Published in final edited form as:

Chem Phys Lipids. 2016 January ; 194: 117–124. doi:10.1016/j.chemphyslip.2015.07.006.

Synthesis and photochemical properties of PEGylated coumarin-caged ceramides for cell studies

Young Ah Kim^{a,*}, Jenna Day^{b,c}, Carol Ann Lirette^{b,c}, Willard J. Costain^b, Linda J. Johnston^b, and Robert Bittman^a

^aDepartment of Chemistry and Biochemistry, Queens College of The City University of New York, Flushing, NY 11367, USA

^bNational Research Council of Canada, Ottawa, ON K1A 0R6, Canada

^cDepartment of Chemistry, University of Ottawa, Ottawa, ON K1N 6N5, Canada

Abstract

Caged ceramide analogues (C6-, C16-, C18-, C22- and C24-Cer) have been prepared by introducing a hydrophilic coumarin-based cage bearing a short polyethylene glycol (PEG) chain. (6-Bromo-7-mTEGylated-coumarin-4-yl)methyl (Btc) caged ceramide showed efficient photo-uncaging to release the parent ceramide upon direct exposure to 350 nm UV light; in contrast (7-mTEGylated-coumarin-4-yl) methyl (Tc) caged ceramide was photolysed more slowly. In preliminary experiments, Btc-caged ceramides were taken up by cells and their photolysis led to decreases in cell viability, but not to activation of caspase enzymes, suggesting that either reactive oxygen species or an alternate caspase-independent pathway may be responsible for the decreases in cell viability caused by photolysis of caged ceramides.

Keywords

Ceramide; PEGylated coumarin cages; Photocage; Photolysis

1. Introduction

A caged compound is a biologically inert form of a bioactive molecule that is cell permeable; it possesses a photolabile protecting group (the cage) that upon irradiation releases a biomolecule with a high degree of spatial and temporal control, thus triggering a biological response. Although the *o*-nitrobenzyl photocage has been widely used, a variety of other photocages have been introduced to avoid problems of short wavelength absorption, complex chemistry and slow photorelease (Yu et al., 2010; Brieke et al., 2012; Klán et al., 2013). Since the photolysis of (7-methoxycoumarin-4-yl)methyl (MCM)-caged diethyl phosphate was first reported in 1984 (Givens and Matuszewski, 1984), coumarin-derived caging groups have been used to photoregulate biomolecules with various functional groups,

*Corresponding author at: Department of Chemistry and Biochemistry, Queens College of The City University of New York, Flushing, NY 11367, USA. address: Youngah.Kim@qc.cuny.edu (Y.A. Kim).

Appendix A. Supplementary data: Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chemphyslip.2015.07.006>.

such as hydroxyl, amino, phosphate, phenolate, thiolate, and carboxylate (Furuta, 2005; Lankalapalli et al., 2009). Furthermore, a (6-bromo-7-hydroxycoumarin-4-yl)methyl (Bhc)-based strategy with RNA (Ando et al., 2001) and DNA (Yamaguchi et al., 2010) has been successfully employed in vivo by demonstrating light-triggered gene expression. Coumarins are also compatible with two-photon excitation which make them suitable for a less-damaging photorelease of bioactive molecules in a cellular system (Furuta et al., 1999).

Ceramide (Cer) is an important bioactive sphingolipid that is involved in various cellular processes such as cell cycle arrest, differentiation, apoptosis, senescence, and autophagy (Hannun, 1996; Hannun and Obeid, 2002, 2008). It is a central molecule in sphingolipid metabolism and can be generated from diverse metabolic pathways including de novo synthesis, sphingomyelin hydrolysis, and the salvage pathway. The de novo synthetic pathway starts with a condensation of L-serine and palmitoyl-CoA to form 3-ketosphinganine, which is catalyzed by serine palmitoyl transferase. 3-Ketosphinganine is reduced to sphinganine, mediated by an action of 3-ketosphinganine reductase. Ceramide synthase (CerS) attaches acyl-CoA to sphinganine and the resulting dihydroceramide is converted to ceramide with a double bond in C4–C5 position by dihydroceramide desaturase. In mammals, six isoforms of ceramide synthase (CerS 1–6) display a high specificity toward acyl-CoAs with defined chain length, hence different ceramide species are generated (Levy and Futerman, 2010; Hannun and Obeid, 2011). For instance, CerS1 has been shown to prefer C18 acyl-CoA. CerS2 utilizes C20–C26 acyl-CoAs, thus it is regarded as one of ceramide synthases responsible for very-long-chain ceramide species. CerS5 and CerS6 have specificity for C16 acyl-CoA. The identification of this family of ceramide synthases has focused attention on the roles of specific *N*-acyl chain lengths in ceramide bioactivity. Recent studies indicated that apparent changes in the levels of distinct ceramides occur in neurodegenerative disorders, inflammatory processes, and apoptosis (Ben-David and Futerman, 2010; Mullen et al., 2011; Grösch et al., 2012; Park et al., 2014).

Ceramide can be also generated by enzymatic hydrolysis of the phosphocholine head group from sphingomyelin, which is mediated by sphingomyelinases that can be distinguished by their pH optima and/or subcellular localization. Complex sphingolipids and sphingomyelin can be degraded to ceramide, which is further deacylated to generate sphingosine and free fatty acid by ceramidases. The resulting sphingosine is reconverted to ceramide through the salvage pathway. On the other hand, sphingosine is also a substrate for sphingosine kinases, which produce sphingosine-1-phosphate (Hannun and Obeid, 2008, 2011).

Ceramide may be further metabolized to generate the more complex sphingolipids; examples include the formation of sphingomyelin by sphingomyelin synthases and the production of ceramide-1-phosphate by phosphorylation by ceramide kinase. Ceramide may also be converted to glycosphingolipids by glucosylceramide synthase or galactosylceramide synthase via addition of a sugar group at the C1 hydroxyl position (Hannun and Obeid, 2008, 2011).

The low water solubility of long-chain ceramides and their low cell membrane permeability necessitates the employment of unusual cell delivery methods or the use of unnatural analogs of ceramide. Exogenous long-chain ceramides are frequently delivered to cells in an

ethanol/dodecane mixture (Ji et al., 1995). Unfortunately, as little as 1 μ M dodecane induces membrane defects (Sinkó et al., 2009). Cell-permeable, unnatural short-chain (C2–C8) ceramides have been used as endogenous long-chain ceramide mimics (Ogretmen et al., 2002; Petrache et al., 2005; Flowers et al., 2012), but may not be faithful mimics of natural ceramide since they can be metabolized to long-chain ceramides (Ogretmen et al., 2002; Kitatani et al., 2008). Because of the limitations of the current methodologies for biological investigations of highly hydrophobic lipids, the development of cell membrane permeable caged lipids is an attractive approach if efficient photocontrolled lipid release and biocompatibility can be achieved.

We have recently reported the synthesis of Bhc-caged ceramide analogs and demonstrated that they are compatible with uptake by cells and photorelease of the parent lipid (Kim et al., 2011). We have also shown that photorelease of ceramide can be accomplished with spatial and temporal control for studies of ceramide-induced membrane reorganization in supported bilayers (Ramirez et al., 2013a,b). As an extension of these efforts, we have now developed short polyethylene glycol (PEG) conjugated coumarin cages, which are tethered to the 1-hydroxyl group of ceramide by a carbonate linkage (Fig. 1). The (7-mTEGylated-coumarin-4-yl) methyl (Tc) and (6-bromo-7-mTEGylated-coumarin-4-yl)methyl (Btc) cages have a short PEG chain attached to the phenolic moiety with an ether linkage in order to enhance the aqueous solubility of the long- and very-long-chain caged ceramides. Herein, we describe the synthesis and photochemical characterization of novel photocleavable ceramide analogs (C6-, C16-, C18-, C22- and C24-Cer). Preliminary studies aimed at assessing their suitability for photorelease of ceramide in cells are also reported.

2. Materials and methods

2.1. General

High-resolution mass spectra were recorded on an Agilent Technologies G6520A Q-TOF mass spectrometer using electrospray ionization. NMR spectra were recorded on Bruker Avance I and III spectrometers (400 or 500 MHz), and chemical shifts are expressed in parts per million (δ) relative to chloroform (7.26 ppm for ^1H and 77.2 ppm for ^{13}C) or DMSO (2.50 ppm for ^1H and 39.5 ppm for ^{13}C) as an internal reference. TLC was performed on 0.25- μ m silica gel 60 F₂₅₄ aluminum-backed plates. Silica gel 60 (230–400 mesh) was used for flash column chromatography. The compounds were visualized by UV light and by spraying with 10% phosphomolybdic acid in ethanol. THF and toluene were distilled from sodium and benzophenone. D-erythro-Sphingosine, N-stearoyl-D-erythro-sphingosine, and N-lignoceroyl-D-erythro-sphingosine were purchased from Avanti Polar Lipids. Flash column chromatography for **10–15** was performed using a Biotage Isolera One automated system with Flash KP-Sil™ silica cartridges. Microwave-assisted experiments were carried out in a microwave instrument (Smithcreator™) in Pyrex vials using standard procedures.

2.2. Absorption and fluorescence spectroscopy

Absorption spectra were measured with a Cary 5000 UV–vis–NIR spectrophotometer. Emission spectra were recorded using a Horiba Jobin Yvon FL-3 21 2tau spectrofluorometer with slit widths of 1 nm. All spectral measurements were performed at 22 °C using a 1-cm

pathlength quartz cuvette. Fluorescence quantum yields were calculated with reference to Coumarin 440 in ethanol ($\Phi_{\text{fl}} = 0.56$ (Pal et al., 2013)); solutions with absorbances 0.25 were matched to the coumarin standard at 330 nm.

2.3. Photolysis and photoproduct analysis

Solutions of 20 μM caged substrate in 10 mM KMops (potassium 3-(*N*-morpholino)propanesulfonate) buffer containing 10% ethanol were irradiated at 350 nm in a Rayonet photochemical reactor (Southern New England Ultraviolet Co., Branford, CT, USA) equipped with four lamps. Aliquots of 20 μL were removed periodically for analysis; Coumarin 480 was added to each aliquot as an internal standard. Loss of starting material was monitored by reversed-phase HPLC with a SunFire C18 column (Waters Corp., Milford, MA, USA), and eluted with an ethanol/water gradient at a flow rate of 0.5–0.8 mL/min. Fluorescence detection at 480 nm was used to monitor loss of starting material and formation of the free coumarin photoproduct using coumarin 480 as an internal standard for quantification. The photolysis plots shown are for loss of starting material since the peak due to the product coumarin was broader than that of the caged coumarin. Note that the concomitant production of ceramide has been demonstrated by HPLC-MS in our previous work (Kim et al., 2011). Some photolyses were done in 4-mL quartz cuvettes rotated in the Rayonet reactor. In other cases the solutions were irradiated in microwell plates; the Rayonet reactor was turned on its side and the plate was positioned in the centre with two lamps above and two below for uniform irradiation.

2.4. Synthesis of caged ceramides

2.4.1. (2*S*,3*R*,4*E*)-2-Hexanoylamido-3-hydroxyoctadec-4-enyl (7-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)coumarin-4-yl)methyl carbonate (10)—

Triphosgene (177 mg, 0.596 mmol) and tricaprylylmethylammonium chloride (Aliquat® 336, 21 μL , 46.6 μmol) were dissolved in 2 mL of dry hexane. The solution was stirred at rt for 16 h in a pressure tube to generate phosgene. To a solution of **9a** (10 mg, 29.6 μmol) in dry THF (1 mL) and toluene (0.5 mL) was added 0.1 mL of the prepared phosgene solution at 0 °C. The resulting mixture was warmed to rt and stirred for 14 h. The solvent was removed to afford Tc-chloroformate, which was used in the next step without further purification: R_f 0.69 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1); ^1H NMR (400 MHz, CDCl_3) δ 3.38 (3H, s), 3.56 (2H, m), 3.65 (2H, m), 3.69 (2H, m), 3.75 (2H, m), 3.90 (2H, t, $J = 4.7$ Hz), 4.20 (2H, t, $J = 4.7$ Hz), 5.44 (2H, m), 6.38 (1H, s), 6.88 (1H, d, $J = 2.5$ Hz), 6.92 (1H, dd, $J = 2.5, 8.8$ Hz), 7.36 (1H, d, $J = 8.8$ Hz); ^{13}C NMR (100 MHz, CDCl_3) δ 59.2, 67.7, 68.2, 69.5, 70.7, 70.8, 71.0, 72.1, 102.3, 110.2, 111.4, 113.5, 124.4, 146.5, 150.8, 155.7, 160.5, 162.5. To a solution of (2*S*,3*R*)-*N*-hexanoylsphingosine (9.4 mg, 23.6 μmol) in dry THF (2 mL) and toluene (1 mL) were added Tc-chloroformate (29.6 μmol), DIPEA (9.3 μL , 53.3 μmol), and 4-(dimethylamino) pyridine (DMAP, 2.9 mg, 23.6 μmol) at 0 °C. The reaction mixture was warmed to rt and was stirred for 14 h. After the reaction mixture was concentrated under reduced pressure, the residue was purified on a Biotage Isolera One flash purification system with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient elution to afford Tc-C6-ceramide **10** (7.1 mg, 40%): R_f 0.58 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1); ^1H NMR (400 MHz, CDCl_3) δ 0.88 (6H, m), 1.25 (26H, m), 1.63 (2H, m), 2.04 (2H, m), 2.20 (2H, m), 2.43 (1H, br), 3.38 (3H, s), 3.55 (2H, m), 3.66 (2H, m), 3.69 (2H, m), 3.75 (2H, m), 3.89 (2H, t, $J = 4.7$ Hz), 4.19 (2H, t, $J = 4.7$ Hz), 4.25 (2H, m), 4.34

(1H, dd, $J = 3.2, 11.2$ Hz), 4.47 (1H, dd, $J = 5.4, 11.2$ Hz), 5.31 (2H, s), 5.50 (1H, dd, $J = 6.5, 15.4$ Hz), 5.78 (1H, m), 5.84 (1H, d, $J = 8.0$ Hz), 6.38 (1H, s), 6.87 (1H, m), 6.90 (1H, dd, $J = 2.4, 8.8$ Hz), 7.39 (1H, d, $J = 8.8$ Hz); ^{13}C NMR (100 MHz, CDCl_3) δ 14.1, 14.3, 22.5, 22.8, 25.5, 29.2, 29.4, 29.5, 29.6, 29.7, 29.8, 31.5, 32.1, 32.4, 36.9, 52.7, 59.2, 64.8, 67.3, 68.2, 69.5, 70.7, 70.8, 71.1, 72.1, 73.1, 102.1, 110.3, 110.6, 113.3, 124.5, 128.4, 135.4, 148.5, 154.7, 155.6, 160.9, 162.3, 173.7; HRMS $[\text{M} + \text{H}]^+$ m/z calcd for $\text{C}_{42}\text{H}_{68}\text{NO}_{11}$ 762.4787, found 762.4783.

2.4.2. (2S,3R,4E)-2-Palmitoylamido-3-hydroxyoctadec-4-enyl (7-(2-(2-methoxyethoxy)ethoxy)ethoxy)coumarin-4-yl)methyl carbonate (11)—Tc-C16-ceramide **11** was prepared from **9a** and (2S,3R)-*N*-palmitoylsphingosine **2** for 36 h using the procedure for the synthesis of **10**. Yield = 29%; ^1H NMR (400 MHz, CDCl_3) δ 0.87 (6H, t, $J = 6.8$ Hz), 1.24 (46H, m), 1.62 (2H, m), 2.03 (2H, m), 2.19 (2H, m), 2.48 (1H, br), 3.38 (3H, s), 3.55 (2H, m), 3.66 (2H, m), 3.69 (2H, m), 3.74 (2H, m), 3.89 (2H, t, $J = 4.6$ Hz), 4.19 (2H, t, $J = 4.6$ Hz), 4.24 (2H, m), 4.33 (1H, dd, $J = 3.3, 11.2$ Hz), 4.47 (1H, dd, $J = 5.4, 11.2$ Hz), 5.30 (2H, s), 5.50 (1H, m), 5.77 (1H, m), 5.87 (1H, d, $J = 8.1$ Hz), 6.37 (1H, s), 6.86 (1H, m), 6.90 (1H, dd, $J = 2.4, 8.8$ Hz), 7.38 (1H, d, $J = 8.8$ Hz); ^{13}C NMR (100 MHz, CDCl_3) δ 14.3, 22.8, 25.8, 29.2, 29.4, 29.5, 29.7, 29.8, 32.1, 32.4, 36.9, 52.7, 59.2, 64.7, 67.3, 68.2, 69.5, 70.7, 70.8, 71.0, 72.1, 73.0, 102.1, 110.3, 110.5, 113.3, 124.4, 128.5, 135.4, 148.5, 154.6, 155.6, 160.9, 162.3, 173.7; HRMS $[\text{M} + \text{H}]^+$ m/z calcd for $\text{C}_{52}\text{H}_{88}\text{NO}_{11}$ 902.6352, found 902.6345.

2.4.3. (2S,3R,4E)-2-Palmitoylamido-3-hydroxyoctadec-4-enyl (6-bromo-7-(2-(2-methoxyethoxy)ethoxy)ethoxy)coumarin-4-yl)methyl carbonate (12)—Btc-C16-ceramide **12** was prepared from **9b** and (2S,3R)-*N*-palmitoylsphingosine **2** for 36 h using the procedure for the synthesis of **10**. Yield = 31%; ^1H NMR (400 MHz, CDCl_3) δ 0.87 (6H, t, $J = 6.8$ Hz), 1.25 (46H, m), 1.62 (2H, m), 2.04 (2H, q, $J = 6.8$ Hz), 2.20 (2H, m), 2.38 (1H, br), 3.38 (3H, s), 3.56 (2H, m), 3.66 (2H, m), 3.69 (2H, m), 3.80 (2H, m), 3.96 (2H, t, $J = 4.7$ Hz), 4.24 (4H, m), 4.34 (1H, dd, $J = 3.2, 11.2$ Hz), 4.48 (1H, dd, $J = 5.4, 11.2$ Hz), 5.27 (2H, m), 5.50 (1H, dd, $J = 6.6, 15.4$ Hz), 5.78 (1H, m), 5.84 (1H, d, $J = 8.1$ Hz), 6.41 (1H, s), 6.90 (1H, s), 7.65 (1H, s); ^{13}C NMR (125 MHz, CDCl_3) δ 14.3, 22.8, 25.8, 29.2, 29.4, 29.5, 29.7, 29.8, 32.1, 32.4, 36.9, 52.6, 59.2, 64.5, 67.4, 69.3, 69.7, 70.7, 70.9, 71.4, 72.1, 73.1, 101.6, 108.5, 111.2, 111.4, 127.5, 128.4, 135.5, 147.6, 154.6, 158.3, 160.2, 173.7; HRMS $[\text{M} + \text{H}]^+$ m/z calcd for $\text{C}_{52}\text{H}_{87}\text{BrNO}_{11}$ 980.5457, found 980.5453.

2.4.4. (2S,3R,4E)-2-Stearoylamido-3-hydroxyoctadec-4-enyl (6-bromo-7-(2-(2-methoxyethoxy)ethoxy)ethoxy)coumarin-4-yl)methyl carbonate (13)—Btc-C18-ceramide **13** was prepared from **9b** and (2S,3R)-*N*-stearoylsphingosine for 36 h using the procedure for the synthesis of **10**. Yield = 28%; ^1H NMR (400 MHz, CDCl_3) δ 0.88 (6H, t, $J = 6.8$ Hz), 1.25 (50H, m), 1.62 (2H, m), 2.04 (2H, q, $J = 6.8$ Hz), 2.20 (2H, m), 2.38 (1H, br), 3.38 (3H, s), 3.56 (2H, m), 3.67 (2H, m), 3.69 (2H, m), 3.80 (2H, m), 3.96 (2H, t, $J = 4.7$ Hz), 4.25 (4H, m), 4.34 (1H, dd, $J = 3.2, 11.2$ Hz), 4.48 (1H, dd, $J = 5.3, 11.2$ Hz), 5.27 (2H, m), 5.50 (1H, dd, $J = 6.6, 15.4$ Hz), 5.78 (1H, m), 5.84 (1H, d, $J = 8.0$ Hz), 6.41 (1H, s), 6.90 (1H, s), 7.65 (1H, s); ^{13}C NMR (125 MHz, CDCl_3) δ 14.3, 22.8, 25.8, 29.2, 29.4, 29.5, 29.7, 29.9, 32.1, 32.4, 36.9, 52.6, 59.2, 64.5, 67.4, 69.3, 69.7, 70.8, 70.9, 71.4, 72.1, 73.1, 101.6,

108.5, 111.2, 111.4, 127.5, 128.4, 135.5, 147.6, 154.6, 158.3, 160.2, 173.7; HRMS [M + H]⁺ *m/z* calcd for C₅₄H₉₁BrNO₁₁ 1008.5770, found 1008.5769.

2.4.5. (2S,3R,4E)-2-Behenoylamido-3-hydroxyoctadec-4-enyl (6-bromo-7-(2-(2-methoxyethoxy)ethoxy)ethoxy)coumarin-4-yl) methyl carbonate (14)—Btc-C22-ceramide **14** was prepared from **9b** and (2*S*,3*R*)-*N*-behenoylsphingosine **3** for 36 h using the procedure for the synthesis of **10**. Yield = 23%; ¹H NMR (400 MHz, CDCl₃) δ 0.87 (6H, t, *J* = 6.8 Hz), 1.25 (58H, m), 1.62 (2H, m), 2.04 (2H, m), 2.20 (2H, m), 2.38 (1H, d, *J* = 4.2 Hz), 3.38 (3H, s), 3.56 (2H, m), 3.67 (2H, m), 3.69 (2H, m), 3.80 (2H, m), 3.96 (2H, t, *J* = 4.7 Hz), 4.23 (4H, m), 4.34 (1H, dd, *J* = 3.2, 11.2 Hz), 4.48 (1H, dd, *J* = 5.3, 11.2 Hz), 5.27 (2H, m), 5.50 (1H, dd, *J* = 6.3, 15.4 Hz), 5.78 (1H, m), 5.84 (1H, d, *J* = 8.0 Hz), 6.41 (1H, s), 6.89 (1H, s), 7.65 (1H, s); ¹³C NMR (125 MHz, CDCl₃) δ 14.3, 22.8, 25.8, 29.2, 29.4, 29.5, 29.7, 29.9, 32.1, 32.4, 36.9, 52.6, 59.2, 64.5, 67.4, 69.3, 69.7, 70.7, 70.9, 71.3, 72.1, 73.1, 101.6, 108.5, 111.2, 111.4, 127.5, 128.4, 135.5, 147.6, 154.5, 158.3, 160.2, 173.7; HRMS [M + H]⁺ *m/z* calcd for C₅₈H₉₉BrNO₁₁ 1064.6396, found 1064.6403.

2.4.6. (2S,3R,4E)-2-Lignoceroylamido-3-hydroxyoctadec-4-enyl (6-bromo-7-(2-(2-methoxyethoxy)ethoxy)ethoxy)coumarin-4-yl)methyl carbonate (15)—Btc-C24-ceramide **15** was prepared from **9b** and (2*S*,3*R*)-*N*-lignoceroylsphingosine for 36 h using the procedure for the synthesis of **10**. Yield = 22%; ¹H NMR (400 MHz, CDCl₃) δ 0.88 (6H, t, *J* = 6.9 Hz), 1.25 (62H, m), 1.62 (2H, m), 2.04 (2H, m), 2.20 (2H, m), 2.37 (1H, br), 3.38 (3H, s), 3.56 (2H, m), 3.67 (2H, m), 3.69 (2H, m), 3.80 (2H, m), 3.96 (2H, t, *J* = 4.7 Hz), 4.25 (4H, m), 4.34 (1H, dd, *J* = 3.3, 11.2 Hz), 4.48 (1H, dd, *J* = 5.4, 11.2 Hz), 5.27 (2H, m), 5.50 (1H, dd, *J* = 6.6, 15.4 Hz), 5.78 (1H, m), 5.83 (1H, d, *J* = 8.1 Hz), 6.41 (1H, s), 6.90 (1H, s), 7.65 (1H, s); ¹³C NMR (125 MHz, CDCl₃) δ 14.3, 22.8, 25.8, 29.2, 29.4, 29.5, 29.7, 29.8, 32.1, 32.4, 36.9, 52.6, 59.2, 64.4, 67.4, 69.3, 69.7, 70.7, 70.8, 71.3, 72.0, 73.0, 101.6, 108.5, 111.1, 111.4, 127.5, 128.4, 135.4, 147.7, 154.6, 158.3, 160.3, 173.7; HRMS [M + H]⁺ *m/z* calcd for C₆₀H₁₀₃BrNO₁₁ 1092.6709, found 1092.6709.

2.5. Cellular studies

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM glutamine and 100 units/mL of penicillin/streptomycin in a humidified incubator with 5% CO₂. Cells were subcultured periodically in T75 flasks when the confluence was around 80%. Bright field and fluorescence microscopy were performed on an IX81 inverted optical microscope (Olympus Corporation, Tokyo, Japan) with a high resolution CoolSNAP CCD camera (Photometrics, Tucson, AZ). Epifluorescence images were obtained using lamp excitation with an Olympus UPlanSApo (100×, NA = 1.4) oil immersion objective and a DAPI filter set (Semrock, Rochester, NY). Image analysis was performed using Image J freeware (NIH, Bethesda, MD).

For uptake experiments, HeLa cells were seeded at a density of 6 × 10⁴ cells per well in Ibidi μ-slide 8 well dishes (Ibidi, Verona, WI) and cultured for 24 h prior to adding caged ceramides (20 μM). The cells were incubated with caged ceramides in serum-free medium (DMEM) for 1–3 h and washed once with PBS buffer (1 ×) and imaged. An untreated (vehicle) control in which the cells were exposed to serum-free medium containing only

matched amounts (1%) of the organic solvents used to solubilize the compounds was included in all experiments.

For the WST-1 (water soluble tetrazolium salt) cell viability assay, HeLa cells were seeded at a density of 5000 cells per well and cultured for 24 h or 2500 cells per well and cultured for 48 h in 96-well plates before being treated with exogenous ceramides (20 μ M) or caged ceramides (20 μ M) in serum-free medium for 24 h (2 h of incubation before 6 or 9 min of UV irradiation + 22 subsequent hours of treatment). The medium was then removed and replaced with 100 μ L of the WST-1 reagent diluted 11-fold in serum-free medium. The cells were incubated with the reagent for 3 h and the absorbance at 450 nm was measured in each well with a FLUOstar Omega microplate reader (BMG Labtech, Cary, NC). Relative cell viability was determined for each treatment group by dividing the mean absorbance for the group by the mean absorbance of the untreated control ($\times 100\%$). In most cases, the UV and no UV categories were analyzed separately (i.e., the values are relative to untreated control for the respective category). The error on the calculated values represents the standard deviation between independent experiments or the standard deviation between sample wells (for single experiments).

For the caspase 3/7 activity assays, HeLa cells were seeded at a density of 2500 cells per well in opaque 96-well plates and cultured for 48 h before being treated with exogenous ceramides (20 μ M) or caged ceramides (20 μ M) in serum-free medium. UV irradiation was performed after 2 h of incubation with the ceramide species. At the end of the treatment period, the medium was removed from the wells and replaced with 50 μ L of the caspase 3/7 assay reagent, which was prepared as per the manufacturer's instructions. After 1 h of incubation, luminescence was measured on a FLUOstar omega microplate reader in orbital averaging mode. The fold increase in caspase 3/7 activity was calculated for each treatment group by dividing the mean luminescence value of the group by that of the untreated control. In most cases, the UV and no UV categories were analyzed separately (i.e., the values are relative to untreated control for the respective category). The error on the calculated values represents the standard deviation between sample wells.

3. Results and discussion

3.1. Synthesis

As shown in Scheme 1, ceramides **2** and **3** were prepared from commercially available D-erythro-sphingosine (**1**) and an appropriate fatty acid in one step. The amide functionality was introduced by using *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetra-methyluronium hexafluorophosphate (HATU) (Han and Kim, 2004) and DIPEA in a 1:1 mixture of DMF/THF, affording **2** and **3** in good yields.

Photocleavable compounds **10–15** bearing the short PEG-functionalized coumarin cage were synthesized, as illustrated in Scheme 2. The Pechmann reaction is one of the most widely applied synthetic routes for coumarin derivatives (Daru and Stirling, 2011; Kalita and Kumar, 2012). It involves the condensation of phenols with β -ketoesters under acid-catalyzed conditions, which proceeds via electrophilic attack, trans-esterification, and dehydration. For the condensation of methyl 4-chloroacetoacetate **4** with substituted phenols

5, catalysis by methanesulfonic acid (Hagen et al., 2010; Kim et al., 2011) gave coumarins **6**. Acetates **7** (86–88%) were prepared in the presence of 3 equiv of KF and 1.1 equiv of AcOH in DMF at rt. In comparison, esterification of **6** under previously reported condition (4 equiv of DBU, 3 equiv of AcOH, benzene, reflux) (Furuta et al., 1999) gave **7** in moderate yields. A microwave-assisted Mitsunobu reaction was accomplished within 30 min at 60 °C, producing short PEG-conjugated compounds **8**. Hydrolysis of **8** with potassium carbonate in a 1:1 mixture of THF/MeOH afforded **9** in nearly quantitative yields.

The PEGylated coumarin cages **9** were coupled to ceramides *via* chloroformate intermediates (Suzuki et al., 2003; Kim et al., 2011) in the presence of DIPEA and DMAP at rt to afford Tc-caged compounds **10–11** (40% and 29%, respectively) and Btc-caged compounds **12–15** (22–31%) without any protecting group manipulation. The yield of the coupling reaction was probably affected by steric hindrance caused by the long fatty acyl chain; however, this route allows a convenient access to a variety of the caged ceramide analogs.

3.2. Photochemical characterization

The absorption and fluorescence emission spectra and fluorescence quantum yields were measured for the coumarin cages **9a–b** and caged ceramides **10–15** in either KMops buffer or KMops plus 10% ethanol (Table 1; representative spectra are shown in Figs. S1 and S2 in Supporting information). The bromo-substituted compounds (**9b** and **12–15**) had absorption maxima at ~327–331 nm, slightly red-shifted from the maxima for the remaining compounds at 318–321 nm. The fluorescence maxima varied from 398 to 408 nm. The parent PEGylated coumarin **9a** had the highest fluorescence quantum yield (0.30) with addition of a bromo-substituent resulting in a 30-fold reduction, consistent with enhanced intersystem crossing due to the heavy atom substituent. Tc-caged ceramides (**10** and **11**) had lower quantum yields (0.06 and 0.05) than the parent coumarin as expected for the addition of a competing photocleavage pathway and similar to our previous observations for Bhc-caged dhCer (Kim et al., 2011). Each of the Btc-caged ceramides (**12–15**) had low fluorescence quantum yields that were indistinguishable from those of the parent coumarin. Note that the *N*-acyl chain length is not expected to affect the photochemical behavior of the caged ceramides.

The time course for photolysis of the caged ceramide at 350 nm in quartz cuvettes was monitored by HPLC analysis of the disappearance of the starting material and the concomitant formation of the coumarin photoproduct. As shown in Fig. 2A for Btc-C22-Cer **14**, the irradiation of Btc-caged compound resulted in efficient photorelease within ~10 min. Under matched conditions, the non-brominated Tc-caged analogs **10** (C6-Cer) and **11** (C16-Cer) were photolysed less efficiently with ~70% and 40% loss of starting material after 30 min. These results indicated that the 6-bromo-substituent on the coumarin increased the photochemical reactivity, in agreement with previous studies (Furuta et al., 1999; Furuta, 2005). For comparison with cellular studies, several of the caged ceramides were also photolysed in micro-well plates and analyzed by HPLC (Fig. 2B). The results are comparable with bromo-substituted compounds **12** (C16-Cer) and **14** (C22-Cer) photolysed completely in ~5 min with much slower photolysis for the non-brominated compound **11**

(C16-Cer). The different irradiation geometries for cuvettes and well plates lead to modest differences in the time required for photorelease.

3.3. Lipophilicity

Since the concept of PEGylation was first introduced in the 1970s, PEGylation has been widely used for modification of proteins, polypeptides, and small molecules. Many studies show that the attachment of PEG increases the solubility of biomolecules in aqueous solutions (Harris and Chess, 2003; Knop et al., 2010; Pandey et al., 2010; Li et al., 2013). PEG exhibits several unique properties such as high solubility in water, lack of toxicity, and approval by Food and Drug Administration (FDA) for internal and topical applications (Harris and Chess, 2003; Knop et al., 2010; Li et al., 2013).

In view of these favorable properties of PEG, we synthesized PEGylated coumarin caged ceramides **10–15**, and calculated their log *D* values along with non-PEGylated compound **16** using the ALOGPS program (Tetko and Bruneau, 2004; Tetko and Poda, 2004; Tetko et al., 2005). The resulting log *D* values ranged from 7.4 to 10.0 as shown in Table 2. As expected, log *D* was lowest for short-acyl chain C6-Cer analog **10**. The other compounds (caged C16-Cer to C24-Cer) exhibited an increase in log *D* value. The Btc-C16-Cer **12** (9.1) had a log *D* value similar to that of the Tc-C16-Cer **11** (9.0), indicating that the bromo-substituent did not significantly alter the hydrophobicity. On the other hand, the PEGylated coumarin caged compounds **12–15** (C16-Cer to C24-Cer) showed lower log *D* values compared with non-PEGylated coumarin caged compound **16** (C16-Cer), which may be attributed increased hydrophilicity due to the PEG moiety. It is known that ether oxygens of the PEG chain are associated with water molecules by forming hydrogen bonding (Tasaki, 1996; Lutz, 2008).

3.4. Cellular studies

The uptake of Btc-C22-Cer **14** in HeLa cells was examined by bright field and fluorescence microscopy, as illustrated in Fig. 3. Control cells showed very weak fluorescence whereas cells that were treated with **14** for 2.5 h and then washed to remove caged ceramide from the medium surrounding the cells showed a significant increase in fluorescence. We attribute the increased fluorescence intensity to uptake of **14** by the cells, and possibly also to a small amount of product coumarin released during imaging. Cells incubated with **14** for a shorter time period prior to washing and imaging showed an intermediate intensity.

With demonstration of cellular permeability using microscopy, we next tested whether photorelease of ceramide from Btc-caged ceramides (**12–14**) in HeLa cells led to changes in cell viability using the WST-1 assay. Fig. 4A shows the effects of photolysis (6 or 9 min irradiation) of the caged ceramides on cell viability, in comparison to control (unirradiated) cells. The effects of the coumarin cage (**9b**) were also examined and C2-Cer was used as a positive control. C2-Cer produced a large decrease in cell viability in both unirradiated and irradiated cells, consistent with expectations for this short chain ceramide, which has been shown to be cell-permeant and to decrease cell viability (Pozuelo-Rubio, 2010). In contrast, the caged ceramides showed little effect on viability (viability >90%) after 6 min irradiation, whereas decreased viability (75–80% viable cells) was observed after 9 min irradiation. However, control experiments involving treatment with the coumarin cage (**9b**) under the

same conditions showed a similar change in viability. Therefore, it is not possible to assess whether the changes in viability are due to the effects of ceramide released by photolysis or photodamage associated with coumarin irradiation.

To further probe the effects of photolysis of the caged ceramides, a luminescent caspase assay was used to evaluate caspase activation, which is frequently observed as a consequence of upregulated ceramide production in cells (Lamkanfi and Dixit, 2010; Samokhvalov et al., 2015). Fig. 4B shows that treatment of HeLa cells with C2-Cer produced an approximately 4-fold increase in caspase activity, independent of cellular irradiation. In contrast, treatment with either **13** or **14** did not increase caspase activity relative to the untreated control cells in the absence or presence of irradiation. This indicates that neither photoreleased long- or very-long-chain ceramides nor coumarin irradiation activate effector caspases.

4. Conclusions

In summary, we have synthesized and characterized a series of photocleavable ceramides with *N*-acyl lengths ranging from C6 to C24. The Btc cage with bromo- and short PEG substituents at the 6- and 7-positions on the coumarin ring was used to cage long- and very-long-chain ceramides, which showed rapid release of the bioactive ceramide by photocleavage of the caging moiety. The photolysis times are slightly longer than observed in our earlier study for Bhc-ceramide (Kim et al., 2011); however, the two experiments were not done under matched conditions (lamp intensity, alignment) and quantum yield measurements at low conversion would be required to establish whether there are significant differences in photolysis efficiency when the hydroxyl group is PEGylated. The Tc-caged ceramides were photolysed more slowly than the Btc-caged analogs (matched conditions) and are therefore less useful for cellular studies.

Preliminary studies indicate that the PEGylated caged ceramides are taken up by HeLa cells and that photo-uncaging leads to decreased cell viability. However, a similar reduction in cell viability occurs when the coumarin cage is irradiated. This observation and the fact that photo-uncaging does not activate caspase enzymes suggest that the decreased cell viability may be due to irradiation-induced reactive oxygen species generation. Alternatively, it is possible that ceramide released by photo-uncaging decreases cell viability by a caspase-independent pathway, as documented in previous studies (Thon et al., 2005; Ardestani et al., 2013). Additional studies to test the effects of antioxidants and to probe the involvement of caspase independent mechanisms are required to determine the suitability of the present approach for mechanistic studies of ceramide activity in cells. Furthermore, the effects of irradiation of the parent coumarin highlight the importance of eliminating complications due to the photolysis products derived from the caging moiety.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by NIH Grant HL-08317 (RB) and a Natural Sciences and Engineering Research Council Grant (LJJ).

References

- Ando H, Furuta T, Tsien RY, Okamoto H. Photo-mediated gene activation using caged RNA/DNA in zebrafish embryos. *Nat Genet.* 2001; 28:317–325. [PubMed: 11479592]
- Ardestani S, Deskins DL, Young PP. Membrane TNF- α -activated programmed necrosis is mediated by ceramide-induced reactive oxygen species. *J Mol Signal.* 2013; 8:12. [PubMed: 24180579]
- Ben-David O, Futerman AH. The role of the ceramide acyl chain length in neurodegeneration: involvement of ceramide synthases. *Neuromol Med.* 2010; 12:341–350.
- Brieke C, Rohrbach F, Gottschalk A, Mayer G, Heckel A. Light-controlled tools. *Angew Chem Int Ed Engl.* 2012; 51:8446–8476. [PubMed: 22829531]
- Daru J, Stirling A. Mechanism of the Pechmann reaction: a theoretical study. *J Org Chem.* 2011; 76:8749–8755. [PubMed: 21932799]
- Flowers M, Fabriás G, Delgado A, Casas J, Abad JL, Cabot MC. C6-ceramide and targeted inhibition of acid ceramidase induce synergistic decreases in breast cancer cell growth. *Breast Cancer Res Treat.* 2012; 133:447–458. [PubMed: 21935601]
- Furuta T. Coumarin-4-ylmethyl phototriggers. In: Goeldner, M.; Givens, RS., editors. *Dynamic Studies in Biology: Phototriggers, Photoswitches, and Caged Biomolecules.* Wiley-VCH; Weinheim: 2005. p. 29–54.
- Furuta T, Wang SSH, Dantzker JL, Dore TM, Bybee WJ, Callaway EM, Denk W, Tsien RY. Brominated 7-hydroxycoumarin-4-ylmethyls: photolabile protecting groups with biologically useful cross-sections for two photon photolysis. *Proc Natl Acad Sci U S A.* 1999; 96:1193–1200. [PubMed: 9990000]
- Givens RS, Matuszewski B. Photochemistry of phosphate esters: an efficient method for the generation of electrophiles. *J Am Chem Soc.* 1984; 106:6860–6861.
- Grösch S, Schiffmann S, Geisslinger G. Chain length-specific properties of ceramides. *Prog Lipid Res.* 2012; 51:50–62. [PubMed: 22133871]
- Hagen V, Kilic F, Schaal J, Dekowski B, Schmidt R, Kotzur N. [8-[Bis (carboxymethyl) aminomethyl]-6-bromo-7-hydroxycoumarin-4-yl]methyl moieties as photoremovable protecting groups for compounds with COOH, NH₂, OH, and C=O functions. *J Org Chem.* 2010; 75:2790–2797. [PubMed: 20356068]
- Han SY, Kim YA. Recent development of peptide coupling reagents in organic synthesis. *Tetrahedron.* 2004; 60:2447–2467.
- Hannun YA. Functions of ceramide in coordinating cellular responses to stress. *Science.* 1996; 274:1855–1859. [PubMed: 8943189]
- Hannun YA, Obeid LM. The ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J Biol Chem.* 2002; 277:25847–25850. [PubMed: 12011103]
- Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol.* 2008; 9:139–150. [PubMed: 18216770]
- Hannun YA, Obeid LM. Many ceramides. *J Biol Chem.* 2011; 286:27855–27862. [PubMed: 21693702]
- Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov.* 2003; 2:214–221. [PubMed: 12612647]
- Ji L, Zhang G, Uematsu S, Akahori Y, Hirabayashi Y. Induction of apoptotic DNA fragmentation and cell death by natural ceramide. *FEBS Lett.* 1995; 358:211–214. [PubMed: 7828738]
- Kalita P, Kumar R. Solvent-free coumarin synthesis via Pechmann reaction using solid catalysts. *Microporous Mesoporous Mater.* 2012; 149:1–9.

- Kim YA, Ramirez DMC, Costain WJ, Johnston LJ, Bittman R. A new tool to assess ceramide bioactivity: 6-bromo-7-hydroxycoumarinyl-caged ceramide. *Chem Commun.* 2011; 47:9236–9238.
- Kitatani K, Idkowiak-Baldys J, Hannun YA. The sphingolipid salvage pathway in ceramide metabolism and signaling. *Cell Signal.* 2008; 20:1010–1018. [PubMed: 18191382]
- Klán P, Šolomek T, Bochet CG, Blanc A, Givens R, Rubina M, Popik V, Kostikov A, Wirz J. Photoremovable protecting groups in chemistry and biology: reaction mechanisms and efficacy. *Chem Rev.* 2013; 113:119–191. [PubMed: 23256727]
- Knop K, Hoogenboom R, Fischer D, Schubert US. Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. *Angew Chem Int Ed.* 2010; 49:6288–6308.
- Lamkanfi M, Dixit VM. Manipulation of host cell death pathways during microbial infections. *Cell Host Microbe.* 2010; 8:44–54. [PubMed: 20638641]
- Lankalapalli RS, Ouro A, Arana L, Gómez-Muñoz A, Bittman R. Caged ceramide 1-phosphate analogues: synthesis and properties. *J Org Chem.* 2009; 74:8844–8847. [PubMed: 19908915]
- Levy M, Futerman AH. Mammalian ceramide synthases. *IUBMB Life.* 2010; 62:347–356. [PubMed: 20222015]
- Li W, Zhan P, Clercq ED, Lou H, Liu X. Current drug research on PEGylation with small molecular agents. *Prog Polym Sci.* 2013; 38:421–444.
- Lutz JF. Polymerization of oligo(ethylene glycol) (meth) acrylates: toward new generations of smart biocompatible materials. *J Polym Sci Part A.* 2008; 46:3459–3470.
- Mullen TD, Jenkins RW, Clarke CJ, Bielawski J, Hannun YA, Obeid LM. Ceramide synthase-dependent ceramide generation and programmed cell death: involvement of salvage pathway in regulating postmitochondrial events. *J Biol Chem.* 2011; 286:15929–15942. [PubMed: 21388949]
- Ogretmen B, Pettus BJ, Rossi MJ, Wood R, Usta J, Szulc Z, Bielawska A, Obeid LM, Hannun YA. Biochemical mechanisms of the generation of endogenous long chain ceramide in response to exogenous short chain ceramide in the A549 human lung adenocarcinoma cell line: role for endogenous ceramide in mediating the action of exogenous ceramide. *J Biol Chem.* 2002; 277:12960–12969. [PubMed: 11815611]
- Pal H, Nad S, Kumbhakar M. Photophysical properties of coumarin-120: unusual behavior in nonpolar solvents. *J Chem Phys.* 2013; 119:443–452.
- Pandey MK, Balwani S, Sharma PK, Parmar VS, Ghosh B, Watterson AC. Design, synthesis and anti-inflammatory evaluation of PEGylated 4-methyl and 4,8-dimethylcoumarins. *Eur J Pharm Sci.* 2010; 39:134–140. [PubMed: 19948217]
- Park JW, Park WJ, Futerman AH. Ceramide synthases as potential targets for therapeutic intervention in human diseases. *Biochim Biophys Acta.* 2014; 1841:671–681. [PubMed: 24021978]
- Petrache I, Natarajan V, Zhen L, Medler TR, Richter AT, Cho C, Hubbard WC, Berdyshev EV, Tudor RM. Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. *Nat Med.* 2005; 11:491–498. [PubMed: 15852018]
- Pozuelo-Rubio M. Proteomic and biochemical analysis of 14-3-3-binding proteins during C2-ceramide-induced apoptosis. *FEBS J.* 2010; 277:3321–3342. [PubMed: 20618440]
- Ramirez DMC, Kim YA, Bittman R, Johnston LJ. Lipid phase separation and protein-ganglioside clustering in supported bilayers are induced by photorelease of ceramide. *Soft Matter.* 2013a; 9:4890–4899. [PubMed: 23667384]
- Ramirez DMC, Pitre SP, Kim YA, Bittman R, Johnston LJ. Photouncaging of ceramides promotes reorganization of liquid-ordered domains in supported lipid bilayers. *Langmuir.* 2013b; 29:3380–3387. [PubMed: 23402522]
- Samokhvalov V, Zlobine I, Jamieson KL, Jurasz P, Chen C, Lee KSS, Hammock BD, Seubert JM. PPAR δ signaling mediates the cytotoxicity of DHA in H9c2 cells. *Toxicol Lett.* 2015; 232:10–20. [PubMed: 25300478]
- Sinkó B, Kökösi J, Avdeef A, Takács-Novák K. A PAMPA study of the permeability-enhancing effect of new ceramide analogues. *Chem Biodivers.* 2009; 6:1867–1874. [PubMed: 19937821]
- Suzuki AZ, Watanabe T, Kawamoto M, Nishiyama K, Yamashita H, Ishii M, Iwamura M, Furuta T. Coumarin-4-ylmethoxycarbonyls as phototriggers for alcohols and phenols. *Org Lett.* 2003; 5:4867–4870. [PubMed: 14653694]

- Tasaki K. Poly(oxyethylene)–water interactions: a molecular dynamics study. *J Am Chem Soc.* 1996; 118:8459–8469.
- Tetko IV, Bruneau P. Application of ALOGPS to predict 1-octanol/water distribution coefficients, log *P*, and log *D*, of AstraZeneca in-house database. *J Pharm Sci.* 2004; 93:3103–3110. [PubMed: 15514985]
- Tetko IV, Gasteiger J, Todeschini R, Mauri A, Livingstone D, Ertl P, Palyulin VA, Radchenko EV, Zefirov NS, Makarenko AS, Tanchuk VY, Prokopenko VV. Virtual computational chemistry laboratory—design and description. *J Comput Aided Mol Des.* 2005; 19:453–463. [PubMed: 16231203]
- Tetko IV, Poda GI. Application of ALOGPS 2. 1 to predict log *D* distribution coefficient for Pfizer proprietary compounds. *J Med Chem.* 2004; 47:5601–5604. [PubMed: 15509156]
- Thon L, Möhlig H, Mathieu S, Lange A, Bulanova E, Winoto-Morbach S, Schütze S, Bulfone-Paus S, Adam D. Ceramide mediates caspase-independent programmed cell death. *FASEB J.* 2005; 19:1945–1956. [PubMed: 16319138]
- Yamaguchi S, Chen Y, Nakajima S, Furuta T, Nagamune T. Light-activated gene expression from site-specific caged DNA with a biotinylated photolabile protection group. *Chem Commun.* 2010; 46:2244–2246.
- Yu H, Li J, Wu D, Qiu Z, Zhang Y. Chemistry and biological applications of photo-labile organic molecules. *Chem Soc Rev.* 2010; 39:464–473. [PubMed: 20111771]

Fig. 1.
Structures of cages and photocleavable ceramides.

Fig. 2.

Time course for photolysis of caged ceramides in KMops buffer containing 10% ethanol using 350-nm irradiation in a Rayonet reactor. Loss of starting material was analyzed by HPLC. (A) Photolysis of **10** (◆), **11** (■) and **14** (▲) in quartz cuvettes. (B) Photolysis of **11** (■), **12** (◆) and **14** (▲) in micro-well plates.

Fig. 3. Bright field (top) and fluorescence images (bottom) of HeLa cells treated without (control) and with 20 μM Btc-C22-Cer (**14**) in serum-free media and then washed with PBS buffer and imaged. (A) control, untreated cells. (B) 1 h incubation. (C) 2.5 h incubation. The two-fold increase in fluorescence intensity after the 2.5 h incubation indicates cellular uptake of **14**.

Fig. 4.

(A) Relative viability of HeLa cells treated with Btc-caged ceramides (**12-14**) and Btc cage (**9b**), and the positive control C2-Cer. Viability was measured using the WST-1 assay after 24 h of treatment. (B) Caspase 3/7 activity assay results for HeLa cells treated with C2-Cer or with the Btc-caged ceramides (**13** and **14**) at a concentration of 20 μ M in serum-free media. Samples were incubated in the dark for 2 h, irradiated in a UV reactor (350 nm) for 9 min and assayed after 24 h.

Scheme 1.
Synthesis of ceramides.

Scheme 2.

Synthesis of the caged ceramide analogs (**10–15**) with a short-, long-, or very-long *N*-acyl chain.

Table 1

Photochemical characterization of cages **9a–b**, Tc-caged ceramides **10–11**, and Btc-caged ceramides **12–15**.

Compound	λ_{abs} (nm)	λ_{em} (nm)	Φ_{fl}^a	Solvent ^b
9a	321	398	0.30	A
9b	327	398	0.01	A
10	320	408	0.06	B
11	318	408	0.05	B
12	329	408	0.01	B
13	330	407	0.01	B
14	331	408	0.01	B
15	328	408	0.005	B

^aQuantum yields of fluorescence for excitation at 330 nm or at 350 nm, with reference to Coumarin 440 ($\Phi_{\text{fl}}=0.56$).

^bSolvent A: KMops (pH 7.4), solvent B: KMops (pH 7.4) containing 10% ethanol.

Table 2

Log *D* values of the photocleavable ceramides.

	10	11	12	13	14	15	16 ^b
Log <i>D</i> ^a	7.4	9.0	9.1	9.3	9.6	9.7	10.0

^a Log *D* values, the distribution coefficient between water and 1-octanol, were calculated with ALOGPS 2.1 program.

^b Bhc-C16-Cer.