Supplementary Information: A blend of small molecules differentially regulates both mating and development in *Caenorhabditis elegans*.

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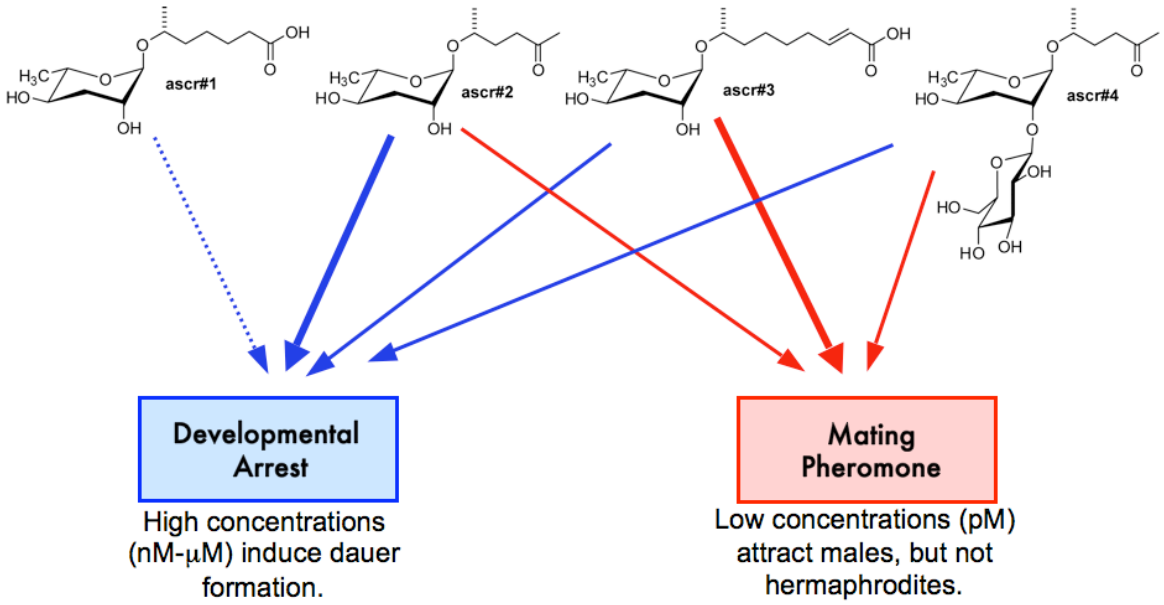
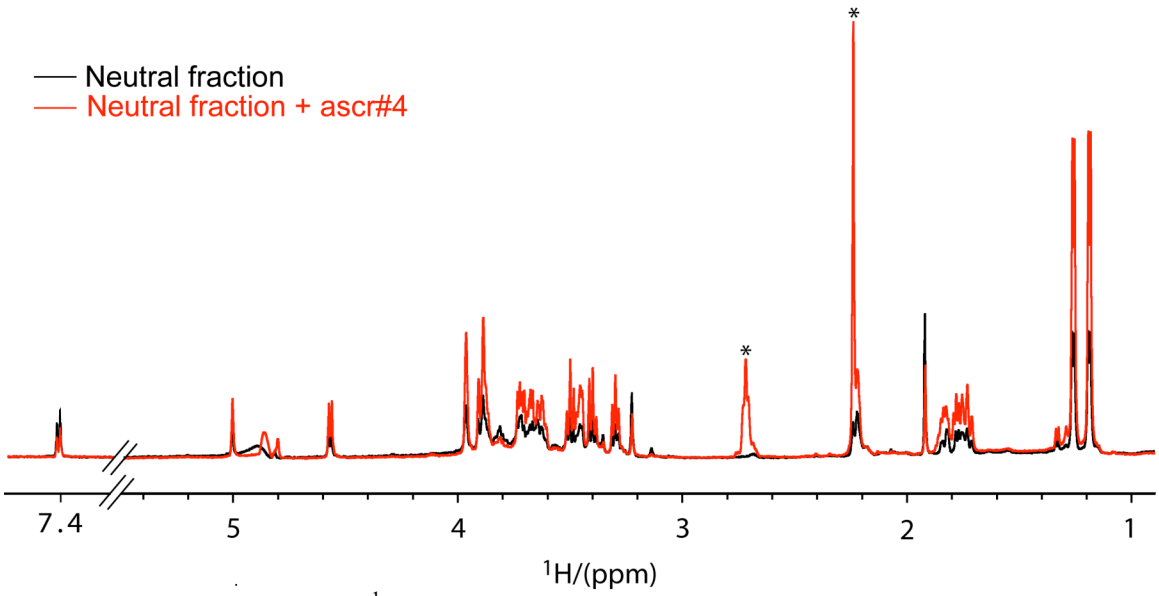
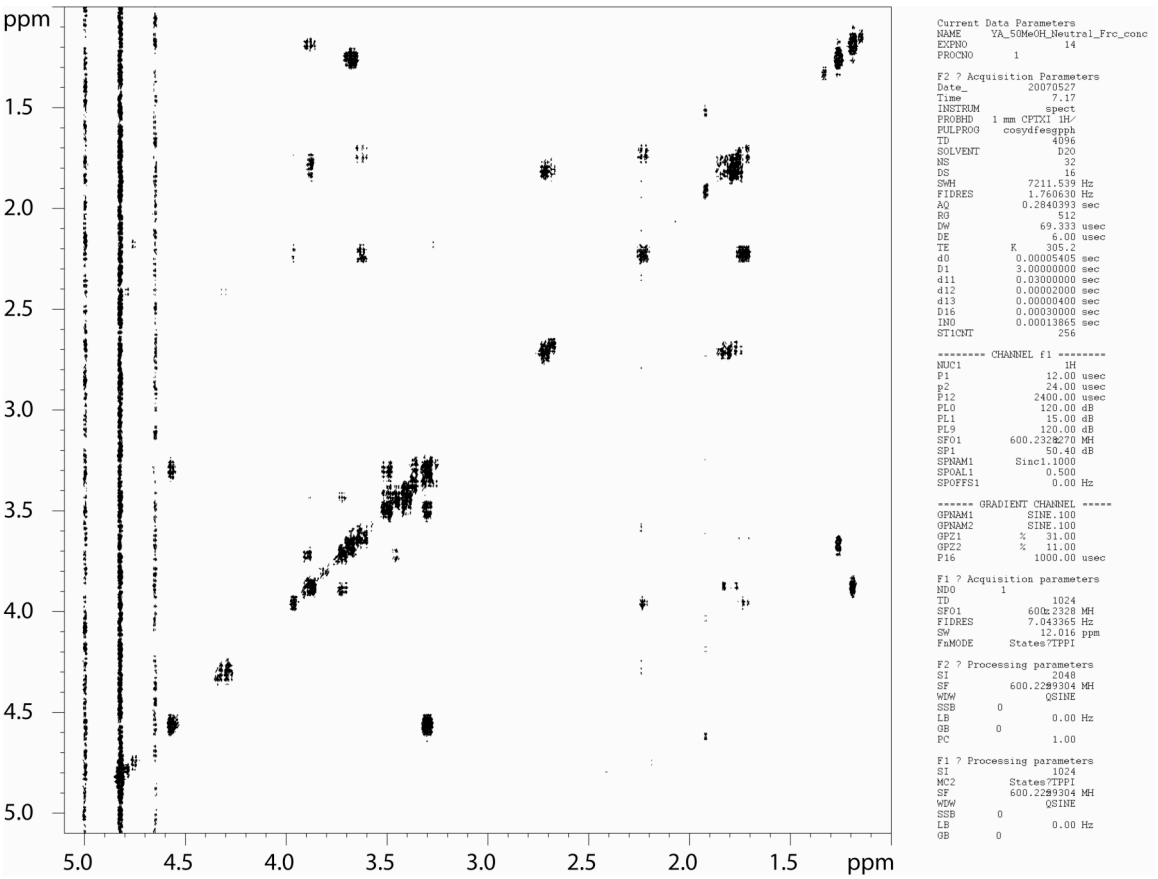
Supplementary Figures



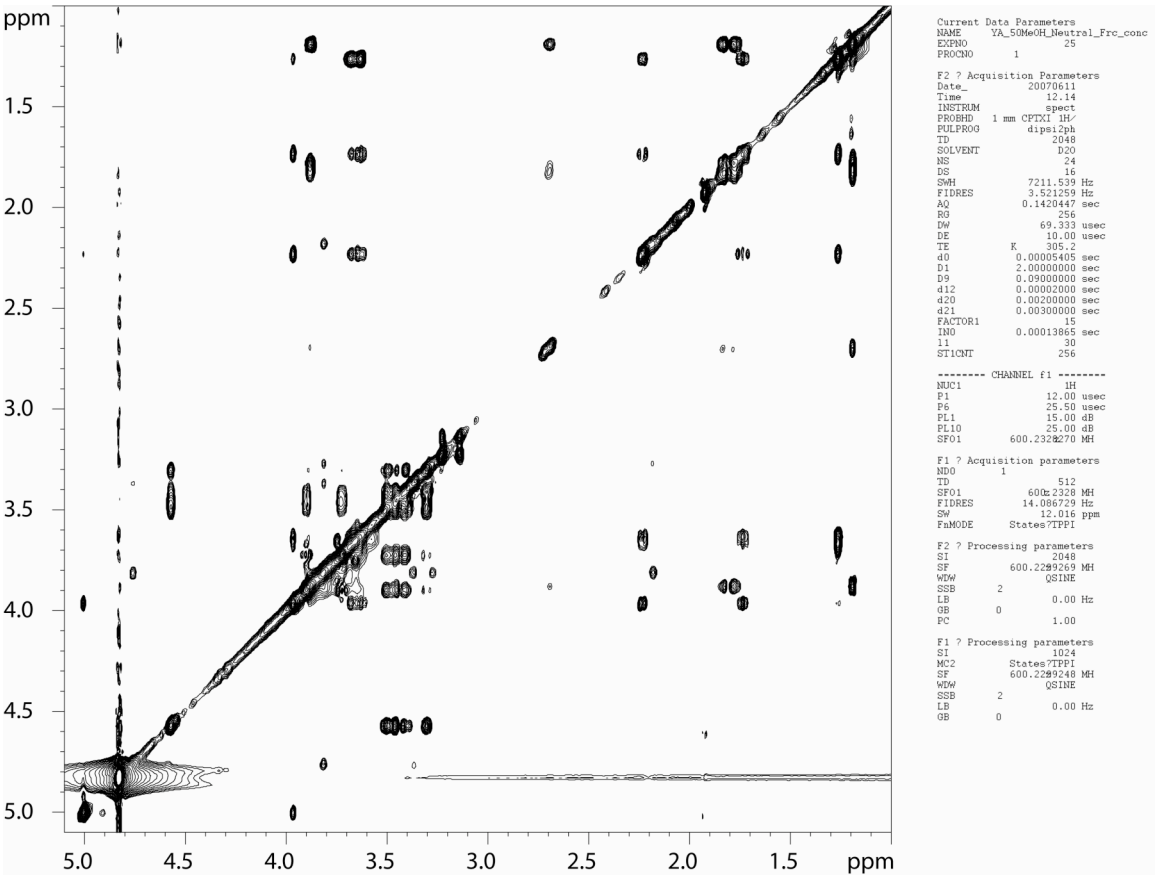
Supplementary Figure 1: Ascarosides ascr#2, ascr#3, and ascr#4 differentially regulate development and mating behavior. Ascr#2 is the most potent dauer inducer, whereas ascr#3 is the most potent component of the mating pheromone.



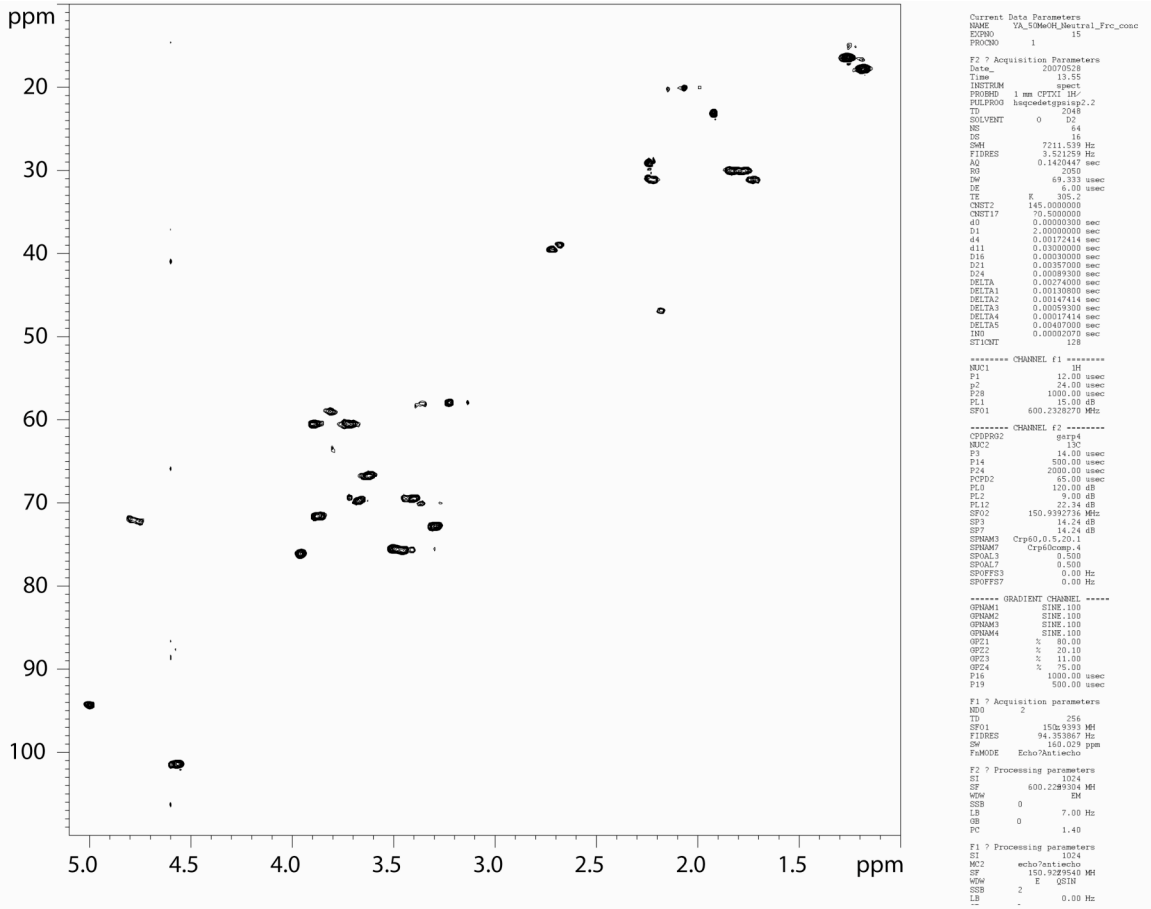
Supplementary Figure 2: ^{1}H NMR spectroscopic spiking experiment to confirm that synthetic ascr#4 is identical with the major component of ion-exchange fraction A. The spectrum obtained for fraction A is shown in black. Subsequently, synthetic ascr#4 was added to this sample, which produced the spectrum shown in red. The resonances at 2.2 and 2.7 ppm (marked with an asterisk) correspond to exchangeable protons (1-H and 3-H, see Supplementary Table 1) in the structure of ascr#4 and, at the time when fraction A was analyzed, had exchanged with the D_2O solvent. The resonance at 7.4 ppm is from another unrelated small molecule also contained in fraction A.



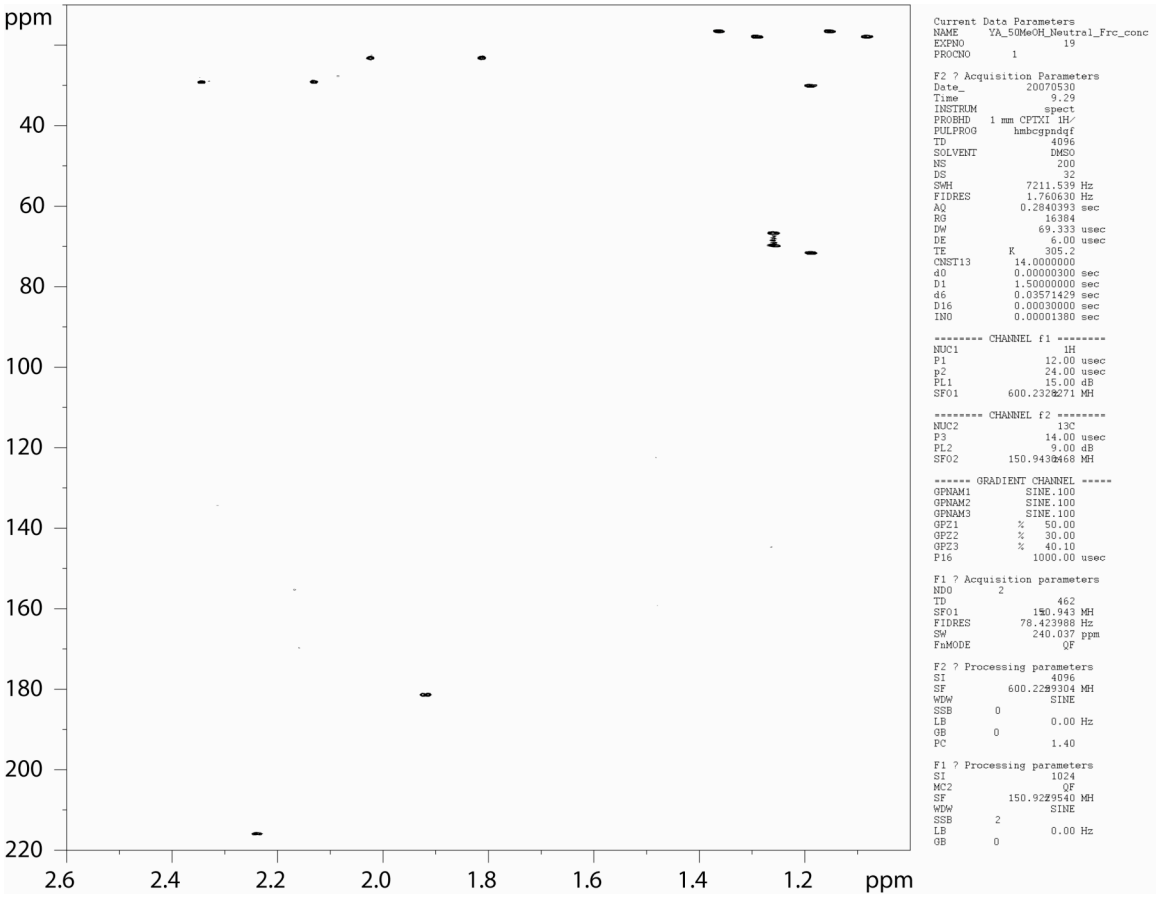
Supplementary Figure 3: COSY spectrum of fraction **A**.



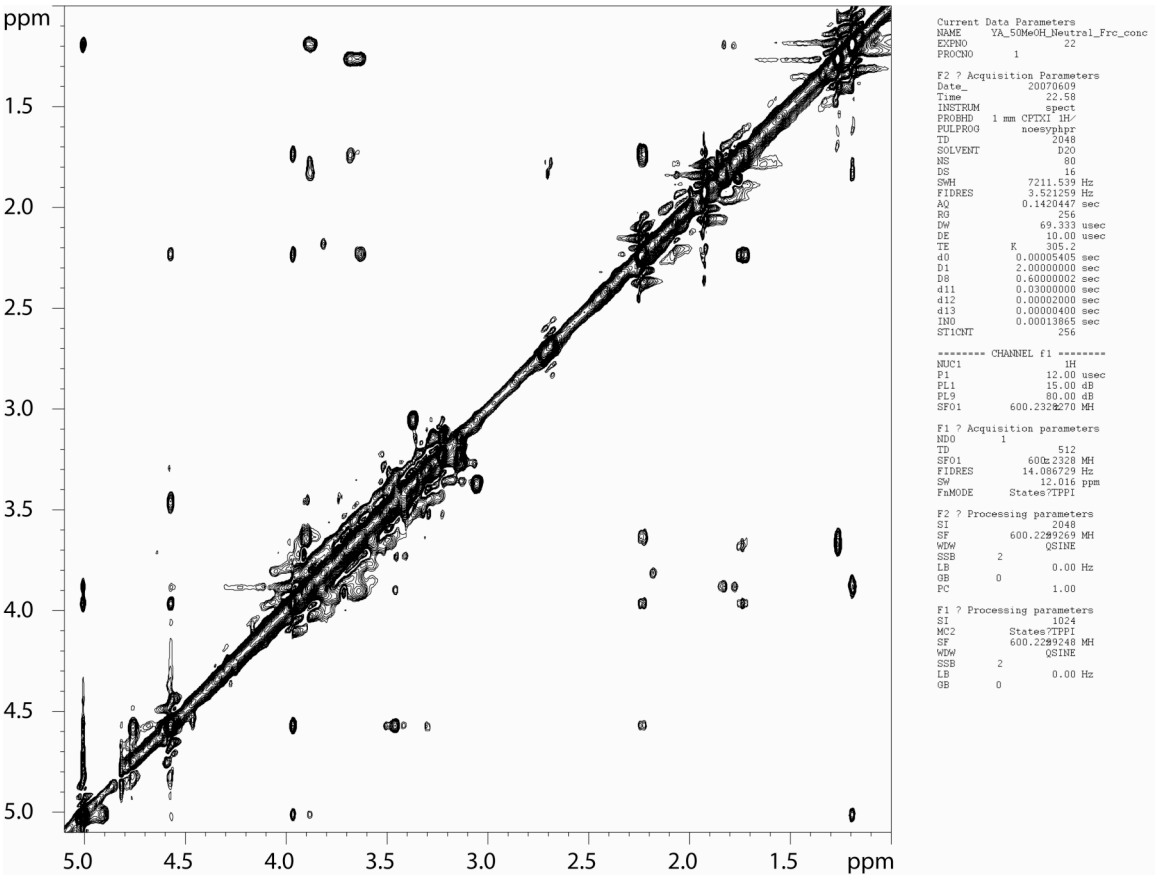
Supplementary Figure 4: TOCSY spectrum of fraction **A**.



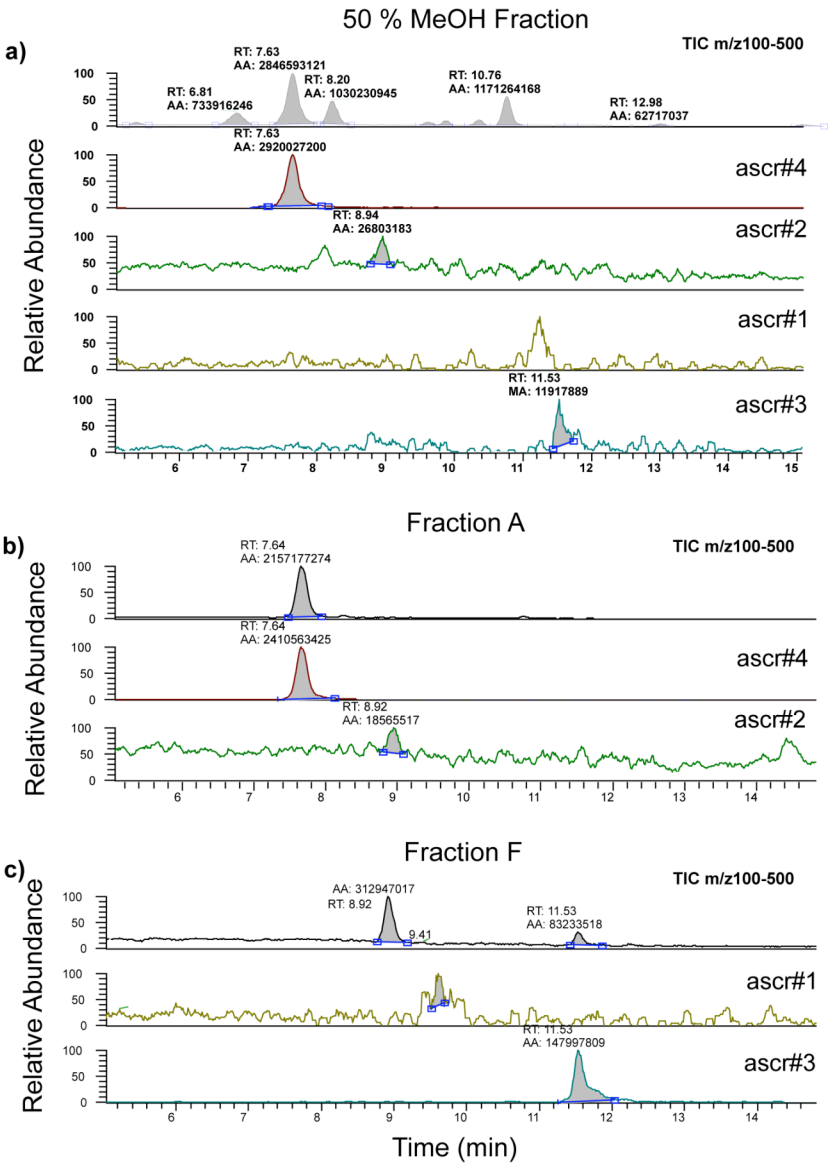
Supplementary Figure 5: ^{13}C-HSQC spectrum of fraction **A**.



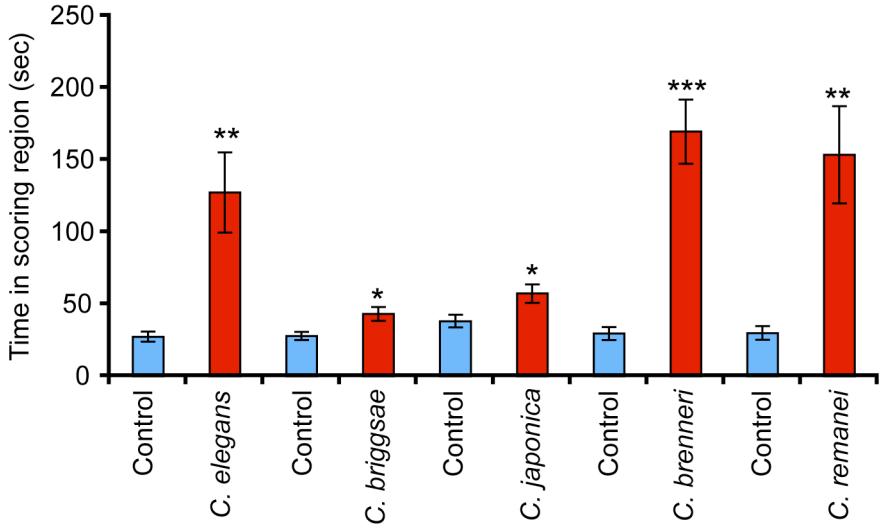
Supplementary Figure 6: ^{13}C-HMBC spectrum of fraction **A**.



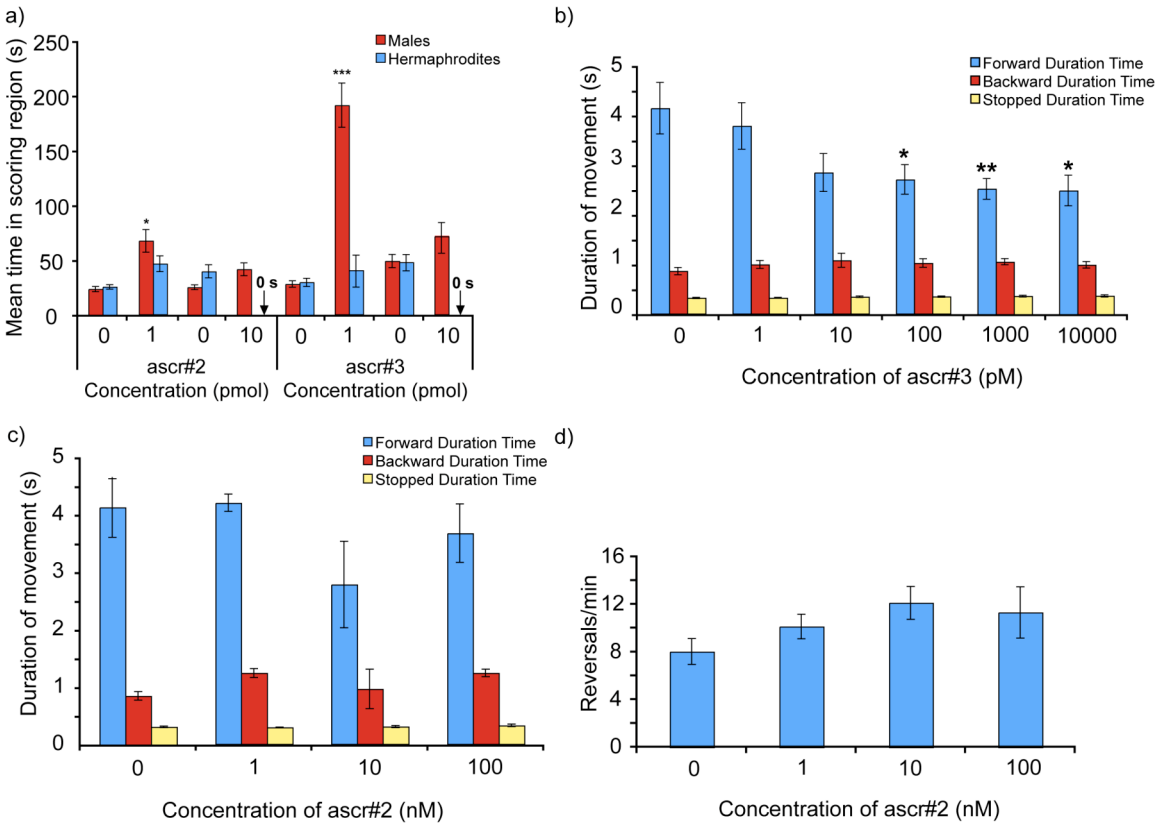
Supplementary Figure 7: NOESY spectrum of fraction **A**.



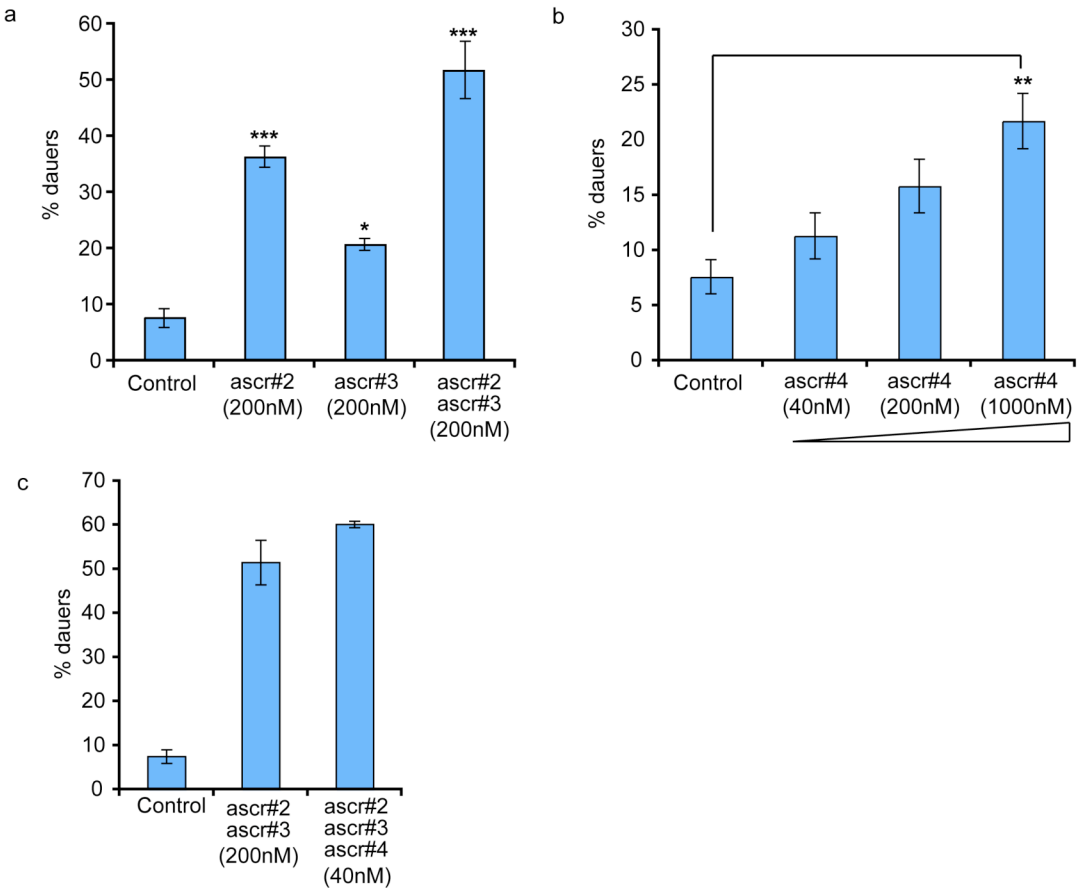
Supplementary Figure 8: LC/MS analysis of the sub-fractions of young adult worm secreted metabolites. a) Total-ion current (TIC) chromatogram and chromatograms of $[M+NH_4]$ (M+18) for ascarosides ascr#1, ascr#2, ascr#3, and ascr#4 in the 50% Methanol sub-fraction of young adult conditioned water showing the different ascarosides and their derivatives. b) Fraction A (neutral fraction) shows the presence of both ascr#4 and also a small amount of ascr#2. None of the other ascarosides were present in this fraction. c) Fraction F (1M KCl cation fraction) shows the presence of ascr#3 along with trace amounts of ascr#1. None of the other ascarosides were present.



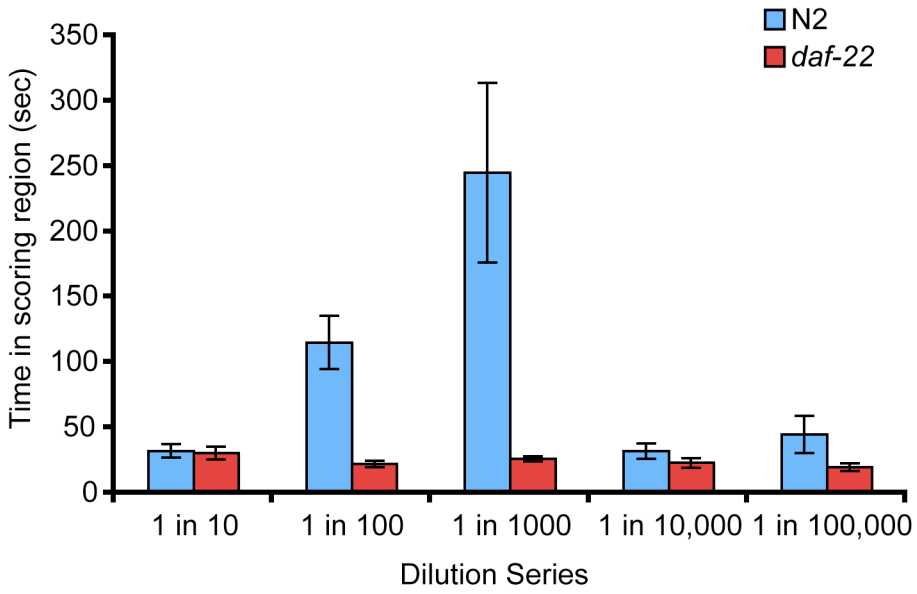
Supplementary Figure 9: Response to the ternary mix of ascr#2, ascr#3 and ascr#4 by different *Caenorhabditis* species: We tested 20 fmol ascr#2, 20 fmol ascr#3, and 1 pmol ascr#4 on the different species. *C. elegans, C. brenneri* and *C. japonica* responded robustly to the mixture (*** $p<0.0001$, ** $p<0.001$ compared to control, unpaired t-test with Welch's correction). *C. briggsae* and *C. japonica* had a weak but significant response to the mixture compared to the other three species (* $p<0.01$ compared to control, unpaired t-test with Welch's correction).



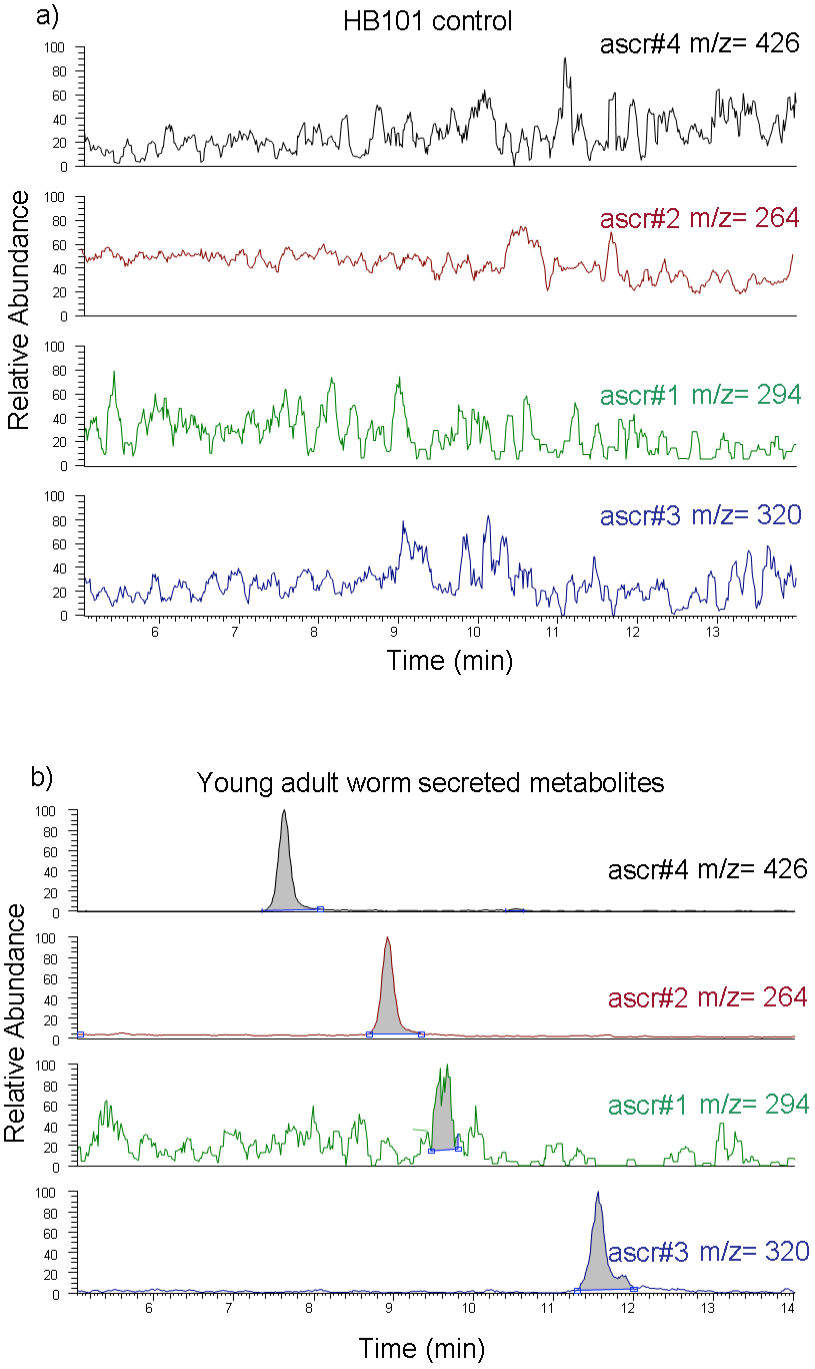
Supplementary Figure 10: ascr#3 is the most potent male attractant. a) Synthetic dauer-inducing ascarosides were tested on males (red bars) and hermaphrodites (blue bars) for attraction. ascr#3 (*** $p<0.0001$ compared to control, unpaired t-test) resulted in the strongest attraction of males at 1 pmol of compound. ascr#2 resulted in weak attraction in males (* $p<0.01$ compared to control, unpaired t-test). Hermaphrodites are not attracted at concentrations that attract males, suggesting that the attraction is sex-specific. At higher concentrations of both ascarosides, males reduce the time spent in the scoring region and hermaphrodites are deterred completely. Arrows indicate that none of hermaphrodites tested spent any time in the spotted region. b) Duration of movement by males on plates containing different concentrations of ascr#3. Forward duration decreases with increasing concentrations of ascr#3. (* $p<0.05$, ** $p<0.01$ compared to control, 1-factor ANOVA with Dunnett's post-test). c) Duration of movement by males on plates containing different concentrations of ascr#2. We did not observe any significant changes duration of forward movement between males on control plates and plates containing ascr#2. d) Reversal frequency of males is only weakly affected by ascr#2.



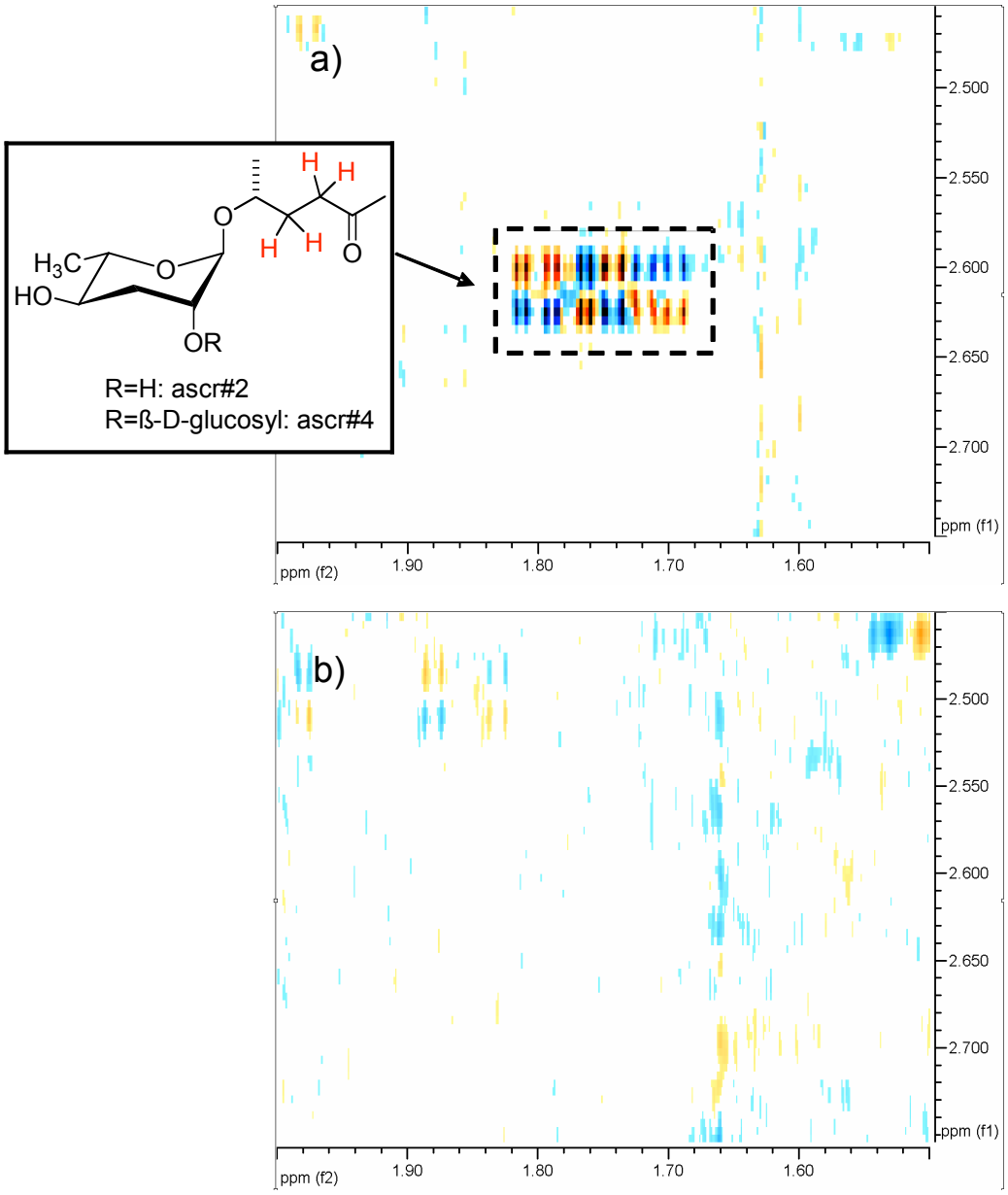
Supplementary Figure 11: Dauer formation by ascr#2, ascr#3 and ascr#4.
a) Additive effect of ascr#2 and ascr#3 in N2 liquid cultures. At 200 nM, ascr#2 is a stronger dauer inducer than ascr#3, and the activities of ascr#2 and ascr#3 are roughly additive. b) ascr#4 is a weak dauer-inducing ascaroside. ascr#4 weakly induces dauer formation at higher concentrations. c) ascr#4 does not significantly enhance the activity of ascr#2 and ascr#3 at the concentration tested. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control, 1- factor ANOVA with Dunnett's post-test).



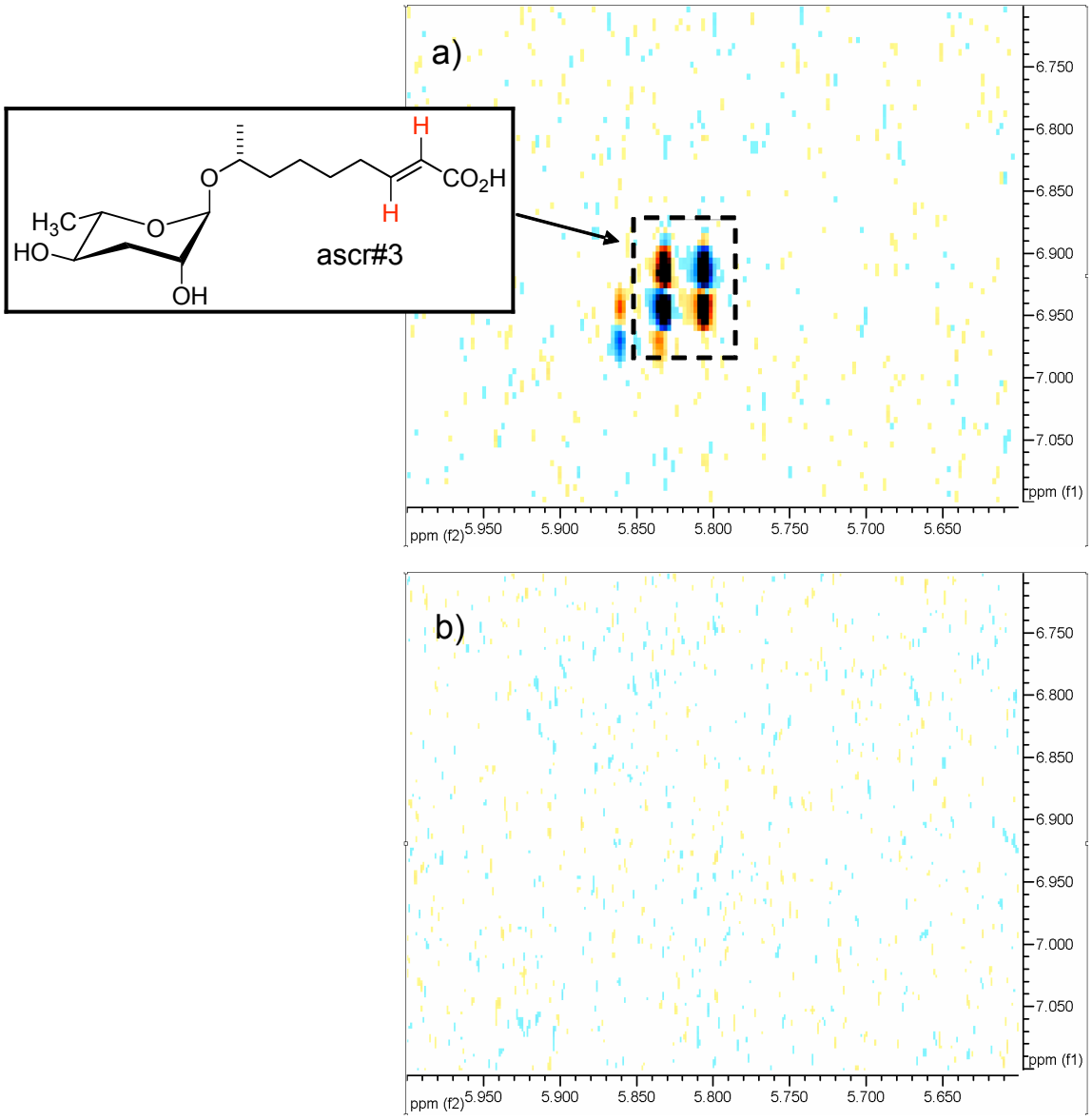
Supplementary Figure 12: *daf-22* media extract does not exhibit any activity in the male attraction assay. Male attraction assays run for a series of dilutions of wild-type (N2) and *daf-22*-media extracts (prepared as described in Supplementary Information section 3) revealed no activity for the *daf-22* extract, whereas the wild-type extract showed strong activity.



Supplementary Figure 13: LC/MS analysis of *E. coli* (HB101) control and young adult worm secretions for the presence of ascr#1, 2, 3, and 4. a) Ion chromatograms obtained for *E. coli* control extracts. Chromatograms represent $[M+NH_4]^+ = [M+18]^+$ of ascr#1, 2, 3 and 4. Figure represents data from three independent experiments, of which none revealed any of the ascarosides detected in young adult secretions. b) Young adult worm secretion showing peaks for $[M+NH_4]^+ = [M+18]^+$. Figure represents five independent experiments.



Supplementary Figure 14: Two-dimensional NMR spectroscopic analysis of bacterial extract (*E. coli* OP50) and OP50-fed *C. elegans* (N2) liquid culture extract for the presence of ascr#2. a) Diagnostic section of the double-quantum filtered COSY spectrum of a *C. elegans* (N2) liquid culture showing strong signals derived from protons 3-H and 4-H in the side chain of ascr#2 and ascr#4 (marked red in the accompanying structure). b) Complete absence of signals corresponding to ascr#2 or ascr#4 in the corresponding region of a double-quantum filtered COSY spectrum of the bacterial extract. The amount of *E. coli* culture (4 L) used for b) was roughly equal to the amount of *E. coli* used to rear the N2 *C. elegans* culture analyzed in a). Extracts were prepared as described in Supplementary Information sections 2 and 3.



Supplementary Figure 15: Two-dimensional NMR spectroscopic analysis of bacterial extract (*E. coli* OP50) and OP50-fed *C. elegans* (N2) liquid culture extract for the presence of ascr#3. a) Diagnostic section of the double-quantum filtered COSY spectrum of a *C. elegans* (N2) liquid culture showing strong signals derived from protons 2-H and 3-H in the side chain of ascr#3 (marked red in the accompanying structure). The spectrum also shows weak signals due to the presence of small amounts of a derivative of ascr#3. b) Complete absence of signals corresponding to ascr#3 in the corresponding region of a double-quantum filtered COSY spectrum of the bacterial extract. The amount of *E. coli* culture (4 L) used for b) was roughly equal to the amount of *E. coli* used to rear the N2 *C. elegans* culture analyzed in a). Extracts were prepared as described in Supplementary Information sections 2 and 3.

Supplementary Methods

1. Worm strains and maintenance: Wild-type *C. elegans* var. N2 Bristol and males from the CB1490 *him-5(e1490)* mutant were used in our bioassays. The *him-5(e1490)* mutant segregates XO male progeny by X chromosome nondisjunction during meiosis[1]. All strains were maintained at 20°C unless mentioned otherwise. Other strains used in our study are AF16 *C. briggsae*, PB4641 *C. remanei*, DF5081 *C. japonica,* PB2801 *C. brenneri*, CU5248 (*smIs26; him-5(e1490); ceh-30(tm272)*)[2], PS3981 *osm-6(p811); him-8(e1489)*, PS299 *osm-3(e1806);him-5(e1490)*, CB1489 *him-8(e1489)*, DR476 *daf-22(m130)*.

2. Preparation of N2 and *daf-22* media extracts: Worms were grown in liquid culture in 50 ml of S-media on a rotary shaker (220 rpm) for 10-12 days at 22 ºC, until a worm density of about 4,000 worms per ml of media was reached. *E. coli* (OP50) was added as needed. Subsequently, the culture was centrifuged (140 g, 20 min) and the supernatant and worm pellets were lyophilized separately. The dry residues representing supernatant and worm pellet were powdered using a mortar and extracted separately with 95% ethanol at 20 ºC for 16 h. After filtration, the filtrates were evaporated to dryness and suspended in methanol. These mixtures were filtered again and the filtrates adjusted to a volume of 1 mL each (see section 15 for details on the spectroscopic analysis).

3. Preparation of bacterial control samples: *E. coli* (HB101) cultures were subjected to a mock treatment identical to the procedure described in Supplementary Information section 2 for the collection of *C. elegans* secreted metabolites. Samples were analyzed by

LC-MS (see section 15 and Supplementary Figure 12 for details). In addition, *E. coli* (OP50) grown in LB broth at 37 ºC were centrifuged, the bacterial pellet lyophilized, and subsequently extracted as described in Supplementary Information section 3 for the preparation of N2 and *daf-22* media extracts (see section 15 and Supplementary Figures 13-14 for details on the spectroscopic analysis).

4. Data analysis for mating assay: Each time a worm entered and left the scoring region it was scored as a worm event. The time the worm entered the scoring region and left the scoring region was recorded. Once inside the scoring region, the average time spent by each worm was noted and the average time spent by the different worms in the scoring region during the 15 minute interval (control and the conditioned water) were calculated..

5. Automated tracker assays for measuring reversal frequency: For the tracker assays, we prepared standard nematode growth medium and added the different ascarosides to the required concentrations into the agar. Plates containing the ascarosides were then allowed to cool and stored at 20°C overnight. Control plates were treated the same way except that instead of the ascaroside, an equivalent quantity of 100% ethanol was added to the agar. Assay plates were seeded with a thin lawn of an overnight grown *E. coli* OP50 culture and allowed to dry overnight. 10-15 L4 males were picked 14-16 hours before the experiment on *E. coli* OP50 seeded plates. For each assay, individual males were picked and placed on assay plates. After 5 minutes, males were assayed for 15 min using the automated tracker to get the different locomotion parameters[4,5]. For each concentration of ascaroside, an equal number of males were tested on control plates.

After the recordings, worms were processed using automated tracker software and the results were analyzed using in-house matlab scripts.

6. Laser ablations and behavioral assays: We used the L1 larva stage for our neuronal ablations. 10 mM sodium azide was used as an anesthetic and ablations were performed as described previously[6]. For ablating males, we looked for the presence of the B cell in the tail region to confirm the animal was a male [7]. ASI, ASK, AWA and AWC neurons were identified using DIC microscopy using the neuronal map of the L1 larva [8]. For CEM neuron ablation, we used the *ceh-30* loss of function mutant which results in cell death of the all four CEM neurons in the male [2]. To remove all three neurons (CEM, ASI, ASK or CEM, AWA, AWC) we ablated the ASI and ASK neurons or the AWA and AWC neurons in the *ceh-30* mutant background. A successful ablation was confirmed after a few hours post recovery and did not exhibit any damage to neighboring neurons. We found that ablated males have a tendency to crawl up the side of the Petri plate and desiccate. To overcome this, we cut a 1" diameter copper pipe into thin rings. These rings were heated slightly and placed on standard NGM plates to create a vacuum seal. The plates were allowed to cool for 1 hour before putting 2-3 drops of *E. coli* OP50 in the middle of the ring. Plates were dried overnight at 20^{o}C. Both mock-ablated and ablated animals were placed on these plates at 20^{o}C for 3 days to ensure consistency before assaying them.

Given the fact that our bioassay is population-based, we tested 10 ablated individuals in our mating assay at least 3-4 times. After each assay, we transferred the ablated animals from the assay plates onto plates containing the copper rings for one hour

to reacclimatize. The same procedure was used for the mock-treated animals. As described previously in the Supplementary Information, the mean time spent in scoring region was computed for both sets of animals. Each ablation set was repeated at least on two separate days to ensure the validity of the data.

7. Dauer Formation assays in liquid cultures: Standard methods were used to grow worms in liquid cultures [9]. The different ascarosides were added at various concentrations and the proportion of dauers were quantified using the 1% SDS resistance assay [10].

8. Statistical Analysis: Different statistical tests were used to validate the data obtained from the different experiments. For each figure, the method that was most appropriate and conservative is described below. Unpaired t-test was used for the data in Figure 1b-d, Figures 2c-d, and Supplementary Figure 10a. Unpaired t-test with Welch's correction was used for Supplementary Figure 9. One-factor ANOVA with Dunnett's post-test was used on the data presented in Figure 3a and c, Supplementary Figure 10b-d, and Supplementary Figure 11a-b .

9. Syntheses of ascr#2, ascr#3: Ascr#2 and ascr#3 were prepared as described previously[12]. **Synthesis of ascr#4 (5R-(3'-O-[β-D-glucosyl]-tetrahydro-3'R,5'R-dihydroxy-6'S-methyl-2H-pyran-2'R-yloxy)-2-hexanone):** Silver carbonate (200 mg) was added to a stirred solution of ascr#2 (7 mg, 0.03 mmol) in dichloromethane (2 ml) at 22 ºC. To the resulting suspension, a solution of acetobromoglucose (100 mg, 0.24 mmol) in 2 ml of dichloromethane was added over a period of 5 h via syringe pump.

After the addition was complete, stirring was continued for additional 2 h. Subsequently, the mixture was filtered, the filter cake washed with ethyl acetate and the combined filtrates evaporated *in vacuo*. The resulting oil was dissolved in acetonitrile (0.5 ml) and purified via HPLC, using an Agilent (Santa Clara, CA, USA) 1100 Series HPLC equipped with a quaternary pump, diode array detector, autosampler, and a reversed phase 25 cm x 10 mm Supelco (Bellefonte, PA, USA) Discovery HS C18 column. A solvent gradient was used, starting with 45% methanol in water for 3 min, followed by a linear increase of methanol content to 100% at 25 min, at a flow rate of 3.4 ml/min. Under these conditions, the tetraacetate of ascr#4 eluted at 12.6 min, whereas its 4-glucosylated isomer eluted at 14.2 min. Fractions containing ascr#4 from several HPLC runs were combined, and methanol and water were removed *in vacuo*, yielding 2 mg of the tetraacetate of ascr#4 as a viscous oil. This material was re-dissolved in methanol and a solution of potassium carbonate (10 mg) in water (0.2 ml) was added. This mixture was stirred for 16 h at 22 ºC. After evaporation of the methanol, the residue was diluted with water (0.3 ml) and purified using the HPLC system described above and a solvent gradient starting with 5% methanol in water for 3 min, followed by a linear increase of methanol content reaching 100% at 25 min, at a flow rate of 3.4 ml/min. Under these conditions, ascr#4 eluted at 11.5 min. Fractions containing ascr#4 from several HPLC runs were combined and evaporated *in vacuo*, yielding 1.2 mg of pure ascr#4.

10. Stability of ascr#2 and ascr#3: Agar plates (total volume: 20 ml) prepared from Nobel agar and S-media containing ascr#2 and ascr#3 at a concentration of 200 nM were stored at room temperature for three months and subsequently lyophilized. The residue

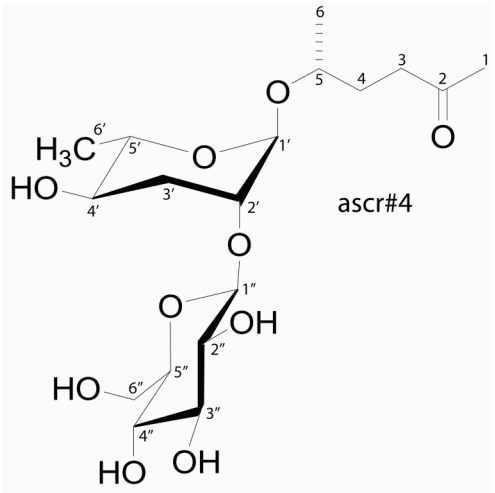
was powdered using a mortar and extracted with ethanol. The extract was evaporated to dryness, redissolved in 1 ml of methanol, and analyzed via LC-MS, using an Agilent 1100 HPLC system equipped with a 10 x 250 mm Supelco 5 μ ODS preparative column eluted at a flow rate of 3.4 ml/min, and a Micromass Quattro II triple-quadrupole mass spectrometer operated in positive-electrospray ionization mode. A solvent gradient was used starting with a solvent composition of 5% methanol and 95% water, which was maintained for three minutes and then progressed linearly to 100% methanol by 40 minutes, resulting in retention times of 21.3 min (ascr#3) and 25.6 min (ascr#2). The LC-MS analyses revealed concentrations of 3.2 μM (ascr#3) and 3.5 μM (ascr#2) in the agar extract, corresponding to 80% (ascr#3) and 87% (ascr#2) recovery.

11. Analyses of wild-type media extracts, *daf-22* media extracts, and *E. coli* (OP50) control extracts for the presence of ascarosides: Wild-type (N2) media extract, *daf-22* media extract, or bacterial control (OP50) extract (prepared as described in Supplementary Information sections 3 and 4) were analyzed by LC-MS for the presence of ascr#2 and ascr#3 as described in Supplementary Information section 14. Of six separately grown *daf-22* cultures, none contained detectable quantities of either ascr#2, ascr#3, or ascr#4, whereas all eight wild-type samples analyzed in this way contained ascr#2-ascr#4 in amounts that correspond to nanomolar concentrations in the original media. None of the three *E. coli* (OP50) control samples analyzed contained either ascr#2, ascr#3, or ascr#4. The absence of ascr#2 and ascr#3 or any derivative thereof in *daf-22* media extracts and bacterial (OP50) controls was also confirmed via 2D-NMR spectroscopic analyses of methanol-d_4 solutions of the total, unfractionated extracts,

employing methodology based on double-quantum filtered COSY (dqfCOSY) spectra as described in reference[12] (see Supplementary Figures 13-14). For these analyses a Varian INOVA 600 MHz NMR spectrometer equipped with a 5 mm inverse-detection HCN-probe was used.

Supplementary Table 1: ^{1}H and ^{13}C NMR spectroscopic data for ascr#4 (600 MHz, D_2O). For details, see NMR methods above.

#	δH [ppm]	*J* [Hz]	δC [ppm]
1	2.22		31.74
2			218.90
3	2.70		42.28
4a	1.76		32.89
4b	1.82		
5	3.86	$J_{6,5}$ = 6.1	74.31
6	1.17		20.57
1'	4.99	$J_{1',2'}$ = 2, $J_{1',3'b}$ = 1.5	97.11
2'	3.95	$J_{2',3'a}$ = 3.0, $J_{2',3'b}$ = 3.6	78.80
3'a	1.71	$J_{3'a,3'b}$ = 13.9, $J_{3'a,4'}$ = 11.3	33.99
3'b	2.21	$J_{3'b,4'}$ = 4.4	33.99
4'	3.61	$J_{4',5'}$ = 9.7	69.44
5'	3.66	$J_{5',6'}$ = 6.1	72.55
6'	1.24		19.29
1"	4.57	$J_{1'',2''}$ = 7.9	104.20
2"	3.28	$J_{2'',3''}$ = 9.4	75.59
3"	3.48	$J_{3'',4''}$ = 9.3	78.37
4"	3.38	$J_{4'',5''}$ = 9.8	72.30
5"	3.44	$J_{5'',6''a}$ = 2.5, $J_{5'',6''b}$ = 5.9	78.53
6"a	3.88	$J_{6''a,6''b}$ = 12.5	63.34
6"b	3.71		63.34



Supplementary Movie S1: Mate response assay showing males responding to YA worm metabolites. The movie is sped up ten times the normal speed. Conditioned water (left side of frame) and control water (right side of frame) were added to the scoring region (See Supplementary methods) and allowed to dry for 5 min. After drying, males were added in the designated spots. After 5 minutes, worms were recorded for 15 minutes. The males enter the conditioned water spot and tend to remain longer than in the control spot. Males also reverse more in the conditioned spot region than in the control water region.

Supplementary Movie S2: Mate response assay (movie is sped up ten times the normal speed) showing *Cel-him-5* males responding to a mixture of the 2 synthetic ascarosides ascr#2 (100 fmol) and ascr#3 (10 fmol). The mixture was added to the scoring region on

the right and control water to the scoring region on the left. Males spent significantly more time in the scoring region on the right and reverse more frequently than on the rest of the agar plate.

References:

1. Hodgkin, J., Horvitz, H. R. & Brenner, S. Nondisjunction Mutants of the Nematode *Caenorhabditis elegans*. Genetics 91, 67-94 (1979).
2. Peden, E., Kimberly, E., Gengyo-Ando, K., Mitani, S. & Xue, D. Control of sex-specific apoptosis in *C. elegans* by the BarH homeodomain protein CEH-30 and the transcriptional repressor UNC-37/Groucho. Genes Dev 21, 3195-207 (2007).
3. Simon, J. M. & Sternberg, P. W. Evidence of a mate-finding cue in the hermaphrodite nematode *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 99, 1598-603 (2002).
4. Cronin, C. J., Feng, Z. & Schafer, W. R. Automated imaging of *C. elegans* behavior. Methods Mol Biol 351, 241-51 (2006).
5. Cronin, C. J. et al. An automated system for measuring parameters of nematode sinusoidal movement. BMC Genet 6, 5 (2005).
6. Bargmann, C. I. & Avery, L. Laser killing of cells in *Caenorhabditis elegans*. Methods Cell Biol 48, 225-50 (1995).
7. Chamberlin, H. M. & Sternberg, P. W. Multiple cell interactions are required for fate specification during male spicule development in *Caenorhabditis elegans*. Development 118, 297-324 (1993).
8. Sulston, J. E. & Horvitz, H. R. Abnormal cell lineages in mutants of the nematode *Caenorhabditis elegans*. Dev Biol 82, 41-55 (1981).
9. Lewis, J. A. & Fleming, J. T. Basic culture methods. Methods Cell Biol 48, 3-29 (1995).
10. Golden, J. W. & Riddle, D. L. The *Caenorhabditis elegans* dauer larva: developmental effects of pheromone, food, and temperature. Dev Biol 102, 368-78 (1984).
11. Brey, W. W. et al. Design, construction, and validation of a 1-mm triple-resonance high-temperature-superconducting probe for NMR. J Magn Reson 179, 290-3 (2006).
12. Butcher, R. A., Fujita, M., Schroeder, F. C. & Clardy, J. Small-molecule pheromones that control dauer development in *Caenorhabditis elegans*. Nat Chem Biol 3, 420-2 (2007).