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Post-print of a peer-reviewed article published by Portland Press Ltd. – a wholly owned subsidiary of the Biochemical Society.
The final version of record is available at: http://dx.doi.org/10.1042/BST20110769

Cite as:
Lowther, J., Naismith, J. H., Dunn, T. M., & Campopiano, D. J. (2012). Structural, mechanistic and regulatory studies of serine palmitoyltransferase. Biochemical Society Transactions, 40(3), 547-54.

Manuscript received: 08/03/2012; Article published: XX/06/2012

Structural, mechanistic and regulatory studies of serine palmitoyltransferase**

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[**]Work on SPT and SLs by D.J.C., J.L. and J.H.N. is supported by the Biotechnology and Biological Sciences Research Council [grant numbers BB/F009739/1 and BB/I013687/1]. The collaboration between D.J.C. and T.M.D. is supported by a Biotechnology and Biological Sciences Research Council United States Partnering Award (USPA) [grant number BB/G53045X/1].

Keywords:
external aldimine, pyridoxal 5′-phosphate (PLP), quinonoid intermediate, serine palmitoyltransferase (SPT), sphingolipid, *Sphingomonas* sp.

Abbreviations:
ACP, acyl-carrier protein; AOS, α-oxoamine synthase; ER, endoplasmic reticulum; GSL, glycosphingolipid; HSAN1, hereditary sensory and autonomic neuropathy type 1; LCB, long-chain base; ORM, orosomucoid; ORMDL, orosomucoid-like; PLP, pyridoxal 5′-phosphate; SL, sphingolipid; SPT, serine palmitoyltransferase; *Sm*SPT, *Sphingomonas multivorum* SPT; *Sp*SPT, *Sphingomonas paucimobilis* SPT; *Sw*SPT,*Sphingomonas wittichii* SPT; ssSPT, SPT small subunit.

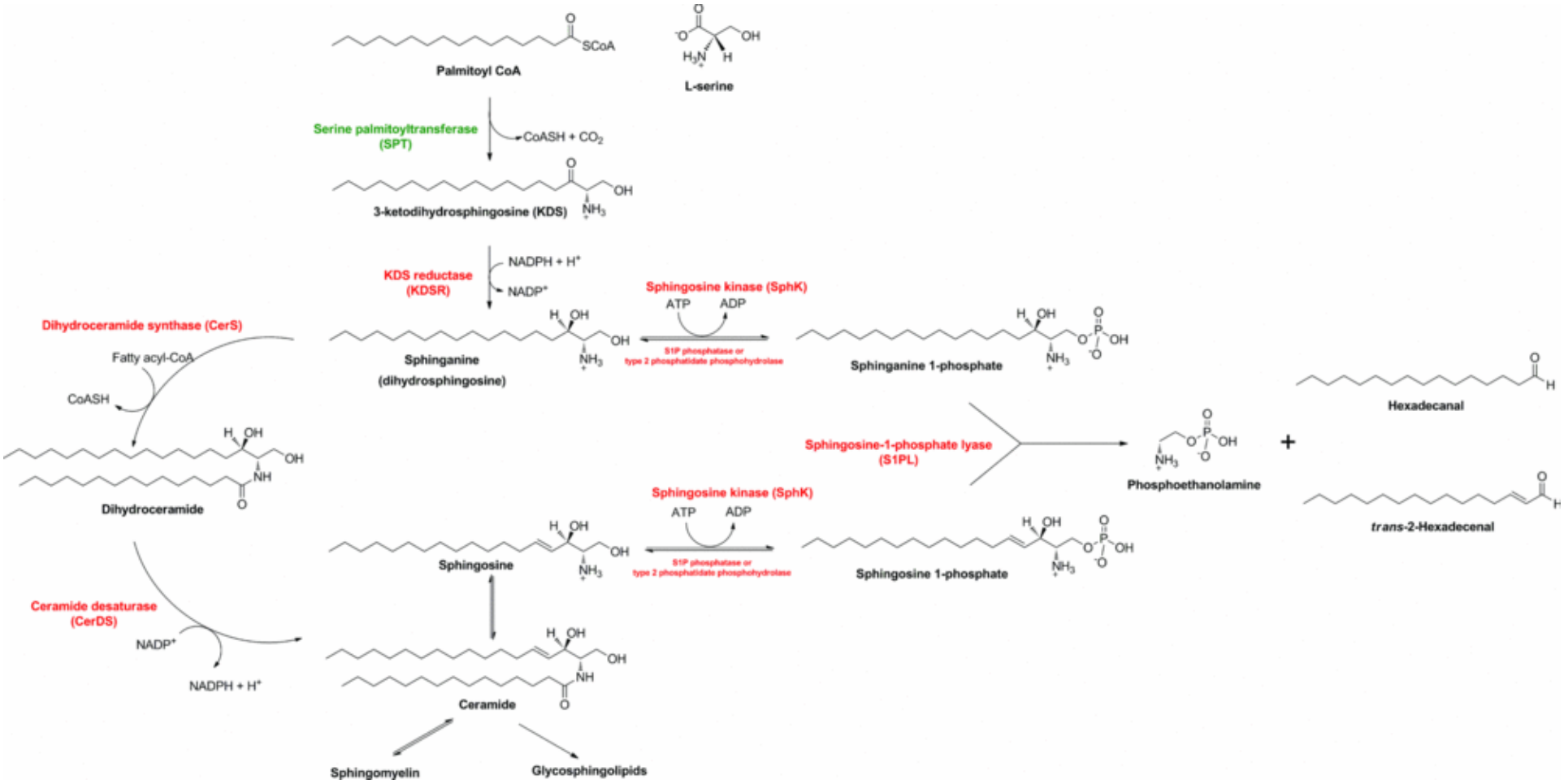
Abstract

Sphingolipids (SLs) are composed of fatty acids and a polar head group derived from L-serine. SLs are essential components of all eukaryotic and many prokaryotic membranes but sphingosine 1-phosphate (S1P) is also a potent signalling molecule. Recent efforts have sought to inventory the large and chemically complex family of SLs (LIPID MAPS Consortium). Detailed understanding of SL metabolism may lead to therapeutic agents specifically directed at SL targets. We have studied the enzymes involved in SL biosynthesis; later stages are species-specific, but all core SLs are synthesized from the condensation of L-serine and a fatty acid thioester such as palmitoyl-CoA that is catalysed by serine palmitoyltransferase (SPT). SPT is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that forms 3-ketodihydrosphingosine (3-KDS) through a decarboxylative Claisen-like condensation reaction. Eukaryotic SPTs are membrane-bound multi-subunit enzymes, whereas bacterial enzymes are cytoplasmic homodimers. We use bacterial SPTs (e.g. from *Sphingomonas*) to probe their structure and mechanism. Mutations in human SPT cause a neuropathy (HSAN1), a rare SL metabolic disease. How these mutations perturb SPT activity is subtle and bacterial SPT mimics of HSAN1 mutants affect the enzyme activity and structure of the SPT dimer. We have also explored SPT inhibition using various inhibitors (e.g. cycloserine). A number of new subunits and regulatory proteins that have a direct impact on the activity of eukaryotic SPTs have recently been discovered. Knowledge gained from bacterial SPTs sheds some light on the more complex mammalian systems. In the present paper, we review historical aspects of the area and highlight recent key developments.

Introduction

SLs are one of eight categories of lipid of the LIPID MAPS classification system [1]. Mammalian cells produce a vast array of SLs that function not only as structural elements of cellular membranes, but also as potent bioactive signalling molecules (Scheme 1). They comprise a sphingoid base that can be amide-linked to a second fatty acid to give ceramides and derivatized further by the addition of a headgroup such as a sugar or phosphate. Structural diversity in the SL family arises from variations in the acyl chains and the large number of possible headgroups [2].

SLs are also produced by plants, fungi and some bacteria, but the biosynthetic pathway in each can differ. For example, sphingosine is the major sphingoid base found in mammals, whereas phytosphingosine is found in fungi (Figure 1A). SL biosynthesis can also vary between species; the yeast *Saccharomyces cerevisiae* produces solely complex SLs containing a phosphoinositol headgroup, whereas *Pichia pastoris* produces both phosphoinositol-based and glucosylceramide-based GSLs (glycosphingolipids) [3]. SL biosynthesis also varies between bacterial species. GSLs are ceramides isolated from the Gram-negative bacterium *Sphingomonas paucimobilis* that contain either saturated and unsaturated sphingoid bases derived from palmitic acid that are attached to a second fatty acyl group derived from myristic acid [4].



Scheme 1. **The series of enzymatic steps that makes up SL *de novo* biosynthesis in mammals.** SPT (green) catalyses the first rate-limiting step; the enzyme names for other major steps in the pathway are in red.

By comparison, SLs in the dental pathogen*Porphyromonas gingivalis* contain unusual iso-branching on both the sphingoid base and the amide-linked fatty acid [5]. Therefore some organisms express anabolic enzymes for SL biosynthesis not found in others. Conversely, the first enzymatic step of *de novo* SL biosynthesis is common in all SL-producing organisms studied to date (Figure 1A). This step is carried out by the pyridoxal 5′-phosphate (PLP)-dependent serine palmitoyltransferase (SPT) [6]. The SPT gene is indispensable and thus can be used as a marker for identifying species of the SL-producing bacterium *Sphingomonas* in environmental samples [7]. SPT catalyses a Claisen-like condensation between L-serine and an acyl (typically palmitol) coenzyme A (CoASH) or an acyl-ACP (acyl-carrier protein) thioester substrate, to form the first sphingolipid metabolite 3-ketodihydrosphingosine (3-KDS) (Figure 1B). Hence this condensation reaction forms the sphingoid base or LCB (long-chain base) found in all subsequent intermediate SLs and complex SLs in the organism. An abridged version of the SPT enzymatic reaction shown in Figure 2(A) indicates three important intermediates: (I) the holo-form or internal aldimine of the enzyme with PLP cofactor bound to the active-site Lys^{265} (*S. paucimobilis* SPT) numbering] via a Schiff base; (II) the external aldimine with L-serine following transimination; and (III) the quinonoid intermediate (carbanion equivalent) following deprotonation at C_α that attacks the incoming thioester substrate.

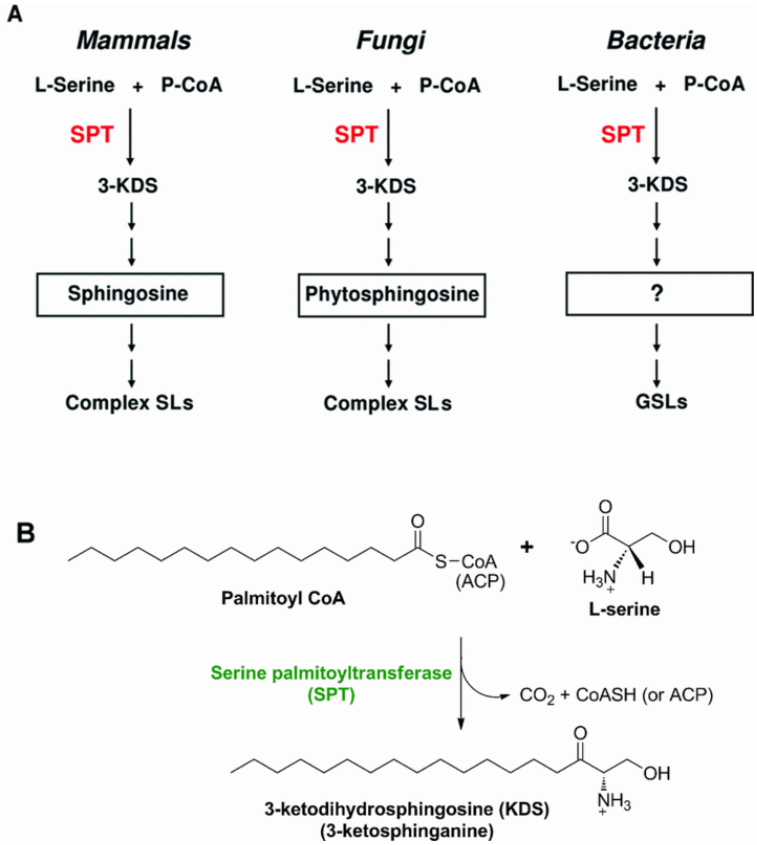


Figure 1. **The key step in sphingolipid biosynthesis is conserved across all organisms studied to date.** (**A**) The *de novo* pathway for SL biosynthesis can differ between species, but the first step, catalysed by SPT, is common across mammals, plants, fungi and bacteria. 3-KDS, 3-ketodihydrosphingosine; P-CoA, palmitoyl-

CoA. (**B**) All SPTs condense L-serine with a fatty acyl derived from either an acyl-CoA substrate or an acyl-ACP substrate.

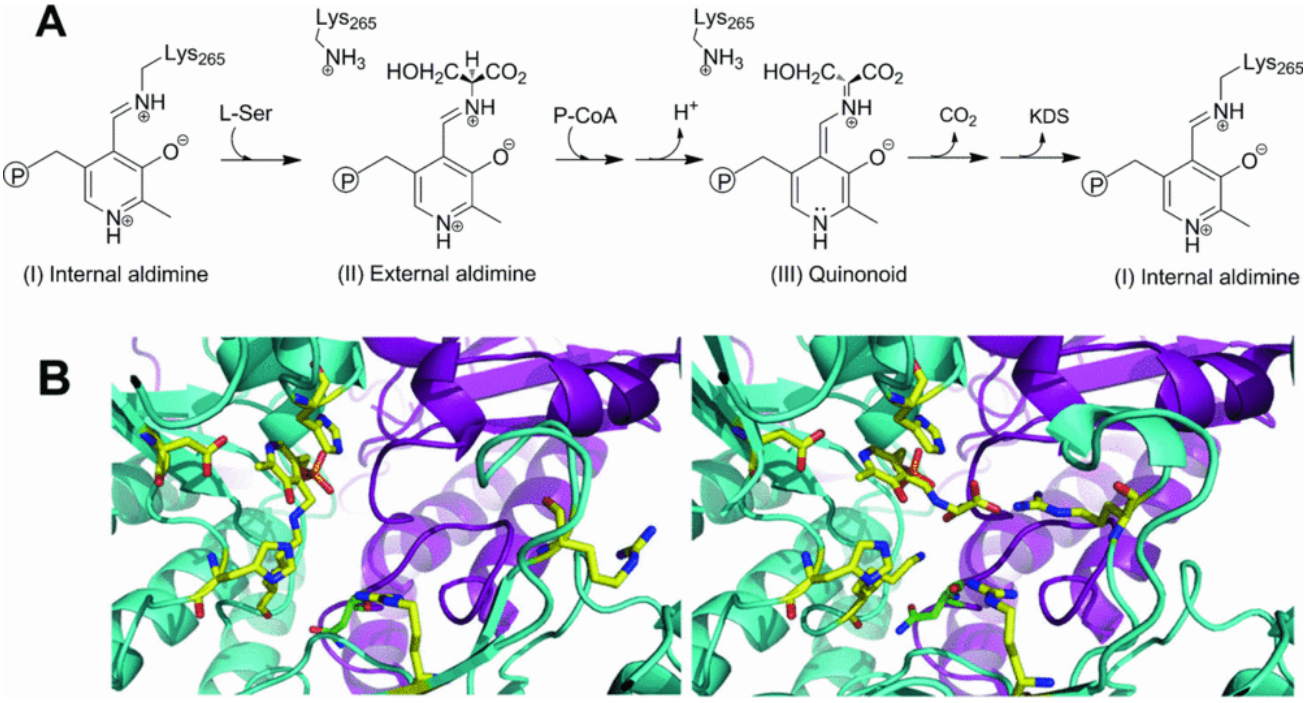


Figure 2. **Mechanism and structure of bacterial SPT.** (**A**) Abridged mechanism for the reaction catalysed by SPT. The reaction is dependent on its active-site PLP cofactor. (**B**) Structural comparison of the SPT internal aldimine with the L-serine-bound external aldimine form.

Three-dimensional structures of bacterial SPTs

SPT is a member of the alpha-oxoamine synthase (AOS) family of PLP-dependent enzymes and is classified in the fold-type I family of which aspartate aminotransferase is the prototype [8]. Three-dimensional crystal structures of several AOS enzymes have been solved including 8-amino-7-oxononanoate synthase (AONS) [9], 5-aminolaevulinate synthase (ALAS) [10], 2-amino-3-oxobutyrate:CoA ligase (KBL) [11] and cholera quorum-sensing autoinducer-1 synthase (CqsA) [12,13]. Isolation of a homodimeric and water-soluble native SPT from the Gram-negative bacterium *S. paucimobilis* (*Sp*SPT) [14] paved the way for structural studies on this enzyme (Table 1). The first crystal structure of the holoenzyme at 1.3 Å resolution (PDB code 2JG2) revealed a symmetrical SPT homodimer with PLP cofactor bound to a conserved Lys265 in each subunit [15] (Figure 2B). The PLP cofactor is held in place via several interactions with active-site residues (at the dimeric interface from both subunits), the most important being π-stacking between the PLP pyridine ring and a conserved His159. Correct positioning of the PLP cofactor in the active site is vital, since addition of the L-serine

substrate to SPT His159 mutants led to an abortive transamination reaction [16]. Subsequent studies captured the enzyme with the L-serine substrate bound as an external aldimine Schiff base following transimination at Lys265 [17]. In this structure, a non-conserved arginine residue (Arg378) had undergone a large swing into the active site to interact with the carboxy moiety of the PLP:L-serine external aldimine substrate (Figure 2B). Active-site arginine residues are crucial for recognition of the L-serine carboxy moiety during the catalytic cycle in this SPT homologue. In fact, mutation of a second active-site arginine residue in *Sphingomonas wittichii* SPT (*Sw*SPT) (Arg370 equivalent to Arg390 in *Sp*SPT), conserved in all SPTs and across the AOS family, resulted in an enzyme that could not form the subsequent quinonoid intermediate (species (III) in Figure 2A) in the presence of a palmitoyl-CoA analogue [18].

Table 1. List of bacterial SPT homodimers and the current components of the human and yeast SPT complexes.

SPT homodimer	**UniProt number**	**Reference(s)**
Bacterial SPT homodimers		
Sphingomonas paucimobilis	Q93UV0	[**14**,**15**]
Sphingomonas wittichii	A5VD79	[**20**]
Sphingobacterium multivorum	A7BFV6	[**19**,**22**]
Sphingobacterium spiritivorum	A7BFV7	[**22**]
Bdellovibrio stolpii	A7BFV8	[**22**]
Bacteroides fragilis	Putative	[**27**]
Eukaryotic SPT complexes		
Human		
LCB1	O15269	[**34**]
LCB2	O15270	[**33**,**34**]
LCB3	Q9NUV7	[**31**]
Small subunit A	Q969W0	[**47**]
Small subunit B	Q8NFR3	[**47**]
ORMDL1/ORMDL2/ORMDL3	Q9P0S3, Q53FV1, Q8N138	[**48**,**50**]
Saccharomyces cerevisiae		
LCB1	P25045	[**29**,**32**,**35**]
LCB2	P40970	[**32**]
Tsc3	Q3E790	[**23**]
ORM1/ORM2	P53224, Q06144	[**48**–**50**]

The three-dimensional structures of two other SPT homologues, *Sphingobacterium multivorum* SPT (*Sm*SPT, 38% homology) and *Sw*SPT (70% sequence identity), have since been elucidated. The external aldimine form of*Sm*SPT revealed an enzyme with an architecture very similar to that of *Sp*SPT [19]. One major difference was

that the carboxy group of the PLP:L-serine external aldimine formed was bound via two water molecules to two residues (Ser81 and Met271) on the opposite monomer, indicating that structural variations occur at the active site among homologous SPTs. Elucidation of the holoenzyme structure of *Sw*SPT, from the dioxin-degrading bacterium *S. wittichii*, uncovered further variations [20]. For example, the entrance to the active site is wider than in its *Sp*SPT homologue, suggesting that *Sw*SPT may utilize a larger acylated-ACP thioester substrate rather than an acyl-CoA thioester. This prediction is strengthened by the fact that a gene encoding a putative bacterial Type II ACP resides immediately upstream of the *Sw*SPT gene. Phylogenetic analysis of AOS enzymes has since found six bacterial SPT genes, including *Sw*SPT, associated with ACP genes [21].

Other bacterial SPT isoforms

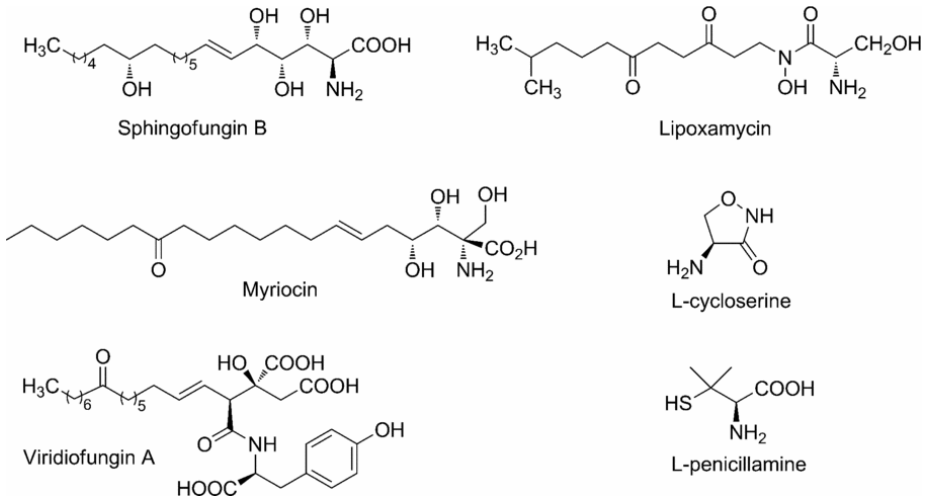
Along with the structural characterization of SPTs from the *Sphingomonas* strains described above, other SPTs have been characterized in terms of PLP-binding and substrate specificity from bacterial species including *S. multivorum*, *Sphingobacterium spiritivorum* and *Bdellovibrio stolpii* (see Table 1) [22]. The PLP spectrum for each of these homologues was different from their *S. paucimobilis* counterpart. The SPTs from *S. multivorum* and *B. stolpii*were found to be peripheral proteins associated with the inner cell membrane, and therefore may more closely resemble the endoplasmic reticulum (ER)-bound eukaryotic SPTs. Furthermore, the SPT from *B. stolpii* displayed substrate inhibition by palmitoyl-CoA, a characteristic also found in the yeast [23] and mammalian SPTs [24–26].

Recently, a study investigating the function of SLs in the gut-dwelling commensal bacterium *Bacteroides fragilis*suggested that SLs play a key role in survival strategies that allow the bacterium to persist under harsh conditions in the intestine [27]. Bacterial cultures grown in the presence of the SPT inhibitor myriocin were more susceptible to stressful conditions, suggesting a role for SLs in survival. Also in this study, a putative SPT gene was identified in *B. fragilis* by BLAST search [27]. Studies in our laboratory have confirmed that a purified recombinant version of this enzyme has SPT activity (Bower, Lowther, Campopiano et al, unpublished data). Kinetic evaluation revealed an overall slower turnover compared with SPTs from *Sphingomonas*strains, as well as substrate inhibition by the acyl-CoA substrate similar to the SPT from *B. stolpii*.

Inhibitors of SPT

Several natural products (e.g. myriocin) have been identified as potent SPT inhibitors (Scheme 2) and are routinely used as ‘blockers’ of sphingolipid biosynthesis in cells. Surprisingly, few complete investigations into the exact mechanism of inhibition by each have been undertaken. We previously carried out a detailed study on inhibition of SPT by the antibiotic L-cycloserine. This broad-spectrum inhibitor usually disables its

target by forming an irreversible 3-hydroxyisoxazole:PLP adduct at the active site of several PLP-dependent enzymes such as alanine aminotransferase. However, prolonged incubation with SPT resulted in formation of pyridoxamine 5′-phosphate (PMP) and a small aldehyde product, β-amino-oxyacetaldehyde, both observed at the enzyme active site using X-ray crystallography and confirmed by MS [28]. Elucidation of the structure of another *Sp*SPT crystal grown in the presence of cycloserine revealed an elongated Lys265 side chain, in keeping with *in situ* modification by the cycloserine-generated aldehyde (Lowther, Naismith, Campopiano et al work). Studies into the mechanism of inactivation by the SPT inhibitors L-penicillamine, β-chloro-L-alanine and myriocin are ongoing.



Scheme 2. **Natural product inhibitors of SPT.**

HSAN1 and deoxy-SLs

It is thought that human SPT contains a core heterodimer that spans the outer membrane of the ER [28] comprising a "non-PLP-binding" LCB1 subunit (encoded by the SPTLC1 gene) together with a PLP-containing LCB2 subunit (encoded by either SPTLC2 or SPTLC3). Here we describe LCB1 as not be able to bind PLP but this has yet to be proven since the PLP binding capacity of each subunit has not been analysed. We infer this from sequence homology/alignment studies; LCB2 contains all of the residues conserved among AOS family members involved in PLP binding and catalysis, i.e. a key lysine residue, two histidine residues and an aspartate residue, whereas LCB1 has none of these residues. Nevertheless, LCB2 is not expressed (and may not be stable) in the absence of LCB1, so the active catalytic SPT complex is thus a LCB1/LCB2 heterocomplex. Whether LCB1 contributes catalytic residues to the PLP active site remains to be determined. Specific mutations identified in either *SPTLC1* or *SPTLC2* cause a rare genetic disorder called hereditary sensory and autonomic neuropathy type 1 (HSAN1) [37–39]. Three-dimensional structures of two bacterial

*Sp*SPT mimics, containing mutations similar to those identified in the LCB1 subunit of HSAN1 patients, revealed subtle structural changes at the enzyme active site [17]. Dunn and colleagues showed that yeast or CHO cells expressing human heterotrimeric SPT (an LCB1–LCB2 heterodimer along with an ssSPT small subunit that is required for optimal SPT activity, see below) carrying the HSAN1-causing mutation (C133W) in the LCB1 subunit gave rise to an enzyme with enhanced ability to condense L-alanine with the acyl-CoA substrate [40]. It therefore appears that HSAN1 mutations cause structural perturbations in the human enzyme, resulting in an altered specificity for the amino acid substrate. This relaxed specificity for amino acid substrate is consistent with the 'gain-of-function' phenotype of elevated levels of deoxysphingoid bases (deoxy-LCBs) arising from condensation of alanine and glycine with palmitoyl-CoA that is observed in HSAN1 patients and in HSAN1 transgenic mice [41].

The absence of the C1-OH in the deoxy-LCBs precludes phosphorylation by LCB kinase and degradation by the LCB-phosphate lyase in the only known pathway for LCB degradation. This may be in keeping with a late onset of disease in HSAN1 patients, i.e. these lipids gradually accumulate to toxic levels over time. It is also interesting to note that deoxysphingoid bases were also observed in 'healthy' cells, suggesting that wild-type SPT has some degree of amino acid substrate freedom to generate these lipids normally. An exciting promising pilot study results suggest that a treatment may be on the horizon for HSAN1 sufferers. A new report shows that oral administration of the natural substrate L-serine could compete with L-alanine and glycine, prevented accumulation of deoxy-SLs and improved HSAN1 symptoms [42].

It is important to stress that deoxy-SLs are natural metabolites that occur at low concentrations in all mammalian cells with a wild-type SPT. For example, deoxysphinganine was identified in kidney cells following addition of the ceramide synthase (CerS) inhibitor fumonisin B1 [43]. More recently, deoxyceramide has been found attached to an isolated human CD1b receptor [44]. Specific functions for deoxy-SLs are still unknown and, because they cannot be phosphorylated, the mechanism by which they are degraded or removed from the cell has yet to be revealed. This will be a particular interesting avenue of study, since it appears that their accumulation over a long period of time causes cytotoxicity in HSAN1. Further research is also required to understand the natural promiscuity of wild-type SPT and whether this phenomenon is unique to SPT among all AOS enzymes.

Identification of new subunits and regulators of the eukaryotic SPT complex

The SPT complex from higher organisms appears to comprise a core LCB1/LCB2 heterodimer (Table). The PLP-containing LCB2 subunit carries out the condensation reaction and although the LCB1 subunit appears to lack the residues that bind PLP it is still required for activity [38]. Hornemann *et al* identified a second isoform of LCB2 (LCB3, encoded by the *SPTLC3* gene) that is expressed only in certain tissues.[31] These two isoforms are functionally redundant in plants [45], but may have distinct functions in mammals since mice

lacking the *Sptlc2* gene were embryonic lethal [46]. It is interesting that some higher eukaryotes have two LCB2-like isoforms and others (e.g.*Drosophila*) have only one. Identification of novel components of SPT other than the core LCB1/LCB2 heterodimer has meant that the current model of the human SPT complex has advanced greatly in recent years. A third small subunit (Tsc3) associated with the heterodimer and required for maximal SPT activity was initially found by Dunn and colleagues in yeast [23], but bioinformatic approaches failed to identify candidate activators of the LCB1–LCB2 heterodimers from higher eukaryotes. However, poor correlation of SPT activity with increased *SPTLC1*/*SPTLC2*expression in mammalian cells and the failure to reconstitute SPT activities comparable with those in mammalian microsomes upon expression of mammalian *SPTLC1* and *SPTLC2* in yeast led Dunn and her collaborators to search for an additional factor similar to the activity-enhancing Tsc3 subunit they had found previously in yeast [47]. Intriguingly, two novel small subunits (ssSPTa and ssSPTb) with no homology with Tsc3p were identified. Either of these small subunits can enhance activity >10-fold when bound to the LCB1/LCB2 (*SPTLC1*/*SPTLC2* or*SPTLC1*/*SPTLC3*) heterodimer. Furthermore, these small activating subunits of SPT also confer distinct specificities for acyl-CoA substrates. The recent exciting discovery by two independent groups (led by Jonathan Weismann [48] and Amy Chang [49]) that the yeast ORM (orosomucoid) 1/ORM2 proteins also associate with and negatively regulate SPT activity has added an additional layer of complexity and prompted the authors to coin the term “SPOTS” complex [serine palmitoyltransferase, ORM1/2, Tsc3, Sac1 (phosphatase)] to incorporate these novel components (Table). The ORMDL (orosomucoid-like) proteins are part of a family of endoplasmic reticulum proteins that were found to play a role in lipid homoeostasis and control of protein quality and trafficking [50]. The ORMs themselves are controlled in yeast through phosphorylation various kinases and Sac1 phosphatase [51]. It appears that the human ORMDL1/2/3 homologues also interact with the LCB1/LCB2 heterodimers (and associated ssSPT, ORM and Sac1 subunits??) embedded in the membrane [48] raises further questions about the organization and regulation of SPT. Exactly how the cellular sphingolipid/ceramide levels are relayed back to this SPOTS complex to turn it on and off is not clear. Detailed studies of the topological organization and interactions between the components of the SPOTS complex will shed light on how these components interact to insure that this committed and rate-limiting enzyme of SL synthesis is properly regulated.

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