Biosynthesis of Modular Ascarosides in *C. elegans*

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Abstract

The nematode Caenorhabditis elegans uses simple building blocks from primary metabolism and a strategy of modular assembly to build a great diversity of signaling molecules, the ascarosides, which function as a chemical language in this model organism. In the ascarosides, the dideoxysugar ascarylose serves as a scaffold to which diverse moieties from lipid, amino acid, neurotransmitter, and nucleoside metabolism are attached. However, the mechanisms that underlie the highly specific assembly of ascarosides are not understood. We show that the acyl-CoA synthetase ACS-7, which localizes to lysosome-related organelles, is specifically required for the attachment of different building blocks to the 4′-position of ascr#9. We further show that mutants lacking lysosome-related organelles are defective in the production of all 4′-modified ascarosides, thus identifying the waste disposal system of the cell as a hotspot for ascaroside biosynthesis.

Keywords

Nematodes, including the model organisms *Caenorhabditis elegans* and *Pristionchus pacificus*, produce a great diversity of ascaroside derivatives[1] (Figure 1). These signaling molecules are involved in virtually all aspects of nematode life history, including the regulation of aging,[2] dauer development,[3] morphology,[4] and behavior,[5] often acting through evolutionarily conserved signaling pathways.[1,6] The identification of the modular ascarosides demonstrated not only unexpected biosynthetic capabilities in an animal, but also provided an example of combinatorial generation of structural diversity that is reminiscent of synthetic combinatorial libraries in medicinal chemistry.[1]

Assembly of the ascarosides identified from *C. elegans* and other nematodes proceeds with high selectivity. For example, the likely folate-derived *p*-aminobenzoic acid moiety in ascr#8 is selectively attached to an unsaturated 7-carbon sidechain, although the simple ascarosides based on saturated 7-carbon and unsaturated 9-carbon sidechains, ascr#1 and ascr#3, are much more abundant (Figure 1).[3c,7] Specific biological functions and highly selective assembly indicate that the modular ascarosides are products of dedicated biosynthetic pathways. Ultimately, the elucidation of these pathways will reveal how input from conserved primary metabolism is transduced to create signals that regulate development and behavior.

Previous studies have demonstrated that the fatty acid like side chains in the ascarosides are derived from very long-chain precursors (**1**) through peroxisomal β-oxidation (Figure 1c).[5d,7,8] Other building blocks, for example, the indole carboxy moiety in icas#3 or the octopamine moiety in osas#10, are derived from canonical primary metabolites, for example, tryptophan in the case of icas#3[7] and tyrosine in the case of osas#10.[5c] Feeding experiments (see Methods in the Supporting Information and Ref.[7]) demonstrated that synthetic ascr#1 and ascr#3 are converted into 4′-substituted derivatives icas#1 and icas#3, thus suggesting that 4′-modification occurs subsequent to and independent of side-chain β-oxidation (Figure 1c). However, the enzymes and cellular compartments involved in the attachment of diverse moieties via ester and amide bonds remained unknown.

The 4′-acylated ascarosides include several important signaling molecules, for example, icas#3 (Figure 1) and icas#9, which are components of the *C. elegans* aggregation pheromone,[5b] and osas#9, a larval dispersal signal (Figure 2a).[5c] In search of enzymes that attach different acyl moieties to the 4′-position of ascarylose, we pursued a two-pronged approach, screening candidate genes based on either predicted function or subcellular localization. The *C. elegans* genome includes 61 genes annotated as O-acyltransferases, and for about 30 of these, likely loss-of-function mutants were available (Table S1 in the Supporting Information). Comparative metabolomic analysis of liquid cultures of these mutants and wildtype controls by LC-MS did not reveal any reduction in the amounts of 4′-modified ascarosides. Since shortening of the fatty acid like side chains of the ascarosides takes place in peroxisomes, we additionally screened several mutants of genes bearing a peroxisome targeting sequence (PTS),[9] including three putative acyl-CoA synthetases. In a pilot screen, we found that the production of two 4′-acylated derivatives of ascr#9, the aggregation pheromone icas#9 and the larval dispersal signal osas#9, was abolished in *acs-7(tm6781)* mutants (Figures 2a, b), whereas production of the structurally related icas#3, icas#10, and osas#10 was similar or elevated compared to the wildtype (Figures 1 and 2a, and Figure S2). The abundances of all other known ascarosides, including ascr#9, were close to those found in wildtype controls (Figure 2a). Similarly, the abundances of *N*-succinyl octopamine (**5**) and indole-3-carboxylic acid (**7**), which are plausible building blocks of osas#9 and icas#9, respectively, were not significantly changed in *acs-7* mutants (Figure S1 in the Supporting Information). Ascaroside production was largely wildtype-like in the other mutants in our pilot screen (Figure S3a).

These results suggested that the enzyme ACS-7 is involved in the biosynthesis of icas#9 and osas#9, specifically. *acs-7* encodes a putative acyl-CoA synthetase with no known function in *C. elegans* and approximately 70% sequence homology to human acyl-CoA synthetase family member 2, a mitochondrial fatty acyl-CoA synthetase with a preference for medium-chain substrates. To confirm the role of ACS-7 in the biosynthesis of icas#9 and osas#9, we expressed green fluorescent protein (*gfp*)-tagged *acs-7* (*gfp::acs-7*) under the native *acs-7* promoter in an *acs-7(tm6781)* mutant background. LC-MS analysis of *gfp::acs-7* cultures revealed that the biosynthesis of icas#9 and osas#9 was restored, whereas the amounts of other ascarosides remained unchanged (Figure 2b and Figure S3b). Additional untargeted metabolomics revealed no other significant differences between the metabolomes of *acs-7* and wildtype worms. This observation confirmed that ACS-7 is involved specifically in the production of the 4′-modified ascarosides icas#9 and osas#9.

Based on sequence homology, we hypothesized that ACS-7 may activate indole-3-carboxylic acid (**7**) and *N*-succinyl octopamine (**5**), as building blocks of icas#9 and osas#9, respectively, for subsequent 4′-acylation of ascr#9. To test this, we purified recombinant his_6–ACS-7 from *E. coli*. Incubation of the ACS-7 with indole-3-carboxylic acid, ATP, and coenzyme A rapidly produced indole-3-carboxy-AMP (**8**) ($K_m = 270 \pm 90$ μM, Figure 2c and Figure S4), whereas formation of indole carboxy-CoA was not observed. Similarly, *N*-succinyl octopamine (**5**) was converted into the corresponding adenylate **6**. However, addition of ascr#9 or ascr#9-CoA into these assays did not lead to production of icas#9, osas#9, or the corresponding CoA-derivatives.

Similarly, ACS-7 did not convert ascr#9 or aliphatic fatty acids, which we included as control substrates, into either the corresponding adenylates or CoA derivatives (Figure S5). These results indicate that ACS-7 contributes to the biosynthesis of icas#9 and osas#9 by activating indole carboxylic acid and *N*-succinyl octopamine, respectively; however, 4′-attachment to ascr#9 or ascr#9-CoA may involve additional factors or require a specific cellular environment not mimicked in our assays.

Next we asked whether ACS-7 localizes to peroxisomes, as predicted by its PTS. Surprisingly, we found that GFP::ACS-7 is primarily localized to punctate organelles ("gut granules")[10] in intestinal cells, rather than peroxisomes (Figures 3a,b). *C. elegans* intestinal cells are known to contain several different types of gut granules, including acidic lysosome-related organelles (LROs).[10] LROs play a central role in the digestion of cellular waste and the regeneration of basic building blocks, but they are also suspected to contribute to the biosyntheses of diverse metabolites.[11] Staining with LysoTracker Red, a dye that selectively stains LROs in *C. elegans*,[10,12] revealed that GFP::ACS-7 localizes specifically to the LROs in the intestinal cells (Figures 3c,d). This suggests that the biosynthesis of the modular ascarosides icas#9 and osas#9 may proceed in the LROs and not in the peroxisomes.

To test whether acidic LROs are required for the biosynthesis of icas#9, osas#9, and potentially other modular ascarosides, we investigated the ascaroside profiles of several mutants that are defective in specific classes of gut granules. *glo-1* encodes a 24 kDa member of the Rab family of small GTPases, which is required for the biogenesis of acidic LROs.[10] Correspondingly, *glo-1(zu437)* mutants, which lack LROs, do not show any staining with LysoTracker Red.[10] *glo-4* encodes a guanine nucleotide exchange factor that contributes to GLO-1 function.[10] Both *glo-1* and *glo-4* act downstream of *apb-3*, which encodes the worm orthologue of the β3 subunit of the mammalian adaptor protein complex AP-3.[13] We found that the biosynthesis of all known 4′-modified ascarosides is largely abolished in *glo-1(zu437)* mutants, which lack LROs (Figure 3e), and reduced in *glo-4(ok623)* and *apb-3(ok429)* mutants, in which LRO formation is reduced but not abolished (Figure S6). The amounts of other ascarosides in these mutants were similar to those in wildtype controls, except for the glycosylated derivatives glas#1, glas#3, and glas#10, which were starkly increased in *glo-1(zu437)* mutants relative to the wildtype (Figure 3f,g). Glycosylation generally serves as a detoxification mechanism in *C. elegans*,[14] and the buildup of glycosylated ascarosides in *glo-1* worms may reflect the loss of alternative waste processing pathways owing to the lack of LROs in this mutant. Importantly, the abundances of indole-3-carboxylic acid and *N*-succinyl octopamine in *glo-1* mutants were similar or only slightly reduced relative to the wildtype, suggesting that abolishment of 4′-modified ascarosides in this mutant is not due to depletion of any of the building blocks (Figure S1 in the Supporting Information). In contrast, the amounts of 4′-modified ascarosides in *cup-5(ar465)*, *haf-4(ok1042)*, and *haf-9(gk23)* mutants, which are defective in the formation of non-acidic gut granules but do have acidic LROs,[15] are similar or increased relative to the wildtype (Figure S6). These results indicate that acidic LROs are required for the biosynthesis of all 4′-modified ascarosides, including icas#9 and osas#9, the biosynthesis of which specifically requires ACS-7 (Figure 4).

The acidic LROs in *C. elegans* share many characteristics with lysosomes in higher animals.[10] The lysosome, classically known as the waste disposal system of the cell, is rapidly gaining attention as a central hub for intra- and intercellular chemical signaling.[11b,16] Lysosomal acid lipases play critical roles under starvation conditions to generate small-molecule signals such as polyunsaturated fatty acids[17] and oleoylethanolamide,[11c] which in turn activate diverse downstream responses, including autophagy, a starvation-activated process by which targeted components of the cell are transferred to the lysosome for recycling.[11b] We found that in *atg-18(gk378)* mutants,[18] which are deficient in autophagosome assembly, the abundances of most 4′-modified ascarosides are significantly reduced relative to wildtype (Figure S7), thus suggesting that autophagy contributes to the biosynthesis of modular ascarosides in the LROs.

Taken together, our results indicate that in *C. elegans*, homologues of genes from canonical metazoan metabolism act in different cell compartments to produce modular specialized metabolites. The high specificity of ACS-7, which is required only for the biosynthesis of 4′-modified versions of the 5-carbon side-chain ascaroside ascr#9, that is, icas#9 and osas#9, but not the corresponding 7-carbon or 9-carbon side-chain versions, for example, icas#3 or osas#10 (Figures 1, 2a), indicates that other enzymes must exist that serve equivalent roles in the biosynthesis of 4′-modified ascarosides with longer fatty acid side chains. The specific localization of ACS-7 to the LROs, which serve as part of the cell waste disposal system, and the finding that LROs are required for the biosynthesis of all 4′-modified ascarosides will motivate organelle-specific proteomics and metabolomics studies to identify other components in the biosynthesis of modular ascarosides. Of particular interest will be the mechanisms that underlie the selection of different building blocks and govern the side-chain-length specificity of the modifications, thereby ultimately integrating metabolic state in a complex chemical message.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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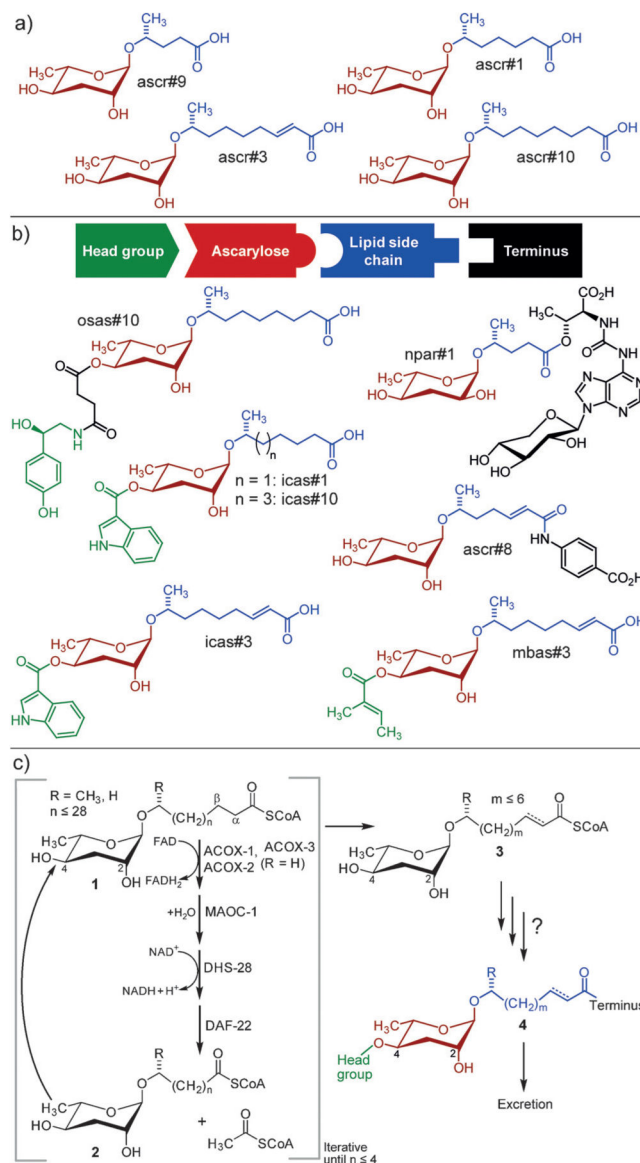
19. See www.smid-db.org for compound naming.

Figure 1.
Structures and biosynthesis of the ascarosides. a) The simple ascarosides ascr#1, ascr#3, ascr#9, and ascr#10, which serve signaling functions in *C. elegans* and many other nematode species. b) Modular ascarosides incorporating building blocks from folate (ascr#8), neurotransmitter (osas#10), tryptophan (icas#3, icas#10), and short-chain fatty acid (mbas#3) metabolism from *C. elegans*, and npar#1 from the satellite model organism *P. pacificus*, which features a nucleoside-derived building block. c) A model for the biosynthesis of ascarosides. Iterative 4-step β-oxidation of long-chain ascarosides (**1**) produces short-chain precursors (**3**) of modular ascarosides (**4**).[19]
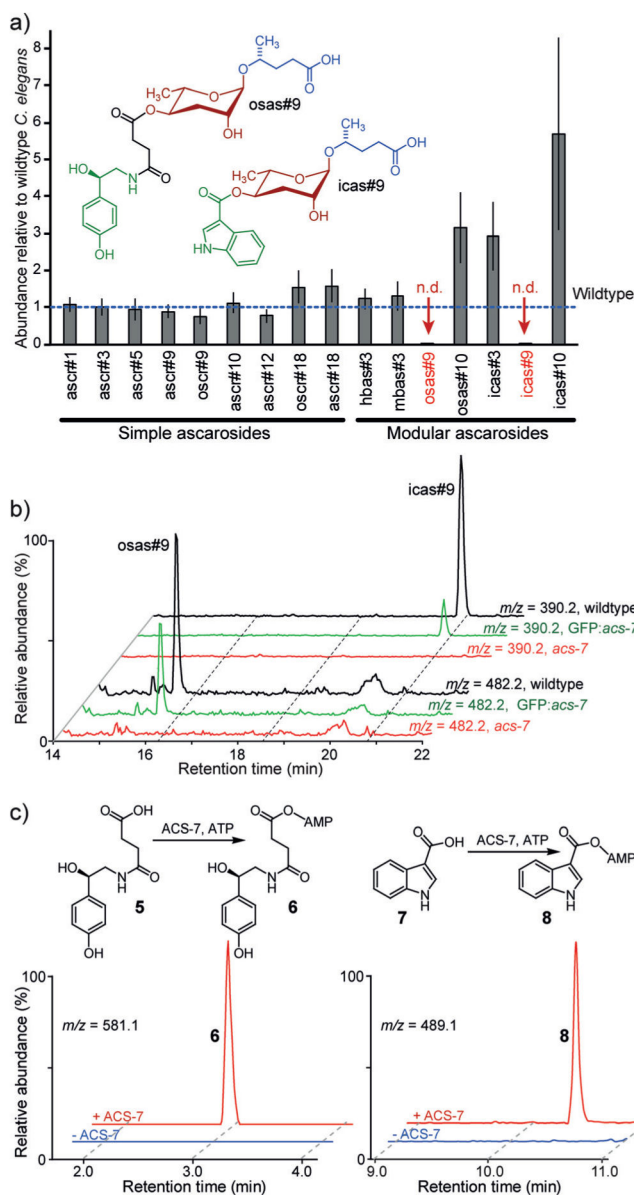
Figure 2.
ACS-7 contributes to the biosynthesis of 4′-acylated ascarosides. a) Abundances of ascarosides in *acs-7*(*tm6781*) mutants relative to wildtype *C. elegans*. Error bars =standard error of three biological replicates. The variability in ascaroside biosynthesis in wildtype *C. elegans* is assessed in Figure S2. b) HPLC–MS (ESI⁻) ion chromatograms for icas#9 and osas#9 in wildtype, *acs-7*(*tm6781*) mutants, and *acs-7*(*tm6781*) mutants expressing *gfp::acs-7*. c) HPLC–MS (ESI⁻) ion chromatograms showing ACS-7-dependent formation of the adenylates of *N*-succinyl octopamine and indole-3-carboxylic acid (also see Figure S4).
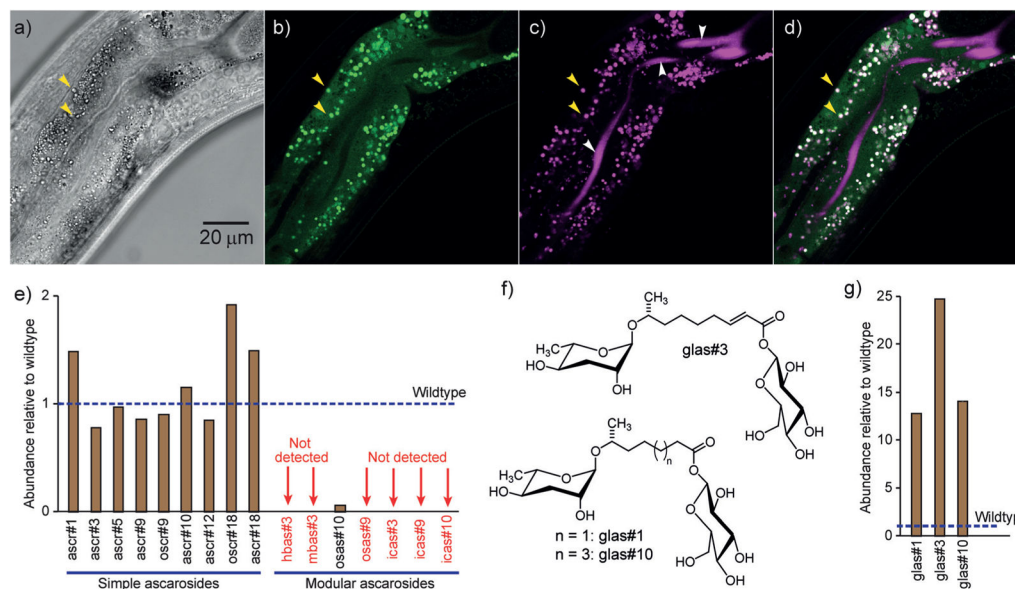
Figure 3.
Identification of LROs as the site of the biosynthesis of modular ascarosides. a) A bright-field image of the midsection of a transgenic worm expressing *gfp::acs-7* under the *acs-7* promoter in *acs-7* mutant background (young adult stage). b) GFP expression is localized to punctate organelles (e.g., yellow arrows). c) Staining of LROs with LysoTracker Red (white arrows: background staining of the intestinal lumen). d) Overlay of images in (b) and (c). e) Abundances of ascarosides in *glo-1(zu437)* mutants relative to wildtype *C. elegans*. f) Structures of ascaroside glucosyl conjugates. g) Abundances of the glucosyl conjugates glas#1, glas#3, and glas#10 are greatly increased in *glo-1* knockout mutants. In (e) and (g), the values shown represent the averages of two replicates.
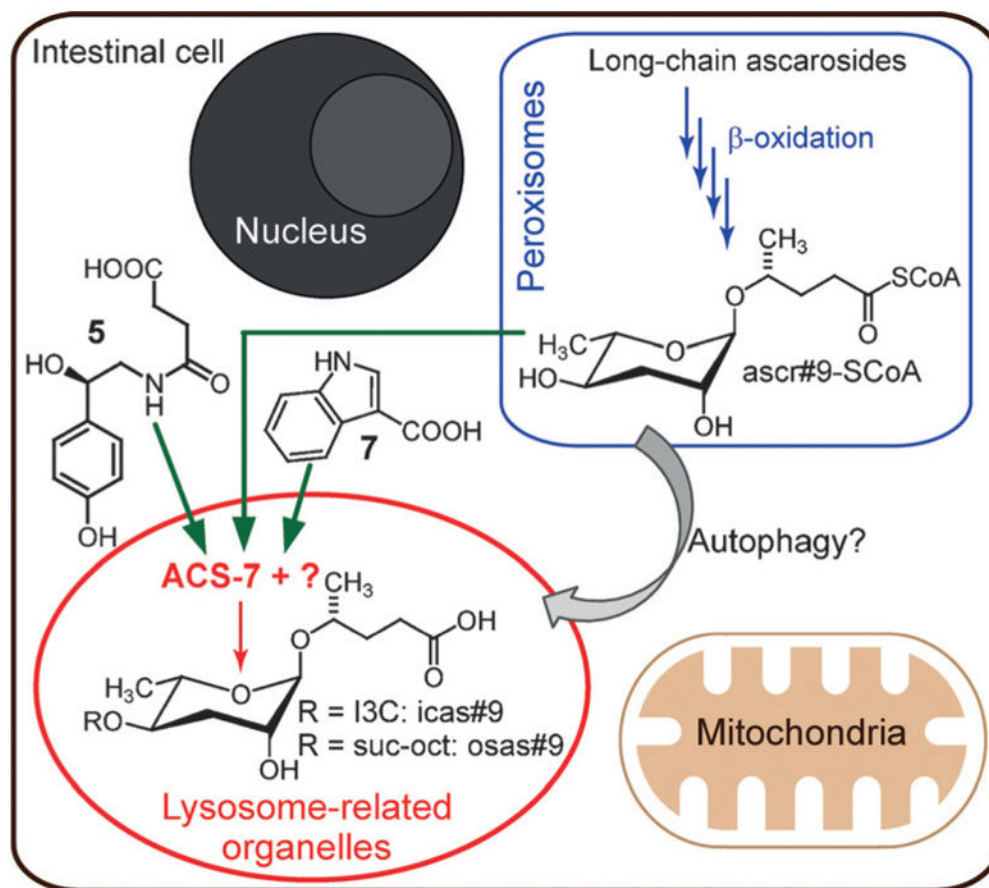
Figure 4.
Simplified model for the biosynthesis of modular ascarosides in *C. elegans*. Long-chain ascaroside precursors undergo multiple rounds of peroxisomal β-oxidation to produce Coenzyme A esters of short-chain ascarosides such as ascr#9. Subsequently, short-chain ascarosides (or their CoA esters) may undergo 4′-modification in acidic lysosome-related organelles (LROs). I3C =indole-3-carboxy, suc-oct =*N*-succinyl octopamine.