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Lipidomic "Deep Profiling": An Enhanced Workflow to Reveal New Molecular Species of Signaling Lipids

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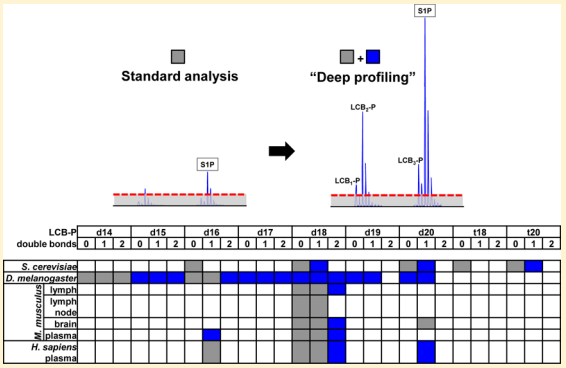
Supporting Information

ABSTRACT: Current mass spectrometry-based lipidomics aims to comprehensively cover wide ranges of lipid classes. We introduce a strategy to capture phospho-monoester lipids and improve the detection of long-chain base phosphates (LCB-Ps, e.g., sphingosine-1-phosphate). Ten novel LCB-Ps (d18:2, t20:1, odd carbon forms) were discovered and characterized in tissues from human and mouse, as well in *D. melanogaster* and *S. cerevisiae*. These findings have immediate relevance for our understanding of sphingosine-1-phosphate biosynthesis, signaling, and degradation.



Lipids are small molecules with large structural and chemical diversity. Over the past two decades, technologies such as chromatography and mass spectrometry have driven the biochemical analyses of complex lipid mixtures, tremendously advancing our knowledge of lipid diversity. Lipid extraction (preceding analysis), however, is still largely based on partitioning procedures developed in the 1950s. Although appropriate for many abundant components, these approaches result in variable recovery of the less-abundant and highly charged lipids, including phosphorylated signaling lipids.

Phosphate is common among biological lipids and present in either monoester and/or diester configurations, with most biologically active lipids containing at least one phospho-monoester, for example, sphingosine-1-phosphate (S1P). Long-chain amino alcohols, generally referred to as long-chain bases (LCB),[1] and their phosphorylated forms (LCB-P) display particularly diverse chemistries across biological species and tissues. In human plasma, the phosphorylated derivative of the LCB with a linear 18 carbon chain, 1,3-dihydroxy, and C4 double bond, i.e., d18:1 S1P [(2S, 3R, 4E)-2-aminooctadec-4-ene-1,3-diol], is the most abundant LCB-P. However, little is known about the existence of other LCB-Ps of different chemical and structural composition, despite the presence of various nonphosphorylated LCB precursors. Here, we sought to experimentally explore this potentially uncharacterized landscape using a capture/targeting approach ("deep profiling").

Enabling this work was the use of a novel polymeric capture phase containing bis-imidazolium moieties capable of complexing phosphate (Supplementary Figure 1, Supporting Information); low molecular weight bis-imidazolium hosts have been extensively studied for the recognition of inorganic phosphate.[2] The phase was prepared by radical copolymerization of (i) bis-imidazolium monomer and (ii) ethylenglycol dimethacrylate to give a macroporous monolith that was crushed, sieved, and used directly after solvent extraction for solid phase extraction (imidazolium polymer, IMP). A complex mixture (Figure 1a), derived from a methanol extraction of murine brain tissue and thus expected to contain phospho-diester and phospho-monoester lipids in addition to lipids without phosphate, was mixed with lipid standards (see Supporting Information) and loaded (in isopropanol) onto the immobilized imidazolium

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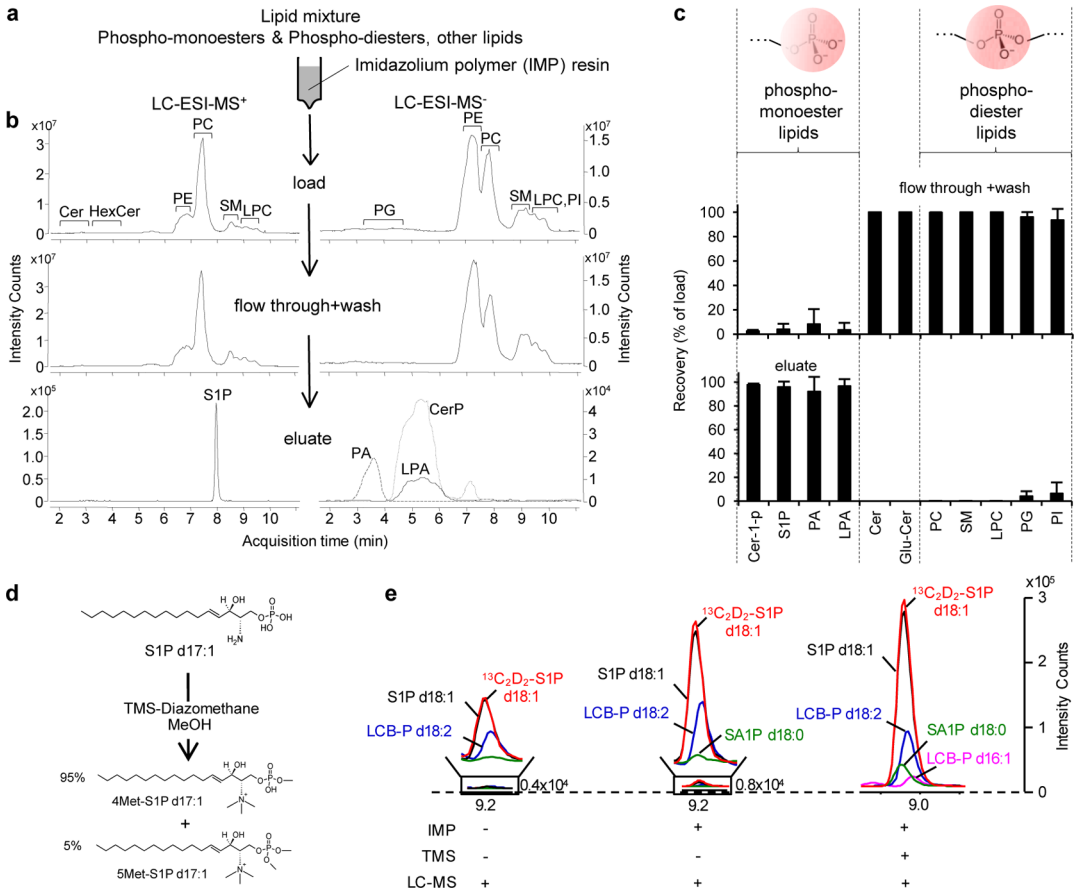

Figure 1. Substantially improved detection of LCB-P via an analytical workflow using phospho-monoester capture and derivatization. (a) Methanol extracts from different types of starting materials were loaded onto an imidazolium polymer (IMP, Supplementary Figure 1, Supporting Information) after protein precipitation (see Supporting Information for detailed protocol). (b) Extracted ion chromatograms (EIC) of the load, flow-through/wash, and eluate fractions as analyzed by liquid chromatography-nanoelectrospray ionization quadrupole time of flight mass spectrometry in either positive (left panel) or negative (right panel) ionization modes. (c) IMP selectively captures phospho-monoester lipids (Ceramide-1-phosphate, Cer-1-P; sphingosine-1-phosphate, S1P; phosphatidic acid, PA; lysophosphatidic acid, LPA). Lipids with no phosphate (Ceramide, Cer; Glucosyl-Ceramide, Glu-Cer) and those with phosphorus bound in diester configuration (phosphatidylcholine, PC; sphingomyelin, SM; lyso-phosphatidylcholine, LPC; phosphatidylglycerol, PG; and phosphatidylinositol, PI) do not efficiently bind to IMP and are recovered in the flow through and wash fractions instead (Supplementary Table 1, Supporting Information). (d) Structure of d17:1 S1P standard before and after derivatization with TMS-diazomethane. The 4Met-S1P derivative (95% of the reaction product) and 5Met-S1P (5%) are drawn based on results from high-resolution product ion spectra (Supplementary Figure 2, Supporting Information). (e) Two-step analytical enhancement using IMP enrichment (~2-fold increase in signal intensity, and importantly, removal of matrix interference, Supplementary Figure 3, Supporting Information) and TMS derivatization (~30-fold increase in signal intensity) leading to substantially improved detection of long-chain base phosphates (LCB-P) with different aliphatic compositions (e.g., the diene form of S1P, LCB-P d18:2) in human blood plasma with excellent analytical precision and linearity (Supplementary Table 2, Supporting Information).

polymer. The "load", "flow-through"/"wash", and "eluate" fractions were analyzed by liquid chromatography-nanoelectrospray ionization mass spectrometry in positive (LC-ESI-MS^+) and negative (LC-ESI-MS^-) polarity. All components with either no phosphate or with diester phosphates in their structures were recovered in the flow-through and wash fractions (Figure 1b,c). Acidification of the solvent system (chloroform/methanol, 1% trifluoroacetic acid; see Supporting Information) led to the quantitative elution of phospho-monoester lipids (Figure 1b,c; Supplementary Table 1, Supporting Information). Therefore, this new IMP resin can separate phospho-monoesters (typically <5% of the total membrane lipid mass) from the more abundant phospho-diester lipids (≥60%). This enrichment step is beneficial for subsequent MS analysis in terms of improvement of detection of phospho-monoesters, due to a lower suppression of other compounds possibly coeluting during a common LC separation and/or to the removal of other small molecules with a similar molecular weight. Note that we did not evaluate the specificity of this material, for example, for the discrimination of sulfate (which closely resembles phosphate in charge and geometry). This fractionation does not require any chemical modification, nor does it lead to quasi-irreversible complex formation.[3]

In a second step of method optimization, we decided to neutralize the negative charge of the zwitterionic LCB-P by derivatization with trimethylsilyldiazomethane (TMS-diazomethane), a commonly used methylating reagent.[4] Methylation conditions were optimized using synthetic d17:1 S1P standard (Figure 1d, Supplementary Figure 2a, Supporting Information), and the reaction products were characterized by LC-MS (Supplementary Figure 2b,c, Supporting Information). Incubation of S1P standard in methanol at 25 °C for 20 min yielded 95% tetra-methyl-S1P (Met_4-S1P) and 5% penta-methyl-S1P (Met_5-S1P, Supplementary Figure 2b, Supporting

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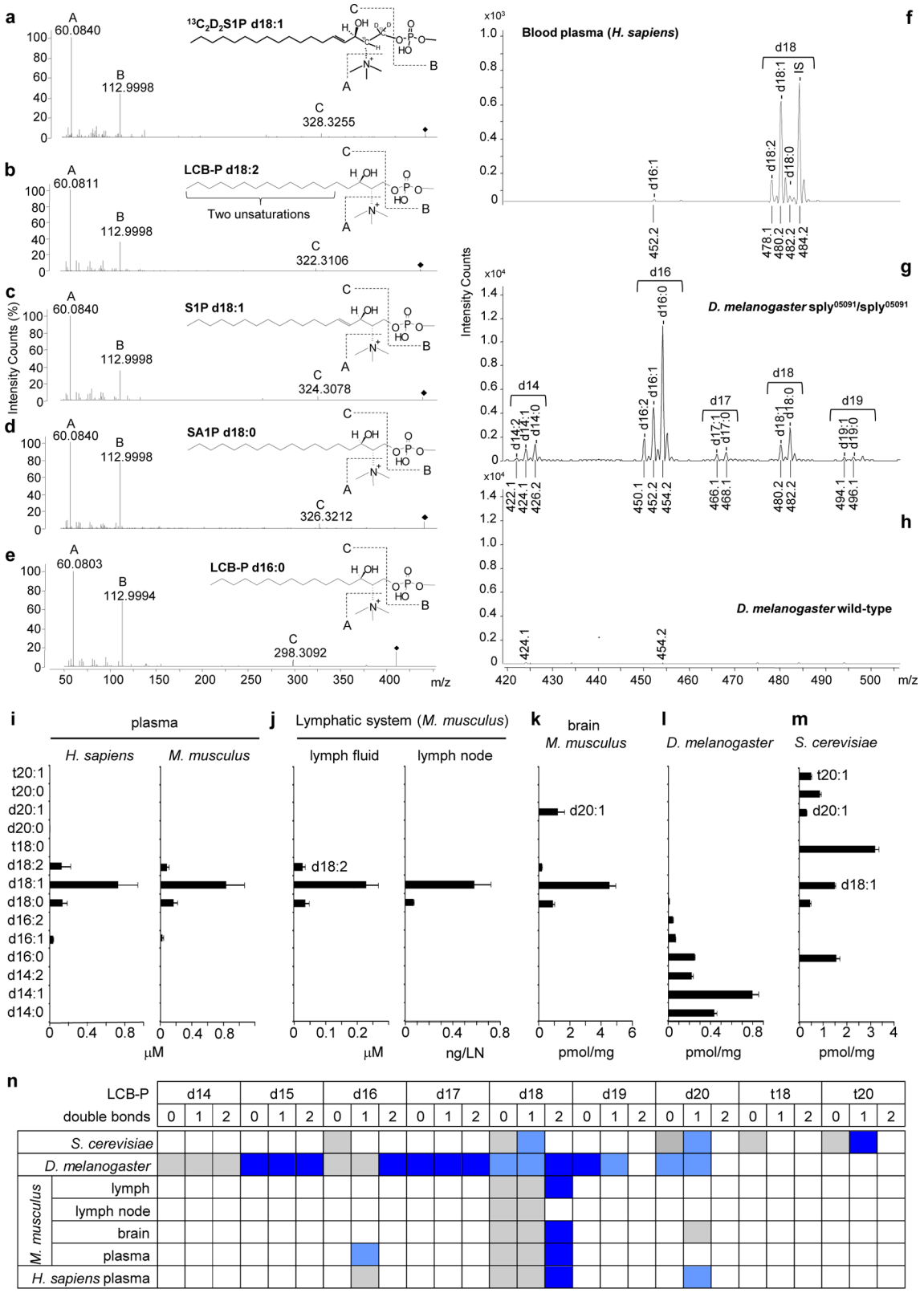

Figure 2. Discovery, characterization, and quantification of new LCB-P in complex mixtures from diverse biological species. (a) Collision-induced dissociation product ion mass spectra (in positive ionization mode) of TMS-derivatized synthetic stable isotope standard (d18:1 $^{13}C_2D_2$-S1P, m/z 440, diamond symbol) yielded expected ions at m/z 60 (fragment A), m/z 113 (fragment B), and m/z 328 (fragment C). The former two fragments are expected to be invariant for LCB-P with different aliphatic compositions which is indeed the case as shown for corresponding product ion spectra of LCB-P d18:2 (b), LCB-P d18:1 (c), and LCB-P d18:0 (d), all derived from extracts of human blood plasma, and d16:0 (e) from whole fly extract. The C fragment, instead, is characteristic of individual species of LCB-P, thus allowing for identification by targeted tandem mass spectrometry (Supplementary Figure 6, Supporting Information). Targeted mass spectrometric analysis based on scanning for neutral loss of the methylated amine fragment (m/z 60) allows for determination of LCB-P pattern in extracts derived from human plasma (f) and *D. melanogaster* (g and h).

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Figure 2. continued

Sphingosine-phosphate lyase (sply in *Drosophila*) is the key enzyme in the irreversible degradation of LCB-P. Deletion of this gene in *Drosophila* leads to an accumulation of a multitude of LCB-Ps, many of which have not been described before (g, Supplementary Table 3, Supporting Information). Quantification using multiple reaction monitoring (Supplementary Table 4, Supporting Information) of LCB-P in mammalian blood plasma (i) and lymphatic system (j), as well as extracts from model organisms (k−m), led to the discovery of new LCB-Ps (Supplementary Table 5, Supporting Information). (n) LCB-P species detected in this study. Dark blue, new LCB-P not described so far in the literature; light blue, LCB-P not known to exist in the respective biological species/tissue; gray, LCB-P previously described and measured with comparable results in this study (see Supplementary Table 6, Supporting Information).

Information), with no other reaction products detected. Derivatization led to a sensitivity gain of approximately 25−30-fold compared with the nonderivatized S1P (Figure 1e), possibly explained by a more effective ionization of the methylated form, which carries a permanent positive charge on the amine group (Figure 1d).

By introducing and optimizing the above two steps (phospho-monoester capture via IMP and derivatization with TMS), we (i) improved the signal intensity of LCB-Ps derived from plasma by ∼60-fold compared with LC-MS alone (Figure 1e); (ii) eliminated the carry-over effects commonly encountered with LC-MS of native LCB-Ps;[5] and (iii) reduced matrix interference that could suppress LCB-Ps ionization or be considered erroneously as a specific signal when using low resolution MS. This last experimental improvement is dependent on the starting material and is particularly important in very complex samples, as in murine brain (see interfering ions in Supplementary Figure 3, Supporting Information, that could compromise the reliability of the analysis) or whole fly extracts, in which no LCB-P signal could be detected without the use of the IMP-based enrichment. This enhanced workflow led to the discovery of previously undescribed LCB-P species unable to be detected/quantified by LC-MS alone, for example, d16:1 in human plasma (Figure 1e). This new detection method was further validated in various biological matrices for its accuracy and precision, reproducibility, and linearity (Supplementary Table 2, Supporting Information). Moreover, the number of species detected and their relative abundance were conserved after the derivatization reaction (Supplementary Figure 4, Supporting Information). The aforementioned characterizations involved nanoelectrospray ionization and high-resolution MS after chromatographic separation of the mixtures by hydrophilic interaction chromatography (HILIC) built into a microfluidic chip. This was important to improve the coelution of S1P standards and endogenous LCB-Ps for qualitative (Supplementary Figure 5, Supporting Information) and quantitative characterizations[6] of new LCB-P species. Indeed, few high-resolution mass spectra of LCB-Ps have been published, possibly because of the low signal intensities that hamper the precise characterization of ion peaks. The intrinsic characteristics of the HPLC chip contributed to the high sensitivity (nanospray ionization) and reproducibility (increased spray stability) of these studies. As both the enrichment and analytical columns present inside the chip were based on HILIC resins, the most hydrophobic molecules could be depleted from the sample by initially loading on the enrichment column before switching to the analytical and starting the elution process. This step further decreased matrix suppression effects.

We next extended our analysis to various biological species known to have different LCB (and thus possibly also LCB-P) inventories. When fragmented by collision-induced dissociation (CID), derivatized d18:1 $^{13}C_2D_2$-S1P standard (m/z 440) yielded three product ions, denoted ions A−C. Originating from the methylated amine group (fragment A at m/z 60; Figure 2a) and methyl-phosphate (fragment B at m/z 113), both A and B ions are expected to be invariant for different LCB-Ps compared with ion C (m/z 328), which originated from the aliphatic portion. Indeed, when LCB-Ps from plasma were analyzed, the C fragment yielded m/z values as predicted for d18:2 (Figure 2b), d18:1 (Figure 2c), d18:0 (Figure 2d), and d16:0 for a lipid extract from fly (Figure 2e, Supplementary Figure 6, Supporting Information, for summary).

This product ion information, collected using a high-resolution time of flight mass spectrometer with high mass accuracy (<5 ppm), provides a firm basis for targeted approaches using tandem-MS. Scanning for neutral loss of m/z 60 (methylated amine group, fragment A) using a triple quadrupole mass spectrometer (with otherwise comparable analytical conditions) is a convenient mode to rapidly reveal LCB-Ps. Human plasma gave rise to strong signals of the d18 series (Figure 2f), including the newly discovered 18:2 derivative (HCCO adduct at m/z 478.1, Figure 2f) not known to have been described previously, to the best of our knowledge.

S1P lyase is the key enzyme involved in the irreversible degradation of S1P; its impaired function in *D. melanogaster* ($sply^{05091}$) leads to an accumulation of LCB-Ps.[7] Here, with a starting sample of only five flies, we demonstrate a 200-fold accumulation in not only d14 and d16 (the major LCB-Ps in *Drosophila*) but also d15, d17, d18, d19, and d20 LCB-Ps in $sply^{05091}/sply^{05091}$ when compared with wild type (Figure 2g,h and Supplementary Table 3, Supporting Information). Genetic background effects were controlled for by also analyzing changes in heterozygous animals that resulted from a cross between $sply^{05091}$ and a control strain, w^{1118} (wild type for sply). Heterozygous $sply^{05091}/+$ showed much less extreme changes than sply/sply (Supplementary Table 3, Supporting Information), suggesting that the differences in sply/sply were indeed due to the mutation rather than the genetic background.

Finally, we established multiple reaction monitoring (MRM) conditions (Supplementary Table 4, Supporting Information) for quantification of LCB-P in extracts from different biological origins (Figure 2i−m, Supplementary Table 5, Supporting Information) with a limit of detection of 0.3 fmol on the column at a signal-to-noise (S/N) ratio of 120 and 20 with and without IMP, respectively, in human plasma (Supplementary Figure 7, Supporting Information). The overall levels measured for d18:1 S1P and 18:0 LCB-P in murine and human plasma corresponded well with published reports.[8] Further, d18:2 LCB-P was present at levels comparable to SA1P (Figure 2i). Lymph node (but not lymph fluid) is devoid of this form of LCB-P (Figure 2j), which could be biologically relevant for the regulation of immune cell function in gradients of LCB-P.[9] We show for the first time that baker's yeast (*S. cerevisiae*) contains

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LCB-P with double bonds (Figure 2m) in addition to the reported fully saturated LCB-P.

Collectively, we have identified, characterized (coelution with standard, exact mass, product ion analysis), and quantified ten LCB-P species not described previously and six LCB-P not known to be present in the tested biological samples, roughly doubling the number of known LCB-Ps (Figure 2n, Supplementary Table 6, Supporting Information). Although there is high confidence in the chemical identity of the newly described LCB-Ps based on comparisons with standards (d17:1 and d18:1 S1P), we cannot unambiguously assign structures (position of the second double bond in 18:2; discrimination between odd chain vs methyl branched LCB-P). Despite this, "deep profiling" is a promising new avenue in biochemical lipidomics for the discovery of new bioactive lipids.

This work has immediate relevance for our understanding of S1P biology as well as its therapeutic targeting. S1P biosynthesis, trafficking, receptor binding, and degradation are highly controlled, and the targeting of S1P receptors and metabolizing enzymes remains an active field of research and drug development.[10] It is conceivable that part of the LCB-P signaling machinery is influenced by the chemical nature of their aliphatic portion. This factor has so far been neglected, mainly because of the lack of information on S1P/LCB-P diversity. It is likely that LCB-P chain diversity affects receptor binding[11] and impacts S1P/LCB-P gradients,[12] which are known to be important underlying components of immune cell trafficking via the reversible dephosphorylation of S1P.[13] Irreversible cleavage of S1P by S1P lyase, on the other hand, is a critical step that links sphingolipid metabolism with that of other lipids.[14] The results from the sply−/− mutant demonstrate the unexpected accumulation of LCB-Ps when this single degradation step is impaired. Finally, it is possible that some of the chemically diverse LCBs (i.e., the non-phosphoryated precursor of the LCB-P described here) found in fungi, plants, and insects[1] could be substrates for kinases (in the respective organism or in other organisms as a result of infection/diet) leading to even greater diversity among LCB-Ps. Our improved method for LCB-P analysis is therefore expected to have a profound impact in a variety of fields.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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