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A new tool to assess ceramide bioactivity: 6-bromo-7-hydroxycoumarinyl-caged ceramide†

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The bioactivity of natural, long-chain ceramides has until now been studied after its delivery to cells in organic solvent mixtures containing dodecane. We have synthesized ceramides conjugated to a (6-bromo-7-hydroxycoumarin-4-yl)methyl group. The photocaged ceramide is efficiently released with 350 nm light in aqueous solution at neutral pH, thus providing a promising new tool to study ceramide's properties.

Photolabile biomolecules were first designed in the 1970s with an *o*-nitrobenzyl or 2-(*o*-nitrophenyl)ethyl substituent to temporarily mask ionizable phosphate groups in cAMP and ATP in order to transport these compounds across cell membranes. Since then, caged compounds have become an important strategy to achieve cell membrane permeation of an inert form of the bioactive molecule, which is subsequently released in its active form by photolysis.[1] Additionally, caged biomolecules enable investigators to gain temporal and spatial control of the release of the bioactive molecule.

Ceramide (*N*-acyl-D-*erythro*-sphingosine) is a key lipid mediator of many cellular death cascades, and among the sphingolipids, it has attracted the most attention owing to its roles in metabolism and regulation of diverse cellular effects.[2] The level of intracellular ceramide is regulated by many pathways, including the *de novo* sphingolipid pathway and the catabolic pathway through hydrolysis of sphingomyelin by sphingomyelinases. A family of six mammalian ceramide synthases is responsible for the markedly different *N*-acyl chain lengths in natural ceramides; the precise roles of the various *N*-acylated forms of ceramide are not known.[3] To supplement the *in situ* ceramide level, investigators have focused on exogenous addition of natural ceramide; unfortunately, as long-chain ceramide is not membrane permeable, the delivery employs a widely used ethanol/dodecane mixture.[4] However, dodecane alters membrane permeability,[5] and as little as 1 μM dodecane induces membrane defects and nonspecific activation of cell signaling pathways.[6] Alternatively, water-soluble, short-chain ceramides have been used as mimics of their long-chain natural counterparts, especially to trigger apoptosis or inhibit signaling in human cancer cells by ceramide accumulation.[7] The biochemical and biophysical properties of natural ceramide differ dramatically from those of short-chain ceramide in membranes and cells.[8] For example, cell permeable short-chain ceramides undergo metabolic conversion to long-chain ceramides through the salvage pathway, which then mediate cellular signaling pathways,[9] and the effect of ceramides on the mitochondrial permeability transition pore was influenced by the fatty amide chain length.[10] The dihydro analogue of ceramide, which is the metabolic precursor of ceramide in *de novo* sphingolipid biosynthesis, fails to trigger apoptosis, and thus provides yet another example of molecular specificity in ceramide's stress-induced responses.[11]

We envisioned that photo-releasable ceramide analogues with different *N*-acyl chain compositions and degrees of saturation in the sphingoid backbone would be valuable new tools for the analysis of ceramide-mediated signal transduction under light-activated spatio-temporal resolution. Coumarin-based cages have several advantages compared with conventional first generation cages.[1e,12] The 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) chromophore[13] undergoes efficient uncaging at longer wavelengths than cages bearing the 2-nitrobenzyl group and its derivatives, which diminishes cell damage caused by UV irradiation; its two-photon excitation cross-sections are useful for imaging of cellular structures and functions; and the phenol group ($pK_a \sim 6.2$) is predominantly deprotonated at physiological pH, which enhances the hydrophilicity and membrane permeability of the caged compound.[14] We report herein the first photocaged ceramide analogues, which we prepared with a carbonate linkage between Bhc and the primary hydroxyl group of ceramide. We also describe preliminary photolysis data, photochemical characterization, and cell uptake for one of these novel Bhc-caged ceramides.

Bhc-caged ceramides (**9**, **10**, **13**, and **16**) with different amide-linked fatty acyl chains and/or sphingolipid backbone were prepared as outlined in Schemes 1 and 2 (see ESI† for details). Bhc-coumarin **4** was prepared from 4-bromoresorcinol (**1**)

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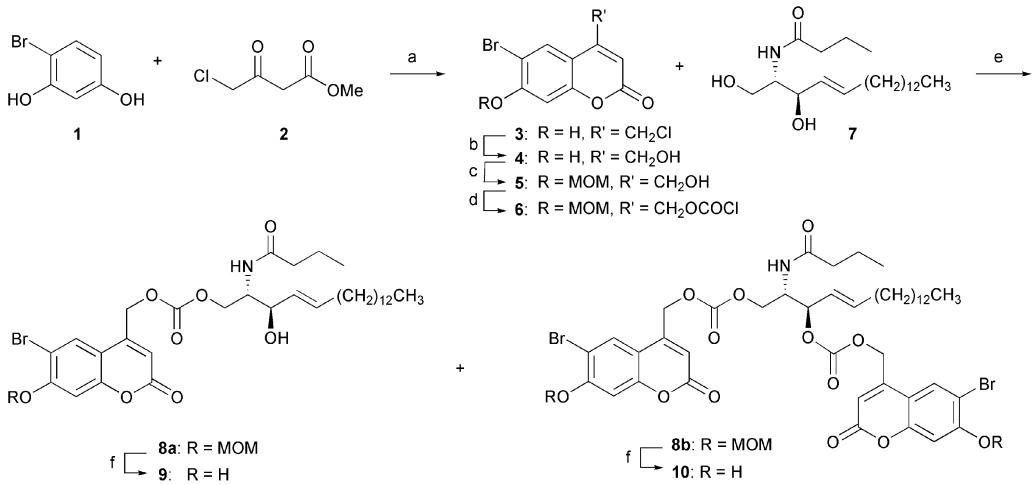
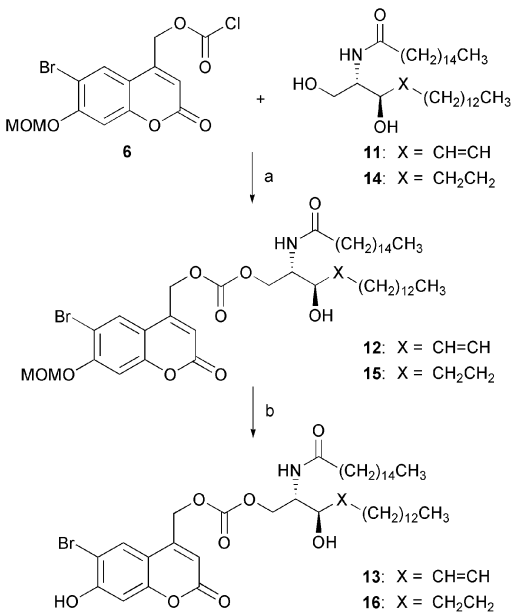


Scheme 1 Synthesis of Bhc-caged short-chain ceramides. (a) CH_3SO_3H, rt, 2 h, 95%; (b) H_2O, reflux, 2 d, 99%; (c) MOMCl, DIPEA, CH_2Cl_2, 0 °C, 2 h, 89%; (d) phosgene, THF/toluene, 3 h, 0 °C, 94%; (e) DIPEA, DMAP, CH_2Cl_2, 0 °C, 2.5 h, 54% for **8a**, 14% for **8b**; (f) $NaHSO_4{\cdot}SiO_2$, CH_2Cl_2, 2 h, rt, 73% for **9**, 68% for **10**.



Scheme 2 Synthesis of Bhc-caged long-chain ceramides. (a) DIPEA, DMAP, THF/CH_2Cl_2 (2 : 1), rt, 36 h, 25% for **12**, 27% for **15**; (b) $NaHSO_4{\cdot}SiO_2$, CH_2Cl_2, 2 h, rt, 70% for **13**, 72% for **16**.

by modification of a reported procedure;[12a] use of methyl 4-chloroacetoacetate (**2**) instead of the ethyl ester of **2** and methanesulfonic acid as catalyst and solvent[15] instead of H_2SO_4 in a Pechmann condensation[16] gave **3** in 95% yield. 4-(Chloromethyl)coumarin **3** was smoothly hydrolyzed in refluxing water, and diol **4** was chemoselectively protected with methoxymethyl chloride (MOMCl) in the presence of DIPEA (*N*,*N*-diisopropylethylamine) to afford phenolic MOM ether **5**. Alcohol **5** was activated by treatment with phosgene solution, which was generated from triphosgene and a catalytic amount of Aliquat^R 336 in hexane.[13a]

The resulting chloroformate **6** was then coupled to unprotected C4-ceramide **7** in the presence of DIPEA and DMAP for 2.5 h at 0 °C in CH_2Cl_2. No product was formed in the absence of DIPEA as a base, presumably because of the diminished nucleophilicity of the hydroxyl group of ceramide by intramolecular hydrogen bonding with the amide.[17] C4-Ceramide gave mono- and di-Bhc-caged products, **8a** and **8b** (ratio 4 : 1), after purification by flash chromatography (hexane/EtOAc gradient elution). The methoxymethyl (MOM) groups were removed with silica-supported sodium hydrogen sulfate ($NaHSO_4{\cdot}SiO_2$) as a heterogeneous catalyst[18] in CH_2Cl_2 to give the corresponding Bhc-caged ceramides **9** and **10**.

Scheme 2 depicts the application of long-chain ceramides (C16-ceramide **11** and C16-dihydroceramide **14**) as the coupling partners. The lower solubility of **11** and **14** compared with C4-ceramide **7** required the use of THF/CH_2Cl_2 (2 : 1) in the coupling reaction, which was carried out at room temperature for 36 h. Notably, no detectable di-Bhc-caged products were observed; selective coupling to the primary hydroxyl group was achieved in the long-chain ceramides without blocking the allylic hydroxyl group. The lower yield of compounds **12** and **15** relative to that of **8** is attributed to steric hindrance by the two long hydrocarbon chains. Finally, the phenolic MOM ether of compounds **12** and **15** was cleaved under mild acidic conditions ($NaHSO_4{\cdot}SiO_2$) at room temperature to afford the Bhc-caged products **13** and **16**.

The photochemical properties of 6-bromo-7-hydroxy-4-hydroxymethylcoumarin (**4**) are similar to those previously reported for Bhc cages,[12a] with absorption/emission maxima at 366 and 466 nm in KMops (potassium 3-(*N*-morpholino)-propanesulfonate) buffer and a fluorescence quantum yield of 0.47 (Table 1). In aqueous ethanol, **4** has an absorption spectrum consistent with a mixture of phenol and phenolate anion, with maxima at 343 and 374 nm, respectively (see ESI†). Caged-ceramide **16** also shows predominantly the protonated form in aqueous ethanol with a weaker long wavelength shoulder due to the anion (Table 1). In KMops buffer, the absorption spectrum for **16** shows predominantly the phenolate form but with residual absorption that we attribute to scattering due to aggregation promoted by the long-chain ceramide. The fluorescence quantum yield of 0.02 for **16** is much lower than that of the parent coumarin **4**,

Table 1 Photochemical properties of (6-bromo-7-hydroxy-4-hydroxymethyl)coumarin **4** and Bhc-caged ceramide **16**

	$\lambda_{abs}(\varepsilon)^a$	λ_{em}	$\Phi_{fl}{}^b$	Solvent^c
4	366 (13 600)	466	0.47	A
	343, 374^d	467	0.50	B
16	338, >380 (sh)	466	0.02	B

^{a} Extinction coefficient (cm^{-1} M^{-1}). ^{b} Quantum yields of fluorescence for excitation at 374 nm, with reference to Coumarin 460 (Φ_{fl} = 0.59). ^{c} Solvent A: KMops (pH 7.4), Solvent B: KMops (pH 7.4) containing 50% ethanol. ^{d} Coexistence of protonated (343 nm) and deprotonated (374 nm) forms of **4**.

consistent with its expected photochemical reactivity. The stability of **16** in aqueous buffer in the dark at 25 °C was examined by HPLC, which showed a half-life of ~30 h (see ESI†), in good agreement with the stability reported for another carbonate-linked photocage.[13a]

Fig. 1A shows the loss of starting material **16** and generation of coumarin **4** as a function of photolysis time for caged-ceramide **16**. These results are consistent with the release of the carbonate anion and the coumarin-4-ylmethyl cation by photocleavage of the C–O bond between the C4 methylene group and the carbonate oxygen. Trapping of the ion pairs by solvent followed by decarboxylation of the carbonic acid product gives ceramide **14** (as confirmed by HPLC/MS, Fig. S5) together with coumarin **4** as the by-product.[12b] The uptake of **16** into the cytosol of J774 macrophages was demonstrated by fluorescence microscopy; on photolysis for 1 to 5 min, the weakly fluorescent **16** is converted to the highly fluorescent free Bhc **4** inside the cells (Fig. 1B and S3). No fluorescence enhancement was observed in cells that were not incubated with **16** (Fig. S4).

In summary, we have synthesized Bhc-caged ceramide analogues and evaluated their photochemical/photophysical behavior and uptake into macrophages. The photolabile group was installed directly into synthetic ceramides with a defined *N*-acyl chain without the use of protecting groups in ceramide. Caged ceramides can be used to analyze the roles of ceramide's *N*-acyl chain structure in distinct intracellular signaling pathways in both physiological and pathophysiological events.

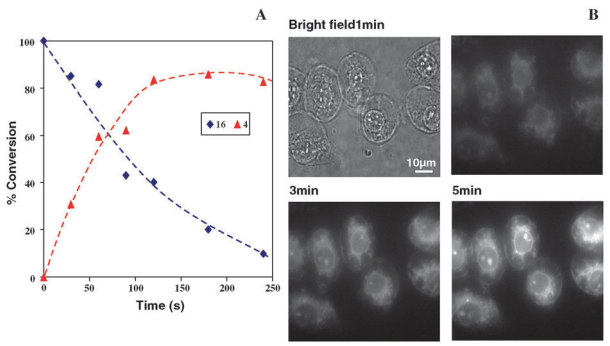


Fig. 1 Time course of photolysis of Bhc-caged ceramide **16** in KMops (pH 7.4) on 350-nm irradiation in a Rayonet photochemical reactor with 4 lamps. (A) Photolysis of **16** (blue) in KMops containing 50% EtOH and formation of by-product **4** (red) were quantified by HPLC analysis. (B) Fluorescence microscopic images of J774 macrophages are shown after uptake of **16** (20 μM in KMops, 5% EtOH) on incubation for 2 h in the dark followed by UV irradiation for the time periods indicated.

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