Chapter 1

INTRODUCTION TO ASCAROSIDE SIGNALING

1.1 *Caenorhabditis elegans*

Caenorhabditis elegans is a free-living soil nematode that is approximately one millimeter in length at full maturity.[1] First introduced as a model organism by Sydney Brenner in 1963, the genes of this multicellular organism are homologous to those of many vertebrates and have been used to uncover breakthroughs in numerous phenomena such as Alzheimer's disease, aging, diabetes, host-pathogen relationships, and metabolism.[2-6]

Several attributes make *C. elegans* a prime target for study. The nematode has a short reproductive cycle and can be cultured easily on agar plates or in liquid cultures when supplied with an *Escherichia coli* food source.[7-8] The worm is also transparent, allowing the cell lineage to be characterized by light microscopy.[9] All hermaphrodite worms contain exactly 959 somatic cells and males contain 1033, yet despite the small number of cells compared to other animals, *C. elegans* contains a number of distinct tissues and organs including an intestine, a hypodermis, muscle tissue, a fully mapped nervous system of 302 neurons, and gonads. Furthermore, with a genome fully sequenced for over a decade, many tools exist for genetic manipulation including RNA interference, tissue-specific promoter expression systems, and CRISPR/Cas9 gene editing.[10-12]

In the typical *C. elegans* life cycle, the worm begins as an embryo encased by a resilient shell and hatches into the first larval stage called L1. Under favorable environmental conditions, the worm will molt and progress through the L2, L3 and L4 larval stages before

finally reaching its reproductive adult form. The entire process spans approximately three days (Figure 1.1).[13] In the normal life cycle, the adult worm can live between two to three weeks; however, under stressful conditions, *C. elegans* exits the L2 larval stage and enters an alternative, developmentally-arrested larval stage called the dauer. Entry and exit into this stress-resistant stage was found to be regulated by a family of small molecules known as the ascarosides.[14]

The term ascaroside was first created to describe a novel lipid detected in the intestinal parasite *Ascaris lumbricoides* over 100 years ago.[15] These lipophilic molecules, containing long aliphatic side chains, formed a layer around *Ascaris* eggs to protect against harsh environmental conditions.[16] More recently, a larger collection of more hydrophilic ascarosides has been identified in *C. elegans* and other related nematodes and has been shown to regulate a multitude of behaviors and aspects of the life history of the worm including development, mate attraction, aggregation, and repulsion.

1.2 Ascaroside Biosynthesis

Ascarosides are defined as glycosides of the dideoxysugar ascarylose (Figure 1.2). At the first position of the sugar core is a lipid side chain that can vary in length, level of saturation, and level of oxidation. Furthermore, this lipid can be joined at either the ultimate (oscr) or penultimate carbon (ascr). Ascarosides can be further modified at the second and fourth positions of the sugar core as well as at the terminal end of the fatty acid side chain. While the biosynthetic machinery of many microorganisms has been deeply studied and characterized, more complex animals were thought to be deficient in their ability to

produce molecule signals with complex structures.[17] This makes ascaroside biosynthesis in *C. elegans* a surprising and interesting topic of study.

The biologically active short-chain ascarosides are derived from long-chain ascaroside precursors through peroxisomal β-oxidation of the lipid side chain, a process that truncates fatty-acids by two carbons in a series of four steps (Figure 1.3).[18-19] Mass spectrometry-based analyses of mutant excretomes, the collection of metabolites and molecules excreted by the animal, revealed that the first step of ascaroside peroxisomal β-oxidation in *C. elegans* is carried out by ACOX-1, an acyl-CoA oxidase, which introduces a site of unsaturation between the α and β carbons of the fatty acid side chain. MAOC-1, a peroxisomal 2-enoyl-CoA hydratase, hydrates the double bond introduced by ACOX-1. The β-hydroxyacyl-CoA intermediate is then converted into a β-ketoacyl-CoA ester by DHS-28, a homolog of the dehydrogenase domain of mammalian peroxisomal multifunctional protein MFE-2. The final truncation step is carried out by DAF-22, a thiolase that shares strong homology to the mammalian peroxisomal 3-ketoacetyl-CoA thiolase SCPx.

Many of the head groups modifying the 4'-position of the ascarylose sugar groups including indole-3-carboxylic acid (icas), *p*-hydroxybenzoic acid (hbas) and (*E*)-2-methyl-2-butenoic acid (mbas) are derived from amino acid metabolism (Figure 1.4). By studying metabolites produced by axenic *C. elegans* cultures supplemented with a specific amino acid, it has been demonstrated that the indole moiety of icas ascarosides is derived from tryptophan, whereas the head groups of hbas and mbas ascarosides could be synthesized from tyrosine and isoleucine, respectively.[18] Similarly, is hypothesized that the *N*-succinylated

octopamine head group decorating osas ascarosides and the tyramine head group of tsas ascarosides may both be derived from tyrosine.[20] Ascaroside biosynthesis is therefore a complex system that incorporates building blocks derived from many parts of primary metabolism including carbohydrates, lipids, and amino acids.

The production of ascarosides is influenced by many factors including stage, environmental conditions, and sex. Whereas the total quantity of ascarosides produced increases as the worm progresses through larval stages, the production of individual ascarosides peak at different stages.[21] For example, ascr#3 production increases as the worm grows from the L1 to the L4 larval stages, but decreases as the worm reaches adulthood. Alternatively, the production of ascr#2 continues to increase as the worm ages and the modified ascarosides osas#9 and osas#10 are predominantly produced by L1 larva. Dauer larva, on the other hand, do not excrete ascarosides.[21]

Studies have shown that ascarosides are also produced in different quantities depending on the temperature at which worms are cultured. Jeong *et al* found that ascr#1 was produced highly in cultures at 20°C, whereas Butcher *et al* found ascr#3 levels to be dominant when worms were raised between 22.5 and 25°C.[22-23] This shift corresponds with worm behavior as dauer formation increases at higher temperatures and ascr#3 is a much more potent dauer pheromone compared to ascr#1.[24-25]

The availability of food also affects ascaroside production. For example, well-fed wildtype worms excrete larger amounts of ascr#3 than starved cultures, which secrete larger amounts of ascr#2.[21] Additionally, it was found that osas#9 is produced predominantly by starved L1 larva, whereas osas#10 is mainly produced by L1 larva when food is plentiful.[20]

The incorporation of various metabolically derived precursors and dependence upon stage and environmental conditions indicate that ascaroside biosynthesis is a complex and highly regulated process.

1.3 Ascarosides in Dauer Formation

Morphologically distinct from larva grown under favorable conditions, the dauer forms a more robust cuticle, forms a plug that blocks its oral orifice, does not pump, and has a higher fat content (Figure 1.5).[26-27] These physical attributes allow the dauer to survive for up to four months compared to the normal worm lifespan of 2-3 weeks.[28] If environmental conditions return to a favorable state, the worm will exit dauer diapause and resume development to reproductive maturity.

In *C. elegans*, ascarosides were first identified as molecules comprising the pheromone that regulates the formation of dauer larva.[29-30] Over 20 years later, using activity-guided fractionation, the structure of ascr#1, also known as daumone, was discovered and found to have a seven-carbon carboxylic acid side chain attached at the first position of the ascarylose sugar (Figure 1.6).[22] However, Gallo *et al* found that the levels of ascr#1 required to induce dauer formation were highly toxic to the worm and therefore indicated the involvement of additional molecules, then unknown.[31] Further studies utilizing activity-guided fractionation unveiled other related molecules involved in dauer formation bearing different fatty-acid like side chains, called ascr#2 and ascr#3.[25] A fourth ascaroside, called ascr#5, was found to act synergistically with ascr#2 and ascr#3.[23]

The synergistic activity of the ascarosides signifies that not all ascarosides may be detected when separated during activity-guided fractionation as certain ascarosides may only influence dauer formation in combination with other ascarosides. To expand the search for new dauer-inducing ascarosides, Pungaliya *et al* conducted NMR spectroscopy-based comparative metabolomic studies that revealed the additional dauer pheromone component called ascr#8, which contains a *p*-aminobenzoic acid group at the terminal end of the unsaturated fatty acid side chain.[32] Additionally, Butcher *et al* discovered an ascaroside containing an unusual indole-3-carbonyl group attached at the fourth position of the ascarylose core called icas#9.[33] Unlike other ascarosides that induce dauer formation in a manner positively correlated to pheromone concentration, icas#9 dauer formation activity decreases at higher concentrations. The synergistic abilities and different activity-concentration relationships between ascarosides may suggest that pheromone perception may involve multiple receptors.

1.4 Ascarosides in Mate Attracting Behavior

C. elegans nematodes are hermaphroditic, producing both oocytes and sperm, and can reproduce by self-fertilization (Figure 1.7). Male worms are naturally produced at low frequency and can fertilize hermaphrodites. Simon and Sternberg found that hermaphrodites emit a chemical cue that attracts males from a distance and it was found that on agar plates, male retention is increased in regions conditioned with hermaphrodite worms.[34]

Using activity-guided fractionation of hermaphrodite-conditioned media, Srinivasan *et al* identified ascr#2 and ascr#3 as major components of the male attracting pheromone.[35]

Although ascr#2 and ascr#3 are also known components of dauer pheromone, the concentrations required to attract males were in the picomolar to nanomolar ranges, and thus much lower than those required to elicit dauer formation. Furthermore, it was found that ascr#4 which is a glucosylated derivative of ascr#2, synergizes with ascr#2 and ascr#3 and enhances male attraction, but does not attract males alone (Figure 1.8). Additional NMR-based metabolomics studies revealed that ascr#8, another component of dauer pheromone, also synergizes with ascr#2 and ascr#3 to enhance male attracting activity. Ascarosides can therefore have distinct or redundant regulatory roles in *C. elegans* behavior.

Mate attracting cues are not exclusively generated by hermaphrodite worms. Analyses of sex-specific cultures demonstrated differences between male and hermaphrodite ascaroside profiles.[36] It was revealed that while hermaphrodite worms primarily produce ascr#3, male worms predominantly produce ascr#10, the α,β-unsaturated version of ascr#3. While ascr#3 demonstrates strong male-attracting activity and repels hermaphrodite worms, ascr#10 is highly attractive to hermaphrodites.

1.5 Ascarosides in Social Behaviors

Foraging behavior naturally varies between different *C. elegans* strains. Although the laboratory strain N2 is considered solitary because it disperses around the bacterial lawn rather than aggregate, other wild type strains are considered social because they tend to gather in areas with a high abundance of food.[37-38] Ascarosides have been shown to influence these social behaviors (Figure 1.9).

Solitary N2 hermaphrodites are strongly repulsed by ascr#2, ascr#3, ascr#5, which were all shown to be attractive to male worms.[39] The studies conducted by Butcher *et al* that discovered the dauer-inducing ascaroside icas#9 also revealed the existence of an entire family of indolated ascaroside derivatives including icas#3 and icas#10.[33] At high concentrations, icas#3 and icas#9 attract both male and hermaphrodite worms, but only hermaphrodites, and not males remain attracted at low concentrations. At concentrations as low at 100 fM, both solitary and social hermaphrodites demonstrate significant levels of attraction, thus indicating icas#3 and icas#9 as effective aggregation pheromones.[40] The *p*-hydroxybenzoyl modified ascaroside hbas#3 was shown to attract hermaphrodites at concentrations as low as 10 fM and is therefore the most potent hermaphrodite aggregation signal reported.[18]

Although not involved in dauer formation, the *N*-succinylated octopamine modified ascaroside osas#9, primarily produced by starved L1 larva, has been identified as a strong deterrent to the worm in all stages of life.[20] It is hypothesized that when food is in limited supply, osas#9 may serve as a component of a dispersal signal.

1.6 Ascaroside Perception

The ascarosides are a complex family of more than 100 molecules with different structures producing many biological effects in *C. elegans*; however, the function of a single ascaroside may differ depending on conditions such as concentration. For example, at high concentrations, ascr#3 induces dauer formation, but at low concentrations, the same

ascaroside acts as a male attracting signal.[25, 35] This poses an interesting question of how worms sense ascarosides and respond accordingly.

Dauer formation signals are perceived and transduced through ciliated chemosensory neurons of the amphids, which are also known to play roles in recognizing soluble social cues and high osmolarity.[41-42] The chemosensory neurons shown to be involved in dauer formation include ADF, ASG, ASI, ASJ, and ASK.[43] Male mating behavior and hermaphrodite behaviors mediated by ascarosides, on the other hand, engage the ASK, ASI, and ADL neurons (Figure 1.10).[35, 39-40, 44]

Previous findings established that two GTP-binding protein (G protein) alpha subunits, GPA-2 and GPA-3, are expressed in chemosensory neurons and are involved in the dauer entry decision.[45] Constitutively active *gpa-2* and *gpa-3* mutants formed dauer larva even when grown under non-dauer-inducing conditions at rates of 99% and 95%, respectively. Conversely, worms containing null mutations in *gpa-2* and *gpa-3* displayed reduced responses to dauer pheromone. These results indicate that dauer formation is controlled, at least partially, by G proteins. It is also therefore suggested that the ciliated chemosensory neurons of the amphids express one or more G protein-coupled receptors (GPCRs) involved in mediating ascaroside perception.

Heterotrimeric G proteins, composed of α, β, and γ subunits, act as molecular switches that activate intracellular signaling pathways in response to the activation of a GPCR by an extracellular stimulus.[46] *C. elegans* encodes more than 1000 putative GPCRs with the majority being expressed in the chemosensory neurons, making them interesting candidates for ascaroside perception.[43]

Previous studies have identified two GPCRs, *srbc-64* and *srbc-66*, that mediate dauer formation in response to dauer pheromone and several of its individual components.[47] Loss-of-function mutations in *srbc-64* and *srbc-66* displayed dauer formation defects in response to several ascarosides. GFP-tagged SRBC-64 and SRBC-66 localized in the sensory cilia of the ASK chemosensory neurons. Furthermore, it was found that in the presence of ascr#2, *srbc-64; gpa-3* and *srbc-66; gpa-3* double loss-of-function mutant worms show similar defects in dauer formation when compared to their single mutation counterparts. Together, these results support the model that ascaroside signals promote dauer formation via SRBC-64 and SRBC-66 chemoreceptors and the GPA-3 Gα protein in the ASK neuron. The precise mechanism by which GPA-2 influences dauer formation remains unkown.

McGrath *et al* determined that the genes *srg-36* and *srg-37* encode GPCRs required for the selective perception of ascr#5.[48] Furthermore, the two genes are partially redundant as rescue of either gene restores ascr#5 sensitivity and both genes are expressed in the ASI neurons, which are shown to be involved in dauer formation. Mutants of two additional genes, *daf-37* and *daf-38* were shown to be defective in ascaroside perception.[49] While a *daf-38* mutant displayed a defective response to ascr#2, ascr#3, and ascr#5, the *daf-37* mutant was only defective in responding to ascr#2. It was later shown through a photo-affinity labeling experiment that *daf-37* directly binds to ascr#2 and serves as the first example of direct binding of an ascaroside to a receptor. This combination of receptor specificity and redundancy indicates that ascaroside perception is a highly complex process.

1.7 Mass Spectrometry-Based Ascaroside Profiling

The *C. elegans* metabolome is complex and therefore, analysis by high performance liquid chromatography tandem mass spectrometry (HPLC-MS) produces crowded chromatograms which makes identifying individual ascarosides a difficult task. To surmount this issue, von Reuss *et al* examined MS/MS fragmentation patterns of various synthetic ascarosides and discovered that under negative-ion electrospray ionization (ESI^-), ascarosides typically fragment to produce a characteristic ion derived from the ascarylose sugar scaffold with a mass-to-charge ratio of 73.0 (Figure 1.11).[18]

By screening for this characteristic ion, HPLC-MS chromatogram peaks become well resolved. Furthermore, by examining fragmentation patterns of known synthetic ascarosides, unknown metabolites can be identified as derivatives or homologs. For example, two peaks in a HPLC-MS chromatogram of a wild type metabolome were found to contain a product ion at *m/z* 301.1651, characteristic of ascr#3. Fractionation and subsequent 2D NMR spectroscopy later revealed these new ascarosides to be hbas#3 and mbas#3, 4'-modified derivatives of ascr#3. Using known retention times derived from synthetic ascarosides and characterized fragmentation patterns, many ascarosides can now be easily detected from the supernatant of *C. elegans* liquid cultures.

1.8 Thesis Summary

Ascarosides constitute a large family of signaling molecules that regulate crucial events in the life history of *C. elegans*. Although the molecular core remains consistent, small structural alterations to the attached moieties appear to be responsible for the vast differences

in biological effects amongst individual ascarosides. However, very little is known about the biological machinery and processes by which ascarosides are synthesized within the worm.

The primary focus of this thesis was to determine where and how ascarosides are produced. In Chapter 2, we found that by selectively driving the expression of DAF-22, an enzyme necessary for the production of biologically active ascarosides, within specific tissues, we were able to assess ascarosides produced within the intestine, hypodermis, and body wall muscles of the worm. Our results revealed that while the intestine is the major site of ascaroside biosynthesis, modest amounts of ascarosides are produced in the hypodermis and body wall muscle that are sufficient in quantity to rescue various ascaroside-regulated *C. elegans* behaviors.

In Chapter 3, we discuss studies on the enzyme ACS-7, which was found to be required for the attachment of head groups to the 4'-position of ascr#9. It was also discovered that although predicted to be peroxisomal, ACS-7 is expressed in lysosome-related organelles found in the intestine of *C. elegans*. These so-called gut granules are necessary for the production of 4'-modified ascarosides. The biosynthesis of ascarosides is a complex process that incorporates many aspects of primary metabolic pathways, and these studies serve to help elucidate how the worm's metabolic status is transduced into worm behavior via ascaroside signaling.

1.9 Figures

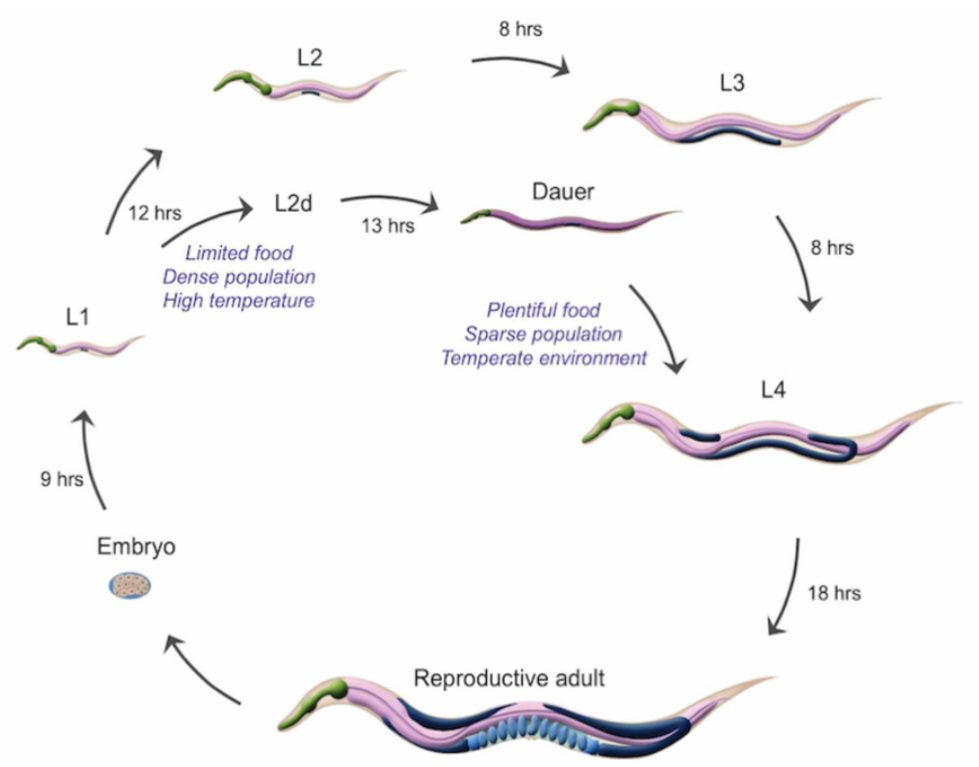

Figure 1.1: The *C. elegans* Life Cycle. Under favorable environmental conditions, *C. elegans* progresses through four larval stages, L1-L4, molting between each stage. In a stressful environment with a high population density, low food, and/or at a high temperature, L1 larva may develop into L2d larva and eventually develop into the alternative stress-resistant L3 larval stage, the dauer. When conditions turn favorable, the worm may exit dauer diapause and return to the normal life cycle as an L4 larva. (Unmodified figure from Wormatlas.[50])

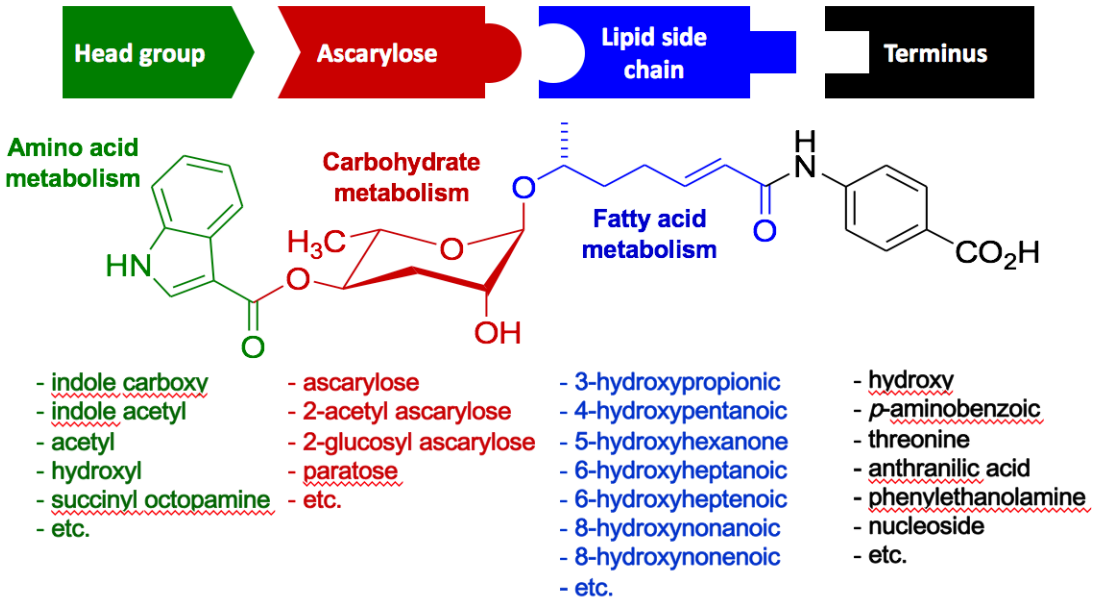

Figure 1.2: Modular Ascaroside Structure. Ascarosides are glycosides of the dideoxysugar ascarylose (red). Attached to the ascarylose core at the 1'-position is a fatty acid moiety that can differ in length, saturation, and oxidation (blue). Ascarosides are modular structures and can have additional groups attached to the 2'- or 4'-positions of the ascarylose sugar (green), or at the terminal end of the lipid side chain (black). (Modified figure from von Reuss *et al.*[18])

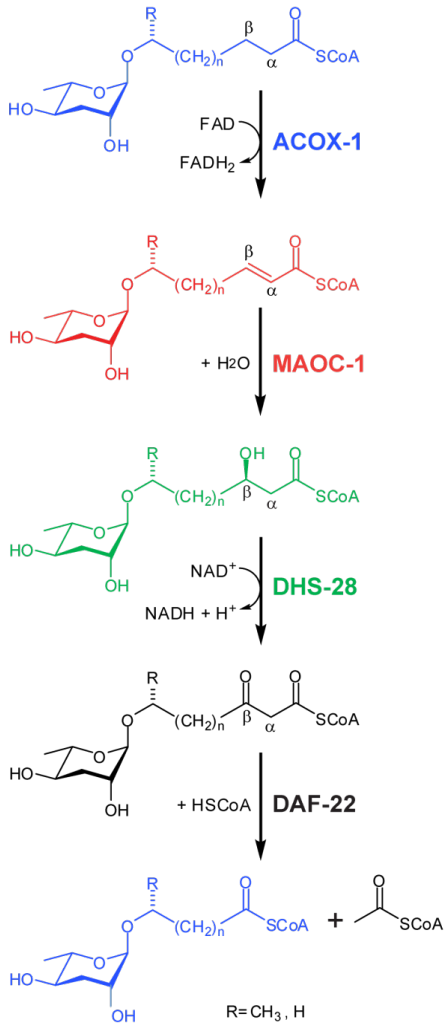

Figure 1.3: Peroxisomal β-Oxidation of Ascaroside Lipid Side Chains. Peroxisomal β-oxidation is a process that truncates a carbon chain by two carbons in a series of four steps. ACOX-1, an acyl-CoA oxidase, introduces a site of α,β-unsaturation (blue). MAOC-1, a peroxisomal 2-enoyl-CoA hydratase, hydrates the double bond (red). DHS-28, a dehydrogenase, oxidizes the hydroxyl group to form a β-ketoacyl-CoA ester (green). DAF-22, a thiolase, truncates the lipid chain to release acetyl-CoA and a shortened ascaroside precursor (black). (Modified figure from von Reuss *et al.*[18])

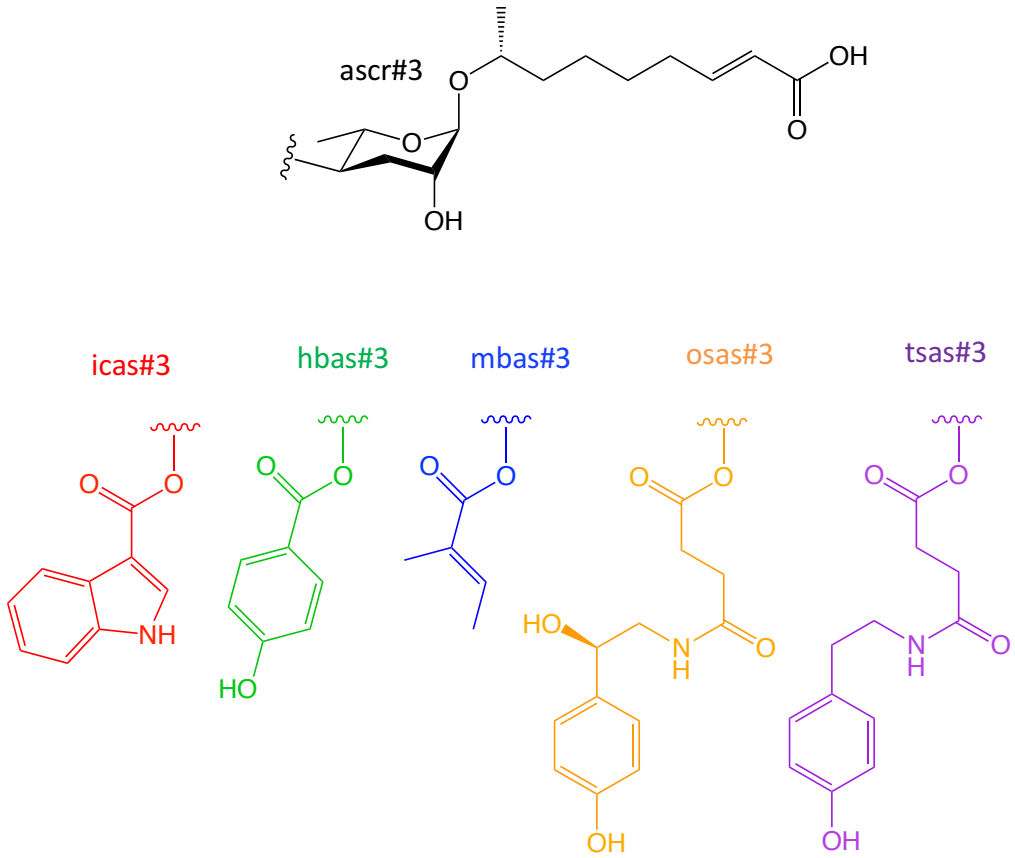

Figure 1.4: 4'-Modification of Ascarosides. Simple ascarosides such as ascr#3 (black) can be modified at the 4'-position with various head groups derived from primary metabolic pathways to form derivatives such as icas (red), hbas (green), mbas (blue), osas (orange), and tsas (purple) ascarosides. Note: tsas#3 is not known to exist, but the tsas head group does modify other simple ascarosides such as ascr#9.

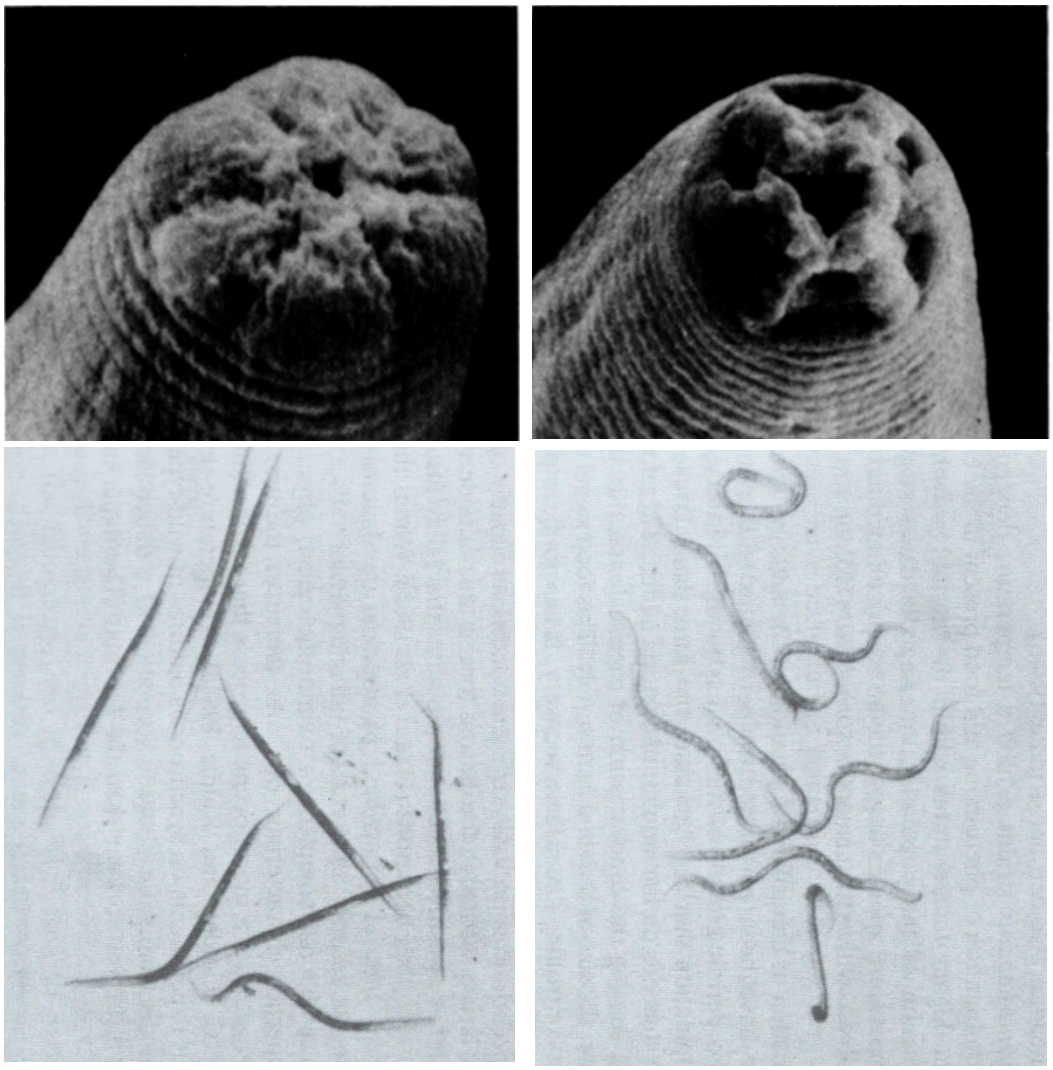

Figure 1.5: The Dauer Larva. The dauer is a stress-resistant alternative larval stage. Scanning electron microscopy reveals that the dauer forms a plug that blocks the mouth, preventing the ingestion of food and toxins from the environment (top panel, left), whereas the mouth of a well fed L2 larva remains open (top panel, right). Dauer larva (bottom panel, left) can be distinguished from L3 larva (bottom panel, right) by light microscopy. With a thicker cuticle, the dauer is rigid and motionless, and appears darker due to an increase in fat storage within the intestine. (Modified figure from Wood *et al.*[13])

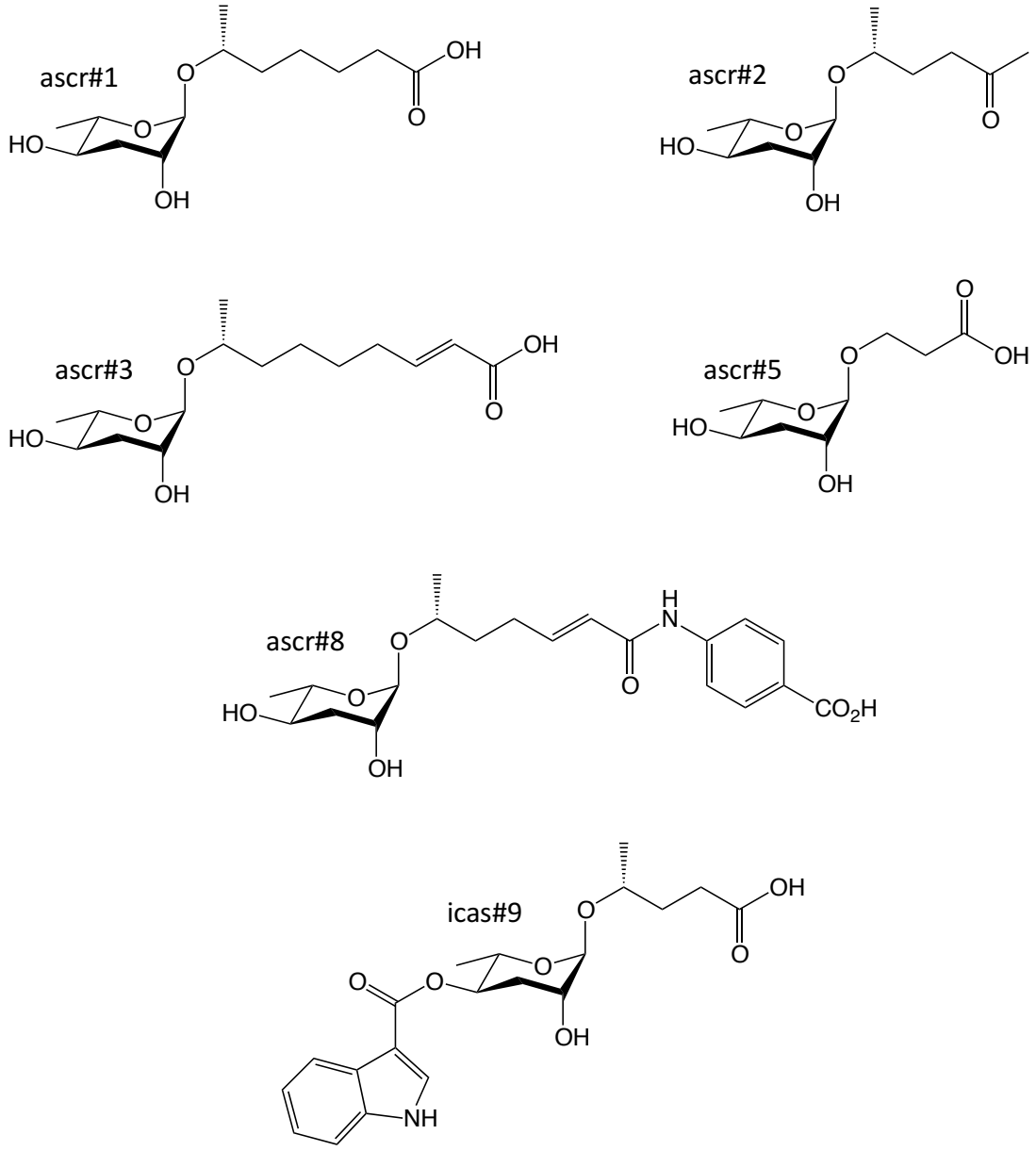

Figure 1.6: Ascarosides Involved in Dauer Formation.

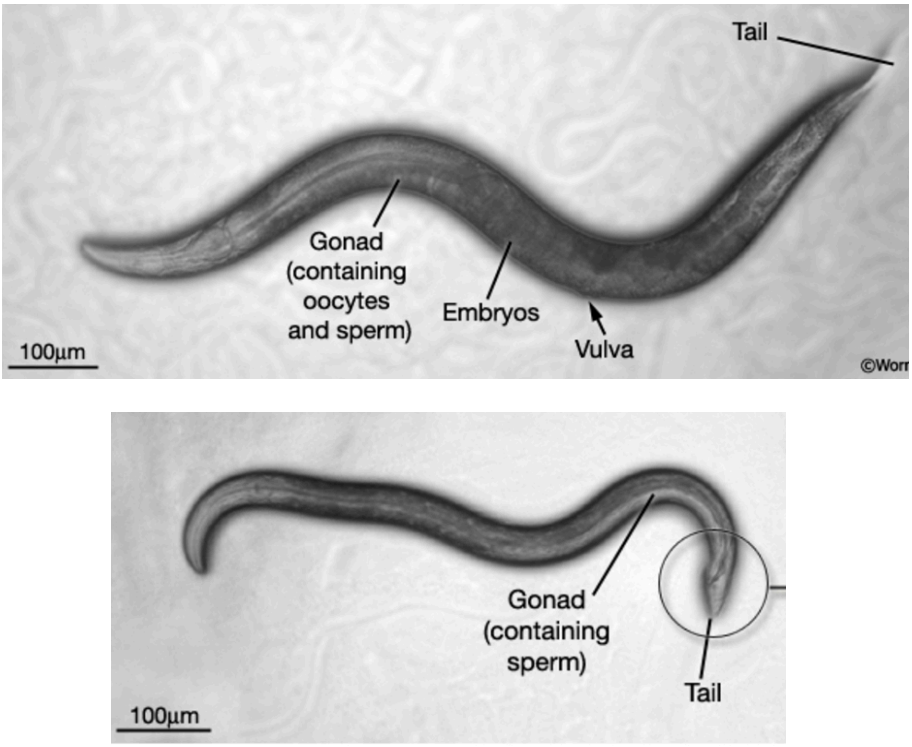

Figure 1.7: Hermaphrodite vs. Male Worm Morphology. *C. elegans* hermaphrodite worms produce both eggs and sperm and can therefore self-fertilize their own oocytes (top panel). Male *C. elegans* worms are smaller than hermaphrodites and have a hook-like copulatory tail that can be inserted into the vulva of a hermaphrodite worm to fertilize eggs (bottom panel). (Modified figure from Wormatlas.[50])

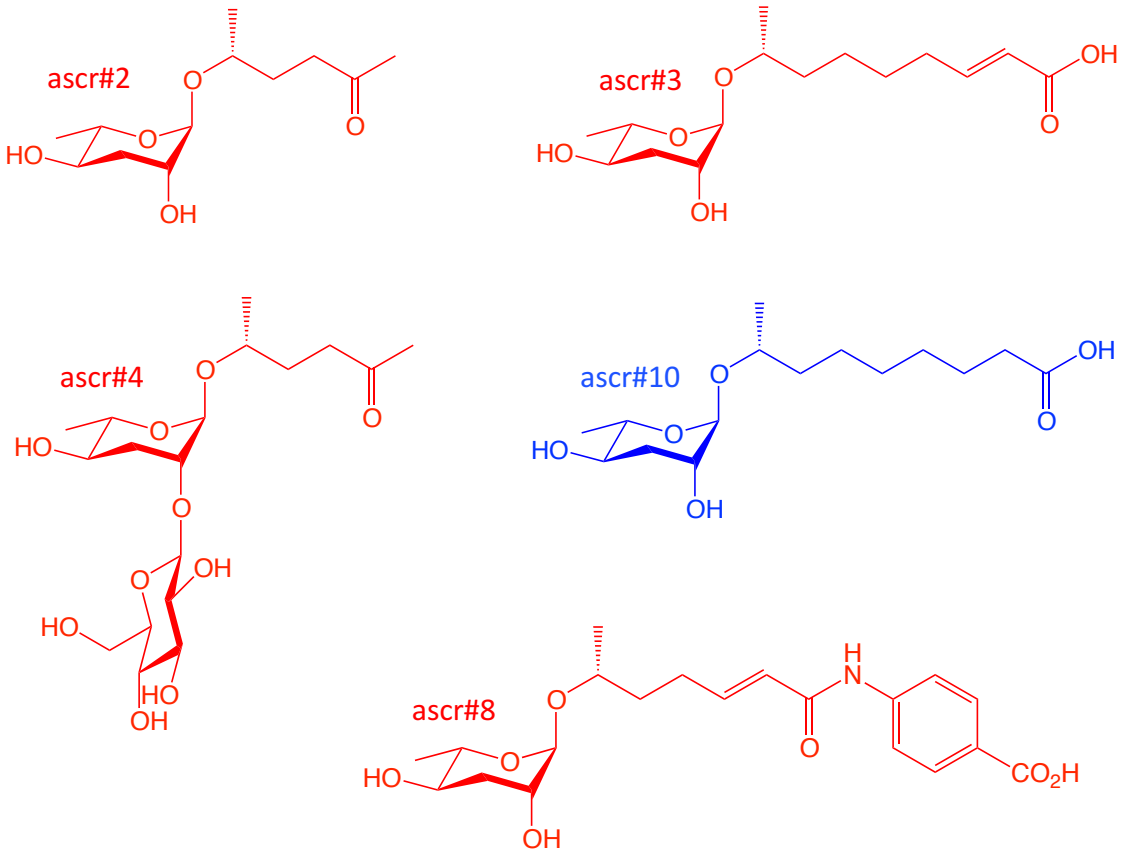

Figure 1.8: Ascarosides Involved in Mate Attraction. Several ascarosides are attractive to male *C. elegans* worms (red), whereas ascr#10, an ascaroside highly produced by male worms, is attractive to hermaphrodite worms (blue).

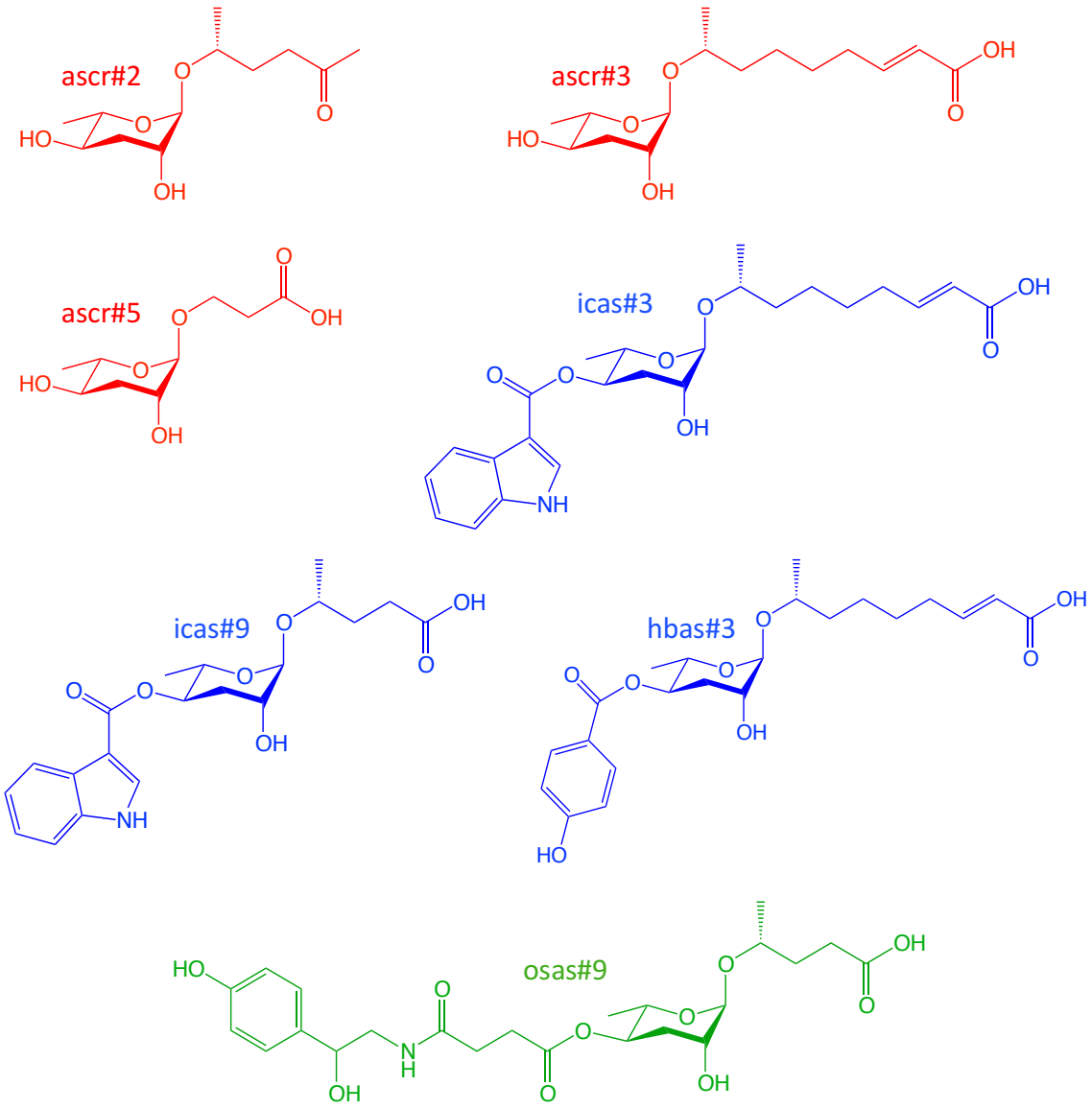

Figure 1.9: Ascarosides Involved in Aggregation and Repulsion. Ascarosides involved in other behaviors such as male attraction and dauer formation are repulsive to *C. elegans* hermaphrodite worms (red). Several 4'-modified ascarosides are hermaphrodite attractants (blue), but osas#9, produced mainly by starved L1 larva, act as deterrents to all larval stages (green).

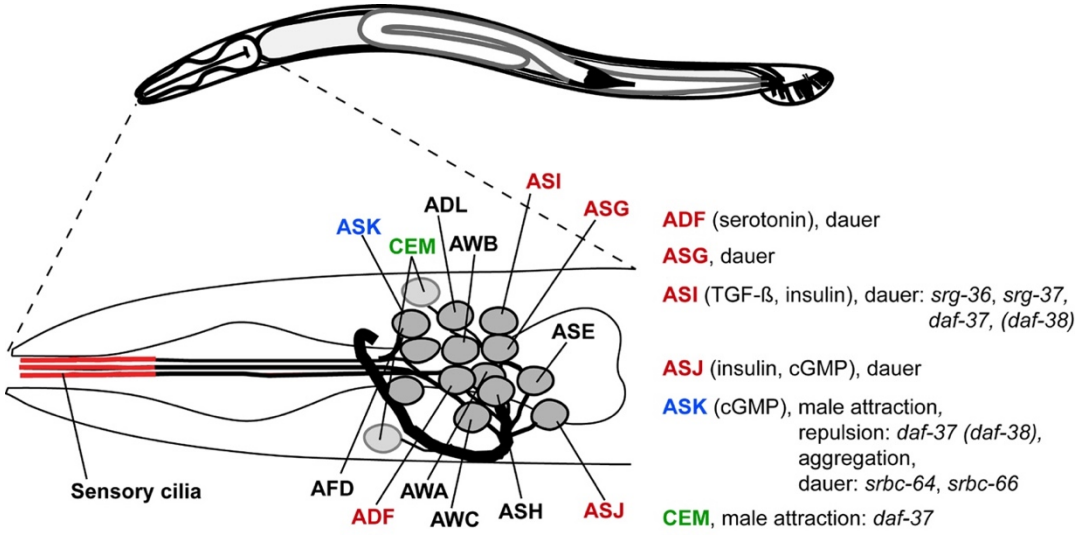

Figure 1.10: Ciliated Sensory Neurons Involved in Ascaroside Perception. *C. elegans* depends on chemosensation in order to locate food, avoid noxious chemicals, to mate, and in development. To do so, the worm relies on chemosensory neurons with cilia exposed to the environment. ADF, ASG, ASI, ASJ, ASK, and ADL neurons have been shown to play roles in perception of dauer pheromone as well as ascaroside-mediated social and mating behaviors. (Modified figure from Wormatlas.[51])

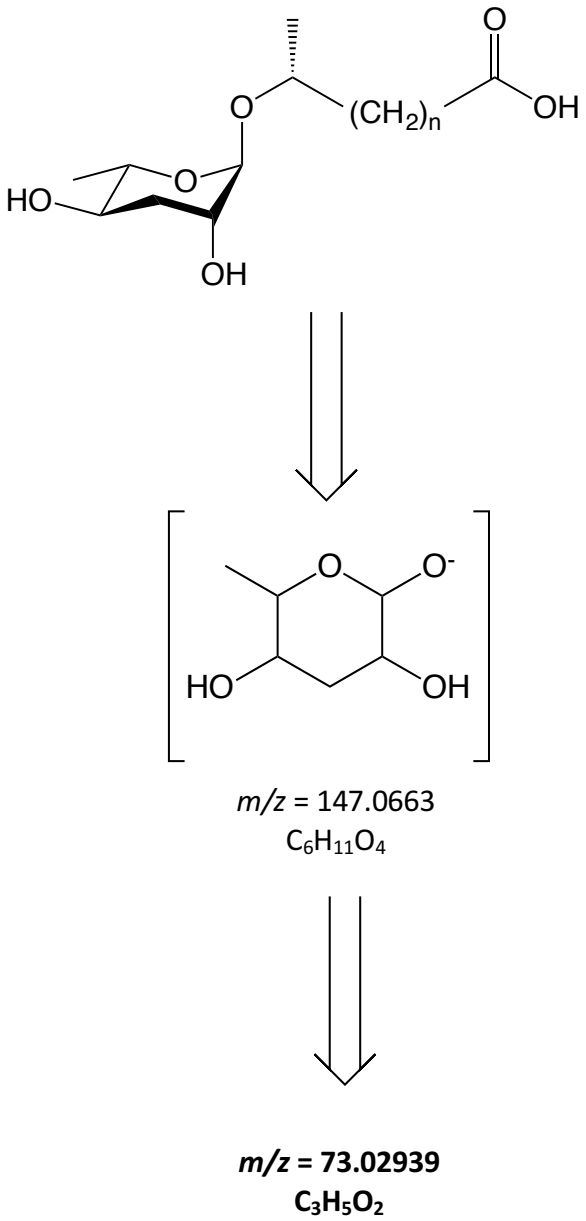

Figure 1.11: MS/MS Fragmentation of Ascarosides. Under ESI$^-$ conditions, most ascarosides produce an ion at *m/z* 73.0 derived from the ascarylose sugar core. (Modified figure from von Reuss *et al.*[18])

1.10 References

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