SUPPLEMENTARY MATERIAL

Full title: Substrate Specificity of the Neutral Sphingomyelinase from *Trypanosoma brucei*

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Running title: *T. brucei* neutral sphingomyelinase substrate specificity

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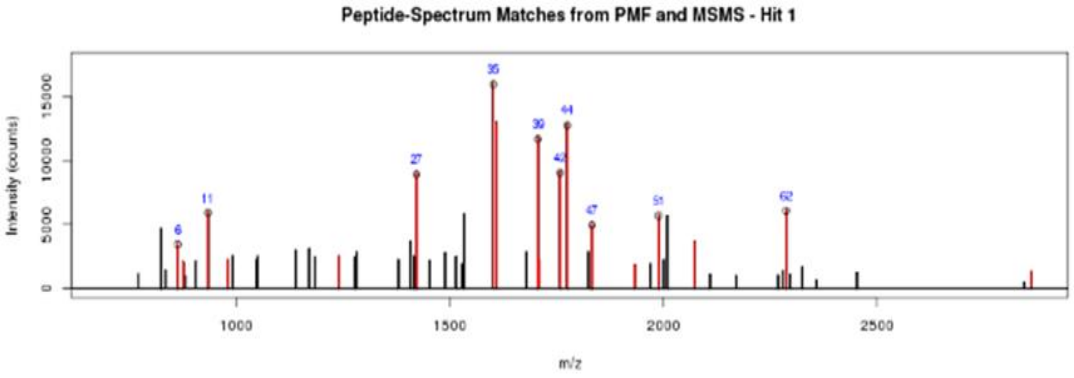
Name: neutral sphingomyelinase [Trypanosoma brucei brucei]
Accession: gi|222350157 Score: 483 Mass: 65798.13 Da



Query	m/z	Delta	E-value	Missed	Ions Score	Sequence (Modifications)
5	862.4139	0.0151	NA	0	0	GHGYCLR
6	862.4139	0.0151	NA	0	14.91	GHGYCLR
7	875.5460	0.0077	NA	0	0	TMLILLR (Oxidation (M))
10	933.5671	0.0155	NA	1	0	RIEQFLK
11	**933.5671**	**0.0155**	**4.03e+02**	**1**	**27.14**	**RIEQFLK**
12	979.5511	0.0708	NA	1	0	NGSSGKVSSR (Deamidated (NQ))
19	1238.6154	0.0143	NA	0	0	DTFSLNLEGSR
26	1421.7278	0.0106	NA	1	0	DWRDEVYLAVR
27	**1421.7278**	**0.0106**	**7.71e-02**	**1**	**65.33**	**DWRDEVYLAVR**
34	1601.8417	0.0095	NA	0	0	YPTAFYGSGIAVISR
35	**1601.8417**	**0.0095**	**2.02e-06**	**0**	**111.24**	**YPTAFYGSGIAVISR**
36	1608.8765	0.0061	NA	0	0	GTAGVRPVPLGEQEAK
38	1705.9209	0.0090	NA	0	0	IISDQITELGNSFLR
39	**1705.9209**	**0.0090**	**3.92e-04**	**0**	**87.69**	**IISDQITELGNSFLR**
40	1708.7527	0.0109	NA	0	0	EMSCPLSDHYGVAAR (Oxidation (M))
41	1757.9336	0.0003	NA	1	0	RYPTAFYGSGIAVISR
42	1757.9336	0.0003	NA	1	15.9	RYPTAFYGSGIAVISR
43	1774.9254	0.0098	NA	0	0	QIMDGQELPAGVVVFR (Oxidation (M))
44	**1774.9254**	**0.0098**	**2.70e+01**	**0**	**39.89**	**QIMDGQELPAGVVVFR (Oxidation (M))**
46	1832.8915	0.0029	NA	0	0	NDEVFLGSQSRPGSSPR
47	1832.8915	0.0029	NA	0	11.78	NDEVFLGSQSRPGSSPR
48	1932.9612	-0.0236	NA	1	0	QIMDGQELPAGVVVFRR (2 Deamidated (NQ); Oxidation (M))
50	1988.9955	0.0058	NA	1	0	RNDEVFLGSQSRPGSSPR
51	1988.9955	0.0058	NA	1	12.61	RNDEVFLGSQSRPGSSPR
55	2072.9229	0.0105	NA	0	0	RPEEGMSEHECIENLAR (Oxidation (M))
56	2072.9229	0.0105	NA	0	0	RPEEGMSEHECIENLAR (Oxidation (M))
61	2287.2439	-0.0006	NA	0	0	DVLVYSTHLVAVYQVPSQLR
62	**2287.2439**	**-0.0006**	**7.40e+02**	**0**	**23.35**	**DVLVYSTHLVAVYQVPSQLR**
68	2861.3623	-0.0311	NA	0	0	SGVFFTFPLQGFPEQVLHGDYYANK

Fig. S1: Presence of GST-TbnSMase in bacterial membrane enriched fractions was confirmed by mass spectrometry analysis. BL21/pLysSGold *E. coli* membrane enriched fractions were separated via SDS-PAGE and stained with Coomassie. The protein band thought to correspond to GST-TbnSMase was excised and submitted for mass spectrometry analysis, which was performed by the Biomedical Sciences Research Complex Mass Spectrometry and Proteomics Facility, University of St Andrews. Highlighted in red are the peptide peaks, detected in the sample, which matched to the *Trypanosoma brucei brucei* neutral sphingomyelinase.

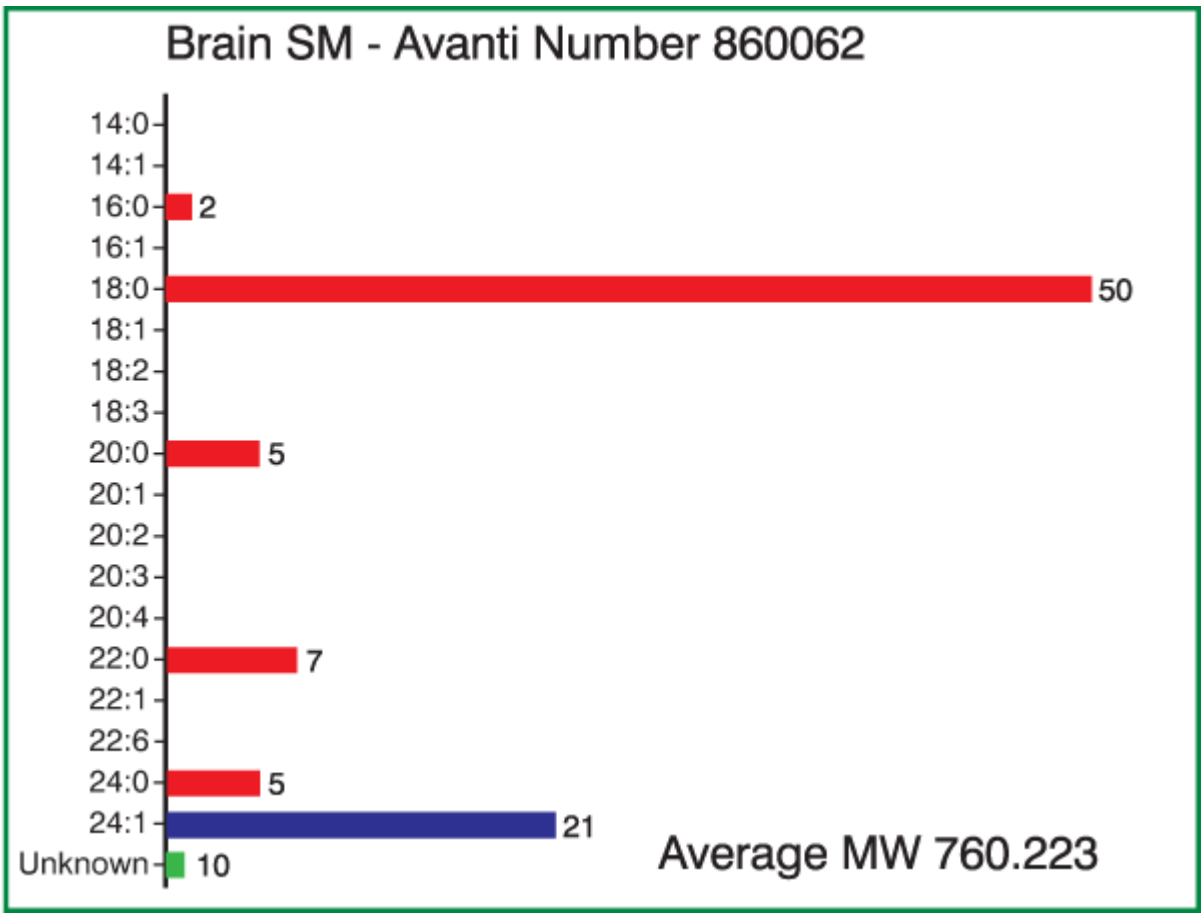


Fig S2: Avanti fatty acid analysis of brain SM product 860062. This product (https://avantilipids.com/product/860062) was used as an SM substrate for GST-TbnSMase (**see Fig. 2 and Fig. S3**). Average fatty acid distribution for SM (d18:1/y) lipid species within the product are shown.

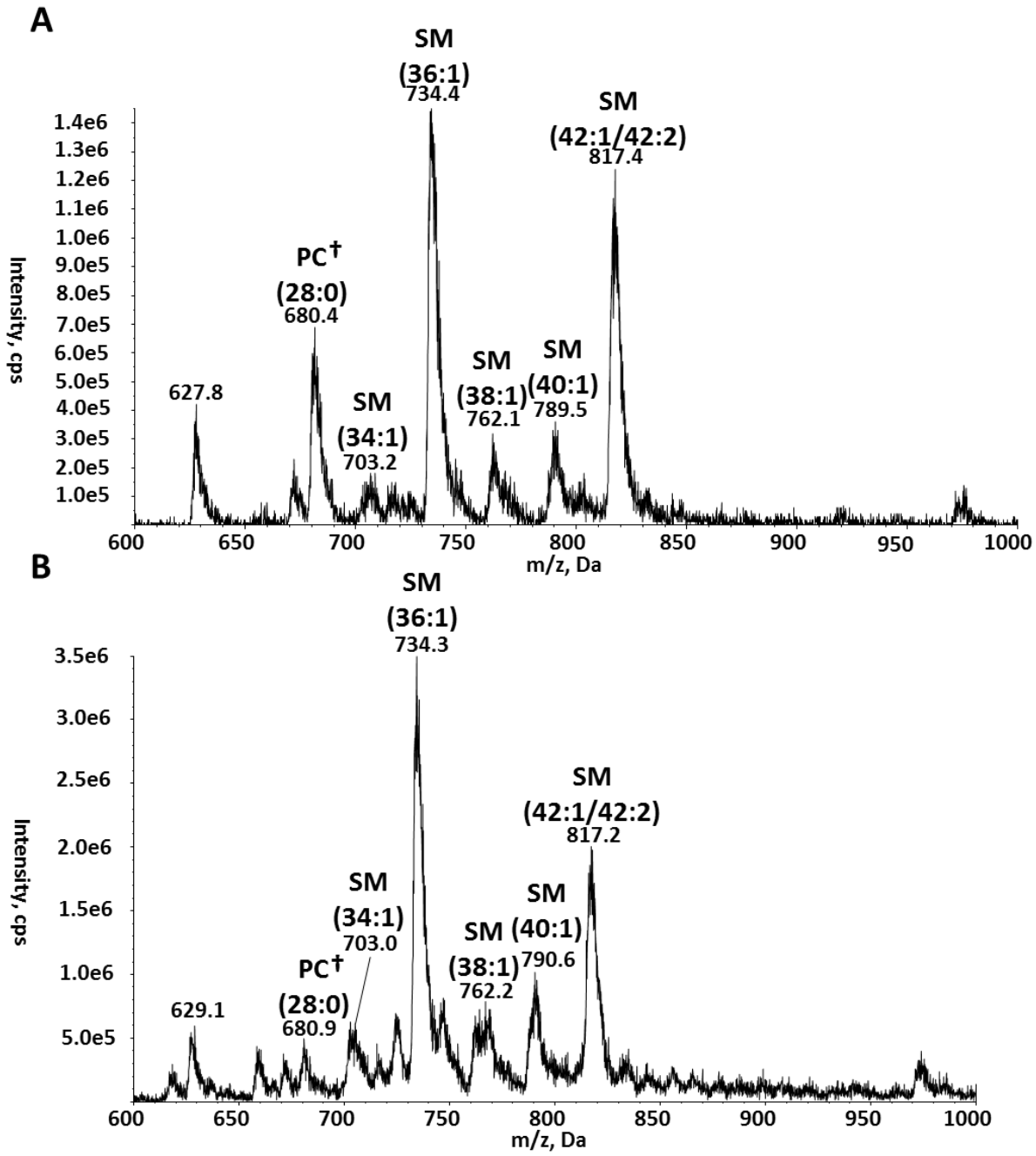


Fig. S3: Additional supporting spectra for GST-TbnSMase catabolism of sphingomyelin. Spectra are ESI-MS/MS precursor ion scans to detect choline-containing lipids (precursors of m/z 184) in positive ion mode. A) SM/Triton X-100 mixed micelle substrate only. B) Non-TbnSMase-expressing bacterial membranes plus SM substrate. (†) Highlights the dimyristoyl-PC (28:0) standard (500 pmoles).

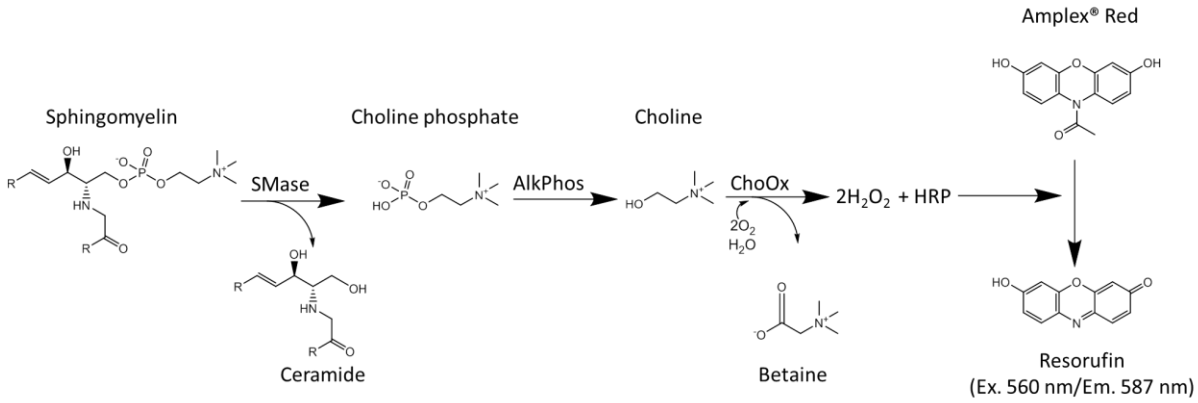


Fig. S4: Amplex® Red assay system. Sphingomyelinase (SMase) activity results in sphingomyelin substrate catabolism, producing ceramide and choline-phosphate (ChoP). ChoP is then hydrolysed by the assay coupling enzyme alkaline phosphatase (AlkPhos), forming choline (Cho). Choline oxidase (ChoOx) oxidises choline to yield betaine and hydrogen peroxide (H_2O_2). As the ChoOx used in the assay is isolated from *Alcaligenes sp.*, Cho is fully oxidised to betaine, via a betaine-aldehyde intermediate (not shown), and 2 moles of hydrogen peroxide (H_2O_2) are produced for every mole of Cho. The H_2O_2 is the oxidising agent for horseradish peroxidase (HRP) that catalyses the conversion of Amplex® Red to red-fluorescent resorufin. The rate of this conversion can be monitored spectrophotometrically (Ex. 560 nm, Em. 587 nm). For the work described here, Amplex® UltraRed, an optimised version of Amplex® Red, was used. However, no chemical structure or formula is available for Amplex® UltraRed and its red-fluorescent conversion product.

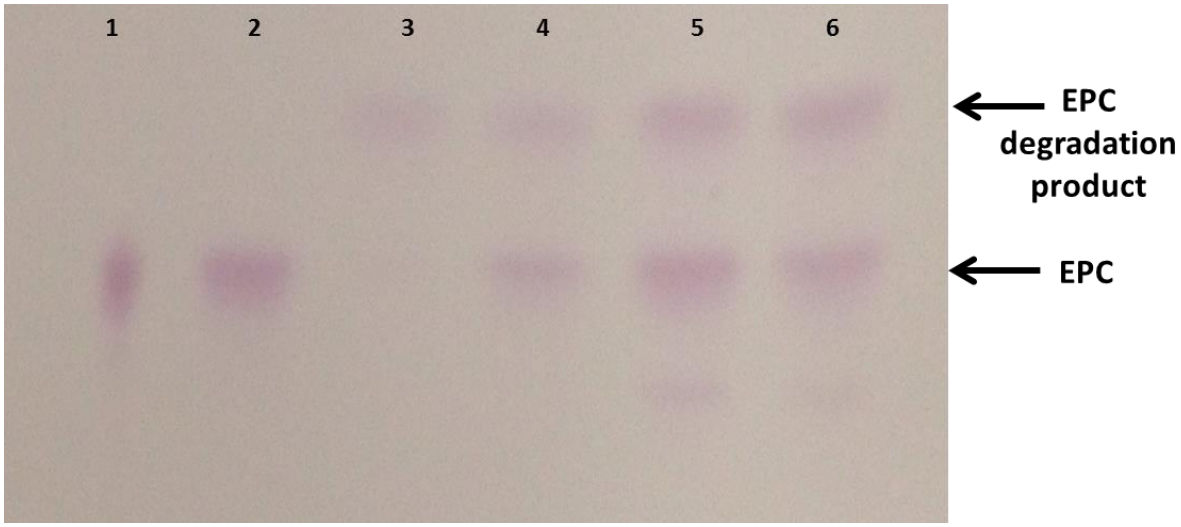


Fig. S5: HPTLC analysis of ethanolamine-phosphoceramide (EPC) substrate reactions.
1) EPC stock solution (no Triton X-100 detergent); 2) EPC/Triton X-100 detergent mixed micelle substrate in reaction buffer; 3) GST-TbnSMase-enriched bacterial membranes plus EPC substrate; 4) non-TbnSMase-expressing bacterial membranes plus EPC substrate; 5) heat-inactivated (HI) GST-TbnSMase-enriched bacterial membranes plus EPC substrate; 6) heat-inactivated (HI) non-TbnSMase-expressing bacterial membranes plus EPC substrate. 'EPC degradation product' refers to a non-specific degradation product of the EPC substrate. This is likely to be sphingosine, based upon its retention factor and the fact that the product must contain an ethanolamine moiety.

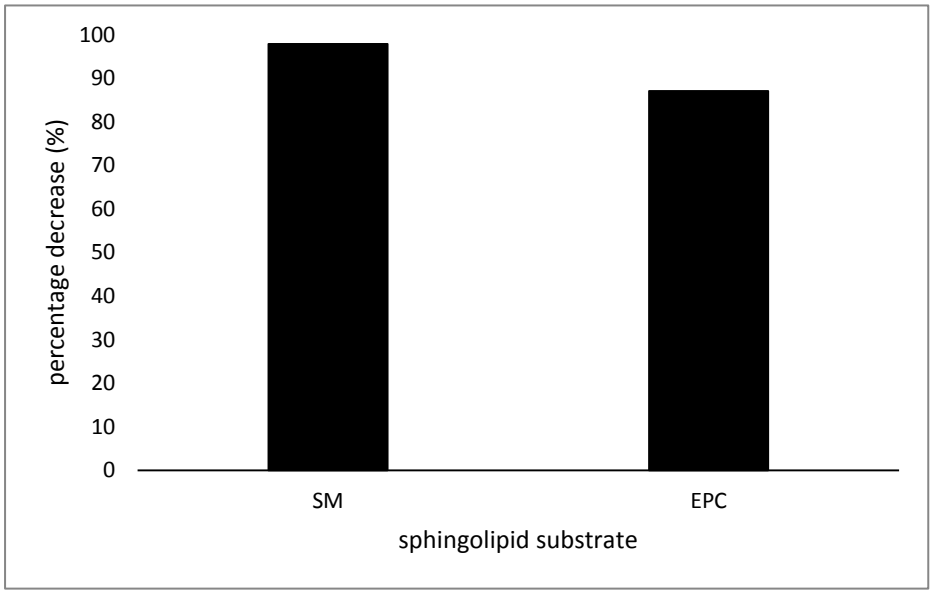


Fig. S6: Sphingomyelin and ethanolamine-phosphoceramide substrate competition assay. A substrate mixture, containing equimolar concentrations (25 nmoles) of sphingomyelin and ethanolamine-phosphoceramide, was incubated with GST-TbnSMase. The percentage decreases (%) in the level of each substrate, relative to a TbnSMase negative control, are shown.

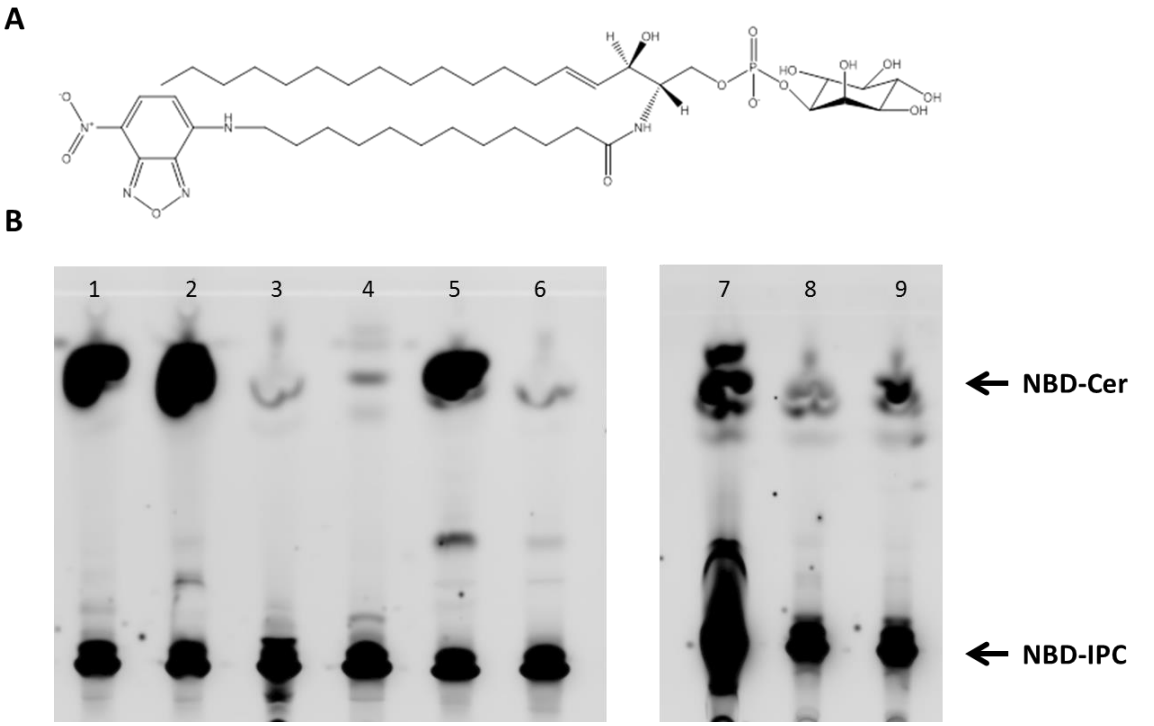


Fig. S7: HPTLC analysis of NBD-IPC substrate reactions. A) Chemical structure of N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] dodecanoyl]-sphingosine-1-phosphoinositol (NBD-IPC) substrate. B) HPTLC analysis. 1) bloodstream form *T. brucei* lysate with NBD-IPC substrate; 2) stumpy form *T. brucei* lysate with NBD-IPC substrate; 3) procyclic form *T. brucei* lysate with NBD-IPC substrate (stored prior to use); 4) NBD-IPC substrate only; 5) GST-TbnSMase-expressing *E. coli* lysate with NBD-IPC substrate; 6) non-GST-TbnSMase-expressing *E. coli* lysate with NBD-IPC substrate; 7) procyclic form *T. brucei* lysate with NBD-IPC substrate (freshly prepared); 8) promastigote form *L. major* lysate with NBD-IPC substrate; 9) epimastigote form *T. cruzi* lysate with NBD-IPC substrate.

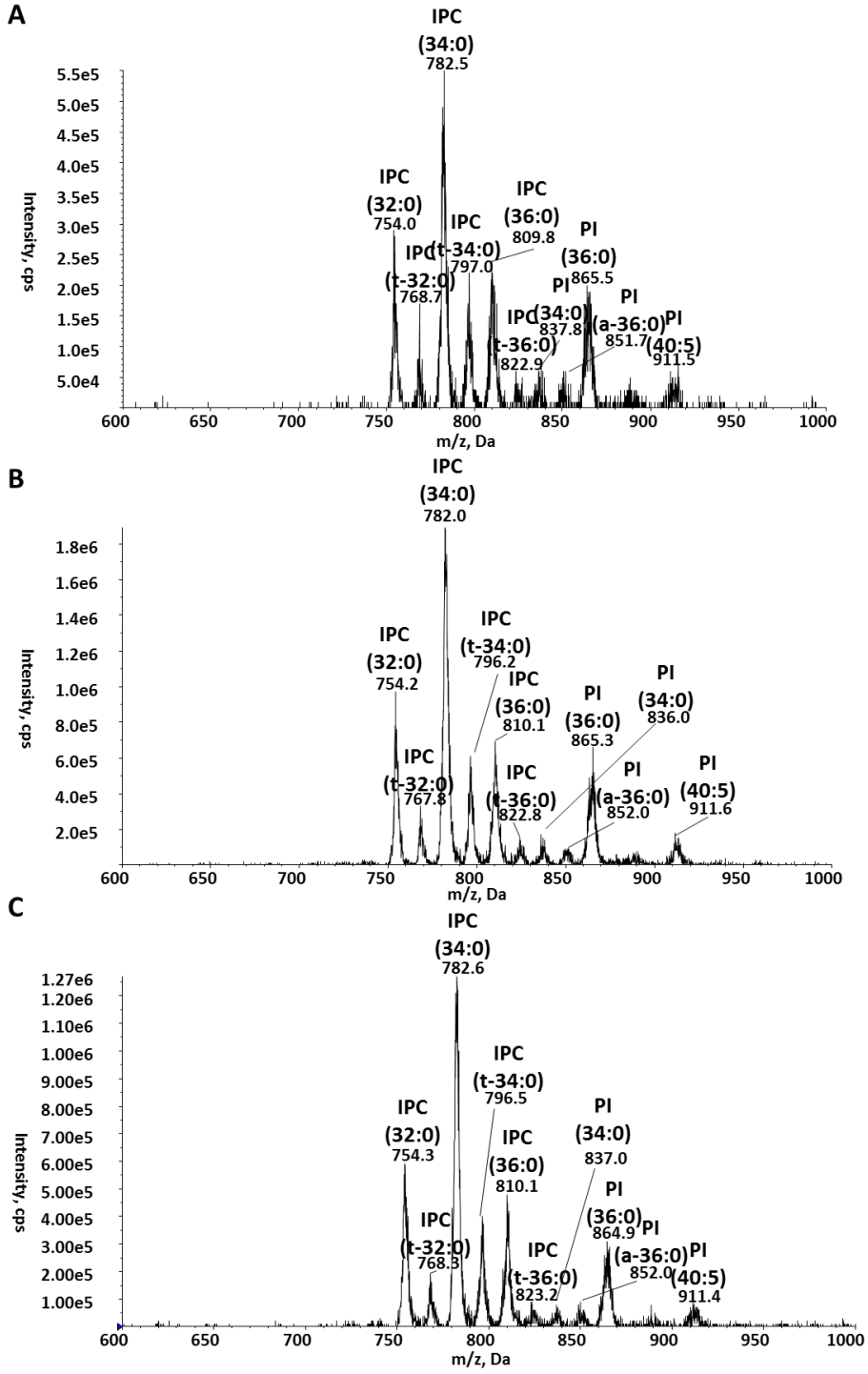


Fig. S8: Additional supporting spectra for *T. brucei* procyclic-extract (inositol-phosphoceramide) reactions. ESI-MS/MS precursor ion scans to detect inositol-containing lipids (precursors of m/z 241) in negative ion mode. A) GST-TbnSMase-enriched bacterial membranes plus procyclic extract (IPC) substrate. B) heat-inactivated GST-TbnSMase-enriched bacterial membranes plus IPC substrate.

C) Non-TbnSMase-expressing bacterial membranes plus IPC substrate reactions. The substrate contains dihydroxylated ceramides, as well as trihydroxylated ceramides, the later denoted as‘t-’.

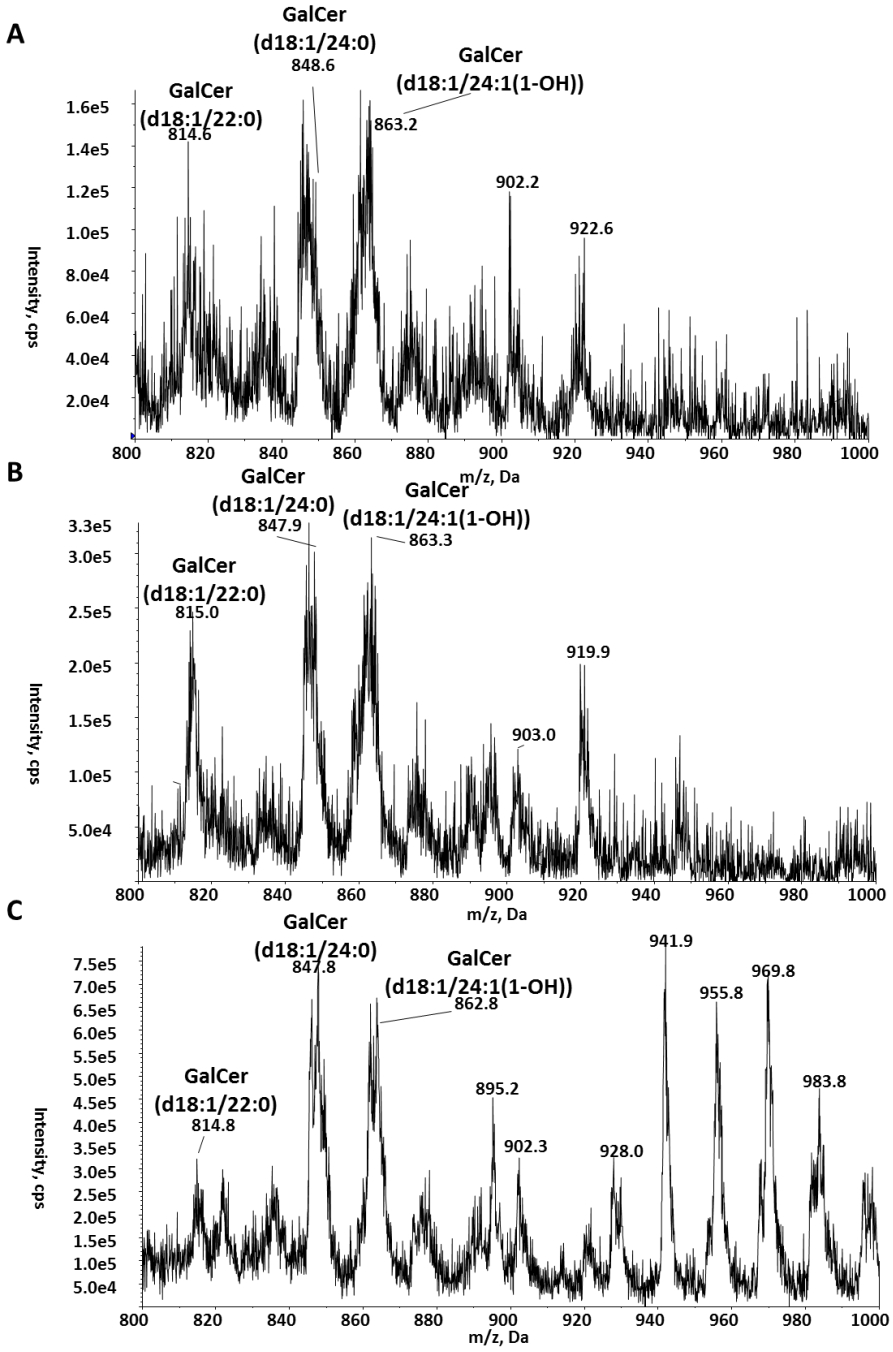


Fig. S9: ESI-MS/MS analysis of galactosylceramide substrate reactions. The product used as a galactosylceramide (GalCer) substrate (Sigma product no. C4905) is a mixture of GalCer species, containing both alpha-hydroxylated and non-hydroxylated fatty acids, mainly C24 in length. ESI-MS/MS survey scans of 800-1000 m/z range in negative ion mode were used to detect galactosylceramide substrate species.
A) galactosylceramide/ Triton X-100 detergent mixed micelle substrate only. B) GST-TbnSMase-enriched bacterial membranes plus galactosylceramide substrate. C) heat-inactivated GST-TbnSMase-enriched bacterial membranes plus galactosylceramide substrate. Annotated galactosylceramides have formed chloride adducts $[M + Cl]^-$.No significant differences were observed when comparing the active GST-TbnSMase spectrum to the controls, leading to the conclusion that GST-TbnSMase does not catabolise galactosylceramide species.