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## Modular assembly of primary metabolic building blocks: a chemical language in *C. elegans*

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### Summary

The metabolome of the nematode *Caenorhabditis elegans*, like that of other model organisms, remained largely uncharacterized until recent studies demonstrated the importance of small molecule-based signaling cascades for many aspects of nematode biology. These studies revealed that nematodes are amazingly skilled chemists: using simple building blocks from primary metabolism and a strategy of modular assembly, nematodes create complex molecular architectures that serve as signaling molecules. These nematode-derived modular metabolites (NDMMs) are based on the dideoxysugars ascarylose or paratose, which serve as scaffolds for attachment of moieties from lipid, amino acid, neurotransmitter, and nucleoside metabolism. Although preliminary biosynthetic studies have confirmed the primary metabolism origin of some of the building blocks incorporated into NDMMs, the mechanisms that underlie their highly specific assembly are not understood. Overall, I argue that identification of new variants of primary metabolism-derived structures that serve important signaling functions in *C. elegans* and other nematodes provides a strong incentive for a comprehensive re-analysis of metabolism in higher animals, including humans.

### Introduction

In contrast to bacteria, fungi, and plants, whose genomes have revealed a great variety of “secondary” small-molecule biosynthetic pathways, animal genomes aren’t generally known to encode biosynthetic machinery for complex secondary metabolites. Many times structurally intriguing metabolites have been identified from animal sources, the compounds ultimately turned out to be derived from microorganisms or plants. Examples include the identification of tetrodotoxin from pufferfish and the great number of polyketides identified from marine sponges (Fisch et al., 2009; Piel, 2009). The great diversity of insect natural products forms a notable exception; however, at least some groups of insect-derived natural products also have been shown to be of microbial origin (Gronquist et al., 2010; Piel, 2009).

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Recent analyses of nematodes, the most abundant animals on earth, have revealed a family of natural products that embodies a new assembly strategy for secondary metabolites. These **nematode-derived modular metabolites** (NDMMs) are based on modular assembly that uses glycosides of the dideoxysugars ascarylose (“ascarosides”) and paratose (“paratosides”) as a central scaffold and attaches different building blocks derived from primary metabolic pathways, for example carbohydrate, amino acid, lipid, nucleoside, or neurotransmitter metabolism, via ester, amide, or glycosidic linkages (Figure 1).

NDMMs serve important signaling functions in nematodes, for example as regulators of organismal development, lifespan, and social communication. The study of these biological activities in the model nematode *C. elegans* has been contributing greatly to our understanding of how conserved signaling pathways (e.g. insulin signaling) regulate aging, metabolism, and behavior (Ludewig and Schroeder, 2013). Notably, available evidence indicates that NDMMs are produced by the nematodes, not associated microbiota, and that conserved primary metabolic pathways contribute to their biosynthesis. In this review, I will begin with a brief description of the biological phenomena that triggered the discovery of the first NDMMs, followed by a summary of NDMM structures and their biological activities, highlighting the role of comparative metabolomics for the discovery of new compounds and activities. Finally, I will summarize current knowledge of NDMM biosynthesis and possible connections to conserved primary metabolism.

## Nematodes as model organisms

Nematodes are arguably the most numerous animals (Blaxter, 1998). They are of great relevance to human health, on one hand, because they infect 25% of the world’s population and significantly impact agricultural crops and animals and, on the other, because the non-parasitic soil nematode *Caenorhabditis elegans* (literally “elegant empty stick”) is an important model organism for biomedical research (Kaletta and Hengartner, 2006). *C. elegans* was selected as the first fully differentiated animal for complete genome sequencing (The *C. elegans* Sequencing Consortium, 1998) and has become a very productive model system, for several reasons. This nematode has a short life cycle of only three days, it is small enough for high-throughput whole-organism screens, and much of its biology is controlled by evolutionarily conserved molecular pathways. This high level of genetic conservation allows ancient features of endocrine pathways to be explored in *C. elegans*. Significantly, *C. elegans* has been developed as a unique platform for the study of conserved mechanisms regulating metabolism, development, reproductive maturation, and longevity, which revealed a deeply intertwined regulatory network that remains, at best, partly understood (Fielenbach and Antebi, 2008). As part of this network, small-molecule signals, including steroids and the NDMMs play major roles in connecting metabolism with behavior, development, and aging.

Similar to *C. elegans*, *Pristionchus pacificus* is a free-living nematode that has been established as a model organism for the study of developmental and evolutionary biology (Dieterich et al., 2008). In contrast to *C. elegans*, *P. pacificus* forms a necromenic association with beetles, which may represent a pre-adaptation to the evolution of true parasitism (Rae et al., 2008). Importantly, *P. pacificus* is used as a “satellite” model

organism alongside *C. elegans*. Satellite models are species that are sufficiently closely related to well-known model organisms so that the genetic regulation of homologous biosynthetic, cellular, and developmental processes can be studied, enabling the identification of the molecular changes that underlie phenotypic and biochemical differences or variation.

## The dauer pheromone: the first ascaroside-based signaling molecules

Studies of longevity and development in *C. elegans* initially focused on one unique feature of its development: an alternative larval stage called “dauer” (German, “enduring”). When exposed to environmental stress, e.g. starvation, high temperature, or excessive crowding, *C. elegans* larvae abort normal reproductive development and enter the highly stress-resistant dauer diapause (Figure 2). The non-feeding dauer larvae are seemingly “non-aging” as they can persist for several months under adverse conditions, compared to a normal adult lifespan of only about 2–3 weeks (Hu, 2007; Riddle and Albert, 1997). When environmental conditions improve, dauer larvae resume reproductive development, resulting in adults with normal physiology and lifespan. Genetic screens for mutants that either cannot attain the dauer stage or form dauer larvae constitutively identified more than three dozen DAF (DAUER Formation) genes (Hu, 2007). Subsequent studies revealed important roles for DAF genes in *C. elegans* lifespan regulation, and orthologs of DAF genes were found to serve similar roles in the regulation of aging in higher organisms (Kenyon, 2010).

More than 30 years ago, Golden and Riddle showed that then unknown small molecule signals (the “dauer pheromone”) that are constitutively excreted by the worms control dauer entry and exit (Golden and Riddle, 1982, 1984). The identification of a mutation, named *daf-22*, that did not produce the dauer pheromone, confirmed that this signal must be worm-derived (Golden and Riddle, 1985). Based on solubility and other physical properties of the dauer-inducing signal, Golden and Riddle suggested that the dauer pheromone may consist of a mixture of short-chain hydroxylated lipid derivatives (Golden and Riddle, 1982, 1984); however, the precise chemical identity of this small-molecule signal remained undetermined for almost 25 years. Finally, in 2005 Jeong et al. described the isolation and identification of “daumone” (also “*ascr#1*”, or “*C7*”, see [www.smid-db.org](http://www.smid-db.org) for ascaroside nomenclature), a glycoside of the dideoxysugar ascarylose featuring a seven-carbon carboxylic acid side chain, as one component of the dauer pheromone (Figure 2) (Jeong et al., 2005). The dideoxysugar ascarylose and derived glycosides (“ascarosides”) had originally been identified several decades earlier from parasitic nematodes of the genus *Ascaris*. The ascarosides obtained from *Ascaris* feature very long aliphatic side chains (predominantly 29 and more carbons in length) and were shown to form a protective coating of *Ascaris* eggs, providing remarkable resilience to harsh environmental conditions (Jezyk and Fairbairn, 1967). More recently, additional variants of such lipophilic ascarosides have been described from *Ascaris suum* (Bartley et al., 1996); however, none of these lipid-like ascarosides are believed to have signaling functions.

More detailed investigations of the biological properties of the ascaroside *ascr#1* revealed that it accounted only for a small portion of the dauer-promoting activity of *C. elegans* metabolite extracts (Butcher et al., 2007; Gallo and Riddle, 2009), suggesting that the dauer

pheromone must include additional components. Subsequent analyses of the *C. elegans* *exo*-metabolome (the entirety of all excreted or secreted metabolites) then demonstrated that the dauer pheromone in fact consists of a mixture of several different ascarosides, including ascr#2, ascr#3, and ascr#5, which are much more potent dauer inducers than the originally identified ascr#1 (Figure 2) (Butcher et al., 2007; Butcher et al., 2008). These dauer-inducing ascarosides are produced constitutively by developing and adult worms and thus function as a population density signal, similar to N-acyl homoserine lactones in bacterial quorum sensing (Dickschat, 2010; Miller and Bassler, 2001).

## Comparative metabolomics reveals modular ascaroside derivatives

Following the identification of ascarosides as components of the *C. elegans* dauer pheromone, it soon became apparent that ascarosides control many other aspects of *C. elegans*' life history, including a variety of social behaviors as well as adult lifespan (Ludewig and Schroeder, 2013). It was shown that ascr#2 and ascr#3 also function as a male-attracting sex pheromone, and that ascr#3 and ascr#5 promote dispersal of hermaphrodites (*C. elegans* forms primarily self-fertile hermaphrodites, as well as small numbers of males). In addition, ascr#2 and ascr#3 were shown to increase adult lifespan in *C. elegans* (Ludewig et al., 2013). However, synthetic samples of the initially identified ascarosides ascr#1-ascr#4 consistently failed to reproduce the full potency of complete, unfractionated *C. elegans* *exo*-metabolome extracts, suggesting that the chemical characterization of *C. elegans* pheromone signals, which had been based on activity-guided fractionation, remained incomplete. The observation that different components of the sex pheromone synergize with each other (Srinivasan et al., 2008) suggested a possible explanation for this loss of activity: chromatographic separation of a synergistic mixture must lead to partial loss of activity, increasing chances that some components of a signal are missed.

The limitations of activity-guided fractionation-based approaches prompted the introduction of comparative metabolomics as a tool for the identification of small-molecule signals, which ultimately revealed a much greater diversity of nematode-produced small molecules. In a first example, Pungaliya *et al.* used 2D NMR-based comparative metabolomics (Differential Analysis by 2D NMR Spectroscopy, DANS) to identify missing components of the dauer and sex pheromone blends. Earlier work had shown that *daf-22* mutant worms do not produce these pheromone signals (Golden and Riddle, 1985; Simon and Sternberg, 2002), and therefore comparison of 2D NMR spectra of the *exo*-metabolomes of wildtype worms with that of *daf-22* mutant worms should reveal NMR signatures (groups of crosspeaks) that represent the sought-after signaling molecules. 2D NMR-based comparison of the *daf-22* mutant- and wildtype-metabolomes revealed a large number of additional ascaroside derivatives in the wildtype-metabolome that were absent in the metabolome of the *daf-22* mutant, suggesting that they may represent additional pheromone signals (Figure 3). Notably, the identified compounds included the first examples for *modular* ascaroside derivatives, NDMMs, in which the ascarylose sugar or side chain is linked to an additional moiety. The first NDMM, ascr#8, includes a *p*-aminobenzoic acid moiety, which is uncommon among known primary and secondary metabolites as a building block, except for its role in folic acid (vitamin B<sub>9</sub>) metabolism. ascr#8 synergizes strongly with the previously

identified components of the dauer and sex pheromones, validating the use of comparative metabolomic approaches to complement activity-guided fractionation.

In addition, DANS-based comparison of the *daf-22* and wildtype *exo*-metabolomes revealed another group of NDMMs, bearing an indole carboxy moiety in position 4 of the ascarylose ring. Although some indole ascarosides were found to contribute to dauer formation (Butcher et al., 2009a), it was initially unclear whether the addition of an indole carboxy moiety to the ascaroside scaffold was associated with any specific signaling function. Screening indole ascarosides in a wide array of behavioral assays, it was eventually shown that indole ascarosides function as potent aggregation pheromones. The two most abundant indole ascarosides, icas#3 and icas#9, were shown to attract *C. elegans* hermaphrodites at concentrations as low as 100 femtomolar (Srinivasan et al., 2012). Notably, the existence of an aggregation pheromone in *C. elegans* had not previously been suspected, and thus this discovery was a direct consequence of chemical analysis of the *C. elegans* metabolome.

### Targeted mass spectrometric analyses reveal additional chemical diversity

Although ascarosides represent only a relatively small fraction of the *C. elegans* metabolome, their important biological roles motivated efforts to identify additional ascarosides, including compounds that are produced in a sex-specific or life stage-specific manner. Serendipitously, it was found that mass-spectrometric fragmentation of ascarosides characteristically produced a fragment  $m/z$  73, corresponding to  $C_3H_5O_2$  (Figure 4) (von Reuss et al., 2012). Screening the entire *C. elegans* *exo*-metabolome for compounds that fragment in this manner, two additional 4-substituted ascarosides were identified, including tiglyl- (mbas#3, Figure 4) and *p*-hydroxybenzoyl (hbas#3, Figure 4) substituted derivatives, which appear to be part of the *C. elegans* aggregation pheromone. The most potent of all so far identified NDMMs, hbas#3 is active at low femtomolar concentrations. To put this into perspective, at this concentration a given worm likely senses no more than one hbas#3 molecule every few minutes (von Reuss et al., 2012). Using the MS/MS screen for the analysis of the *exo*-metabolomes of different larval stages led to the identification of osas#9 (Figure 4), an ascaroside connected to a succinyl octopamine moiety, which is specifically produced during the first larval stage and acts as a dispersal signal under starvation conditions (Artyukhin et al., 2013). Similar to dopamine in higher animals, octopamine in nematodes serves as a neurotransmitter mediating starvation responses, and thus the chemical structure of osas#9 appears to integrate ascaroside and neurotransmitter signaling.

### Multi-modular ascarosides from *Pristionchus pacificus*

The discovery of ascaroside-based signaling molecules as central mediators of *C. elegans* development and behavior suggested that other nematode species may produce similar types of signaling molecules. Given that the *C. elegans* ascarosides are derived from modular assembly of building blocks of conserved primary metabolism, it seemed possible that animal species from other phyla also may employ similar strategies for the biosynthesis of small molecule signals. Therefore, metabolomic analysis of the satellite model *P. pacificus*, a nematode species that diverged from *C. elegans* more than 200 million years ago, has been of particular significance.

As in *C. elegans*, harsh environmental conditions, for example food shortage, trigger developmental arrest as highly stress-resistant dauer larvae (Weller et al., 2010). *P. pacificus* further exhibits a unique dimorphism in mouth development, representing an example for phenotypic plasticity in an adult metazoan (Ogawa et al., 2011). Previous studies had suggested that both dauer formation and mouth dimorphism are regulated by specific small-molecule signals that target evolutionarily conserved downstream pathways, including the nuclear hormone receptor DAF-12, a homolog of mammalian vitamin D and liver-X receptors (Bento et al., 2010; Ogawa et al., 2011; Sommer and Ogawa, 2011).

Detailed 2D NMR-spectroscopic analyses of the *P. pacificus* *exo*-metabolome revealed that dauer induction and mouth-form dimorphism in *P. pacificus* are controlled by a series of structurally unusual small molecules (Figure 5) (Bose et al., 2014; Bose et al., 2012). These NDMMs are based on glycosides of two different deoxy sugars, the ascarosides and paratosides, which are combined with building blocks derived from fatty acid, carbohydrate, amino acid, and nucleoside metabolism. Modular assembly of building blocks from these pathways generates diverse molecular architectures, which are further distinguished from any previously known animal metabolites by several unprecedented structural features, including the sugar L-paratose (e.g. in part#9), dimeric ascarosides such as dasc#1, the 3'-ureido isobutyrate moieties in ubas#1 and ubas#2, and, notably, a xylopyranose-based adenosine as part of npar#1. This nucleoside is likely derived from canonical (ribo)-threonylcarbamoyl adenosine (t6A), a highly conserved nucleoside found directly adjacent to the anticodon triplet of a subset of tRNAs (Deutsch et al., 2012). t6A plays an important role for maintaining translational fidelity; however, it usually accounts for only a very small fraction of tRNA, and the production of large quantities of a xylopyranose derivative in *P. pacificus* is surprising. Similarly, the 3'-ureido isobutyrate sidechains in ubas#1 and ubas#2 likely originate from thymine metabolism (Bose et al., 2012). Different combinations of these NDMM's regulate dauer formation and mouth-form dimorphism in different *P. pacificus* wildtype strains. For example, in some wildtype strains, the nucleoside derivative npar#1 regulates dauer formation, whereas in other strains the *N*-succinyl 1-phenylethanolamide derivative pasc#9 serves this function (Bose et al., 2014). Similarly, the quantitative profiles of NDMMs vary significantly between different strains, suggesting that both NDMM biosynthesis and receptors evolve rapidly in nematodes (Bose et al., 2014).

## NDMM functions and biosyntheses are highly selective

Extensive bioassays with *C. elegans* and *P. pacificus* using synthetic samples of the identified NDMMs have shown that compounds that differ in the presence of a single structural feature may have strikingly different biological effects. For example, ascr#3 strongly induces developmental arrest and attracts male *C. elegans*, whereas a two-carbon shorter variant, ascr#7, has neither activity (Hollister et al., 2013; Ludwig and Schroeder, 2013). Similarly, the indole ascaroside icas#3 functions as a potent aggregation signal, whereas ascr#3, lacking the indole carboxy moiety, acts as a dispersal signal (Srinivasan et al., 2012). In *P. pacificus*, dasc#1, a dimeric NDMM derived from the simple ascaroside ascr#1, regulates adult body shape, whereas the monomer, ascr#1, is completely inactive in this assay (Bose et al., 2012). Even the addition of a single double bond can greatly affect biological activity. For example, whereas ascr#3, the most abundant ascaroside produced by

hermaphrodites, attracts male worms and repulses other hermaphrodites, its saturated derivative, the predominantly male-produced ascr#10, is strongly attractive to hermaphrodites (Izrayelit et al., 2012).

Correspondingly, biosynthesis of the NDMMs identified from *C. elegans* and *P. pacificus* proceeds with high selectivity. For example, even though ascarosides with a 7-carbon sidechain (e.g. ascr#1) are much more abundant than 5-carbon sidechain ascarosides (e.g. ascr#9) in *P. pacificus*, only the 5-carbon variant is further decorated with a 3'-ureido isobutyrate substituent: there are no 7-carbon sidechain homologs of ubas#1 or ubas#2. Even more strikingly, an  $\omega$ -oxygenated 5-carbon sidechain ascaroside is selectively attached to the 2-position in ubas#1, whereas all other identified compounds feature ( $\omega$ -1)-oxygenated sidechains (Bose et al., 2012).

A similarly high level of selectivity is observed for some of the modular *C. elegans* compounds. For example, the likely folate-derived *p*-aminobenzoic acid moiety in ascr#8 is selectively attached to an unsaturated 7 carbon sidechain, although ascarosides based on saturated 7-carbon and unsaturated 9-carbon sidechain are much more abundant (Pungaliya et al., 2009). Similarly, the indole ascarosides icas#9 (5-carbon sidechain) and icas#3 (9-carbon sidechain) are about equally abundant, although the unmodified 5-carbon ascaroside ascr#9 is orders of magnitude less abundant than the 9-carbon variant ascr#3 (Srinivasan et al., 2012).

Lastly, comparison of NDMM profiles in the *exo*-metabolomes of *C. elegans* and *P. pacificus* with those found in the *endo*-metabolome (the entirety of extractable metabolites in the worm bodies) revealed that NDMM excretion is highly selective. For example, *P. pacificus* worm bodies retain large amounts of the simple, unmodified ascarosides ascr#1 and ascr#9, whereas the paratosides part#9 and npar#1 are primarily excreted (Bose et al., 2014; Bose et al., 2012).

## NDMM biosynthesis

The selective combination of building blocks and chain lengths in the NDMMs from *P. pacificus* and *C. elegans*, along with their structure-specific biological functions, suggests that NDMMs are the products of carefully controlled biosynthetic pathways. Considering the structures of their diverse building blocks, it seems likely that these are derived more or less directly from conserved primary metabolism. For example, the incorporation of hydroxybenzoyl and indole carboxyl moieties in hbas#3 and icas#3 is suggestive of the involvement of amino acid metabolism, which was confirmed for the case of icas#3 by feeding *C. elegans* with tryptophan-*d*<sub>5</sub> (Srinivasan et al., 2012). However, the involvement of conserved primary metabolic enzymes has been confirmed only for the biosynthesis of the lipid-derived sidechains, which were shown to be derived from conserved peroxisomal  $\beta$ -oxidation in *C. elegans* (Figure 6) (Butcher et al., 2009b; Joo et al., 2010; von Reuss et al., 2012). Ascaroside-targeted mass spectrometric analysis of null-mutants of the acyl-CoA oxidase ACOX-1, the enoyl-CoA hydratase MAOC-1, and the 3-hydroxyacyl-CoA dehydrogenase DHS-28 revealed almost complete abolishment of the biosynthesis of ascaroside-based signaling molecules as well as accumulation of long-chain ascarosides as

shunt metabolites, consistent with enzymatic functions as predicted by homology (Butcher et al., 2009b; von Reuss et al., 2012). Targeted MS analysis of null-mutants of the predicted 3-ketoacyl-CoA thiolase DAF-22, catalyzing the last step in the 4-step peroxisomal  $\beta$ -oxidation cycle, also indicated abolishment of the biosynthesis of short-chain ascarosides; however, the MS-based analysis did not uncover any shunt metabolites that would directly support the proposed enzymatic function. Ultimately, *daf-22*-dependent shunt metabolites were identified using untargeted NMR-based metabolomics via multivariate DANS (mvaDANS), a program that partially automates comparative 2D NMR spectroscopic analysis (Izrayelit et al., 2013). Using mvaDANS, it was shown that *daf-22* mutant worms accumulate large quantities of long-chain  $\beta$ -keto ethanolamides (**4** and **5**) and methylketones (**6**, Figure 6C) The accumulation of these long-chain ascarosides in *daf-22(0)* worms confirmed that *daf-22* serves as a thiolase in ascaroside biosynthesis, as had previously been proposed based on homology (Butcher et al., 2009b; Pungaliya et al., 2009). Furthermore, this study demonstrated that accumulation of ascaroside ethanolamides in response to inhibited peroxisomal  $\beta$ -oxidation dramatically reduces endocannabinoid levels, revealing an unexpected connection between fatty acid peroxisomal  $\beta$ -oxidation and endocannabinoid signaling (Izrayelit et al., 2013).

Although these results clearly demonstrate that NDMM biosynthesis and primary metabolism are deeply intertwined, the biosynthetic origin of most building blocks of the *C. elegans* and *P. pacificus* NDMMs has not been determined. Similarly, the enzymes that create complex architectures by combining different modules via ester and amide bonds in a highly selective manner remain unknown. Growing *C. elegans* and *P. pacificus* for several generations axenically, i.e. under sterile conditions using a bacteria-free nutrient solution, demonstrated that bacteria are not required for the biosynthesis any of the identified NDMMs (Bose et al., 2012; Srinivasan et al., 2012). Characterization of this biosynthetic machinery may reveal how input from conserved primary metabolism is transduced to create small-molecule signals that regulate aging, development, and behavior in nematode model organisms. If NDMM biosynthesis turns out to rely mostly on conserved enzymes, their identification may have additional implications for the metabolism of other animal groups.

## Downstream pathways and receptors

The NDMM-mediated lifespan and developmental phenotypes have been shown to depend on evolutionarily conserved downstream signaling pathways, including sirtuin and insulin signaling (Ludewig et al., 2013; Ludewig and Schroeder, 2013; Sommer and Ogawa, 2011). As endogenous small molecules, the NDMMs thus provide exciting new opportunities to study the role of conserved signaling cascades in aging and development. To take full advantage of these possibilities will require detailed knowledge of the chemosensory mechanisms that underlie NDMM perception, which given the NDMMs' diverse biological activities, may involve a large diversity of receptor proteins. Notably, the *C. elegans* genome encodes more than 1000 G-protein coupled receptors (GPCRs), of which many are expressed in chemosensory neurons that have been shown to be required for NDMM-mediated phenotypes (Bargmann, 2006; Ludewig and Schroeder, 2013). Several GPCRs have recently been described as receptors of the main components of the *C. elegans* sex and dauer pheromones (Kim et al., 2009; McGrath et al., 2011); however, direct binding to a



GPCR has been demonstrated for only one compound, asc#2 (Park et al., 2012). Moreover, little is known about the mechanisms that underlie the observed structure-activity relationships or the synergistic activity of many compounds.

## Ascaroside production is widely conserved among nematodes

Recent analyses of a larger selection of parasitic and free-living nematodes revealed the presence of ascaroside derivatives in the metabolomes of most of the analyzed species (Choe et al., 2012a; Choe et al., 2012b; Noguez et al., 2012). For example, the ethanolamide easc#18 was shown to regulate development of the insect parasitic *Heterorhabditis bacteriophora* and the side chain-dihydroxylated dhas#18 serves as a mating signal in the sour paste nematode *Panagrellus redivivus* (Figure 7). The use of ascarosides and the related paratosides, neither of which have been found in other animals, as scaffolds for signaling molecules in nematodes may be the result of a peculiarity of nematode lipid metabolism: long-chain ascarosides (chain length 29 carbons and more) have been shown to account for a considerable portion of total lipid content in *C. elegans* and other nematode species, e.g. *Ascaris* spp. (Bartley et al., 1996; Jezyk and Fairbairn, 1967). Therefore, short-chain ascarosides appear as endproducts of lipid metabolism, which then are combined with building blocks from amino acid catabolism and other metabolic pathways. However, the fact that all so-far characterized members of this “modular language” of nematode signaling molecules are based on glycosides of the two deoxysugars, ascarylose and paratose, may also reflect bias toward identifying additional ascaroside derivatives rather than characterizing other groups of nematode metabolites, whose potential relevance for nematode biology may seem less clear. Nonetheless, mass spectrometric and 2D NMR spectroscopic analyses of the *C. elegans* and *P. pacificus* metabolomes show that ascarosides make up for only a small fraction of nematode metabolomes and have provided evidence for the presence of a large diversity of yet uncharacterized, non ascarylose-derived compounds (Bose et al., 2012; von Reuss et al., 2012).

## Outlook and future directions

NDMMs represent a new class of natural products that transcends the dichotomy between “primary” and “secondary” metabolism. Much of the NDMMs’ chemical originality and appeal derives from the fact that they combine well-known primary metabolites into new structures, sometimes with unusual chemical modifications, demonstrating unexpected biosynthetic capabilities in animals (Figure 8).

More than one hundred ascaroside-based NDMMs have been identified over the past few years, and, not surprisingly, the characterization of their biological properties has not kept pace with this high rate of chemical discovery. Given the large number of yet entirely uncharacterized metabolites known to exist in *C. elegans* and other model organisms, one may ask whether this unexplored chemical diversity is likely to be of any significance to the biology of these organisms. The discovery of unexpected phenotypes as a result of the identification of new NDMMs, e.g. the discovery of an aggregation pheromone as a result of the identification of the indole ascarosides, or the finding that ascarosides regulate *C. elegans* lifespan via sirtuin-dependent pathways (see above), suggests that many yet to be

identified metabolites serve important signaling functions. More generally, it should be noted that, in fact, any molecule produced by a living organism – or a community of organisms – carries specific information about the state of the producer(s), simply by virtue of the molecule's biosynthetic history (Meinwald, 2011). Therefore, it is not surprising that many different types of biological small molecules have acquired signaling roles; within a single cell, as hormones or second messengers between different cells or tissues of one organisms, or as pheromones and quorum sensing signals between individuals of the same or several different species. For these small molecule signals, biosynthetic pathways have evolved to transduce specific messages most effectively, along with dedicated perception mechanisms, for example in the form of nuclear or membrane-bound receptor proteins that are finely tuned to respond to specific small molecules, often at very low concentrations.

It seems certain that many more new small molecule structures and functions will be discovered in nematodes, contributing to the value of *C. elegans* and *P. pacificus* as model systems for basic biology, but also providing a clear rationale for a re-analysis of the metabolomes of other groups of animals, including mammals.

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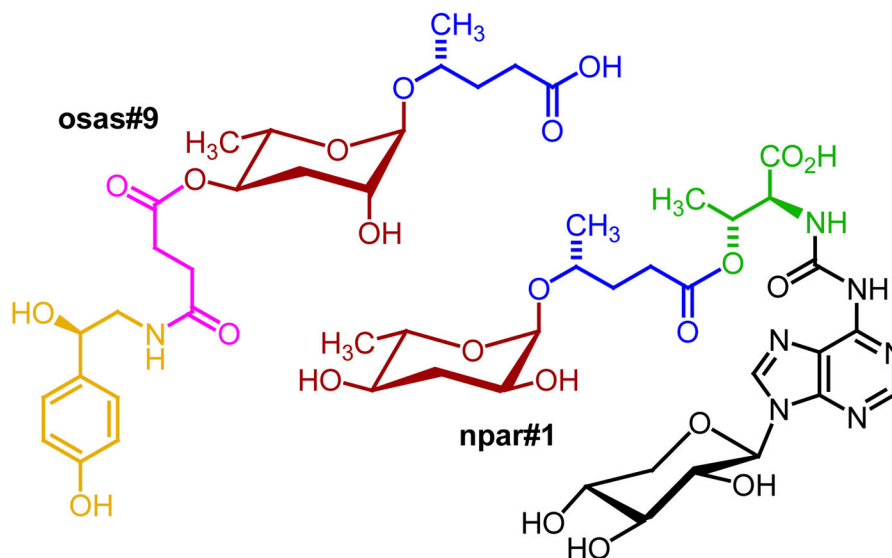
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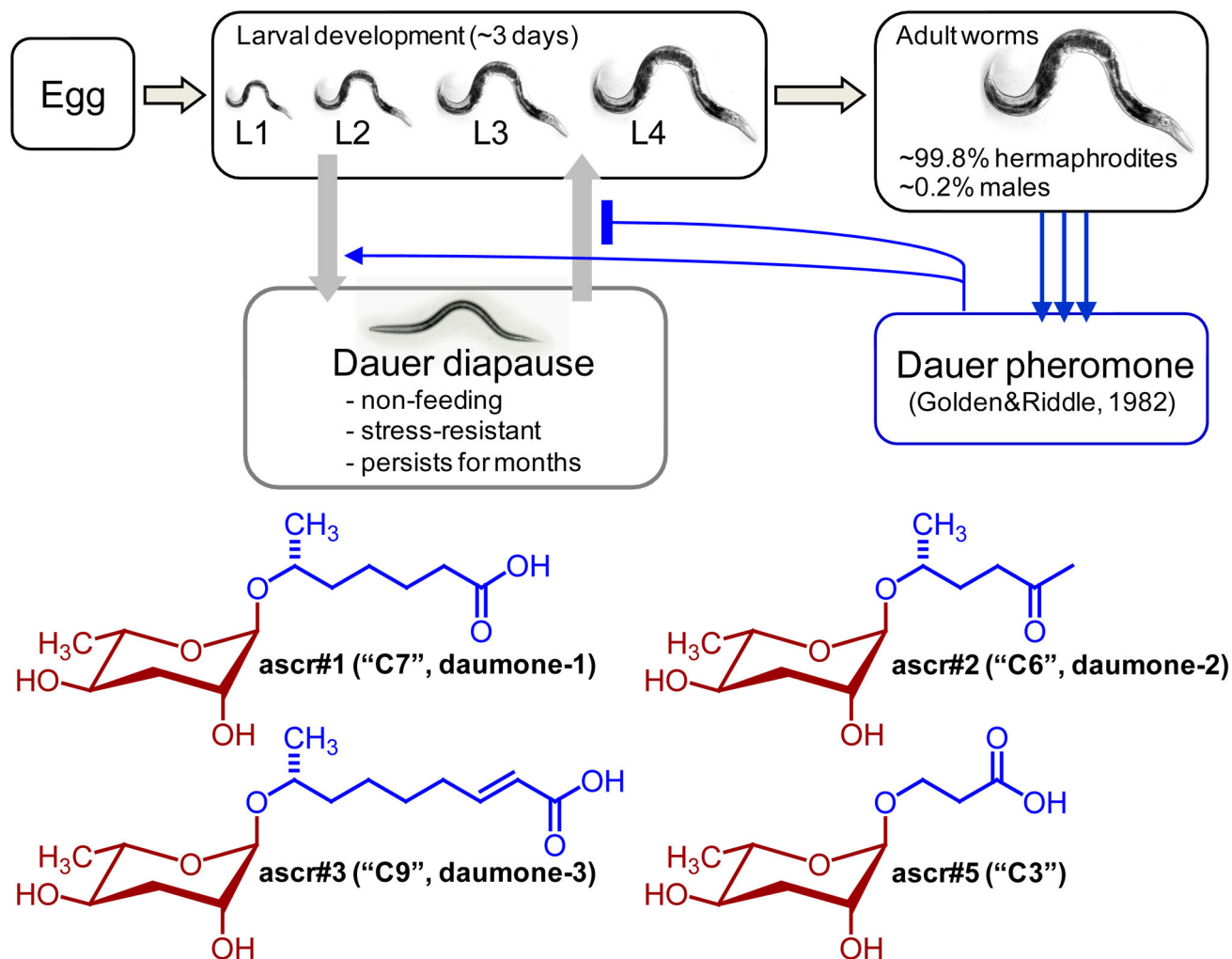
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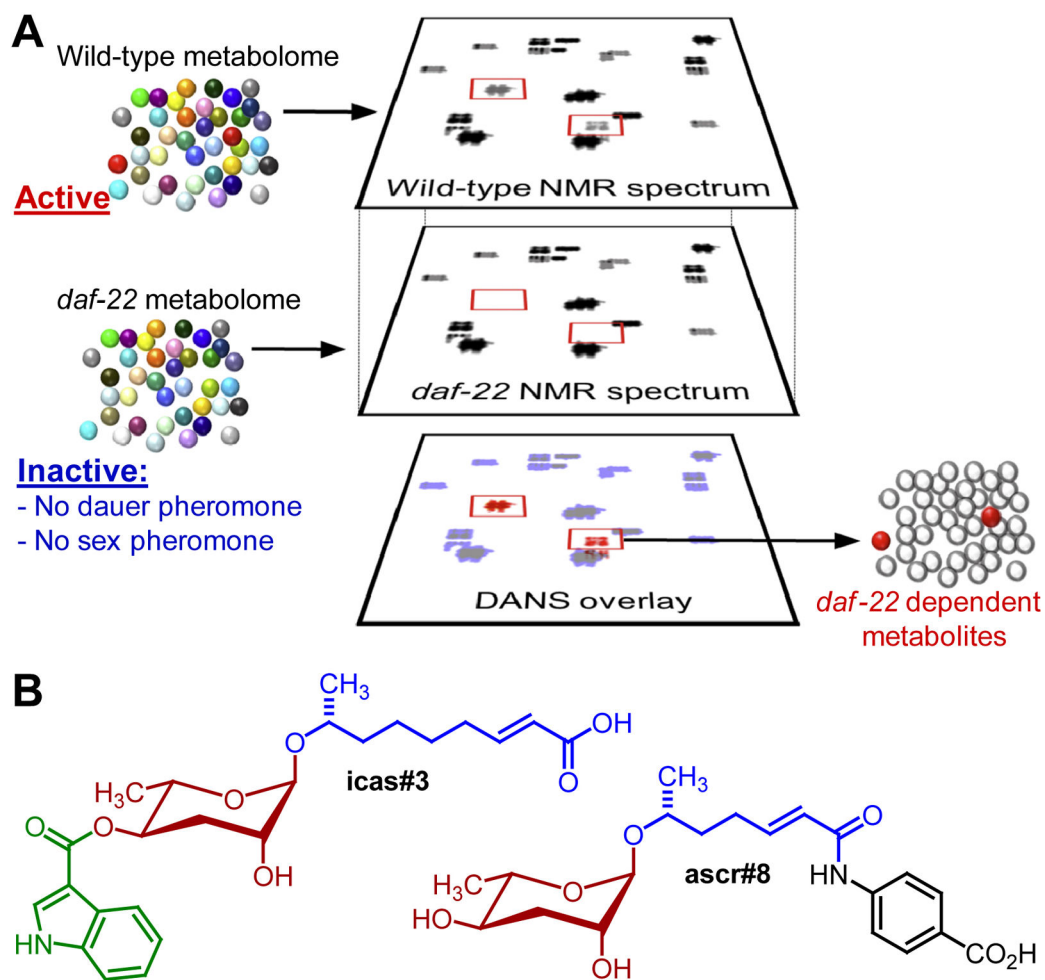
**Figure 1. Examples for nematode-derived modular metabolites (NDMMs)**

The ascaroside osas#9 from *C. elegans* L1 larvae serves as a dispersal signal (Artyukhin et al., 2013), whereas the paratoside npar#1 identified from *P. pacificus* promotes entry into a long-lived and stress resistant larval diapause (dauer larva formation) (Bose et al., 2012). Structures of the two NDMMs incorporate building blocks from lipid (blue), amino acid (green), neurotransmitter (yellow), nucleoside (black), and short-chain fatty acid (purple) metabolism. Ascarosides are named using Small Molecule Identifiers (“SMIDs”), representing searchable, gene-style identifiers that consist of four lower case non-italicized letters followed by a pound sign and a number, e.g., ascr#3, osas#9, or npar#1. See <http://www.smid-db.org/> and the WormBook chapter on ascarosides for additional information on compound naming (Ludewig and Schroeder, 2013).



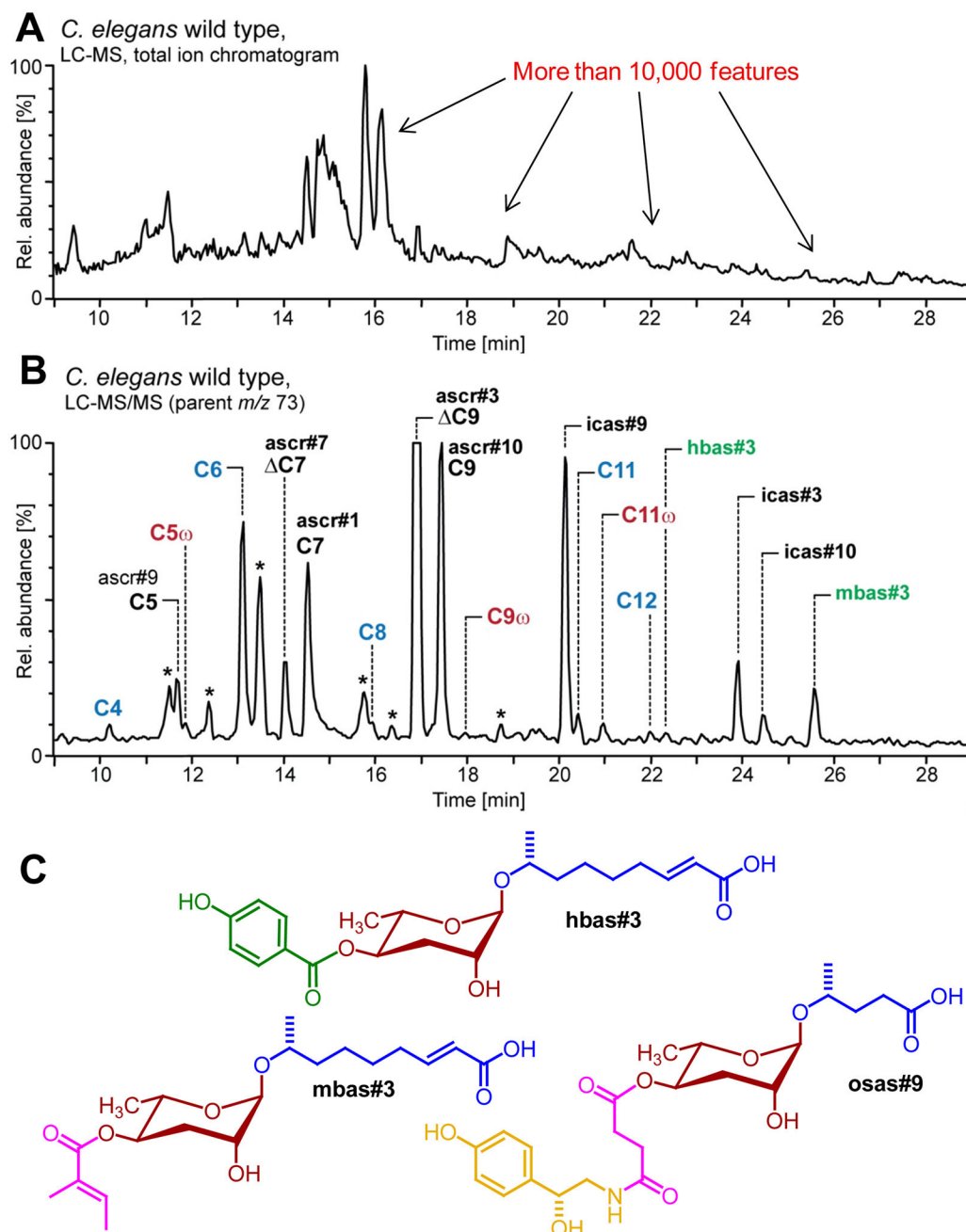
**Figure 2. *C. elegans* life cycle and the dauer pheromone**

Under favorable conditions, *C. elegans* larvae develop rapidly into reproductive adults, whereas environmental stress, in combination with the dauer pheromone, leads to formation of stress-resistant and long-lived dauer larvae. Recent work showed that the dauer pheromone, originally described in 1982 by Golden and Riddle, consists of a mixture of ascarosides, including ascr#1, ascr#2, ascr#3, and ascr#5 (Ludewig and Schroeder, 2013).



**Figure 3. Identification of multi-modular ascarioside derivatives (NDMMS) via 2D NMR-based comparative metabolomics (DANS)**

(A) Schematic representation of differential 2D NMR spectroscopy (DANS) applied to comparing *C. elegans* wildtype *exo*-metabolome with that of *daf-22* mutant worms (Pungaliya et al., 2009). (B) NDMMS detected via DANS, including lipid- (blue), amino acid- (green), and folate- (black) derived building blocks.

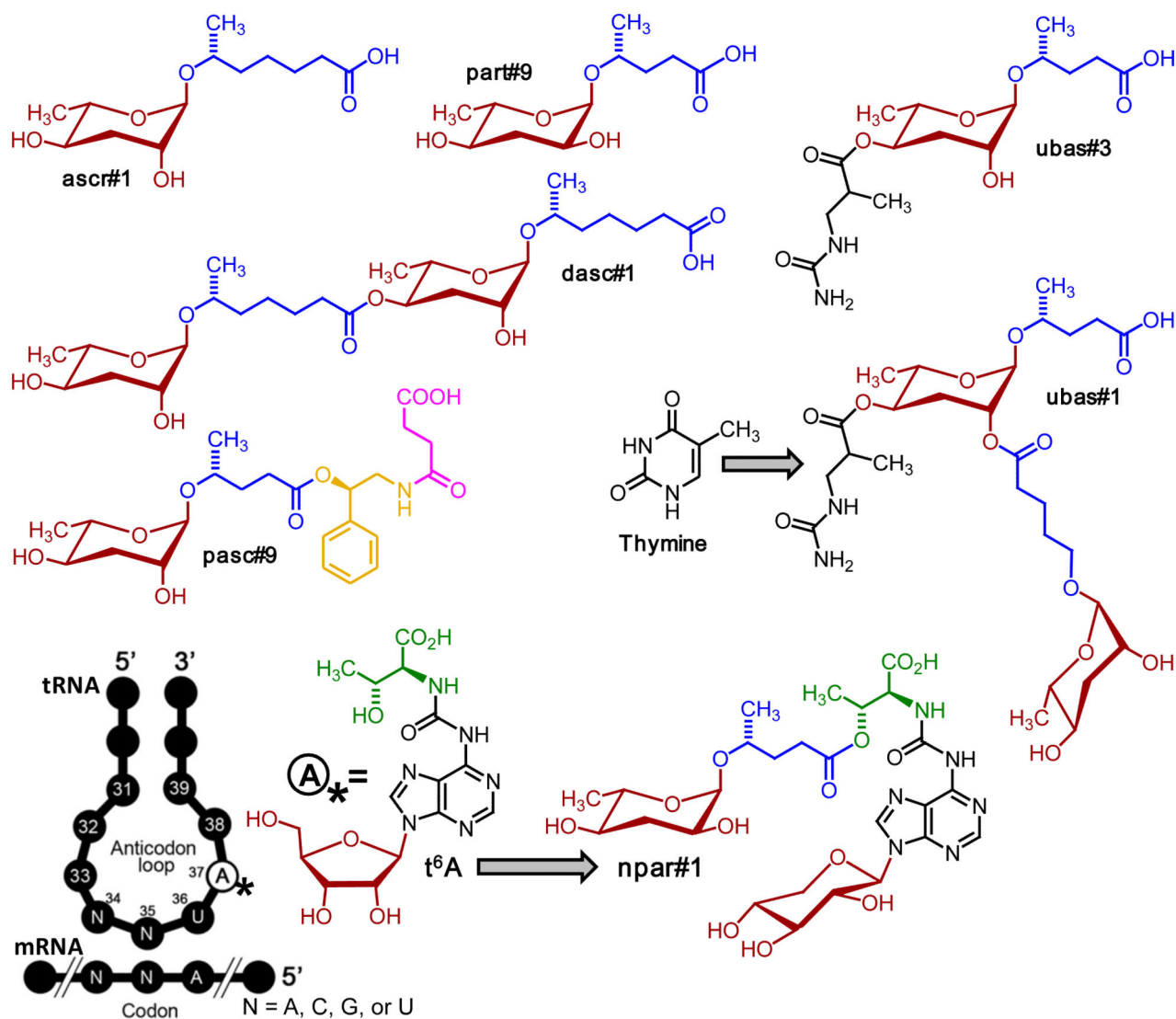


**Figure 4. Ascaroside-targeted HPLC-MS/MS**

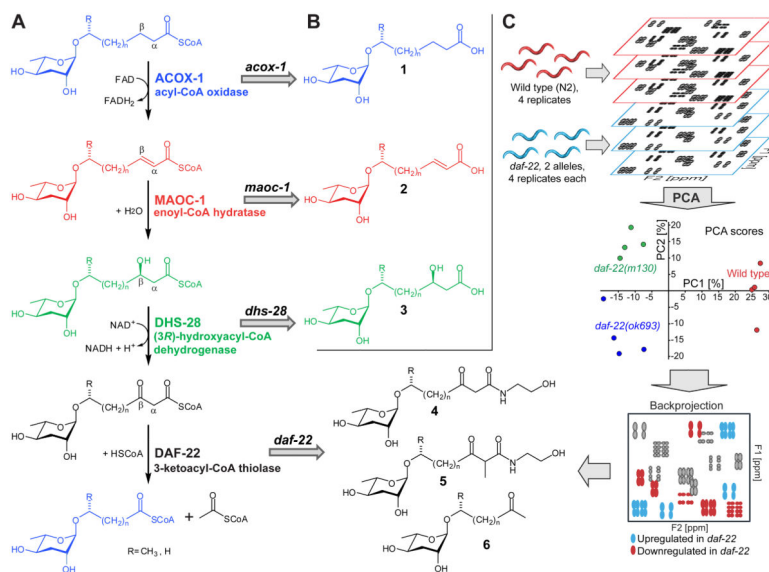
(A) Total ion current chromatogram of *C. elegans* *exo*-metabolome sample, representing more than 10,000 features, most of which belong to yet undescribed compounds (von Reuss et al., 2012). (B) Chromatogram of ascaroside targeted HPLC-MS/MS analysis (precursors of  $m/z$  73) of the same *C. elegans* *exo*-metabolome sample reveals known ascarosides (black), new homologs (blue), new ( $\omega$ )-oxygenated isomers (red) and new 4'-acylated derivatives (green) (\* non-ascarosides). (C) Structures of hbas#3, mbas#3, and osas#9, including building blocks from lipid (blue), amino acid (green), neurotransmitter (yellow),



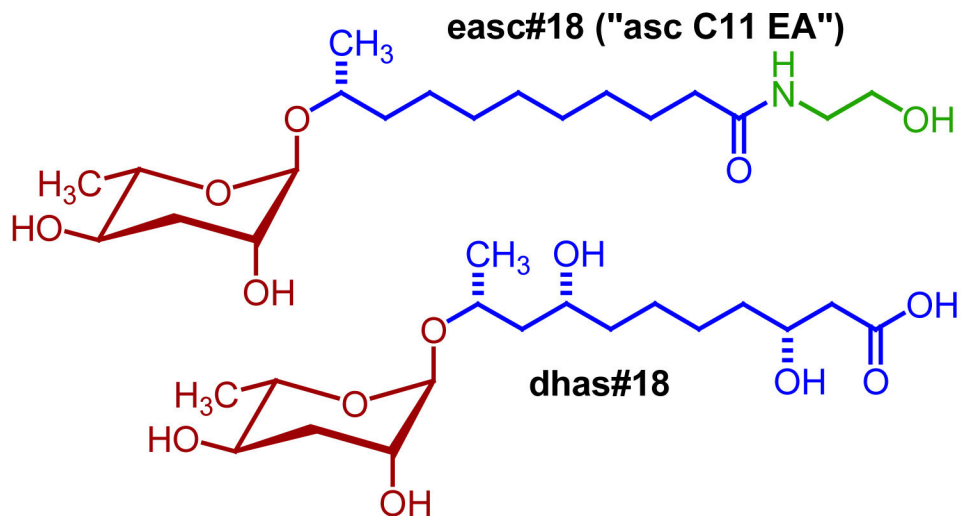
and short-chain fatty acid (purple) metabolism. For compound naming, see <http://www.smid-db.org/>.



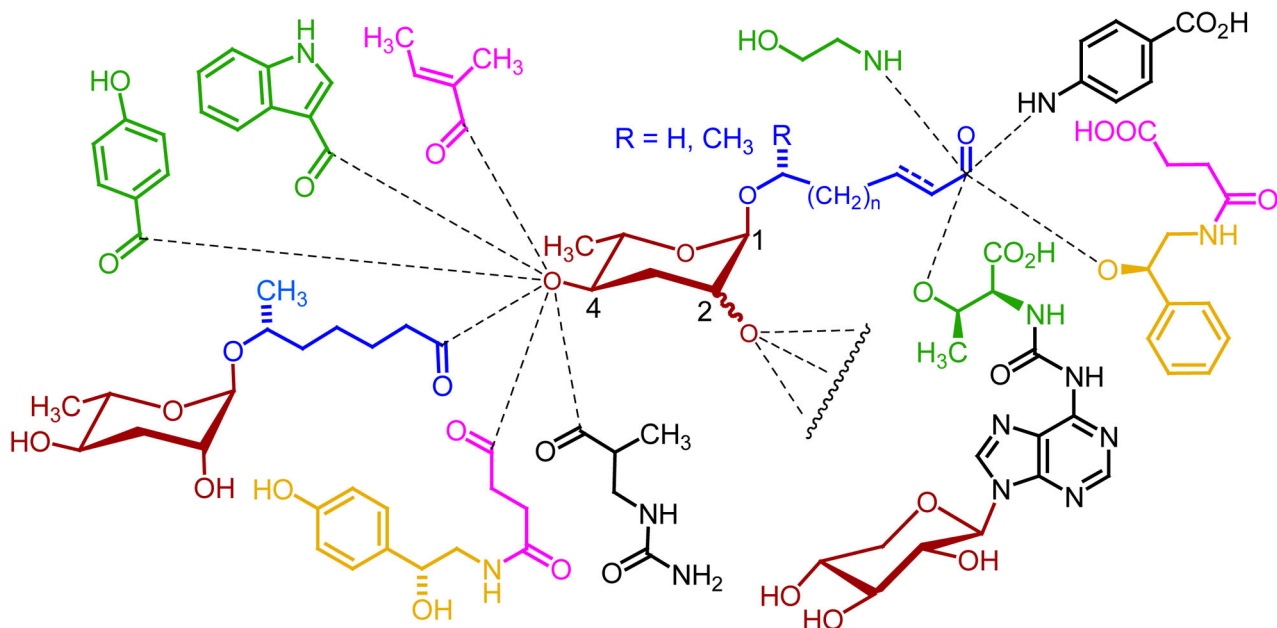
**Figure 5.** Structures of NDMMs from *P. pacificus* and putative origin of building blocks from nucleoside metabolism (Bose et al., 2012). The shown NDMMs incorporate building blocks from lipid (blue), amino acid (green), neurotransmitter (yellow), nucleoside (black), and short-chain fatty acid (purple) metabolism. For compound naming, see <http://www.smid-db.org/>.



**Figure 6. Role of conserved peroxisomal  $\beta$ -oxidation in ascarioside side-chain biosynthesis**  
 (A) Homology-based model of side-chain biosynthesis. (B) Structures of shunt metabolites **1–3** identified in *acox-1*, *maoc-1*, and *dhs-28* mutants via targeted MS-based metabolomics (Butcher et al., 2009b; von Reuss et al., 2012). (C) Use of untargeted NMR-based metabolomics (mvaDANS) for the identification of shunt metabolites **4–6** from *daf-22* mutant worms (Izrayelit et al., 2013).



**Figure 7.** Examples for ascaroside-based signaling molecules identified from the insect parasite *Heterorhabditis bacteriophora* (easc#18, (Noguez et al., 2012)) and the sour paste nematode *Panagrellus redivivus* (dhas#18, (Choe et al., 2012a)).



**Figure 8.**

NDMMs form a modular library of signaling molecules, derived from combination of building blocks from diverse primary metabolic pathways, e.g. neurotransmitter (yellow), amino acid (green), short-chain fatty acid (pink), folate and nucleoside (black), long-chain fatty acid (blue), and carbohydrate (crimson) metabolism. Acylation or glycosylation at position 2 of the ascarbose is also observed, although much less frequently than at position 4.