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Supporting Information

Biosynthesis of Modular Ascarosides in *C. elegans*

*Oishika Panda, Allison E. Akagi, Alexander B. Artyukhin, Joshua C. Judkins, Henry H. Le, Parag Mahanti, Sarah M. Cohen, Paul W. Sternberg, and Frank C. Schroeder**

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***C. elegans* Strains.** Wild type (N2, Bristol), FX04381 *dhs-13*(*tm4381*), FX06263 *C24A3.4*(*tm6263*), FX03584 *ndx-9*(*tm3584*), VC754 *ctl-2*(*ok1137*), RB2147 *acs-13*(*ok2861*), RB2452 *acs-14*(*ok3391*), GS2477 arIs37; *cup-5*(*ar465*); *dpy-20*(*e1282*), RB1080 *haf-4*(ok1042), VC32 *haf-9*(*gk23*), VC893 *atg-18* (*gk378*), GH10 *glo-1* (*zu437*), RB811 *glo-4*(*ok623*), RB662 *apb-3*(*ok429*), FX06781 *acs-7*(*tm6781*), FCS1 *daf-22(ok693)*. Some strains were obtained from the Caenorhabditis Genetics Center (CGC), USA, and the National BioResource Project (NBRP), Japan. GH10 was kindly provided by D. Gems and *daf-22(ok693)* was a gift from H. Y. Mak. See Table S1 for a list of *O*-acyltransferase mutants. FCS10 *acs-7*(*tm6781*) was obtained by outcrossing FX06781 10x against GE1710 *rol-6*(*e187*);*unc-4*(*e120*). FCS10 was used for all experiments reported for *acs-7*(*tm6781*). Worms were maintained on Nematode Growth Medium (NGM) plates seeded with *E. coli* OP50 or HB101.

Nematode Culture and Extraction. Mixed stage worms from a populated 10 cm NGM agar plate seeded with *E. coli* OP50 were washed into 25 ml of S-complete medium and fed OP50 on days 1, 3 and 5 for a 7-day culture period, while shaking at 22 °C, 220 rpm. The cultures were then centrifuged and worm pellets and supernatant frozen separately, lyophilized and extracted with 35 mL of 95% ethanol at room temperature for 12 h. The extracts were dried *in vacuo*, resuspended in 200 µL methanol and analyzed by LC/MS. All cultures were grown in at least two biological replicates.

Mass Spectrometric Analysis. High resolution LC-MS analysis was performed on a Dionex 3000 UPLC coupled with a Thermo Q Exactive high-resolution mass spectrometer as described previously.[1] Metabolites were separated using water–acetonitrile gradient on Agilent Zorbax Eclipse XDB-C18 column (150 mm x 2.1 mm, particle size 1.8 µm) maintained at 40 °C. Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in acetonitrile. A/B gradient started at 5% B for 5 min after injection and increased linearly to 100% B at 12.5 min. Most ascarosides were detected as $[M-H]^-$ ions or $[M+Cl]^-$ adducts in the negative ionization mode (spray voltage 3 kV) and confirmed based on their high-resolution masses (< 1 ppm), fragmentation spectra, and comparison of retention times with those of synthetic standards.

Low resolution LC-MS was performed using the Agilent 1100 Series HPLC system equipped with an Agilent Eclipse XDB-C18 column (250 mm x 9.4 mm, particle size 5 µm), connected to a Quattro II or Quattro Ultima mass spectrometer.[2] Solvent A: 0.1% acetic acid in water; Solvent B: 0.1% acetic acid in acetonitrile. A/B gradient started at 5% B for 5 min after injection and increased linearly to 100% B over a period of 40 min. Ascarosides were detected as $[M-H]^-$ ions in the negative ionization mode (spray voltage 3.5 kV, cone voltage -40 V) and confirmed based on comparison of retention times with those of synthetic standards.

Ascr#1 Feeding Experiment. Mixed stage *daf-22(ok693)* worms from a populated 10 cm NGM agar plate seeded with *E. coli* OP50 were washed into two flasks containing 10 ml of S-complete medium and 2% HB101. One flask additionally contained 10 µM of synthetic ascr#1. HB101 was added on days 1, 3 and 5 for a 7-day culture period, while shaking at 22 °C, 220 rpm. The medium was then collected, processed, and analyzed by high-resolution HPLC-MS as described above, revealing production of icas#1. None of the other known ascarosides were observed. These results are consistent with an earlier study in which ascr#3 was fed to *daf-22* worms and production of icas#3 was observed.[2]

Heterologous Protein Expression and Purification. The protein coding genetic sequence of *acs-7* was synthesized (Biomatik) and cloned into pET-21a(+) with primers 3′-ATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGATATTTCACGGTGAACAAC TTG-5′ and 5′-GTTAGCAGCCGGATCTCAGTGGTGGTGGTGGTGGTGCAATTTAGCC TTTTTTGCATCCA-3 ′ using ligase-free PCR cloning to construct C-terminally tagged hexa-histidine ACS-7. The expression vector was then transformed into BL21(DE3) (New England Biolabs) *E. coli* and grown in Terrific Broth (TB) supplemented with 10 mM $MgCl_2$ and selected with 100 µg/mL ampicillin. 10 mL overnight cultures were diluted into 1 L of TB in a 4 L Erlenmeyer flask and shaken at 200 RPM at 37 °C to an OD of approximately 0.75, cooled to 16 °C and further grown to an OD of roughly 1.0-1.2 and induced with 100 µM IPTG. Cultures were maintained at 16 °C at 200 RPM for an additional 18 hours before harvesting at 5000xg, 4 °C for 10 min and stored at -80 °C until purification. All further steps occur at 4 °C unless otherwise noted.

10 g of frozen pellets were resuspended in 100 mL of 50 mM sodium phosphate pH 7.6, 300 mM NaCl, 0.3 mM PMSF and sonicated. Lysed cells were spun at 20,000x*g* for 20 min and the supernatant was collected and gently stirred with 1 mL HisPur Ni-NTA Resin (Thermo Fisher Scientific) for 30 min. During incubation, 5 uL of Benzonase (EMD Millipore) was added along with 1 mM $MgCl_2$. The slurry was loaded and passed through a column and the resin was washed with 20 column volumes of fresh lysis buffer without PMSF. The protein was then eluted with 20 mL lysis buffer containing 200 mM imidazole and 10% glycerol. The elution was concentrated with an Amicon Ultra-15 30 kDa spin filter (EMD Millipore), flash frozen in liquid nitrogen and stored at -80 °C until further purification. FPLC purification was performed using a HiLoad 16/600 Superdex 200 prep grade column run on a Amersham Biosciences P-920 pump equipped with a UPC-900 detector and a Frac-950 fraction collector (GE Healthcare) with a running buffer of 50 mM sodium phosphate pH 7.6, 300 mM NaCl, and 10% glycerol. Fractions containing ACS-7 were combined and concentrated with an Amicon Ultra-15 30 kDa spin filter and flash frozen in liquid nitrogen and stored at -80 °C until further analysis.

***In vitro* assays.** All assays were performed with ACS-7 assay buffer containing: 100 mM potassium phosphate pH 7.0, 5 mM ATP, 5 mM $MgCl_2$. For representative HPLC chromatograms, assay buffer was incubated with 1.7 µM ACS-7 and 100 µM putative substrate(s) (pentanoic acid, ascr#9, ascr#9-SCoA, indole-3-carboxylic acid, *N*-succinyl octopamine, as well as combinations of indole-3-carboxylic acid or *N*-succinyl octopamine with ascr#9 or ascr#9-SCoA, as well as additional test substrates listed in Figure S4), which were added from concentrated ethanolic stock solutions so that the final ethanol concentration remained below 1%. The reaction was allowed to incubate for 2 hours before analysis by HPLC/MS. For kinetic analysis of ACS-7, various concentration of indole-3-carboxylic acid were added to 170 nM ACS-7 in ACS-7 assay buffer in 1 mL reaction volumes and timed injections were collected and analyzed by HPLC/MS. Kinetic data analysis was performed using GraphPad Prism version 6.0 for Windows.

Synthesis of the CoA-thioester of ascr#9 (ascr#9-SCoA). Synthetic ascr#9 (2 µmol),[2] tetrafluorophenol (6 µmol), DIPEA (12 µmol), EDC·HCl (4 µmol), and DMAP (0.2 µmol) were dissolved in 0.5 mL of DMF and stirred for 36 hours at room temperature under argon. The

reaction mixture was diluted with a 1:1 mixture of ethyl acetate and 0.1 M aqueous HCl (2 mL), and the organic layer was collected and then washed two additional times with 1 mL of 0.1 M HCl. The organic layer was dried and dissolved in 625 μL of a mixture of DMF and aqueous 10 mM potassium phosphate (4:1), containing the sodium salt of coenzyme A (2 μmol), and stirred for 16 hours at room temperature under argon. Subsequently, ascr#9-SCoA was isolated by reverse phase HPLC, using acetonitrile and water, both containing 0.1% acetic acid. A gradient was used starting with 1% acetonitrile for 5 minutes, followed by a linear gradient to 100% acetonitrile over 27.5 minutes. After 2.5 minutes at 100% acetonitrile, the column was re-equilibrated at 1% acetonitrile for 5 minutes. NMR spectra of the purified compound were identical to those published previously.[3]

Microscopy. For LysoTracker staining we followed a published protocol.[4] 0.5 ml of 2 μM Lysotracker Deep Red (obtained from Thermo Fisher as 1 mM stock solution in DMSO) diluted in M9 buffer was added to an NGM plate seeded with *E. coli* OP50 and incubated in the dark at 20 ºC for 24 h. Worms were then added to the plate and allowed to grow overnight in the dark. For imaging, worms were removed from the plate and transferred onto a glass slide with a thin agarose pad containing sodium azide or levamisole to immobilize worms during imaging. Microscopic analysis was performed with Leica TCS SP5 laser scanning confocal microscope. GFP was excited with 488 nm Argon laser line and the emission detector was set at 500-550 nm. LysoTracker Red stain was excited with 561 nm diode-pumped solid state laser, while the detector was set at 570-650 nm. Images were taken at 1024x1024 pixel resolution and 100-400 Hz scanning rate. Dry 40x/0.85 and oil 63x/1.4 objectives were used.

Supporting Figures

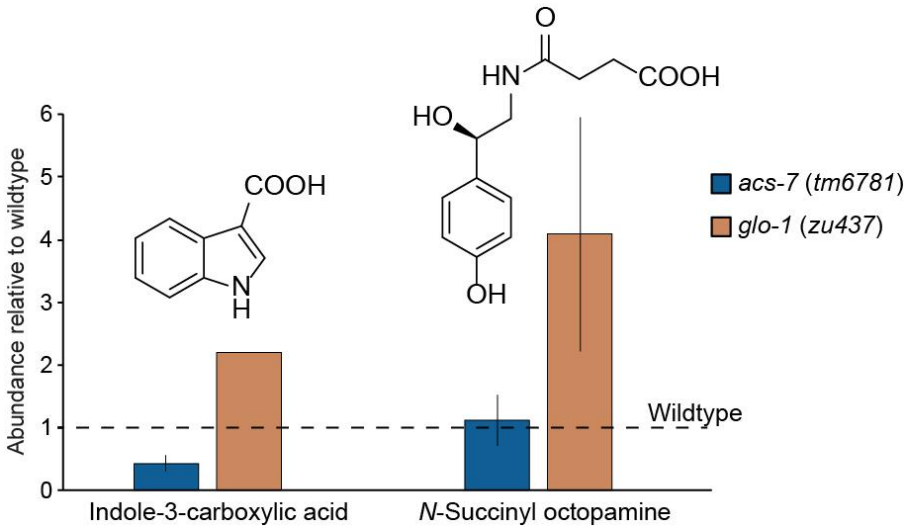

Figure S1. Relative abundances of indole-3-carboxylic acid and *N*-succinyl octopamine in *acs-7* and *glo-1* mutants, as determined by negative-ion ESI HPLC-MS. Samples were prepared from synchronized mixed stage cultures as described above (for measurement of indole-3-carboxylic acid) or L1-stage larvae that were incubated for 2.5 days (for measurement of *N*-succinyl octopamine) .[5] Error bars represent standard deviations of two biological replicates.

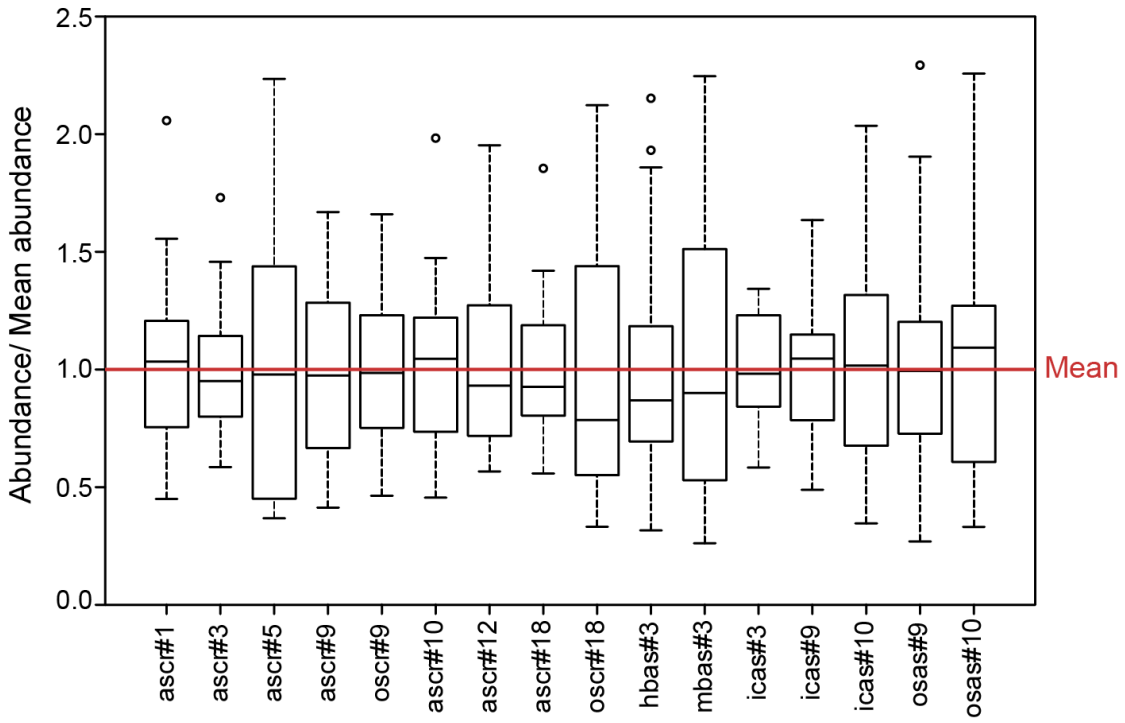

Figure S2. Box plot showing variation in ascaroside abundance in wildtype (N2) normalized to mean abundance for each ascaroside (data from 30 independent biological replicates). The horizontal line within each box indicates the median, boundaries of the box indicate 25th (Q1) and 75th (Q3) percentile, and the whiskers indicate the highest and lowest values of the results. Outliers (values less than Q1 or greater than Q3 by more than 1.5 times the interquartile range) are indicated by hollow black circles.

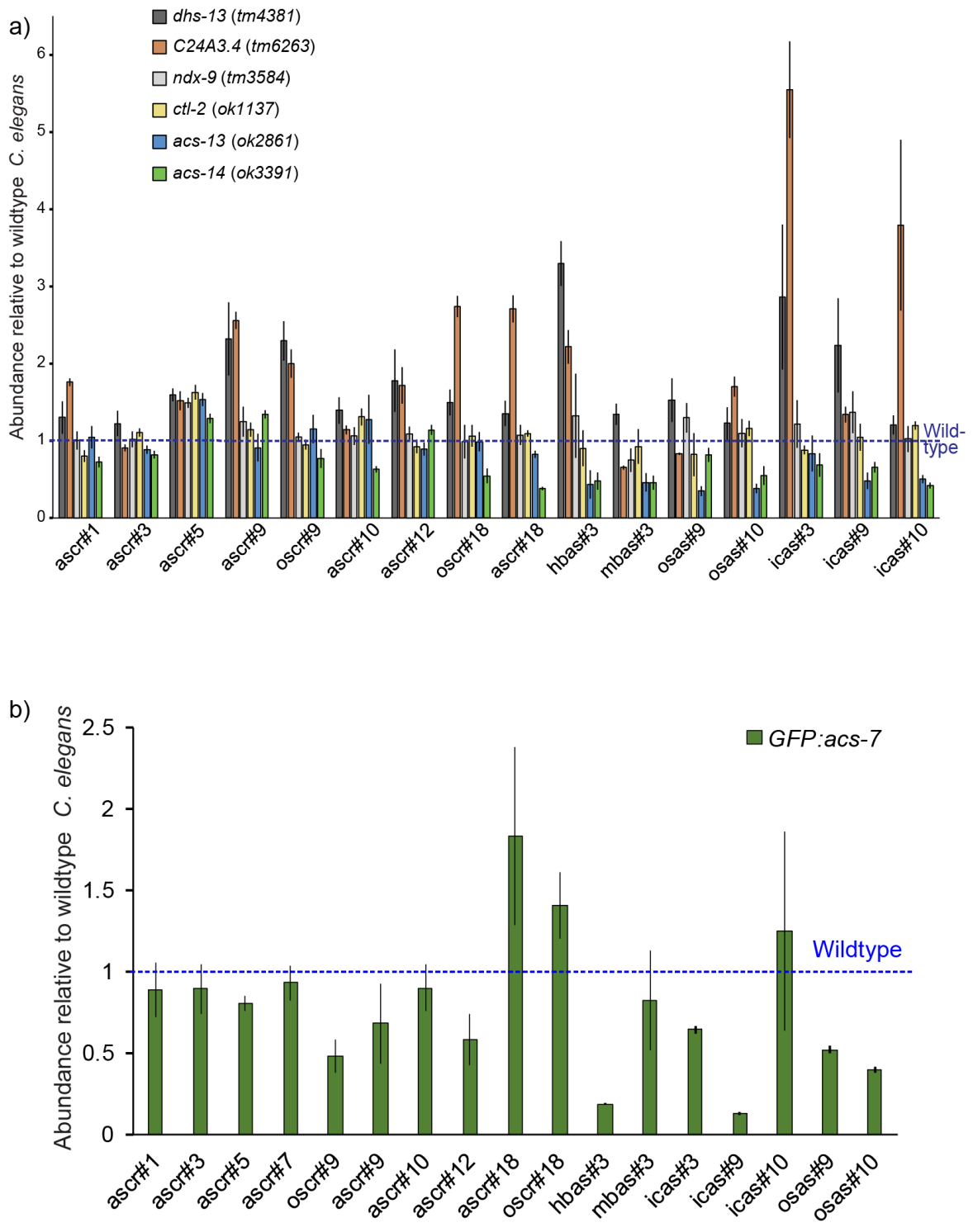

Figure S3. Relative abundances of ascarosides in knock-out mutants of putative peroxisome-targeted genes (**a**) and transgenic worms carrying *acs-7p*::*gfp*::*acs-7* in *acs-7* mutant background (**b**), as determined by negative-ion ESI HPLC-MS. Error bars represent standard error of at least three biological replicates.

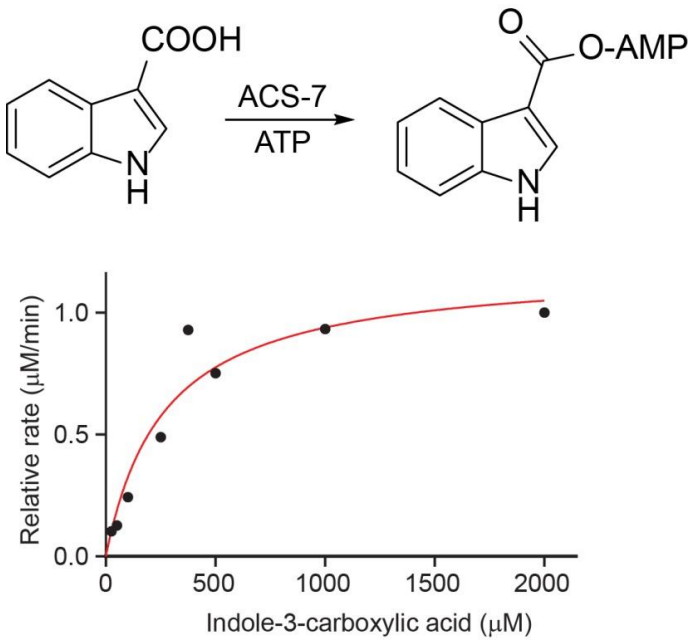

Figure S4. Steady-state kinetics for ACS-7 operating on indole-3-carboxylic acid, K_m = 270±90 μM at 25 °C.

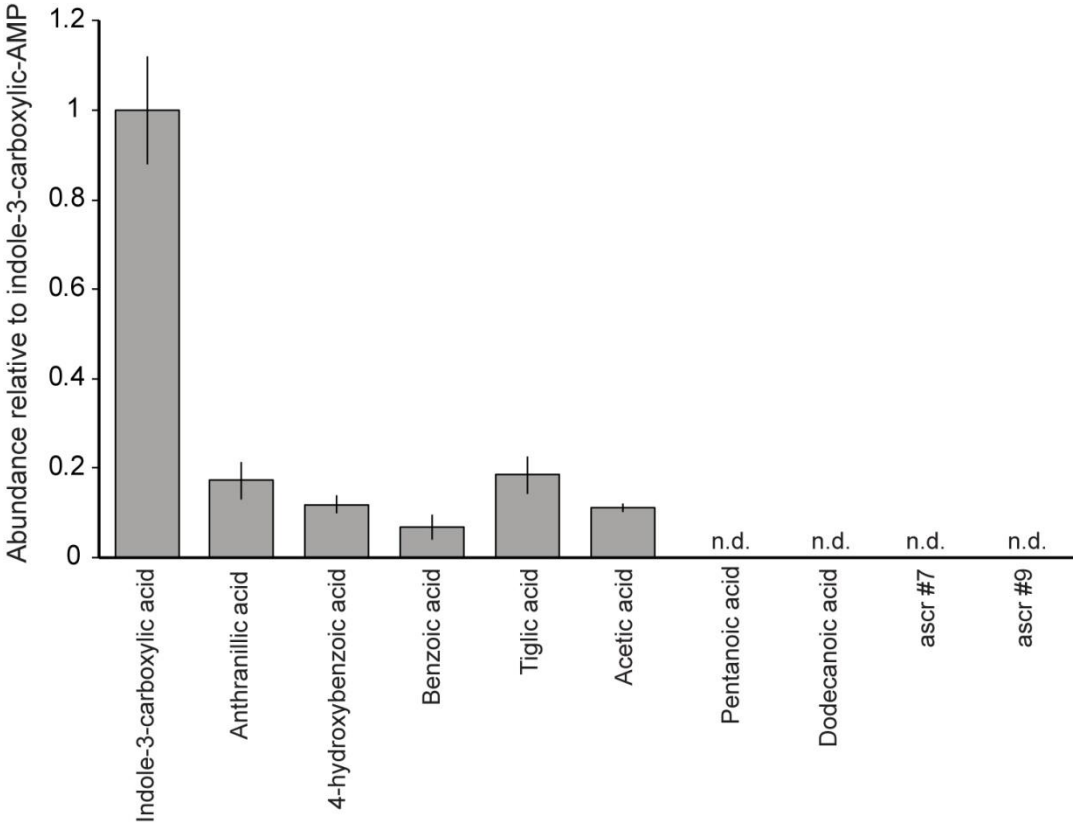

Figure S5. Carboxylic acid adenylation screen for ACS-7. Test substrates were analyzed by negative-ion ESI HPLC-MS after 45 min incubations at 25 °C (see Methods). Relative abundances were calculated from MS peak areas and do not account for differences in ionization efficiency. Error bars represent standard deviation. n.d., not detected.

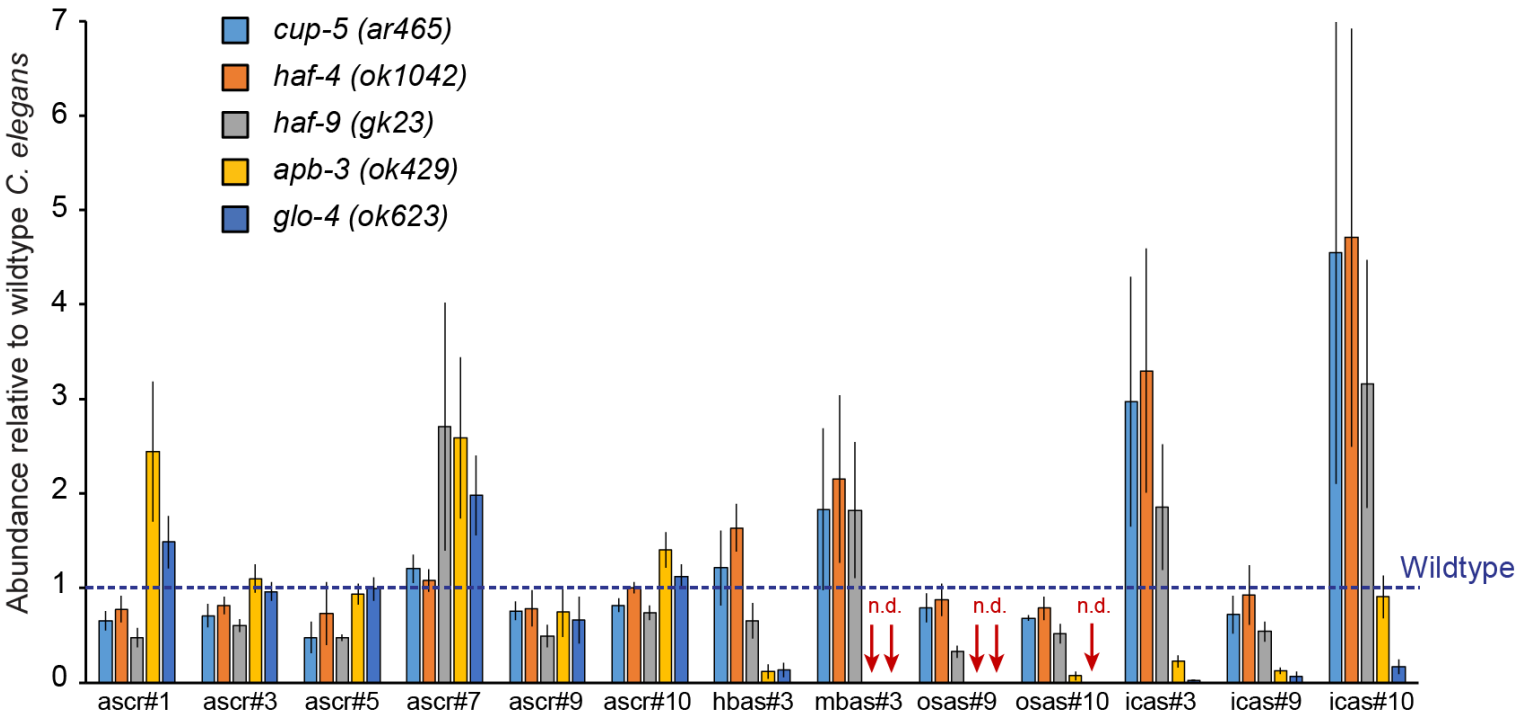

Figure S6. Relative abundances of ascarosides, as determined by negative-ion ESI HPLC-MS, in *glo-4(ok623)* and *apb-3(ok429)* mutants, in which acidic LRO formation is reduced, but not abolished, as well as *cup-5(ar465)*, *haf-4(ok1042)* and *haf-9(gk23)* mutants, which are defective in the formation of non-acidic gut granules, but have normal acidic LROs. Error bars represent standard error of four biological replicates. n.d. not detected.

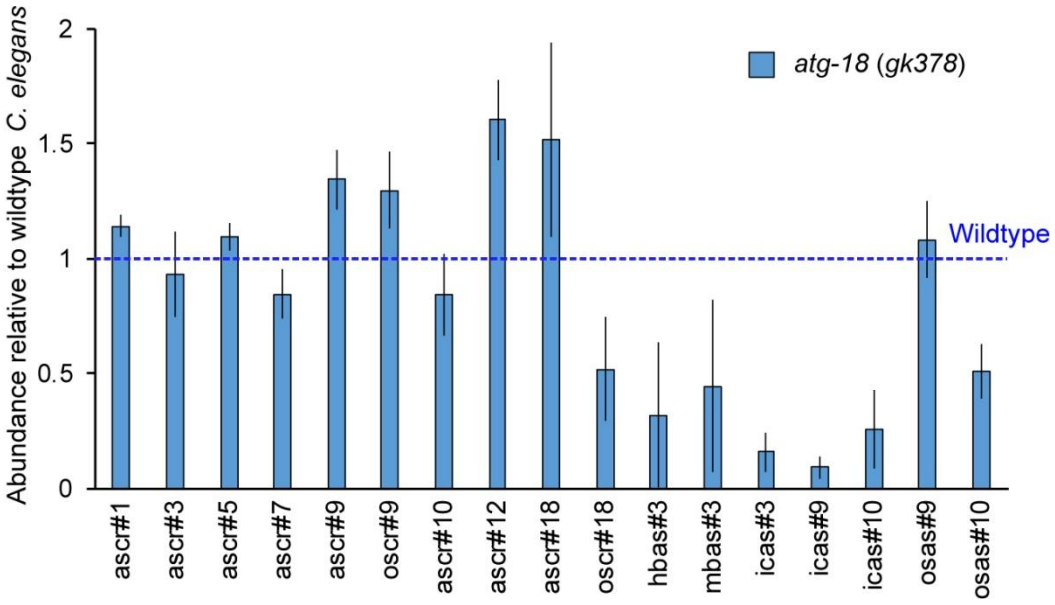

Figure S7. Relative abundances of ascarosides in autophagy-deficient *atg-18* mutants, as determined by negative-ion ESI HPLC-MS. Error bars represent standard error of three biological replicates.

Supporting Table

Table S1. List of *O*-acyltransferase mutants screened.

Gene	**Strain**	**Source**
ndg-4 (*sa529*)	JT529	Deletion mutant from CGC
nrf-6 (*sa525*)	JT525	Deletion mutant from CGC
oac-11 (*gk531381*)	VC40243	Million Mutant Project
oac-14 (*gk519224*)	VC40217	Million Mutant Project
oac-14 (*gk786954*)	VC40738	Million Mutant Project
oac-16 (*gk914989*)	VC40988	Million Mutant Project
oac-20 (*gk256989*)	VC10128	Million Mutant Project
oac-23 (*gk445127*)	VC30240	Million Mutant Project
oac-27 (*gk694121*)	VC40561	Million Mutant Project
oac-29 (*gk646323*)	VC40455	Million Mutant Project
oac-3 (*gk252641*)	VC20209	Million Mutant Project
oac-34 (*gk652397*)	VC40469	Million Mutant Project
oac-35 (*gk883174*)	VC40922	Million Mutant Project
oac-36 (*gk124636*)	VC20551	Million Mutant Project
oac-38 (*gk648702*)	VC40461	Million Mutant Project
oac-39 (*gk145*)	VC247	Deletion mutant from CGC
oac-4 (*gk363869*)	VC20633	Million Mutant Project
oac-40 (*gk242459*)	VC20235	Million Mutant Project
oac-41 (*gk242464*)	VC20211	Million Mutant Project
oac-41 (*gk766757*)	VC40696	Million Mutant Project
oac-42 (*WBVar00026015*)	CB4856	Wild isolate
oac-43 (*gk737013*)	VC40638	Million Mutant Project
oac-49 (*gk264099*)	VC20294	Million Mutant Project
oac-5 (*gk398429*)	VC30020	Million Mutant Project
oac-50 (*gk402144*)	VC20784	Million Mutant Project
oac-51 (*gk533438*)	VC40246	Million Mutant Project
oac-54 (*gk684785*)	VC40540	Million Mutant Project
oac-6 (*gk735518*)	VC40635	Million Mutant Project
oac-7 (*gk586689*)	VC40345	Million Mutant Project
oac-8 (*gk211086*)	VC20046	Million Mutant Project
oac-9 (*gk662463*)	VC40490	Million Mutant Project
oac-26 (*WBVar00158777*)	CB4856	Wild isolate

Supporting References

[1] G. V. Markov, J. M. Meyer, O. Panda, A. B. Artyukhin, M. Claassen, H. Witte, F. C. Schroeder, R. J. Sommer, *Mol Biol Evol* **2016**, *33*, 2506-2514.

[2] S. H. von Reuss, N. Bose, J. Srinivasan, J. J. Yim, J. C. Judkins, P. W. Sternberg, F. C. Schroeder, *J Am Chem Soc* **2012**, *134*, 1817–1824.

[3] X. Zhang, L. Feng, S. Chinta, P. Singh, Y. Wang, J. K. Nunnery, R. A. Butcher, *Proc Natl Acad Sci USA* **2015**, *112*, 3955-3960.

[4] G. J. Hermann, L. K. Schroeder, C. A. Hieb, A. M. Kershner, B. M. Rabbitts, P. Fonarev, B. D. Grant, J. R. Priess, *Mol Biol Cell* **2005**, *16*, 3273-3288.

[5] A. B. Artyukhin, J. J. Yim, M. Cheong Cheong, L. Avery, *Sci Rep* **2015**, *5*, 10647.