Supporting Information

An indole-containing dauer pheromone component with unusual dauer inhibitory activity at higher concentrations

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Supplemental Methods

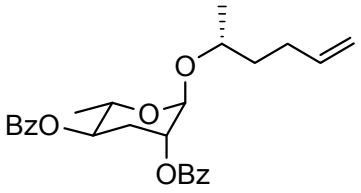
General procedures. NMR spectra for natural and synthetic indolecarboxyl-ascaroside C5 (**5**) were recorded on a Varian VNMRS 600 NMR (600 MHz for ^{1}H, 151 MHz for ^{13}C) or a Varian INOVA 600 NMR equipped with a cryoprobe. NMR spectra for all synthetic intermediates were recorded on a Bruker Avance 300 NMR (300 MHz for ^{1}H, 75 MHz for ^{13}C). Optical rotations were measured on an Autopol IV Automatic Polarimeter (Rudolph Research Analytical). CD spectra were collected in an AVIV model 202 CD spectropolarimeter with a 1 mm-pathlength cuvette. HRMS was performed at the University of Illinois at Urbana-Champaign Mass Spectrometry Facility.

Strains and culture conditions. *C. elegans* variety Bristol, strain N2 (wild type) worms and *daf-22* (*ok693*) worms were grown at room temperature on NGM agar plates, which were made with granulated agar (BD Biosciences) and seeded with OP50 bacteria.

Purification of indolecarboxyl-ascaroside C5 (5). Crude dauer pheromone (obtained from a total of 16.5 L of culture) was fractionated by C_{18} column chromatography with stepwise gradient of aqueous methanol (0% to 100%). The active fractions were followed with the dauer formation assay. The most active fractions were combined and further fractionated on a silica gel column with chloroform and methanol solvent mixtures (10:1 to 1:5), followed by a chloroform, methanol and water solvent mixture (3:7:0.5). Active fractions (that eluted from the silica gel column following ascaroside C6) were further purified by reversed phase HPLC on a C_{18} column (Supelco) using an aqueous acetonitrile gradient (10% to 80%). The final yield was 125 μg indolecarboxyl-ascaroside C5, as estimated using an UV-based standard curve generated with synthetic material. Indolecarboxyl-ascaroside C5 (**5**): UV (MeOH) λ_{max} (log ε) 213 (4.2), 226 (3.9), 245 (3.7), 281 (3.7) nm; NMR spectral data, see Table 1 and Figure S1; HRESIMS, see main text; CD, see below.

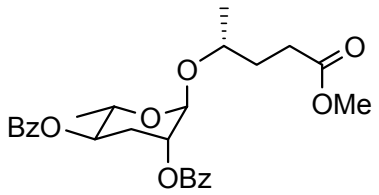
General synthetic methods. All reactions were performed under a nitrogen atmosphere. Chemicals and solvents were purchased from commercial suppliers and used as received. Methylene chloride and tetrahydrofuran were dried by passing through an activated alumina column prior to use. All flash chromatography was performed using 230-400 mesh silica gel (EMD Chemicals).

Synthesis of 8:



To a 0 ^{o}C suspension of 58.4 mg (0.164 mmol) dibenzoyl ascarylose (**6**)[1], 30 μL (0.25 mmol) (*R*)-5-hexen-2-ol (**7**) and 20 mg crushed 3 Å molecular sieves in 2 mL dichloromethane was added 80 μL $BF_3{\cdot}OEt_2$ at once. The resulting mixture was stirred at 0 ^{o}C for 2 h and then 2 mL saturated $NaHCO_3$ solution was added at once. 10 mL dichloromethane was then added and the layers were separated. The aqueous layer was extracted with an additional 2 x 10mL dichloromethane. Organic extracts were dried over anhydrous $MgSO_4$ and filtered. Evaporation of volatiles afforded 77 mg of an oil. Silica gel chromatography (50-67 % dichloromethane in hexanes) afforded 52.8 mg (73 %) of a colorless oil. $[\alpha]_D^{20}$ = -8.0, *c* 0.44 (MeOH); HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{26}H_{30}O_6Na$ 461.1940, found 461.1946; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.12 (d, 1H, J=8.4 Hz); 8.05 (d, 2H, J=8.4 Hz); 7.59 (t, 2H, J=9.0 Hz); 7.47 (m, 4H); 5.88 (m, 1H); 5.19 (td, 1H, J=9.9 Hz, J=4.5 Hz); 5.15 (m, 1H); 5.09 (d, 1H, J=17.1 Hz); 5.02 (d, 1H, J=10.2 Hz); 4.96 (s, 1H); 4.13 (m, 1H); 3.88 (m, 1H); 2.43 (dt, 1H, J=13.5 Hz, J=3.9 Hz); 2.22 (m, 3H); 1.75 (m, 1H); 1.62 (m, 1H); 1.28 (d, 3H, J=6.3 Hz); 1.21 (d, 3H, J=6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.8; 165.7; 138.4; 133.2; 133.1; 130.1; 130.0; 129.9; 129.6; 128.4; 114.7; 93.8; 72.1; 71.3; 70.7; 67.1; 36.4; 30.0; 29.8; 19.1; 17.9.

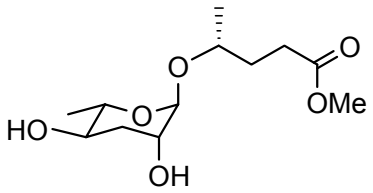
Synthesis of 9:



A solution of 209.0 mg (0.477 mmol) **8** in 10 mL dichloromethane was cooled to –78 ^{o}C in a dry ice/acetone bath. To this solution was added a steady stream of O_3 over the course of 2 min resulting in the formation of a persistent blue color to the solution. Then, a steady stream of dry N_2 was added to this solution over the course of 20 min until the blue color had completely disappeared. 477 mg (~1.43 mmol of phosphine) Janda Jel polymer-supported PPh_3 was then added and the reaction mixture was allowed to warm to 23 ^{o}C and stir for 21 h. The reaction mixture was filtered and the filter cake was washed with 2 x 10 mL dichloromethane. The filtrate was washed with 5 mL saturated $NaHCO_3$ solution and the aqueous wash was then back-extracted with 2 x 10 mL dichloromethane. Organic extracts were dried over anhydrous Na_2SO_4 and filtered. Evaporation of volatiles afforded 223.4 mg of an oil that was redissolved in 10 mL *t*BuOH and 2.5 mL dimethyl sulfoxide. To this solution was added dropwise a solution of 412 mg (4.56 mmol) $NaClO_2$ and 490 mg (3.6 mmol) $NaH_2PO_4 \cdot H_2O$ in 4.5 mL H_2O dropwise over the course of 3 min. The resulting mixture was stirred at 23 ^{o}C for 30 min after which the volatiles were evaporated. 12 mL H_2O was added and the pH of the solution was adjusted to 3 via the addition of 2 N HCl. The resulting mixture was extracted with 6 x 10 mL EtOAc. The extracts were then dried over anhydrous Na_2SO_4 and filtered. Evaporation afforded 292.9 mg of an oil that was then dissolved in 3 mL MeOH. To this solution was added 0.38 mL (0.76 mmol) of a solution of trimethylsilyldiazomethane (2 M in Et_2O) over the course of 2 min. After two min stirring at 23 ^{o}C, an additional 0.2 mL (0.4 mmol) of the trimethylsilyldiazomethane solution was added. The reaction mixture was allowed to stir for an additional 10 min after which glacial acetic acid was added dropwise until the yellow color of the solution had disappeared. Evaporation of volatiles afforded 331 mg of an oil. Silica gel chromatography (CH_2Cl_2) of the oil afforded 149.8 mg of a residue that was suspended in 10 mL hexanes and filtered in order to remove contaminant dimethyl sulfone. Evaporation afforded 135.2 mg (60 %, 3 steps) of an

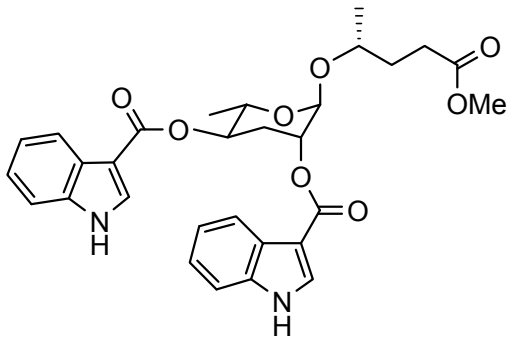
oil. $[\alpha]_D^{20}$ = -11.1, *c* 0.52 (MeOH); HRMS (*m/z*): [M+Na]+ calcd. for $C_{26}H_{30}O_8Na$ 493.1838, found 493.1822; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.11 (d, 2H, J=7.8 Hz); 8.05 (d, 2H, J=7.8 Hz); 7.59 (t, 2H, J=7.5 Hz); 7.47 (m, 4H); 5.18 (td, 1H, J=9.9 Hz, J=4.5 Hz); 5.14 (m, 1H); 4.95 (s, 1H); 4.07 (m, 1H); 3.90 (m, 1H); 3.71 (s, 3H); 2.51 (t, 2H, J=7.4 Hz); 2.42 (dt, 1H, J=13.5 Hz, J=3.9 Hz); 2.18 (t, 1H, J=12.0 Hz); 1.92 (m, 2H); 1.28 (d, 3H, J=6.3 Hz); 1.22 (d, 3H, J=6.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.8; 165.8; 165.7; 133.24; 133.17; 130.1; 129.91; 129.87; 129.7; 128.5; 93.6; 71.4; 71.2; 70.6; 67.2; 51.6; 32.1; 30.3; 29.8; 18.9; 17.9.

Synthesis of 9a:



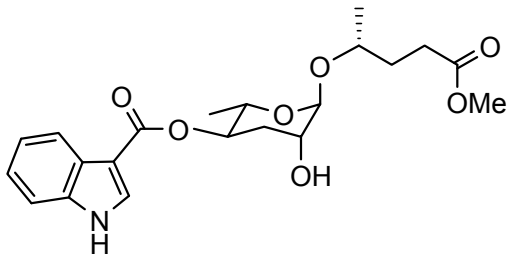
74 mg (3.2 mmol) Na was added to 10 mL MeOH. Consumption of Na by the MeOH resulted in a ~0.32 M solution of NaOMe. To a 23°C solution of 69.5 mg (0.148 mmol) of **9** in 2 mL MeOH was added 2.3 mL (~0.74 mmol) of the aforementioned NaOMe solution at once. The resulting solution was stirred at 23 °C for 2 h after which was added 0.05 mL glacial acetic acid. Volatiles were evaporated and 5 mL H_2O was added. The resulting mixture was extracted with 6 x 5mL EtOAc and dried over anhydrous Na_2SO_4. Filtration and evaporation afforded 69.6 mg of a residue. Silica gel chromatography (3-5 % MeOH in CH_2Cl_2) afforded 35.4 mg (91 %) of a colorless oil. $[\alpha]_D^{20}$ = -96.7, *c* 0.48 (MeOH); HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{12}H_{22}O_6Na$ 285.1314, found 285.1322; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.67 (s, 1H); 3.82 (m, 1H); 3.77 (m, 1H); 3.66 (s, 3H); 3.59 (m, 2H); 2.42 (t, 2H, J=7.2 Hz); 2.20-2.25 (m, 2H); 2.04 (dt, 1H, J=13.2 Hz, J=3.3 Hz); 1.82 (m, 3H); 1.25 (d, 3H, J=6.0 Hz); 1.13 (d, 3H, J=6.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.1; 95.7; 70.2; 70.0; 69.1; 67.9; 51.6; 35.1; 32.1; 30.3; 18.6; 17.6.

Synthesis of 10:



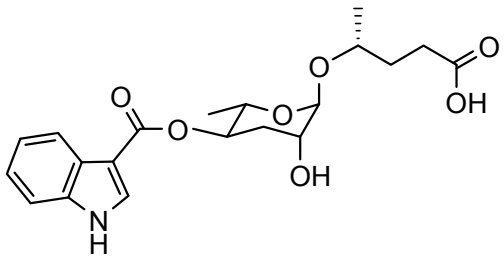
To a 0 °C solution of 54.2 mg (0.207 mmol) **9a** and 0.25 mL (1.5 mmol) diisopropylethylamine in 3 mL THF was added a solution of 223 mg (1.24 mmol) indole-3-carboxyl chloride in 2 mL THF over the course of 7 min. The ice bath was promptly removed and the reaction mixture was allowed to warm to 23 °C and stir for 90 min. 5 mL H_2O was then added at once and the resulting mixture was allowed to stir for an additional 8 min. The mixture was extracted with 4 x 10 mL EtOAc. Organic extracts were dried over anhydrous Na_2SO_4 and filtered. Evaporation afforded 249 mg of a residue. The material was purified with silica gel chromatography (5-20 % Et_2O in CH_2Cl_2). The resulting fractions containing desired product were concentrated and suspended in 5 mL CH_2Cl_2. The suspension was filtered through a plug of cotton and the resulting filtrate was re-purified with silica gel chromatography (5-15 % Et_2O in CH_2Cl_2) which afforded 94.7 mg (83 %) of an oil. $[\alpha]_D^{20}$ = +19.0, *c* 0.31 (MeOH); HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{30}H_{32}N_2O_8Na$ 571.2056, found 571.2033; ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.18 (s, 1H); 9.12 (s, 1H); 8.25 (d, 1H, J=7.5 Hz); 8.17 (m, 1H); 7.92 (d, 1H, J=3.0 Hz); 7.88 (d, 1H, J=3.0 Hz); 7.41 (m, 2H); 7.26 (m, 4H); 5.32 (td, 1H, J=10.8 Hz, J=4.8 Hz); 5.21 (m, 1H); 5.03 (s, 1H); 4.13 (m, 1H); 3.95 (m, 1H); 3.72 (s, 3H); 2.56 (t, 2H, J=7.5 Hz); 2.50 (m, 1H); 2.25 (t, 1H, J=12.6 Hz); 1.96 (m, 2H); 1.34 (d, 3H, J=6.0 Hz); 1.23 (d, 3H, J=6.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.1; 164.5; 164.2; 136.2; 131.8; 131.5; 125.9; 125.8; 123.3; 123.2; 122.2; 121.43; 121.37; 111.7; 111.6; 108.5; 108.2; 94.0; 71.2; 70.0; 69.6; 67.6; 51.6; 32.2; 30.4; 30.2; 18.9; 18.0.

Synthesis of 10a:



143 mg (6.21 mmol) Na was added to 5 mL MeOH resulting in the formation of a ~1.24 M solution of NaOMe in MeOH. To a 23 ^{o}C solution of 39.2 mg (71.5 μmol) **6** in 2 mL MeOH was added 0.17 mL (~0.21 mmol) on the aforementioned NaOMe solution at once. The resulting solution was warmed from 23 ^{o}C to reflux over a period of 4 min. After 4.5 h, the solution was allowed to cool to 23 ^{o}C. Methanolic HCl was carefully added until the solution pH = 2. Evaporation of volatiles afforded an oil that was resissolved in 2 mL 1:1 MeOH/THF. To this 23 ^{o}C solution was added 0.2 mL (0.4 mmol) of a solution of trimethylsilyldiazomethane (2 M in Et_2O) at once. The resulting mixture was stirred for 40 min and then glacial acetic acid was added until the yellow color of the solution had disappeared. The reaction mixture was filtered through a plug of cotton and evaporated to afford 53.7 mg of a solid. Silica gel chromatography (1-2 % MeOH in CH_2Cl_2) afforded 7.6 mg (26 %) of a colorless oil. $[\alpha]_D^{20}$ = -94.2, *c* 0.38 (MeOH); HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{21}H_{27}NO_7Na$ 428.1685, found 428.1686; ^{1}H NMR (300 MHz, CD_3OD): δ 8.04 (m, 1H); 7.97 (s, 1H); 7.45 (m, 1H); 7.20 (m, 2H); 5.13 (td, 1H, J=10.4 Hz, J=4.8 Hz); 4.75 (s, 1H); 4.01 (m, 1H); 3.90 (m, 1H); 3.79 (m, 1H); 3.69 (s, 3H); 2.54 (td, 2H, J=7.8 Hz, J=4.8 Hz); 2.22 (dt, 1H, J=12.9 Hz, J=5.1 Hz); 2.00 (t, 1H, J=12.0 Hz); 1.87 (m, 2H); 1.23 (d, 3H, J=6.3 Hz); 1.19 (d, 3H, J=6.3 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 174.0; 164.2; 136.2; 131.3; 125.8; 123.3; 122.2; 121.6; 111.5; 108.8; 96.2; 70.7; 69.4; 68.9; 67.9; 51.6; 32.4; 32.2; 30.3; 18.8; 17.8.

Synthesis of indolecarboxyl-ascaroside C5 (5):



A solution of 4.0 mg (9.9 μmol) **10a** and 2.0 mg (16 μmol) potassium trimethylsilanolate in 2 mL THF was warmed from 23 ^{o}C to reflux over a period of 2 min. After 1h, an additional 3.2 mg (25 μmol) potassium trimethylsilanolate was added and the mixture was refluxed for an additional 32 min and then allowed to cool to 23 ^{o}C. 2 mL 0.1 N HCl was added and the resulting mixture was extracted with 6 x 5 mL EtOAc. The extracts were dried over anhydrous Na_2SO_4 and filtered. Evaporation of volatiles afforded 4.0 mg of a residue. Silica gel chromatography (2.5-5 % *i*PrOH in CH_2Cl_2) afforded 2.0 mg (52 %) of a colorless oil. $[\alpha]_D^{20}$ = -91.8, *c* 0.17 (MeOH); HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{20}H_{25}NO_7Na$ 414.1523, found 414.1514; ^{1}H NMR (300 MHz, CD_3OD): δ 8.04 (m, 1H); 7.97 (s, 1H); 7.44 (m, 1H); 7.20 (m, 2H); 5.13 (td, 1H, J=10.4 Hz, J=4.8 Hz); 4.76 (s, 1H); 4.05 (m, 1H); 3.93 (m, 1H); 3.80 (m, 1H); 2.50 (m, 2H); 2.22 (dt, 1H, J=12.9 Hz, J=5.1 Hz); 2.03 (t, 1H, J=12.0 Hz); 1.87 (m, 2H); 1.24 (d, 3H, J=6.3 Hz); 1.19 (d, 3H, J=6.3 Hz).

Comparison of natural and synthetic indolecarboxyl-ascaroside C5 (5). Synthetic indolecarboxyl-ascaroside C5 was converted to a salt by incubation with a two molar excess of sodium hydroxide so that it could be compared by NMR to natural indolecarboxyl-ascaroside C5, which was isolated as a salt (see Table 1 and Table S1 and Figure S1 and S3). CD spectra were also obtained to verify that the synthetic and natural molecules share the same absolute configuration. Natural indolecarboxyl-ascaroside C5: CD (MeOH) λ_{max} ($\Delta\varepsilon$) 210 (+14.35), 244 (-5.18). Synthetic indolecarboxyl-ascaroside C5: CD (MeOH) λ_{max} ($\Delta\varepsilon$) 210 (+16.69), 244 (-1.78).

Dauer formation assay. The dauer formation assays were performed as described[2] on NGM-agar plates made with Noble agar (BD Biosciences).

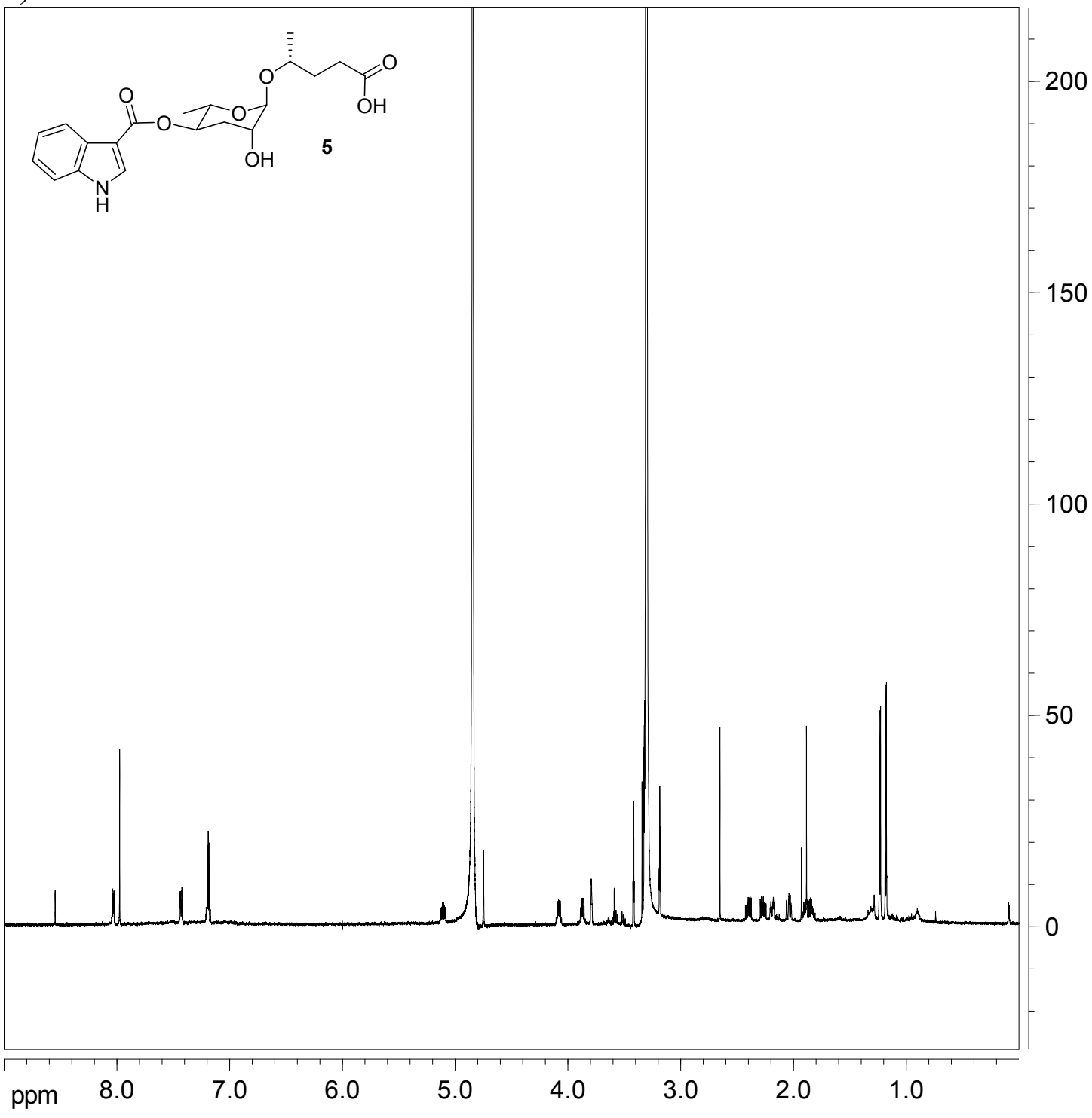
Data analysis. EC_{50} values were determined using Prism software (GraphPad Software). Titration curves for ascarosides C3, C6, and C9 were fit with a sigmoidal curve with variable slope, in which the lower limit was set at 0 and the upper limit was not defined. Titration curves for indolecarboxyl-ascaroside C5 were fit with a bell-shaped curve, in which the lower limit was set at 0 and the upper limit was not defined. EC_{50} was defined as the concentration at which each ascaroside reached half its maximal activity (as calculated by Prism).

References

1. Jeong, P. Y.; Jung, M.; Yim, Y. H.; Kim, H.; Park, M.; Hong, E.; Lee, W.; Kim, Y. H. Kim, K.; Paik, Y. K. *Nature* **2005**, *433*, 541.

2. Butcher, R. A.; Fujita, M.; Schroeder, F. C.; Clardy, J. *Nat Chem Biol* **2007**, *3*, 420.

Figure S1. NMR spectra of natural indolecarboxyl-ascaroside C5 (**5**) salt in methanol-d_4. (a) ^{1}H NMR spectrum of natural indolecarboxyl-ascaroside C5 salt in methanol-d_4**.** (b) dqf-COSY spectrum of natural indolecarboxyl-ascaroside C5 salt in methanol-d_4. (c) gHSQC spectrum of natural indolecarboxyl-ascaroside C5 salt in methanol-d_6. (d) gHMBC spectrum of natural indolecarboxyl-ascaroside C5 salt in methanol-d_6. (e) NOESY spectrum of natural indolecarboxyl-ascaroside C5 salt in methanol-d_6.



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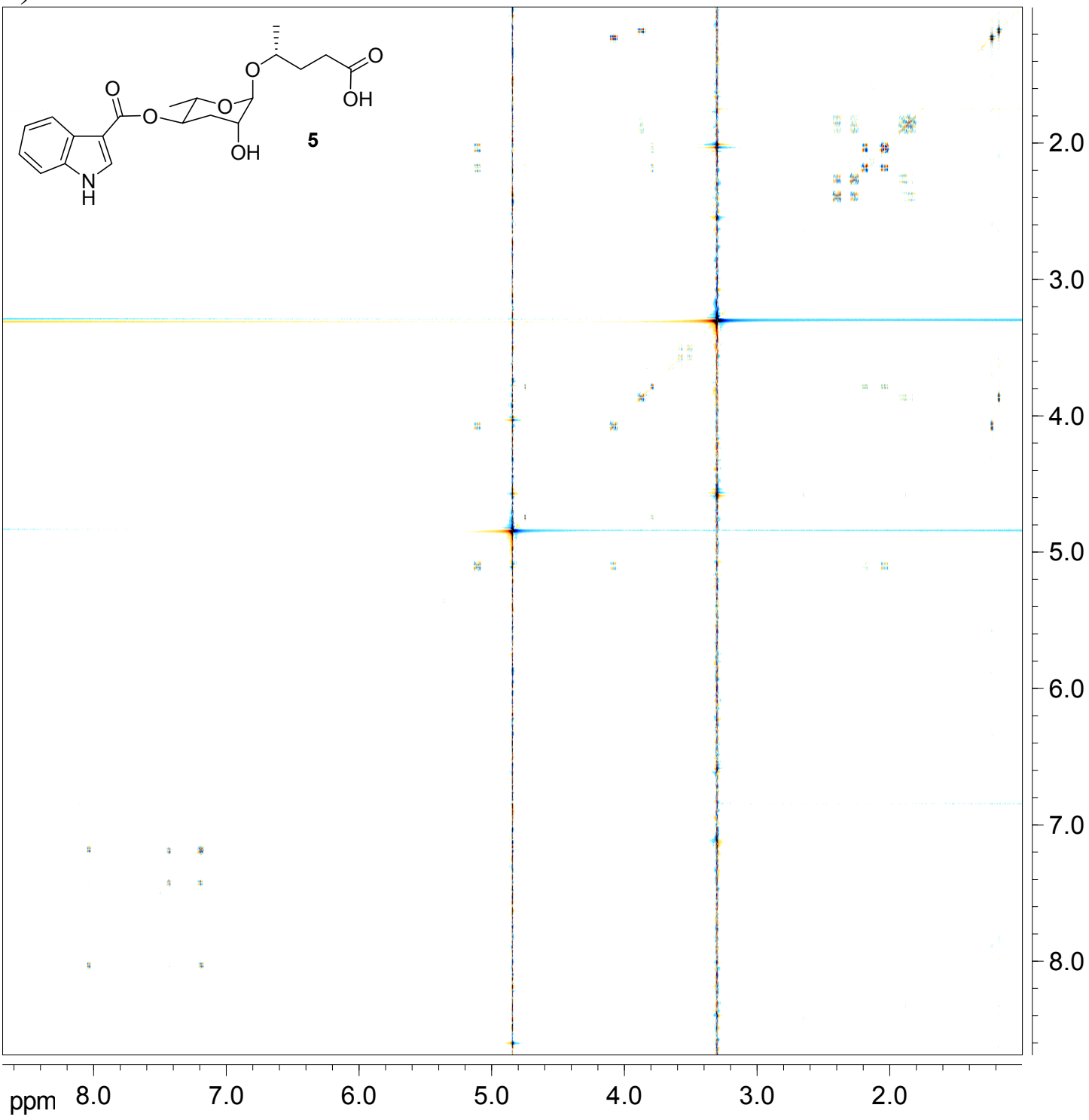

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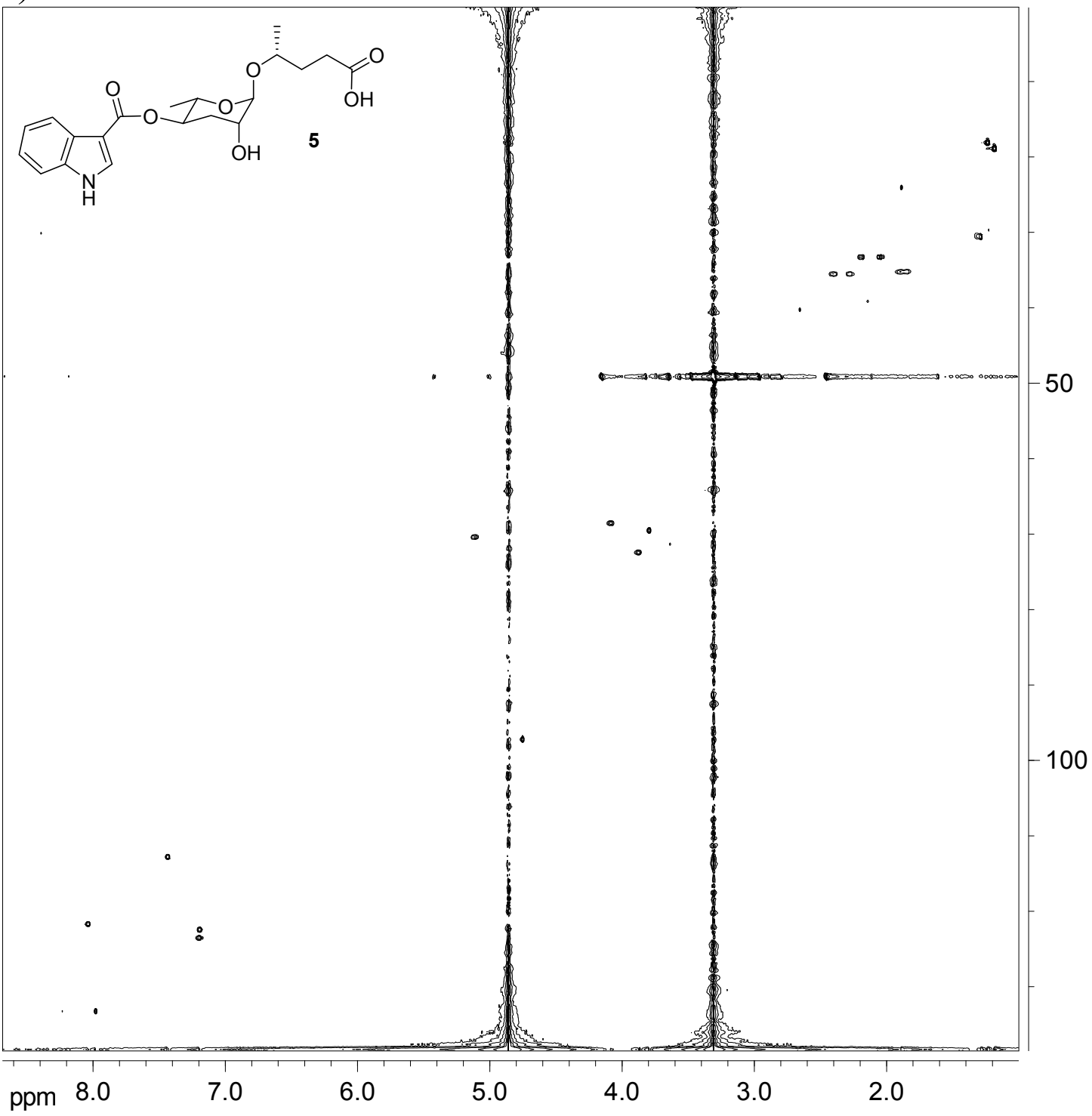

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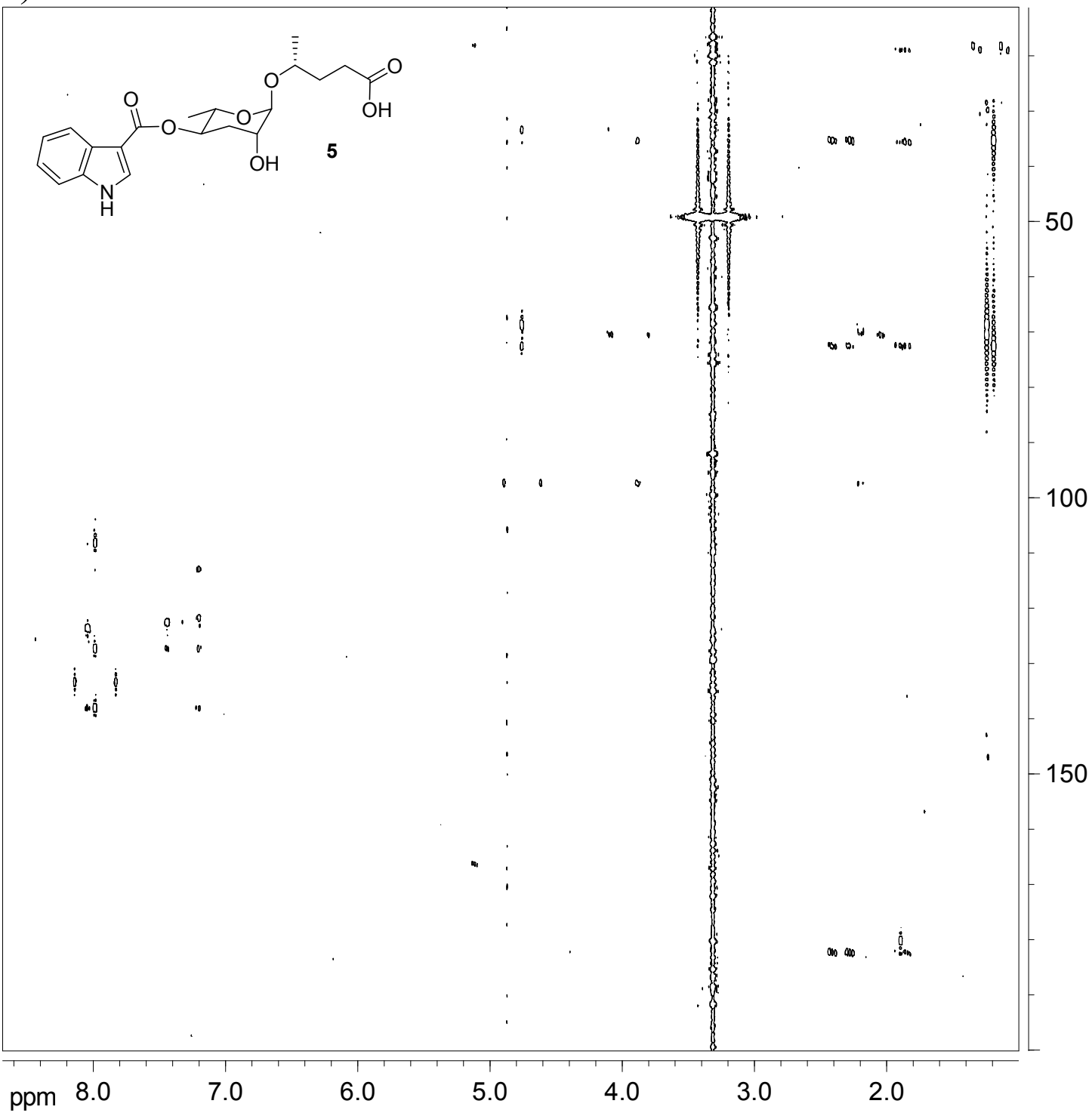

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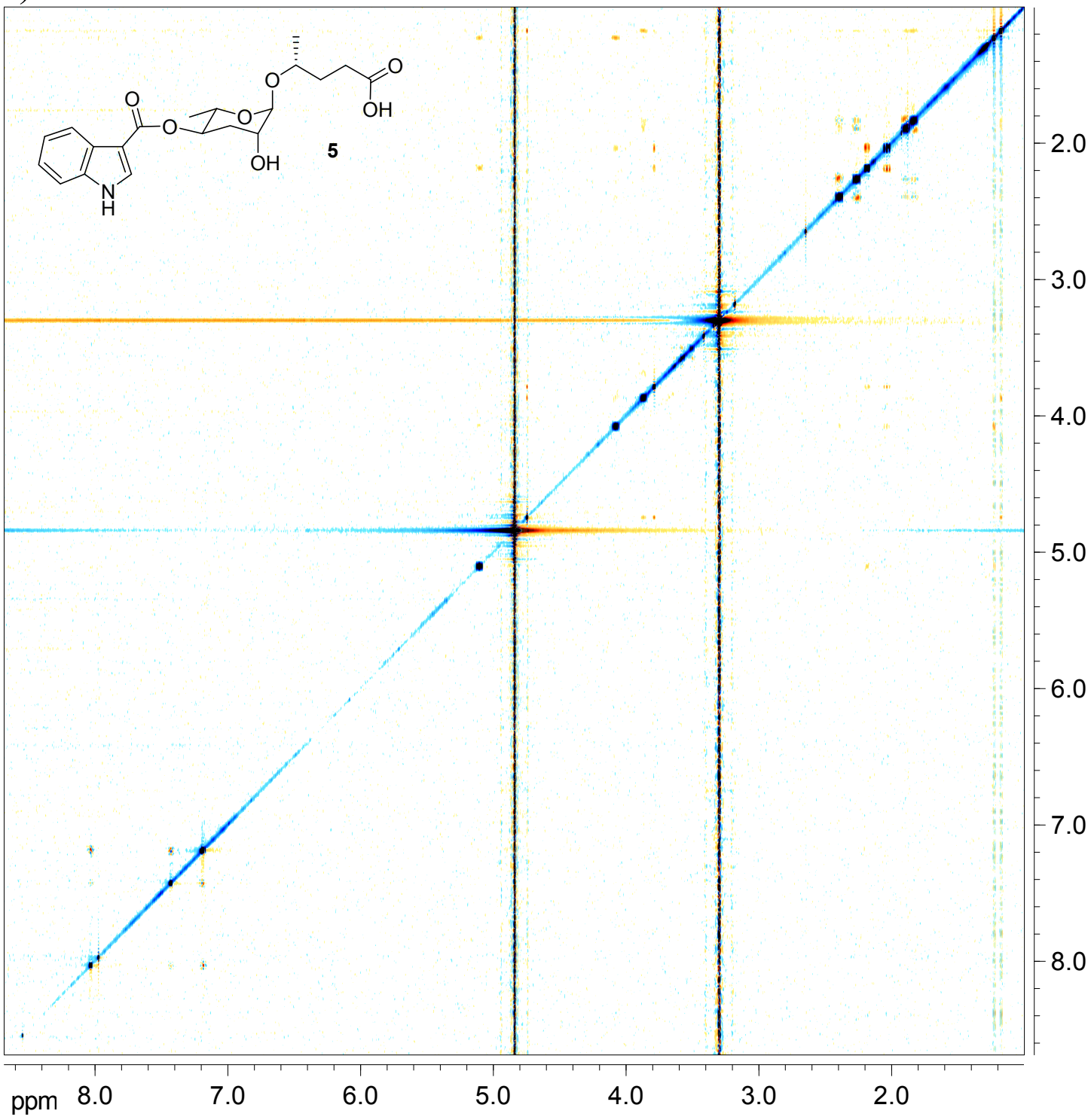


Figure S2. Key correlations for structure elucidation of indolecarboxyl-ascaroside C5 (**5**). (a) Key dqf-COSY and HMBC correlations. (b) Key NOESY correlations.

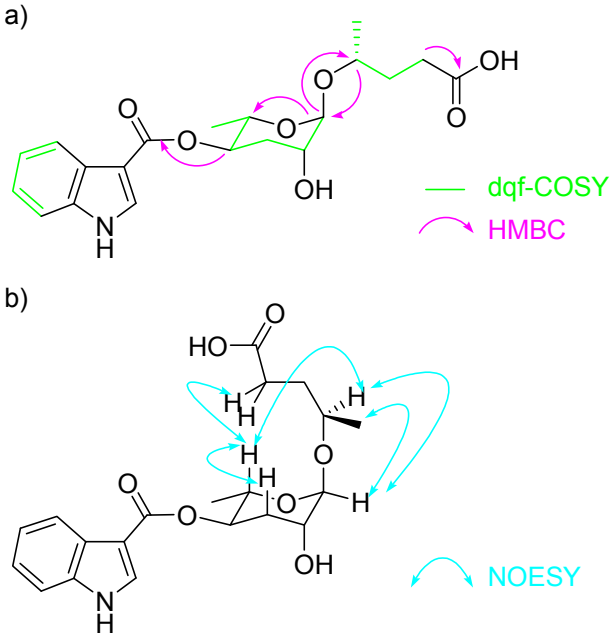
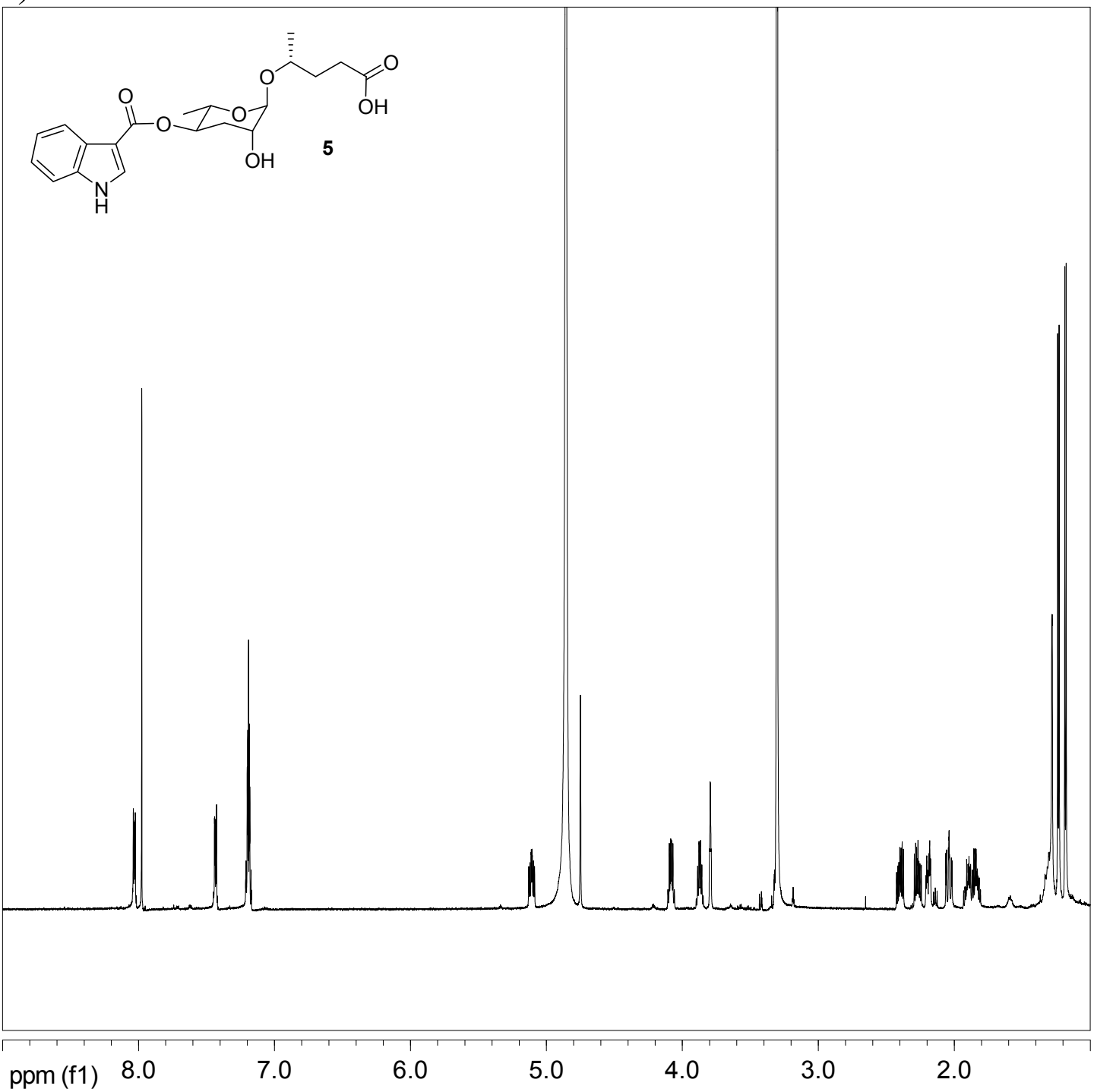


Figure S3. NMR spectra of synthetic indolecarboxyl-ascaroside C5 (**5**) salt in methanol-d_4. (a) ^{1}H NMR spectrum of synthetic indolecarboxyl-ascaroside C5 salt in methanol-d_4**.** (b) dqf-COSY spectrum of synthetic indolecarboxyl-ascaroside C5 salt in methanol-d_4. (c) gHSQC spectrum of synthetic indolecarboxyl-ascaroside C5 salt in methanol-d_6. (d) gHMBC spectrum of synthetic indolecarboxyl-ascaroside C5 salt in methanol-d_6. (e) NOESY spectrum of synthetic indolecarboxyl-ascaroside C5 salt in methanol-d_6.

a)



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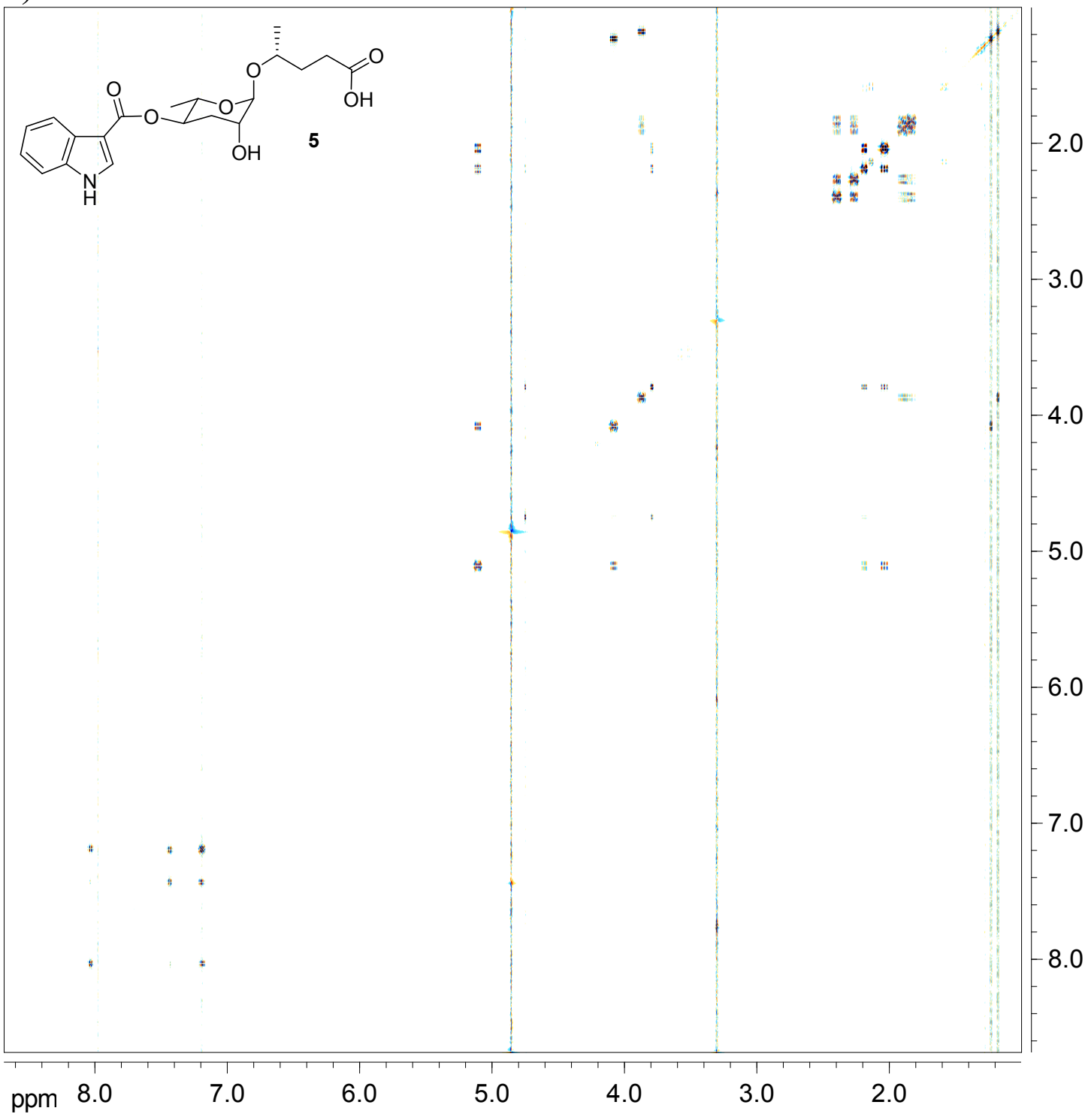

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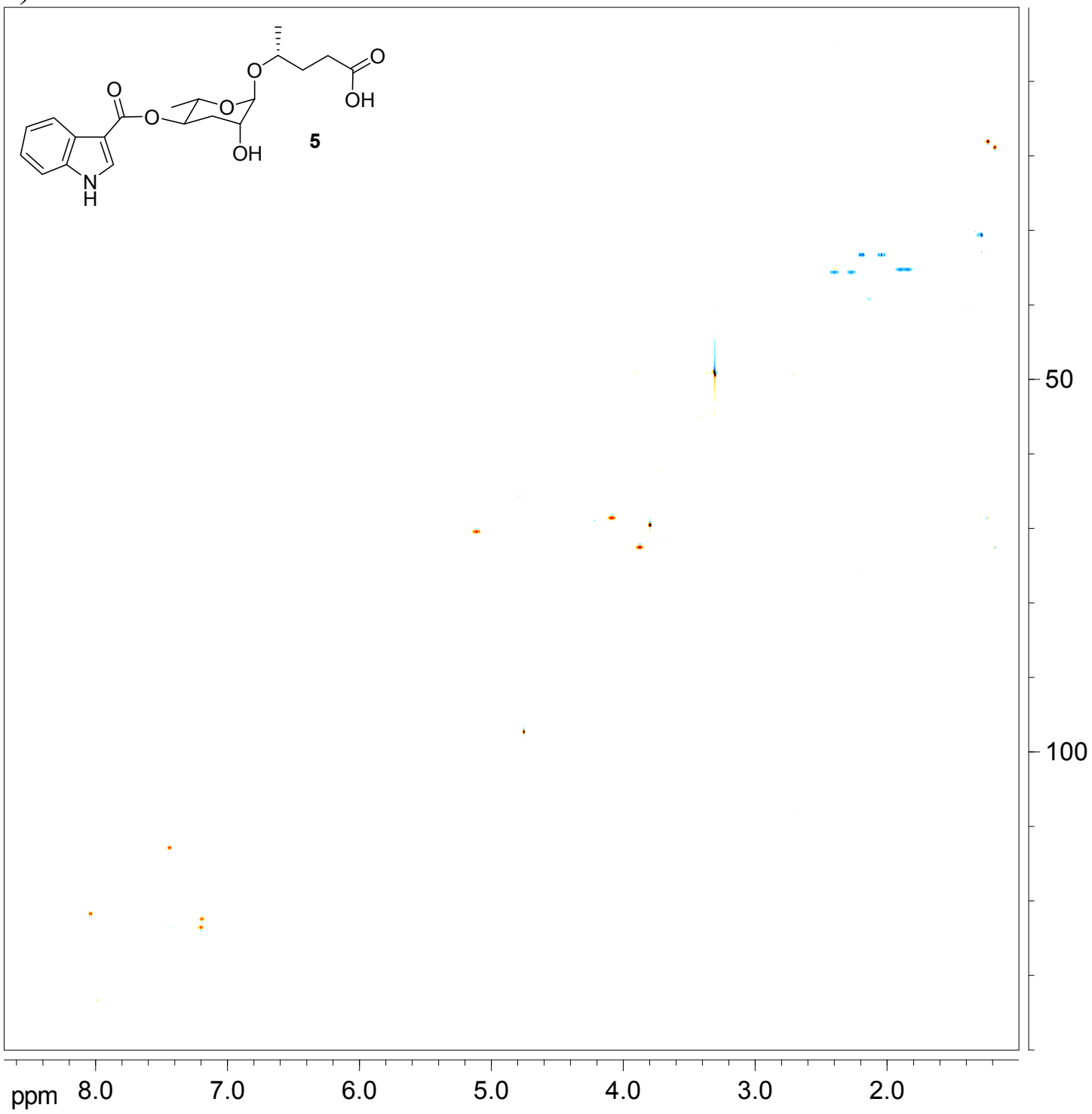

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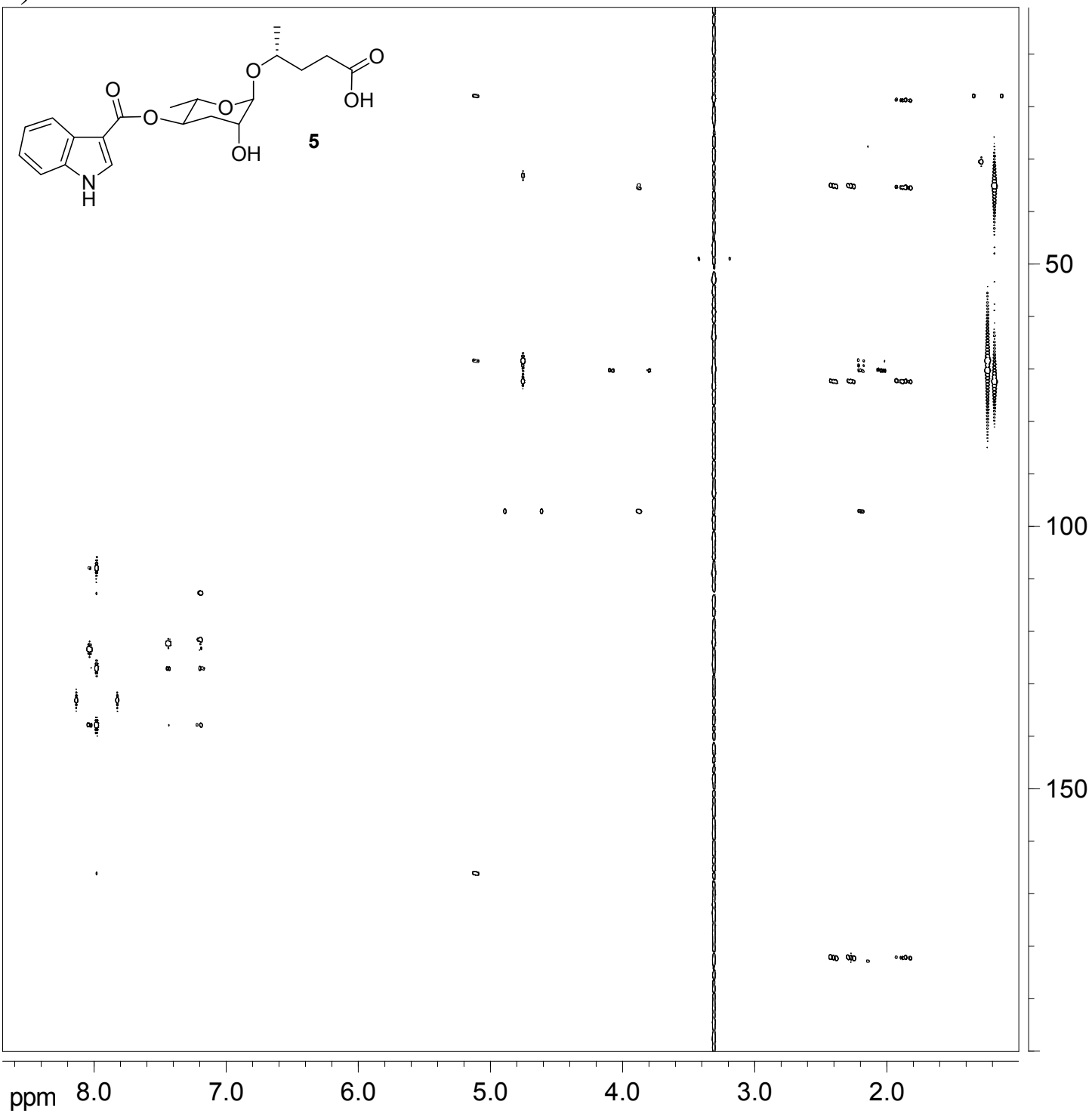

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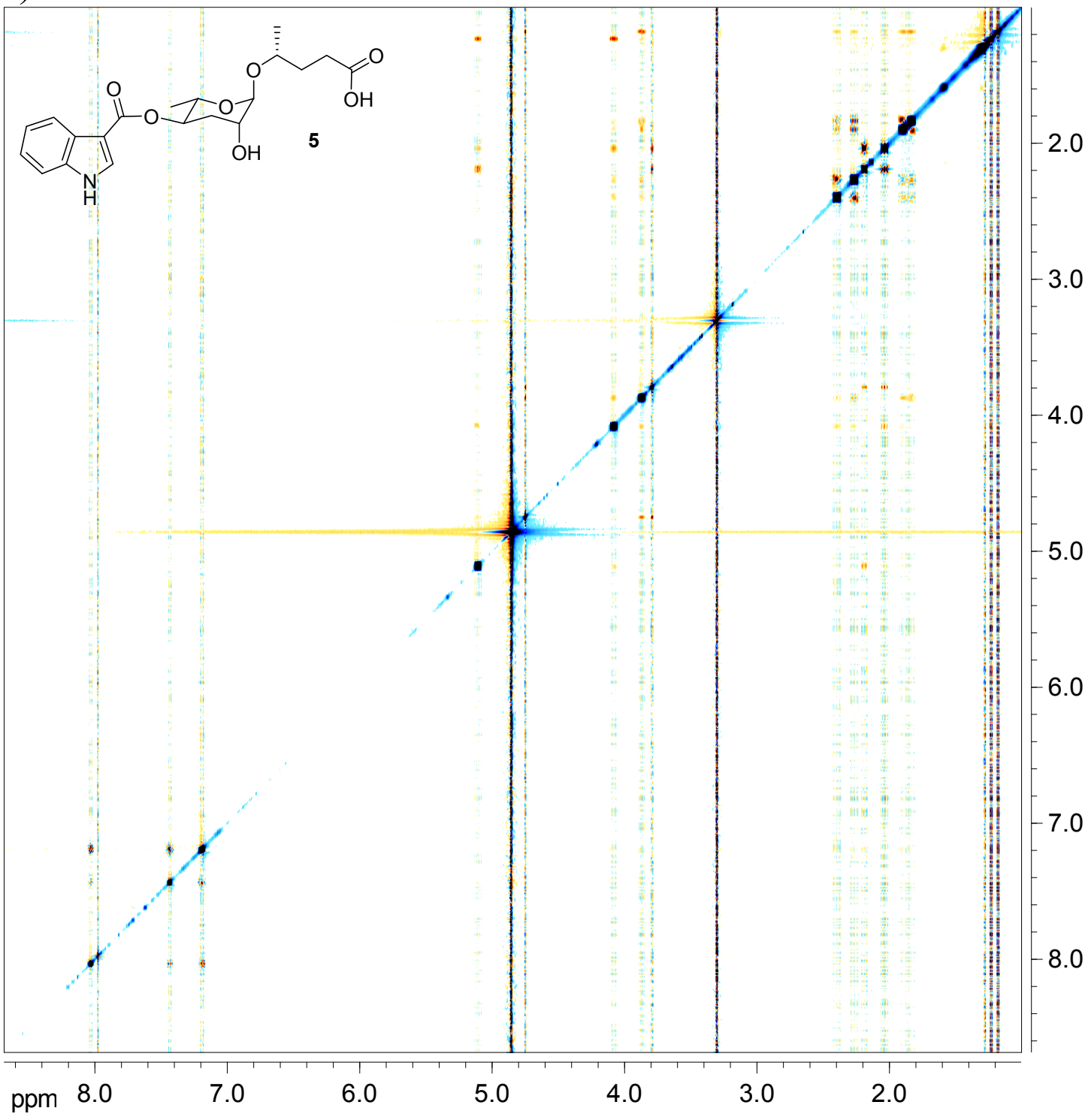

Figure S4. Effect of a high concentration of indolecarboxyl-ascaroside C5 (6 μM) on the activity of ascaroside C3, ascaroside C6, or ascaroside C9 (220 nM) in the dauer formation assay in wild-type worms at 25 °C. The data represent the average of two experiments (± one standard deviation).

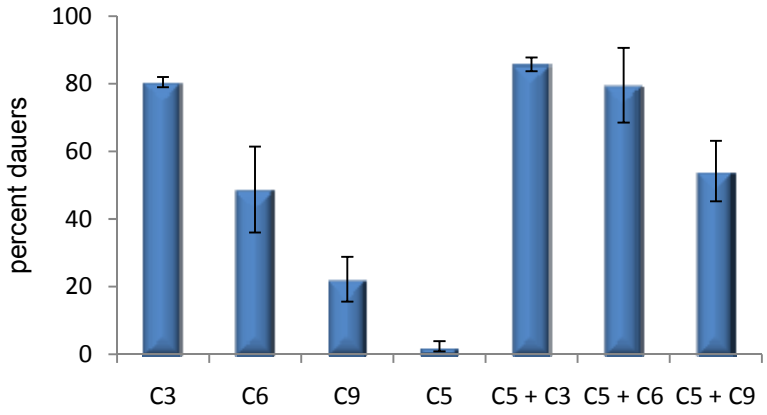

Figure S5. Effect of ascaroside C3, ascaroside C6, ascaroside C9, and indolecarboxyl-ascaroside C5 (at 146 nM alone or at 73 nM each in combination) in the dauer formation assay in *daf-22* (*ok693*) worms (which have a mutation in a fatty acid β-oxidation gene necessary for pheromone biosynthesis) at 20 °C. (We find that synergism is more reproducible in this mutant background, presumably because in the mutant strain natural pheromone production by the worms involved in the assay does not interfere with assay results.) The data represent the average of two experiments (± one standard deviation).

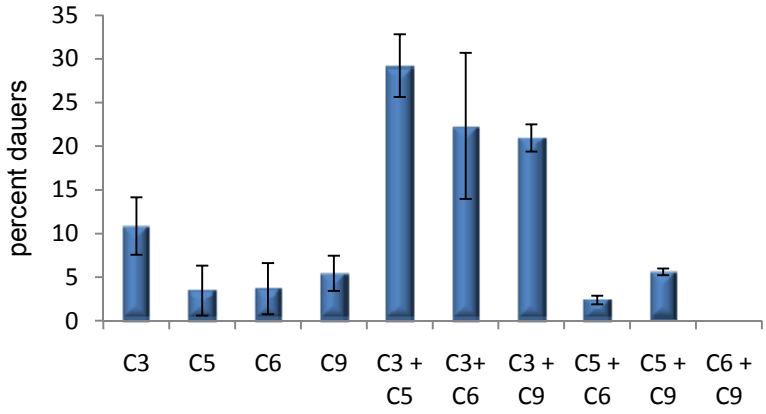

Table S1. NMR shifts derived from ^{1}H, dqf-COSY, gHSQC, and gHMBC spectra of synthetic indolecarboxyl-ascaroside C5 (**5**) salt in methanol-d_4.

no.	δ_H mult. (J (Hz))	δ_C	HMBC
1		182.49	
2a	2.27, m ($J_{2a,2b}$=14.4)	35.51	C-1,3,4
2b	2.41, m		C-1,3,4
3a	1.84, m	35.18	C-1,2,4,5
3b	1.91, m		C-1,2,4,5
4	3.88, m	72.45	C-2,1’
5	1.19, d ($J_{4,5}$=6.0)	18.83	C-3,4
1’	4.76, br s	97.19	C-4,3’,5’
2’	3.80, dt ($J_{1',2'}$=2.6)	69.47	C-4’
3’ax	2.05, ddd ($J_{2',3'ax}$=2.6, $J_{3'ax,3'eq}$=13.0)	33.19	C-4’
3’eq	2.20, ddd ($J_{2',3'eq}$=3.3)		C-1’,4’
4’	5.11, ddd ($J_{3'ax,4'}$=11.1, $J_{3'eq,4'}$=4.7)	70.38	C-5’,6’,1”
5’	4.09, dq ($J_{4',5'}$=9.7)	68.47	C-3’,4’
6’	1.24, d ($J_{5',6'}$=6.2)	18.00	C-4’,5’
1”		166.36	
2”		108.19	
3”	7.98, s	133.22	C-1” (w), 2”,4”,5”
4”		138.19	
5”		127.29	
6”	8.04, m	121.64	C-2”,4”,8”
7”	7.19, m	122.34	C-5”,6”,8”
8”	7.20, m	123.50	C-4”,9”
9”	7.44, m	112.80	C-4”,5”,7”