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Ascaroside Signaling in the Bacterivorous Nematode *Caenorhabditis remanei* Encodes the Growth Phase of Its Bacterial Food Source

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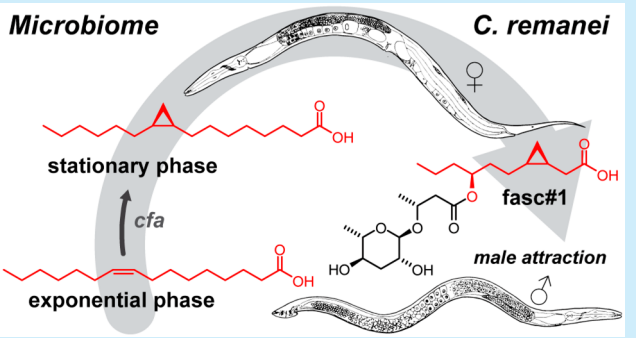
Supporting Information

ABSTRACT: A novel class of species-specific modular ascarosides that integrate additional fatty acid building blocks was characterized in the nematode *Caenorhabditis remanei* using a combination of HPLC−ESI−(−)-MS/MS precursor ion scanning, microreactions, HR-MS/MS, MS^n, and NMR techniques. The structure of the dominating component carrying a cyclopropyl fatty acid moiety was established by total synthesis. Biogenesis of this female-produced male attractant depends on cyclopropyl fatty acid synthase (*cfa*), which is expressed in bacteria upon entering their stationary phase.



In their natural habitat of decaying plant material, bacterivorous nematodes such as the model organism *Caenorhabditis elegans* or its close relative *Caenorhabditis remanei* depend on their microbiome to convert environmental resources into readily accessible biomass. The majority of their associated bacteria support nematode propagation, but a few are detrimental.[1] Bacterial composition affects nematode population growth, lifespan, development, metabolism, foraging behavior, and pathogen resistance.[2] Nematodes exhibit preference for beneficial bacteria, but the underlying molecular mechanisms have remained largely unknown.[3] Recent advances in analytical techniques facilitated the characterization of nematode ascarosides,[4] a modular glycolipid library based on the 3,6-dideoxysugar L-ascarylose, which serve as key regulators in nematode chemical ecology.[5] De novo ascaroside biosynthesis depends on the co-option of a primary metabolic pathway, the peroxisomal β-oxidation cycle of fatty acid metabolism, to furnish a large diversity of homologous aglycones from very long chain precursors.[4a,6] The resulting short-chain ascarosides are highly conserved in nematodes[7] and further serve as scaffolds for species-specific attachment of additional building blocks from diverse primary metabolic pathways. Some of these building blocks appear to originate from microbial metabolism (Figure 1a) such as the L-tryptophan-derived indole-3-carboxylate unit of indole ascarosides (**1**, IC-asc-C#; SMID:[8] icas), a group of potent attraction and aggregation signals in the *Caenorhabditis*[9] or the *p*-aminobenzoic acid unit of *C. elegans*' male-attracting asc-ΔC7-PABA (**2**, SMID:[8] ascr#8).[10] Ascaroside biosynthesis thus appears to integrate the metabolic state of the producing organism along with environmental factors including bacterial food availability and composition, but this has not yet been experimentally demonstrated.

Here, we show that species-specific biogenesis of a potent male-attracting ascaroside signal by females of the gonochoristic *C. remanei* depends on a bacterial food derived cyclopropyl fatty acid building block (Figure 1), the production of which is initiated upon reaching the stationary growth phase.

Comprehensive ascaroside profiling of the *C. remanei* exometabolome using HPLC−ESI−(−)-MS/MS precursor ion scanning for *m/z* 73.0 $[C_3H_5O_2]^-$[4a] revealed common simple ascarosides along with several yet undescribed modular components (Figure 2a) including a novel class of fatty acid ascarosides (SMID:[8] fasc). ESI-(−)-HR-MS/MS analysis of the dominating component, fasc#1 (**3**), indicated the molecular $C_{21}H_{36}O_8$ and suggested the presence of an asc-C4 unit with an uncommon even numbered C4 aglycone along with a hydroxylated C11 fatty acid building block (Figure 2b).

Approximately 100 μg of fasc#1 (**3**) was isolated from 1.5 L of the *C. remanei* culture supernatant using a combination of RP-C18 solid-phase extraction and semipreparative HPLC. Its modular structure including an asc-C4 building block and a 7-

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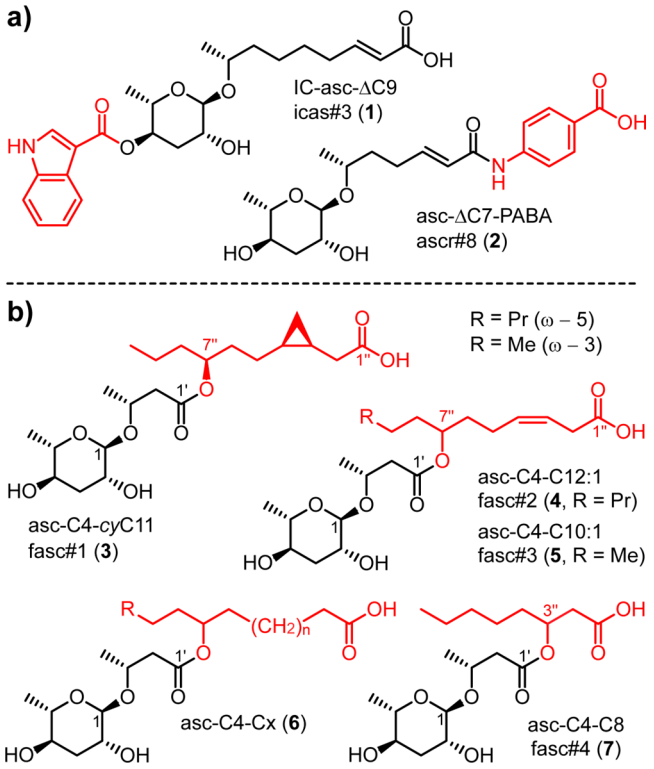

Figure 1. (a) Modular ascarosides including building blocks of microbial origin. (b) Fatty acid ascarosides from *C. remanei* and *C. latens*.

hydroxy-3,4-methylenedecanoic acid moiety was determined by NMR spectroscopy (Table S1). The *cis*-configuration of the cyclopropyl unit was deduced from the ^{1}H NMR chemical shifts along with characteristic NOE interactions (Figure 2c), but the stereochemistry of the (ω-3)-oxymethine group could not be established. The absolute configuration of the 7-hydroxy-*cis*-3,4-methylenedecanoic acid moiety (**8**) was consequently determined using the advanced Mosher method.[11] Alkaline hydrolysis of approximately 75 μg fasc#1 (**3**) gave asc-C4 (ascr#11, **19**) and 7-hydroxy-*cis*-3,4-methylenedecanoic acid (**8**) that was converted to the methyl ester (**9**) (Figure 2c). Comparative ^{1}H NMR analysis of the diastereomeric (*S*)- and (*R*)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) esters (**10**) and determination of $\Delta\delta^{SR}$ values (Table S2) indicated the (7*S*)-configuration (Figure 2d). Furthermore, assuming a staggered conformation, the divergent $\Delta\delta^{SR}$ values of the anisochoric 3,4-methylene bridge protons along with comparatively small $\Delta\delta^{SR}$ values of the *cis*-3,4-methine protons suggested a (3*S*,4*S*,7*S*)-configuration in which the *cis*-3,4-methylene and the MTPA moiety share a syn-facial arrangement in agreement with a potential origin from *E. coli* derived (9*R*,10*S*)-*cis*-9,10-methylenehexadecanoic acid (**25**).[12]

The structure assignment of fasc#1 (**3**) was unambiguously established by total synthesis as shown in Scheme 1. Enantioselective Simmons–Smith cyclopropanation of allyl alcohol **11** using the chiral Charette ligand[13] furnished (−)-(2*S*,3*S*)-*cis*-2,3-methylene-5-TBSO-pentan-1-ol (**12**) with an enantiomeric excess >93% as shown by enantioselective gas chromatography (Figure S1). Oxidation of **12** using Dess–Martin periodinane[14] gave the corresponding aldehyde **13** that was directly submitted to Wittig methylenation. The resulting vinylcyclopropane (−)-**14** was coupled with (−)-(3*S*)-1-hexen-

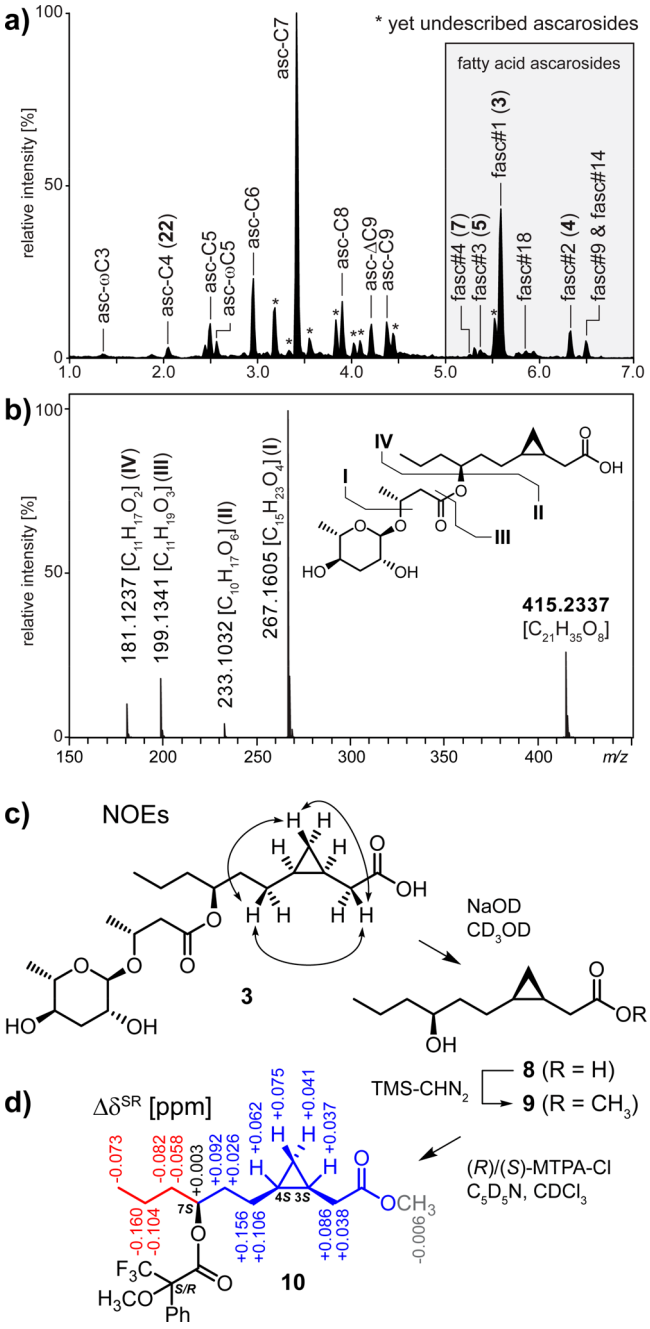

Figure 2. (a) HPLC–ESI–(−)-MS/MS precursor ion scanning of the *C. remanei* exometabolome shows simple ascarosides along with modular components including fatty acid ascarosides (fasc). (b) HR-MS/MS spectrum and fragmentation of fasc#1 (**3**). (c) NOE interactions in *cis*-configured fasc#1 (**3**) and conversion to 7-hydroxy-*cis*-3,4-methylenedecanoic acid (**8**) and its methyl ester (**9**). (d) $\Delta\delta^{SR}$ values of diastereomeric MTPA esters (**10**) indicate a (7*S*)-configuration and a syn-facial arrangement of the *cis*-3,4-methylene and MTPA moiety.

3-yl acetate (**15**), obtained with an enantiomeric excess >98% by kinetic resolution of racemic (±)-1-hexen-3-ol using immobilized *Candida antarctica* lipase B (Novozyme 435) (Figure S2).[15] Cross-metathesis of the chiral building blocks (−)-**14** and (−)-**15** catalyzed by Grubb's second-generation catalyst[16] afforded the cyclopropylalkenyl acetate **16**, which was reduced to **17** using diimide[17] and hydrolyzed under alkaline conditions. Using Steglich esterification,[18] the resulting cyclopropylalkanol

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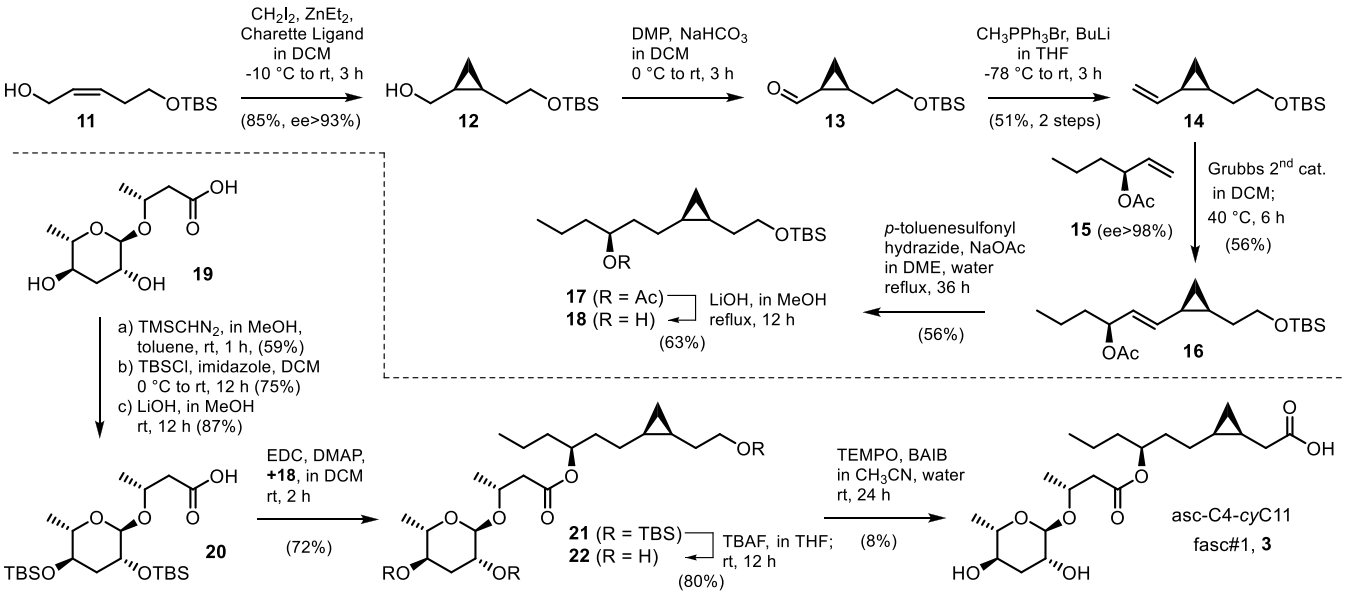
Scheme 1. Total Synthesis of asc-C4-*cy*C11 (fasc#1,3) from *C. remanei*



unit **18** was coupled with a 2,4-di-*O*-*tert*-butyldimethylsilyl-protected asc-C4 building block **20**, obtained in three steps from asc-C4 (SMID:[8] ascr#11, **19**), which was prepared in eight steps from L-rhamnose and ethyl (3*R*)-hydroxybuytyrate as previously described.[4c] The coupling product **21** was deprotected and the resulting triol **22** submitted to (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO)-catalyzed selective oxidation with bis-(acetoxy) iodobenzene (BAIB)[19] to afford asc-C4-*cy*C11 (fasc#1, **3**) identical to the natural product isolated from *C. remanei* (Figures S3–S5 and Table S3).

In addition to dominating fasc#1 (**3**), several related fatty acid ascarosides were detected as minor and trace components (Table 1 and Figure S6). Isolated fasc#2 (**4**) carrying a (ω-5)-linked *cis*-configured 7-hydroxydodec-3-enoic acid building block was identified by NMR spectroscopy (Table S1). Using a combination of microreactions and mass spectrometry additional asc-C4 derivatives carrying saturated, unsaturated, and monocyclic fatty acid residues could be tentatively identified (Tables S5 and S6). Palladium-catalyzed hydrogenation[20] distinguished a series with inert cyclopropyl fatty acids including *cy*C11 (fasc#1, **3**) from those with unsaturated fatty acids including C12:1 (fasc#2, **4**) and C10:1 (fasc#3, **5**) that readily formed dihydro derivatives (Figure S7). The (ω-5) and (ω-3)-linkage of fasc#2 (**4**) and fasc#3 (**5**) was deduced from multiple-stage mass spectra (MS^n) of the corresponding hydroxy fatty acid fragment ions,[21] respectively (Figures S8 and S9). Furthermore, the position of the double bonds in **4** and **5** was deduced by osmium tetraoxide mediated *cis*-1,2-dihydroxylation[22] followed by MS^n (Figure S10). Comparative analysis of five wild-type isolates demonstrated that fatty acid ascaroside biosynthesis is conserved in *C. remanei*, whereas the relative concentrations of individual components show considerable strain specificity (Figure S11). Fatty acid ascarosides dominated by the short chain asc-C4–C8 (fasc#4, **7**) were also identified in *Caenorhabditis latens* (Table S4), a sister species of *C. remanei*,[23] but not in any of the other 13 *Caenorhabditis* species tested (Figure S12), indicating a high degree of species specificity.

Sex-specific analysis revealed that dominating fasc#1 (**3**) is almost exclusively released by *C. remanei* females at a rate of approximately 2.5 fmol·h^{-1} (Figure S13). Its behavioral activity was evaluated using a holding assay that quantifies nematode retention in ascaroside conditioned scoring regions.[24] Ecologically relevant amounts as low as 1 fmol elicit significant responses of males and females (Figure 3), but retention factors are considerably more pronounced for males. Furthermore, males already respond to amounts as low as 100 amol, which are released by a single female in less than 2.5 min. Loss of female susceptibility to increasing concentrations is most likely due to saturation because females that passed through a region conditioned with 10 pmol subsequently became unresponsive to retention by 100 fmol (an otherwise active cue) (Figure S14). The biosynthetic building blocks of fasc#1 (**3**), namely, 7-hydroxy-*cis*-3,4-methylenedecanoic acid (**8**) and asc-C4 (**19**), individually or in combination, did not elicit any response

Table 1. Fatty Acid Ascarosides from *C. remanei* and *C. latens*[a]

	asc-C4-···	SMID[8]	linkage	unsaturation
saturated	C6	fasc#5	(ω-3)	
	C6	fasc#6	(ω-4)	
	C8	fasc#4 (**7**)	(ω-5)	
	C8	fasc#7	(ω-6)	
	C10	fasc#8	(ω-3)	
	C12	fasc#9	(ω-5)	
	C13	fasc#10	(ω-5)	
	C14	fasc#11	(ω-5)	
	C16	fasc#12	(ω-5)	
cyclopropyl	*cy*C10	fasc#13	(ω-3)	$\Delta^{2,3}$
	*cy*C11	fasc#1 (**3**)	(ω-3)	$\Delta^{3,4}$
	*cy*C13	fasc#14	(ω-3)	$\Delta^{5,6a}$
	*cy*C15	fasc#15	(ω-3)	$\Delta^{7,8a}$
unsaturated	C10:1	fasc#3 (**5**)	(ω-3)	Δ^{3}
	C12:1	fasc#2 (**4**)	(ω-5)	Δ^{3}
	C14:1	fasc#16	(ω-5)	Δ^{5}
	C16:1	fasc#17	(ω-5)	Δ^{7a}
	C12:2	fasc#18	(ω-3)	Δ^{3} Δ^{9a}
	C12:2	fasc#19	(ω-5)	Δ^{3} Δ^{6a}

[a]Tentative assignment of position.

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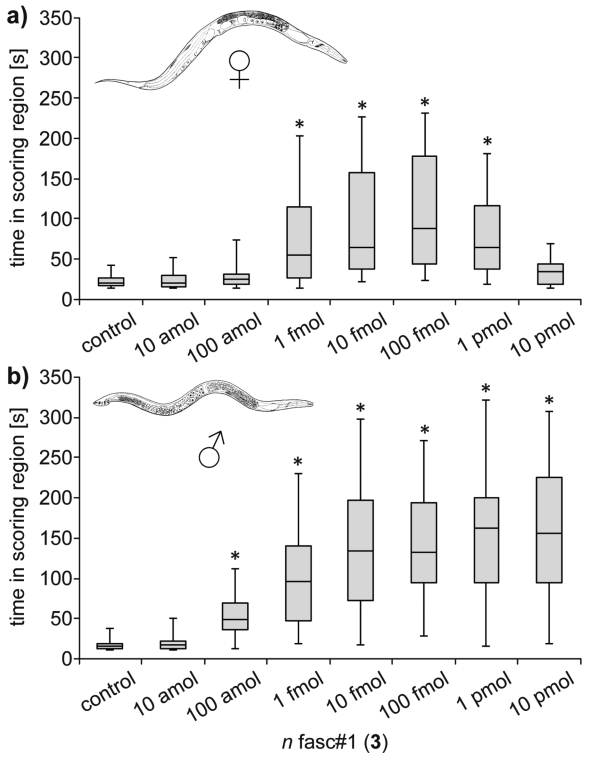

Figure 3. Holding assay shows retention of *C. remanei* females (a) and males (b) in scoring regions conditioned with fasc#1 (**3**) in comparison to solvent control (n = 20, *P < 0.001).

(Figure S15), demonstrating that fasc#1 activity depends on its intact modular structure. Furthermore, isolated fasc#2 (**4**) and partially enriched fasc#3 (**5**) did not retain *C. remanei* at any of the concentrations tested (Figure S16), demonstrating that attraction to fasc#1 (**3**) depends on the presence of the cyclopropyl unit.

Biogenesis of the cyclopropyl group containing *cis*-3,4-methylenedecanoic acid building block (**8**) was hypothesized to depend on (9*R*,10*S*)-*cis*-9,10-methylenehexadecanoic acid (*cy*C17, **25**), a component of the *E. coli* food source[12] that has previously been identified in the lipidome of *C. elegans* and shown to be exclusively derived from the bacterial diet.[25] Cyclopropanation of monounsaturated fatty acids within membrane-forming phospholipids (Figure 4a) by *S*-adenosyl methionine dependent cytosolic cyclopropyl fatty acid synthase (*cfa*) is widespread in bacteria.[26] Expression of the short-lived *cfa* protein is controlled by the stationary phase sigma factor σ^{38} (*RpoS*) and strongly induced upon entrance into the stationary growth phase.[27] The ecological significance of lipid cyclopropanation has long remained enigmatic and is now considered to involve increased survival under environmental stress such as pH shifts.[28]

GC−EIMS analysis of the fatty acid methyl ester (FAME) composition confirmed the presence of *cy*C17 (**25**) in the *C. remanei* lipidome (Figure S17). Feeding with an *E. coli* Δ*cfa* mutant[29] that does not produce any cyclopropyl fatty acids as shown by NMR (Figure S18) and GC−EIMS (Figure S19) resulted in the disappearance of *cy*C17 (**25**) from the lipidome (Figure S17) and the significant attenuation of fasc#1 (**1**) content in the exometabolome (Figure 4c), demonstrating that their biogenesis depends on bacterial *cfa*. Supplementing the Δ*cfa* diet with synthetic (±)-*cis*-9,10-methylenehexadecanoic acid (*cy*C17, **25**)[30] partially rescued its presence in the lipidome (Figure S17) along with fasc#1 (**3**) production (Figure 4c),

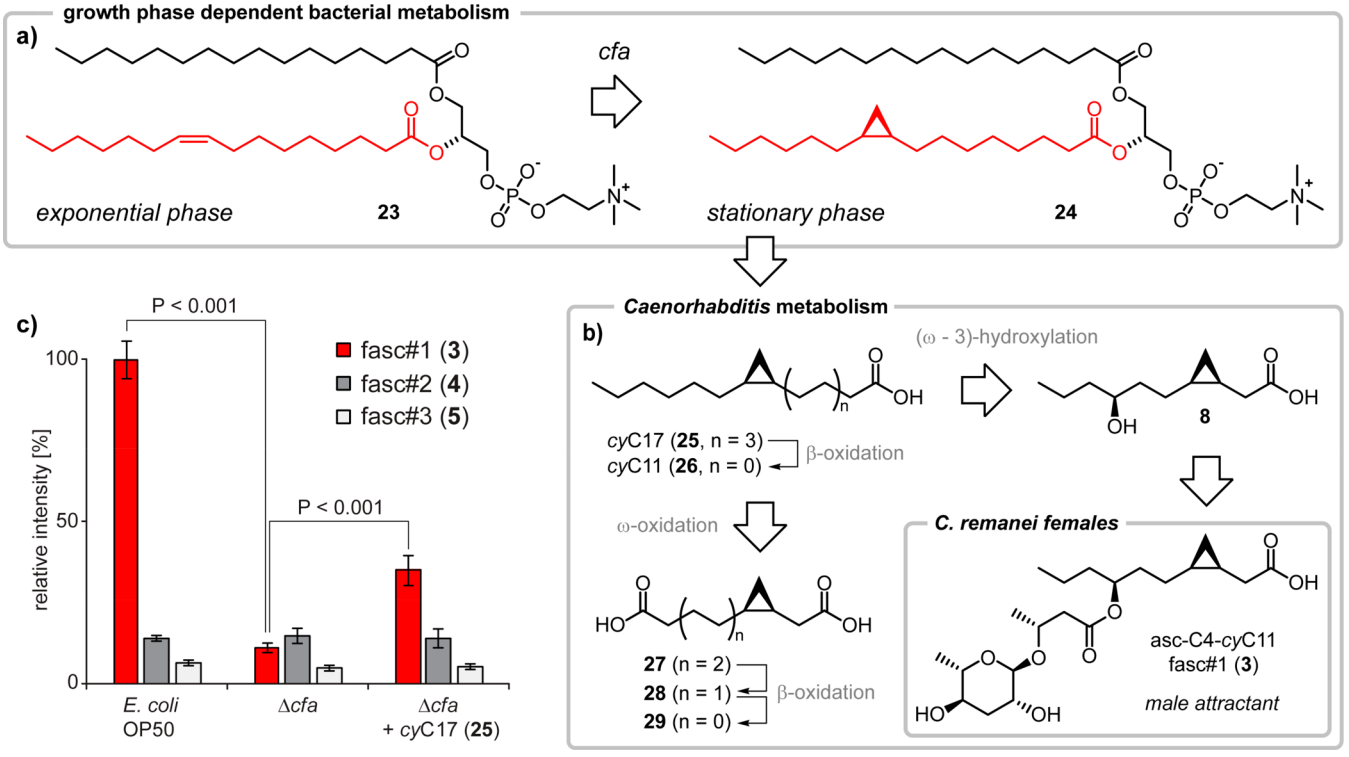

Figure 4. (a) Growth phase dependent cyclopropanation of bacterial phospholipids and (b) their metabolism in *Caenorhabditis* nematodes including the species-specific biogenesis of male attracting fasc#1 (**3**) by *C. remanei* females depends on bacterial cyclopropyl fatty acid synthase (*cfa*) as shown by feeding with the *E. coli* Δ*cfa* mutant (c).

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demonstrating that this indicator of bacterial starvation represents an essential precursor for fasc#1 (**3**) biosynthesis. In contrast, fasc#2 (**4**) and fasc#3 (**5**) were not affected by the *E. coli* Δ*cfa* diet (Figure 4c) despite the significant accumulation of palmitoleic acid (16:1n7) and *cis*-vaccenic acid (18:1n7) (Figure S19) that represents potential precursors for the 7-hydroxydec-3-enoate (10:1n7) moiety of **5**, thus suggesting its de novo biosynthesis in *C. remanei*. Furthermore, oleic acid (18:1n9), the putative precursor for the 7-hydroxydodec-3-enoate (12:1n9) moiety of fasc#2 (**4**), is not present in the bacterial diet, indicating a de novo origin.

Because fasc#1 (**3**) is only produced in small amounts, the dominating pathway for *cis*-cyclopropyl fatty acid metabolism was elucidated using comparative MS analysis of cultures fed with wildtype and Δ*cfa E. coli* (Figure S20). A series of differential compounds were isolated and identified by NMR spectroscopy, which indicated that *E. coli* derived *cy*C17 (**25**) is metabolized via the *β*-oxidation cycle to furnish *cis*-3,4-methylenedecanoic acid (*cy*C11, **26**), whereas a combination of *ω*-oxidation and *β*-oxidation affords the corresponding dicarboxylic acids *cy*C11DA (**27**), *cy*C9DA (**28**), and *cy*C7DA (**29**) (Figure 4b). Comparative MS analysis revealed that cyclopropyl fatty acid metabolism is conserved in the *Caenorhabditis* genus and identical to those in higher animals[31] including humans.[32] Furthermore, 7-hydroxy-*cis*-3,4-methylenedecanoic acid (**8**), the key building block of fasc#1 (**3**), was also observed in some other *Caenorhabditis* species (Figure S21), suggesting that it is not only its biosynthesis via (*ω*-3)-hydroxylation of *cy*C11 (**26**) but also its subsequent linkage with an activated asc-C4 (**19**) unit that controls the extraordinary species-specificity of fasc#1 (**3**) biogenesis in *C. remanei*.

In conclusion, females of the gonochoristic *C. remanei* produce species-specific modular ascarosides that integrate fatty acid building blocks from the bacterial food source or de novo metabolism. Biogenesis of the dominating component fasc#1 (**3**) depends on bacterial *cfa*, the expression of which is controlled by the stationary phase sigma factor σ^{38} (*RpoS*). Fasc#1 translates bacterial growth phase into nematode signaling by integrating a lipid component hidden within the bacterial membrane into an ascaroside signal that is released into the exometabolome. Retention of *C. remanei* males (and females) in fasc#1 conditioned regions induces site preference for habitats in which environmental resources have been made available to bacterivorous nematodes through extensive transformation by the microbiome. Along with a diversity of additional ascarosides (Figure 2a), volatile cues,[33] and other yet unidentified compounds,[34] these signals shape the behavior and development of *C. remanei* in its natural habitat.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b01914.

Detailed experimental procedures, supporting tables and figures as described in the text, and NMR spectra of isolated and synthetic compounds (PDF)

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Notes

The authors declare no competing financial interest.

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