Supplemental information for: A brief overview of the toxic sphingomyelinase Ds of brown recluse spider venom and other organisms, and simple methods to detect production of its signature cyclic ceramide phosphate

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ABBREVIATIONS: C6-, Hexanoyl-; C12-, Dodecanoyl-; C16-, Palmitoyl; C24-, Lignoceroyl; C12-NBD-, (N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-; C16-NBD-, (N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexadecanoyl]-; C24-NBD-, (N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]tetracosanoyl]-; Cer, ceramide; Cer1P, ceramide 1-phosphate; Cer1,3P, ceramide 1,3-cyclic phosphate; DCCD, N,N'-dicyclohexylcarbodiimide; DIPE, diisopropylethylamine; DMSO, Dimethylsulfoxide; FA, fatty acyl-; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; HPLC, High-performance liquid chromatography; LC-, liquid chromatography; MS, mass spectrometry; NHS, N-hydroxysuccinimide; NMR, nuclear magnetic resonance; PLD, phospholipase D; Rf, Retention
factor; S1P, sphingosine 1-phosphate; SM, sphingomyelin; SMase, sphingomyelinase; TLC, thin-layer chromatography; TopFluor, Dipyrrrometheneboron difluoride; UV, Ultraviolet

Introduction

This supplement contains more comments and details about the SMase D/PLD assay, including sources of materials and alternative methodologies. Most of this information is from (Lachmayr, 2021).

Materials

**Fluorescent sphingolipids:** These were obtained from Avanti Polar Lipids (Alabaster, AL): C12-NBD-Cer (N-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-D-erythro-sphingosine, catalog #810211), C11-TopFluor-Cer1P (N-[11-(dipyrrrometheneboron difluoride)undecanoyl]-D-erythro-sphingosine-1-phosphate, ammonium salt, catalog # 810270), C12-NBD-SM (catalog # 810219) and the C12-NBD-fatty acid (NBD-dodecanoic acid) (as a special order without catalog number). C12-NBD-Cer1P and C12-NBD-Cer1,3P were synthesized from S1P and C12-NBD-fatty acid by modifying the previous method for making NBD-sphingolipids (Futerman and Pagano, 1992), as described below.

**Non-fluorescent sphingolipids:** The C12-Cer (catalog # 860512), C24-Cer (catalog # 860524), C12-Cer1P (catalog # 860531), C24-Cer1P (catalog # 860527), C6-Cer(1,3)P (catalog # 860704), C12-Cer1,3P (catalog # 860702), C16-SM (catalog # 860584) and C24:1-SM (catalog # 860584)were obtained from Avanti Polar Lipids (Alabaster, AL). C24-Cer1,3P was synthesized from C24-Cer1P by a modification of the method of (Boudker and Futerman, 1993), as described below.
**Reagents for synthetic procedures and chromatography:** N,N'-dicyclohexylcarbodiimide (99%, catalog # D80002), N-hydroxysuccinimide (catalog # 130672), pyridine (99.8% anhydrous, catalog # 270970) and diisopropylethylamine (DIPE) (#D125806) were from Sigma-Aldrich. Anhydrous dimethylsulfoxide (DMSO) was a gift from Dr. Christoph Fahrni at Georgia Tech, but is available from commercial vendors. Additional solvents for the syntheses and chromatography were obtained from these suppliers: HPLC-grade chloroform (#EM-CX1050) and methol (#EM-MX0475) from VWR (West Chester, PA); ammonium hydroxide (#1303), n-propanol (#1824) and dichloromethane (DX0838) from Millipore-Sigma; n-Butanol (#27,069), diisopropylethylamine (DIPE) (>99%, catalog# D125806), iodine (≥99.8%, catalog#207772) and ethanol (absolute for analysis EMSURE® ACS,ISO,Reag. Ph Eur) from Sigma-Aldrich; ACS-grade acetic acid (catalog#A38C-212) from Fisher Scientific (Waltham, MA); and ethylacetate (99.5% HPLC, catalog#22192) from Alfa Aesar/Thermo Scientific.

**Chromatography materials:** Thin-layer chromatography (TLC) plastic sheets (Silica gel 60, #105719) were from EM Science (Darmstadt, Germany), for which a current commercial supplier is Avantor (Radnor, PA) (TLC SG-60, # EM1.05748.0001). Silica gel for column chromatography (Unisil) was from Clarkson Chromatographic Products (South Westport, PA).

**Syntheses**

The following syntheses were used to prepare the compounds used for these assays. They produced the desired compounds in adequate amounts and purity for these studies; however, the procedures have not been optimized with respect to the best reagent ratios, reaction times, yields, etc.
**Synthesis of C12-NBD-Cer1P and C12-NBD-Cer1,3P.** C12-NBD-Cer1P (N-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-ceramide 1-phosphate) was synthesized from S1P (sphingosine 1-phosphate) by modifying the method of Futerman and Pagano (Futerman and Pagano, 1992) for synthesis of NBD-Cer from sphingosine as follows: To a 13 x 100 mm screw-capped borosilicate glass test tube with Teflon-lined cap was added ~ 10 µmol of C12-NBD-FA, (N-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoic acid) (MW 378 = 3.8 mg) dissolved in 0.1 mL of anhydrous dimethylsulfoxide (DMSO), then ~ 10 µmol of N-hydroxysuccinimide (NHS) (MW 115 = 1.2 mg) and ~ 15 µmol of N,N'-dicyclohexylcarbodiimide (DCCD) (MW 206 = 3.2 mg) were added in 0.4 mL of anhydrous DMSO. The test tube was purged with N₂ gas, tightly capped and sealed with parafilm, then wrapped with aluminum foil to react with rocking for 2 days at room temperature in the dark. The production of the N-hydroxyl succinimidyl ester of the C12-NBD FA was established by TLC using silica plates and CHCl₃:MeOH (9:1, v/v) as the developing solvent (the Rᶠ of the NHS ester was ~ 0.9 versus the free acid at ~ 0.5).

To the test tube was added 10 µmol of S1P (4 mg) that was mostly dissolved in 2 mL of DMSO plus 20 µmol of diisopropylethylamine (DIPE) (2.7 mg), and the test tube was purged with N₂, sealed, covered with aluminum foil, and rocked for 3 days at room temperature in the dark. Examination of the products by TLC indicated that the fluorescence was about equally distributed among unreacted NBD-fatty acid, C12-NBD-Cer1P, and C12-NBD-Cer1,3P (C12-NBD-Cer 1,3-cyclic phosphate) (the latter was apparently formed from excess DCCD). The products were extracted by adding chloroform and water to have two phases, with most of the color in the chloroform layer and interface. The upper layer was reextracted with a small volume of chloroform which was added to the first extract. The pooled extracts were mostly
dried under N₂, (a residue of DMSO remained), then redissolved by addition of chloroform for purification by column chromatography as described below.

*Purification of NBD-C12-Cer1P and NBD-C12-Cer1,3P by silica gel column chromatography.* The desired products of the reaction were separated from most of the other components of the reaction mixture by silica gel column chromatography with elution by chloroform and increasing proportions of methanol. Silica gel (Unisil, Supelco) was suspended in chloroform and added rapidly to a small glass column (ca. 1 cm in width x 6 cm in height) with a glass frit until the settled bed of silica gel was approximately 2/3 the volume of the column in height. Then, the column was washed with several mL of chloroform and drained until the solvent reached the top of the column. Examination of the eluate showed that silica was not leaking through the frit, so without allowing the solvent to run dry at the top of the column, the reaction products (dissolved in ~ 1.5 mL of chloroform and sonicated for 10 minutes) were loaded to the column and washed with several mL of additional chloroform. All but a small about of the reaction products was added to the column; this small amount was reserved for visualization on TLC. Fractions of approximately 10 mL each were collected in glass test tubes (16 x 100 mm) from the time that the reaction mixture was added to the column. In each step, 20 mL of the following solvent mixtures (chloroform with increasing % methanol) were added to the top of the column (with care not to disturb the silica) and the eluate from each collected into test tubes; the percentages of methanol were 0%, 1%, 5%, 10%, 17.5%, 25%, 35%, and 50%. Aliquots of each fraction were spotted onto silica TLC plates along with S1P, Cer1P and Cer1,3P standards, and developed with CHCl₃:methanol:H₂O, 60:30:2, v/v/v. After solvent had evaporated from the TLC plates, the NBD-tagged compounds were visualized with UV light (long wavelength mineral light), and the non-fluorescent compounds were visualized by placing...
the plates in a tank containing solid iodine and the rust-colored spots were noted. As expected, DCCD eluted in the early fractions from the column and later fractions (10%, 25%, and 35%) contained essentially pure C12-NBD-Cer1P, a mixture of C12-NBD-Cer1P and C12-NBD-Cer1,3P, and essentially pure C12-NBD-Cer1,3P, respectively. After the solvent was removed from these test tubes under a stream of nitrogen gas, the residues were redissolved in 0.5 mL of CHCl₃:methanol, 1:1, v/v and re-examined by TLC under conditions that better resolve C12-NBD-Cer1,3P from C12-NBD-Cer1P (see below). The 35% eluate was confirmed to be pure (see the lane containing this standard in the supplemental figure). The final yields of C12-NBD-Cer1P and -Cer1,3P were only 5-10%, but this was judged sufficient for chromatographic standards.

**Synthesis of C24-Cer1,3P.** C24-Cer1,3P (N-lignoceroyl-ceramide-1,3-cyclic-phosphate) was synthesized from C24-Cer1P by substituting dry pyridine (Sigma-Aldrich) for DMF as the solvent in the method of (Boudker and Futerman, 1993). C24-Cer1,3P was obtained in ~50% yield and the identify was confirmed by TLC and mass spectrometry.

**Additional information about these methods, including alternatives**

**Alternative substrates for the assay.** C6-NBD-SM can be used as an alternative substrate for some applications of these methods (it is utilized by the brown recluse venom enzyme). C6-NBD-SM and C6-NBD-Cer are available from a number of vendors and C6-NBD-Cer1P is available from Echelon Biosciences (Salt Lake City, UT) (catalog # S- 500N6); therefore, this chain length NBD-Cer1P can be used as an alternative starting material for synthesis of C6-NBD-Cer1,3P, as in the original method of (Boudker and Futerman, 1993). The shorter chain
length NBD-sphingolipids have the advantage of greater solubility in aqueous media, however, that might also make the analogs less natural substrates for SMase D/PLD from some sources.

Natural chain-length SM can also be used as substrates, but usually with detergents or mixtures with other lipids to form liposomes (Gomes et al., 2011). In preliminary analyses, we have compared the NBD-sphingolipid analogs with non-fluorescent sphingolipids with different chain lengths that are available commercially or by the syntheses described above (i.e., with C16- and C24-chain lengths) and they migrated to nearly the same Rf as the C12-NBD-sphingolipids in the first solvent system described in the text (data not shown) (this was not the case for the second solvent system, where the TLC migration differed considerably with chain length). The products can be detected on the TLC plates using a number of methods, including primuline for fluorescence detection (van Echten-Deckert, 2000), or a recently published reagent for sensitive detection of unlabeled lipids (Asressu and Zhang, 2023).

**Alternative methods for thin-layer chromatography.** In addition to the solvent systems described in the main text, the following were also found to resolve Cer1,3P from SM and Cer1P with an Rf of 0.5-0.6 for Cer1,3P: CH$_2$Cl$_2$:ethanol:aq. 2 N NH$_4$OH (40:60:2, v/v/v) and n-butanol:ethanol:aq 2N NH$_4$OH (60:20:2, v/v/v). These might be preferable for some applications.

Another modification that is useful for analysis of a single sample is to use a Sorbtech Rocket TLC chamber (Sorbent Technologies, Inc., Norcross, GA), for which a plastic-backed TLC plate is cut to the dimensions 2.5 x 7.5 cm and only 1 mL of solvent is needed (CHCl$_3$:methanol:aqueous 2N NH$_4$OH, 60:15:1, v/v/v).

**Alternatives to TLC.** The cleavage of NBD-SM by the venoms can also be examined by reverse-phase liquid chromatography with fluorescence detection (for NBD-SM) (Kok et al.,
or by liquid-chromatography, tandem mass spectrometry to follow disappearance of non-fluorescent SM (Shaner et al., 2009) and/or appearance of the 1,3-cyclic ceramide phosphate products (Lajoie et al., 2013).

**Expanded acknowledgements**

Dr. Merrill is grateful to the many undergraduate students who conducted research projects in his lab with the goal of exploring alternative SMase D assays and to apply them to determine if SMaseD activity is found elsewhere than brown recluse spider venom. These preliminary studies were not as successful as the new assay described in this review because Cer1P and Cer1,3P standards were not yet available; nonetheless, the enthusiasm of these students kept interest alive, and contributed to the success of this work. These students were (with apologies to anyone who has been omitted accidentally): Kara A. Boltz, Humera Chaudhary, R. Brett Fields, Courtney Fox, Saudiqa Hoossainy, Catherine Park, Brent Portz and William C. Weldon III. Others in my lab who assisted the undergraduate students were: Jeremy A. Allegood, Samuel Kelly, Rebecca Shaner and Elaine Wang.

**References**


Lachmayr H (2021) Assay development for and evaluation of sphingomyelinase D and associated activities in venoms from Loxosceles reclusa and Kukulcania hibernalis and in isolated soil bacteria, in School of Biological Sciences p 74, Georgia Institute of Technology, Georgia Tech Theses and Dissertations.

