Microwave-assisted synthesis of near-infrared fluorescent sphingosine derivatives†

Kumar R. Bhushan, Fangbing Liu, Preeti Misra and John V. Frangioni*

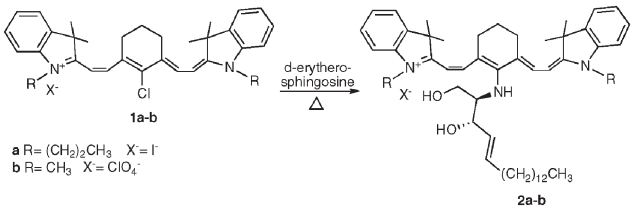
Received (in Cambridge, UK) 9th May 2008, Accepted 12th June 2008
First published as an Advance Article on the web 28th July 2008
DOI: 10.1039/b807930g

Microwave-assisted synthesis of near-infrared fluorescent sphingosine derivatives is described, and the utility of the probes demonstrated by co-localization studies with visible wavelength fluorescent sphingosine derivatives.

Sphingolipids are essential components of the plasma membrane of eukaryotic cells, where they are typically found in the outer leaflet. Sphingolipids differ from phospholipids in being based on a lipophilic amino alcohol rather than glycerol. They play important roles in signal transduction processes,[1–3] and although particularly abundant in mammalian cells, they are also present in *Saccharomyces cerevisiae*,[4] other fungi, and plants. Sphingolipid metabolism typically occurs in the membranes of the Golgi apparatus and endoplasmic reticulum, and fluorescent sphingosine derivatives are important probes for measuring the intracellular distribution and transport of labeled molecules in live cells.[5] Fluorescent analogs of sphingolipids are also important for detecting, at the cellular level,[6,7] genetic defects in sphingolipid synthesis and degradation, such as those caused by type I Gaucher's disease, type A Niemann–Pick disease, and Krabbe's disease.[8–12]

Microwave irradiation (MWI) has proven to be a powerful technique for promoting a variety of chemical reactions.[13–15] The main benefits of performing reactions under MWI conditions are significant rate-enhancements and higher yields.[16,17] Heptamethine cyanine dyes[18–20] have been widely used in biology, with a recent emphasis on image-guided surgery.[21] Their spectra reach the near-infrared (NIR) region, where living cells and tissue exhibit minimal absorption and autofluorescence background.[22–24] The resurgence of interest in imaging and detecting various diseases and analytes by NIR optical methods has led to a need for more efficient synthesis of novel fluorescent probes. With this in mind, we describe the microwave-assisted synthesis of two NIR sphingosine derivatives. We also present *in vitro* cell labeling, and comparison of NIR sphingosine derivatives with visible wavelength derivatives.

To explore the use of MWI with NIR probes, we started with the heptamethine indocyanines IR-780 (**1a**) and IR-786 (**1b**),



Scheme 1 Synthesis of sphingosine derivatives.

which differ in the aliphatic chains of their indole nitrogens (Scheme 1). To the best of our knowledge, this is the first report of the microwave-assisted reaction of NIR dyes using sphingosine as a nucleophile. Reactions were performed under MWI, and the efficiency of NIR sphingosine synthesis‡ was compared with conventional heating. Dimethylformamide was the best solvent in both conditions. There was no reaction rate enhancement observed with addition of 0.1 to 1.0 equivalent tertiary amine bases.

Reaction time under MWI was reduced from days to minutes, with more than two-fold improvement in product yields when compared to conventional methods (Table 1). High performance liquid chromatography (HPLC) methods were developed to isolate the products from the reaction mixture. Purity and characterization of products was performed using liquid chromatography-mass spectrometry (LCMS) (Fig. 1) and nuclear magnetic resonance (NMR) spectroscopy (see ESI†). Calculated/found m/z for **2a** and **2b** were 802.62/802.65 and 746.56/746.56, respectively.

Compounds **2a** and **2b** were fully characterized for their spectral properties§ in methanol (Fig. 2). Relative to the starting materials, peak absorptions of **2a** and **2b** exhibited hypsochromic shifts centered at 670 and 666 nm, respectively. Emission maxima of 744 and 738 nm, respectively, revealed a large Stokes shift. Dye **2a** had a molar extinction coefficient (ε) and quantum yield (QY) of 73 600 M^{-1} cm^{-1} and 4.2%, respectively, compared to 72 900 M^{-1} cm^{-1} and 3.8% for compound **2b**.

For subcellular co-localization studies, 1 μM each of the visible fluorescence sphingosine derivatives BODIPY-FL-C_5-Ceramide and BODIPY-TR-Ceramide, and either **2a** or **2b**, were incubated with living PC-3 prostate cancer cells in either PBS or RPMI-1640 supplemented with 10% calf serum, followed by fluorescence microscopy.¶ Custom filter sets (470/525 nm, 545/605 nm and 650/710 nm) permitted imaging of all three fluorophores in the same cells. As shown in Fig. 3, all sphingosine derivatives labeled intracellular membranes and, to a lesser extent the plasma membrane. There was slight

Division of Hematology/Oncology, Department of Medicine and Department of Radiology, Beth Israel Deaconess Medical Center/ Harvard Medical School, 330 Brookline Avenue, Room SLB-05, Boston, MA 02215, USA. E-mail: jfrangio@bidmc.harvard.edu; Web: http://www.frangionilab.org; Fax: +1 617-667-0981; Tel: +1 617-667-0692

† Electronic supplementary information (ESI) available: Chemical sources, NMR and LCMS data. See DOI: 10.1039/b807930g

Table 1 Reaction conditions and optical properties

Compound	T/°C	MWI t/h (yield %)	Oil-bath t/h (yield %)	Optical properties			
				Abs/nm	Flu/nm	ε^a/M^{-1} cm^{-1}	QY^b (%)
2a	90	0.5 (79)	72 (28)	670	744	73 600	4.2
2b	90	0.5 (83)	72 (30)	666	738	72 900	3.8
1a	—	—	—	780	807	220 000	18.2
1b	—	—	—	781	801	236 600	16.7

[a] Fluorophores were excited at their maximum absorbance. [b] The fluorescence quantum yields (QY) of **2a** and **2b** were determined in methanol in reference to methyl DOTCI (QY = 4% in DMSO),[25] while dye **1a** and **1b** in methanol were referenced to ICG (QY = 13% in DMSO).[26] Abs: Absorbance; Flu: fluorescence; ε: extinction coefficient; QY: quantum yield.

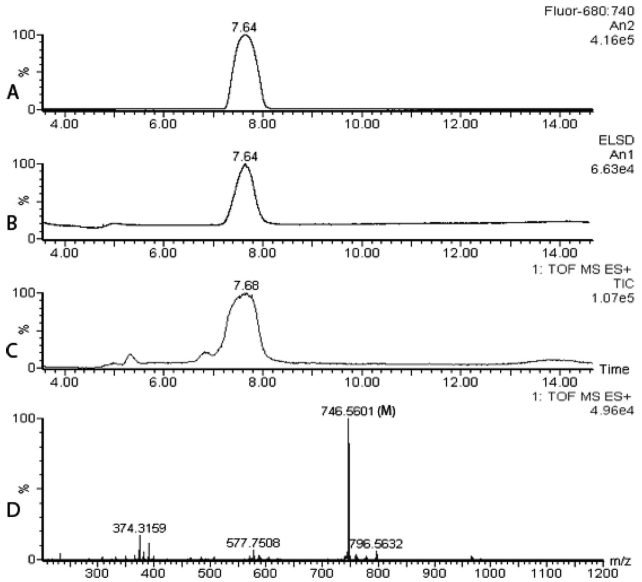


Fig. 1 LCMS of compound **2b**: (A) Fluorescence (680/740 nm). (B) Evaporative light scatter detector (ELSD). (C) Total ion chromatogram (TIC), and (D) Mass spectrum (electrospray positive mode) of 7.6 min peak. m/z: Calculated for $C_{50}H_{72}N_3O_2^+$: 746.56, found 746.56.

intracellular and intercellular variation among the fluorophores, with even the two commercial sphingosine derivatives not being identical. What was identical, however, were results from **2a** *vs*. **2b**, living cells *vs*. cells fixed with 2% paraformaldehyde, and incubation in PBS only *vs*. serum-containing medium (data not shown).

In conclusion, we have developed a microwave-assisted method for the rapid and efficient synthesis of NIR sphingosine derivatives of heptamethine indocyanine dyes. We have also demonstrated that these NIR sphingosine derivatives co-localize with previously described sphingosine derivatives. These new molecules should lay the foundation for the development of nerve-specific NIR fluorescent probes.

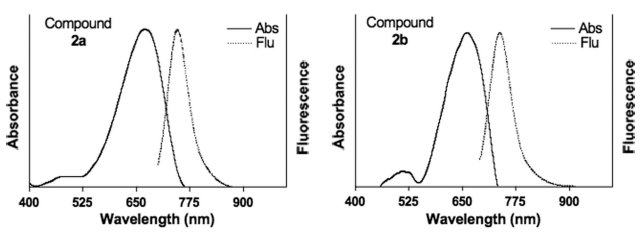


Fig. 2 Absorbance and fluorescence spectra of 10 μM of compound **2a** (left) and **2b** (right) in methanol.

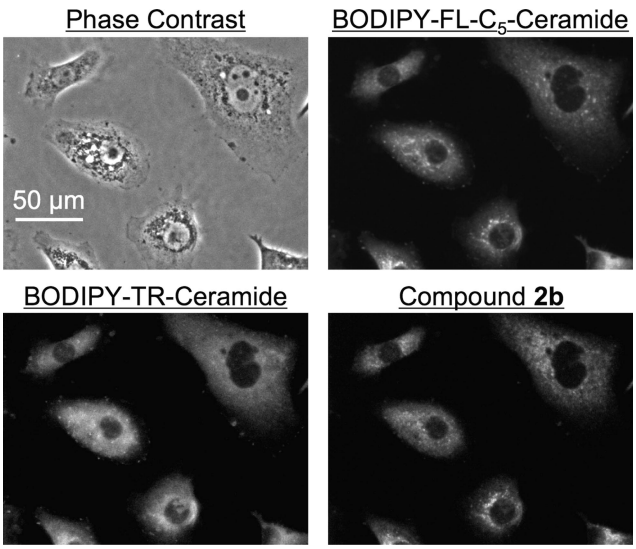


Fig. 3 Fluorescence microscopy of PC-3 cells after incubation with 1 μM BODIPY-FL-C_5-Ceramide, BODIPY-TR-Ceramide, and compound **2b** for 20 min at room temperature, followed by extensive washing.

Funded by NIH grant #R01-CA-115296 to J. V. F.

Notes and references

‡ *Chemical synthesis*: See ESI† for chemical sources. In a typical procedure, sphingosine (5 mg, 16.67 μmol; Sigma-Aldrich, St. Louis, MO) and NIR dye (16.67 μmol; Sigma) were mixed in a 4 mL Teflon-capped tube fitted with stirring bar. 0.4 mL dimethylformamide was added under nitrogen atmosphere and the tube was mounted and agitated in a CEM Explorer microwave system. Progress of the reaction was monitored by LCMS on a Waters system consisting of a 1525 binary HPLC pump with a manual 7725i Rheodyne injector, a 2487 dual wavelength absorbance detector, and a 2475 multi-wavelength fluorescence detector (Waters). The column eluate was divided in two using a flow splitter (Upchurch Scientific). A portion of the eluate flowed into an ELSD (Richards Scientific) while the remainder flowed into a Micromass LCT TOF-ES spectrometer (Waters) equipped with a Symmetry®C18 (4.6 × 150 mm, 5 μm) reverse-phase HPLC column. For mass spectrometry mobile phase was solvent A = H_2O + 0.1% formic acid, solvent B = CH_3CN + 0.1% formic acid, flow rate was 0.6 ml min^{-1}, capillary voltage was −3317 V, and sample cone voltage was −50 V. Gradient 30% B from 0 min to 100% B in 8 min and afterward 100% B used for 15 min. Retention times for compounds **2a** and **2b** were 7.35 and 7.67 min, respectively.

After completion, the reaction mixture was poured over 2 mL of ice-cold water and purified by preparative reverse phase HPLC on a Waters (Milford, MA, USA) prepLC 150 mL fluid handling unit (Waters) equipped with a Symmetry Prep$^{®}$C$_{18}$ column (19 × 150 mm, 7 μm particle size), a manual injector (Rheodyne 3725i) and a 2487 dual wavelength absorbance detector (Waters) outfitted with a semi-preparative flow cell. A flow splitter (Upchurch Scientific, Oak Harbor, WA, USA) diverted a portion of the eluate into an evaporative light scatter detector (ELSD, Richards Scientific, Novato, CA, USA) with the nebulizer modified to reduce band broadening at low flow rates while the other part flowed into a fraction collector (Waters, Fraction Collector II). The ELSD was set to 40 °C, with a nitrogen pressure at 3.5 bar and a gain of 6. For HPLC mobile phase was solvent A = H_2O + 0.1% formic acid, solvent B = CH_3CN + 0.1% formic acid and flow rate was 15 ml min^{-1}. Gradient was 30% B from 0 min to 5 min and afterward 30% B to 100% B for 20 min. Retention times for compounds **2a** and **2b** were 15.8 min and 16.0 min, respectively.

§ *Spectral measurements*: Absorbance spectroscopy was performed in a 1 cm path length quartz cuvette (Starna, Atascadero, CA, USA), mounted in a CUV-ALL-UV four-way cuvette holder (Ocean Optics, Dunedin, FL, USA), and excited with a balanced deuterium–tungsten light source (Ocean Optics) spectrometer with a 1.3 nm resolution from 200 to 850 nm, using 10 μM of NIR fluorophore in the indicated buffer. Fluorescence spectrometry was performed in a three-sided quartz cuvette (Starna) excited with a 5 mW 770 nm laser diode coupled through a 300 μm core diameter, NA 0.22 fiber (Fiberguide Industries, Stirling, NJ, USA), and a 5 mW 680 nm laser diode (Opcom O.E., China). Fluorescence measurements were made on an HR2000 (Ocean Optics) spectrometer with a 7.6 nm resolution from 350 to 1000 nm, using 10 μM NIR fluorophore in the indicated buffer. QYs of compounds **1a** and **1b**, were calculated using ICG in DMSO as calibration standard, under conditions of matched fluorophore absorbance at the 770 nm laser line. QYs of compounds **2a** and **2b**, were calculated using methyl-DOTCI in DMSO as calibration standard, under conditions of matched fluorophore absorbance at the 680 nm laser line.

¶ *Live cell fluorescence*: Exponentially growing PC-3 cells at a confluence of 75% on glass coverslips were incubated with 0.2 mL of medium containing 1 μM **2a** or **2b**, with or without 1 μM BODIPY-FL-C_5-Ceramide (Invitrogen, Carlsbad, CA) and 1 μM BODIPY-TR-Ceramide (Invitrogen), for 20 min at room temperature. Cells were washed three times with PBS and either imaged live, or fixed with 2% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed an additional three times with PBS and mounted with Fluoromount-G. All cells were imaged on a four-channel visible/NIR fluorescence microscope that has been described in detail previously.[27]

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