Exploring *Leishmania major* Inositol Phosphorylceramide Synthase (*Lmj*IPCS): Insights into the ceramide binding domain

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ABSTRACT

The synthesis of set of ceramide analogues exploring hydrophobicity in the acyl chains and the degree and nature of hydroxylation is described. These have been assayed against the parasitic protozoan enzyme *Lmj*IPCS. These studies showed that whilst the C-3 hydroxyl group was not essential for turnover it provided enhanced affinity. Reflecting the membrane bound nature of the enzyme a long (C_{13}) hydrocarbon ceramide tail was necessary for both high affinity and turnover. Whilst the N-acyl chain also contributed to affinity, analogues lacking the amide linkage functioned as competitive inhibitors in both enzyme and cell-based assays. A model that accounts for this observation is proposed.

INTRODUCTION

Protozoan parasites of the order Trypanosomatidae cause a range of human diseases, including the leishmaniases and human African trypanosomiasis (HAT).[1-3] These infections are of increasing prevalence, particularly in developing countries, and have been classified by the World Health Organisation as Category I: emerging or uncontrolled diseases.[3] Moreover the spread and severity of leishmaniasis is exacerbated by its status as an important co-infection of AIDS patients and the overlap in prevalence of HIV and *Leishmania* spp.[4] The treatment of trypanosomatid infections is difficult with the most serious visceral form of leishmaniasis often requiring a long and costly course of drug therapy. The challenge presented by these disease states is heightened by the fact that the few efficacious drugs available often exhibit serious, potentially fatal, side-effects. Moreover, reports of resistance to even the

newer drugs are emerging.[5, 6] This situation renders the discovery and validation of new therapeutic targets a priority in these organisms.

Sphingolipids are essential components of eukaryotic cell membranes having critical roles in a variety of cell processes including signal transduction, intracellular membrane trafficking and the regulation of cell growth and survival.[7-10] The *de novo* biosynthesis of these lipid species is initiated by the condensation of palmitoyl CoA **1** with L-serine via the ubiquitous eukaryotic enzyme serine palmitoyltransferase (*SPT*) to produce 3-ketosphinganine which is then reduced to dihydrosphingosine **2**. Subsequently, there is evolutionary divergence in the pathway. In mammals, dihydrosphingosine is acylated to produce dihydroceramide which is then desaturated to ceramide **3**, a key bioactive molecule. In contrast, fungi and plants first generate hydroxylated sphinganine before acylation to form phytoceramide **4**, Figure 1.[11, 12] Like mammals *Leishmania* spp. also predominantly synthesise ceramide.[13] Beyond this point in the biosynthetic pathway a further dichotomy emerges in the synthesis of the predominant phosphosphingolipids (PSLs). Utilising sphingomyelin synthase (SMS) mammalian cells transfer phosphorylcholine from phosphatidylcholine (PC) to ceramide to give sphingomyelin **5**.[14] In contrast, but like fungi and plants, *Leishmania* spp. (and other trypanosomatids) synthesise inositol phosphorylceramide (IPC) **6** as their primary PSL. One notable difference is that in trypanosomatids the IPC formed is that derived from ceramide **3** whereas in plants and fungi the dominant SL used is phytoceramide **4** leading to the formation of IPC **7**. Whilst the essential fungal enzyme catalysing this reaction, AUR1p or IPC synthase (IPCS), has long been characterised as a novel target for anti-fungals,[12, 15] until recently, the trypanosomatid (and plant)[16] orthologues of this protein remained unknown. However, using complementation strategy we have isolated the gene encoding IPCS in *L. major* (*Lmj*IPCS).[17] Moreover, closely related orthologues are apparent in the genome sequence databases of the other parasitic trypanosomatids, *Trypanosoma cruzi* and *T. brucei*.[17-19]

IPCS catalyses the transfer of the phosphorylinositol group from phosphatidylinositol (PI) to ceramide or phytoceramide with the concomitant release of diacylglycerol (DAG). Consequently, in addition to producing the major PSL this reaction is also important in maintaining homeostasis in the levels of the key signaling components ceramide and DAG. Since the former is pro-apoptotic and the latter mitogenic,[20] modulating the activity of this enzyme can have catastrophic effects on cell architecture and function. Reflecting this the *Trypanosoma brucei* orthologue has recently been shown to be essential for the pathogenic bloodstream form stage of the lifecycle.[18, 19] Given the global impact of these diseases, further study of these putative protozoan drug targets is essential. Like all other PSL synthases, *Lmj*IPCS is an integral membrane protein with six predicted transmembrane spanning domains.[19] This makes structure-function studies using protein crystallisation or spectroscopy a significant challenge. In addition, whilst similarities with related enzymes, notably the lipid phosphate phosphatases (LPPs),[14, 17, 21, 22] have enabled the identification of a conserved active site triad incorporating two histidines and one

aspartate residue,[17] the substrate binding sites have not been identified. Consequently, we have initiated a chemical biology approach to explore the enzyme with the aim of developing a model of the active site that may ultimately inform the design of effective inhibitors. To this end we have established a microtitre plate-based assay and delineated the kinetic parameters and mode of action of *Lmj*IPCS.[23] These studies revealed that the enzyme follows a ping-pong bi-bi mechanism and that, of the two substrates, ceramide has a higher affinity for the enzyme than does PI. This observation, combined with the fact that PI is a relatively abundant substrate in most membraneous environments, suggested that ceramide is the rate-limiting substrate in the *in situ* IPCS reaction. On this basis we opted to explore the binding of this substrate to *Lmj*IPCS and in this report we describe the synthesis and evaluation of a set of ceramide analogues.

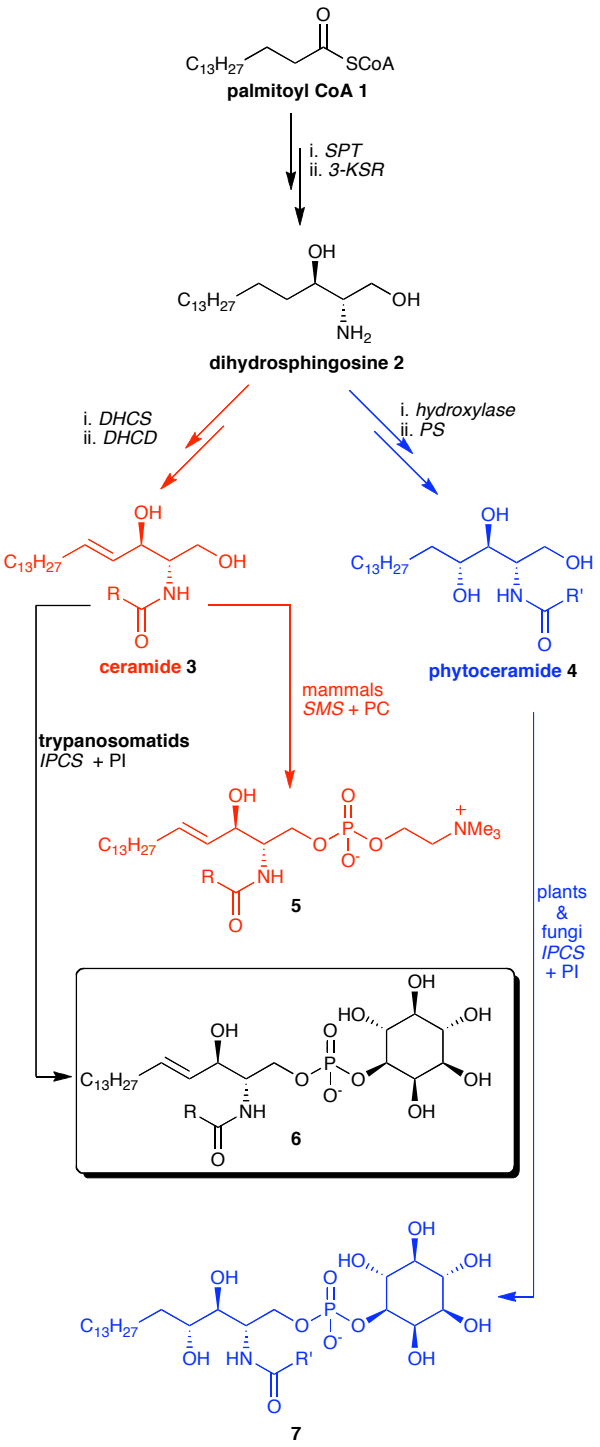


Figure 1: Divergent pathways in phosphosphingolipid biosynthesis (*SPT*, *serine palmitoyl transferase; 3-KSR, 3-ketosphinganine reductase; DHCS, dihydroceramide synthase; DHCD, dihydroceramide desaturase; PS, phytoceramide synthase; SMS sphingomyelin synthase; IPCS inositol ceramide synthase*; PC phosphatidylcholine; PI phosphatidylinositol)

RESULTS AND DISCUSSION

Synthesis of ceramide analogue library

Even excluding functional group variations, simple analysis of the ceramide structure revealed many possible points of variation including the degree of hydroxylation, stereochemistry at the two stereogenic centres and the nature of the fatty acid component. Moreover we have previously demonstrated that, whilst *N*-acetyl sphingosine **8** was an acceptable substrate for *Lmj*IPCS, sphingosine **9** was not and functioned as a competitive inhibitor.[23] Consequently, all the structures initially targeted as potential substrate probes retained an amide linkage. With this requirement we opted to explore the length and substitution in the sphingosine tail, stereochemistry and, more drastically, the presence of the C-1 and C-3 hydroxy groups, Figure 2.

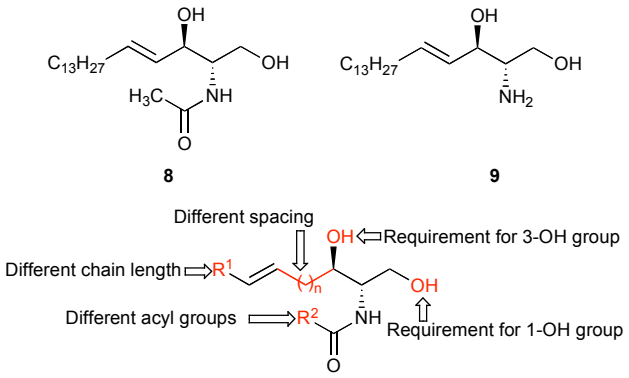
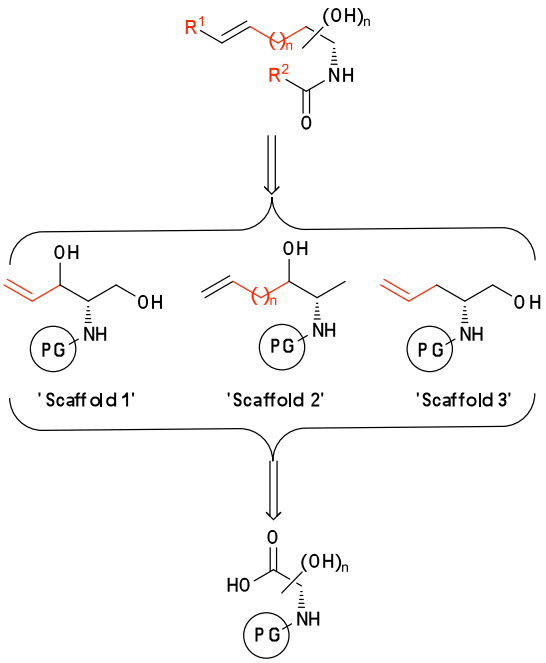


Figure 2: The selected structural features of the ceramide molecule

Whilst many different routes to substituted sphingolipids have been reported, including asymmetric strategies,[24-45] we sought routes that would minimise the need for individual compound purification strategies. Recognising that variation in the sphingosine and fatty acid alkyl chains could be introduced by simple cross metathesis and amine acylation respectively,[46] the problem simplified to generation of a set of core hydroxybutenyl amine scaffolds, which in turn could be accessed from readily available α-amino acids, Scheme 1.[26-34]

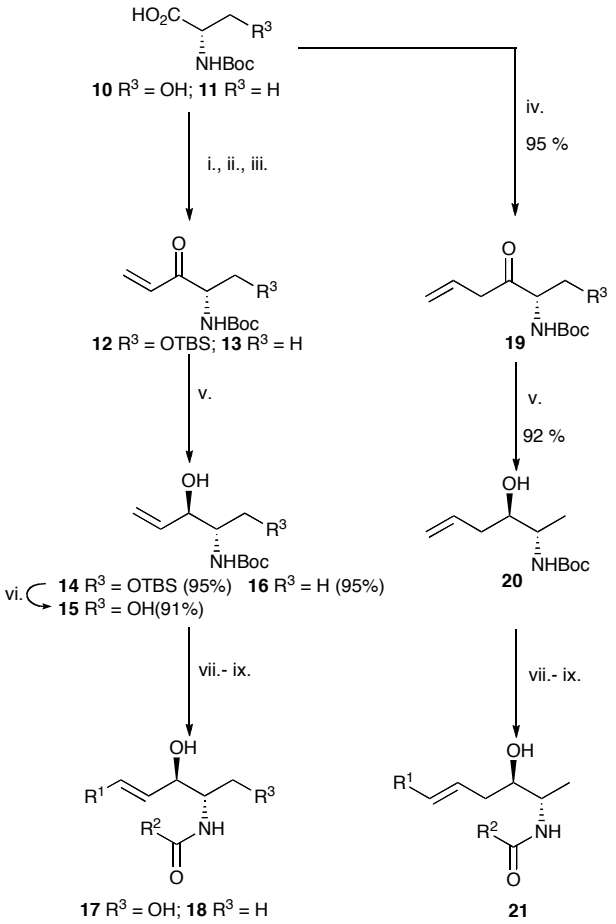


Scheme 1: Retrosynthetic Analysis of the common scaffold

Synthetic work commenced by exploring routes to the fully substituted ceramide core as represented by 'Scaffold 1". This was achieved following an approach developed by Katsumura,[31] based on the stereoselective reduction of the vinyl ketone **12**, Scheme 2. Whilst, in our hands, protection of *N*-Boc Ser **10** as the TBS ether followed by direct addition of a vinyl nucleophile proved not to be viable, a stepwise strategy proceeding via the Weinreb amide proved efficient providing ketone **12** in good yield (74%). Reduction with $LiAl(O^tBu)_3H$ was highly selective affording the desired (2*S*, 3*R*) alcohol **14** with only trace amounts (<1%) of the undesired diastereoisomer being detected in the crude reaction mixture. Selective silyl group deprotection was then achieved using dilute aqueous acid to afford the first core structure **15**. We then explored methods to allow a ceramide array to be constructed in a time efficient fashion minimising chromatography where possible. Cross-metathesis using the Grubbs' second-generation catalyst with a variety of terminal alkenes afforded *N*-Boc sphingosine analogues. Deprotection of the Boc group could be achieved using either TFA-DCM or HCl-dioxane mixtures although the former led to variable amounts of the corresponding trifluoroacetamide. Following removal of volatiles, direct treatment of the crude reaction mixture with the various acid chlorides in the presence of $NaHCO_3$ (pH=8) afforded the desired ceramide analogues **17** in good chemical and stereochemical purities. Whilst final products were purified by chromatography where needed, given the array nature of this synthesis, no attempt was made to optimise reactions in which low conversions were obtained. Importantly, with a view towards future larger library generation, it also proved possible to conduct the last three steps (cross-metathesis, deprotection and acylation) with minimal chromatographic purification. In this case, partition of the final crude reaction mixture between dichloromethane and dilute

aqueous acid (pH 5) by passage through a 'hydrophobic' filter tube (Whatman PTFE) provided product of sufficient purity (≥85 % ^{1}H NMR) to permit screening.

In an identical fashion, commencing from N-Boc Ala **11** allowed the synthesis of ceramide analogues lacking the primary hydroxy group, "Scaffold 2", as well as a homologated series derived from allyl ketone **19**, Scheme 2. Whilst, in this latter series the synthesis could be further shortened by the direct addition of allylmagnesium bromide to the starting *N*-Boc protected amino acid,[47] the reduction of the ketone **19** to the amino alcohol **20** was somewhat less selective producing an 84 : 16 mixture of the (2S, 3R) and (2S, 3S) diastereoisomers respectively. These proved trivial to separate by standard column chromatography and the major isomer was taken through the metathesis and acylation steps as for the other analogues.



	R^1	R^2	17[a]	18[a]	21[a]
az	$C_{13}H_{27}$	CH_3	24 %	51 %	82 %
ay	$C_{13}H_{27}$	Bn	32 %	14 %	86 %
ax	$C_{13}H_{27}$	C_7H_5	18 %	26 %	85 %
bz	C_4H_9	CH_3	44 %	62 %	44 %
by	C_4H_9	Bn	39 %	40 %	48 %
bx	C_4H_9	C_7H_5	41 %	14 %	52 %
cz	$PhCH_2$	CH_3	19 %	51 %	55 %
cy	$PhCH_2$	Bn	53 %	66 %	17 %
cx	$PhCH_2$	C_7H_5	51 %	69 %	45 %

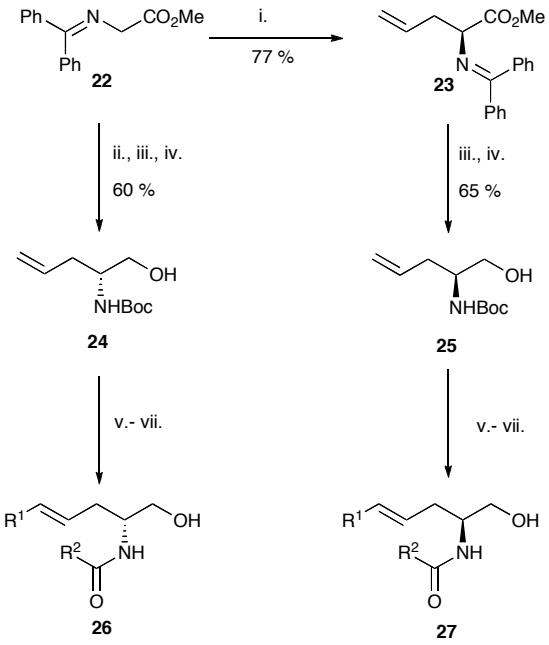
a Yield of purified isolated final product following steps vii.-ix.

Reagents: i. Me(MeO)NH•HCl, NMM, EDCI, DCM, -15 ˚C; ii.TBSCl, imidazole, DMAP, DMF, rt; iii. ^{n}BuLi then CH_2=CHMgBr, (4eq),THF, rt; iv. ^{n}BuLi then CH_2=$CHCH_2MgBr$(4eq),THF, rt; v. $LiAl(O^tBu)_3H$, EtOH, -78 ˚C; vi. HCl, MeOH-H_2O; vii. R^1CH=CH_2, Grubbs 2nd Gen, DCM, 6h; vii. HCl, dioxane or TFA, DCM; viii. R^2COCl, $NaHCO_3$, DCM-H_2O.

Scheme 2: The synthesis and elaborations of 'Scaffolds 1 & 2'

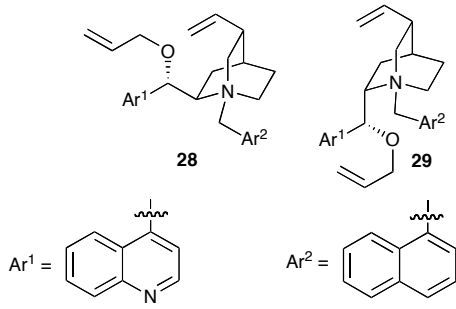
In order to explore the relevance of the secondary hydroxyl group in ceramide we then prepared the corresponding series of compounds in which this group was lacking, "Scaffold 3". These could be accessed by asymmetric alkylation of the benzophenone imine of glycine following the precedents established by Corey, Lygo and others, Scheme 3.[48, 49] Thus reaction of the glycine imine with allyl bromide and KOH in the presence of 5 mol% of the cinchona catalyst **29** derived from cinchonidine afforded the allyl glycine derivative **23** in good yield. Following exchange of the nitrogen protection group

and reduction of the ester, metathesis and acylation, as before, afforded the desired set of ceramide analogues **27**. Replacing the phase-transfer catalyst in the initial alkylation with that **28** derived from cinchonine provided access to the enantiomeric amino alcohol in similarly good yields. Cross-metathesis with 1-alkenes and acylation as before then afforded the enantiomeric analogues **26**.



		R^1	R^2	yield[a]
26	**az**	$C_{13}H_{27}$	CH_3	66 %
26	**ay**	$C_{13}H_{27}$	Bn	35 %
26	**ax**	$C_{13}H_{27}$	C_7H_5	57 %
27	**az**	$C_{13}H_{27}$	CH_3	22 %
27	**ay**	$C_{13}H_{27}$	Bn	27 %
27	**ax**	$C_{13}H_{27}$	C_7H_5	27 %

a Yield of purified isolated final product following steps v.- vii.



Reagents: i. CH_2=$CHCH_2Br$, KOH, **29**, PhMe-DCM, -78 ˚C, 8h (77%); ii. CH_2=$CHCH_2Br$, KOH, **28**, PhMe-DCM, -78 ˚C, 8h (79%); iii. a. HCl, MeOH, 2h; b. Boc_2O, $NaHCO_3$, DCM-H_2O; iv. $LiAlH_4$, Et_2O, rt, 2h; v. R^1CH=CH_2, Grubbs 2nd Gen, DCM, 6h; vi. HCl, dioxane or TFA, DCM; vii. R^2COCl, $NaHCO_3$, DCM-H_2O.

Scheme 3: Synthesis and elaboration of 'Scaffold 3'

BIOLOGICAL EVALUATION

All members of the synthesized analogue library were then evaluated for their ability to inhibit the synthesis of fluorescently labelled IPC using our established microtitre plate-based assay.[23] In this system each compound was incubated with the labelled acceptor substrate NBD-C_6-ceramide, and the donor substrate PI in the presence of *Lmj*IPCS. The amount of labelled product, inositol phosphoryl-NBD-C_6-ceramide (NBD-C_6-IPC), formed was then quantified. The ratio between this value and that for a control reaction, without the addition of an analogue, provided a measure of competitive binding / inhibition. Notably, the calculated *Z*-factor for this system is > 0.5 rating the assay as statistically valid for screening purposes.[50]

This screen identified 24 compounds that reduced the quantity of *Lmj*IPCS synthesized NBD-C_6-IPC by > 40%. Of these, 13 compounds reduced formation of labelled product by > 50% including 3 by > 75%, (Figure 3 & Table 1). The results indicated a proportional increase in the inhibitory effect of the ceramide analogues based on their hydrophobicity. With the exception of three derivatives (Table 1 entries 24, 28 and 48), all compounds that exhibited greater than 40 % inhibition of *Lmj*IPCS with respect to formation of NBD-C_6-IPC, contained a long hydrophobic sphingosine tail (R^1 = $C_{13}H_{27}$). This observation strongly suggests that the chain length of the sphingosine tail is crucial for binding to the enzyme, as might be expected given the hydrophobic nature of *Lmj*IPCS as an integral membrane enzyme. Similarly, increasing the hydrophobicity of the *N*-acyl moiety appeared to favour its affinity for *Lmj*IPCS as determined by the reduction in NBD-C_6-IPC synthesis. However, the effect was smaller and less consistent than that seen when increasing hydrophobicity of the sphingosine residue (Figure 3, n to x). Consistent with these observations, analysis of the *N*-Boc protected cross-metathesis products produced as synthetic intermediates showed similar trends (Table 1 entries 6-16). Whilst those compounds containing a short chain (C_4H_9) or an aromatic residue (CH_2Ph) in the sphingosine backbone showed little or no inhibition (≤ 16 %) of *Lmj*IPCS mediated NBD-C_6-IPC formation, those with a long alkyl tail ($C_{13}H_{27}$) exhibited moderate levels of inhibitory effect (29-59 %).

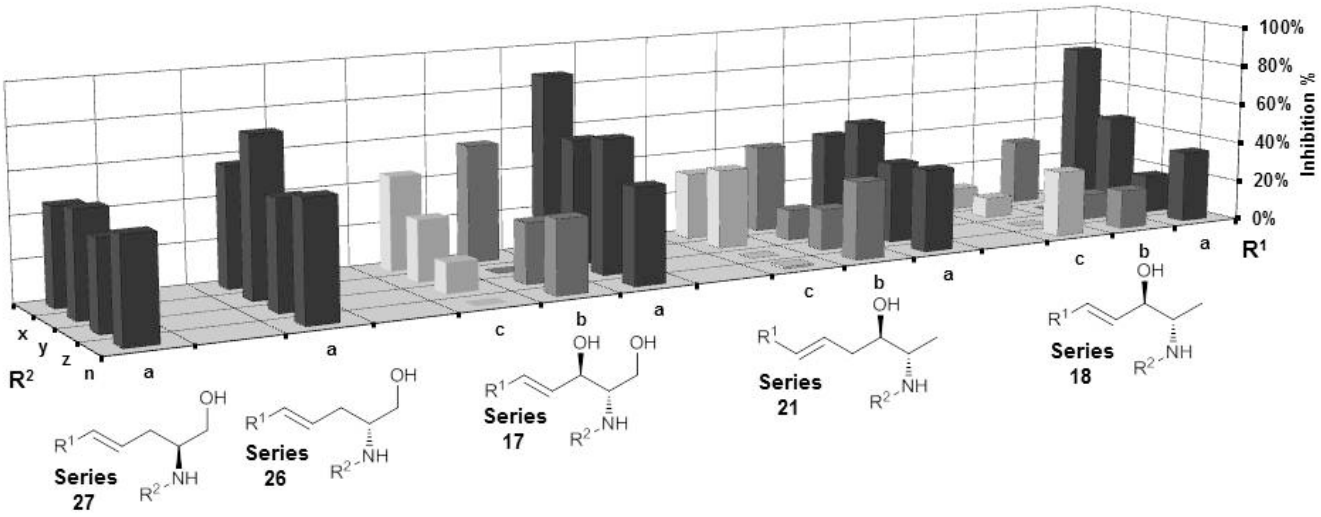
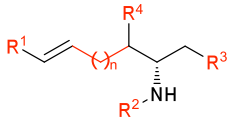


Figure 3: Graphical representation of screening of ceramide analogues against *Lmj*IPCS. Bars show level of inhibition of NBD-C_6-IPC production against control. (R^1: a = $C_{13}H_{27}$, b = C_4H_9 and c = CH_2Ph; R^2: n = NH_2 , x= C_7H_{15} , y = CH_2Ph and z = CH_3)

Table 1: Screening of ceramide analogues against *Lmj*IPCS



Entry	R^1	R^2	R^3	R^4	n	№	Inh ±SE%
1	H	Boc	OH	OH	0	15	9.0 ± 17%
2	H	Boc	H	OH	0	16	25 ± 8%
3	H	Boc	H	OH	1	20	11 ± 19%
4	H	Boc	OH	H	0	24	0 ± 1%
5	H	Boc	OH	H	0	25	24 ± 1%
6	$C_{13}H_{27}$	Boc	OH	OH	0	17a	34.0 ± 8%
7	C_4H_9	Boc	OH	OH	0	17b	0.0 ± 5%
8	CH_2Ph	Boc	OH	OH	0	17c	0 ± 12%
9	$C_{13}H_{27}$	Boc	H	OH	0	18a	0 ± 6%
10	C_4H_9	Boc	H	OH	0	18b	0 ± 3%
11	CH_2Ph	Boc	H	OH	0	18c	0 ± 1%
12	$C_{13}H_{27}$	Boc	H	OH	1	21a	29 ± 3%
13	C_4H_9	Boc	H	OH	1	21b	16 ± 6%
14	CH_2Ph	Boc	H	OH	1	21c	15 ± 9%
15	$C_{13}H_{27}$	Boc	OH	H	0	26a	55 ± 15%
16	$C_{13}H_{27}$	Boc	OH	H	0	27a	59 ± 4%
17	$C_{13}H_{27}$	H	OH	OH	0	17an	46 ± 10%
18	$C_{13}H_{27}$	$COCH_3$	OH	OH	0	17az	64 ± 5%
19	$C_{13}H_{27}$	$COCH_2Ph$	OH	OH	0	17ay	60 ± 1%
20	$C_{13}H_{27}$	COC_7H_{15}	OH	OH	0	17ax	87 ± 4%
21	C_4H_9	H	OH	OH	0	17bn	35 ± 3%
22	C_4H_9	$COCH_3$	OH	OH	0	17bz	29 ± 3%

23	C_4H_9	$COCH_2Ph$	OH	OH	0	17by	0 ± 3%
24	C_4H_9	COC_7H_{15}	OH	OH	0	17bx	56 ± 1%
25	CH_2Ph	H	OH	OH	0	17cn	0 ± 10%
26	CH_2Ph	$COCH_3$	OH	OH	0	17cz	14 ± 8%
27	CH_2Ph	$COCH_2Ph$	OH	OH	0	17cy	29 ± 2%
28	CH_2Ph	COC_7H_{15}	OH	OH	0	17cx	45 ± 4%
29	$C_{13}H_{27}$	H	H	OH	0	18an	36 ± 4%
30	$C_{13}H_{27}$	$COCH_3$	H	OH	0	18az	19 ± 7%
31	$C_{13}H_{27}$	$COCH_2Ph$	H	OH	0	18ay	45 ± 11%
32	$C_{13}H_{27}$	COC_7H_{15}	H	OH	0	18ax	79 ± 2%
33	C_4H_9	H	H	OH	0	18bn	19 ± 8%
34	C_4H_9	$COCH_3$	H	OH	0	18bz	13 ± 6%
35	C_4H_9	$COCH_2Ph$	H	OH	0	18by	0 ± 2%
36	C_4H_9	COC_7H_{15}	H	OH	0	18bx	32 ± 5%
37	CH_2Ph	H	H	OH	0	18cn	32 ± 8%
38	CH_2Ph	$COCH_3$	H	OH	0	18cz	0 ± 9%
39	CH_2Ph	$COCH_2Ph$	H	OH	0	18cy	10 ± 8%
40	CH_2Ph	COC_7H_{15}	H	OH	0	18cx	10 ± 9%
41	$C_{13}H_{27}$	H	H	OH	1	21an	40 ± 2%
42	$C_{13}H_{27}$	$COCH_3$	H	OH	1	21az	39 ± 6%
43	$C_{13}H_{27}$	$COCH_2Ph$	H	OH	1	21ay	55 ± 5%
44	$C_{13}H_{27}$	COC_7H_{15}	H	OH	1	21ax	45 ± 4%
45	C_4H_9	H	H	OH	1	21bn	38 ± 1%
46	C_4H_9	$COCH_3$	H	OH	1	21bz	20 ± 9%
47	C_4H_9	$COCH_2Ph$	H	OH	1	21by	15 ± 6%
48	C_4H_9	COC_7H_{15}	H	OH	1	21bx	42 ± 4%
49	CH_2Ph	H	H	OH	1	21cn	1 ± 6%
50	CH_2Ph	$COCH_3$	H	OH	1	21cz	0 ± 1%
51	CH_2Ph	$COCH_2Ph$	H	OH	1	21cy	38 ± 17%
52	CH_2Ph	COC_7H_{15}	H	OH	1	21cx	32 ± 8%
53	$C_{13}H_{27}$	H	OH	H	0	26an	57 ± 7%
54	$C_{13}H_{27}$	$COCH_3$	OH	H	0	26az	52 ± 1%
55	$C_{13}H_{27}$	$COCF_3$	OH	H	0	26af	65 ± 5%
56	$C_{13}H_{27}$	$COCH_2Ph$	OH	H	0	26ay	76 ± 4%
57	$C_{13}H_{27}$	COC_7H_{15}	OH	H	0	26ax	57 ± 4%
58	$C_{13}H_{27}$	H	OH	H	0	27an	48 ± 6%
59	$C_{13}H_{27}$	$COCH_3$	OH	H	0	27az	42 ± 11%
60	$C_{13}H_{27}$	$COCH_2Ph$	OH	H	0	27ay	50 ± 5%
61	$C_{13}H_{27}$	COC_7H_{15}	OH	H	0	27ax	46 ± 3%

Switching from a $\Delta^{4,5}$ to a $\Delta^{5,6}$ alkene only had a small effect except when in conjunction with a long chain *N*-acyl unit where this change resulted in a twofold decrease in inhibition of NBD-C_6-IPC production (Table 1 entries 32 & 44). Previous work has indicated that the *trans* double bond exerts a considerable

effect on the hydrogen bonding interactions of the 3-OH group in ceramide.[51] Whilst the loss of this effect may account for the observed reduction in apparent affinity for *Lmj*IPCS in series 21, it is possible that a simple conformational change enforced by the alkene position is also responsible. In support of the latter suggestion, when comparing 1,3-dihydroxy analogues (series 17) with derivatives lacking the 3-OH group (series 26 and 27), it is clear that the 3-hydroxyl group exerts a minimal influence on substrate binding.

Whilst a number of analogues significantly reduced the level of NBD-C_6-IPC production in the assay, this could arise through one of two functions; either true inhibition of *Lmj*IPCS or compounds behaving as an alternative competitive substrates and being processed to non-labelled IPC analogues. In order to determine the extent of each possibility a small subset of the assay reactions, involving both good and poor modulators of NBD-C_6-IPC formation, were analysed in greater detail using mass spectrometry to look for the formation of the correspondingly unlabelled but modified sphingolipid. Following reaction and fractionation as described, organic phases were subjected to positive and negative ion mass spectrometry. The resultant MS spectra (see ESI) were searched for the mass peaks [M+241] corresponding to the hypothetical PSL products. Whilst compounds **17ax**, **26af**, and **26ax** all showed signals for the phosphorylated product indicating that these were viable substrates, no evidence for phosphosphingolipid formation could be detected for compounds **18ax**, **21bx**, **17bx**, **27a**, **26an** and **26ay** suggesting that these functioned as true inhibitors of the enzyme.

These observation are consistent with the predicted reaction mechanism,[23] in that analogues that lack the primary (C1) hydroxyl group (e.g. **18ax**) showed no evidence of phosphorylation and the presence of an inositol head group. However, these compounds do function as effective inhibitors suggesting that the oxygen atom is non-essential for binding (comparing series 17 with 18). Not surprisingly, the *N*-octanoyl ceramide analogue, **17ax**, functioned as an alternative substrate as did its derivative **26ax** which lacked a 3-hydroxy group further confirming that this latter group is not essential for turnover by *Lmj*IPCS. Additionally, although ceramide analogues with a shorter sphingosine backbone ($R^1 = C_4H_9$) resulted in moderate inhibition suggesting competitive binding to *Lmj*IPCS, such analogues (e.g. **17bx**) were not processed by *Lmj*IPCS indicating the importance of a long backbone for effective substrate binding and IPC synthesis. Moreover, incorporation of steric bulk into either the sphingosine backbone or the *N*-acyl moiety appears to result in these compounds acting as true enzyme inhibitors (compare **27a** or **26ay** with **26ax**). In this respect it is pertinent to note that α-branched *N*-pivaloyl phytoceramide analogues have been shown to exhibit relatively high inhibition of *S. cerevisiae* IPCS turnover. However, in this case it was not stated whether these were acting as true inhibitors or alternative substrates.[29]

To further investigate the interaction of ceramide with *Lmj*IPCS four of the most active true-inhibitors were analysed in dose-inhibition assays and the respective IC_{50} values determined (Figure 4). The most

effective inhibitor as identified in the screen (Table 1), *N*-octanoyl 1-deoxyceramide **(18ax)**, had an IC_{50} of 4.79 μM is, not surprisingly, structurally the closest to the natural substrate. Consistent with the initial screening data incorporation of steric bulk into the N-acyl unit led to higher IC_{50} values (Figure 4 C&D)

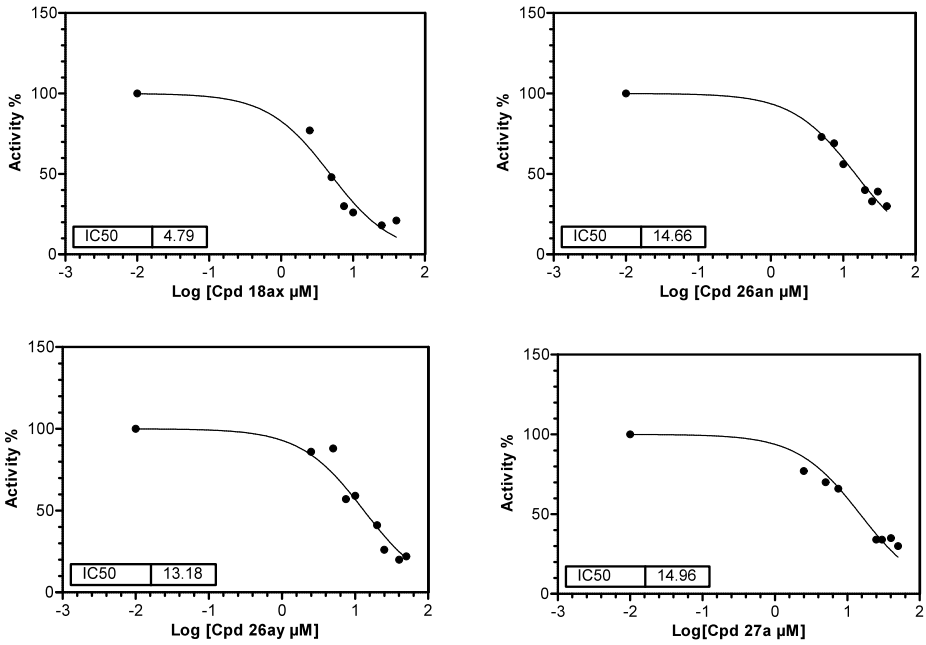


Figure 4: Inhibition curves and IC_{50} values of selected inhibitors. Activity refers to percentage activity relative to untreated control. Calculated IC_{50} values A (**18ax**) 4.8 μM; B (**26an**) 14.7 μM; C (**26ay**) 13.2 μM; D (**27a**) 15.0 μM

Previously we have demonstrated that whilst *N*-acetyl-D-*erythro*-sphingosine was an acceptor substrate, D-*erythro*-sphingosine was not.[23] Similarly here, MS analyses revealed that whilst *N*-octanoyl-3-deoxyceramide **(26ax)** was turned over by the enzyme, its derivative containing a free amine **(26an)** was not, and functioned as an inhibitor with an IC_{50} of 14.7 μM (Figure 4B). Collectively, these results suggest that the free-amino group of sphingosine contributes strongly to the observed inhibition by these compounds potentially through an electrostatic interaction with a positively charged ammonium salt that would be formed at physiological relevant pH.[23]

Similar observations have been previously reported, e.g. the inhibitory effect of sphingosine analogues on the *S. cerevisiae* phosphatidate phosphatase,[52] a member of the LPP enzyme superfamily believed to share a mechanism of action with the sphingolipid synthases.[14] Moreover, *Sigal et al.*[21] have proposed a generalised hypothetical mechanism of action for such phosphoryl transferases, Figure 5B. This mechanism, in addition to the catalytic triad, involves one lysine and two arginine residues. These three residues are presumed to be in a protonated state and are therefore able to stabilise the transition state structure during phosphate group transfer. Moreover, Sigal demonstrated that one of these arginine residues is conserved across different families of enzymes and organisms. This residue is located close (5 amino acids away) to the nucleophilic histidine residue of the active site. Investigation of the *Lmj*IPCS

sequence identified Arg262 (in *Lmj*IPCS) as a conserved residue in all the identified orthologous IPC synthases in the TriTryp genome,[17] Figure 5A.[17] Consequently, Arg262 can be presumed a potential candidate residue involved in the stabilisation of the transition state during the phosphorylinositol group transfer by *Lmj*IPCS, Figure 5C. This speculation is consistent with the hypothesis that the protonated amino group of sphingosine can electrostatically interfere with the protonated Arg262 resulting in inefficient stabilisation of the transition state and inhibition of the catalytic transfer mechanism.

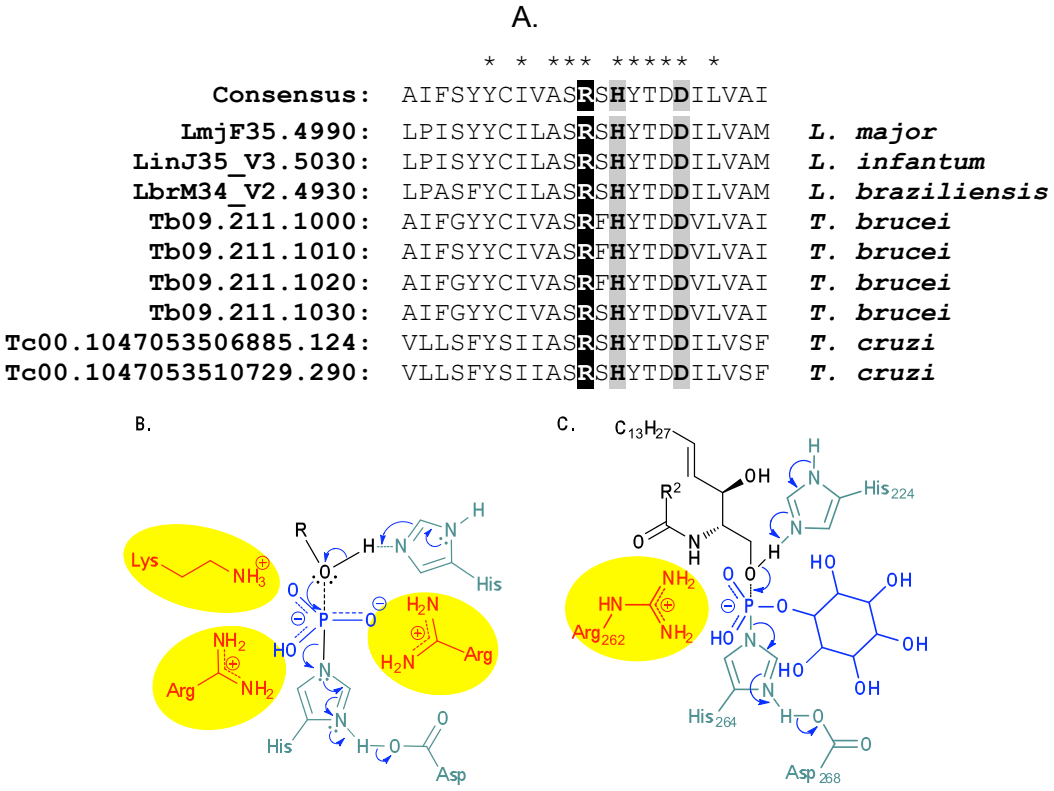


Figure 5: A. Sequence alignment of active site residues of trypanosomatid IPCSs. B. Proposed mechanism of action of phosphoryl transferases adapted from Sigal *et al* (ref 21). C. Proposed mechanism of action of *Lmj*IPCS

Significantly, analyses of the efficacy of the four compounds above against cultured wild type *L. major* promastigotes identified the free amino-derivative **(26an)** as the only one to reproducibly demonstrate significant anti-protozoal effects at the concentrations analysed. Under the experimental conditions described maximal cytotoxicity was recorded at 12.5 μM, where cell viability was assessed as 4.6±0.5 and 3.1±0.7% of an untreated control in 2 independent experiments. In contrast, equivalent analyses of a *L. major* serine palmitoyltransferase (SPT) mutant line[53] demonstrated cell viability to be 14.6±0.02 and 13.8±0.7% of the control. Loss of SPT function, which catalyses the first and rate limiting step in sphingolipid biosynthesis pathway, is tolerated by insect stage *L. major* promastigotes and renders *Lmj*IPCS redundant.[53] Therefore, this line will be resistant to specific *Lmj*IPCS inhibitors. A similar

strategy has been used to identify specific *S. cerevisiae* IPCS inhibitors.[54] Although these mutants remain sensitive to **26an** indicating off target effects, potentially an S1P ripple response,[55-57] they are 3-4 fold less sensitive than the wild type line. This suggests that targeting *Lmj*IPCS is a viable strategy for anti-parasitic agents.

CONCLUSION

A library of ceramide derivatives built around a set of hydroxybutenyl amine cores has been prepared exploring variations in the sphingosine tail, *N*-acyl unit and the degree of hydroxylation. The ability of these compounds to perturb the conversion of NBD-C_6-ceramide to NBD-C_6-IPC mediated by *Lmj*IPCS has been assessed using a microtitre plate-based assay. The dominant factor for effective binders, as determined by the reduced levels of NBD-C_6-IPC produced, was the possession a long chain lipophilic sphingosine tail. Competitive substrates and inhibitors could be distinguished by MS analysis of the reaction products. Whilst the presence of the hydroxyl groups and a long chain *N*-acyl unit were beneficial for activity, they were not essential for binding to the active site of *Lmj*IPCS. Notably, a free amino group conferred a true inhibitory effect (rather than function as an alternative substrate) and this is consistent with previously reported models of the mechanism of action of this class of enzymes. Furthermore, this class of analogue demonstrated anti-leishmanial activity *in cellulo* with a significant proportion of this activity indicated to be due to on-target effects.

These SAR studies will contribute to the development of a pharmacophore model of the active site of this membrane bound enzyme and help guide the design of future inhibitors of this essential enzyme as potential new drug treatments for leishmaniasis. In this respect it is pertinent to note that through these studies the microitre plate-based assay has been statistically validated for a future high-throughput screening purposes with a *Z*-value > 0.5. Studies in this direction are in progress and will be reported in due course.

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Graphical Abstract

A series of ceramide analogues have been synthesised and evaluated as substrates for the protozoan enzyme inositolphosphoryl ceramide synthase from *Leishmania Major*