Modular metabolite assembly in *Caenorhabditis elegans* depends on carboxylesterases and formation of lysosome-related organelles

Henry H Le[1†], Chester JJ Wrobel[1†], Sarah M Cohen[2], Jingfang Yu[1], Heenam Park[2], Maximilian J Helf[1], Brian J Curtis[1], Joseph C Kruempel[3], Pedro Reis Rodrigues[1], Patrick J Hu[4], Paul W Sternberg[2]*, Frank C Schroeder[1]*

[1]Boyce Thompson Institute and Department of Chemistry and Chemical Biology, Cornell University, Ithaca, United States; [2]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, United States; [3]Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, United States; [4]Departments of Medicine and Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, United States

Abstract Signaling molecules derived from attachment of diverse metabolic building blocks to ascarosides play a central role in the life history of *C. elegans* and other nematodes; however, many aspects of their biogenesis remain unclear. Using comparative metabolomics, we show that a pathway mediating formation of intestinal lysosome-related organelles (LROs) is required for biosynthesis of most modular ascarosides as well as previously undescribed modular glucosides. Similar to modular ascarosides, the modular glucosides are derived from highly selective assembly of moieties from nucleoside, amino acid, neurotransmitter, and lipid metabolism, suggesting that modular glucosides, like the ascarosides, may serve signaling functions. We further show that carboxylesterases that localize to intestinal organelles are required for the assembly of both modular ascarosides and glucosides via ester and amide linkages. Further exploration of LRO function and carboxylesterase homologs in *C. elegans* and other animals may reveal additional new compound families and signaling paradigms.

*For correspondence:
pws@caltech.edu (PWS);
fs31@cornell.edu (FCS)

†These authors contributed equally to this work

Introduction

Recent studies indicate that the metabolomes of animals, from model systems such as *Caenorhabditis elegans* and Drosophila to humans, may include >100,000 of compounds (*da Silva et al., 2015*; *Artyukhin et al., 2018*). The structures and functions of most of these small molecules have not been identified, representing a largely untapped reservoir of chemical diversity and bioactivities. In *C. elegans* (*Girard et al., 2007*), a large modular library of small-molecule signals, the ascarosides, are involved in almost every aspect of its life history, including aging, development, and behavior (*Schroeder, 2015*; *Butcher, 2017*; *Butcher et al., 2007*; *Jeong et al., 2005*). The ascarosides represent a structurally diverse chemical language, derived from glycosides of the dideoxysugar ascarylose and hydroxylated short-chain fatty acid (*Figure 1a*; *von Reuss et al., 2012*). Structural and functional specificity arises from optional attachment of additional moieties to the sugar, for example indole-3-carboxylic acid (e.g. icas#3 (1)), or carboxy-terminal additions to the fatty acid chain, such as *p*-aminobenzoic acid (PABA, as in ascr#8 (2)) or *O*-glucosyl uric acid (e.g. uglas#11 (3), *Figure 1b*; *Artyukhin et al., 2018*; *Artyukhin et al., 2013*; *Bose et al., 2014*; *Aprison and*

Ruvinsky, 2017; *Curtis et al., 2020*; *Pungaliya et al., 2009*). Given that even small changes in the chemical structures of the ascarosides often result in starkly altered biological function, ascaroside biosynthesis appears to correspond to a carefully regulated encoding process in which biological state is translated into chemical structures (*Panda et al., 2017*). Thus, the biosynthesis of ascarosides and other *C. elegans* signaling molecules (e.g. nacq#1) (*Ludewig et al., 2019*) represents a fascinating model system for the endogenous regulation of inter-organismal small-molecule signaling in metazoans. However, for most of the >200 recently identified *C. elegans* metabolites (*Artyukhin et al., 2018*; *von Reuss et al., 2012*; *Artyukhin et al., 2013*), biosynthetic knowledge is sparse. Previous studies have demonstrated that conserved metabolic pathways, for example peroxisomal β-oxidation (*Artyukhin et al., 2013*; *Bose et al., 2014*) and amino acid catabolism (*von Reuss et al., 2012*; *Srinivasan et al., 2012*; *Figure 1a*), contribute to ascaroside biosynthesis; however, many aspects of the mechanisms underlying assembly of multi-modular metabolites remains unclear.

Recently, metabolomic analysis of mutants of the Rab-GTPase *glo-1*, which lack a specific type of lysosome-related organelles (LROs, also referred to as autofluorescent gut granules), revealed complete loss of 4′-modified ascarosides (*Panda et al., 2017*). The *glo-1*-dependent LROs are acidic, pigmented compartments that are related to mammalian melanosomes and drosophila eye pigment organelles (*Coburn and Gems, 2013*; *Hermann et al., 2005*). LROs form when lysosomes fuse with other cellular compartments, for example peroxisomes, and appear to play an important role for recycling proteins and metabolites (*Coburn and Gems, 2013*). Additionally, it has been suggested that LROs may be involved in the production and secretion of diverse signaling molecules (*Dell'Angelica et al., 2000*; *Luzio et al., 2014*), and the observation that *glo-1* mutant worms are deficient in 4′-modified ascarosides suggested that intestinal organelles may serve as hubs for their assembly (*Figure 1a*; *Panda et al., 2017*). In addition to the autofluorescent LROs, several other types of intestinal granules have been characterized in *C. elegans*, including lipid droplets (*Cao et al., 2019*) and lysosome-related organelles that are not *glo-1*-dependent (*Tanji et al., 2016*).

Parallel studies of other *Caenorhabditis* species (*Dong et al., 2016*; *Bergame et al., 2019*; *Dolke et al., 2019*) and *Pristionchus pacificus* (*Falcke et al., 2018*), a nematode species being developed as a satellite model system to *C. elegans* (*Rae et al., 2008*), revealed that production of modular ascarosides is widely conserved among nematodes. Leveraging the high genomic diversity of sequenced *P. pacificus* isolates, genome-wide association studies coupled to metabolomic analysis revealed that *uar-1*, a carboxylesterase from the α/ß-hydrolase superfamily with homology to cholinesterases (AChEs), is required for 4′-attachment of an ureidoisobutyryl moiety to a subset of ascarosides, e.g. ubas#3 (4, *Figure 1c*; *Falcke et al., 2018*). Homology searches revealed a large expansion of <u>carboxyl</u><u>ester</u>ase (*cest*) homologs in *P. pacificus* as well as *C. elegans* (*Figure 1—figure supplement 1*), and recently it was shown that in *C. elegans*, the *uar-1* homologs *cest-3*, *cest-8*, and *cest-9.2* are involved in the 4′-attachment of other acyl groups in modular ascarosides (*Faghih et al., 2020*). Based on these findings, we posited that *cest* homologs localize to *glo-1*-dependent intestinal granules where they control assembly of modular ascarosides, and perhaps other modular metabolites. In this work, we present a comprehensive assessment of the impact of *glo-1*-deletion on the *C. elegans* metabolome and uncover the central role of *cest* homologs that localize to intestinal granules in the biosynthesis of diverse modular metabolites.

Results

Novel classes of LRO-dependent metabolites

To gain a comprehensive overview of the role of *glo-1* in *C. elegans* metabolism, we employed a fully untargeted comparison of the metabolomes of a *glo-1* null mutant and wild-type worms (*Figure 1d*). HPLC–high-resolution mass spectrometry (HPLC–HRMS) data for the *exo*-metabolomes (excreted compounds) and *endo*-metabolomes (compounds extractable from the worm bodies) of the two strains were analyzed using the Metaboseek comparative metabolomics platform, which integrates the *xcms* package (*Tautenhahn et al., 2008*). These comparative analyses revealed that the *glo-1* mutation has a dramatic impact on *C. elegans* metabolism. For example, in negative ionization mode, we detected >1000 molecular features that were at least 10-fold less abundant in the *glo-1 exo-* and *endo*-metabolomes, as well as >3000 molecular features that are 10-fold upregulated
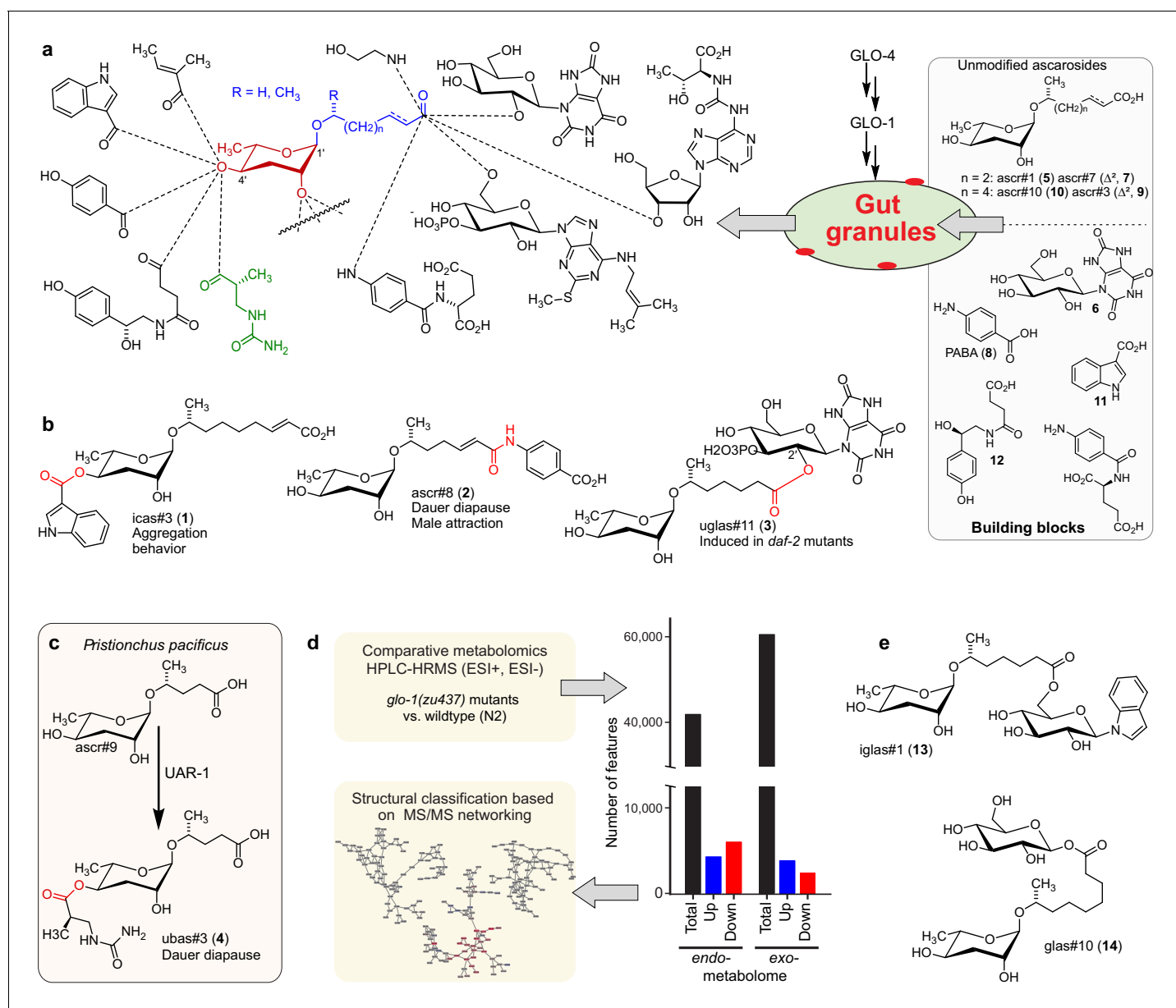
Figure 1. Modular ascarosides in nematodes and proposed role of the Rab-GTPase GLO-1. (**a**) Modular ascarosides are assembled from simple ascarosides, e.g. ascr#1 (5) or ascr#3 (9), and building blocks from other metabolic pathways, e.g. glucosyl uric acid (6), *p*-aminobenzoic acid (PABA, 8) indole-3-carboxylic acid (11), or succinyl octopamine (12). We hypothesize that *glo-1*-dependent gut granules play a central role in their biosynthesis. (**b**) Examples for modular ascarosides and their biological context. (**c**) UAR-1 in *P. pacificus* converts simple ascarosides into the 4'-ureidoisobutyric-acid-bearing ascarosides, for example ubas#3 (4). (**d**) Strategy for comparative metabolomic analysis of LRO-deficient *glo-1* mutants. (**e**) Example for modular ascarosides whose production is increased in *glo-1* mutants.

The online version of this article includes the following source data and figure supplement(s) for figure 1:

Source data 1. Source data for *Figure 1d*.
Figure supplement 1. Dendrogram of serine hydrolase annotated in *C. elegans* and *Ppa-uar-1* (marked blue).
Figure supplement 2. MS peak areas relative to wildtype (N2) of several building blocks of modular ascarosides.
Figure supplement 2—source data 1. Source data for *Figure 1—figure supplement 2*.

in *glo-1* mutants. For further characterization of differential features, we employed tandem mass spectrometry (MS^2) based molecular networking, a method which groups metabolites based on shared fragmentation patterns (*Figure 1d*, *Figure 2—figure supplements 1–4*; *Wang et al., 2016*). The resulting four MS^2 networks – for data obtained in positive and negative ionization mode for the *exo*- and *endo*-metabolomes – revealed several large clusters of features whose abundances were largely abolished or greatly increased in *glo-1* worms. Notably, although some differential MS^2 clusters represented known compounds, for example ascarosides, the majority of clusters were found to represent previously undescribed metabolite families.

In agreement with previous studies (*Panda et al., 2017*), biosynthesis of most modular ascarosides was abolished or substantially reduced in *glo-1* mutants, including all 4′-modified ascarosides, e.g. icas#3 (**1**) (*Figure 1b*, *Figure 2—figure supplement 5a*). Similarly, production of ascarosides modified at the carboxy terminus, e.g. uglas#11 (**3**) derived from ester formation between ascr#1 (**5**) and uric acid glucoside (*Curtis et al., 2020*) (**6**), and ascr#8 (**2**), derived from formation of an amide bond between ascr#7 (**7**) and of *p*-amino benzoic acid (**8**), was largely abolished in *glo-1* mutants (*Figure 1a–b*, *Figure 2—figure supplement 5a*). Metabolites plausibly representing building blocks of these modular ascarosides were not strongly perturbed in *glo-1* mutants (*Figure 1—figure supplement 2*). For example, abundances of unmodified ascarosides, for example ascr#3 (9) and ascr#10 (10), or metabolites representing 4′-modifications, for example indole-3-carboxylic acid (11) and octopamine succinate (12), were not significantly perturbed in the mutant (*Figure 1a*, *Figure 2—figure supplement 5a*, *Figure 1—figure supplement 2*). In contrast, a subset of modular ascaroside glucose esters (e.g. iglas#1 (13) and glas#10 (14), *Figure 1e*), was strongly increased in *glo-1* mutants (*Figure 2—figure supplement 5b*). These results suggest that *glo-1*-dependent intestinal organelles function as a central hub for the biosynthesis of most modular ascarosides, with the exception of a subset of ascarosylated glucosides, whose increased production in *glo-1* mutants may be indicative of a shunt pathway for ascarosyl-CoA derivatives (*Zhang et al., 2015*; *Zhang et al., 2016*; *Zhang et al., 2018*), which represent plausible precursors for modular ascarosides modified at the carboxy terminus.

Next, we analyzed the most prominent MS^2 clusters representing previously uncharacterized metabolites whose production is abolished or strongly reduced in *glo-1* mutants (*Figure 2*). Detailed analysis of their MS^2 spectra indicated that they may represent a large family of modular hexose derivatives incorporating moieties from diverse primary metabolic pathways. For example, MS^2 spectra from clusters **I**, **II**, and **III** of the positive-ionization network suggested phosphorylated hexose glycosides of indole, anthranilic acid, tyramine, or octopamine, which are further decorated with a wide variety of fatty acyl moieties derived from fatty acid or amino acid metabolism, for example nicotinic acid, pyrrolic acid, or tiglic acid (*Figure 2*, *Appendix 1—table 1*; *Coburn and Gems, 2013*; *Stupp et al., 2013*). Given the previous identification of the glucosides iglu#1/2 (15/16, *Figure 2e*) and angl#1/2 (17/18), we hypothesized that clusters I, II, and III represent a modular library of glucosides, in which *N*-glucosylated indole, anthranilic acid, tyramine, or octopamine (*O'Donnell et al., 2020*) serve as scaffolds for attachment of diverse building blocks. To further support these structural assignments, a series of modular metabolites based on *N*-glucosylated indole ('iglu') were selected for total synthesis. Synthetic standards for the non-phosphorylated parent compounds of iglu#4 (19), iglu#6 (20), iglu#8 (21), and iglu#10 (22) matched HPLC retention times and MS^2 spectra of the corresponding natural compounds (*Figure 2—figure supplement 6*), confirming their structures and enabling tentative structural assignments for a large number of additional modular glucosides, including their phosphorylated derivatives, e.g. iglu#12 (23), iglu#41 (24), angl#4 (cluster II, 25), and tyglu#4 (cluster III, 26) (*Figure 2*). The proposed structures include several glucosides of the neurotransmitters tyramine and octopamine, whose incorporation could be verified by comparison with data from a recently described feeding experiment with stable isotope-labeled tyrosine (*O'Donnell et al., 2020*). Similar to ascaroside biosynthesis, the production of modular glucosides is life stage dependent; for example, production of specific tyramine glucosides peaks at the L3 larval stage, whereas production of angl#4 increases until the adult stage (*Figure 2—figure supplement 8*). Notably, modular glucosides were detected primarily as their phosphorylated derivatives, as respective non-phosphorylated species were generally less abundant. In contrast to most ascarosides, the phosphorylated glucosides are more abundant in the *endo*-metabolome than the *exo*-metabolome, suggesting that phosphorylated glucosides may be specifically retained in the body (*Figure 2—figure supplement 7*).
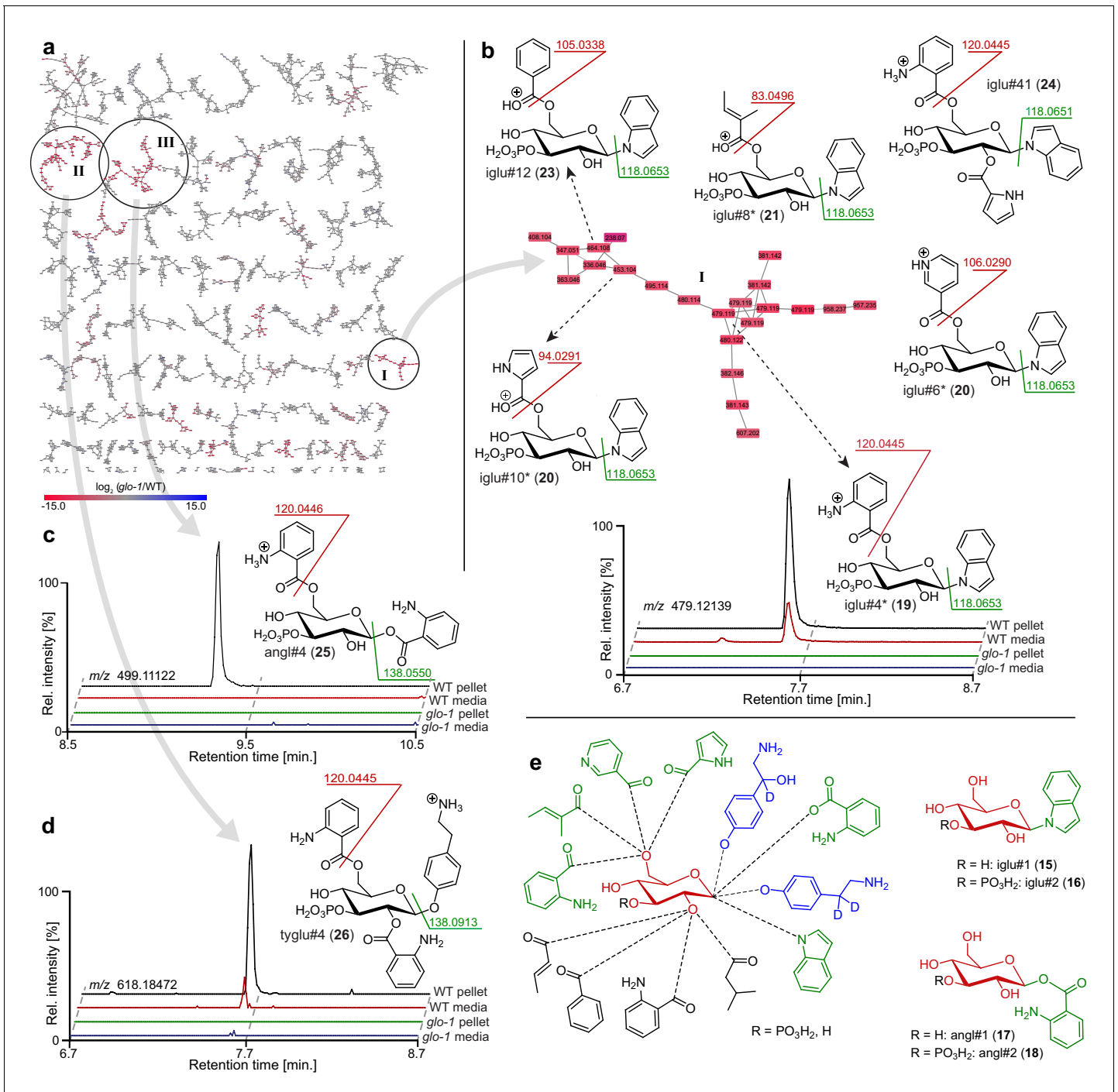
Figure 2. Comparative metabolomic analysis of glo-1 mutants. (a) Partial MS² network (positive ion mode) for *C. elegans endo*-metabolome highlighting three clusters of modular glucosides that are down regulated in the *glo-1* mutants (also see *Figure 2—figure supplements 1–4*). Red represents downregulated and blue upregulated features compared to wildtype *C. elegans*. (b) Cluster I feature several modular indole glucoside derivatives. Structures were proposed based on MS² fragmentation patterns, also see *Appendix 1—table 1*. Compounds whose non-phosphorylated analogs were synthesized are marked (*). Shown ion chromatograms demonstrate loss of iglu#4 in *glo-1* mutants. (c,d) Examples for modular glucosides detected as part of clusters II and III. Ion chromatograms show abolishment of angl#4 (25) (c) and tyglu#4 (26) (d) production in *glo-1* mutants. (e) Modular glucosides are derived from combinatorial assembly of a wide range of building blocks. Incorporation of moieties was confirmed via total synthesis of example compounds (green) or stable isotope labeling (blue). For all compounds, 3-phosphorylation was proposed based on the established structures of iglu#2 (16), angl#2 (18), and uglas#11 (3).

The online version of this article includes the following source data and figure supplement(s) for figure 2:

Figure 2 continued on next page

Figure 2 continued

Figure supplement 1. Full MS^2 molecular network of *endo*-metabolome acquired in positive ion mode (left).

Figure supplement 2. Full MS^2 molecular network of *endo*-metabolome acquired in negative ion mode.

Figure supplement 3. Full MS^2 molecular network of *exo*-metabolome acquired in positive ion mode.

Figure supplement 4. Full MS^2 molecular network of *exo*-metabolome acquired in negative ion mode.

Figure supplement 5. MS peak areas relative to wildtype (N2) of simple and modular ascarosides, glucosylated ascarosides, and phosphorylated ascarosides in *glo-1* (a, b, c) and *glo-4* (d, e, f) mutant worms.

Figure supplement 5—source data 1. Source data for *Figure 2—figure supplement 5a–f*.

Figure supplement 6. Identification of iglu metabolites.

Figure supplement 7. Concentration of simple and modular glucosides in the *endo*- or *exo*-metabolomes wild-type *C. elegans*.

Figure supplement 7—source data 1. Source data for *Figure 2—figure supplement 7*.

Figure supplement 8. Production of modular glucosides is life-stage-dependent.

Figure supplement 8—source data 1. Source data for *Figure 2—figure supplement 8a–d*.

Figure supplement 9. Peak area relative to wildtype (N2) of building blocks of modular glucosides in *glo-1* mutant worms.

Figure supplement 9—source data 1. Source data for *Figure 2—figure supplement 9*.

Figure supplement 10. Representative ion chromatograms and MS^2 spectra of upregulated leucine- and proline-containing peptides.

As in the case of modular ascarosides, the abundances of putative building blocks of the newly identified modular glucosides were not strongly perturbed in *glo-1* mutants. For example, abundances of anthranilic acid, indole, octopamine, and tyramine were not significantly affected in *glo-1* null animals (*Figure 2—figure supplement 9*). Notably, abundances of the glucosides scaffold, e.g. iglu#1 and angl#1, were also largely unaltered or even slightly increased in *glo-1* mutants (*Figure 2—figure supplement 9*). In addition, production of some of the identified modular glucosides, e.g. iglu#5, is reduced but not fully abolished in *glo-1* worms (*Figure 2—figure supplement 6*).

To confirm our results, we additionally compared the *glo-1* metabolome with that of *glo-4* mutants. *glo-4* encodes a predicted guanyl-nucleotide exchange factor acting upstream of *glo-1*, and like *glo-1* mutants, *glo-4* worms do not form LROs (*Hermann et al., 2005*). We found that the *glo-4* metabolome closely resembles that of *glo-1* worms, lacking most of the modular ascarosides and ascarosides detected in wildtype worms (*Figure 2—figure supplement 5c*). Correspondingly, similar sets of compounds are upregulated in *glo-1* and *glo-4* mutants relative to wild type, including ascarosyl glucosides and ascaroside phosphates. Compounds accumulating in *glo-1* and *glo-4* mutant worms further include a diverse array of small peptides (primarily three to six amino acids), consistent with the proposed role of LROs in the breakdown of peptides derived from proteolysis (*Figure 2—figure supplement 10*; *Bird et al., 2009*). Taken together, our results indicate that, in addition to their roles in the degradation of metabolic waste, the LROs serve as hotspots of biosynthetic activity, where building blocks from diverse metabolic pathways are attached to glucoside and ascaroside scaffolds (*Figure 1a*).

Carboxylesterases are required for modular assembly

Comparing the relative abundances of different members of the identified families of modular glucosides and ascarosides, it appears that combinations of different building blocks and scaffolds are highly specific, suggesting the presence of dedicated biosynthetic pathways. For example, uric acid glucoside, gluric#1 (6), is preferentially combined with an ascaroside bearing a seven-carbon side chain (to form uglas#11, 3), whereas ascarosides bearing a nine-carbon side chain are preferentially attached to the anomeric position of free glucose, as in glas#10 (14) (*Artyukhin et al., 2018*; *von Reuss et al., 2012*). Similarly, tiglic acid is preferentially attached to indole and tyramine glucosides but not to anthranilic acid glucosides (*Appendix 1—table 1*). Given that 4′-modification of ascarosides in *P. pacificus* and *C. elegans* require *cest* homologs, we hypothesized that the biosynthesis of other modular ascarosides as well as the newly identified glucosides may be under the control of *cest* family enzymes (*Falcke et al., 2018*; *Faghih et al., 2020*). From a list of 44 *uar-1* homologs from BLAST analysis (*Appendix 1—table 2*), we selected seven for further study (*Figure 3a*, *Appendix 1—table 3*). The selected homologs are predicted to have intestinal expression, one primary site of small molecule biosynthesis in *C. elegans* (*Artyukhin et al., 2018*), and are closely related to the UAR-1 gene, while representing different sub-branches of the phylogenetic tree. Utilizing a recently optimized CRISPR/Cas9 method, we obtained two null mutant strains for

Figure 3. Carboxylesterases are required for modular assembly. (**a**) Serine hydrolase dendrogram relating *P. pacificus uar-1* to homologous predicted genes in *C. elegans*. *Ppa-uar-1*, *cest-3*, *cest-8*, *cest-9.2* (green) mediate ester formation at the 4'-position of ascarosides in *P. pacificus* and *C. elegans*. Genes shown in red color were selected for the current study. (**b,c**) Production of ascr#8 (**2**), ascr#81 (27), and ascr#82 (28) is abolished in *cest-2.2* mutants Isogenic revertant strains of the *cest-2.2* null mutants in which the STOP-IN cassette was precisely excised, demonstrate wild-type-like recovery of the associated metabolite. (**d,e**) Production of uglas#1 and uglas#11 is abolished in *cest-1.1*(null) mutants and recovered in genetic revertants. (**f**) Biosynthesis of positional isomers uglas#14 (31) and uglas#15 (32) is unaltered or increased in *cest-1.1* mutants (**f**). (**g**) Production of uglas#1 and uglas#11, but not gluric#1, is abolished in *cest-1.1*(S213) mutants. (**h,i**) Production of the anthranilic-acid-modified glucoside iglu#4 is largely abolished in *cest-4* mutants and fully recovered in genetic revertants. (**j**) Production of iglu#6 (36) and iglu#8 (37), whose structures are closely related to that of iglu#4, is not abolished in *cest-4* mutants. Ion chromatograms in panels b, d, and g further demonstrate abolishment in *glo-1* mutants. n.d., not detected. Error bars are standard deviation of the mean, and p-values are depicted in the Figure.

Figure 3 continued on next page

Figure 3 continued

The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. Source data for *Figure 3c,e,f,g,I,j*.
Figure supplement 1. Relative abundances of ascr#8 (**2**) and related metabolites in *cest-1.1, cest-2.2, cest-4* mutants, and wild type (N2).
Figure supplement 1—source data 1. Source data for *Figure 3—figure supplement 1*.
Figure supplement 2. Ion chromatograms demonstrating that abundances of potential precursors of (**a**) *cest-1.1*-dependent, (**b**) *cest-2.2*-dependent, and (**c**) *cest-4*-dependent metabolites is large unchanged in the corresponding mutants.
Figure supplement 3. Ion chromatograms demonstrating recovery of (**a**) *cest-1.1*-dependent, (**b**) *cest-8*-dependent, (**c**) *cest-2.2*-dependent, (**d**) *cest-4*-dependent metabolites from CRISPR/Cas9 reversions of the corresponding null mutants.
Figure supplement 4. Relative abundance of other indole containing glucosides in *cest-4* mutants, demonstrating that *cest-4* is specifically required for the production of iglu#3 (**34**) and #4 (**19**).
Figure supplement 4—source data 1. Source data for *Figure 3—figure supplement 4*.

five of the selected genes (*Wang et al., 2018*). Mutants for the remaining two homologs, *ges-1* and *cest-6*, had been previously obtained (*Appendix 1—table 3*). We then analyzed the *exo*- and *endo*-metabolomes of this set of mutant strains by HPLC-HRMS to identify features that are absent or strongly downregulated in null mutants of a specific candidate gene compared to wildtype worms and all other mutants in this study. We found that two of the seven tested homologs (*cest-1.1, cest-2.2*) are defective in the production of two different families of modular ascarosides, whereas *cest-4* mutants were defective in the biosynthesis of a specific subset of modular indole glucosides (*Figure 3*). The metabolomes of mutants for the remaining four *cest* homologs did not exhibit any significant differences compared to wild type under the tested conditions.

Analysis of the metabolomes of the two *cest-2.2* null mutants revealed loss of dauer pheromone component and male attractant ascr#8 (**2**) as well as of the closely related ascr#81 (**27**) and ascr#82 (**28**) (*Figure 3b*, *Figure 3—figure supplements 1* and *2b*). Biosynthetically, the ascr#8 family of ascarosides are derived from amide formation between ascr#7 (ΔC7, **7**) and folate-derived *p*-aminobenzoic acid (PABA, **8**), PABA-glutamate (**29**), or PABA-diglutamate, respectively. We did not detect any significant reduction in the production of plausible ascr#8 precursors, including PABA and PABA-glutamate, or ascr#7 (*Figure 3b*, *Figure 3—figure supplement 2b*). Biosynthesis of ascr#8, ascr#81, and ascr#82 was recovered in *cest-2.2* mutant worms in which the *cest-2.2* sequence had been restored to wild type using CRISPR/Cas9 (*Figure 3c*, *Figure 3—figure supplement 3b*). These results indicate that CEST-2.2 is required specifically for biosynthesis of the amide linkage between the carboxy terminus of ascr#7 and PABA derivatives, in contrast to the implied functions of UAR-1, CEST-8, CEST-3, and CEST-9.2, which are involved in the formation of ester bonds between various head groups and the 4′-hydroxy group of ascarylose (*Falcke et al., 2018*; *Faghih et al., 2020*).

In *cest-1.1* null mutants (*cest-1.1*(null)), biosynthesis of the nucleoside-like ascaroside uglas#1 (**30**) and its phosphorylated derivative uglas#11 (**3**) was abolished (*Figure 3d*, *Figure 3—figure supplement 2a*). uglas#1 and uglas#11 are derived from the attachment of ascr#1, bearing a seven carbon (C7) side chain, to the uric acid gluconucleoside gluric#1 (**6**). Production of ascr#1 (**5**) and gluric#1 (**6**), representing plausible building blocks of uglas#1 (**30**), was not reduced (*Figure 3—figure supplement 2a*). Furthermore, production of uglas#14 (**31**) and uglas#15 (**32**), isomers of uglas#1 and uglas#11 bearing the ascarosyl moiety at the 6′ position instead of the 2′ position, was not abolished but rather slightly increased in *cest-1.1*(null) (*Figure 3d–e*). These results indicate that CEST-1.1 is required for the formation of the ester bond specifically between ascr#1 (**5**) and the 2′-hydroxyl group in gluric#1. As in the case of *cest-2.2*, biosynthesis of uglas#1 and uglas#11 was fully recovered in *cest-1.1* mutant worms in which the *cest-1.1* sequence had been restored to wild type using CRISPR/Cas9 (*Figure 3f*, *Figure 3—figure supplement 3a*).

Sequence alignment with human AChE suggested that serine 213 is part of the conserved catalytic serine-histidine-glutamate triad of CEST-1.1 (*Figure 4—figure supplement 1*). To test whether disruption of the catalytic triad would affect production of *cest-1.1*-dependent metabolites, we generated a point mutant, *cest-1.1*(S213A). As in *cest-1.1*(null), production of uglas#1 (**30**) and uglas#11 (**3**) was fully abolished in *cest-1.1*(S213A), whereas production of gluric#1 was not affected (*Figure 3g*).

Previous work implicated *cest-1.1* with longevity phenotypes associated with argonaute-like gene 2 (*alg-2*) (*Aalto et al., 2018*). *alg-2* mutant worms are long lived compared to wild type and their

long lifespan was further shown to require *daf-16*, the sole ortholog of the FOXO family of transcription factors in *C. elegans*, as well as *cest-1.1*. Moreover, uglas#11 biosynthesis is significantly increased in mutants of the insulin receptor homolog *daf-2*, a central regulator of lifespan in *C. elegans* upstream of *daf-16* (*Curtis et al., 2020*). These findings suggest the possibility that the production of uglas ascarosides underlies the *cest-1.1*-dependent extension of adult lifespan in *C. elegans*.

In contrast to our results for *cest-1.1* and *cest-2.2* mutants, comparative metabolomic analysis of the *cest-4* mutant strains did not reveal any defects in the biosynthesis of known ascarosides. Instead, we found that the levels of a specific subset of modular anthranilic acid (33) bearing indole glucosides, including iglu#3 (34) and its phosphorylated derivative iglu#4 (35) were abolished in the *cest-4* mutant worms (*Figure 3h*). Abundances of the putative precursor glucosides, iglu#1 (15) and iglu#2 (16), were not significantly changed in *cest-4* (*Figure 3i*, *Figure 3—figure supplement 2c*). Notably, production of other indole glucosides, e.g. iglu#6 (36) and iglu#8 (37), was not significantly reduced in *cest-4* worms (*Figure 3j*, *Figure 3—figure supplement 4*). Biosynthesis of iglu#3 and iglu#4 was restored to wild-type levels in genetic revertant strains for *cest-4* (*Figure 3i*, *Figure 3—figure supplement 3c*). Therefore, it appears that *cest-4* is specifically required for attachment of anthranilic acid to the 6′ position of glucosyl indole precursors, whereas attachment of tiglic acid, nicotinic acid, and other moieties is *cest-4*-independent (*Figure 3j*, *Figure 3—figure supplement 4*). The role of *cest-4* in the biosynthesis of the iglu family of modular glucosides thus parallels that of *cest-1.1* in the biosynthesis of the uglas ascarosides: whereas *cest-4* appears to be required for the attachment of anthranilic acid (33) to the 6′ position of a range of indole glucosides, *cest-1.1* appears to be required for attaching the ascr#1 side chain to the 2′ position in uric acid glucosides.

CEST-2.2 localizes to intestinal granules

All *cest* homologs selected for this study exhibit domain architectures typical of the α/ß-hydrolase superfamily of proteins, including a conserved catalytic triad, and further contain a predicted disulfide bridge, as in mammalian AChE (*Soreq and Seidman, 2001*; *Figure 4—figure supplement 1*). The *cest* genes also share homology with neuroligin, a membrane bound member of the α/ß-hydrolase fold family, that mediates the formation and maintenance of synapses between neurons (*Bemben et al., 2015*). Sequence analysis suggests that five of the seven CEST homologs studied here are membrane anchored, given the presence of a predicted *C*-terminal transmembrane domain (*Krogh et al., 2001*) (consisting of ~20 residues), with the *N* terminus on the luminal side of a vesicle or organelle (*Figure 4—figure supplement 2*). Since the production of all so far identified *cest*-dependent metabolites is abolished in *glo-1* mutants, it seemed likely that the CEST proteins localize to intestinal granules. To test this idea, we created a mutant strain that express *cest-2.2 C*-terminally tagged with mCherry at the native genomic locus to avoid potentially confounding effects of overexpression. The red fluorescent mCherry was chosen because of the strong green autofluorescence of the LROs (*Coburn and Gems, 2013*). We confirmed that production of all *cest-2.2*-dependent metabolites, including ascr#8 (**2**), ascr#81 (**27**), and ascr#82 (**28**) was not significantly altered in *cest-2.2*-mCherry mutants (*Figure 4a*), indicating that CEST-2.2 remained functional. Imaging of wild-type adult worms revealed strong green and weaker red autofluorescence in circular features in intestinal cells, consistent with LROs. In addition, *cest-2.2*-mCherry-tagged worms showed red fluorescence in a distinct set of intestinal granules that showed little if any autofluorescence (*Figure 4b*, *Figure 4—figure supplements 3–4*). It is unclear whether mCherry also localizes to the strongly autofluorescent granules, as we cannot distinguish the mCherry signal from the red component of the autofluorescence, given relatively low CEST-2.2-mCherry expression in this non-overexpressing strain. Taken together, it appears that CEST-2.2-mCherry localizes to a subset of intestinal organelles that is partly distinct from the autofluorescent LROs. Further studies are required to determine if CEST-2.2-mCherry co-localizes with other intestinal granule markers, specifically GLO-1 and the lysosomal marker LMP-1.

Glo-1-dependent metabolites in *C. briggsae*

In addition to *C. elegans* and *P. pacificus*, modular ascarosides have been reported from several other *Caenorhabditis* species (*Dong et al., 2020*; *Kanzaki et al., 2018*), including *C. briggsae* (*Dong et al., 2016*; *von Reuss, 2018*). To assess whether the role of LROs in the biosynthesis of modular metabolites is conserved across species, we created two *Cbr-glo-1* (CBG01912.1) knock-

out strains using CRISPR/Cas9. As in *C. elegans*, *Cbr-glo-1* mutant worms lacked autofluorescent LROs, which are prominently visible in wild-type *C. briggsae* (*Figure 5—figure supplement 1*). Comparative metabolomic analysis of the *endo-* and *exo-*metabolomes of wild-type *C. briggsae* and the *Cbr-glo-1* mutant strains revealed that biosynthesis of all known modular ascarosides is abolished in *Cbr-glo-1* worms, including the indole carboxy derivatives icas#2 (35) and icas#6.2 (36), which are highly abundant in wild-type *C. briggsae* (*Figure 5a*; *Dong et al., 2016*). In addition, the *C. briggsae* MS^2 networks included several large *Cbr-glo-1*-dependent clusters representing modular glucosides,

a

R = OH: ascr#8 (**2**)
R = glutamate: ascr#81 (**27**)
R = Glu-Glu: ascr#82 (**28**)

b

Autofluorescence (green channel)   mCherry   Merge   DIC

Wildtype
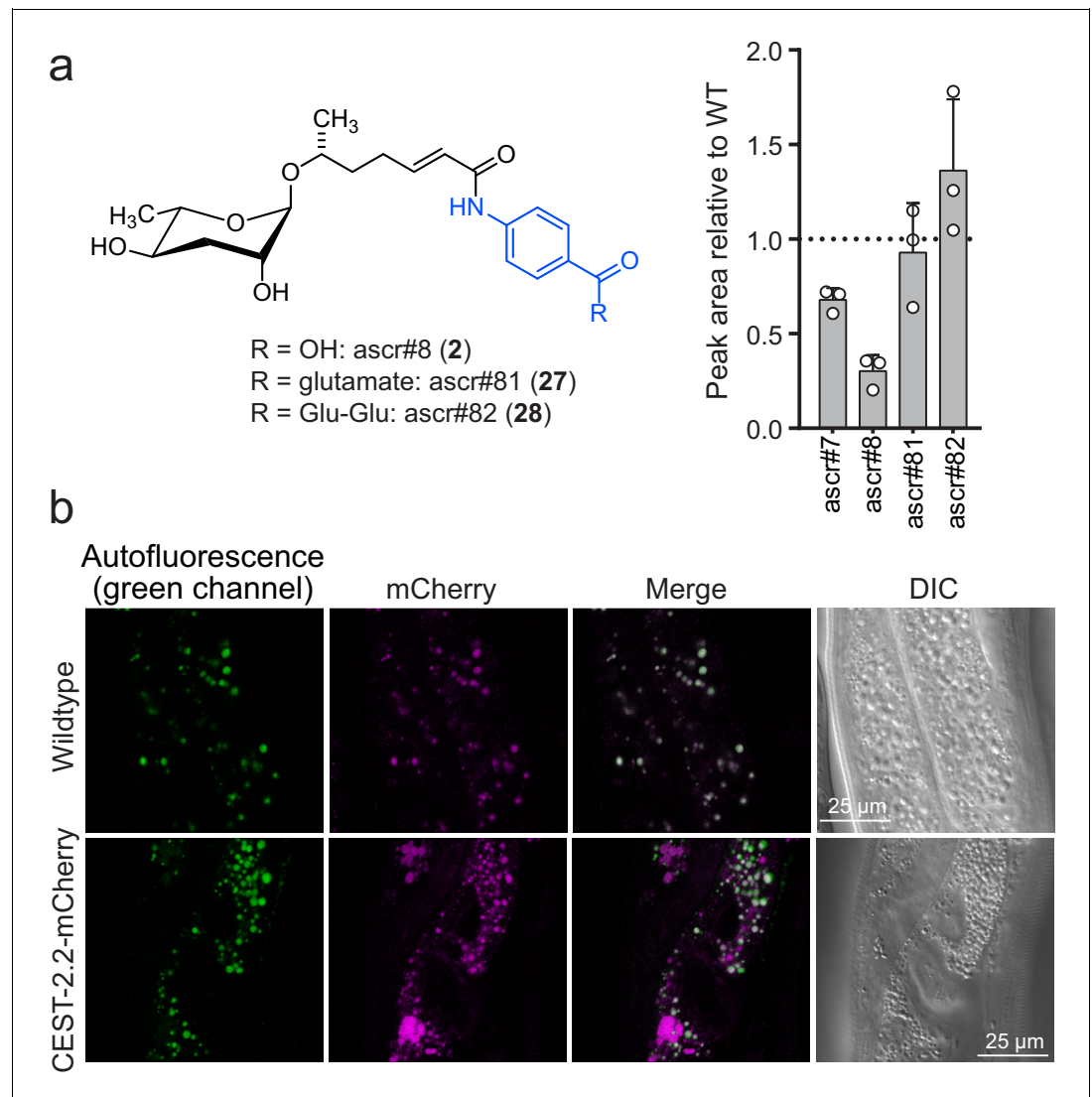
CEST-2.2-mCherry

Figure 4. CEST-2.2 localizes to intestinal granules. (**a**) Relative amounts of *cest-2.2*-dependent metabolites in worms expressing *C*-terminally mCherry-tagged CEST-2.2. (**b**) Red fluorescence in intestinal granules in wild-type and *cest-2.2*-mCherry gravid adults. Top, wild-type (N2) control; bottom, *cest-2.2*-mCherry worms.

The online version of this article includes the following source data and figure supplement(s) for figure 4:

Source data 1. Source data for *Figure 4a*.
Figure supplement 1. Amino acid sequence alignments of human acetyl cholinesterase (hAChE), *P. pacificus* UAR-1, and *C. elegans* CEST-1.1, CEST-2.2, and CEST-4.
Figure supplement 2. Transmembrane domain prediction for CEST proteins in this study (*cest-1.1, cest-2.2, cest-4, cest-6, cest-19, cest-33, ges-1*).
Figure supplement 3. Red fluorescence in intestinal granules in gravid adults, expressing *C*-terminally mCherry-tagged CEST-2.2.
Figure supplement 4. Co-localization of green and red autofluorescence in wild-type (N2) gravid adults.
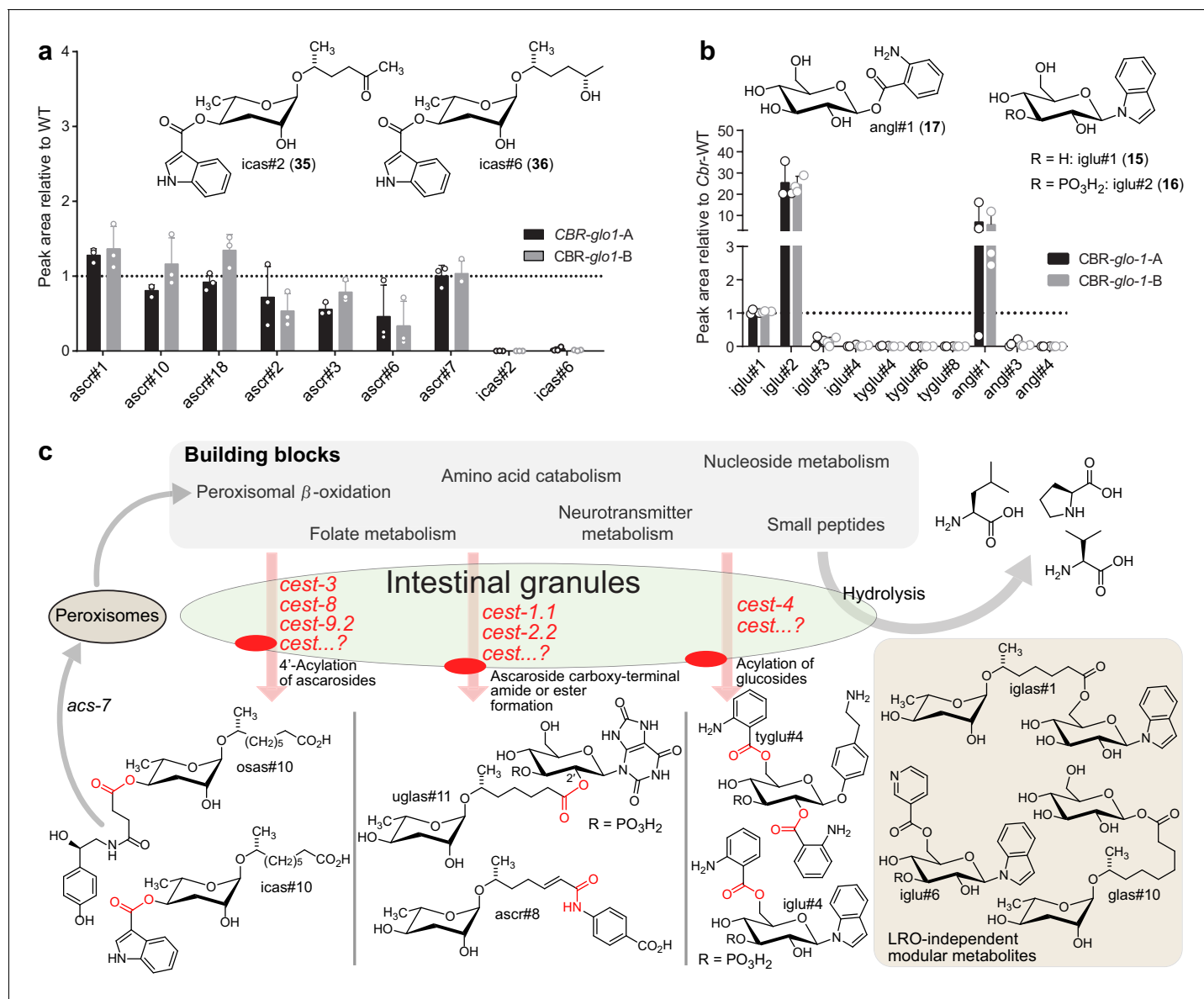
Figure 5. Relative abundance of (a) simple and modular ascarosides and (b) simple and modular glucosides in the *endo*-metabolome of *Cbr-glo-1* mutants relative to wild-type *C. briggsae*. n.d., not detected. (c) Model for modular metabolite assembly. CEST proteins (membrane-bound in the LROs, red) mediate attachment of building blocks from diverse metabolic pathways to glucose scaffolds and peroxisomal β-oxidation-derived ascarosides via ester and amide bonds. Some of the resulting modular ascarosides may undergo additional peroxisomal β-oxidation following activation by *acs-7* (**Dolke et al., 2019**).

The online version of this article includes the following source data and figure supplement(s) for figure 5:

Source data 1. Source data for *Figure 5a–b*.

Figure supplement 1. Gut granules in *C. briggsae*.

including many of the compounds also detected in *C. elegans*, for example iglu#4 and angl#4. As in *C. elegans*, production of unmodified glucoside scaffolds, e.g. iglu#1 (15) and angl#1 (17), was not reduced or increased in *Cbr-glo-1* mutants, whereas biosynthesis of most modular glucosides derived from attachment of additional moieties to these scaffolds was abolished (*Figure 5b*). Taken together, these results indicate that the role of LROs as a central hub for the assembly of diverse small molecule architectures, including modular glucosides and ascarosides, may be widely conserved among nematodes (*Figure 5c*).

Discussion

Our results indicate that in *C. elegans* the Rab-GTPase *glo-1,* which is required for formation of intestinal LROs, plays a central role in the biosynthesis of several large compound families derived from modular assembly via *cest* homologs. Formation of the autofluorescent LROs via *glo-1* is reminiscent of the roles of its human orthologs RAB32 and RAB38, which are required for the formation of melanosomes, and perhaps other LROs (*Wasmeier et al., 2006*; *Marks et al., 2013*). Lysosomes and LROs are generally presumed to function in autophagy, phagocytosis, and the hydrolytic degradation of proteins, and Rab32 family GTPases have been shown to be required for these processes in diverse organisms (*Morris et al., 2018*). Consistent with the notion that lysosomes and LROs are degradation hotspots, many of the building blocks of the identified modular ascarosides and glucosides are derived from catabolic pathways, for example, anthranilic acid is derived from tryptophan catabolism, uric acid stems from purine metabolism, and the short chain ascarosides are the end products of peroxisomal β-oxidation of very long-chain precursors. Importantly, although our results indicate that carboxylesterases participate in *glo-1*-dependent modular metabolite assembly, additional studies are required to clarify whether the intestinal compartments that carboxylesterases localize to also contain GLO-1 and the lysosomal marker LMP-1, as is the case for the autofluorescent LROs (*Tanji et al., 2016*).

Further, our results demonstrate that the modular assembly paradigm extends beyond ascarosides. The modular glucosides represent a previously unknown family of nematode metabolites. In contrast to the well-established role of modular ascarosides as pheromones, it is unknown whether modular glycosides serve specific biological functions, for example as signaling molecules; however, their specific biosynthesis via *cest-4* as well as their life-stage-dependent production strongly supports this hypothesis (*Figure 2—figure supplement 8*). Like the ascaroside pheromones, some modular glucosides are excreted into the media, suggesting that they could be involved in inter-organismal communication. Identifying developmental and environmental conditions that affect modular glucoside production, as well as a more comprehensive understanding of their biosyntheses, may help uncover potential signaling and other biological roles. In particular, the apparent peroxisomal origin of the ascaroside scaffolds suggests a link between peroxisome and gut granule activity, perhaps via pexophagy (*Sakai et al., 2006*), and characterization of the role of autophagy for gut granule-dependent metabolism may contribute to uncovering the functions of modular glucoside and ascarosides. A connection to autophagy is also suggested by our previous finding (*Panda et al., 2017*) that production of modular ascarosides is reduced in mutants of *atg-18* (*Palmisano and Meléndez, 2019*), which is essential for autophagy.

The high degree of selectivity in which different building blocks are combined in the modular ascarosides and glucosides strongly suggests that these compounds, despite their numbers and diversity, represent products of dedicated enzymatic pathways, as has recently been established for 4'-acylated ascarosides. Our results revealed a wider range of biosynthetic functions associated with *cest* homologs, including esterification and amide formation at the carboxy terminus of ascarosides and acylation of glucosides (*Figure 5c*). Notably, all *cest* null mutants whose metabolomes have been characterized so far are defective in the biosynthesis of one or a few compounds sharing a specific structural feature, further supporting the view that these selectively assembled molecular architectures serve dedicated functions.

All CEST proteins that so far have been associated with modular metabolite assembly contain membrane-anchors and exhibit domain architectures typical of serine hydrolases of the AChE family, including an α/β-hydrolase fold, a conserved catalytic serine-histidine-glutamate triad, and bridging disulfide cysteines (*Figure 4—figure supplement 1*; *Soreq and Seidman, 2001*). While our efforts at heterologous expression of CEST proteins were unsuccessful, the finding that mutation of the catalytic serine in *cest-1.1*(S213A) abolished production of all *cest-1.1*-dependent compounds suggests that CEST enzymes directly participate in the biosynthesis of modular metabolites. Therefore, we hypothesize that CEST proteins, after translating from the endomembrane system to *glo-1*-dependent intestinal organelles, partake in the assembly of diverse ascaroside or glucoside-based architectures via acyl transfer from corresponding activated intermediates, e.g. CoA or phosphate esters (*Soreq and Seidman, 2001*; *Vaz and Wanders, 2002*). α/β-hydrolase fold enzymes are functionally highly diverse (*Rauwerdink and Kazlauskas, 2015*) and include esterases, peptidases, oxidoreductases, and lyases, serving diverse biosynthetic roles in animals, plants (*Mindrebo et al., 2016*), and

bacteria (*Zheng et al., 2016*). While acyltransferase activity is often observed as a side reaction for esterases and lipases, α/β-hydrolase fold enzymes can function as dedicated acyltransferases, for example in microbial natural product biosyntheses (*Rauwerdink and Kazlauskas, 2015*; *Lejon et al., 2008*). Additional biochemical studies will be required to delineate the exact mechanisms by which *cest* homologs contribute to modular metabolite assembly in nematodes.

Finally, although our results indicate that *glo-1* is required for the biosynthesis of most modular metabolites we have detected so far, it is notable that some modular ascarosides, e.g. iglas#1 (13), and modular glucosides, e.g. iglu#6 (20) and iglu#8 (21), do not appear to be *glo-1*-dependent (*Figure 2—figure supplement 7*). This suggests that diverse cell compartments contribute to modular metabolite biosynthesis and may also indicate that not all CEST proteins are delivered to the same cellular compartment. Similarly, *glo-1* mutants continue to generate the simple glucosides and ascarosides that serve as scaffolds for further elaboration via CEST proteins, which may be derived from UDP-glycosyltransferases (*Mackenzie et al., 2005*).

Reminiscent of the role of AChE for neuronal signal transduction in animals, it appears that, in *C. elegans*, carboxylesterases with homology to AChE have been co-opted to establish additional signal transduction pathways that are based on a modular chemical language, for inter-organismal communication, and perhaps also intra-organismal signaling. The biosynthetic functions of most of the 200 serine hydrolases in *C. elegans*, including more than 30 additional *cest* homologs, remain to be assessed, and it seems likely that this enzyme family contributes to the biosynthesis of a large number of additional, yet unidentified compounds. Similarly, the exact enzymatic roles of many families of mammalian serine hydrolases have not been investigated using HRMS-based untargeted metabolomics. Our results may motivate a systematic characterization of metazoan *cest* homologs and other serine hydrolases, with regard to their roles in metabolism and small molecule signaling, associated enzymatic mechanisms, and cellular localization.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain, strain background *Caenorhabditis elegans*	N2	Caenorhabditis Genetics Center (CGC)		Wild type
Strain, strain background *Caenorhabditis elegans*	GH10	David Gems		*glo-1(zu437)*
Strain, strain background *Caenorhabditis elegans*	RB811	Caenorhabditis Genetics Center (CGC)		*glo-4(ok623)*
Strain, strain background *Caenorhabditis elegans*	RB2053	Caenorhabditis Genetics Center (CGC)		*ges-1(ok2716)*
Strain, strain background *Caenorhabditis elegans*	PS8031	This work		*cest-1.1(sy1180)*
Strain, strain background *Caenorhabditis elegans*	PS8032	This work		*cest-1.1(sy1181)*
Strain, strain background *Caenorhabditis elegans*	DP683	This work		*cest-1.1(dp683) (S213A)*
Strain, strain background *Caenorhabditis elegans*	PS8259	This work		*cest-1.1(sy1180 sy1250)*
Strain, strain background *Caenorhabditis elegans*	PS8260	This work		*cest-1.1(sy1180 sy1251)*
Strain, strain background *Caenorhabditis elegans*	PS8261	This work		*cest-1.1(sy1181 sy1252)*
Strain, strain background *Caenorhabditis elegans*	PS8262	This work		*cest-1.1(sy1181 sy1253)*
Strain, strain background *Caenorhabditis elegans*	PS8008	This work		*cest-2.2(sy1170)*

Continued on next page

Continued

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain, strain background *Caenorhabditis elegans*	PS8009	This work		*cest-2.2 (sy1171)*
Strain, strain background *Caenorhabditis elegans*	PS8236	This work		*cest-2.2(sy1170 sy1236)*
Strain, strain background *Caenorhabditis elegans*	PS8238	This work		*cest-2.2(sy1171 sy1238)*
Strain, strain background *Caenorhabditis elegans*	FCS02	SunyBiotech		*cest-2.2-mCherry*
Strain, strain background *Caenorhabditis elegans*	PS8116	This work		*cest-4(sy1192)*
Strain, strain background *Caenorhabditis elegans*	PS8117	This work		*cest-4(sy1193)*
Strain, strain background *Caenorhabditis elegans*	PS8781	This work		*cest-4(sy1192)*
Strain, strain background *Caenorhabditis elegans*	PS8782	This work		*cest-4(sy1193)*
Strain, strain background *Caenorhabditis elegans*	PS8783	This work		*cest-4(sy1194)*
Strain, strain background *Caenorhabditis elegans*	PS8784	This work		*cest-4(sy1195)*
Strain, strain background *Caenorhabditis elegans*	RB1804	Caenorhabditis Genetics Center (CGC)		*cest-6(ok2338)*
Strain, strain background *Caenorhabditis elegans*	PS8029	This work		*cest-19(sy1178)*
Strain, strain background *Caenorhabditis elegans*	PS8030	This work		*cest-19(sy1179)*
Strain, strain background *Caenorhabditis elegans*	PS8033	This work		*cest-33(sy1182)*
Strain, strain background *Caenorhabditis elegans*	PS8034	This work		*cest-33(sy1183)*
Strain (*Caenorhabditis briggsae*)	PS8515	This work		CBR-*glo-1(sy1382)*
Strain (*Caenorhabditis briggsae*)	PS8516	This work		CBR-*glo-1(sy1383)*
Peptide, recombinant protein	Proteinase K	New England Biolabs		New England Biolabs: P8107S
Software, algorithm	Metaboseek	Metaboseek (metaboseek.com)		Version 0.9.6
Software, algorithm	GraphPad Prism	GraphPad Prism (graphpad.com)		Version 8.4.3

General information

Unless noted otherwise, all reagents were purchased from Sigma-Aldrich. All newly identified compounds were assigned four letter 'SMID's (a search-compatible, Small Molecule IDentifier) for example 'icas#3' or 'ascr#10'. For a list of all compounds referred to in the text and figures, see *Appendix 1—table 9*. The SMID database (www.smid-db.org) is an electronic resource maintained in collaboration with WormBase (www.wormbase.org). A complete list of SMIDs can be found at www.smid-db.org/browse, and example structures for different SMIDs at www.smid-db.org/smidclasses.

BLAST analysis of *uar-1*

Amino acid sequence of *Ppa*-UAR-1 was used as previously published (*Falcke et al., 2018*). BLASTp was run from the WormBase engine at (https://wormbase.org/tools/blast_blat). E-value threshold was set to 1E0. Database was set to WS269 and species was set to *C. elegans*. Results of BLASTp search are listed in *Appendix 1—table 2*.

Amino acid sequence alignment

hAChE was aligned with *Ppa*-UAR-1, CEST-1.1, CEST-2.2, and CEST-4 was done using T-Coffee Multiple Sequence alignment (*Notredame et al., 2000*). Protein sequences for *C. elegans* CEST proteins are from WormBase. The AChE sequence was obtained from NCBI (accession number P22303). Amino acids were colored based on chemical properties: AVFPMILW = red (small + hydrophobic), DE = blue (acidic), RHK = magenta (basic), STYHCNGQ = green (hydroxyl + sulfhydryl + amine + glycine). See *Figure 4—figure supplement 1* for results.

Phylogenetic tree

The protein sequence of Ppa-UAR1 was submitted to an NCBI BLASTp search (*Altschul et al., 2005*) (restricted to species *C. elegans*, conditional compositional BLOSUM62, gap open cost:11, gap extension cost: 1, word size: 6) using Geneious software (Biomatters Inc). The top BLAST hits by E-value up to and including *ace-3* were selected, and only the best scoring transcript variant was kept for each protein sequence hit. A total of 28 sequences were then imported into MEGA7 (*Kumar et al., 2016*) and aligned using MUSCLE (*Edgar, 2004*) (settings: gap open penalty: −2.9, gap extend 0, hydrophobicity multiplier 1.2, max. iterations 8, clustering method for all iterations: UPGMB, minimal diagonal length: 24). From this alignment, an Maximum Likelihood tree was built based on the JTT matrix-based model (*Jones et al., 1992*). Initial trees were built by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model assuming uniform substitution rates across positions. Phylogeny confidence was tested using 200 bootstrap replications. The tree with the highest log likelihood (−22299.9282) is shown. At each branch, the percentage of bootstrap replicates containing the same branching event is denoted. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (*Jones et al., 1992*). The tree with the highest log likelihood (−22299.9282) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 427 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (*Kumar et al., 2016*; *Felsenstein, 1985*).

Nematode strains

Wild-type (N2) and *glo-1(zu437)* null animals were provided by the Caenorhabditis Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). *cest-2.2* mutant strains integrating N-terminal (mCherry-*cest-2.2*) or C-terminal mCherry (*cest-2.2*-mCherry) were generated by SunyBiotech. Generation of *C. elegans and C. briggsae* null mutants and revertants as well as generation of the *cest-1.1* point mutant is described below. See *Appendix 1—table 3* for a complete list of strains used in this study.

C. elegans CRISPR mutagenesis for generation of *cest* null mutants

CRISPR/Cas9 mutagenesis was performed as in *Wang et al., 2018*. Briefly, *C. elegans* strain N2 was gene-edited by insertion of a 43-base-pair insertion that disrupts translation *Appendix 1—table 8*. Independent homozygous mutants were picked among the progeny of heterozygous F1 progeny of injected hermaphrodites and given distinct unique allele names. Reversion of mutants was accomplished in the same way.

C. briggsae CRISPR mutagenesis for generation of *glo-1* null mutants

The *C. briggsae glo-1* mutants *sy1382* and *sy1383* were both created using the briggsae adaptation of the STOP-IN cassette method as described in *Cohen and Sternberg, 2019* and *Wang et al., 2018*. Both strains were made using a successful insertion of the STOP-IN cassette into the middle of the first exon using the guide AACAAATCTCCGGATGATTG. To detect the insertion, we used forward primer GGGTGACCGCCCATTTATTG and reverse primer AAAGGCGCACATCTTGCTTC.

C. elegans CRISPR mutagenesis for generation of the cest-1.1(dp683) allele encoding the S213A catalytic mutant

cest-1.1(dp683) was generated as previously described (Paix et al., 2015). Briefly, daf-2(e1368) mutant animals were injected with in-vitro-assembled Cas9-crRNA-tracrRNA complexes targeting cest-1.1 and the dpy-10 co-CRISPR gene and two 100 bp repair oligonucleotides containing the desired cest-1.1 mutation and the dpy-10(cn64) co-CRISPR mutation (Arribere et al., 2014). Sequences of the cest-1.1 crRNA and repair oligonucleotide are 5' acctacCGCTACTATCATAC 3' and 5' GAAA TTGAAAACTTTGGAGGAAATAAAAACAGAATTACATTGGCAGGGCATGCCGCTGGAGCAAGTA TGATAGTAGCGgtaggtcacataaatgatacatttttg 3', respectively. F1 Rol progeny of injected animals were picked and screened for the presence of the cest-1.1(dp683) mutation after egglay. F2 broods of F1 Rol animals that were heterozygous for cest-1.1(dp683) were screened for animals that were homozygous for cest-1.1(dp683) and either wild-type or heterozygous for cn64 at the dpy-10 locus. Subsequent broods were screened for wild-type dpy-10 animals to remove the co-CRISPR mutation.

Nematode imaging

To image, gravid adult C. elegans were transferred to an agarose pad on a glass slide with 10 µM of levamisole to immobilize the worms. Microscopic analysis was performed using a Leica TCS SP5 Laser Scanning Confocal Microscope. Green autofluorescence was excited at 488 nm and the emission detector was set to 490–540 nm. mCherry was excited with 561 nm and the emission detector was set to 590–650 nm. Worms were imaged using the 100x objective.

C. briggsae imaging

0.5 mL of 2 µM Lysotracker Deep Red (Thermo Fisher 1 mM stock in DMSO) was added to a 6 cm NGM plate seeded with 0.1 mL of E. coli OP50 and incubated in the dark for 24 hr at 20°C. L4 larvae of C. briggsae were added to the plate and allowed to grow in the dark for 24 hr at 20°C. To image, C. briggsae were transferred to an agarose pad on a glass slide with 10 µM of levamisole to immobilize the worms. Microscopic analysis was performed using a Zeiss Axio Imager Z2 florescence microscope with Apotome.

Nematode cultures, mixed stage

Culturing began by chunking C. elegans or C. briggsae onto 10 cm NGM plates (each seeded with 800 µL of OP50 E. coli grown to stationary phase in Lennox Broth) and incubated at 22°C. Once the food was consumed, the cultures were incubated for an additional 24 hr. Each plate was then washed with 25 mL of S-complete medium into a 125 mL Erlenmeyer flask, and 1 mL of OP50 E. coli was added (E. coli cultures were grown to stationary phase in Terrific Broth, pelleted and resuspended at 1 g wet mass per 1 mL M9 buffer), shaking at 220 RPM and 22°C. After 70 hr, cultures were centrifuged at 5000 G for 1 min. After discarding supernatant, 24 mL H_2O was added, along with 6 mL bleach, 900 µL 10 M NaOH and the mixture was shaken for 3 min to prepare eggs. Eggs were centrifuged at 5000 G, the supernatant was removed, and the egg pellet washed with 35 mL M9 buffer twice and then suspended in a final volume of 5 mL M9 buffer in a 50 mL centrifuge tube. Eggs were counted and placed on a rocker and allowed to hatch as L1 larvae for 24 hr at 22°C. 70,000 L1 larvae were seeded in 25 mL cultures of S-complete with 1 mL of OP50 and incubated at 220 RPM and 22°C in a 125 mL Erlenmeyer flask. After 72 hr, cultures were fed an additional 1 mL of OP50 and incubation continued. After an additional 48 hr, worms were spun at 1000 G 5 min and spent medium was separated from worm body pellet. Separated medium and worm pellet were flash frozen over liquid nitrogen until further processing. At least three biological replicates were grown for all mutant strains. Mutants were grown with parallel wildtype controls, and biological replicates were started on different days.

Metabolite extraction

Lyophilized pellet and media samples were crushed and homogenized by shaking with 2.5 mm steel balls at 1300 rpm for 3 min in 30 s pulses while chilled with liquid nitrogen (SPEX sample prep miniG 1600). Thus powdered media and pellet samples were extracted with 15 mL methanol in 50 mL centrifuge tubes, rocking overnight at 22°C. Extractions were pelleted at 5000 g for 10 min at 4°C, and supernatants were transferred to 20 mL glass scintillation vials. Samples were then dried in a

SpeedVac (Thermo Fisher Scientific) vacuum concentrator. Dried materials were resuspended in 1 mL methanol and vortexed for 1 min. Samples were pelleted at 5000 g for 5 min and 22°C, and supernatants were transferred to 2 mL HPLC vials and dried in a SpeedVac vacuum concentrator. Samples were then resuspended in 200 µL of methanol, transferred into 1.7 mL Eppendorf tubes, and centrifuged at 18,000 G for 20 min at 4°C. Clarified extracts were transferred to fresh HPLC vials and stored at −20°C until analysis.

Preparation of *exo*-metabolome samples from staged starved and fed cultures

40,000 synchronized L1 larvae were added to 125 mL Erlenmeyer flasks containing 30 mL of S-complete medium. Worms were fed with 4 mL of concentrated OP-50 and incubated at 20°C with shaking at 160 RPM for: 12 hr (L1), 24 hr (L2), 32 hr (L3), 40 hr (L4) and 58 hr (gravid adults). For preparation of starved samples, each of the stages was starved for 24 hr after reaching their desired developmental stage in S-complete without OP-50. After incubation for the desired time, liquid cultures were centrifuged (1000 x g, 22°C, 1 min) and supernatants were collected. Supernatant was separated from intact OP-50 cells by centrifuging (3000 x g, 22°C, 5 min) and the resulting supernatants (*exo*-metabolome) were lyophilized. Lyophilized samples were homogenized with a dounce homogenizer in 10 mL methanol and extracted on a stirring plate (22°C, 12 hr). The resulting suspension was centrifuged (4000 g, 22°C, 5 min) to remove any precipitate before carefully transferring to an LC-MS sample vial. Three biological replicates were started on different days.

Mass spectrometric analysis

High resolution LC-MS analysis was performed on a Thermo Fisher Scientific Vanquish Horizon UHPLC System coupled with a Thermo Q Exactive HF hybrid quadrupole-orbitrap high-resolution mass spectrometer equipped with a HESI ion source. 1 µL of extract was injected and separated using at water-acetonitrile gradient on a Thermo Scientific Hypersil GOLD C18 column (150 mm x 2.1 mm 1.9 um particle size 175 Å pore size, Thermo Scientific) and maintained at 40°C. Solvents were all purchased from Fisher Scientific as HPLC grade. Solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile. A/B gradient started at 1% B for 5 min, then from 1% to 100% B over 20 min, 100% for 5 min, then down to 1% B for 3 min. Mass spectrometer parameters: 3.5 kV spray voltage, 380°C capillary temperature, 300°C probe heater temperature, 60 sheath flow rate, 20 auxiliary flow rate, one spare gas; S-lens RF level 50.0, resolution 240,000, *m/z* range 100–1200 m/z, AGC target 3e6. Instrument was calibrated with positive and negative ion calibration solutions (Thermo-Fisher) Pierce LTQ Velos ESI pos/neg calibration solutions.

Feature detection and characterization

LC−MS RAW files from each sample were converted to mzXML (centroid mode) using MSConvert (ProteoWizard), followed by analysis using the XCMS (*Smith et al., 2006*) analysis feature in METABOseek (metaboseek.com). Peak detection was carried out with the centWave algorithm (*Tautenhahn et al., 2008*), values set as: 4 ppm, 320 peakwidth, 3 snthresh, 3100 prefilter, FALSE fitgauss, 1 integrate, TRUE firstBaselineCheck, 0 noise, wMean mzCenterFun, −0.005 mzdiff. XCMS feature grouping values were set as: 0.2 minfrac, 2 bw, 0.002 mzwid, 500 max, 1 minsamp, FALSE usegroup. METABOseek peak filling values set as: 5 ppm_m, 5 rtw, TRUE rtrange. Resulting tables were then processed with the METABOseek Data Explorer. Molecular features were filtered for each particular null mutant against all other mutants. Filter values were set as: 10 to max minFoldOverCtrl, 15000 to max meanInt, 120 to 1500 rt, 0.95 to max Peak Quality as calculated by METABOseek. Features were then manually curated by removing isotopic and adducted redundancies. Remaining masses were put on the inclusion list for MS/MS (ddMS2) characterization. Positive and negative mode data were processed separately. In both cases we checked if a feature had a corresponding peak in the opposite ionization mode, since fragmentation spectra in different modes often provide complementary structural information. To acquire MS2 spectra, we ran a top-10 data dependent MS2 method on a Thermo QExactive-HF mass spectrometer with MS1 resolution 60,000, AGC target 1×10^6, maximum IT (injection time) 50 ms, MS2 resolution 45,000, AGC target 5×10^5, maximum IT 80 ms, isolation window 1.0 m/z, stepped NCE (normalized collision energy) 25, 50, dynamic exclusion 3 s.

Statistical analysis

Peak integration data from HPLC-MS analysis were log-transformed (*Karpievitch et al., 2012*) prior to statistical analysis. Significance of differences between average peak areas were then assessed using unpaired t-tests.

MS²-based molecular networking

For the differential featuresidentified above, MS^2 data was acquired. To generate the MS^2 molecular network, Metaboseek version 0.9.6 was used. Using the MS2scans function, differential features were matched with their respective MS^2 scan, using an *m/z* window of 5 ppm, and a retention time window of 15 s. To construct the molecular network, tolerance of the fragment peaks was set to *m/z* of 0.002 or 5 ppm, minimum number of peaks was set to 5, with a 2% noise level. Once the network was constructed, a cosine value of 0.8 was used, and the number of possible connections was constrained to 5.

Serine hydrolase dendrogram

The serine hydrolase list was reported previously (*Chen et al., 2019*). From this list, sequences were inputted into Geneious Prime (version 2020.1.2 Biomatters). Sequences were aligned using Clustal Omega, neighbor joining alignment. Dendrogram tree was generated using the Geneious Tree Builder; Genetic distance model Jukes-Cantor, Tree build method UPGMA, no outgroup, Bootstrap resampling, random seed 508,949, 300 interactions, support threshold of 1. CEST enzymes were colored red and PPA-UAR-1 was colored blue (*Figure 1—figure supplement 1*).

Synthetic procedures

Synthesis of iglu#1 (15). iglu#1 was synthesized as described previously (*Messaoudi et al., 2004*).

Synthesis of angl#1 (17). angl#1 was synthesized as described previously (*Coburn et al., 2013*).
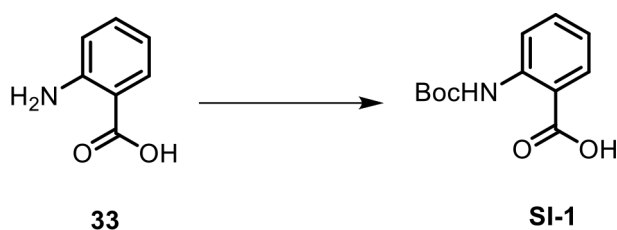
Scheme 1. Synthesis of 2-((*tert*-butoxycarbonyl)amino)benzoic acid (Boc-AA, SI-1).

To a solution of anthranilic acid (**33**, 300 mg, 2.18 mmol) in 4 mL of THF and H$_2$O (1:1), Boc-anhydride (521 mg, 2.39 mmol) was added, and 2 M NaOH was added to the mixture until pH 10 was reached. The reaction mixture was stirred at room temperature. After 23 hr, the solution was concentrated in vacuo, and 15% citric acid aqueous solution was added until pH 4 was reached. The white precipitate was filtered off and dried under vacuum to provide 2-((*tert*-butoxycarbonyl)amino) benzoic acid (**SI-1**, 497 mg, 96%) as a white solid. ^{1}H NMR, 600 MHz, chloroform-*d*: δ (ppm) 10.06 (s, 1H), 8.47 (dd, *J* = 8.7, 0.9 Hz, 1H), 8.08 (dd, *J* = 7.9, 1.5 Hz, 1H), 7.57 (dt, *J* = 7.9, 1.5 Hz, 1H), 7.03 (dt, *J* = 7.2, 1.2 Hz, 1H), 1.55 (s, 9H).
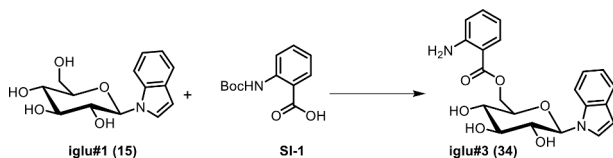

Scheme 2. Synthesis of *N-β*-(6-(2'-aminobenzoyl)-glucopyranosyl) indole (iglu#3, 34).

To a stirred solution of *N*-(*tert*-butoxycarbonyl)anthranilic acid (*Krueger et al., 2008*) (SI-1, 10 mg, 0.042 mmol) in dimethylformamide, 1-(3-dimethylaminopropyl)−3-ethylcarbodiimide

hydrochloride (EDC·HCl, 20.1 mg, 0.105 mmol) was added. The mixture was stirred at room temperature for 5 min, and 4-dimethylaminopyridine (DMAP, 18.1 mg, 0.105 mmol) and N-β-glucopyranosyl indole (iglu#1, **15**, 9.8 mg, 0.0351 mmol) were added. The reaction mixture was stirred at room temperature. After 5 hr, the mixture was concentrated in vacuo to yield a viscous oil, which was dissolved in 1.4 mL of a 5:2 mixture of dichloromethane and methanol. Trifluoroacetic acid (TFA, 0.5 mL) was added slowly and the reaction mixture was stirred at room temperature. After 3 hr, the mixture was concentrated in vacuo. Preparative HPLC provided a pure sample of iglu#3 (**34**, 0.8 mg, 5.7%). See *Appendix 1—table 4* for NMR spectroscopic data of iglu#3.

HRMS (ESI) m/z: $[M - H]^-$ calcd for $C_{21}H_{21}N_2O_6^-$ 397.13938; found 397.14017.
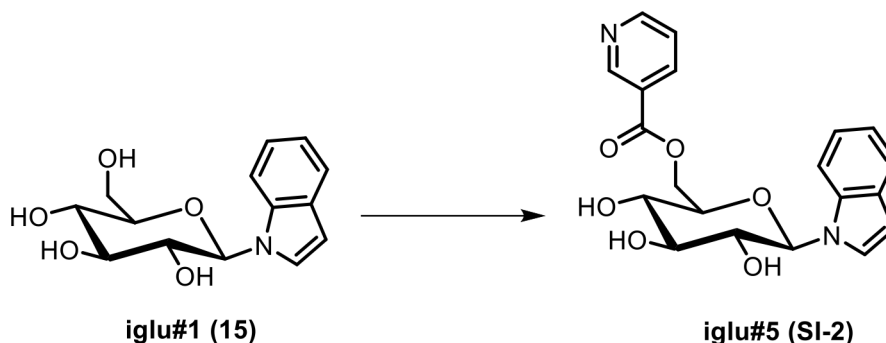
Scheme 3. Synthesis of N-β-(6-nicotinoylglucopyranosyl) indole (iglu#5, SI-2).

To a stirred solution of nicotinic acid (7.3 mg, 0.059 mmol) in a mixture of dimethylformamide and dichloromethane (1:1), EDC·HCl (28.4 mg, 0.148 mmol) was added. The mixture was stirred at room temperature for 30 min, before DMAP (18.1 mg, 0.148 mmol) and N-β-glucopyranosyl indole (iglu#1, **15**, 13.8 mg, 0.0494 mmol) were added. The reaction mixture was stirred at room temperature for 20 hr, the mixture was concentrated in vacuo, and flash column chromatography on silica using a gradient of 0–25% methanol in dichloromethane afforded **iglu#5** (**SI-2**, 2.5 mg, 13.9%) as a colorless oil. See *Appendix 1—table 5* for NMR spectroscopic data of iglu#5.

HRMS (ESI) m/z: $[M + H]^+$ calcd for $C_{20}H_{21}N_2O_6^+$ 385.13941; found 385.14038.
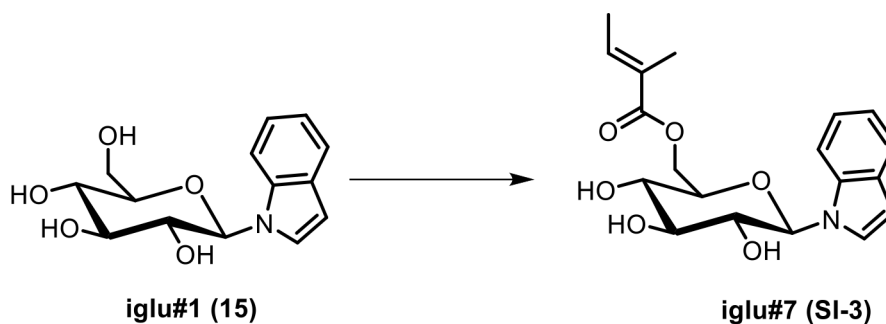
Scheme 4. Synthesis of N-β-(6-(2'-methylbut-2'E-enoyl)-glucopyranosyl) indole (iglu#7, SI-3).

To a stirred solution of tiglic acid (5.0 mg, 0.050 mmol) in a 1:1 mixture of dimethylformamide and dichloromethane, EDC·HCl (23.9 mg, 0.125 mmol) was added. The mixture was stirred at room temperature for 30 min, and DMAP (15.2 mg, 0.125 mmol) and N-β-glucopyranosyl indole (iglu#1, **15**, 11.6 mg, 0.0416 mmol) were added. The reaction mixture was stirred at room temperature for 22 hr and then concentrated in vacuo. Flash column chromatography on silica using a gradient of 0–30% methanol in dichloromethane afforded **iglu#7** (**SI-3**, 2.5 mg, 11.3%) as a colorless oil. See *Appendix 1—table 6* for NMR spectroscopic data of iglu#7.

HRMS (ESI) m/z: $[M + H]^+$ calcd for $C_{19}H_{24}NO_6^+$ 362.15981; found 362.16025.
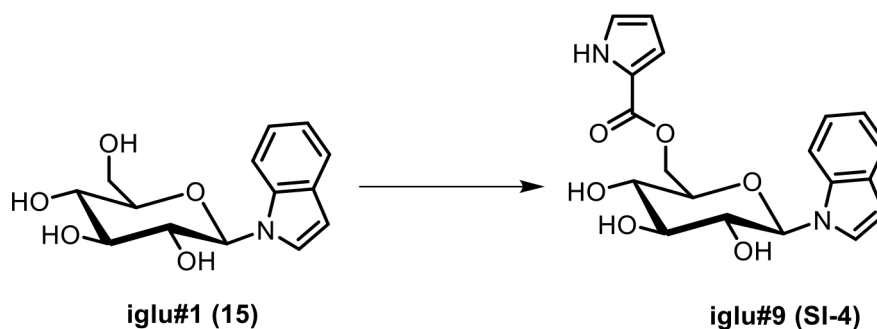
Scheme 5. Synthesis of *N-β*-(6-(pyrrole-2'-carbonyl)-glucopyranosyl) indole (iglu#9, SI-4).

To a suspension of pyrrole-2-carboxylic acid (6.0 mg, 0.054 mmol) in dichloromethane, oxalyl chloride (14 µL, 0.163 mmol) was added slowly, followed by dimethylformamide (1 µL, 0.0129 mmol). The mixture was stirred at room temperature for 18 hr and then concentrated to dryness in vacuo. The residue was re-dissolved in dimethylformamide (2 mL) containing *N-β*-glucopyranosyl indole (iglu#1, **15**, 10.8 mg, 0.0387 mmol). Triethylamine (45 µL, 0.324 mmol) was added, and the reaction was stirred at 35°C for 7 days. Subsequently the mixture was concentrated in vacuo, and flash column chromatography on silica using a gradient of 0–30% methanol in dimethylformamide afforded **iglu#9** (**SI-4**, 1.5 mg, 10.4%) as a colorless oil. See *Appendix 1—table 7* for NMR spectroscopic data of iglu#9.

HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{19}H_{21}N_2O_6^+$ 373.13941; found 373.14026.
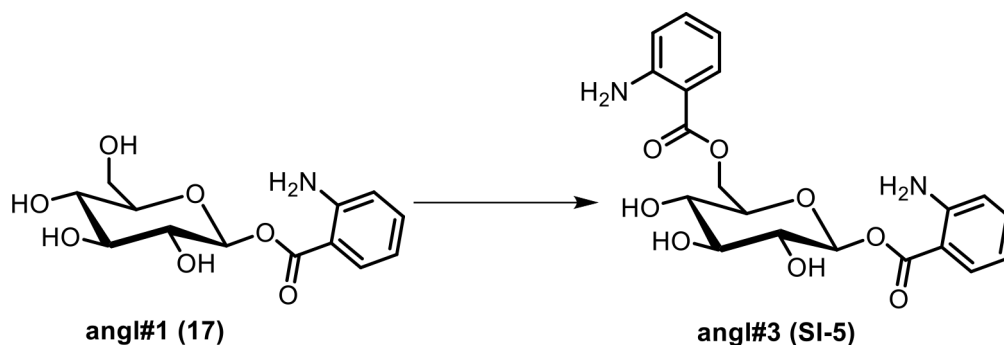
Scheme 6. Synthesis of an HPLC standard of ((2*R*,3*S*,4*S*,5*R*,6*S*)−6-((2-aminobenzoyl)oxy)−3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl 2-aminobenzoate (angl#3, SI-5).

To a stirred solution of Boc-AA (2 mg, 0.00 84 mmol) in dimethylformamide, 1-(3-dimethylaminopropyl)−3-ethylcarbodiimide hydrochloride (3.9 mg, 0.0203 mmol) was added. The mixture was stirred at room temperature for 5 min, and 4-dimethylaminopyridine (2.5 mg, 0.0203 mmol) and angl#1 (**17**, 2 mg, 0.0068 mmol) were added. The reaction mixture was stirred at room temperature. After 5 hr, the mixture was concentrated in vacuo. The crude product was dissolved in 0.55 mL dichloromethane and methanol (10:1), and trifluoroacetic acid (500 µL) was added slowly. The reaction mixture was stirred at room temperature for 3 hr and then was concentrated in vacuo, affording **angl#3** (**SI-5**).

HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{20}H_{23}N_2O_7^+$ 403.14998; found 403.15100.
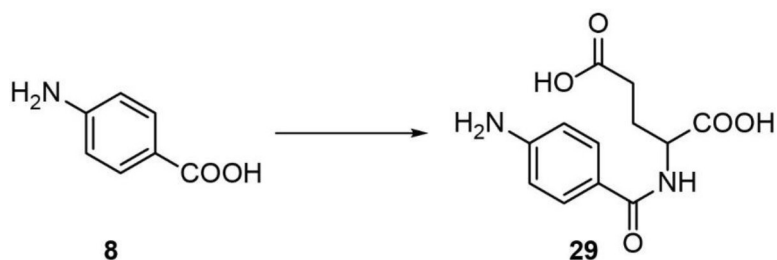
Scheme 7. Synthesis of an HPLC standard of *N*-(*p*-aminobenzoyl)glutamate (PABA-glutamate) (29).

p-Aminobenzoic acid (Chem-Impex) (**8**) was dissolved in warm dichloromethane (DCM) containing triethylamine (0.1 eq). EDC·HCl (Amresco Biochemicals) (1 eq), and di-*tert*-butyl glutamate (1 eq) was added to the reaction mixture. *N*,*N*-Dimethylaminopyridine (1.1 eq) was then added and the resulting mixture was stirred at room temperature for 24 hr and then extracted with ethyl acetate. The combined extractswere dried with sodium sulfate and evaporated to dryness *in vacuo*. The crude product was dissolved in DCM, and trifluoroacetic acid was added (100 eq). The reaction was then stirred for 6 hr at room temperature. TFA and DCM were evaporated off to yield crude PABA-glutamate (**29**). ^{1}H NMR, 600 MHz, methanol-d_4: δ (ppm) 7.93 (d, *J* = 8.6 Hz, 2H), 7.37 (d, *J* = 8.5 Hz, 2H), 4.61 (dd, *J* = 5.0, 9.3 Hz, 1H), 2.09–2.28 (m, 4H).

NMR spectra appendix. NMR spectra of synthetic intermediates and newly identified metabolites.

Acknowledgements

Additional information

Funding

Author contributions

Henry H Le, Chester JJ Wrobel, Conceptualization, Data curation, Formal analysis, Investigation, Writing - original draft, Writing - review and editing; Sarah M Cohen, Conceptualization, Resources, Methodology; Jingfang Yu, Resources, Formal analysis; Heenam Park, Resources, Methodology; Maximilian J Helf, Software, Methodology; Brian J Curtis, Resources, Investigation; Joseph C Kruempel, Patrick J Hu, Resources; Pedro Reis Rodrigues, Data curation, Investigation; Paul W Sternberg, Conceptualization, Funding acquisition, Writing - original draft, Project administration, Writing - review and editing; Frank C Schroeder, Conceptualization, Formal analysis, Supervision, Funding acquisition, Writing - original draft, Project administration, Writing - review and editing

Author ORCIDs

Henry H Le http://orcid.org/0000-0003-2942-2357
Jingfang Yu http://orcid.org/0000-0003-1770-5368
Paul W Sternberg https://orcid.org/0000-0002-7699-0173
Frank C Schroeder https://orcid.org/0000-0002-4420-0237

Additional files

Supplementary files

• Supplementary file 1. NMR spectra appendix. NMR spectra of synthetic intermediates and newly identified metabolites.

• Transparent reporting form

Data availability

All data generated or analysed during this study are included in the manuscript and supporting files. MS/MS data is available via MassIVE under accession number: MSV000086293.

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Le HH, Wrobel CJJ, Cohen SM, Yu J, Park H, Helf MJ, Curtis BJ, Kruempel JC, Rodrigues PR, Hu PJ, Sternberg PW, Schroeder FC	2020	Modular metabolite assembly in C. elegans depends on carboxylesterases and formation of lysosome-related organelles	https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=715e60ce44ae4ecea2-b84e28dd336c01	MassIVE, MSV0000 86293

References

Aalto AP, Nicastro IA, Broughton JP, Chipman LB, Schreiner WP, Chen JS, Pasquinelli AE. 2018. Opposing roles of microRNA argonautes during *Caenorhabditis elegans* aging. *PLOS Genetics* **14**:e1007379. DOI: https://doi.org/10.1371/journal.pgen.1007379, PMID: 29927939

Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schäffer AA, Yu YK. 2005. Protein database searches using compositionally adjusted substitution matrices. *FEBS Journal* **272**:5101–5109. DOI: https://doi.org/10.1111/j.1742-4658.2005.04945.x, PMID: 16218944

Aprison EZ, Ruvinsky I. 2017. Counteracting ascarosides act through distinct neurons to determine the sexual identity of *C. elegans* Pheromones. *Current Biology* **27**:2589–2599. DOI: https://doi.org/10.1016/j.cub.2017.07.034, PMID: 28844646

Arribere JA, Bell RT, Fu BX, Artiles KL, Hartman PS, Fire AZ. 2014. Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in *Caenorhabditis elegans*. *Genetics* **198**:837–846. DOI: https://doi.org/10.1534/genetics.114.169730, PMID: 25161212

Artyukhin AB, Yim JJ, Srinivasan J, Izrayelit Y, Bose N, von Reuss SH, Jo Y, Jordan JM, Baugh LR, Cheong M, Sternberg PW, Avery L, Schroeder FC. 2013. Succinylated octopamine ascarosides and a new pathway of biogenic amine metabolism in *Caenorhabditis elegans*. *Journal of Biological Chemistry* **288**:18778–18783. DOI: https://doi.org/10.1074/jbc.C113.477000, PMID: 23689506

Artyukhin AB, Zhang YK, Akagi AE, Panda O, Sternberg PW, Schroeder FC. 2018. Metabolomic "Dark Matter" Dependent on Peroxisomal β-Oxidation in *Caenorhabditis elegans*. *Journal of the American Chemical Society* **140**:2841–2852. DOI: https://doi.org/10.1021/jacs.7b11811, PMID: 29401383

Bemben MA, Shipman SL, Nicoll RA, Roche KW. 2015. The cellular and molecular landscape of neuroligins. *Trends in Neurosciences* **38**:496–505. DOI: https://doi.org/10.1016/j.tins.2015.06.004, PMID: 26209464

Bergame CP, Dong C, Sutour S, von Reuß SH. 2019. Epimerization of an Ascaroside-Type glycolipid downstream of the canonical β-Oxidation cycle in the nematode *Caenorhabditis nigoni*. *Organic Letters* **21**:9889–9892. DOI: https://doi.org/10.1021/acs.orglett.9b03808, PMID: 31809061

Bird PI, Trapani JA, Villadangos JA. 2009. Endolysosomal proteases and their inhibitors in immunity. *Nature Reviews Immunology* **9**:871–882. DOI: https://doi.org/10.1038/nri2671, PMID: 19935806

Bose N, Meyer JM, Yim JJ, Mayer MG, Markov GV, Ogawa A, Schroeder FC, Sommer RJ. 2014. Natural variation in dauer pheromone production and sensing supports intraspecific competition in Nematodes. *Current Biology* **24**:1536–1541. DOI: https://doi.org/10.1016/j.cub.2014.05.045, PMID: 24980503

Butcher RA, Fujita M, Schroeder FC, Clardy J. 2007. Small-molecule pheromones that control dauer development in *Caenorhabditis elegans*. *Nature Chemical Biology* **3**:420–422. DOI: https://doi.org/10.1038/nchembio.2007.3, PMID: 17558398

Butcher RA. 2017. Decoding chemical communication in Nematodes. *Natural Product Reports* **34**:472–477. DOI: https://doi.org/10.1039/C7NP00007C, PMID: 28386618

Cao Z, Hao Y, Fung CW, Lee YY, Wang P, Li X, Xie K, Lam WJ, Qiu Y, Tang BZ, Shui G, Liu P, Qu J, Kang BH, Mak HY. 2019. Dietary fatty acids promote lipid droplet diversity through seipin enrichment in an ER subdomain. *Nature Communications* **10**:2902. DOI: https://doi.org/10.1038/s41467-019-10835-4, PMID: 31263173

Chen AL, Lum KM, Lara-Gonzalez P, Ogasawara D, Cognetta AB, To A, Parsons WH, Simon GM, Desai A, Petrascheck M, Bar-Peled L, Cravatt BF. 2019. Pharmacological convergence reveals a lipid pathway that regulates *C. elegans* lifespan. *Nature Chemical Biology* **15**:453–462. DOI: https://doi.org/10.1038/s41589-019-0243-4, PMID: 30911178

Coburn C, Allman E, Mahanti P, Benedetto A, Cabreiro F, Pincus Z, Matthijssens F, Araiz C, Mandel A, Vlachos M, Edwards SA, Fischer G, Davidson A, Pryor RE, Stevens A, Slack FJ, Tavernarakis N, Braeckman BP, Schroeder FC, Nehrke K, et al. 2013. Anthranilate fluorescence marks a calcium-propagated necrotic wave that promotes organismal death in *C. elegans*. *PLOS Biology* **11**:e1001613. DOI: https://doi.org/10.1371/journal.pbio.1001613, PMID: 23935448

Coburn C, Gems D. 2013. The mysterious case of the *C. elegans* gut granule: death fluorescence, anthranilic acid and the kynurenine pathway. *Frontiers in Genetics* **4**:151. DOI: https://doi.org/10.3389/fgene.2013.00151, PMID: 23967012

Cohen S, Sternberg P. 2019. Genome editing of Caenorhabditis briggsae using CRISPR/Cas9 co-conversion marker dpy-10. *microPublication Biology* **2019**:000171. DOI: https://doi.org/10.17912/micropub.biology.000171

Curtis BJ, Kim LJ, Wrobel CJJ, Eagan JM, Smith RA, Burch JE, Le HH, Artyukhin AB, Nelson HM, Schroeder FC. 2020. Identification of uric acid Gluconucleoside-Ascaroside conjugates in *Caenorhabditis elegans* by Combining Synthesis and MicroED. *Organic Letters* **22**:6724–6728. DOI: https://doi.org/10.1021/acs.orglett.0c02038, PMID: 32820938

da Silva RR, Dorrestein PC, Quinn RA. 2015. Illuminating the dark matter in metabolomics. *PNAS* **112**:12549–12550. DOI: https://doi.org/10.1073/pnas.1516878112, PMID: 26430243

Dell'Angelica EC, Mullins C, Caplan S, Bonifacino JS. 2000. Lysosome-related organelles. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* **14**:1265–1278. DOI: https://doi.org/10.1096/fj.14.10.1265, PMID: 10877819

Dolke F, Dong C, Bandi S, Paetz C, Glauser G, von Reuß SH. 2019. Ascaroside signaling in the bacterivorous nematode *Caenorhabditis remanei* Encodes the Growth Phase of Its Bacterial Food Source. *Organic Letters* **21**:5832–5837. DOI: https://doi.org/10.1021/acs.orglett.9b01914, PMID: 31305087

Dong C, Dolke F, von Reuss SH. 2016. Selective MS screening reveals a sex pheromone in Caenorhabditis briggsae and species-specificity in indole ascaroside signalling. *Organic & Biomolecular Chemistry* **14**:7217–7225. DOI: https://doi.org/10.1039/C6OB01230B, PMID: 27381649

Dong C, Dolke F, Bandi S, Paetz C, von Reuß SH. 2020. Dimerization of conserved ascaroside building blocks generates species-specific male attractants in Caenorhabditis nematodes. *Organic & Biomolecular Chemistry* **18**:5253–5263. DOI: https://doi.org/10.1039/D0OB00799D, PMID: 32614033

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**:1792–1797. DOI: https://doi.org/10.1093/nar/gkh340, PMID: 15034147

Faghih N, Bhar S, Zhou Y, Dar AR, Mai K, Bailey LS, Basso KB, Butcher RA. 2020. A large family of enzymes responsible for the modular architecture of nematode pheromones. *Journal of the American Chemical Society* **142**:13645–13650. DOI: https://doi.org/10.1021/jacs.0c04223, PMID: 32702987

Falcke JM, Bose N, Artyukhin AB, Rödelsperger C, Markov GV, Yim JJ, Grimm D, Claassen MH, Panda O, Baccile JA, Zhang YK, Le HH, Jolic D, Schroeder FC, Sommer RJ. 2018. Linking genomic and metabolomic natural variation uncovers nematode pheromone biosynthesis. *Cell Chemical Biology* **25**:787–796. DOI: https://doi.org/10.1016/j.chembiol.2018.04.004, PMID: 29779955

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**:783–791. DOI: https://doi.org/10.1111/j.1558-5646.1985.tb00420.x, PMID: 28561359

Girard LR, Fiedler TJ, Harris TW, Carvalho F, Antoshechkin I, Han M, Sternberg PW, Stein LD, Chalfie M. 2007. WormBook: the online review of *Caenorhabditis elegans* biology. *Nucleic Acids Research* **35**:D472–D475. DOI: https://doi.org/10.1093/nar/gkl894, PMID: 17099225

Hermann GJ, Schroeder LK, Hieb CA, Kershner AM, Rabbitts BM, Fonarev P, Grant BD, Priess JR. 2005. Genetic analysis of lysosomal trafficking in *Caenorhabditis elegans*. *Molecular Biology of the Cell* **16**:3273–3288. DOI: https://doi.org/10.1091/mbc.e05-01-0060, PMID: 15843430

Jeong PY, Jung M, Yim YH, Kim H, Park M, Hong E, Lee W, Kim YH, Kim K, Paik YK. 2005. Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. *Nature* **433**:541–545. DOI: https://doi.org/10.1038/nature03201, PMID: 15690045

Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. *Bioinformatics* **8**:275–282. DOI: https://doi.org/10.1093/bioinformatics/8.3.275, PMID: 1633570

Kanzaki N, Tsai IJ, Tanaka R, Hunt VL, Liu D, Tsuyama K, Maeda Y, Namai S, Kumagai R, Tracey A, Holroyd N, Doyle SR, Woodruff GC, Murase K, Kitazume H, Chai C, Akagi A, Panda O, Ke HM, Schroeder FC, et al. 2018. Biology and genome of a newly discovered sibling species of *Caenorhabditis elegans*. *Nature Communications* **9**:3216. DOI: https://doi.org/10.1038/s41467-018-05712-5, PMID: 30097582

Karpievitch YV, Dabney AR, Smith RD. 2012. Normalization and missing value imputation for label-free LC-MS analysis. *BMC Bioinformatics* **13 Suppl 16**:S5. DOI: https://doi.org/10.1186/1471-2105-13-S16-S5, PMID: 23176322

Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. *Journal of Molecular Biology* **305**:567–580. DOI: https://doi.org/10.1006/jmbi.2000.4315, PMID: 11152613

Krueger EB, Rawson TE, Burdick DJ, Liang J, Zhu B-Y. 2008. Pyrimidine kinase inhibitors. *GENENTECH, INC.* WO/2008/079719. https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2008079719.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**:1870–1874. DOI: https://doi.org/10.1093/molbev/msw054, PMID: 27004904

Lejon S, Ellis J, Valegård K. 2008. The last step in cephalosporin C formation revealed: crystal structures of deacetylcephalosporin C acetyltransferase from acremonium chrysogenum in complexes with reaction intermediates. *Journal of Molecular Biology* **377**:935–944. DOI: https://doi.org/10.1016/j.jmb.2008.01.047, PMID: 18279889

Ludewig AH, Artyukhin AB, Aprison EZ, Rodrigues PR, Pulido DC, Burkhardt RN, Panda O, Zhang YK, Gudibanda P, Ruvinsky I, Schroeder FC. 2019. An excreted small molecule promotes *C. elegans* reproductive development and aging. *Nature Chemical Biology* **15**:838–845. DOI: https://doi.org/10.1038/s41589-019-0321-7, PMID: 31320757

Luzio JP, Hackmann Y, Dieckmann NM, Griffiths GM. 2014. The biogenesis of lysosomes and lysosome-related organelles. *Cold Spring Harbor Perspectives in Biology* **6**:a016840. DOI: https://doi.org/10.1101/cshperspect.a016840, PMID: 25183830

Mackenzie PI, Bock KW, Burchell B, Guillemette C, Ikushiro S, Iyanagi T, Miners JO, Owens IS, Nebert DW. 2005. Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. *Pharmacogenetics and Genomics* **15**:677–685. DOI: https://doi.org/10.1097/01.fpc.0000173483.13689.56, PMID: 16141793

Marks MS, Heijnen HF, Raposo G. 2013. Lysosome-related organelles: unusual compartments become mainstream. *Current Opinion in Cell Biology* **25**:495–505. DOI: https://doi.org/10.1016/j.ceb.2013.04.008, PMID: 23726022

Messaoudi S, Sancelme M, Polard-Housset V, Aboab B, Moreau P, Prudhomme M. 2004. Synthesis and biological evaluation of oxindoles and benzimidazolinones derivatives. *European Journal of Medicinal Chemistry* **39**:453–458. DOI: https://doi.org/10.1016/j.ejmech.2004.01.001, PMID: 15110971

Mindrebo JT, Nartey CM, Seto Y, Burkart MD, Noel JP. 2016. Unveiling the functional diversity of the alpha/beta hydrolase superfamily in the plant kingdom. *Current Opinion in Structural Biology* **41**:233–246. DOI: https://doi.org/10.1016/j.sbi.2016.08.005, PMID: 27662376

Morris C, Foster OK, Handa S, Peloza K, Voss L, Somhegyi H, Jian Y, Vo MV, Harp M, Rambo FM, Yang C, Hermann GJ. 2018. Function and regulation of the *Caenorhabditis elegans* Rab32 family member GLO-1 in lysosome-related organelle biogenesis. *PLOS Genetics* **14**:e1007772. DOI: https://doi.org/10.1371/journal.pgen.1007772, PMID: 30419011

Notredame C, Higgins DG, Heringa J. 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology* **302**:205–217. DOI: https://doi.org/10.1006/jmbi.2000.4042, PMID: 10964570

O'Donnell MP, Fox BW, Chao PH, Schroeder FC, Sengupta P. 2020. A neurotransmitter produced by gut Bacteria modulates host sensory behaviour. *Nature* **583**:415–420. DOI: https://doi.org/10.1038/s41586-020-2395-5, PMID: 32555456

Paix A, Folkmann A, Rasoloson D, Seydoux G. 2015. High efficiency, Homology-Directed genome editing in *Caenorhabditis elegans* using CRISPR-Cas9 ribonucleoprotein complexes. *Genetics* **201**:47–54. DOI: https://doi.org/10.1534/genetics.115.179382, PMID: 26187122

Palmisano NJ, Meléndez A. 2019. Autophagy in *C. elegans* development. *Developmental Biology* **447**:103–125. DOI: https://doi.org/10.1016/j.ydbio.2018.04.009, PMID: 29709599

Panda O, Akagi AE, Artyukhin AB, Judkins JC, Le HH, Mahanti P, Cohen SM, Sternberg PW, Schroeder FC. 2017. Biosynthesis of modular ascarosides in *C. elegans*. *Angewandte Chemie* **56**:4729–4733. DOI: https://doi.org/10.1002/anie.201700103, PMID: 28371259

Pungaliya C, Srinivasan J, Fox BW, Malik RU, Ludewig AH, Sternberg PW, Schroeder FC. 2009. A shortcut to identifying small molecule signals that regulate behavior and development in *Caenorhabditis elegans*. *PNAS* **106**:7708–7713. DOI: https://doi.org/10.1073/pnas.0811918106, PMID: 19346493

Rae R, Schlager B, Sommer RJ. 2008. Pristionchus pacificus: a genetic model system for the study of evolutionary developmental biology and the evolution of complex Life-History traits. *Cold Spring Harbor Protocols* **2008**:pdb emo102. DOI: https://doi.org/10.1101/pdb.emo102, PMID: 21356685

Rauwerdink A, Kazlauskas RJ. 2015. How the same core catalytic machinery catalyzes 17 different reactions: the Serine-Histidine-Aspartate catalytic triad of α/β-Hydrolase fold enzymes. *ACS Catalysis* **5**:6153–6176. DOI: https://doi.org/10.1021/acscatal.5b01539, PMID: 28580193

Sakai Y, Oku M, van der Klei IJ, Kiel JA. 2006. Pexophagy: autophagic degradation of peroxisomes. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research* **1763**:1767–1775. DOI: https://doi.org/10.1016/j.bbamcr.2006.08.023, PMID: 17005271

Schroeder FC. 2015. Modular assembly of primary metabolic building blocks: a chemical language in *C. elegans*. *Chemistry & Biology* **22**:7–16. DOI: https://doi.org/10.1016/j.chembiol.2014.10.012, PMID: 25484238

Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. 2006. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Analytical Chemistry* **78**:779–787. DOI: https://doi.org/10.1021/ac051437y, PMID: 16448051

Soreq H, Seidman S. 2001. Acetylcholinesterase–new roles for an old actor. *Nature Reviews Neuroscience* **2**:294–302. DOI: https://doi.org/10.1038/35067589, PMID: 11283752

Srinivasan J, von Reuss SH, Bose N, Zaslaver A, Mahanti P, Ho MC, O'Doherty OG, Edison AS, Sternberg PW, Schroeder FC. 2012. A modular library of small molecule signals regulates social behaviors in *Caenorhabditis elegans*. *PLOS Biology* **10**:e1001237. DOI: https://doi.org/10.1371/journal.pbio.1001237, PMID: 22253572

Stupp GS, von Reuss SH, Izrayelit Y, Ajredini R, Schroeder FC, Edison AS. 2013. Chemical detoxification of small molecules by *Caenorhabditis elegans*. *ACS Chemical Biology* **8**:309–313. DOI: https://doi.org/10.1021/cb300520u, PMID: 23163740

Tanji T, Nishikori K, Haga S, Kanno Y, Kobayashi Y, Takaya M, Gengyo-Ando K, Mitani S, Shiraishi H, Ohashi-Kobayashi A. 2016. Characterization of HAF-4- and HAF-9-localizing organelles as distinct organelles in *Caenorhabditis elegans* intestinal cells. *BMC Cell Biology* **17**:4. DOI: https://doi.org/10.1186/s12860-015-0076-2, PMID: 26817689

Tautenhahn R, Böttcher C, Neumann S. 2008. Highly sensitive feature detection for high resolution LC/MS. *BMC Bioinformatics* **9**:504. DOI: https://doi.org/10.1186/1471-2105-9-504, PMID: 19040729

Vaz FM, Wanders RJ. 2002. Carnitine biosynthesis in mammals. *Biochemical Journal* **361**:417–429. DOI: https://doi.org/10.1042/bj3610417, PMID: 11802770

von Reuss SH, Bose N, Srinivasan J, Yim JJ, Judkins JC, Sternberg PW, Schroeder FC. 2012. Comparative metabolomics reveals biogenesis of Ascarosides, a modular library of small-molecule signals in *C. elegans*. *Journal of the American Chemical Society* **134**:1817–1824. DOI: https://doi.org/10.1021/ja210202y, PMID: 22239548

von Reuss SH. 2018. Exploring modular glycolipids involved in nematode chemical communication. *CHIMIA International Journal for Chemistry* **72**:297–303. DOI: https://doi.org/10.2533/chimia.2018.297, PMID: 29789066

Wang M, Carver JJ, Phelan VV, Sanchez LM, Garg N, Peng Y, Nguyen DD, Watrous J, Kapono CA, Luzzatto-Knaan T, Porto C, Bouslimani A, Melnik AV, Meehan MJ, Liu WT, Crüsemann M, Boudreau PD, Esquenazi E, Sandoval-Calderón M, Kersten RD, et al. 2016. Sharing and community curation of mass spectrometry data with global natural products social molecular networking. *Nature Biotechnology* **34**:828–837. DOI: https://doi.org/10.1038/nbt.3597, PMID: 27504778

Wang H, Park H, Liu J, Sternberg PW. 2018. An efficient genome editing strategy to generate putative null mutants in *Caenorhabditis elegans* Using CRISPR/Cas9. *G3: Genes, Genomes, Genetics* **8**:3607–3616. DOI: https://doi.org/10.1534/g3.118.200662, PMID: 30224336

Wasmeier C, Romao M, Plowright L, Bennett DC, Raposo G, Seabra MC. 2006. Rab38 and Rab32 control post-Golgi trafficking of melanogenic enzymes. *Journal of Cell Biology* **175**:271–281. DOI: https://doi.org/10.1083/jcb.200606050, PMID: 17043139

Zhang X, Feng L, Chinta S, Singh P, Wang Y, Nunnery JK, Butcher RA. 2015. Acyl-CoA oxidase complexes control the chemical message produced by *Caenorhabditis elegans*. *PNAS* **112**:3955–3960. DOI: https://doi.org/10.1073/pnas.1423951112, PMID: 25775534

Zhang X, Li K, Jones RA, Bruner SD, Butcher RA. 2016. Structural characterization of acyl-CoA oxidases reveals a direct link between pheromone biosynthesis and metabolic state in *Caenorhabditis elegans*. *PNAS* **113**:10055–10060. DOI: https://doi.org/10.1073/pnas.1608262113, PMID: 27551084

Zhang X, Wang Y, Perez DH, Jones Lipinski RA, Butcher RA. 2018. Acyl-CoA oxidases Fine-Tune the production of ascaroside pheromones with specific side chain lengths. *ACS Chemical Biology* **13**:1048–1056. DOI: https://doi.org/10.1021/acschembio.7b01021, PMID: 29537254

Zheng Q, Wang S, Duan P, Liao R, Chen D, Liu W. 2016. An α/β-hydrolase fold protein in the biosynthesis of thiostrepton exhibits a dual activity for endopeptidyl hydrolysis and epoxide ring opening/macrocyclization. *PNAS* **113**:14318–14323. DOI: https://doi.org/10.1073/pnas.1612607113, PMID: 27911800

Appendix 1

Supporting tables

Appendix 1—table 1. MS[2] data of *glo-1*-dependent features presented in this manuscript.

						Representative MS/MS spectra of modular glucosides.			
Formula	RT [min.]	Compound number	SMID	m/z (M +H)	m/z (M-H)	ms/ms fragments, positive ionization mode	ms/ms fragments, negative ionization mode	Substituents on glucose	Stable isotope labeling
C26H26N3O12P	9.30		angl#10	604.13381	602.11813	105.03366 (C7 H5 O+) 120.04469 (C7 H6 O N+)	96.96870 (H2 O4 P-) 121.02911 (C7 H5 O2-) 136.03983 (C7 H6 O2 N-)	anthranilic acid, nicotinic acid	
C20H22N2O7	8.67	SI-5	angl#3	403.14998	401.13542	120.04459 (C7 H6 O N+) 138.05496 (C7 H8 O2 N+)		anthranilic acid, anthranilic acid	
C20H23N2O11P	9.26	25	angl#4	499.11235	497.09667	120.04463 (C7H6ON+)	96.96868 (H2 O4 P-) 78.95800 (O3 P-) 136.03999 (C7 H6 O2 N-) 223.00078 (C6 H8 O7 P-)	anthranilic acid, anthranilic acid	
C19H21N2O9P	9.59	22	iglu#10	453.10574	451.09119	94.02916 (C5 H4 O N+) 118.06535 (C8 H8 N+) C14 H12 O2 N (C14 H12 O2 N+)	78.95802 (O3 P-) 96.96867 (H2 O4 P-) 110.02444 (C5 H4 O2 N-) 116.05042 (C8 H6 N-)	indole, nicotinic acid	
C21H22NO9P	10.79	23	iglu#12	464.11049	462.09594	105.03382 (C7 H5 O+) 118.06538 (C8 H8 N+) 226.08620 (C14 H12 O2 N+) 348.12271 (C21 H18 O4 N+)	78.95801 (O3 P-) 96.96865 (H2 O4 P-)	indole, benzoic acid	
C14H18NO8P	6.05	16	iglu#2	360.08541	358.06973	98.98453 (H4 O4 P +) 118.06536 (C8 H8 N+) 244.09660 (C14 H14 O3 N+)	78.95802 (O3 P-) 96.96869 (H2 O4 P-)	indole	
C21H22N2O6	10.69	34	iglu#3	399.15506	397.13938		116.05032 (C8 H6 N-) 136.04002 (C7 H6 O2 N-) 215.09431 (C13 H13 O2 N-)	indole, anthranilic acid	
C21H23N2O9P	10.29	19	iglu#4	479.12252	477.10684	118.06536 (C8 H8 N+) 120.04456 (C7 H6 O N+) 138.05490 (C7 H8 O2 N+) 226.08612 (C14 H12 O2 N+)	78.95801 (O3 P-) 96.96867 (H2 O4 P-) 116.05042 (C8 H6 N-) 136.03970 (C7 H6 O2 N-) 358.06805 (C14 H17 O8 N P-)	indole, anthranilic acid	
C27H26N3O10P	10.49	41	iglu#41	584.14398	582.1283	96.04494 (C5 H6 O N+) 120.04456 (C7 H6 O N+) 124.03937 (C6 H6 O2 N+) 166.04985 (C8 H8 O3 N+) 228.06477 (C13 H10 O3 N+) 330.03705 (C12 H13 O8 N P+)	78.95801 (O3 P-) 96.96867 (H2 O4 P-) 122.02431 (C6 H4 O2 N-) 136.04013 (C7 H6 O2 N-)	indole, anthranilic acid, nicotinic acid	

Continued on next page

Appendix 1—table 1 continued

Representative MS/MS spectra of modular glucosides.

Formula	RT [min.]	Compound number	SMID	m/z (M+H)	m/z (M-H)	ms/ms fragments, positive ionization mode	ms/ms fragments, negative ionization mode	Substituents on glucose	Stable isotope labeling
C26H29N2O10P	10.48	20	iglu#42	561.16439	559.14871	83.04974 (C5 H7 O +) 118.06553 (C8 H8 N+) 120.04465 (C7 H6 O N+) 202.08635 (C12 H12 O2 N+)	78.95805 (O3 P-) 96.96868 (H2 O4 P-)136.03995 (C7 H6 O2 N-)	indole, antranilic acid, tiglic acid	
C20H20N2O6	8.93	SI-2	iglu#5	385.13941	383.12373	106.02911 (C6 H4 O N+) 118.06535 (C8 H8 N+) 124.03936 (C6 H6 O2 N+) 268.08124 (C12 H14 O6 N+)		indole, nicotinic acid	
C20H21N2O9P	8.29	20	iglu#6	465.10687	463.09119	106.02907 (C6 H4 O N+) 118.06532 (C8 H8 N+) 124.03942 (C6 H6 O2 N+) 226.08630 (C14 H12 O2 N+) 250.07079 (C12 H12 O5 N+)	78.95802 (O3 P-) 96.96868 (H2 O4 P-) 122.02421 (C6 H4 O2 N-) 340.05878 (C14 H15 O7 N P-)	indole, nicotinic acid	
C19H23NO6	11.24	SI-3	iglu#7	362.15981	360.14413	83.04967 (C5 H7 O+) 101.06001 (C5 H9 O2+) 118.06536 (C8 H8 N+) 198.09097 (C13 H12 O N+) 226.08626 (C14 H12 O2 N+)		indole, tiglic acid	
C19H24NO9P	10.48	21	iglu#8	442.12727	440.11159	83.04967 (C5 H7 O +) 101.06020 (C5 H9 O2+) 118.06538 (C8 H8 N+) 226.08621 (C14 H12 O2 N+)	78.95798 (O3 P-) 96.96864 (H2 O4 P-) 116.05011 (C8 H6 N-)	indole, tiglic acid	
C19H20N2O6	6.33	SI-4	iglu#9	373.13941	371.12486		110.02437 (C5 H4 O2 N-) 116.05027 (C8 H6 N-)	indole, nicotinic acid	
C21H27N2O11P	4.31		oglu#4	515.14365	513.12797	120.04459 (C7 H6 O N+) 136.07550 (C8 H10 O N+) 138.05511 (C7 H8 O2 N+) 216.06795 (C12 H10 O3 N+)	78.95781 (O3 P-) 96.96854 (H2 O4 P-) 136.03995 (C7 H6 O2 N-) 223.00067 (C6 H8 O7 P-) 376.07953 (C14 H19 O9 N P-)	octopamine, anthranilic acid	d1 from d2-L-Tyrosine
C18H24N2O7	4.79		sgnl#1	381.16563	379.14995		217.09767 (C12 H13 O2 N2-)	n-acetylserotonin	
C25H29N3O8	7.34		sgnl#3	500.20274	498.18706	120.04427 (C7H6NO+) 160.07555 (C10H10NO+)		n-acetylserotonin, anthranilic acid	
C25H30N3O11P	7.89		sgnl#4	580.1702	578.15452	120.04459 (C7 H6 O N+) 138.05498 (C7 H8 O2 N+) 160.07590 (C10 H10 O N+) 219.11266 (C12 H15 O2 N2+)	(O3 P-) 96.96865 (H2 O4 P-) 136.04048 (C7 H6 O2 N-) 223.00072 (C6 H8 O7 P-)	n-acetylserotonin, anthranilic acid	

Continued on next page

Appendix 1—table 1 continued

Representative MS/MS spectra of modular glucosides.

Formula	RT [min.]	Compound number	SMID	m/z (M+H)	m/z (M-H)	ms/ms fragments, positive ionization mode	ms/ms fragments, negative ionization mode	Substituents on glucose	Stable isotope labeling
C29H33N2O11P	8.22		tyglu#12	617.1906	615.17492	120.04458 (C7 H6 O N+) 238.08728 (C15 H12 O2 N+)	78.95803 (O3 P-) 96.96867 (H2 O4 P-) 136.04008 (C7 H6 O2 N-) 135.04503 (C8 H7 O2-) 360.08469 (C14 H19 O8 N P-) 478.12738 (C29 H20 O6 N-)	tyramine, anthranilic acid, phenylacetic acid	d2 from d2-L-Tyrosine
C26H35N2O11P	7.92		tyglu#14	583.20625	581.19057	109.02870 (C6 H5 O2+) 120.04459 (C7 H6 O N+) 138.05489 (C7 H8 O2 N+) 204.10226 (C12 H14 O2 N+) 257.12808 (C15 H17 O2 N2+) 348.14429 (C18 H22 O6 N+)	78.95802 (O3 P-) 96.96866 (H2 O4 P-) 101.05991 (C5 H9 O2-) 136.04047 (C7 H6 O2 N-) 444.14252 (C19 H27 O9 N P-)	tyramine, anthranilic acid, (iso)valeric acid	
C28H31N2O11P	7.97		tyglu#16	603.17495	601.15927	105.03380 (C7 H5 O+) 120.04455 (C7 H6 O N+) 138.05487 (C7 H8 O2 N+) 224.07047 (C14 H10 O2 N+) 257.12775 (C15 H17 O2 N2+) 368.11160 (C20 H18 O6 N+)	78.95805 (O3 P-) 96.96869 (H2 O4 P-) 121.02914 (C7 H5 O2-) 136.03978 (C7 H6 O2 N-) 464.11099 (C21 H23 O9 N P-)	tyramine, anthranilic acid, carboxy-benzyl	d2 from d2-L-Tyrosine
C21H27N2O10P	5.40		tyglu#2	499.14874	497.13306	120.04459 (C7 H6 O N+) 138.05487 (C7 H8 O2 N+) 138.09137 (C8 H12 O N+) 257.12814 (C15 H17 O2 N2+) 264.08633 (C13 H14 O5 N+)	78.95802 (O3 P-) 96.96870 (H2 O4 P-) 136.04005 (C7 H6 O2 N-) 223.00053 (C6 H8 O7 P-) 360.08472 (C14 H19 O8 N P-)	tyramine, anthranilic acid	d2 from d2-L-Tyrosine
C28H32N3O11P	7.65	26	tyglu#4	618.18585	616.17017	120.04459 (C7 H6 O N+) 138.09137 (C8 H12 O N+)	78.95802 (O3 P-) 96.96867 (H2 O4 P-) 136.03989 (C7 H6 O2 N-) 479.12198 (C21 H24 O9 N2 P-)	tyramine, anthranilic acid (x2)	
C27H30N3O11P	6.55		tyglu#6	604.1702	602.15452	106.02901 (C6 H4 O N+) 120.04460 (C7 H6 O N+) 124.03939 (C6 H6 O2 N+) 138.05513 (C7 H8 O2 N+) 166.04988 (C8 H8 O3 N+) 257.12781 (C15 H17 O2 N2+)	78.95781 (O3 P-) 96.96851 (H2 O4 P-) 223.00017 (C6 H8 O7 P-) 381.09375 (C16 H17 O9 N2-) 534.17279 (C22 H33 O12 N P-)	tyramine, anthranilic acid, nicotinic acid	d2 from d2-L-Tyrosine
C26H33N2O11P	7.67		tyglu#8	581.1906	579.17492	83.04968 (C5 H7 O+) 120.04460 (C7 H6 O N+) 138.05479 (C7 H8 O2 N+) 257.12848 (C15 H17 O2 N2+)	78.95779 (O3 P-) 96.96852 (H2 O4 P-) 99.04408 (C5 H7 O2-) 136.03972 (C7 H6 O2 N-) 442.12637 (C19 H25 O9 N P-)	tyramine, anthranilic acid, tiglic acid	d2 from d2-L-Tyrosine

Appendix 1—table 2. BLASTp results from the WormBase BLAST engine when searching against the amino acid sequence of UAR-1 and CRISPR/Cas9 targets for this study (red).

Sequence	Score	E-value
C01B10.10	280	2e-75
C01B10.4a	260	2e-69
T22D1.11	248	7e-66
C42D4.2	233	4e-61
C17H12.4	231	1e-60
C23H4.4a	225	8e-59
C23H4.7	199	6e-51
C23H4.3	194	1e-49
E01G6.3	193	3e-49
C23H4.2	168	1e-41
T02B5.1	157	2e-38
F15A8.6a	154	1e-37
F15A8.6b	154	1e-37
ZC376.3	153	3e-37
T02B5.3	150	2e-36
ZC376.2b	148	1e-35
ZC376.2a	147	2e-35
F56C11.6b	141	1e-33
F56C11.6a	137	2e-32
Y71H2AM.13	136	5e-32
ZC376.1	135	1e-31
R173.3 r	129	6e-30
T07H6.1a	127	2e-29
T28C12.4a	124	1e-28
T28C12.4b	124	2e-28
K07C11.4	119	6e-27
R12A1.4	118	1e-26
K11G9.2	116	4e-26
02B12.4	115	8e-26
Y75B8A.3	114	3e-25
Y48B6A.8	113	4e-25
F13H6.3	111	2e-24
Y48B6A.7	109	5e-24
09B12.1	108	9e-24
K11G9.1	108	2e-23
ZC376.2c	105	7e-23
F07C4.12b	105	7e-23
C52A10.1	101	1e-21
Y44E3A.2	101	2e-21
K11G9.3	99	1e-20
C52A10.2	97	3e-20

Continued on next page

Appendix 1—table 2 continued

Sequence	Score	E-value
C40C9.5d	96	6e-20
C40C9.5b	96	6e-20
C40C9.5a	96	6e-20
F55D10.3	96	1e-19
C40C9.5f	94	2e-19
C01B10.4b	94	2e-19
C40C9.5g	94	2e-19
C40C9.5c	94	3e-19
C40C9.5e	94	3e-19
B0238.7	93	4e-19
B0238.1	92	1e-18
F55F3.2b	83	6e-16
F55F3.2a	83	7e-16
C23H4.4b	50	5e-06
Y43F8A.3a	42	0.002
Y43F8A.3b	35	0.18

Appendix 1—table 3. List of *C. elegans* strains used in this study.

Strain name	Identifier	Description	Associated metabolites
PS8031	*cest-1.1(sy1180)*	*cest-1.1* null	uglas#1 uglas#11
PS8032	*cest-1.1(sy1181)*	*cest-1.1* null	uglas#1 uglas#11
PS8259	*cest-1.1(sy1180 sy1250)*	*cest-1.1* null reverted to WT sequence	uglas#1 uglas#11
PS8260	*cest-1.1(sy1180 sy1251)*	*cest-1.1* null reverted to WT sequence	uglas#1 uglas#11
PS8261	*cest-1.1(sy1181 sy1252)*	*cest-1.1* null reverted to WT sequence	uglas#1 uglas#11
PS8262	*cest-1.1(sy1181 sy1253)*	*cest-1.1* null reverted to WT sequence	uglas#1 uglas#11
PS8008	*cest-2.2(sy1170)*	*cest-2.2* null	ascr#8, ascr#81, ascr#82
PS8009	*cest-2.2(sy1171)*	*cest-2.2* null	ascr#8, ascr#81, ascr#82
PS8236	*cest-2.2(sy1170 sy1236)*	*cest-2.2* null reverted to WT sequence	ascr#8, ascr#81, ascr#82
PS8238	*cest-2.2(sy1171 sy1238)*	*cest-2.2* null reverted to WT sequence	ascr#8, ascr#81, ascr#82
PS8116	*cest-4(sy1192)*	*cest-4* null	iglu class modular glucosides
PS8117	*cest-4(sy1193)*	*cest-4* null	iglu class modular glucosides
JJ1271	*glo-1(zu437)*	*glo-1* null	Most known modular ascarosides/glucosides
PS8781	*cest-4(sy1192)*	*cest-4* null reverted to WT sequence	iglu class modular glucosides
PS8782	*cest-4(sy1193)*	*cest-4* null reverted to WT sequence	iglu class modular glucosides
PS8783	*cest-4(sy1194)*	*cest-4* null reverted to WT sequence	iglu class modular glucosides
PS8784	*cest-4(sy1195)*	*cest-4* null reverted to WT sequence	iglu class modular glucosides
PS8515	CBR-*glo-1*-A (*sy1382*)	*C. briggsae glo-1* null	Most known modular ascarosides/glucosides

Continued on next page

Appendix 1—table 3 continued

| Strain name	Identifier	Description	Associated metabolites
PS8516	CBR-*glo-1*-B (*sy1383*)	C. briggsae *glo-1* null	Most known modular ascarosides/glucosides
PS8029	*cest-19(sy1178)*	*cest-19* null	Undetermined
PS8030	*cest-19(sy1179)*	*cest-19* null	Undetermined
PS8033	*cest-33(sy1182)*	*cest-33* null	Undetermined
PS8034	*cest-33(sy1183)*	*cest-33* null	Undetermined
RB2053	*ges-1 (ok2716)*	*ges-1* null	Undetermined
RB1804	*cest-6(ok2338)*	*cest-6* null	Undetermined
DP683	*cest-1.1(dp683)*	*cest-1.1* (S213A) point mutant	uglas#1 uglas#11
FCS02	*cest-2.2*-mCherry	*cest-2.2* C-terminal mCherry	ascr#8, ascr#81, ascr#82

Appendix 1—table 4. NMR spectroscopic data for iglu#3 (34).
^{1}H (600 MHz), HSQC, and HMBC NMR spectroscopic data were acquired in methanol-d_4. Chemical shifts were referenced to δ(C$\underline{H}$D$_2$OD)=3.31 ppm and δ(^{13}CHD$_2$OD)=49.00 ppm.
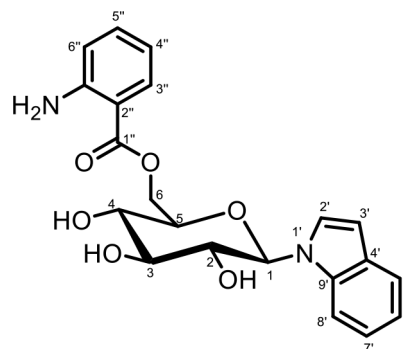
Position	δ ^{13}C [ppm]	δ ^{1}H ([ppm] J_{HH}[Hz])	HMBC
1	86.9	5.51 ($J_{1,2}$ = 9.3)	C-2, C-3, C-5, C-2′, C-9′
2	73.0	3.99 ($J_{2,3}$ = 9.0)	C-1, C-3
3	78.7	3.65 ($J_{3,4}$ = 9.0)	C-4
4	71.3	3.64 ($J_{4,5}$ = 9.1)	C-3
5	77.5	3.91 ($J_{5,6a}$ = 5.5)	C-4
6a	64.1	4.43 ($J_{6a,6b}$ = 12.1)	C-5, C-1″
6b		4.67 ($J_{5,6b}$ = 2.2)	C-4, C-1″
2′	126.3	7.37 ($J_{2′,3′}$=3.3)	C-1 (weak), C-3′, C-4′, C-8′ (weak), C-9′
3′	102.9	6.48	
4′	130.4		
5′	121.4	7.52 ($J_{5′,6′}$=8.0)	C-3′, C-7′, C-9′
6′	120.8	7.03 ($J_{6′,7′}$=7.4, $J_{3′,6′}$=1.1)	C-4′, C-8′
7′	122.4	7.06	C-5′, C-9′
8′	111.5	7.53	C-4′, C-6′

Continued on next page

9′	137.5		
1″	168.6		
2″	112.8		
3″	132.1	7.90 ($J_{3'',4''}$=8.2, $J_{3'',5''}$=1.4)	C-1″, C-5″, C-7″
4″	118.2	6.73 ($J_{4'',5''}$=7.6)	C-2″, C-6″
5″	135.0	7.32 ($J_{5'',6''}$=7.8)	C-3″, C-7″
6″	118.6	6.84	C-2″, C-4″
7″	149.9		

Appendix 1—table 5. NMR spectroscopic data for iglu#5 (SI-2).

^{1}H (600 MHz), HSQC, and HMBC NMR spectroscopic data were acquired in methanol-d_4. Chemical shifts were referenced to δ(CHD$_2$OD)=3.31 ppm and δ([13]CHD$_2$OD)=49.00 ppm.
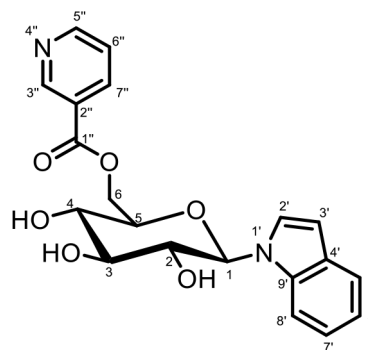
Position	δ [13]C [ppm]	δ [1]H ([ppm] J_{HH}[Hz])	HMBC
1	86.9	5.51 ($J_{1,2}$ = 9.2)	C-2, C-3, C-5, C-2′, C-9′
2	73.0	4.00 ($J_{2,3}$ = 9.0)	C-1, C-3
3	78.7	3.65 ($J_{3,4}$ = 9.0)	C-4
4	71.4	3.63 ($J_{4,5}$ = 8.9)	C-3
5	77.4	3.95 ($J_{5,6a}$ = 5.8)	C-4
6a	65.3	4.51 ($J_{6a,6b}$ = 12.1)	C-4, C-5, C-1″
6b		4.75 ($J_{5,6b}$ = 2.3)	C-4, C-5, C-1″
2′	126.4	7.37 ($J_{2',3'}$=3.5)	C-3′, C-4′, C-9′
3′	103.1	6.47	C-2′, C-4′, C-9′
4′	130.5		
5′	121.4	7.51 ($J_{5',6'}$=7.9)	C-4′, C-6′, C-9′
6′	120.8	7.01 ($J_{6',7'}$=7.5, $J_{3',6'}$=1.2)	C-4′, C-8′
7′	122.5	7.05	C-4′, C-5′, C-8′, C-9′
8′	111.4	7.49	C-4′, C-6′
9′	137.6		
1″	165.8		
2″	127.7		
3″	150.8	9.12 ($J_{3'',6''}$=0.5, $J_{3'',7''}$=2.0)	C-2″, C-5″, C-7″

Continued on next page

5''	153.7	8.74 ($J_{5'',6'''}$=4.9, $J_{5''',7''}$=1.7)	C-3'', C-6'', C-7''
6''	125.1	7.54 ($J_{6''',7''}$=8.0)	C-2'', C-5''
7''	138.9	8.37	C-1'', C-2'', C-5''

Appendix 1—table 6. NMR spectroscopic data for iglu#7 (SI-3).
^{1}H (600 MHz), HSQC, and HMBC NMR spectroscopic data were acquired in methanol-d_4. Chemical shifts were referenced to δ(C$\underline{H}$D$_2$OD)=3.31 ppm and δ(^{13}CHD$_2$OD)=49.00 ppm.
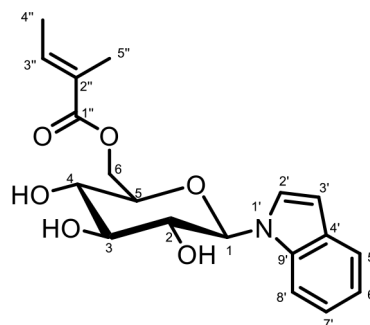
Position	δ ^{13}C [ppm]	δ ^{1}H ([ppm] J_{HH}[Hz])	HMBC
1	86.9	5.46 ($J_{1,2}$ = 9.1)	C-2, C-3, C-5, C-2', C-9'
2	73.2	3.96 ($J_{2,3}$ = 9.0)	C-1, C-3
3	78.9	3.61 ($J_{3,4}$ = 9.0)	C-2, C-4
4	71.4	3.55 ($J_{4,5}$ = 9.6)	C-3, C-5, C-6
5	77.6	3.81 ($J_{5,6a}$ = 5.6)	C-1 (weak), C-3, C-4
6a	64.5	4.27 ($J_{6a,6b}$ = 11.9)	C-4, C-5, C-1''
6b		4.49 ($J_{5,6b}$ = 2.2)	C-4, C-5, C-1''
2'	126.6	7.35 ($J_{2',3'}$=3.5)	C-1 (weak), C-3', C-4', C-5' (weak), C-8' (weak), C-9'
3'	103.2	6.48	
4'	130.6		
5'	121.6	7.53 ($J_{5',6'}$=7.9)	C-3', C-7', C-9'
6'	120.9	7.05 ($J_{6',7'}$=7.5, $J_{3',6'}$=1.1)	C-4', C-8', C-9' (weak)
7'	122.5	7.11	C-5', C-8' (weak), C-9'
8'	111.7	7.50	C-4', C-6'
9'	137.6		
1''	169.2		
2''	129.3		
3''	138.9	6.87 ($J_{3'',4''}$=6.8)	C-1'', C-4'', C-5''
4''	14.2	1.79	C-2'', C-3''
5''	11.9	1.81	C-1'', C-2'', C-3''

Appendix 1—table 7. NMR spectroscopic data for iglu#9 (SI-4).
^{1}H (600 MHz), HSQC, and HMBC NMR spectroscopic data were acquired in methanol-d_4. Chemical shifts were referenced to δ(C$\underline{H}$D$_2$OD)=3.31 ppm and δ(^{13}CHD$_2$OD)=49.00 ppm.
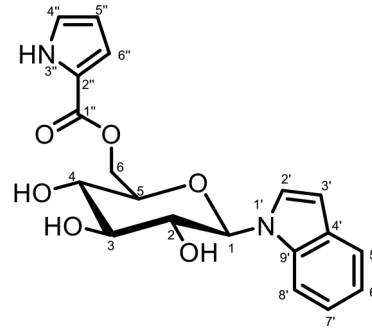
Position	δ ^{13}C [ppm]	δ ^{1}H ([ppm] J_{HH}[Hz])	HMBC
1	86.9	5.47 ($J_{1,2}$ = 9.1)	C-2, C-3, C-5, C-2′, C-9′
2	73.2	3.96 ($J_{2,3}$ = 9.0)	C-1, C-3
3	78.7	3.62 ($J_{3,4}$ = 9.8)	C-4
4	71.3	3.61 ($J_{4,5}$ = 9.7)	C-3
5	77.9	3.86 ($J_{5,6a}$ = 5.7)	
6a	63.9	4.38 ($J_{6a,6b}$ = 11.9)	C-5, C-1″
6b		4.68 ($J_{5,6b}$ = 2.1)	C-4, C-1″
2′	126.6	7.36 ($J_{2′,3′}$=3.4)	C-3′, C-4′, C-9′
3′	103.1	6.47	C-2′, C-4′, C-9′
4′	130.6		
5′	121.4	7.52 ($J_{5′,6′}$=7.8)	C-7′, C-9′
6′	120.8	7.02 ($J_{6′,7′}$=7.3, $J_{3′,6′}$=1.2)	C-4′, C-8′
7′	122.4	7.05	C-5′, C-9′
8′	111.6	7.50	C-4′, C-6′
9′	137.4		
1″	162.4		
2″	123.0		
4″	124.7	6.96 ($J_{4″,5″}$=2.5, $J_{4″,6″}$=1.4)	C-2″, C-5″, C-6″
5″	110.6	6.20 ($J_{5″,6″}$=3.8)	C-2″(weak), C-4″(weak)
6″	116.8	6.90	C-2″, C-4″, C-5″

Appendix 1—table 8. DNA oligonucleotides used for this study.

Target gene	Sequence name	Strain	Allelle	Guide sequence	ssDNA repair oligonucleotide sequence
cest-1.1	T02B5.1	PS8031, PS8032	sy1180, sy1181	ACTCCTTCCCATGA TTTCGG	TATTCATTTGTTACCAAAACTCCTTCCCA TGATTTG CTAGCTTATCACTTAGTCACCTCTGCTC TGGACAAA CTTCCCCGGTGGACGGGGTTTTCGATA TCGAAGGTCTCCAATTG
cest-2.2	ZC376.2	PS8008, PS8009	sy1170, sy1171	GGAGGCGAAGGAG TATAAAG	CCCTGGGACGGAG TTTTGGAGGCGAAGGAGTATA GGGAAGTTTGTCCAGAGCAGAGGTGAC TAAGTGATAA GCTAGCAAGCGGCTTGTATGAGTGATCA- GAAGTAAGAGATA

Continued on next page

Appendix 1—table 8 continued
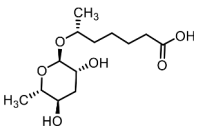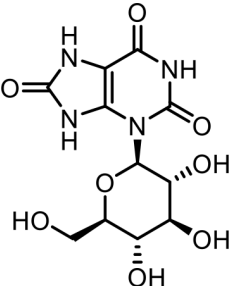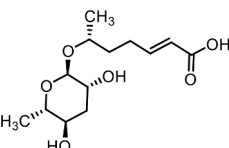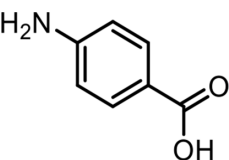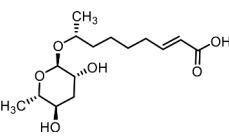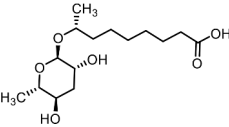
Target gene	Sequence name	Strain	Allelle	Guide sequence	ssDNA repair oligonucleotide sequence
cest-4	C17H12.4	PS8116, PS8117	*sy1192, sy1193*	ACTCCGGTCCA TTTCTCAGG	CATACCTTTTGCATTTCTCACTCCGGTCCA TTTCTCGCTAGC TTATCACTTAGTCACCTCTGCTCTGGA-CAAACTTCCCAGGCGG TTCTGGTTTTTGAAATCTTAATTTTCCAA TTG

Appendix 1—table 9. List of all modular metabolites referred to in the text and Figures.

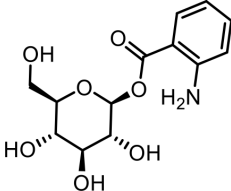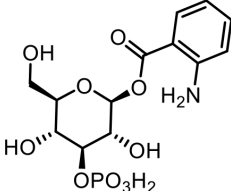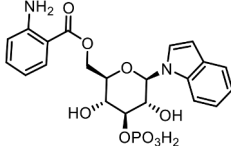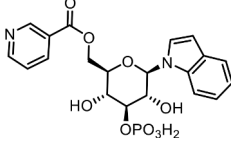| Compound number	SMID ID	IUPAC Name	Evidence	Structure
1	icas#3	(*R*)−8-(((2*R*,3*R*,5*R*,6*S*)−5-((1*H*-indole-3-carbonyl) oxy)−3-hydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)nonanoic acid	Previously identified via synthesis (*Srinivasan et al., 2012*)	
2	ascr#8	4-((*R*,*E*)−6-(((2*R*,3*R*,5*R*,6*S*)−3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)hept-2-enamido)benzoic acid	Previously identified via synthesis (*Pungaliya et al., 2009*)	
3	uglas#11	(2*R*,3*R*,4*S*,5*R*,6*R*)−5-hydroxy-6-(hydroxymethyl)−4-(phosphonooxy)−2-(2,6,8-trioxo-1,2,6,7,8,9-hexahydro-3*H*-purin-3-yl) tetrahydro-2*H*-pyran-3-yl (*R*)−6-(((2*R*,3*R*,5*R*,6*S*)−3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy) heptanoate	Previously identified via synthesis (*Curtis et al., 2020*)	
4	ubas#3	(*R*)−4-(((2*R*,3*R*,5*R*,6*S*)−3-hydroxy-6-methyl-5-(((*R*)−2-methyl-3-ureidopropanoyl)oxy) tetrahydro-2*H*-pyran-2-yl)oxy)pentanoic acid	Previously inferred via tandem mass spectrometry (*Falcke et al., 2018*)	
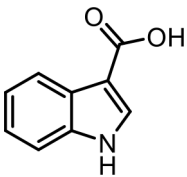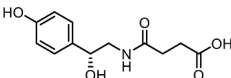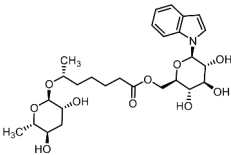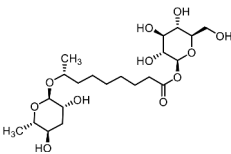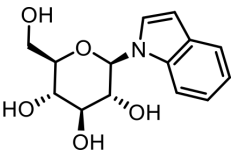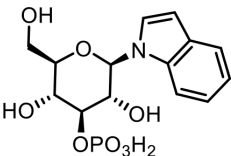
Continued on next page

Appendix 1—table 9 continued

Compound number	SMID ID	IUPAC Name	Evidence	Structure
5	ascr#1	(*R*)−6-(((2*R*,3*R*,5*R*,6*S*)−3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)heptanoic acid	Previously identified via NMR and synthesis (*Jeong et al., 2005*)	
6	gluric#1	3-((2*R*,3*R*,4*S*,5*S*,6*R*)−3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)−7,9-dihydro-1*H*-purine-2,6,8 (3*H*)-trione	Previously identified via synthesis (*Curtis et al., 2020*)	
7	ascr#7	(*R,E*)−6-(((2*R*,3*R*,5*R*,6*S*)−3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)hept-2-enoic acid	Previously identified via synthesis (*Pungaliya et al., 2009*)	
8	PABA	4-Aminobenzoic acid	Commercial product (Sigma-Aldrich)	
9	ascr#3	(*R,E*)−8-(((2*R*,3*R*,5*R*,6*S*)−3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)non-2-enoic acid	Previously identified via synthesis (*Butcher et al., 2007*)	
10	ascr#10	(*R*)−8-(((2*R*,3*R*,5*R*,6*S*)−3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)nonanoic acid	Previously identified via synthesis (*Srinivasan et al., 2012*)	
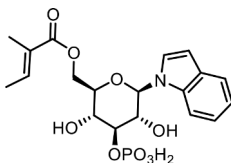
Continued on next page

Appendix 1—table 9 continued

Compound number	SMID ID	IUPAC Name	Evidence	Structure
11		1*H*-indole-3-carboxylic acid	Commercial product (Sigma-Aldrich)	
12		(*R*)—4-((2-hydroxy-2-(4-hydroxyphenyl)ethyl)amino)—4-oxobutanoic acid	Identified via synthesis (This manuscript)	
13	iglas#1	((2*R*,3*S*,4*S*,5*R*,6*R*)—3,4,5-trihydroxy-6-(1*H*-indol-1-yl)tetrahydro-2*H*-pyran-2-yl)methyl (*R*)—6-(((2*R*,3*R*,5*R*,6*S*)—3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)heptanoate	Previously identified via synthesis (*Artyukhin et al., 2018*)	
14	glas#10	(2*S*,3*R*,4*S*,5*S*,6*R*)—3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl (*R*)—8-(((2*R*,3*R*,5*R*,6*S*)—3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)nonanoate	Previously identified via NMR and synthesis (*Coburn et al., 2013*)	
15	iglu#1	(2*R*,3*S*,4*S*,5*R*,6*R*)—2-(hydroxymethyl)—6-(1*H*-indol-1-yl)tetrahydro-2*H*-pyran-3,4,5-triol	Previously identified via NMR and synthesis (*Coburn et al., 2013*)	
16	iglu#2	(2*R*,3*R*,4*S*,5*R*,6*R*)—3,5-dihydroxy-2-(hydroxymethyl)—6-(1*H*-indol-1-yl)tetrahydro-2*H*-pyran-4-yl dihydrogen phosphate	Previously identified via NMR (*Coburn et al., 2013*)	

Continued on next page

Appendix 1—table 9 continued

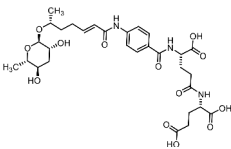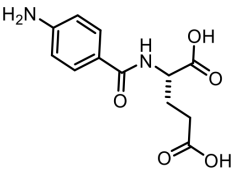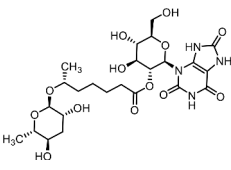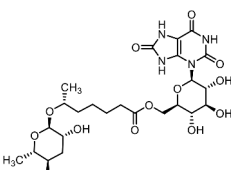| Compound number	SMID ID	IUPAC Name	Evidence	Structure
17	angl#1	(2S,3R,4S,5S,6R)—3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl 2-aminobenzoate	Previously identified via NMR and synthesis (*Coburn et al., 2013*)	
18	angl#2	(2S,3R,4S,5R,6R)—3,5-dihydroxy-6-(hydroxymethyl)—4-(phosphonooxy)tetrahydro-2H-pyran-2-yl 2-aminobenzoate	Previously identified via NMR (*Coburn et al., 2013*)	
19	iglu#4	(2R,3R,4S,5R,6R)—3,5-dihydroxy-6-(1H-indol-1-yl)—4-(phosphonooxy)tetrahydro-2H-(pyran-2-yl)methyl 2-aminobenzoate	Proposed structure, based on identification of non-phosphorylated derivative (**34**) via synthesis (This manuscript)	
20	iglu#6	((2R,3R,4S,5R,6R)—3,5-dihydroxy-6-(1H-indol-1-yl)—4-(phosphonooxy)tetrahydro-2H-pyran-2-yl)methyl nicotinate	Proposed structure, based on identification of non-phosphorylated derivative (**SI-2**) via synthesis (This manuscript)	
21	iglu#8	((2R,3R,4S,5R,6R)—3,5-dihydroxy-6-(1H-indol-1-yl)—4-(phosphonooxy)tetrahydro-2H-pyran-2-yl)methyl (E)—2-methylbut-2-enoate	Proposed structure, based on identification of non-phosphorylated derivative (**SI-3**) via synthesis (This manuscript)	
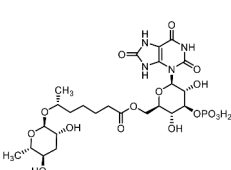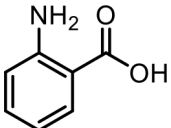
Continued on next page

Appendix 1—table 9 continued

Compound number	SMID ID	IUPAC Name	Evidence	Structure
22	iglu#10	((2R,3R,4S,5R,6R)—3,5-dihydroxy-6-(1H-indol-1-yl)—4-(phosphonooxy)tetrahydro-2H-pyran-2-yl)methyl 1H-pyrrole-2-carboxylate	Proposed structure, based on identification of non-phosphorylated derivative (**SI-4**) via synthesis (This manuscript)	
23	iglu#12	((2R,3R,4S,5R,6R)—3,5-dihydroxy-6-(1H-indol-1-yl)—4-(phosphonooxy)tetrahydro-2H-pyran-2-yl)methyl benzoate	Proposed structure. Inferred via tandem mass spectrometry (This manuscript)	
24	iglu#41	(2R,3R,4S,5R,6R)—6-(((2-aminobenzoyl)oxy)methyl)—5-hydroxy-2-(1H-indol-1-yl)—4-(phosphonooxy)tetrahydro-2H-pyran-3-yl 1H-pyrrole-2-carboxylate	Proposed structure. Inferred from iglu#3 (**34**) via tandem mass spectrometry (This manuscript)	
25	angl#4	((2R,3R,4S,5R,6S)—6-((2-aminobenzoyl)oxy)—3,5-dihydroxy-4-(phosphonooxy)tetrahydro-2H-pyran-2-yl)methyl 2-aminobenzoate	Proposed structure. Inferred from angl#3 (**SI 5**) via tandem mass spectrometry (This manuscript)	
26	tyglu#4	((2R,3R,4S,5R,6R)—5-((2-aminobenzoyl)oxy)—3-hydroxy-6-((4-(2-aminoethyl)phenoxy)—4-(phosphonooxy)tetrahydro-2H-pyran-2-yl))methyl 2-aminobenzoate	Proposed structure. Initially described (*O'Donnell et al., 2020*) and further inferred via tandem mass spectrometry (This manuscript)	
27	ascr#81	(4-((R,E)—6-(((2R,3R,5R,6S)—3,5-dihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)hept-2-enamido)benzoyl)-L-glutamic acid	Identified via synthesis (*Artyukhin et al., 2018*)	

Continued on next page

Appendix 1—table 9 continued

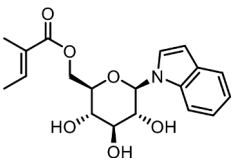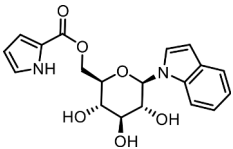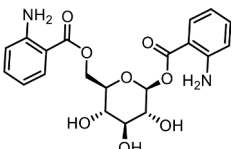| Compound number	SMID ID	IUPAC Name	Evidence	Structure
28	ascr#82	((S)−4-carboxy-4-(4-((R,E)−6-(((2R,3R,5R,6S)−3,5-dihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)hept-2-enamido)benzamido)butanoyl)-L-glutamic acid	Previously inferred via tandem mass spectrometry (*Artyukhin et al., 2018*)	
29	PABA-glu	(4-aminobenzoyl)-L-glutamic acid	Identified via synthesis (This manuscript)	
30	uglas#1	(2R,3R,4S,5S,6R)−4,5-dihydroxy-6-(hydroxymethyl)−2-(2,6,8-trioxo-1,2,6,7,8,9-hexahydro-3H-purin-3-yl)tetrahydro-2H-pyran-3-yl (R)−6-(((2R,3R,5R,6S)−3,5-dihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)heptanoate	Identified via synthesis (*Curtis et al., 2020*)	
31	uglas#14	((2R,3S,4S,5R,6R)−3,4,5-trihydroxy-6-(2,6,8-trioxo-1,2,6,7,8,9-hexahydro-3H-purin-3-yl)tetrahydro-2H-pyran-2-yl)methyl (R)−6-(((2R,3R,5R,6S)−3,5-dihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)heptanoate	Identified via synthesis (*Curtis et al., 2020*)	
32	uglas#15	((2R,3R,4S,5R,6R)−3,5-dihydroxy-4-(phosphonooxy)−6-(2,6,8-trioxo-1,2,6,7,8,9-hexahydro-3H-purin-3-yl)tetrahydro-2H-pyran-2-yl)methyl (R)−6-(((2R,3R,5R,6S)−3,5-dihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)heptanoate	Previously inferred via tandem mass spectrometry (*Artyukhin et al., 2018*; *Curtis et al., 2020*)	
33		2-Aminobenzoic acid	Commercial product (Sigma-Aldrich)	

Continued on next page

Appendix 1—table 9 continued

Compound number	SMID ID	IUPAC Name	Evidence	Structure
34	iglu#3	((2R,3S,4S,5R,6R)—3,4,5-trihydroxy-6-(1H-indol-1-yl)tetrahydro-2H-pyran-2-yl)methyl 2-aminobenzoate	Identified via synthesis (This manuscript)	
35	icas#2	(2S,3R,5R,6R)—5-hydroxy-2-methyl-6-(((R)—5-oxohexan-2-yl)oxy)tetrahydro-2H-pyran-3-yl 1H-indole-3-carboxylate	Identified via synthesis (*Dong et al., 2016*)	
36	icas#6.2	(2S,3R,5R,6R)—5-hydroxy-6-(((2R,5S)—5-hydroxyhexan-2-yl)oxy)—2-methyltetrahydro-2H-pyran-3-yl 1H-indole-3-carboxylate	Identified via synthesis (*Dong et al., 2016*)	
SI 1		2-((tert-butoxycarbonyl)-amino)benzoic acid	Characterized via synthesis (This manuscript)	
SI 2	iglu#5	((2R,3S,4S,5R,6R)—3,4,5-trihydroxy-6-(1H-indol-1-yl)tetrahydro-2H-pyran-2-yl)methyl nicotinate	Identified via synthesis (This manuscript)	

Continued on next page

Appendix 1—table 9 continued

Compound number	SMID ID	IUPAC Name	Evidence	Structure
SI 3	iglu#7	((2*R*,3*S*,4*S*,5*R*,6*R*)—3,4,5-trihydroxy-6-(1*H*-indol-1-yl)tetrahydro-2*H*-pyran-2-yl)methyl (*E*)—2-methylbut-2-enoate	Identified via synthesis (This manuscript)	
SI 4	iglu#9	((2*R*,3*S*,4*S*,5*R*,6*R*)—3,4,5-trihydroxy-6-(1*H*-indol-1-yl)tetrahydro-2*H*-pyran-2-yl)methyl 1*H*-pyrrole-2-carboxylate	Identified via synthesis (This manuscript)	
SI 5	angl#3	((2*R*,3*S*,4*S*,5*R*,6*S*)—6-((2-aminobenzoyl)oxy)—3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl 2-aminobenzoate	Proposed structure based on synthesis of a reference sample for MS (This manuscript)	
SI 6	tyglu#6	(2*R*,3*R*,4*S*,5*S*,6*R*)—6-(((2-aminobenzoyl)oxy)methyl)—2-((4-(2-aminoethyl)-phenoxy))—5-hydroxy-4-(phosphonooxy)-tetrahydro-2*H*-pyran-3-yl nicotinate	Proposed structure. Initially described (*O'Donnell et al., 2020*) and further inferred via tandem mass spectrometry (This manuscript)	