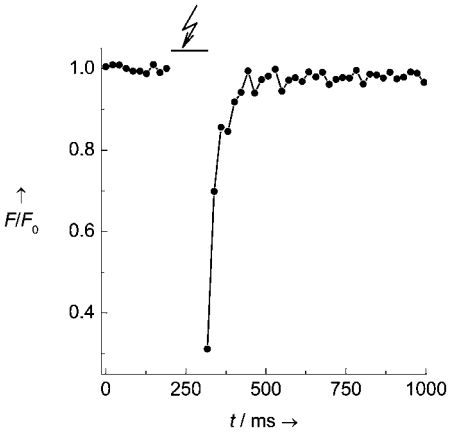

Figure 2. Photorelease of H^+ from **6** upon two-photon excitation. A solution of **6** (500 μM) was mixed with the pH-sensitive fluorescence indicator FITC dextran (750 nM) in CAPSO buffer solution (pH 9.0) and irradiated for 106 ms (black bar; CAPSO = 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid, sodium salt). The fluorescence intensity F of FITC dextran was recorded before and after irradiation. The fluorescence recovery was due to proton diffusion out of the irradiated area. F/F_0 = relative fluorescence intensity.

The pK_a values of the corresponding acids of the sulfate (1.98),[20] diethyl phosphate (0.71),[21] and methanesulfonate group (−1.54)[22] suggest that **1**–**3** are potent H^+ sources in solutions down to pH 2 or 1. Acidifications by photolysis of compounds **4**–**6** are somewhat restricted by the buffering capacity of the dimethylamino group in **4**–**6** or in the released alcohol **8** ($pK_a = 2.0$), but the photolytic pH drop is not

notably diminished as long as pH > 2. Large pH jumps may well be achieved with **1**–**6**. Thus, an aqueous 200 μM solution of **6** at pH 7.0 gave pH changes of three units in representative H^+ photorelease experiments (data not shown).

Despite their excellent photocharacteristics, **2** and **5** are less useful proton sources because their half-life in aqueous buffers (pH 7.2) is only 26.8 and 28.6 h, respectively. In contrast, within 24 h less than 0.5% of **1**, **3**, **4**, and **6** was hydrolyzed in 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (pH 7.2) as indicated by HPLC. Since **3** and **6** are easily soluble in HEPES buffer and both **1** and **4** exhibit reasonable solubility in acetonitrile/HEPES (5:95; Table 1), their application for biophysical studies seems promising.

We have probed membrane partitioning of phototriggers **1**, **3**, **4**, and **6** by isothermal titration calorimetry (details see Supporting Information). Figure 3 shows dilution-corrected

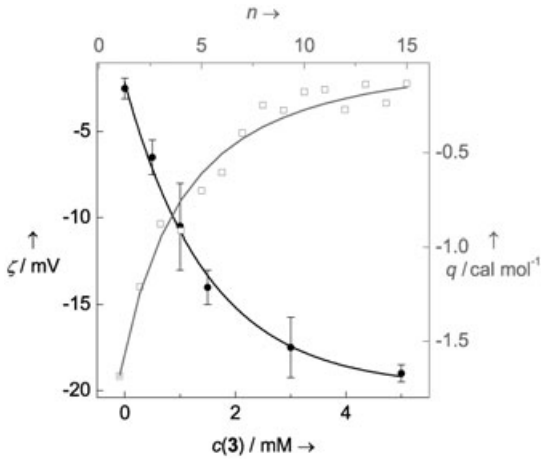

Figure 3. Membrane adsorption of the phototriggers. By titration of 20 μM **1** with 20 μL aliquots of 40 mM DPhPC (100 mM NaCl, 10 mM CAPSO, pH 9.0, 25 °C) the heats of reaction (*q*, squares) could be measured, normalized with respect to the molar amount of injectant, and plotted versus the injection number *n*. The best fit (gray line) yielded $\Delta H \approx -1$ kcal mol^{-1}, $\Delta G^0 \approx -6$ kcal mol^{-1}, and $\Delta S^0 \approx 16$ cal mol^{-1} K^{-1}. For **3** and **6** (not shown): $\Delta H \approx -2$ kcal mol^{-1}, $\Delta G^0 \approx -5$ kcal mol^{-1}, and $\Delta S^0 \approx 10$ cal mol^{-1} K^{-1}. Adsorption of **3** increased the electrophoretic mobility (monitored by a zeta-sizer, model DELSA 440 SX, Coulter Electronics, USA) of large unilamellar DPhPC vesicles (suspended in 20 mM NaCl, 10 mM HEPES, pH 7.1, 23 °C), which was used to calculate their ζ-potentials (circles with standard deviations).

data for compound **1**, from which the adsorption constant[23] was obtained. For **1** and **4**, $K = 300 \pm 150\,\text{M}^{-1}$, whereas the two ionic species **3** and **6** bound somewhat less strongly with $K = 100 \pm 50\,\text{M}^{-1}$. The calorimetric data are consistent with measurements of electrophoretic mobility, which revealed roughly the same adsorption constants (Figure 3). The charge brought by adsorption of **3** to diphytanoyl-phosphatidylcholine (DPhPC) vesicles was monitored and used to calculate the electrical potential (ζ) at the shear plane of the vesicles.[24,25] With increasing concentrations of **3,** the ζ-potential saturated at −20 mV (in 20 mM NaCl), indicating that the binding site for one molecule of **3** is at least 20 lipid molecules in size.

Proton release from membrane-anchored coumarin derivatives is an excellent tool to study the kinetics of acid-sensing membrane channels or other processes taking place at the membrane–water interface, for example, proton migration along the membrane surface.[12] To quantify membrane surface acidification, horizontal planar lipid bilayers containing fluorescein-labeled lipids were placed on the top of an inverse fluorescence microscope.[12] After membrane exposure to a UV flash, the response of the pH-sensitive dye (*N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) was monitored from one leaflet or both leaflets, depending on whether the membrane was permeable for the caged compound.

Due to the high energetic barrier for membrane diffusion of charged compounds, photolysis of **6** mediated a rapid fluorescence decrease of fluorescein in one membrane leaflet only (Figure 4, α). Subsequent proton diffusion out of the

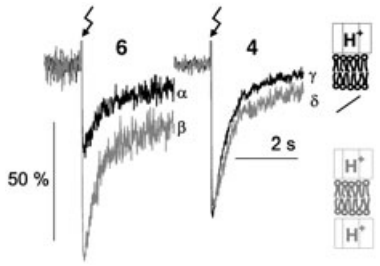

Figure 4. Sidedness of proton release upon flash photolysis. Addition of **6** (α) and **4** (γ) exclusively to the upper membrane leaflet induced a rapid drop in fluorescence (from F_0 to F) of the membrane-anchored pH-sensitive dye. In contrast to **4** (δ), **6** doubled the fluorescence drop (β) when added also to the compartment facing the lower membrane leaflet. The difference indicates that the membrane-impermeant compound **6** can be used to generate a transmembrane pH gradient, whereas the membrane-permeant compound **4** always acidifies both membrane leaflets similarly. After an initial acidification, fluorescence recovery was observed due to buffer or proton diffusion. The buffer solution contained 0.1 mM CAPSO, 100 mM NaCl, pH 9.0. Membranes were formed from a mixture of DPhPC and the fluorescent pH-sensitive dye (mass ratio 95:5) dissolved in *n*-decane. Fluorescence was collected from the central membrane region used for proton release by a UV flash.

region irradiated led to fluorescence recovery. Addition of **6** to the aqueous solutions on both sides of the membrane doubled the fluorescence drop because both interfaces were acidified (Figure 4, β). In contrast to the charged compounds **3** and **6**, we expected the hydrophobic diethyl phosphates **1** and **4** to be membrane-permeant. Consistently, addition of **4** to one or both compartments revealed the same pH drop (Figure 4, γ and δ).

The experiments in Figure 4 imply that the caged compound located in the aqueous phase contributes only negligibly to the interfacial acidification. This hypothesis was confirmed: the shift induced by **3** vanished when c_i (interfacial concentration) was diminished from ≈0.3 to about 0.04 mM while the bulk aqueous concentration was held constant (0.3 mM). To this end, a charged membrane was formed containing 50 mol% negatively charged lipids (diphytanoyl-phosphatidylserine, DPhPS). The Boltzmann distribution[26]

predicts that the bulk concentration must be augmented to 2 mM to compensate for the electrostatic repulsion caused by the surface potential of −50 mV and to reestablish the initial c_i, which is in accord with our experimental observation (Figure 5). The same considerations hold for the sulfate **6**, which, due to the higher quantum yield, produced larger pH responses at the same concentration (Figure 5).

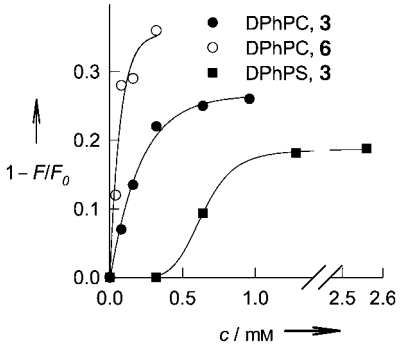

Figure 5. The increase in the H^+ concentration on the bilayer surface results exclusively from photolysis of phototriggers bound to the membrane. The relative fluorescence drop measured for negatively charged DPhPS bilayers (squares) was smaller than that for electroneutral DPhPC membranes (circles) due to electrostatic repulsion of the negatively charged **3** (filled symbols). As a result of the higher quantum yield, the efficiency of acidification mediated by **6** (open symbols) was higher. For conditions and a definition of F and F_0 see Figure 4.

Thus, coumarinylmethyl diethyl phosphates and coumarinylmethyl sulfates act as very sensitive and ultrafast phototriggers for protons, which can induce large pH jumps. Their membrane affinity can be exploited for investigation of the impact of membrane surface pH on signaling, folding, and transport. Leaflet specificity is achieved by choosing membrane-permeant (**1**, **4**) or membrane-impermeant (**3**, **6**) compounds. Moreover, the proton precursors **1**, **3**, **4**, and **6** will extend decisively the tools available for the study of spatial- and time-dependent aspects of other H^+-triggered processes. Their biological applications are aided by long-wavelength UV/Vis-activation, which should minimize cell-damaging side effects.

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Keywords: membranes · photoactivatable compounds · photolysis · protecting groups · protons

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