Phytosphingosine as a Specific Inhibitor of Growth and Nutrient Import in Saccharomyces cerevisiae

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In the yeast Saccharomyces cerevisiae, we have demonstrated a necessary role for sphingolipids in the heat stress response through inhibition of nutrient import (Chung, N., Jenkins, G. M., Hannun, Y. A., Heitman, J., and Obeid, L. M. (2000) J. Biol. Chem. 275, 17229–17232). In this study, we used a combination of pharmacological and genetic approaches to determine which endogenous sphingolipid is the likely mediator of growth inhibition. When cells were treated with exogenous phytosphingosine (PHS, 20 μM) or structurally similar or metabolically related molecules, including 3-ketodihydrosphingosine, dihydrophosphoingosine, C2-phytoceramide (PHC), and stearylamine, only PHS inhibited growth. Also, PHS was shown to inhibit uptake of uracil, tryptophan, leucine, and histidine. Again this effect was specific to PHS. Because of the dynamic nature of sphingolipid metabolism, however, it was difficult to conclude that growth inhibition was caused by PHS itself. By using mutant yeast strains defective in various steps in sphingolipid metabolism, we further determined the specificity of PHS. The elo2Δ strain, which is defective in the conversion of DHS to PHS, was shown to have slower biosynthesis of ceramides and to be hypersensitive to PHS (5 μM), suggesting that PHS does not need to be converted to PHS. The lcb4Δ lcb5Δ strain is defective in the conversion of DHS to PHS 1-phosphate, and it was as sensitive to PHS as the wild-type strain. The syr2Δ mutant strain was defective in the conversion of DHS to PHS. Interestingly, this strain was resistant to high concentrations of DHS (40 μM) that inhibited the growth of an isogenic wild-type strain, demonstrating that DHS needs to be converted to PHS to inhibit growth. Together, these data demonstrate that the active sphingolipid species that inhibits yeast growth is PHS or a closely related and yet unidentified metabolite.

Certain sphingolipid metabolites including ceramide, sphingosine, and sphingosine 1-phosphate have pleiotropic effects on cellular growth and proliferation. The yeast Saccharomyces cerevisiae has emerged as an excellent model system for studying sphingolipid-mediated signal transduction. First, compared with over 300 different kinds of sphingolipids found in mammalian cells, there is only a limited number of sphingolipid species in the yeast, which simplifies lipid analysis (2, 3). Moreover, the basic structure, biosynthesis, and metabolism of sphingolipids are well conserved between mammalian and yeast systems. Second, many yeast genes in the sphingolipid biosynthetic and metabolic pathways have been cloned, providing opportunities for studying the effects of endogenous sphingolipids using genetics tools. Finally, although this is not exclusive to sphingolipid studies, yeast genetics provide excellent tools to identify and characterize components in signal transduction pathways (4–6).

Evidence for conservation of the sphingolipid signaling pathway in yeast comes from several studies. These include demonstrating that d-erythro-ceramide inhibited yeast cell growth in liquid culture and activated a protein phosphatase 2A that could be inhibited by okadaic acid (7). Later, Nickels and Broach (8) showed that ceramide inhibited yeast cell growth by arresting cell cycle at G1 phase and that ceramide-activated protein phosphatase is composed of three protein phosphatase 2A components encoded by the TPD3, CDC55, and SIT4 genes.

More recent studies showed that upon heat stress, cellular levels of DHS1 and PHS rapidly increase severalfold within 10–20 min and then slowly return to basal levels over 30–60 min (9, 10). The levels of ceramide also increased severalfold but with slow kinetics corresponding to 60–120 min. On the other hand, the levels of complex sphingolipids show little if any change in response to heat stress. This increase in sphingoid bases derives primarily from de novo synthesis initiated by serine palmitoyltransferase. More recently, DHS and PHS were shown to inhibit yeast growth by inhibiting tryptophan import (11). These studies suggest important signaling and regulatory functions for sphingolipids, their phosphates, and/or ceramide, but they do not provide insight into which specific sphingolipids are involved in what specific cellular functions.

In this report, we set out to determine which sphingolipid (among various sphingolipid species including 3-KDS, DHS, PHS, PHC, and PHS 1-phosphate) is the primary inducer of growth inhibition. Through pharmacological and genetic approaches, we found that DHS inhibits growth, but it needs to be first converted to PHS to do so. PHS, on the other hand, does not need to be converted to PHS 1-phosphate or PHC, and PHS itself is sufficient to inhibit growth. Our data demonstrate that PHS inhibits growth in a specific manner, suggesting that this

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sphingoid base may play a specific role in growth regulation of _S. cerevisiae_ and that it targets a specific pathway responsive only to PHS and not to any of its known precursors or subsequent metabolites.

**EXPERIMENTAL PROCEDURES**

**Strains**—All the yeast strains used in this study are isogenic to a normal laboratory strain JK9-3d (MATa trpl1 his3 leu2-3,112 ura3-52 rme1 HMLa; see Ref. 12: NC85-1a (TRP1), NC65 (ser2::G418<sup>res</sup>), NC75 (elo2::G418<sup>res</sup>), NC75-3 (elo2::G418<sup>res</sup> SEL1-1), NC78 (TRP1 elo2::G418<sup>res</sup>), NC127-1b (lcb4::H16<sup>res</sup>), NC128-1c (lcb5::G418<sup>res</sup>), NC129 (lcb4::G418<sup>res</sup> lcb5::Hyg<sup>res</sup>), CM1 (var3::G418<sup>res</sup> var5::URA3; see Ref. 13), and JS16 (bst1::G418<sup>res</sup>; see Ref. 14).

**Media and Genetic Manipulations**—Recipes for media and yeast genetic methods followed standard protocols (15). Yeast transformation followed the protocol developed by Gietz et al. (16). Gene disruptions were carried out as described previously (17, 18). In short, open reading frames were replaced by the polymerase chain reaction products consisting of the G418<sup>res</sup> or Hyg<sup>res</sup> cassette flanked by 43-base pair sequence homology to targeted genes on both sides of an open reading frame. Each gene disruption was confirmed by polymerase chain reaction, which was designed to amplify the specific chimeric junction of the target gene and the G418<sup>res</sup> or Hyg<sup>res</sup> cassette.

**Preparation of Sphingolipid Derivatives**—PHS and STA were purchased from Sigma, and DHS from Biomol. KDS and C2-PHC were kind gifts from Dr. Alicja Bielawska (Medical University of South Carolina, Charleston, SC). The quality of these sphingolipid derivatives was evaluated by thin layer chromatography. These lipids were dissolved in ethanol at 20 mM stock solutions and stored at −20 °C as described previously (19). They were warmed to 30 °C before use. For solid medium, sphingolipid derivatives were added to the medium that had been autoclaved and cooled down to 50 °C, together with 0.05% Tergitol (Nonidet P-40; Sigma) to help even distribution of lipids in solid agar (20). For liquid medium, warmed up stock solutions of the lipids were directly added to medium, vigorously shaken, and equilibrated before use. Tergitol did not affect the biological activities of sphingolipid derivatives in liquid culture, and the results shown here represent experiments with and without the use of Tergitol.

**Measurement of Yeast Growth**—Measurement of yeast growth was carried out as described previously (19). Briefly, in liquid culture an overnight culture of cells at exponential growth phase was diluted into fresh medium containing indicated lipids or ethanol (0.1% at the final concentration) as a control. While incubating at 30 °C in the dark as described previously (19). They were warmed to 30 °C before use. For solid medium, sphingolipid derivatives were added to the medium that had been autoclaved and cooled down to 50 °C, together with 0.05% Tergitol (Nonidet P-40; Sigma) to help even distribution of lipids in solid agar (20). For liquid medium, warmed up stock solutions of the lipids were directly added to medium, vigorously shaken, and equilibrated before use. Tergitol did not affect the biological activities of sphingolipid derivatives in liquid culture, and the results shown here represent experiments with and without the use of Tergitol.

**Uptake of amino acids or uracil** was measured for 2 h, and total proteins were extracted and subjected to base hydrolysis to remove non-sphingolipid serine-labeled products. Radioactive bands of sphingolipids and their intermediates were visualized by a PhosphorImager (Molecular Dynamics) after exposure to a tritium screen.

**Immuno blotting**—The wild-type JK9-3d strain was transformed with a low copy plasmid containing either the GAP1 or the TAT2 gene that is tagged with the hemagglutinin epitope at its carboxyl terminus. Transformed cells were grown to an early-to-mid log phase and treated with indicated lipids for 2 h, and total proteins were extracted and quantitated, and immunoblotting was performed as described previously (1). The permease proteins were detected by using mouse monoclonal antibody against hemagglutinin (Covance, 1:1,000).

**RESULTS**

**Specificity of Growth Inhibition by PHS**—Previous studies (7, 14) suggested that growth inhibition by sphingolipids and their derivatives is conserved in _S. cerevisiae_ as well as in mammalian cells, and later (11) it was shown in liquid culture that PHS inhibits yeast growth. An additional study showed that yeast sphingolipids are necessary for heat-induced down-regulation of nutrient permeases (1). However, these studies did not resolve questions regarding the specificity of growth inhibition by sphingolipids and what are the endogenous mediators of growth suppression. This is further complicated by the interconversion of these lipids. For example, PHS can be further converted to other sphingolipid species including inositol phosphoceramide, mannose inositol phosphoceramide, and mannose diinositol phosphoceramide (Fig. 1). Therefore, even when cells are treated with PHS, it is difficult to determine whether growth inhibition is caused by PHS or by other sphingolipid species that are converted from PHS (or precursors of the pathway). Moreover, DHS was claimed to have an inhibitory potential as PHS (11). We therefore set out to determine whether PHS itself or other sphingolipid species inhibits growth.

We first confirmed and extended previous observations by

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showing that PHS inhibits growth of a normal laboratory strain JK9-3d in both liquid and solid media (Fig. 2A). In fact, cells in liquid medium were more sensitive to PHS than those in solid medium, such that 15–20 μM PHS was required to inhibit growth in solid medium, whereas 10–15 μM PHS was sufficient to attain a similar degree of growth inhibition in liquid medium (data not shown). This range of concentrations was comparable to the ranges of sphingosine and ceramide concentrations used for mammalian studies. On YPD medium containing 20 μM PHS, plating of more than 10⁴ cells per 9-cm plate failed to yield any viable colonies; in liquid medium, more than 10⁷ cells/ml still showed immediate growth inhibition in response to 20 μM PHS (data not shown). This demonstration of growth inhibition by PHS in diverse experimental conditions enabled us to use multidisciplinary approaches, especially the genetic approach, to determine the specificity of PHS.

PHS can be converted to PHC by ceramide synthase in vivo (24), raising a possibility that apparent growth inhibition by PHS could actually be due to PHC. Also, in mammalian cells, ceramide plays an important role in cell cycle regulation, apoptosis, and cellular senescence (25, 26). Therefore, we decided to test whether PHC has an equivalent function in yeast cells and, if so, whether PHC has a comparable potency to PHS. For this purpose, we used C₂-PHC because we reasoned C₂-PHC, a natural PHC to yeast, will not be efficiently delivered into cells due to strong hydrophobicity, and short chain ceramides such as C₂- and C₆-ceramide have been commonly used in mammalian studies. C₂-PHC (20 μM) had little effect on yeast growth (Fig. 2B).

We then tested other sphingolipid derivatives that are structurally similar and/or metabolically related. At concentrations up to 100 μM, KDS and STA did not inhibit growth (Fig. 2B). It was noteworthy that DHS, at the 20 μM concentration, showed weak but some degree of growth inhibitory effects (see below).

Specificity of Yeast Sphingolipids

Specificity of Nutrient Import Inhibition by PHS—In a previous study, PHS was shown to inhibit the growth of tryptophan- auxotrophic yeast strains and to inhibit tryptophan import (11). Thus, it was hypothesized that a primary cause of growth inhibition by PHS is inhibition of tryptophan import activities. Again, it was not shown whether inhibition of tryptophan import was specific for PHS; to the contrary, both PHS and DHS were suggested to be equally active in inhibiting tryptophan import (11). We believe this is an important issue that needs to be resolved because it could help determine whether such inhibition involves specific mechanisms.

Therefore, we measured tryptophan import activity in the presence of PHS or various analogs that were used for studying the specificity of growth inhibition. In this test, only PHS inhibited tryptophan import, whereas other analogs including DHS, KDS, C₂-PHC, and STA (20 μM each) did not inhibit tryptophan import (Fig. 3A, left panel). The inhibition of tryptophan uptake activity by PHS primarily reflected the decrease in the levels of the general amino acid permease (Gap1) rather than a tryptophan-specific permease (Tat2; Fig. 3B, right)
Again, the decrease in the permease protein was specific for PHS, compatible with the decrease in the uracil permease levels by PHS (1).

While experimenting with various different auxotrophic strains for PHS sensitivity, we found that, in addition to tryptophan-auxotrophic strains (trp1), certain other auxotrophs are also sensitive to PHS. At 20 μM PHS, any tryptophan-prototrophic strains (TRP1), regardless of other auxotrophic status, grew as well as they did in control medium (Fig. 3B). Leucine-prototrophic strains (LEU2) were also found to be somewhat resistant to PHS. At 60 μM PHS, the TRP1 strains became partially sensitive and the LEU2 strains became as sensitive as auxotrophic strains. The TRP1 LEU2 strains were more resistant to PHS than the TRP1 leu2 and trp1 LEU2 strains. However, all auxotrophic strains showed some degree of sensitivity to PHS, and only the TRP1 LEU2 HIS4 fully prototrophic strains showed full resistance to 60 μM PHS. From these observations, we concluded that the greater the auxotrophic requirement of a strain, the more sensitive it is to PHS.

These results also suggested that PHS inhibits the import of multiple nutrients. To test this idea, we measured nutrient import activities for leucine, tryptophan, histidine, and uracil in the presence of 20 μM PHS (Fig. 3C). PHS inhibited the import of all four nutrients.

DHS Needs to Be Converted to PHS to Inhibit Growth—To assess the physiological significance of the above pharmacolog-
chemical studies, we next examined the specificity of growth inhibition by genetically modulating the levels of cellular DHS. As shown in Fig. 1, exogenous DHS could be converted to PHS, PHC to PHC, and PHC to PHS. The possibilities of such inter-conversions raise the question as to which endogenous sphingolipid derivative mediates the growth-inhibitory effects of exogenous PHS.

Because we saw that at higher concentrations (40–60 μM) DHS inhibited growth to some extent, we first tried to resolve whether DHS is a bona fide inhibitor of yeast growth, or whether its conversion to PHS is necessary for growth inhibition. To answer this question, we used a mutant strain that is defective in the conversion of DHS to PHS. In previous studies (27, 28), the syr2 mutant strain was shown to be defective in the C-4 hydroxylation of DHS and dihydroceramide to PHS and PHC, respectively, and the SYR2 gene was proposed to encode a lipid hydroxylase. Therefore, we first determined whether the syr2Δ mutant strain shows defects in the conversion of DHS to PHS in the JKH9-3d strain background. When we tried to analyze sphingolipid profiles of the syr2Δ and isogenic wild-type strain using TLC after [3H]DHS labeling, we could hardly detect differences between the wild-type and the syr2Δ strains (Fig. 4A, lanes 1 and 3). The levels of DHS in the syr2Δ strain seemed to be higher than in the wild-type strain. In these studies, we could not detect free DHS, probably due to its rapid conversion to PHS or due to lack of direct hydroxylation of DHS. Therefore, we attempted to resolve this issue and trap PHS by utilizing fumonisin B1, an inhibitor of ceramide synthase (24, 29). Under these conditions, the wild-type strain was capable of converting DHS to PHS and accumulated PHS in the presence of fumonisin B1, whereas the syr2Δ strain failed to accumulate PHS. In other words, the use of fumonisin B1 enabled us to detect the accumulation of PHS in the wild-type strain but not in the syr2Δ strain (Figs. 1A and 4A). Thus, syr2Δ is defective in hydroxylation of exogenous DHS to PHS.

If DHS is by itself sufficient for growth inhibition, this should be the case regardless of the status of the SYR2 gene. On the other hand, if DHS needs to be first converted to PHS to inhibit growth, DHS would inhibit the growth of only wild-type cells but not syr2Δ mutant cells. Indeed, whereas PHS (20 and 40 μM) inhibited both the wild-type and syr2Δ mutant strains, DHS (40 μM) only inhibited growth of the wild-type strain and failed to inhibit growth of the syr2Δ strain (Fig. 4B). We have found that DHS is as efficiently as or better taken up than PHS by yeast cells and comparably metabolized into complex sphingolipids (data not shown). It is therefore unlikely that the requirement for high concentration of DHS for growth inhibition was due to slow internalization of DHS compared with PHS. Tetrad analysis of a syr2Δ/SYR2 heterozygous diploid strain showed co-segregation of the syr2Δ allele with resistance to 40 μM DHS. Also, when the syr2Δ mutant strain was restored with a single copy of the wild-type SYR2 gene, it became as sensitive to DHS as the original wild-type strain (data not shown). In conclusion, DHS does not by itself inhibit growth and requires conversion to PHS by Syr2p.

PHS Does Not Need to Be Converted to PHC and Is Sufficient for Growth Inhibition—Next we attempted to distinguish between PHS and PHC. PHS can be converted to PHC by ceramide synthase, and PHS can be reverted to PHS by ceramidase (24, 30). Treating cells with an excessive amount of PHS could shift the equilibrium toward PHC making it difficult to distinguish the effects of PHS from those of PHC. In the above section, we showed that C26-PHC only weakly inhibited growth, but the data were not conclusive since C26-PHC may not be an adequate substitute for the long chain natural PHC.

To avoid using natural C26-PHC, which might cause solubility and permeability problems, we looked for other ways to differentiate the effects of PHS from those of PHC. The production of PHC requires two substrates: PHS and C24- or C26-very long chain fatty acid (VLCFA) (Fig. 1). Therefore, if the supply of VLCFA is blocked, there will be less production of PHC from PHS even when PHS is present in excess. VLCFA is the result of sequential addition of acetyl groups to the more commonly found normal length fatty acids like palmitic acid (C16). The key steps in this process involve the conversion of C22- to C24/VLCFA by the ELO2 gene product and C24- to C26/VLCFA by the ELO3 gene product (Fig. 1) (22). When we labeled the elo2Δ mutant strain with [3H]serine and analyzed by TLC, we could indeed observe the increase in the levels of PHS and its upstream precursors including DHS and KDS and the decrease in the levels of PHC and complex sphingolipids (Fig. 5A).

If PHS is by itself capable of inhibiting growth, then the growth of elo2Δ mutant cells will be inhibited by PHS treatment. On the other hand, if PHS needs to be converted to PHC to inhibit growth, then elo2Δ mutant cells will be resistant to PHS. In fact, the elo2Δ mutant strain was hypersensitive to...
PHS such that its growth was inhibited by only 0.5 mM PHS, a concentration at which the growth of wild-type cells was unaffected (Fig. 5B). Notably, the elo2Δ mutant strain grew slowly even without PHS treatment (data not shown), probably due to the accumulation of endogenous PHS.

The sensitivity of the elo2Δ mutant strain to PHS was tightly linked to the mutant allele of the TRP1 gene (trp1); the elo2Δtrp1 strain was sensitive to PHS, but the elo2ΔTRP1 strain was resistant (Fig. 6B). Because these two elo2Δ strains showed essentially identical TLC profiles including PHS levels, and their only difference was the status of TRP1 allele (TRP1 versus trp1), we concluded that the wild-type TRP1 allele enabled the elo2Δ mutant strain to overcome deleterious effects of PHS accumulation. Also, when the elo2Δtrp1 strain was grown on medium containing 0.5 mM PHS plus excess tryptophan, it became as resistant to PHS as the elo2ΔTRP1 strain (Fig. 5B).

These data confirm that PHS inhibits growth of the trp1 mutant strain by inhibiting tryptophan import and further support the hypothesis that the hypersensitivity of elo2 cells is due to accumulation of endogenous PHS since it was reversed by excess tryptophan, indicating a similar mechanism as wild-type cells.

Interestingly and probably due to selective pressure stemming from growth defects, spontaneous suppressor mutations frequently arose in the elo2Δ trp1 strain. One of these suppressors (SEL1-1 for suppressor of elo2Δ) was a dominant mutant that restores the levels of PHS and complex sphingolipids to normal levels (Fig. 5A) and cures PHS hypersensitivity (Fig. 5B). The data therefore suggest that the reduction in the levels of PHS results in the suppression of elo2Δ growth defects.

TABLE I
Summary of PHS phenotypes of sphingolipid metabolic mutant strains

| Defects Genotype        | PHS phenotype |
|-------------------------|--------------|
| CONTROL WT              | S            |
| DHS → PHS syr2Δ         | S            |
| PHS → PHS elo2Δ         | HS           |
| PHS → PHS-1-P lcb4Δ     | S            |
| PHS → PHS-1-P lcb5Δ     | S            |
| PHS → PHS-1-P lcb4Δ lcb5Δ| S          |
| PHS-1-P → PHS ysr2Δ     | S            |
| PHS-1-P → PHS ysr3Δ     | S            |
| PHS-1-P → PHS ysr2Δ ysr3Δ| S         |
| PHS-1-P → degradation dpl1Δ| S      |
of endogenous PHS to a normal level in the SEL1-1" suppressor mutant relieved the PHS hypersensitivity resulting from the elo2Δ mutation.

**PHS 1-Phosphate Does Not Inhibit Growth**—The conclusion from a series of observations suggests that sphingosine 1-phosphate, but not sphingosine itself, inhibits yeast growth. First, the dpl1Δ mutant strain accumulates sphingosine 1-phosphate and shows growth inhibition when treated with sphingosine (14). Second, the overexpression of the YSR2 gene, which encodes for sphingosine 1-phosphate phosphatase, in the dpl1Δ mutant strain reverses sphingosine 1-phosphate accumulation and restores wild-type growth (13). Finally, the lcbΔ dpl1Δ double mutant strain does not accumulate sphingosine 1-phosphate and is resistant to sphingosine (31). A consensus that can be drawn from these data is that any strain that allows the accumulation of sphingosine 1-phosphate is sensitive to exogenous sphingosine. One may assume that PHS, too, needs to be converted to PHS 1-phosphate to inhibit growth.

We therefore set out to determine the differences between PHS and PHS 1-phosphate. When the SYR2 gene is deleted, DHS can still be converted to DHS 1-phosphate. Because the syr2Δ mutant strain was resistant to DHS, DHS 1-phosphate does not appear to inhibit growth. Does PHS then inhibit growth via PHS 1-phosphate? We next tested if PHS 1-phosphate mediates the effects of PHS in two independent experiments, using mutant yeast strains defective in two enzymes involved in PHS metabolism, sphingoid base kinase and phosphorylated sphingoid base lyase. There are two sphingoid base kinase isoenzymes encoded by two highly homologous genes, LCB4 and LCB5 (31). The lcbΔ lcb5Δ double mutant strain, which cannot convert PHS to PHS 1-phosphate, was as sensitive to PHS as a wild-type strain (Table I). This demonstrates that PHS does not need to be converted to PHS 1-phosphate to inhibit growth. In a second experiment, we tested PHS sensitivity of the dpl1Δ mutant strain, which lacks phosphorylated sphingoid base lyase. The dpl1Δ mutant strain did not show hypersensitivity to PHS, suggesting that the accumulation of PHS 1-phosphate does not lead to growth inhibition. In short, unlike the case with sphingosine, PHS does not need to be converted to PHS 1-phosphate and by itself inhibits growth.

**DISCUSSION**

In this report, we demonstrated the specificity of growth inhibition by PHS through several approaches. PHS inhibited yeast growth at a low micromolar concentration range. It was specific to PHS, in that other metabolically and structurally related compounds did not inhibit growth. By using various mutants involved in sphingolipid biosynthesis and metabolism, we demonstrated that DHS needs to be converted to PHS, and PHS does not need to be converted to PHC or PHS 1-phosphate to inhibit growth. Therefore, PHS is a likely *bona fide* growth-inhibitory sphingolipid derivative.

In addition to the above conclusion, the data presented in this report also suggest that de novo synthesis of PHS is important in growth inhibition. The gene products of both SYR2 and ELO2 are involved in de novo sphingolipid synthesis, and the data drawn from the mutant strains defective in these genes support the conclusion that the accumulation of PHS via de novo synthesis results in growth inhibition. Previously, it was suggested that heat stress signaling could also be mediated via de novo synthesis of sphingolipids (9, 10). Also in mammalian cells, de novo synthesis of ceramides has been suggested to be important in apoptosis (32–35). Despite our data, we cannot rule out the possibility that the generation of PHS by hydrolysis of other sphingolipids such as PHC, inositol phosphocholine, and others may also play a role in growth inhibition. In our data, C2-PHC did not inhibit growth. In addition, labeled C2-dihydroceramide in *S. cerevisiae* was rapidly internalized, metabolized, and incorporated into complex sphingolipids. Thus, C2-PHC is also probably internalized, converted to PHS by ceramidases (23, 36), and incorporated into complex sphingolipids. Therefore, C2-PHC does not likely cause accumulation of PHS and consequently does not play a role in PHS-mediated growth inhibition. On the contrary, as in mammalian cells, both biosynthetic and catabolic pathways to generate PHS may be important for growth inhibition, differing in temporal order of PHS generation and/or cellular context (26).

Considering the structural similarities between PHS and other metabolically related molecules including PHC, DHS, and KDS, the specificity of growth inhibition by PHS is remarkable. PHS differs from PHC in that the amino group at the C-2 position is acylated in PHC, and it differs from DHS in that the hydroxyl group at the C-4 position is absent in DHS (Fig. 2). Computer-simulated three-dimensional modeling of PHS showed that the amino group at the C-2 position and the hydroxyl group at the C-4 position are clustered in close proximity at one end of the hydrocarbon chain (data not shown). It is likely that PHS is embedded in membrane bilayers with these functional groups protruding out of the membrane. The combined amino and hydroxyl groups could provide an interface to other macromolecules that relay growth-inhibitory signals, and the abolishment of these features could result in failure to recruit signaling macromolecules.

We used genetics methodology to demonstrate the specificity of PHS. We believe this kind of approach should be more extensively utilized in many other studies requiring the specificity of molecular actions. Because of the dynamics of many signaling molecules in the context of metabolism, it is not guaranteed whether the biological effects of a particular molecule really originated from itself. The combination of pharmacological and genetic tools could eliminate these doubts.

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