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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ABSTRACT

A special category of phospholipase Ds (PLD) in the venom of the brown recluse spider (*Loxosceles reclusa*) and several other Sicariid spiders accounts for the dermonecrosis and many of the other clinical symptoms of envenomation. Related proteins are produced by other organisms including fungi and bacteria. These PLDs are often referred to as sphingomyelinase Ds (SMase D) because they cleave sphingomyelin (SM) to choline and "ceramide phosphate." The lipid product has actually been found to be a novel sphingolipid: ceramide 1,3-cyclic phosphate (Cer1,3P). Since there are no effective treatments for the injury induced by the bites of these spiders, SMase D/PLDs are attractive targets for therapeutic intervention, and some of their features will be described in this minireview. In addition, two simple methods are described for detecting the characteristic SMase D activity using a fluorescent SM analog, (N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-SM (C12-NBD-SM), that is cleaved to C12-NBD-Cer1,3P, which is easily separated from other potential metabolites by thin-layer chromatography and visualized under ultraviolet light. Besides confirming that C12-NBD-Cer1,3P is the only product detected upon incubation of C12-NBD-SM with brown recluse spider venom, the method was also able to detect for the first time very low levels of activity in venom from another spider, *Kukulcania hibernalis*. The simplicity of the methods makes it relatively easy to determine this signature activity of SMase D/PLD.

SIGNIFICANCE STATEMENT

The sphingomyelinase D/PLDs that are present in the venom of the brown recluse spider and other sources cause considerable human injury, but detection of the novel sphingolipid product, ceramide 1,3-cyclic phosphate, is not easy by previously published methods. This minireview describes simple methods for detection of this activity that will be useful for studies of its
occurrence in spider venoms and other biological samples, perhaps including lesions from suspected spider bites and infections.
Running title: The sphingomyelinase D of spider venom and its assay

ABBREVIATIONS:  C12-, dodecanoyl; C12-NBD-, (N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]-dodecanoyl]-; Cer, ceramide; Cer1P, ceramide 1-phosphate; Cer1,3P, ceramide 1,3-cyclic phosphate; CerPE, ceramide phosphoethanolamine; cLPA, cyclic lysophosphatidic acid; FA, fatty acyl-; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MS, mass spectrometry; NMR, nuclear magnetic resonance; PLD, phospholipase D; PMN, polymorphonuclear leukocytes; Rf, Retention factor; S1P, sphingosine 1-phosphate; SM, sphingomyelin; SMase, sphingomyelinase; TLC, thin-layer chromatography; TopFluor, dipyrrrometheneboron difluoride; UV, Ultraviolet
Introduction

Sphingomyelinases (SMase) cleave one of the two phosphodiester bonds of sphingomyelins (SM), either between the backbone ceramide (Cer) and phosphocholine for SMase Cs, or between the backbone lipid phosphate and headgroup choline for SMase Ds (Fig. 1). These enzymes have long been of interest as therapeutic targets, beginning with acid SMase (a SMase C), which was discovered to be defective in Niemann Pick disease types A and B (Brady et al., 1966; Schneider and Kennedy, 1967) and is still a therapeutic challenge (Pfrieger, 2023). Three classes of SMase Cs are now known--acidic, neutral and basic--and all are of therapeutic interest (Shanbhogue and Hannun, 2020; Xiang et al., 2021) because they play important roles in SM digestion and metabolic homeostasis, modulation of the properties of membranes and lipoproteins, and diverse aspects of cell signaling via Cer and its subsequent metabolites sphingosine, sphingosine 1-phosphate (S1P), and Cer 1-phosphate (Cer1P). SMase Ds have also been considered therapeutic targets since their discovery as toxins produced by pathogenic Corynebacteria (Barksdale et al., 1981; Soucek and Souckova, 1974) and brown recluse spiders (Loxosceles reclusa) (Forrester et al., 1978). SMase D/PLD are also found in venom from other Loxosceles species and related spiders, and account for many or all of the clinical symptoms of bites by these spiders (termed loxoscelism) (Chaim et al., 2006; Gremski et al., 2020; Tambourgi et al., 2010; Tambourgi et al., 1998a). The lipid product of this category of SMase D/PLD was initially thought to be Cer1P, but has been discovered instead to be the novel sphingolipid Cer 1,3-cyclic phosphate (Cer1,3P) (Fig. 1) (Lajoie et al., 2013).

This minireview will give a short overview of the SMase D/PLD of the brown recluse spider, related sicariid species and other organisms, and it will discuss some of the pathological effects of envenomation and how they are thought to arise. It will then compare methods that
have been used to assay the activity of this special category of SMase and describe simple methods that can be used to detect if a biological sample with putative SMase D/PLD activity produces the signature product Cer1,3P.

**Spiders and other organisms that produce SMase D**

The brown recluse spider and dozens of other spiders, with most categorized as species of the genera *Loxosceles* and *Sicarius*, display toxic SMase D/PLD activity among the spectrum of components of their venoms (Binford et al., 2009; Cordes and Binford, 2018; Kuhn-Nentwig et al., 2011). These SMase D/PLDs belong to a protein domain family known as the GDPD-like PLDs (for glycerophosphoryl diester phosphodiesterase, GDPD); furthermore, many cleave phospholipids beyond just SM, as will be discussed in the next section, so are sometimes referred to as toxic PLDs rather than just SMase Ds. Another name that has been used is SicTox (Binford et al., 2009) since *Loxosceles* and *Sicarius* are both in the *Sicariidae* family, broadly termed "sicariid spiders"). There is further subdivision by clade and other features (for example, classes I and II are defined by the presence of a single disulphide bond in class I and two disulphide bonds in class II). Spiders can produce more than one toxin subtype (for example, brown recluse spiders have two toxins designated αIA1 and αIB1) (Binford et al., 2009). Another scheme connotes the toxin from a particular spider species (and other information, such as whether it has been expressed as a recombinant protein) by an abbreviation for the species; for example, the *Loxosceles intermedia* recombinant dermonecrotic toxin is abbreviated LiRecDT (Chaim et al., 2006; da Silveira et al., 2006). For simplicity, this review will refer to this enzyme family collectively as SMase D/PLD.
Sicariid spiders have been found on all continents except Australia (Gremski et al., 2014; Lopes et al., 2020; Vetter, 2015), with the major species for different regions being: in North America, *Loxosceles reclusa* in the south and central United States, *Loxosceles deserta* in central Arizona west to California and south to Mexico, *Loxosceles devia* and *Loxosceles blanda* in Texas, and several others of more limited distribution; in Central and South America, *Loxosceles laeta* (found also in a few areas in California), *Loxosceles intermedia*, *Loxosceles gaucho* and others (South American spiders are generally referred to as "brown spiders"); in several Mediterranean countries, the Mediterranean recluse (*Loxosceles rufescens*); and others for the West Indies, Africa, China and other countries. Most of these spiders are rarely found outside of their region, but *Loxosceles rufescens* has been described as "one of the most invasive spiders of the world," although this has not been declared a call for alarm because it "rarely bites humans and the bites are less harmful than often described" (Nentwig et al., 2017).

There have been occasional observations of apparent SMase D/PLDs in venoms of other spiders, such as *Kukulcania hibernalis*, based on cross-reactivity to an antigenic assay for *L. reclusa* venom (Aran-Sekul et al., 2018; Gomez et al., 2002). However, this is not thought to be a dangerous spider, and an analysis of case reports of five verified envenomations from *Kukulcania* spiders noted that all were with minor manifestations (Vetter, 2022). The reason SMase D/PLDs appear in other spiders is not clear, but might be for digestion of prey (Binford et al., 2009), as highlighted by a recent report of SMase D/PLD in *Uloboridae* spiders, which are the only spider family that has lost its venom glands secondarily during evolution (Valladao et al., 2023).

In addition to spiders, SMase D/PLDs (and homologous genes/peptides) have been found in many other organisms (Cordes and Binford, 2018), although apparently not (so far) mammals
(McDermott et al., 2020). These include bacteria (Flores-Diaz et al., 2016; Mariutti et al., 2017), fungi (Dias-Lopes et al., 2013), and some other arthropods, most notably tick saliva (Alarcon-Chaidez et al., 2009; Rajendran et al., 2021; Regmi et al., 2020) and scorpions (Ben Yekhlef et al., 2020; Borchani et al., 2011). Some of these SMases D/PLDs have been established to have activities similar to the sicariid spiders, but most have not been fully characterized. This distribution information has been given because SMase D/PLDs from different sources have varying degrees of similarities and differences, so this should be borne in mind in approaching the literature. It also creates a complication in attributing a patient's dermonecrotic lesion and other symptoms to envenomation unless the spider responsible for the bite has been accurately identified, which is often not the case (Vetter, 2015). So far, there has been limited success in developing an assay to establish that a patient with the symptoms of loxoscelsism has, indeed, been bitten by a Sicariid spider (Lopes et al., 2020).

**Biochemical properties of the brown recluse SMase D/PLD**

The SMaseD/PLD enzymes have molecular weights of approximately 30 to 35 kDa and have substantial sequence homology, especially with respect to amino acids found in the active site (Murakami et al., 2006). Some of the common features are a TIM-barrel structure (named after triose-phosphate isomerase) comprised of eight $\alpha$ helices and eight $\beta$-strands that alternate along the peptide backbone; two conserved active site histidines that function in acid-base catalysis; and active site carboxyl groups that bind Mg$^{2+}$ (Masood et al., 2018; Moutoussamy et al., 2022).

The SMaseD/PLD enzymes have different substrate specificities related to whether the toxins are categorized $\alpha$- or $\beta$-clade: $\alpha$-clade enzymes have high activity against SM whereas $\beta$-
clade enzymes may have little or no activity with SM (Moutoussamy et al., 2022). *Loxosceles* venoms contain both α and β clade paralogs but venoms from other spiders, in the *Sicarius* and *Hexophthalma* genera, for example, have only β clade enzymes. A comparison of the utilization of SM and ceramide phosphoethanolamine (CerPE) by α and a β clade enzymes from *Loxosceles arizonica* (Lajoie et al., 2015) found that La_αIB2bi displayed one to two orders of magnitude higher activity against SM than CerPE, but La_βID1 showed little discrimination between them (Lajoie et al., 2015). The enzymatic activity with CerPE was at least three orders of magnitude higher than with SM for the β-clade enzyme St_β1B1 from *Sicarius terrosus*.

The utilization of SM and/or CerPE is sensible because both are sphingolipids found in insect prey and other spiders (Panevska et al., 2019). Crude venom and a purified recombinant SMase D/PLD from *Loxosceles arizonica* (abbreviated Laz-SMase D) have been shown to be highly insecticidal (Zobel-Thropp et al., 2012), causing rapid paralysis followed by death of the test insect (crickets) at between 2-3 μg/g insect weight. In addition to the toxic SMase D/PLDs in venom, expression of these enzymes and homologs has been detected in other parts of the spiders, such as saliva and digestive juices, and might have originally functioned in digestion and evolved to be toxic components of venom (Valladao et al., 2023).

Some lysophospholipids are also cleaved by venom PLDs: lysophosphatidylcholine (LPC) (van Meeteren et al., 2004), lysophosphatidylinositol, lysophosphatidylserine, lyso-PAF and cyclic phosphatidic acid (Lee and Lynch, 2005) and lysophosphatidyl ethanolamine (LPE) (Lajoie et al., 2015). Comparison of LPC and LPE with the α- and β-clade enzymes of the preceding paragraph found the same headgroup selectivities--i.e., choline was preferred by α-clade PLD whereas the β-clade enzymes utilize both, or only ethanolamine. One of the factors that accounts for the preference for choline headgroups in the α-clade is a "cage" of three
tyrosines that establish cation-π interactions with the choline (Chaves-Moreira et al., 2023; Moutoussamy et al., 2022). This interaction was seen in both substrate selection and in the binding of the enzymes to liposomes of different composition and molecular dynamics simulations.

In addition to headgroup preferences, SMase D/PLD also display differences in activity depending on the lipid backbones (Chaves-Moreira et al., 2023). Assays of the recombinant SMase D/PLD from L. intermedia venom, LiRecDT1, using SM and LPC with different fatty acyl chains found highest activity with d18:1/C6:0 SM (i.e., an 18-carbon sphingosine with an N-acyl fatty acid of 6 carbons) and decreasing activities as the N-acyl chain increased in length. For lysoPC's, the highest activities were for C12 and C16, and lower for both shorter and longer chains.

As noted above, a distinctive feature of SMase D/PLD's from the brown recluse spider and several other sicariid species (for the cases studied thus far) is that they cleave SM to choline plus Cer 1,3-cyclic phosphate (Cer1,3P) (Fig. 1) (Lajoie and Cordes, 2015; Lajoie et al., 2015; Lajoie et al., 2013). This sphingolipid has not been found, so far, in mammals, but one would presume it appears in wounds of persons bitten by these spiders. Cer1,3P is also produced by SMase D/PLD from some bacteria and fungi (Lajoie and Cordes, 2015), but this has not yet been established for all of the organisms that have SMase D/PLDs or homologous proteins. The lysophosphatidic acid formed from LPC has also been established to be a cyclic phosphate (cyclic lysophosphatidic acid, cLPA or sometimes abbreviated CPA) (Lajoie et al., 2015; Lajoie et al., 2013).

The ability of some PLD to catalyze transphosphatidylation reactions has been known for some time (Selvy et al., 2011), although, in most cases, they occur in the presence of small
primary alcohols that compete with water rather than being the usual products of the enzyme-catalyzed reaction. For those enzymes, an active site His forms a covalent adduct with the headgroup phosphate, displacing choline as the leaving group. Next, water (or an alcohol) is activated by an active site His to hydrolyze the intermediate to the free lipid phosphate. This has also been hypothesized for SMase D/PLD (Fig. 2, Mech #1) (Gremski et al., 2014; Masood et al., 2018; Murakami et al., 2006; Murakami et al., 2005). Fig. 2 also illustrates an alternative mechanism (Fig. 2, Mech #2) that has been proposed for the SicTox SMase D/PLD in consideration of the product being a cyclic phosphate (Lajoie et al., 2015). The authors' diagram illustrated cyclization of lysoPC (Fig. 8F in Lajoie et al., 2015) and we have substituted SM to show that the first step could be for His' to activate the 3-OH of the Cer for attack of the phosphodiester of SM (which is coordinated with Mg$$^{2+}$$ and an active site lysine), with His" providing a proton for the leaving group oxygen of choline. For both of these mechanisms, formation of Cer1,3P rather than Cer1P depends on a more favorable active site orientation of the intramolecular hydroxyl than an exogenous water molecule (and the analogous situation for production of cLPA from lysoPC).

There are precedents among the bacterial PLD for enzymes that make both cyclic and monoester products. SMase D/PLD of *Streptomyces chromofuscus* (which has a broad substrate specificity encompassing SM, PC, lysoPC and other phospholipids) (Imamura and Horiuti, 1979; Martin et al., 2000) provides an interesting example of an enzyme that ultimately forms lysophosphatidic acid (LPA), but first produces the cyclic intermediate cLPA that is subsequently hydrolyzed (Friedman et al., 1996). The ring opening hydrolysis reaction was also found when a synthetic cLPA was added to the enzyme. The authors noted that their synthetic cLPA (1-octanoyl 2,3-cyclic glycerophosphate) was stable at pH 6 – 8 for at least 10 h.
(Friedman et al., 1996). Although cyclic phosphates might be chemically unstable, a fluorescent Cer1,3P analog (C12-NBD-Cer1,3P) was also reported to remain intact, even when incubated with mammalian cells in culture. This suggests that Cer1,3P are relatively chemically stable and that mammalian cells do not have enzymes to cleave them (Boudker and Futerman, 1993).

**Characteristics of loxoscelism and roles of SMase D/PLD**

Loxoscelism is the term for the clinical manifestations of envenomation by numerous *Loxosceles* and related spiders. It can be mild to very severe, and is usually divided into two categories that are described as cutaneous versus systemic (da Silva et al., 2004; Swanson and Vetter, 2006) or cutaneous versus cutaneous-hemolytic/cutaneous-visceral loxoscelism (Lopes et al., 2020). The latter nomenclature appears to give emphasis to the fact that some patients have systemic effects in addition to the dermonecrotic lesion. Cutaneous loxoscelism has been estimated to account for between 70% (or more) (Gremski et al., 2022) to 84 to 97% (Manzoni-de-Almeida et al., 2018) of cases. It usually appears as a slowly progressing lesion that may be associated with pain, edema, erythema, and sometimes nonspecific systemic symptoms such as headache, vomiting, nausea, diffuse rash and fevers, and may evolve into dermonecrosis with gravitational spreading of the lesion (Lopes et al., 2020). Other names for the cutaneous form are "Dermonecrotic Loxoscelism" and "Gangrenous Arachnidism" (Antunes et al., 2022). Systemic (cutaneous-hemolytic/cutaneous-visceral) loxoscelism is less common (ranging from about 1 to 27% of victims depending on *Loxosceles* species and other factors) (Gremski et al., 2022) and symptoms include intravascular hemolysis, disseminated intravascular coagulation, systemic inflammatory response syndrome, thrombocytopenia, platelet aggregation,
nephrotoxicity and sometimes cardiotoxicity, and can result in death (Lopes et al., 2020; Vetter, 2015).

**Pathophysiological mechanisms**

Although venom is comprised of many enzymatic activities that probably contribute to some of the pathophysiology of envenomation (hyaluronidases, metalloproteases, serine proteases, knottins, serpins, allergens and others) (Gremski et al., 2021), it is asserted (Gremski et al., 2020) "...that the data described in the literature are quite strong and prove the participation of *Loxosceles* venom PLDs as molecules involved in the dermonecrotic, inflammatory, hemolytic, nephrotoxic and thrombocytopenic effects described in the injured patients".

Evidence for this connection includes the ability of purified SMase D/PLD to recapitulate many of the symptoms of whole venom, loss of toxin potency when active site amino acid(s) that are necessary for SMase D/PLD activity are missing (due to genetic differences between species or by preparation of mutant recombinant enzymes) and neutralization of SMase D/PLD activity with antibodies.

Cutaneous loxoscelism has been attributed to the SMase D/PLD induced activation of blood vessel endothelial cells so they express E-selectin and secrete granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-8 (IL-8/CXCL8), which results in a massive and persistent infiltration and activation of neutrophils (Patel et al., 1994) concomitant with dissociation of the collagen fibers in the dermis (Tambourgi et al., 2005). Venom SMase D/PLD also induces IL-6, IL-8, CXCL1/GRO-α, and CCL2/MCP-1 production by skin fibroblasts and migration of monocytes that release additional inflammatory mediators (IL-6, MCP-1 and others) (Dragulev et al., 2007; Rojas et al., 2017). The net effect of this
inflammation and tissue damage is a difficult to heal dermonecrosis. The earliest mediators of the downstream events are not yet known, but have been speculated to be the bioactive metabolites of SM and lysoPC that are produced by SMase D/PLD (Dragulev et al., 2007; Rojas et al., 2017).

Systemic loxoscelism also involves inflammation (de Oliveira-Lima et al., 2016; Tambourgi et al., 1998b), but its hallmark (Lopes et al., 2020) is intravascular hemolysis which releases free hemoglobin and can lead to nephrotic acute tubular necrosis. The hemolytic effects of SMase D/PLD in vivo have been studied in depth because they can be recapitulated with red blood cells in vitro. The conversion of SM to Cer1,3P disrupts lipid bilayer morphology and integrity (Stock et al., 2012) and changes membrane asymmetry with exposure of phosphatidylserine to the cell surface (Tambourgi et al., 2002; Tambourgi et al., 2010; van den Berg et al., 2012). These disruptions trigger complement-dependent hemolysis via classical and alternative pathways (Tambourgi et al., 2002; Tambourgi et al., 2005).

But what specifically happens as SM is converted to Cer1,3P (and, perhaps, likewise conversion of lysoPC to cLPA) to initiate these responses? In the case of inflammation, the discussion (Rivera et al., 2015) has focused mainly on the signaling pathways for Cer1P and its bioactive metabolites, Cer, sphingosine and sphingosine 1-phosphate (Hannun and Obeid, 2018). This is an attractive hypothesis because processes that are known to be modulated by Cer1P include inflammation, cell growth and survival, cell migration (Rivera et al., 2015) and more recently inhibition of wound healing (Berwick et al., 2019) and stimulation of MMP-1 and MMP-3 expression (Shin et al., 2022). These might be involved, but the finding that the product of the SMase D/PLD reaction is Cer1,3P (Lajoie et al., 2013) necessitates that either Cer1,3P is able to interact with Cer1P receptors and/or other partners, or that Cer1,3P somehow alters the
levels of endogenous Cer1P. It isn't known if Cer1,3P can serve as an agonist or antagonist for Cer1P targets, although either (or both) might occur. Mammals do not appear to have an enzyme that cleaves Cer1,3P to Cer1P (Boudker and Futerman, 1993), nor does venom (see below); therefore, once Cer1,3P is produced, it would be expected to persist for a long time rather than be converted to Cer1P. High amounts of Cer1,3P might elevate Cer1P, nonetheless, by another mechanism: it is a weak inhibitor of Cer1P hydrolysis (Boudker and Futerman, 1993) and might suppress turnover of endogenous Cer1P, which has been found in relatively high amounts in skin (Yamashita et al., 2016).

In addition to Cer1,3P, cyclic LPA is also produced by SMase D/PLD from lysoPC, a lysophospholipid regularly present in circulation (Fujiwara, 2008). The product cLPA is anti-mitogenic, induces stress fiber formation, inhibits tumor cell invasion and metastasis, promotes differentiation and survival of cultured embryonic hippocampal neurons and inhibits LPA-induced platelet aggregation. The potential roles of these cyclic lipid mediators deserve further investigation.

**Therapeutic strategies**

Two general approaches have been taken for treatment of loxoscelism: treat the symptoms as they progress, and attempt to block the SMase D/PLD activity. The therapeutic strategy also depends on whether the patient exhibits cutaneous or systemic loxoscelism, or both. Although there is some consistency in treatment protocols, definitive therapeutic approaches have not yet been established in the medical literature (Calhoun et al., 2022), and treatments vary depending on the region of the world, with most of the envenomizations occurring in South America, the United States, and occasionally in the Mediterranean region (Malaque et al., 2022)
(furthermore, some treatments also have risk of deleterious effects) (Tambourgi et al., 2010). In one survey (Lopes et al., 2020), the most frequently used drugs were antibiotics (66%), corticosteroids (35%), antihistamines (11%) and dapsone (9%); other therapies included transfusion (23%), surgical debridement (13%), skin graft (9%), dialysis (5%), antivenom therapy (2.5%), hyperbaric oxygen (2.5%), therapeutic plasma exchange (1.7%) and negative pressure wound therapy (1%). An added complication is that diagnosis of loxoscelism is difficult, and patients with similar presentations are often misdiagnosed (Swanson and Vetter, 2006).

For cutaneous loxoscelism, many patients in some South America countries are treated with antivenom, which animal studies indicate as the best therapy when administered within the first few hours after envenomation (Pauli et al., 2009). Since most patients are not seen by physicians until much later, this practice is currently considered controversial and not performed in the United States of America (da Silva et al., 2021). Nonetheless, a prospective observational study of patients suggested that this reduces necrosis (Malaque et al., 2022), which might reflect inhibition and/or accelerated elimination of the toxins. To address the complication that antivenom production is expensive using native venom SMase D/PLD, an alternative has been to use a chimeric protein that contains four immunodominant epitopes from *L. intermedia* and *L. laeta* SMase D/PLD (Souza et al., 2018). An immunization approach is particularly attractive in areas where there is a high risk of being bitten, and as proof of concept, immunization of rabbits with inactive recombinant PLDs (due to active site mutations) reduced edema and dermonecrosis caused by *Loxosceles intermedia* crude venom (Antunes et al., 2022).

Antibiotics are administered in a high percentage of cases because secondary infections are of concern, and because many lesions are initially suspected to be due to infection rather than...
envenomation by a *Loxosceles* spider (Lopes et al., 2020). Corticosteroids are commonly administered, but have limited benefit in cutaneous loxoscelism (Sams et al., 2001), and are more standard for hemolytic anemia and to protect the kidney (Calhoun et al., 2022). Other treatments that are sometimes applied include administration of dapsone to reduce activation of polymorphonuclear leukocytes, PMN (although there is a risk of hemolysis), antihistamines to reduce platelet aggregation and ischemia, hyperbaric oxygen therapy to reduce PMN recruitment and activation as well as promote healing in ischemic nonhealing wounds (and perhaps inactivate SMase D/PLD by oxidizing sulfhydryl bonds), electric shock therapy, surgical excision, and vacuum-assisted wound closure (Swanson and Vetter, 2006; Tambourgi et al., 2010). For systemic loxoscelism, blood transfusion and plasma exchange therapy may be used to address hemolysis and hematological alterations to reduce the risk of clinical shock and renal failure, and dialysis may be used when there is evidence of severe renal failure (Gremski et al., 2022). Tetracycline is also being tested because it reduces renal cell death and synthesis and secretion of metalloproteinases (MMP-2 and MMP-9) (Okamoto et al., 2017).

Because SMase D/PLD is thought to play important roles in both cutaneous and systemic loxosceliam, there is considerable interest in finding inhibitors of its activity. Some of the compounds that have been found are: Suramin, Vu0155056, and Vu0359595, which inhibit SMase D/PLD and reduce the hemolytic, dermonecrotic, and inflammatory activities of the venom toxin in biological assays (Chaves-Moreira et al., 2017), and 6-amino-2-((4-cyanobenzyl)thio)pyrimidin-4-yl 4-methylbenzenesulphonate; 4-bromo-N-[(E)-(2-methyl-1H-indol-3-yl) methyleneamino] benzenesulfonamide and 4-methyl-3-oxo-2-(3-pyridylmethylene)benzo[3,4-b]furan-6-yl 4-chlorobenzenesulphonate which inhibit activity and enzyme binding to the surface of human erythrocytes (the middle compound was most effective.
at inhibiting *Loxosceles* venom-induced dermonecrosis in rabbits) (Lopes et al., 2019). Diethyl azelate is another inhibitor of SMase D/PLD that has been suggested for treatment of brown recluse spider bites (Streeper and Izbicka, 2022).

**Methods that have been used to assay SMase D/PLD activity**

Early studies of venom utilized thin-layer chromatography (TLC) to demonstrate that brown recluse spider venom has a SMase activity that produced a more polar product (i.e., "ceramide phosphate") instead of Cer, and thus fell in the PLD family (Forrester et al., 1978). Most of the subsequent studies used easier assays that followed release of the product choline using commercially available kits, such as the Amplex™ Red Phospholipase D Assay Kit, (Molecular Probes/Invitrogen, Eugene, OR)(Binford et al., 2009; Chaves-Moreira et al., 2023; Mariutti et al., 2017; Zobel-Thropp et al., 2012). Another assay (Felicori et al., 2006; Young and Pincus, 2001) utilized TNPAL-SM (trinitrophenylaminolauryl-SM), which is cleaved to release TNPAL-Cer that is extracted into a heptane-rich phase for detection of its absorbance (Gatt et al., 1981).

Immunoaassays have also been utilized (Gomez et al., 2002) and 40 ng of *Loxosceles reclusa* venom was detectable in swabs of skin from rabbits for two weeks and as long as 21 days in some cases after being injected subcutaneously (McGlasson et al., 2009). When higher amounts of venom were assayed (2,000 ng), venoms from *Scytodes fusca* and *Kukulcania hibernalis* also cross-reacted with the assay (as did several other venoms at much higher amounts). These findings are not surprising now that SMase D/PLD is thought to be present for digestion as well as envenomation (Valladao et al., 2023). More surprising was the finding of heterophilic antibodies capable of generating a cross-reaction against the venom of *L. laeta* and
Sicarius spiders in people without contact with spider venom (Aran-Sekul et al., 2018), which the authors caution might interfere with immunoassays intended to detect Loxosceles venom. Assuming that these people had not been bitten without their knowledge, they note that these results might indicate that humans have antibodies against similar PLDs that are produced by other pathogenic organisms (Dias-Lopes et al., 2013).

Therefore, facile enzymatic assays based on detection of the lipid product(s) of SMase D/PLD would help establish that activity is present as well as whether or not Cer1,3P is made. Such analyses can be conducted by mass spectrometry (MS) and NMR (Lajoie et al., 2013), but the equipment and expertise is not available to all laboratories. A simple alternative would be to use an assay based on production of fluorescent lipid products that can be detected by TLC, as has been available for SMase Cs for over forty years (Gatt et al., 1981) and more recently with a commercially available substrate (C12-NBD-SM) and product (C12-NBD-Cer) standard (Loidl et al., 2002). We are not aware that this approach has been used for SMase D's, perhaps due to the lack of many standards for NBD-Cer1P and the absence of any for NBD-Cer1,3P. However, relatively straightforward methods for synthesis of these compounds are in the literature (Boudker and Futerman, 1993; Futerman and Pagano, 1992) (see Supplemental Information). Using these and other reagents (see below), TLC conditions were developed (Lachmayr, 2021) to resolve C12-NBD-Cer1,3P from the other possible metabolites of C12-NBD-SM by capitalizing on the difference in charge between Cer1,3P (-1) and Cer1P (-2) at basic pH. There was already a precedent for this type of TLC solvent in a study that used egg SM as the substrate and analyzed the products using silica TLC plates developed with CHCl₃:methanol:methylamine (65:35:10, v/v/v) (Vuitika et al., 2016). Although standards were not used to verify the identity of the product in that study, the Rₚ was similar to the method that we describe below, so it was
probably Cer1,3P. A basic solvent has also been used to study the production of lysoPA from lysoPC (Chaim et al., 2011).

In addition to this new TLC method, there is a published method for analysis of Cer1P (Wijesinghe et al., 2007) that we have found to also separate C12-NBD-Cer1,3P from C12-NBD-Cer1P and C12-NBD-SM (see below); therefore, we conclude this brief review with a description of this assay and these two TLC methods for examining the products.

**Simple methods for detecting ceramide 1,3-cyclic phosphate production**

**Materials and Methods**

**Materials.** The sources for the fluorescent sphingolipids were: Avanti Polar Lipids (Alabaster, AL) for C12-NBD-SM (N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-D-erythro-sphingosine 1-phosphorylcholine, catalog # 810219), C12-NBD-Cer (catalog #810211), C11-TopFluor-Cer1P (N-[11-(dipyrrometheneboron difluoride)undecanoyl]-D-erythro-sphingosine-1-phosphate, ammonium salt, catalog # 810270) and NBD-dodecanoic acid (C12-NBD-fatty acid) (as a special order without catalog #); and C12-NBD-Cer1P and C12-NBD-Cer1,3P were synthesized from sphingosine 1-phosphate (Avanti, catalog # 860492) and C12-NBD-fatty acid by modifying a previous method for making NBD-sphingolipids (Futerman and Pagano, 1992), as described in Supplemental Information (which also contains information about possible alternatives for some of the materials and methods described here).

Thin-layer chromatography (TLC) plastic sheets (Silica gel 60, #105719) were from EM Science (Darmstadt, Germany). The TLC plates were cut to the dimensions 10 x 10 cm and stored in a dessicator box until use. Solvents were obtained from these suppliers: HPLC-grade
chloroform (#EM-CX1050), methanol (#EM-MX0475) and ACS grade glacial acetic acid (#BDH3092) were from VWR (West Chester, PA); acetone (AX0115) and ammonium hydroxide were from Millipore-Sigma (#1303) and the latter was diluted to 2N with deionized water; and ethanol (absolute for analysis EMSURE® ACS,ISO,Reag. Ph Eur) from Sigma-Aldrich.

Lyophilized venoms from *L. reclusa* and *K. hibernalis* were obtained from Spider Pharm (Yarnell, AZ) and redissolved in HEPES buffer from Teknova (Hollister, CA).

**Preparation of stock solutions of the fluorescent sphingolipids.** Stock solutions of the fluorescent sphingolipids were prepared in ethanol and the concentrations were estimated from their absorbance using the Beer-Lambert law and a molar extinction coefficient for NBD of ~25,000 M$^{-1}$cm$^{-1}$ at 480 nm, as cited in (Ladokhin et al., 2002), and for TopFluor of ~97,000 M$^{-1}$cm$^{-1}$ at 495 nm using technical information provided by Avanti (https://avantilipids.com/news/tech-talks/excitation-emission-d-extinction). All procedures with fluorescent compounds were conducted in dim light to minimize photodecomposition.

**Thin-layer chromatography.** The TLC conditions for optimal separation of C12-NBD-Cer1,3P from the other sphingolipids of interest are shown in Fig.3. Samples (in 5 to 10 µL of solvent) were applied to the plate as narrow lines 1 cm from the bottom using a Hamilton syringe. One lane (labeled "C" for "cocktail") contained a mixture of C12-NBD-SM, C12-NBD-Cer1,3P, C12-NBD-Cer and C12-NBD-fatty acid and C12-NBD-Cer1P (panel B) or the same NBD-standards except C11-TopFluor-Cer1P was used in place of C12-NBD-Cer1P (labeled C' in panel A). This substitution was made because the migration of C12-NBD-Cer1P overlaps that of C12-NBD-SM too much to be distinguished in a mixture (see asterisk in Fig.3A), whereas, the different fluorescent color of C11-TopFluor-Cer1P (which migrates slightly higher) allows it's
position to be seen despite the overlap (see Fig. 3A and 4A). After evaporation of the application solvent, the plates were placed in a Twin Trough Chamber with a stainless steel lid (catalog #022.5155 for 10 x 10 cm plates, CAMAG, Wilmington, NC) that had been pre-equilibrated with 10 to 18 mL of freshly prepared developing solvent (depending on whether one or both wells of the TLC tank are used): CHCl₃:methanol:aqueous 2N NH₄OH; 60:25:2, v/v/v) for panel A and CHCl₃:acetone:methanol:glacial acetic acid:distilled-deionized water; 10:4:3:2:1, v/v/v/v/v) for panel B. When the solvent had migrated within several mm of the top of the plate, plates were removed from the tank and air dried. The fluorescent spots were detected using a long-wavelength ultraviolet mineral light and the images were photographed with a cell phone camera.

**Incubations of venoms with NBD-SM.** The substrate and venoms were prepared as follows: C12-NBD-SM was added to a 13 x 100 mm borosilicate glass test tube as an ethanol solution, the solvent was evaporated under a stream of nitrogen to provide a thin coating of the bottom of the test tube, followed by placing the test tube in a dessicator under vacuum for several h to remove any residual solvent. Enough 50 mM HEPES buffer (pH 7.5) was added to the test tube to yield a final concentration of 2.4 mM C12-NBD-SM, and the buffer was gently swirled over the lipid film to hydrate it. After ~15 min, the test tube was vortexed and sonicated in a warm water bath (~37 °C) for ~10 min to disperse the C12-NBD-SM as a milky yellow suspension, from which aliquots were removed quickly for assays. If some of the C12-NBD-SM remains attached to the test tube, it can be dislodged with a glass rod so all of the substrate will be suspended by sonication. Just prior to use, 5 µL of freeze-dried *L. reclusa* or *K. hibernalis* venom was redissolved at 1:20 and 1:10 dilutions, respectively, with ice-cold 50 mM HEPES buffer (pH 7.5).
To begin the assay, 10 µL of substrate and 10 µL of diluted venom were mixed in microEppendorf tubes and incubated at room temperature for 1 h or longer, if detection of very weak activities is desired. For multiple timepoints, extra tubes can be prepared or the volume of the assay increased and aliquots removed at each timepoint. For our analyses, incubations were conducted for 1 h or overnight, then 100 µL of ethanol was added to stop the reaction, and 10 µL from each venom incubation were spotted onto two adjacent lanes of a 10 x 10 cm silica-gel plate (see Fig. 4A). After the solvent had dried, one of these lanes was co-spotted with 10 µL of the standards cocktail and an adjacent lane was spotted with the incubation mix (everything minus venom, to see where the substrate SM migrates) and one lane with the standards cocktail alone (to compare with the co-spotted standards and products of the incubation with venom). After the solvent had evaporated, the plate was either developed with the solvent CHCl₃:methanol:2N NH₄OH (60:25:2, v/v/v) or CHCl₃:acetone:methanol:glacial acetic acid:distilled-deionized water (10:4:3:2:1, v/v/v/v/v). The fluorescent spots were detected using a long-wavelength ultraviolet mineral light and the images were photographed with a cell phone camera.

Results and Discussion

The TLC solvent system shown in Fig. 3A provides good separation of C12-NBD-Cer1,3P (Rf 0.56) from the fluorescent Cer1P and SM standards (Rf between 0.05 and 0.1) from the less polar C12-NBD-FA (Rf 0.81) and C12-NBD-Cer (Rf 0.85). However, this system does not separate C12-NBD-SM and C12-NBD-Cer1P (which migrates at the position of the asterisk in Fig. 3A, data from a separate plates, not shown). Instead, a fluorescent standard with a different color (C11-TopFluor-Cer1P) was used to visualize the approximate migration of Cer1Ps and verify their separation from C12-NBD-Cer1,3P despite the presence of C12-NBD-
SM (this will be most important for the enzymatic assays described below). The other TLC system (Fig. 3B), which was optimized for Cer1Ps (Wijesinghe et al., 2007), gave good separation of C12-NBD-Cer1,3P (Rf 0.58) from C12-NBD-Cer1P (Rf 0.37) and C12-NBD-SM (Rf 0.21) as well as C12-NBD-FA (Rf 0.90) and C12-NBD-Cer (Rf 0.88), although the latter two are not resolved well enough to be distinguished in a mixture.

Thus, either of these systems can be used to detect C12-NBD-Cer1,3P but the first has the advantage of a greater distance between C12-NBD-Cer1,3P and the substrate -SM and the product that might be produced by some categories of PLD, -Cer1P. It will additionally detect if a SMase C activity is present, since C12-NBD-Cer is clearly resolved. A limitation of the first system is that it will not rule out the production of C12-NBD-Cer1P unless the C12-NBD-SM has been completely consumed. To evaluate that possibility, the products can be re-analyzed using the second system.

Results from incubation of C12-NBD-SM with the venoms from two spider species and analysis using the first solvent system are shown in Fig. 4A. Incubation of L. reclusa venom (0.5 μL) with 2.4 nmol of C12-NBD-SM for 1 h effected an essentially complete conversion of the substrate to C12-NBD-Cer1,3P (c.f. the left two lanes of Fig. 3). Note that the third lane is the reaction mixture co-spotted with a mixture of standards, so the presence of the venom, buffer, etc. in the incubation mixture did not have a substantial effect on migration of the products. Furthermore, the venom does not appear to contain additional enzymes that degrade C12-NBD-SM or C12-NBD-Cer1,3P to other fluorescent products under these assay conditions.

Since there have been several indications in the literature that venom from Kukulcania hibernalis might contain SMase D/PLD (as discussed above) (Aran-Sekul et al., 2018; Gomez et al., 2002), the same incubation was conducted using 1 μL of venom from that species. The two
right lanes of Fig. 4A show that *K. hibernalis* venom also effected some cleavage of C12-NBD-SM to NBD-Cer1,3P. Thus, there is detectable SMase D/PLD activity in *K. hibernalis* venom, but it is obviously much lower than that of *L. reclusa* venom.

There was no evidence for production of C12-NBD-Cer nor NBD-FA from C12-NBD-SM by the *K. hibernalis* venom, but since the Kh venom did not hydrolyze all of the C12-NBD-SM in the timeframe of this assay, one cannot rule out the possibility that there might also have been production of C12-NBD-Cer1P. Therefore, the assay with *K. hibernalis* venom was left to proceed overnight then the reaction products were examined in the second TLC system (Fig. 4B). This was sufficient time for the venom to convert nearly all of the C12-NBD-SM to C12-NBD-Cer1,3P and there was only a trace of fluorescence migrating at the position for C12-NBD-Cer1P. Therefore, the major product of the *K. hibernalis* SMase D/PLD is Cer1,3P, similar to the enzyme in *L. reclusa* venom.

Preliminary investigations of venoms from other species of spiders (including several from *Lycosoidea* and *Theraphosidae*) using these incubation conditions did not detect the production of fluorescent metabolites from C12-NBD-SM. Therefore, as is already thought (Vetter, 2015), it does not appear that all spiders have easily detectible SMase D/PLD activity in venom.

These results illustrate that it is relatively easy to look for C12-NBD-Cer1,3P production by SMase D/PLD's using these methods. They should also be useful for quantitative analyses after the types of optimizations described for the assay of SMase C using C12-NBD-SM (Loidl et al., 2002). An important caveat is that the assay is based on a fluorescent SM analog that might not be accommodated by all enzymes. Since most investigations will probably already have evidence for SMase D activity using one of the easier assays (such as ones that follow
release of choline), those assays could be used to determine if choline is also released from the fluorescent SM analogue, and if so, examine the lipid products by TLC. If a SMase D/PLD is encountered that does not have activity with the fluorescent SM analog, it is likely that the assay could be conducted with a natural alkyl chain SM (see comments in Supplemental Information) with detection of the products after TLC using a sensitive reagent such as primulin (van Echten-Deckert, 2000). Co-migration of unknowns and standards in two different TLC solvent conditions is considered strong evidence for identification, but the ultimate proof of the identity of the products would be analysis by mass spectrometry and/or NMR (Lajoie et al., 2013).

**Closing comments**

SMase D/PLD is a fascinating and important family of enzymes for many reasons, but one of its most intriguing features is the production of Cer1,3P-- a highly unique sphingolipid that plausibly might be an important determinant of the pathophysiologic effects of this enzymatic activity. The TLC assay described in this review provides an easy method to determine if SMase D/PLD produces Cer1,3P or Cer1P (or both) without having to rely on more sophisticated instruments (MS and NMR) (Lajoie and Cordes, 2015; Lajoie et al., 2015; Lajoie et al., 2013). This would help investigators determine if Cer1,3P is made by the many SMase D/PLDs that have not yet been characterized as well as explore if there are cases where the product changes from Cer1,3P to Cer1P when enzymes are mutated *in vivo* or by genetic manipulation in the lab. One can easily envision how subtle structural changes in a SMase D/PLD could enable water to become positioned near the active site histidine (His' in Fig. 2, Mech #2) such that water becomes the activated nucleophile instead of the 3-hydroxyl- of Cer, and thereby produce Cer1P instead of Cer1,3P.
It is highly plausible that Cer1,3P plays a profound role in some of the events triggered by envenomation since it resembles another sphingolipid signaling molecule, Cer1P, and might be an agonist or antagonist of Cer1P signaling. Although there have been no published studies of the biological activity of Cer1,3P at the time this article was written, two highly relevant findings are that Cer1,3P is an inhibitor of Cer1P phosphatase, and itself appears to be stable in mammalian cells (Boudker and Futerman, 1993). Therefore, if mammals do not have an enzyme that can cleave the cyclic phosphate, Cer1,3P could reach high amounts and be very long lived in skin, blood and internal organs, which might account for the long-term symptoms in persons who have been bitten. Therefore, it would be useful to know which SMase D/PLDs produce Cer1,3P.

Because a fluorescence assay is both simple and sensitive, it might be able to detect SMase D/PLD activity in spider bites in a field or clinical setting. *Loxosceles* bites are barely noticed initially and the spider is rarely captured (Gremski et al., 2014), so there is a need for an easy method of detecting envenomation before patients develop symptoms that are sufficiently severe for them to contact a physician. A collateral finding might be that clinical severity is related to the presence of a SMase D/PLD that produces Cer1,3P regardless of whether it is from venom or bacterial or fungal infection, and thus help explain why many dermonecrotic lesions are incorrectly diagnosed as loxoselism (Vetter, 2015). Indeed, if the most important determinant of clinical symptoms is the production of Cer1,3P from SM by SMase D/PLDs, perhaps the better descriptor for these disorders, irrespective of whether they are cause by spider bites or infection, would be "SMase D/PLD toxicity syndrome."
Authorship Contributions

*Participated in research design:* Lachmayr and Merrill.

*Conducted experiments:* Lachmayr and Merrill.

*Contributed new reagents or analytic tools:* Merrill.

*Performed data analysis:* Lachmayr and Merrill.

*Wrote or contributed to the writing of the manuscript:* Lachmayr and Merrill.

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Data availability. The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data. If additional information is needed, please contact the corresponding author.
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Footnotes:

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Figure legends

**Fig. 1.** Distinction of the potential cleavage products of SMase C versus SMase D. R₁ and R₂ represent varying alkyl chain or other substituents (such as fluorophors).

**Fig. 2.** General schemes for the possible roles of the two active site histidines and other active site groups in formation of Cer₁,₃P from SM by SMaseD/PLD. In the first mechanism (Mech #1), based on analogy with other PLD, His’ attacks the SM phosphodiester (dotted arrow) to displace choline followed by displacement of His’ by water (not shown). In a mechanism that accounts for the production of Cer₁,₃P (Mech #2), His’ activates the 3-OH of the Cer for attack of the phosphodiester (solid arrow) and His” protonates the leaving group oxygen of choline. Mg²⁺ and an active site lysine assist by coordinating with the phosphodiester. R₁ and R₂ refer to the alkyl chains of the sphingoid base and fatty acyl moieties of Cer. This scheme was inspired from the suggested mechanism (Mech #2) for cyclization of lysoPC by SicTox enzymes (Fig. 8F in Lajoie et al., 2015).

**Fig. 3.** Separation of fluorescent sphingolipids relevant to cleavage of SM by SMase D by thin-layer chromatography. The sphingolipid standards were prepared as ~10 μM stocks of the individual compounds in ethanol plus a mixture of all five, and approximately 10 μL of each was spotted on two 10 x 10 cm plastic-backed TLC plates coated with Silica gel 60, as shown in the figure. C and C’ refer to a cocktail mix of all of the other internal standards on each plate (with the latter having C₁₁-TopFluorCer₁P instead of C₁₂-NBD-Cer₁P). After the plates were developed with the shown solvent mix, they were removed from the tank, air dried, and fluorescence was detected using a long wavelength UV mineral light. The images were photographed with a cell phone camera. The yellow asterisk indicates where C₁₂-NBD-Cer₁P would migrate in the system in panel A (from on a separate plate).
Fig. 4. Visualization of the production of NBD-Cer1,3P from NBD-SM by venoms from L. reclusa (Lr) and K. hibernalis (Kh). The assay was conducted by mixing 10 µL of an emulsion of 2.4 mM C12-NBD-SM in 50 mM HEPES buffer (pH 7.5) with 10 µL of buffer or venom (Lh at 1:20 dilution; Kh at 1:10 dilution) and incubated with gentle shaking at 37 °C for 1 h (Panel A) and panel B shows a 24 h incubation of Kh venom in the alternative solvent system. After each incubation, 100 µL of ethanol was added and 10 µL of each incubation mixture was spotted on a 10 x 10 cm plastic-backed TLC plate coated with Silica gel 60, as shown in the figure. The other lanes of the plate contained the same standard cocktails as Fig. 3 spotted individually and co-spotted over one lane for each venom incubation. After the plates were developed with indicated solvents, they were air dried and fluorescence was detected using a long wavelength UV mineral light. The images were photographed with a cell phone camera. The lane labeled "SM Pre" in panel A reflects the incubation cocktail with only buffer.
Fig. 1

Phosphohydrolase vs Transphosphatidylation

SMase C

SMase D

Cer

Cer1P

Cer1,3P

Choline-P

Choline
Fig. 2
Developing solvents:

CHCl₃:Methanol:2N NH₄OH (60:25:2)

CHCl₃:Acetone:Methanol:Acetic acid:H₂O (10:4:3:2:1)

Origin

Solvent front

Standards applied

Fig. 3
Developing solvents:
CHCl₃: Methanol: 2N NH₄OH
(60:25:2)
CHCl₃: Acetone: Methanol: Acetic acid: H₂O
(10:4:3:2:1)

Samples and standards applied
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
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-benzoazol-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-benzoazadiazol-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-benzoazadiazol-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 identity 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.,
1997), 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).

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