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Succinylated Octopamine Ascarosides and a New Pathway of Biogenic Amine Metabolism in Caenorhabditis elegans

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Background: Ascarosides play central roles regulating C. elegans behavior and development. Results: L1 larvae produce starvation-dependent ascarosides based on succinylated octopamine. Conclusion: Succinylation is an important pathway for metabolism of biogenic amines in C. elegans. Significance: Octopamine ascarosides connect neurotransmitter and ascaroside signaling in C. elegans.

The ascarosides, small-molecule signals derived from combinatorial assembly of primary metabolism-derived building blocks, play a central role in Caenorhabditis elegans biology and regulate many aspects of development and behavior in this model organism as well as in other nematodes. Using HPLC-MS/MS-based targeted metabolomics, we identified novel ascarosides incorporating a side chain derived from succinylation of the neurotransmitter octopamine. These compounds, named osas#2, osas#9, and osas#10, are produced predominantly by L1 larvae, where they serve as part of a dispersal signal, whereas these ascarosides are largely absent from the metabolomes of other life stages. Investigating the biogenesis of these octopamine-derived ascarosides, we found that succinylation represents a previously unrecognized pathway of biogenic amine metabolism. At physiological concentrations, the neurotransmitters serotonin, dopamine, and octopamine are converted to a large extent into the corresponding succinates, in addition to the previously described acetates. Chemically, bimodal deactivation of biogenic amines via acetylation and succinylation parallels posttranslational modification of proteins via acetylation and succinylation of L-lysine. Our results reveal a small-molecule connection between neurotransmitter signaling and interorganismal regulation of behavior and suggest that ascaroside biosynthesis is based in part on co-option of degradative biochemical pathways.

Small-molecule signals that incorporate glycosides of the dideoxy sugar ascaroylase (“ascarosides”) play a central role in the biology of the model organism Caenorhabditis elegans, regulating several aspects of development and behavior. Ascaroside-based signaling molecules were initially identified as components of the dauer pheromone, a population density signal that induces entry into the dauer stage, a highly stress-resistant alternate larval stage that can survive for months without food intake before resuming normal development (1, 2). Subsequent studies showed that ascarosides also regulate C. elegans lifespan (3), as well as several different behaviors, including sex-specific attraction avoidance, dispersal, and aggregation (4–10) (see Fig. 1A). The structures of the ascarosides are highly modular, integrating building blocks from amino acid, lipid, and other primary metabolic pathways (11). Ascaroside signaling is highly conserved among nematodes, including both parasitic and non-parasitic species (12–14).

The biosynthesis of nematode ascarosides is only partially understood. Although the lipid-like side chains have been shown to derive from peroxisomal β-oxidation of longer chained precursors (11, 15–17), site(s) and enzymes involved in attaching substituents to the 2- and 4-positions of the ascaroylose or the side chain have not been characterized. Here we describe the identification of a new series of modular ascarosides, in which the 4-position of the ascaroylose is connected to a side chain derived from succinylation of the neurotransmitter octopamine. These octopamine ascarosides are specifically produced by L1 larvae, which had not previously been studied in detail. The incorporation of N-succinylated octopamine in these ascarosides then led us to investigate whether succinylation plays a general role in biogenic amine metabolism in C. elegans.

**EXPERIMENTAL PROCEDURES**

C. elegans Strains and General Culture Methods—Wild-type C. elegans (N2, Bristol), MT13113 tdc-1, MT9455 tbh-1, RB859 daf-22, and VS8 dhs-28 mutant strains were obtained from the Caenorhabditis Genetics Center (CGC). Strains were maintained at 20 °C on NGM plates with bacteria (Escherichia coli OP50 or HB101) as food.

Preparation of L1 Exo-metabolome Samples—Worms were grown at 20–22 °C either on NGM plates seeded with E. coli OP50 or seeded with food in the absence of bacteria. L1 exo-metabolome samples were prepared by culturing synchronized L1 worms (3–5 days post-emergence) at 20 °C on NGM plates seeded with food, washing them into a growth medium (GM) (50% NGM broth, 50% GM500, pH 6.0), and sonicating (10 s at 100 W) for 4 cycles. The resulting supernatants were concentrated using Centricon-30 centrifugal filters (pore size 30-kDa; Millipore) and desalted using reverse-phase (RP)-C18 Sep-Pak cartridges (Waters).

This article contains supplemental Figs. S1–S11.

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HB101 or in liquid culture (S-complete medium supplemented with 3% HB101). Cultures were started with synchronized L1 larvae. Gravid worms were collected and treated with 20% bleach and 0.3 M NaOH for 6 min to yield a clean egg suspension. The eggs were washed twice with M9 buffer, resuspended in fresh M9 at a density of 30–500 eggs/μl, and incubated with shaking at 25 °C for 24 h. Hatched L1 larvae were spun down, and the supernatant (starved L1 medium) was either used directly for HPLC-MS analysis in single ion recording mode or frozen, lyophilized, extracted with methanol, and then analyzed by HPLC-MS or MS/MS. For preparation ofexo-metabolome samples from fed L1 larvae, eggs were prepared similarly but resuspended in M9 with HB101. Sample for ascaroside profiling was taken 14 h after egg preparation to assure that worms are still in L1 stage.

**HPLC-MS Analysis**—HPLC-MS and HPLC-MS/MS were performed using an Agilent 1100 series HPLC system equipped with an Agilent Eclipse XDB-C18 column (9.4 × 250 mm, 5-μm particle diameter) connected to a Quattro II spectrometer (Micromass/Waters) using a 10:1 split. For HPLC, a 0.1% acetic acid-acetonitrile solvent gradient was used at a flow rate of 3.6 ml/min, starting with an acetonitrile content of 5% for 5 min, which was increased to 100% over a period of 40 min. Exo-metabolome samples were analyzed by HPLC-electrospray ionization-MS in negative ion mode using a capillary voltage of 3.5 kV and a cone voltage of −40 V. HPLC-MS/MS screening for precursor ions of m/z = 73.0 was performed using argon as collision gas at 2.1 mtorr and 40 eV. Analysis and characterization of osas ascarosides and N-acetylated derivatives of serotonin, dopamine, and octopamine were performed in both negative and positive ionization modes with cone voltages of −40 V and −20 V for negative and +30 V for positive electrospray ionization mode. High resolution HPLC-MS was performed using a Waters nanoACQUITY UPLC system equipped with Waters ACQUITY UPLC HSS C-18 column (2.1 × 100 mm, 1.8-μm particle diameter) connected to a Xevo G2 QTRap mass spectrometer.

**Identification**—High resolution HPLC-MS analysis demonstrated a molecular formula of putative tsas#9 in the natural sample (calculated mass of molecular ion 466.2077 and ascr#5 fragment 247.1182; measured masses 466.2082 and 247.1184, respectively). To confirm the identity of tsas#9 in tbh-1 L1 medium, we compared its retention time with the retention time of synthetic tsas#9. Both have the same retention time (17.0 min under the conditions described above) and upon co-injection elute as one peak.

**Avoidance Assays**—A previously described avoidance assay was used to test the behavior of animals to osas#9 and octopamine (18). For these experiments, osas#9 was diluted into doubly distilled water, and water was used as a control. Assays were conducted on unseeded NGM plates using animals of different stages. To test animals for avoidance to osas#9, worms were washed from mixed stage plates in M9 buffer to remove bacteria. After a couple of washes, the worms were suspended in a small volume of buffer and dispensed onto unseeded NGM plates. For avoidance measurements, a drop of assay solution containing osas#9 or control was delivered near the tail of a moving animal. The drop surrounds the animal and reaches the anterior amphid sensory neurons. Avoidance of osas#9 is indicated by a backward motion away from the drop. Such a response was scored as a positive response. The avoidance index was calculated by dividing the number of positive responses to the total number of trials (18). The worms were allowed to freely move on the plate for about 20–30 min before testing. After 30 min, worms were tested for response to distilled water. For all the stages of worms tested, we observed an avoidance index of less than 0.1 for water. 50 individual animals per life stage were tested on each day using freshly prepared osas#9 concentrations. Shown data represent the mean from assays conducted on 3 or more days.

**Dauer Assay**—Dauer assays were performed in liquid as described previously with modifications (10). Eggs were suspended in 3 ml of S-complete medium in 20-ml scintillation vials at a concentration of 1 egg/μl, and E. coli (HB101) was added for a final concentration of 1.3 mg/ml. Aliquots of ascaroside solutions were added to achieve 200 nm ascr#2, 200 nm ascr#5, and 1 μM osas#9, and the worms were grown on a 180-rpm shaker at 20 °C for 6 days. Dauer formation was assessed by soaking the worms in 1% SDS for 50 min and counting surviving L4 worms after 24 h of recovery on HB101 plates.

**L1 Survival Assay**—Eggs were suspended in 6 ml of M9 buffer or osas#9 solution in M9 in 40-ml glass vials at a concentration of 0.8 egg/μl and incubated in a shaker at 210 rpm and 25 °C. Triplicate aliquots from each sample were taken every day or every other day and seeded on HB101 plates for recovery. Starvation survival was assessed by counting worms that resumed growth after recovery at 20 °C for 2–3 days.

**RESULTS AND DISCUSSION**

**L1 Larvae Produce Octopamine Ascarosides**—We started our investigations with an analysis of chemical signals produced by starved L1 larvae, which enter a distinct state of developmental arrest that confers enhanced stress resistance and extended starvation survival (19–23). To screen for ascarosides specifically produced by starved L1 worms, we used comparative HPLC-MS analysis combined with an MS/MS screen for precursor ions of the ascaroside-characteristic fragment ion at m/z 73, a technique that greatly simplifies recognition of ascaroside derivatives in complex metabolome samples (11). Comparison of ascaroside profiles in the exo-metabolomes of starved L1 larvae, adult worms, or mixed stage cultures showed remarkable differences. ascr#3, one of the most abundant ascarosides in mixed stage cultures, is produced in L1 stage only in trace amounts. Instead, HPLC-MS/MS analysis of the L1 exo-metabolome revealed a strong peak with MS/MS precursor ions at m/z 482 and m/z 247, which did not match the molecular ions of any known ascaroside and therefore suggested the presence of a yet undescribed compound (Fig. 1B). High resolution MS analyses indicated molecular formulae of C_{25}H_{32}N_{10}O_{10} for the molecular ion ([M+H]^{+}, calculated 482.2026; observed 482.2030) and C_{11}H_{19}O_{6} (calculated 247.1182; observed 247.1186), suggesting that this compound may be derived from...
the addition of a C_{12}H_{13}NO_{4} moiety to the known ascr#9, a simple ascaroside featuring a saturated five-carbon side chain with a terminal carboxyl group that has been identified in exo-metabolome samples from mixed stage cultures (11). Analysis of exo-metabolome samples from fed L1 larvae and fed mixed stage cultures revealed only trace amounts of the compound with m/z 482, whereas it is consistently produced in large amounts by starved L1 larvae. Detailed examination of HPLC-MS data from fed and starved L1 larvae revealed two additional compounds at m/z 538 and 480, whose MS fragmentation patterns were similar to the fragmentation of the compound with m/z 482 (supplemental Fig. S1), suggesting that they are chemically related. The molecular formulae of C_{27}H_{40}NO_{10} ([M-H]^{-}, calculated 538.2652; observed 538.2658) and C_{24}H_{34}NO_{9} ([M-H]^{-}, calculated 480.2234; observed 480.2243) obtained for these ions suggested that they represent ascarosides derived from the addition of a C_{12}H_{13}NO_{4} moiety to ascr#10 (featuring a 9-carbon side chain) and the keto ascaroside ascr#2, respectively.

To obtain larger quantities of the detected compounds, we fractionated large scale exo-metabolome samples by preparative HPLC (11). Fractions containing the unknown compounds were analyzed by two-dimensional NMR spectroscopy, using high resolution double quantum filtered correlation spectroscopy spectra as described previously for the analysis of complex metabolite mixtures (10, 24). NMR spectroscopic analysis revealed cross-peaks representing the (ω-1)-oxygenated side chain of an ascaroside derivative. Furthermore, cross-peaks indicative of a 4-O-acylated ascarosyl, as well as spin systems suggesting a p-hydroxy substituted phenyl group, a likely N- and O-substituted CH—CH₂ moiety, as well as signals suggestive of a succinyl group were observed (supplemental Fig. S2). Based on the chemical shifts of protons in these tentative fragments and a putative molecular formula of C_{23}H_{33}NO_{10}, we proposed a structure in which ascr#9 is connected via a succinyl moiety to the nitrogen of octopamine, a biogenic amine that serves as a neurotransmitter in C. elegans (25) (Fig. 1C). We named this structure osas#9 (octopamine succinyl ascaroside, see Small Molecule Identifier Database (SMID DB) for nomenclature), named its putative longer chained homolog osas#10, and named the ascr#2-derived member of this series osas#2 (Fig. 1C).

For confirmation of the proposed structure of osas#9 and to determine its stereochemistry, we developed a short synthesis as shown in Fig. 2A (see supplemental data for methods). Bis-succinylation of the benzyl ester 1 of ascr#9 provided intermediate 2, which was reacted with (R)-octopamine 3 (26), the naturally occurring enantiomer (27). The resulting bis-octopamine derivative 5 was then hydrogenated to remove the benzyl group and subsequently treated with lithium hydroxide, producing a mixture of osas#9 and its 2-O-substituted isomer, from which pure synthetic osas#9 was isolated via preparative HPLC. Comparison of HPLC retention times (supplemental Fig. S3), mass spectra, and NMR spectroscopic data confirmed the structure of the natural compound with m/z 482 as that of osas#9 (supplemental Fig. S4). In parallel, we repeated the synthesis shown in Fig. 2A using racemic octopamine. The mixture of diastereomers of osas#9 obtained from racemic octopamine showed distinct NMR spectroscopic signals for the methylene group of the octopamine, which enabled us to confirm R-con-
figuration of the octopamine side chain in natural osas#9 via comparison of double quantum filtered correlation spectroscopy spectra (supplemental Fig. S4).

Comparison of starved and fed L1 exo-metabolomes revealed that osas#9 is most abundantly produced by starved L1 larvae, whereas osas#10 is dominant in fed L1 cultures (Fig. 2B) and osas#2 is observed in smaller, highly variable amounts. We noticed a general shift from ascarosides with 9-carbon side chains (ascr#10, osas#10) in the presence of food to ascarosides with a shorter 5-carbon side chain in starved conditions (ascr#9, osas#9, icas#9) (Fig. 2B).

Biosynthesis of Octopamine Aascarosides—We then considered possible biogenetic origins of osas#9. Previous work demonstrated that the fatty acid-derived side chains of the ascarosides are derived from peroxisomal β-oxidation (11). Correspondingly, we found that osas#9 is not produced by null mutants of daf-22 and dhs-28, encoding the peroxisomal 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase, respectively (11) (supplemental Fig. S5). Octopamine is derived from decarboxylation of tyrosine (catalyzed by tyrosine decarboxylase), and hydroxylation of the resulting tyramine from decarboxylation of tyrosine (catalyzed by tyrosine decarboxylase) (11) (supplemental Fig. S5). Octopamine is derived from worm-produced octopamine, which do not produce tyramine or octopamine, as well as of tbc-1 mutant worms, which produce tyramine, but not octopamine. We found that both tdc-1 and tbc-1 mutant worms do not produce osas#9, whereas production of other ascarosides, e.g. ascr#9 and icas#9, is not abolished in these mutants (supplemental Fig. S6). In addition, we found that, instead of osas#9, tbc-1 mutant worms produce large amounts of a corresponding tyramine derivative, tsas#9 (supplemental Fig. S6, supplemental data for structure elucidation). We only detected trace amounts (1–2% of osas#9) of tsas#9 in starved wild-type L1 medium, suggesting that the great abundance of this compound in tbc-1 medium is a result of the roughly 20-fold increased tyramine levels in this mutant (28). These findings demonstrated that osas ascarosides are synthesized from worm-produced octopamine.

osas#9 Induces Avoidance Behavior—In most metazoans, monoamine neurotransmitters such as octopamine and serotonin interact with neuropeptides to modulate behavioral state (29). In C. elegans, octopamine down-regulates pharyngeal pumping (and thereby food intake) as well as egg laying (25, 28, 30), opposing the effects of serotonin. In addition, octopamine signaling is required for avoidance behavior in response to specific chemical stimuli (29), although octopamine itself is not known to be deterrent. Because we found that osas#9 is specifically associated with starved L1 larvae, we asked whether this compound promotes dauer entry (a starvation response) or
otherwise affected larval survival. However, synthetic osas#9 did not induce dauer or affect lifespan of starved L1 larvae (supplementary Fig. S7). Next, we tested behavioral responses of larval and adult worms to synthetic osas#9 and found that all larval stages are strongly deterred by this compound (Fig. 2D). Notably, the addition of E. coli as food overrides osas#9 deterrence. Octopamine itself did not elicit avoidance behavior (supplementary Fig. S8). We then tested whether the presence of osas#9 contributes to avoidance responses elicited by starved L1 medium. For this purpose, we compared avoidance responses to medium from starved L1 larvae with those to medium from starved tdc-1 mutant L1 larvae, which do not produce osas#9 or tsas#9. We found that although starved L1 medium from wild-type worms is strongly deterrent, the deterrence of medium from tdc-1 mutant worms is significantly weaker, especially at higher dilutions (Fig. 2E). The residual deterrence of the tdc-1 medium likely results from the presence of other ascarosides, which are produced by wild-type and tdc-1 mutant larvae in roughly equal amounts (data not shown) and have previously been shown to be deterrent at high concentrations (6, 7). Taken together, these results suggest that osas#9 is part of a dispersal signal that indicates unfavorable conditions, specifically the absence of food.

_C. elegans Succinylates Biogenic Amines—_Acetylation of the neurotransmitters octopamine, dopamine, and serotonin is generally thought of as a deactivation or degradation mechanism and has been described for insects, nematodes, and other invertebrates (31, 32), and several N-acetyl transferases with octopamine-acetylating activity have been described (33, 34). Our identification of succinylated octopamine (osas) and tyramine (tsas) ascarosides led us to ask whether succinylation, in addition to acetylation, could represent a general pathway of biogenic amine metabolism. In fact, HPLC-MS analysis of the metabolomes of worm pellets from mixed stage cultures revealed large quantities of _N_succinyl octopamine (supplementary Fig. S9), in addition to much smaller quantities of _N_acyetyl octopamine as well as the _N_acyetyl and _N_succinyl derivatives of serotonin and dopamine. To test whether worms can succinylate added biogenic amines, we incubated starved L1 larvae with dopamine and serotonin (5-hydroxytryptamine) and profiled their metabolites by HPLC-MS. These analyses revealed _N_acyetyl and _N_succinyl derivatives of both dopamine and serotonin, of which the _N_acyetyl derivatives dominated at high (millimolar) amine concentrations, whereas relatively larger amounts of _N_succinyl serotonin and dopamine were observed at lower (micromolar), more physiological concentrations (Fig. 2F, supplementary Fig. S10). Intriguingly, amounts of all osas ascarosides dropped dramatically at high concentration of added dopamine, serotonin, and most surprisingly, octopamine, whereas the concentrations of other ascarosides did not change appreciably (Fig. 2G, supplementary Fig. S11). These results suggest that _N_acyetylation and _N_succinylination are competing mechanisms of biogenic amine metabolism, and that at high (millimolar) concentrations of added biogenic amines, the succinate pool becomes exhausted, resulting in increased formation of the corresponding _N_acyetyl derivatives. Thus, succinylation is a part of biogenic amine metabolism in _C. elegans_ and perhaps other organisms, where it may serve as a deactivation mechanism or, as in the case of octopamine in _C. elegans_, lead to the creation of new signaling molecules. Notably, competing succinylation and acetylation of biogenic amines appear to parallel posttranslational modification of proteins via acetylation and succinylation of _l_-lysine (35). Use of primary metabolite-derived building blocks in the biosynthesis of more complex signaling molecules provides a direct link between metabolic status of the organism and small-molecule signaling. Competition for shared building blocks (e.g., succinate) by different biochemical reactions may result in interdependence of different pathways, creating the potential for additional layers of regulation.

The octopamine ascarosides thus connect intraorganismal neurotransmitter-based signaling pathways with interorganis-}

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## REFERENCES


REPORT: Biogenic Amine Metabolism via Succinylation

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