Natural products as chemical tools to dissect complex biology in *C. elegans*

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The search for novel pheromones, hormones, and other types of natural products in the nematode *Caenorhabditis elegans* has accelerated over the last 10-15 years. Many of these natural products perturb fundamental processes such as developmental progression, metabolism, reproductive and somatic aging, and various behaviors and have thus become essential tools for probing these processes, which are difficult to study in higher organisms. Furthermore, given the similarity between *C. elegans* and parasitic nematodes, these natural products could potentially be used to manipulate the development and behavior of parasitic nematodes and target the infections caused by them.

Introduction

C. elegans is an ideal model system for exploring the role of small-molecule signals in controlling complex processes from development to metabolism to fertility to lifespan, as well as diverse behaviors. Using chemosensory neurons, *C. elegans* senses pheromones and other types of small-molecule signals in order to respond to changes in its environment and alter its development and behavior accordingly. For example, *C. elegans* secretes a class of pheromones known as ascarosides to communicate with other worms, enabling it to gauge its population density [1-5], to detect mates [6,7], and to perform a host of other functions essential for its survival. Meanwhile, the worm uses a neuroendocrine network to respond to various environmental inputs by synthesizing downstream hormones and modulating signaling pathways in different target tissues. *C. elegans* offers many advantages for the study of the role of small-molecule signals, including a fully mapped nervous system and well-understood developmental program. Because *C. elegans* can be manipulated genetically and grown in culture with ease, comparative metabolomics can be used to discover the small-molecule signals that are produced

by specific biosynthetic genes in the worm [8,9]. Furthermore, the identification of the protein targets of these small-molecule signals can be facilitated by crossing worm strains that are sensitive and resistant to the small molecules and correlating the sensitivity of the cross progeny to the small molecules with their genomic sequence [10-12].

Since 40% of *C. elegans* proteins have homologs in humans, and many signaling pathways are conserved between the two organisms [13], studying the roles of small molecules in *C. elegans* could potentially translate to a better understanding of human biology. The insulin / insulin-like growth factor-1 (IGF-1) and TGFβ pathways in *C. elegans* are homologous to pathways in humans and play a central role in controlling the worm's development, metabolism, and lifespan in response to environmental cues [14,15]. When the worm population density is low and food is adequate, these pathways promote the biosynthesis of the dafachronic acids (DAs), which are hormones that bind to the nuclear hormone receptor DAF-12 and promote development of the worm through four larval stages (L1-L4) to the reproductive adult (**Figure 1A**) [16]. As the worm population density overwhelms available food [17], a group of ascarosides known as the dauer pheromone accumulate in the surroundings and downregulate the insulin and TGFβ pathways, forcing the worm to enter an alternative L3 larval stage called the dauer (**Figure 1B**). The dauer is a stress-resistant, non-feeding larval stage that survives by metabolizing fat stores and enables the worm to survive for prolonged periods until conditions improve.

The discovery of small-molecule signals in *C. elegans* could have implications for the control of parasitic nematodes. The dauer stage in *C. elegans* is analogous to the infective stage iL3 in parasitic nematodes, which enables these nematodes to survive while outside their host [18]. The

insulin and DAF-12 pathways (but not TGFβ pathway) play an important role in controlling development of the iL3 stage [18]. Furthermore, many of the small-molecule signals discovered in *C. elegans* play conserved roles across nematode evolution. For example, ascarosides have been detected in a wide range of nematode species and have been shown to affect development and behavior in a number of them [19-22].

This review will focus on recent developments regarding ascarosides and DAs, as well as the discovery of several new types of nematode natural products, including phosphorylated glycosphingolipids and nemamides. It will emphasize the technologies that are facilitating the discovery of these natural products, as well as emerging areas of research.

Ascarosides and their roles in development, aging, and behavior

The first ascaroside pheromones were discovered through activity-guided fractionation of conditioned worm medium to purify and structurally characterize compounds that would induce the dauer stage [1-4] (**Figure 2A**). Many additional ascarosides that induce mating attraction [6,7], suppress foraging [12], enhance aggregation [23], or cause avoidance [24,25] in worms have since been discovered using a variety of different approaches. These ascarosides have become important tool compounds that are helping to dissect the connections between G protein coupled receptors, neurons, signaling pathways, and downstream processes and behaviors [11,12,23,26-32]. The identification of the ascarosides has enabled discovery of several of the GPCRs that they target, including GPCRs that regulate dauer development [11,27,28] and foraging [12,30]. The ascarosides are also revealing roles for pheromones that were previously unknown, such as interactions between the sexes. For example, males produce specific

ascaroside mixtures that delay the loss of germline progenitor cells in hermaphrodites that occurs with aging, thereby extending the reproductive lifespan of the hermaphrodites [33]. On the other hand, male-produced ascarosides and possibly other compounds contribute to making hermaphrodites age faster and die younger [33,34]. As another example, in hermaphrodites that sense unfavorable ascarosides, TGFβ signaling is reduced, inhibiting oocyte production of prostaglandins that are used to attract sperm [35].

Structural similarities between the ascarosides have facilitated the discovery of new ascarosides [23,24,36], and *C. elegans* has now been shown to produce hundreds of ascarosides although the function of most of these compounds remains unknown [9]. The ascarosides share a common modular structure with a central ascarylose sugar derivatized with a fatty acid-derived side chain (**Figure 2A**). The ascarosides can be modified with various head groups on the ascarylose sugar or terminus groups at the end of the side chain, and these modifications are often derived from pathways in primary metabolism such as amino acid metabolism and sugar metabolism. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for rapidly profiling known and novel ascarosides in crude worm extracts [24,36]. This method uses a triple quadrupole mass spectrometer operating in precursor ion scanning mode to detect ascarosides based on an ascaroside-derived product ion that is produced by those ascarosides that ionize in negative mode (that is, those containing a carboxylic acid, which constitute many of the ascaroside pheromones) [36]. More recently, a gas-chromatography-mass spectrometry (GC-MS) method was developed that monitors for an ascarylose-derived product ion in TMS-derivatized crude worm extracts and that can detect both acidic and non-acidic ascaroside pheromones [37]. This method is particularly useful for detecting ascarosides with a wide range

of side-chain lengths (3-33 carbons) and side-chain modifications (such as hydroxylation), but unlike the LC-MS/MS method, cannot detect ascarosides modified with various head groups.

Comparative studies of the metabolomes of wild-type worms and mutant worms with defects in ascaroside biosynthesis have further stimulated the discovery of novel ascarosides. Peroxisomal β-oxidation cycles shorten the side chains of long-chain ascarosides in order to biosynthesize the short- and medium-chain ascaroside pheromones [36,38,39]. DAF-22, which is the last enzyme in these β-oxidation cycles, is essential for the biosynthesis of the ascaroside pheromones [38]. Comparisons of the 2D NMR spectra of wild-type and *daf-22* culture extracts have been used to discover a number of novel ascarosides [5,23]. More recent work has used untargeted LC-MS-based metabolomics to compare wild-type and *daf-22* culture extracts, as well as molecular networking to group the *daf-22*-dependent metabolites based on the similarity of their MS/MS fragmentations patterns [9]. This work has shown that *C. elegans* produces a broader diversity of ascarosides than was previously thought, with additional types of terminus group modifications, including those derived from nucleoside metabolism [9]. The challenge now lies in connecting these compounds with biological roles in the worm.

DAs and their roles in development, reproduction, and lifespan

DAs bind to and promote the transcriptional activity of DAF-12, which plays a role in regulating dauer development, developmental progression, metabolism, and lifespan in nematodes [40] (**Figure 2B**). In order to discover the DAs, potential precursors to the DAF-12 ligand, lathosterone and 4-cholesten-3-one, were treated *in vitro* with DAF-9, a cytochrome P450 enzyme known to be in the ligand's biosynthetic pathway [41]. Lathosterone and 4-cholesten-3-

one were converted into, respectively, Δ^7-DA and Δ^4-DA, which both bound to DAF-12 *in vitro*, stimulated DAF-12 transcriptional activity, and could be detected in extracts from wild-type worms but not *daf-9* mutant worms. Later work using partial purification of the ligand via activity-guided fractionation suggested that Δ^7-DA was likely the more physiologically relevant hormone and identified an additional DAF-12 ligand that was just as potent as Δ^7-DA, $\Delta^{1,7}$-DA [42].

Like the ascarosides, DAs have become important tool compounds, and they are being used to dissect the role of DAF-12 in nematode biology. DAs have been instrumental in demonstrating how DAF-12 directs changes in stem cell differentiation during larval progression by inducing microRNAs of the conserved *let-7* family [43]. In addition to developmental gene expression, DA/DAF-12 signaling also promotes a transcriptional program that promotes the oxidation of fatty acids and thereby supports reproductive growth [44]. In response to removal of the worm germline, DA/DAF-12 signaling extends adult lifespan by inducing *let-7* microRNAs and suppressing insulin signaling [45]. DAs have also been used to show that DAF-12 controls lysosomal pH in post-reproductive, adult worms, possibly improving proteolysis and reducing the deterioration of somatic tissue that occurs with aging [46].

As the interaction between DAF-12 and DAs is well-conserved in many parasitic nematode species, DAs have been particularly useful for probing the role of DAF-12 in these species, which are not amenable to genetic manipulation [47-49]. For example, DAs have been used to block not only the development of dauers in *C. elegans*, but also development of the iL3 stage in animal parasitic nematodes [47,50-52] and the dispersal L_{IV} stage in the plant parasitic pinewood

nematode [53]. *Strongyloides stercoralis*, which infects over 100 million people worldwide, can undergo two different developmental pathways: one in which L1 larvae are excreted in the feces, go through one free-living generation, and then develop into iL3 that can then infect the host, and one in which autoinfective L1 larvae stay in the host, directly develop into iL3, and establish a continual, often untreatable infection. Importantly, Δ^7-DA blocks the development of free-living and autoinfective L1 larvae into iL3 *in vitro* and was recently shown to reduce *S. stercoralis* hyperinfection in severely immunocompromised mice [47,51,52].

Novel phosphorylated glycosphingolipids and their roles in cholesterol mobilization

In *C. elegans*, phosphoethanolamine glycosylceramides (PEGCs), a novel type of phosphorylated glycosphingolipids, promote the mobilization of internal pools of cholesterol, which is essential for nematode development and growth (**Figure 2C**) [54]. *C. elegans* cultured in the absence of cholesterol will arrest after one generation as L2-like larvae [55]. Boland *et al.* identified the PEGCs through activity-guided fractionation of worm lipid extracts to purify molecules that would block this larval arrest. The PEGCs also suppress the dauer arrest that occurs in Niemann-Pick C1 (NPC1) mutant worms, which have defects in cholesterol trafficking and form dauers constitutively due to their inability to make DAs from cholesterol [54]. Endogenous endocannabinoids in *C. elegans*, including 2-arachidonoyl glycerol and anandamide, have been shown to work synergistically with the PEGCs to promote cholesterol mobilization [56]. These compounds are helping to elucidate the nature of cholesterol trafficking in *C. elegans*, as well as its role in nematode development and growth, and could have implications for cholesterol trafficking in humans.

Nemamides and their roles in starvation survival

The nemamides are hybrid polyketide-nonribosomal peptides that are produced in an assembly-line manner by two huge, multidomain enzymes, PKS-1 and NRPS-1 (**Figure 2D**) [8]. The nemamides represent the first of this type of natural product to be discovered in an animal and were identified through unbiased, comparative LC-MS-based metabolomics between wild-type, *pks-1* mutant, and *nrps-1* mutant worms. Mass-guided fractionation of wild-type worm extracts then enabled the purification of the nemamides, the structures of which were subsequently determined through NMR spectroscopy and mass spectrometry. Experiments with the *pks-1* and *nrps-1* mutant worms suggest that the nemamides may promote survival during the larval arrest that occurs when eggs are hatched in the absence of bacterial food [8]. Although the exact mechanism of action of the nemamides is not known, they are produced in two poorly understood, but essential neurons in the worm, the canal-associated neurons, and are not thought to be secreted. Importantly, *pks-1* and *nrps-1* are conserved in most nematode species, suggesting that the nemamides may play an important biological role that is conserved across nematode evolution.

Conclusions

C. elegans has over 1000 GPCRs, the majority of which have unknown ligands, and 284 nuclear hormone receptors, only a handful of which have known ligands [41,42,57-59]. Thus, there is a lot to be done in terms of discovering novel small-molecule signals in the worm, as well as identifying their receptor targets. Recent work in the worm has established evidence for a number of uncharacterized signals. *C. elegans* males [33] and hermaphrodites [60] have been shown to secrete unknown chemical signals that shorten the time for hermaphrodites to develop

from an egg to an egg-laying adult. An uncharacterized steroid hormone has been shown to regulate the size of triacylglycerol-containing lipid droplets in *C. elegans* [61]. Although the hormone appears to function via DAF-12, it does not appear to correspond to Δ^7-DA. In addition to the pheromones and hormones produced by *C. elegans*, the worm is also an excellent tool to study the small-molecules signals that mediate interactions between *C. elegans* and its microbiome, as well as pathogens in its environment [62], and very little work has been done in this area. Beyond *C. elegans*, there are tens of thousands of other nematodes species, and we know virtually nothing about their chemistry. For example, although a number of entomopathogenic, plant-parasitic, and animal parasitic nematode species have been shown to produce ascarosides [19-22], suggesting that these molecules are conserved through nematode evolution, only a handful of nematode species have been investigated in depth in order to detect structurally novel ascarosides and other types of compounds. Furthermore, while DAs have been shown to bind homologs of *C. elegans* DAF-12 in other nematodes and influence their larval development [44,47,48,52], endogenous DAs or similar compounds have not yet been reported in these nematode species. The discovery of novel natural products in *C. elegans* and other nematode species will undoubtedly increase in the coming years, aided by an increased understanding of the biosynthetic potential of *C. elegans*, the development of genome editing strategies in non-*C. elegans* nematodes, as well as improvements in mass spectrometry and comparative metabolomics techniques.

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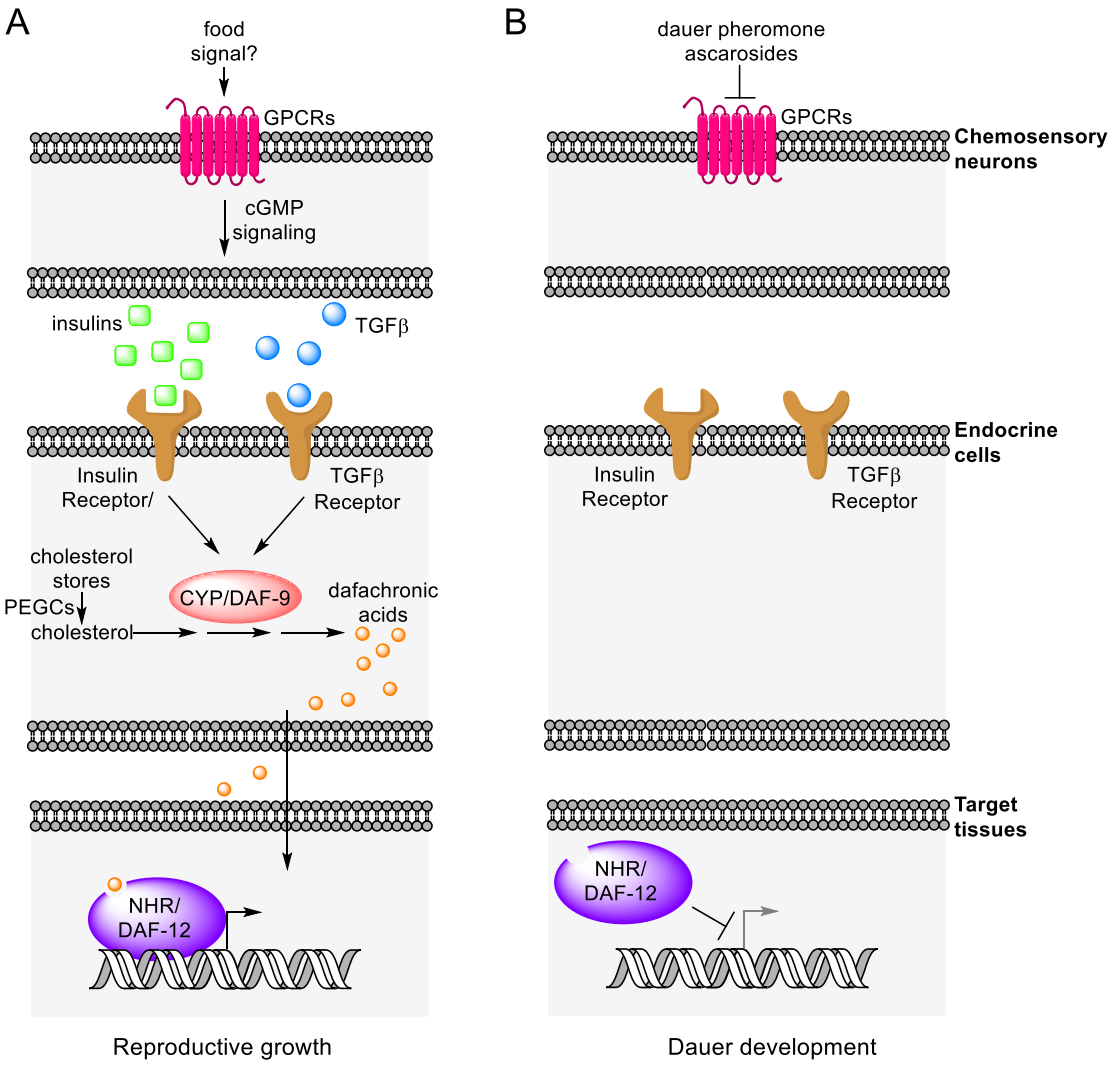

Figure 1. Signaling pathways that control dauer development in *C. elegans*. (A) Under favorable conditions (low population density and high food), cGMP signaling in chemosensory neurons promotes the secretion of insulin and TGFβ ligands that bind to the insulin and TGFβ receptors. This insulin and TGFβ signaling induces the expression of the cytochrome P450 DAF-9, which promotes the biosynthesis of dafachronic acids (DAs) from cholesterol. Phosphoethanolamine glycosylceramides (PEGCs) and endocannabinoids promote the mobilization/trafficking of cholesterol required for this process. (B) In contrast, under unfavorable conditions (high population density and low food), the dauer pheromone ascarosides target specific GPCRs and block cGMP signaling, thereby suppressing insulin and TGFβ signaling and the production of DAs and promoting dauer formation.

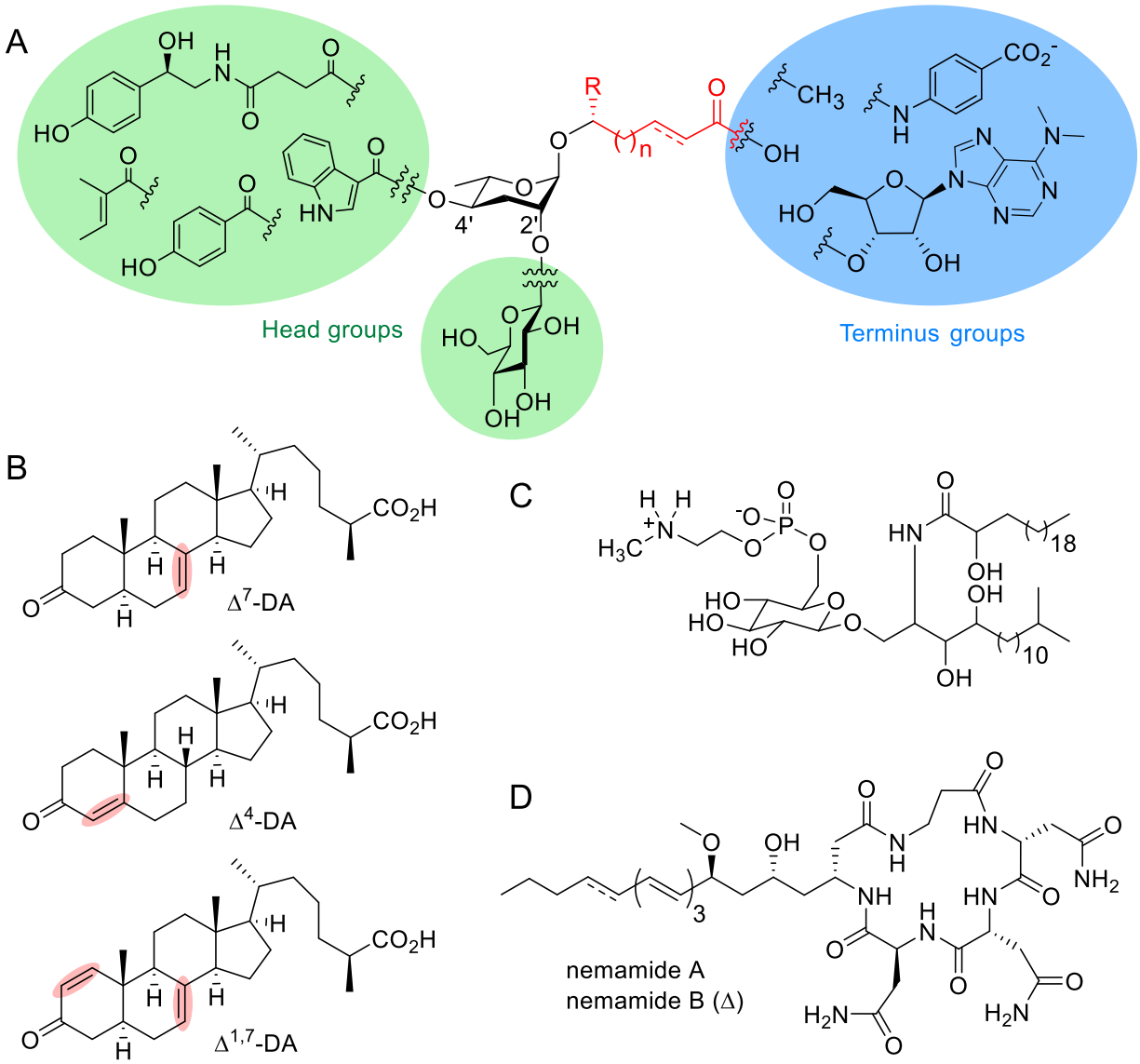

Figure 2. Examples of natural products discovered in nematodes. (A) The ascarosides have a central 3,6-dideoxysugar ascarylose that is attached to the terminal (R=H) or penultimate (R=CH_3) carbon of fatty acids of various lengths. Ascarosides with specific side-chain lengths are modified with various head groups on the 2' and 4'-positions of the ascarylose sugar and with various terminus groups on the end of the side chain. The head and terminus groups shown are a small sampling of those that are known. (B) The structures of the dafachronic acids (DAs). (C) The structures of the phosphoethanolamine glycosylceramides (PEGCs). (D) The structures of the nemamides.

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