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 fates.[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 α-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) diethyl phosphate (4), DMACM methanesulfonate (5), and sodium DMACM sulfate (6) as a new class of phototrigger 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]

**Photoactivatable Compounds**

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>![Structure 1]</td>
</tr>
<tr>
<td>2</td>
<td>![Structure 2]</td>
</tr>
<tr>
<td>3</td>
<td>![Structure 3]</td>
</tr>
<tr>
<td>4</td>
<td>![Structure 4]</td>
</tr>
<tr>
<td>5</td>
<td>![Structure 5]</td>
</tr>
<tr>
<td>6</td>
<td>![Structure 6]</td>
</tr>
</tbody>
</table>

**Scheme 2. Photolysis of 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 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]**

<table>
<thead>
<tr>
<th>Phototrigger</th>
<th>λ_{max} [nm]</th>
<th>ε_m [M⁻¹ cm⁻¹]</th>
<th>ψ</th>
<th>λ_f{max} [nm]</th>
<th>ψ_f</th>
<th>ε</th>
<th>[μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>346</td>
<td>11400</td>
<td>0.08</td>
<td>447</td>
<td>0.005</td>
<td>&gt;10000</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>348</td>
<td>10700</td>
<td>0.23</td>
<td>437</td>
<td>0.002</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>345</td>
<td>11000</td>
<td>0.09</td>
<td>445</td>
<td>0.007</td>
<td>&gt;10000</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>386</td>
<td>16100</td>
<td>0.16</td>
<td>497</td>
<td>0.006</td>
<td>3700</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>388</td>
<td>17100</td>
<td>0.79</td>
<td>487</td>
<td>0.002</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>382</td>
<td>14700</td>
<td>0.46</td>
<td>506</td>
<td>0.008</td>
<td>&gt;10000</td>
<td></td>
</tr>
</tbody>
</table>

[a] Long-wavelength absorption maxima λ_{abs}, extinction coefficient ε_{max}, photochemical quantum yield ψ, fluorescence quantum yield ψ_F, fluorescence quantum yield  ψ_F, and concentration at saturation ϵ_F. [b] In acetonitrile/HEPES (5:95), pH 7.2. [c] In acetonitrile/HEPES (20:80), pH 7.2. [d] In HEPES, pH 7.2. [e] Present address: Institut für Biophysik Johannes Kepler Universität Linz (Austria) The Supporting Information for this article is available on the WWW under http://www.angewandte.org or from the author.
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 photolysis proceeds in analogy to the conversion of MCM esters by means of an S_N1 mechanism, including singlet-singlet excitation. The photochemical quantum yields were expected to be higher for the DMACM esters than for the DMCM esters and to increase in the order diethyl phosphates < 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 q multiplied by the molar absorbivity (ε) as a criterion for the overall sensitivity of photocleavage, the DMACM compounds 4-6 were confirmed to be the most sensitive phototriggers described so far. The g^max values of 1-3 (900-2400 mol^{-1}cm^{-1}) and of 4-6 (5800-13500 mol^{-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).

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.

Proton sources relying on the 2-nitrobenzyl rearrangement provide H^+ photorelease with rate constants of 2 \times 10^7 s^{-1} and > 4 \times 10^7 s^{-1} for unbuffered and buffered solutions, respectively. 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 \times 10^9 s^{-1}, that is, H^+ was liberated within two nanoseconds or even faster.

The pK_a values of the corresponding acids of the sulfate (1.98), diethyl phosphate (0.71), and methanesulfonate group (−1.54) 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 µmol 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 acetoni trile/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 ΔH = −1 kcal mol⁻¹, ΔG = −6 kcal mol⁻¹, and ΔS = 16 cal mol⁻¹ K⁻¹. For 3 and 6 (not shown): ΔH = −2 kcal mol⁻¹, ΔG = −5 kcal mol⁻¹, and ΔS = 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, 25°C), which was used to calculate their ζ-potentials (circles with standard deviations).

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 F₀ to F) of the membrane-anchored pH-sensitive dye. In contrast to 4 (β), 6 doubled the fluorescence drop (b) 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, b). 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 b).

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 (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 (diphytanoylphosphatidylserine, DPhPS). The Boltzmann distribution[28]...
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 $c_0$, 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](image-url) 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.

Received: August 6, 2004

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