# Long-Chain *O*-Ascarosyl-alkanediols Are Constitutive Components of *Caenorhabditis elegans* but Do Not Induce Dauer Larva Formation

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Two long-chain ascarosides, *O*-ascarosylnonacosane-2,28-diol (**1**) and *O*-ascarosyluntriacontane-2,30-diol (**2**), were isolated from *Caenorhabditis elegans* and detected in all developmental stages of the worm. The long-chain ascarosides were shown to be minor lipid components, and it was also shown that they do not induce dauer larva formation.

**Introduction.** – Ascarosides, glycolipids containing the 3,6-dideoxysugar ascarylose (3,6-dideoxy-L-arabino-hexose), have been described in parasitic nematodes of the genus *Ascaris*, almost a century ago [1][2]. Based on the structure of the side chain, they are classified as long-chain monool glycosides, diol monoglycosides and diol diglycosides [3]. Ascarosides of *Ascaris* are present in embryos, mainly in the form of esters of acetic, propylic, and isovaleric acids [2]. Upon fertilization, the ascarosides are deesterified and translocated to the inner layer of the newly formed eggshell, being a major constituent of this layer and providing eggshell impermeability [4]. Ascarosyl compounds were also described in *Caenorhabditis elegans*, where they are dauer larva-inducing and sexual pheromones [5]. The aglycones of these *C. elegans* ascarosides are short-chain alkanes with COO and C=O groups [6][7]. Recently, it has been reported that long-chain *O*-ascarosyl-(2,$\omega-1$) alkanediols with 29 and 31 C-atoms were present in growth media of this nematode [8]. In this study, we addressed the question whether long-chain ascarosides are present in different life-stages of the worm, and investigated their ability to induce dauer larva formation.

**Results and Discussion.** – First, we tried to answer the question whether long-chain ascarosides are present in the developmental stages of *C. elegans*. For this purpose, a lipid extract of *C. elegans* mixed populations was prepared according to *Bartley et al.* [3] with modifications. After saponification, the hydrophobic compounds were extracted with $CHCl_3$ and separated by RP-HPLC/MS, to check for the presence of molecular weights corresponding to long-chain ascarosides, previously described in different *Ascaris* species. Molecular ions potentially corresponding to *O*-ascarosylnonacosane-2,28-diol (**1**; *m/z* 569.6 ($[M-H]^-$)) and *O*-ascarosyluntriacontane-2,30-diol (**2**; *m/z* 597.6 ($[M-H]^-$)) were observed.

To confirm that these molecular ions correspond to long-chain ascarosides, we have isolated them from the saponified lipid extract of *C. elegans*, using preparative 2D thin layer chromatography (TLC). After visualization of the control TLC plate, corre-

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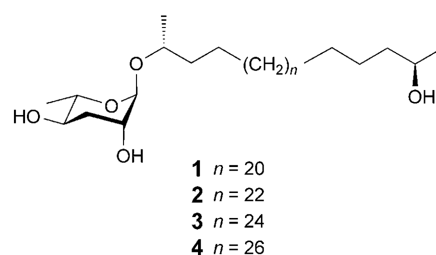


sponding regions of the preparative plates were back-extracted and checked for the presence of long-chain ascarosides with 2D HPTLC and HPLC/MS, using long-chain *O*-ascarosyl alkanediols isolated from *Ascaris suum* as a reference. The TLC behavior of the long-chain ascarosides from *C. elegans* corresponded to that of the reference, with $R_f$ 0.3 in 1D and 0.1 in 2D TLC, respectively (*Fig. 1,a* und *c*). When the mixture of ascarosides from *C. elegans* was analyzed by HPLC/MS (gradient elution, see *Exper. Part*), the peaks of **1** and **2** were observed at *m/z* 569.6 ($[M-H]^-$) and 597.6 ($[M-H]^-$), respectively (*Fig. 1,b*). The chromatographic behavior of **2** ($t_R$ 22.0 min) isolated from *C. elegans* corresponded to that of **2** isolated from *A. suum* (*Fig. 1,b* and *d*). As expected, from *A. suum*, also *O*-ascarosyltritriacontane-2,32-diol (**3**) and *O*-ascarosylpentatriacontane-2,34-diol (**4**) were isolated. The differences between the $t_R$ of compounds **2**, **3**, and **4** isolated from *A. suum* were the same as that between compounds **1** ($t_R$ 20.0 min) and **2** isolated from *C. elegans.* This confirmed the assumption that **1** is *O*-ascarosylnonacosane-2,28-diol.

To definitively establish the identity of the sugar residue, long-chain ascarosides of *C. elegans* were subjected to acidic hydrolysis, yielding ascarylose, as determined by HPLC/MS ($t_R$ 2.3 min; ESI-MS (negative-ion mode): *m/z* 207 ($[M+CH_3COO]^-$)), using synthetic ascarylose as a reference (*Fig. 2*). When performing MS/MS fragmentation of parent masses of **1** and **2**, neutral losses of sugar (130 Da) and two $H_2O$ residues (147 and 164 Da) were observed, confirming their identity. Thus, in *C. elegans*, diol monoglycosides **1** and **2** constituted the major types of long-chain ascarosides.

It is known that long-chain ascarosides in *Ascaris* are esterified. Therefore, the question was addressed, whether the same is true for *C. elegans.* The concentrations of **1** and **2** were compared in the equivalent lipid extracts of adult worms before and after saponification, using the same HPLC/MS system as for the detection of the ascarosides. The amounts of **1** and **2** were *ca.* eight times higher after saponification ($P<0.05$). Thus, in *C. elegans*, ascarosides are mainly present as esters. Their presence in the culture media in the unesterified state [8] might be due to the release by enzymatic hydrolysis, similar to the way it occurs during the formation of the eggshell in *Ascaris* [4].

Moreover, it was investigated whether the ascarosides are constitutive components during the life cycle or whether they accumulate at a certain developmental stage. The relative amounts of ascarosides in the different life stages were measured by HPLC/MS, using the same system as for their detection, and the amounts were normalized either to the number of worms (*Fig. 3,a*) or to the protein content (*Fig. 3,b*). The amount of ascarosides per worm steadily increased, reaching a maximum at the adult stage.

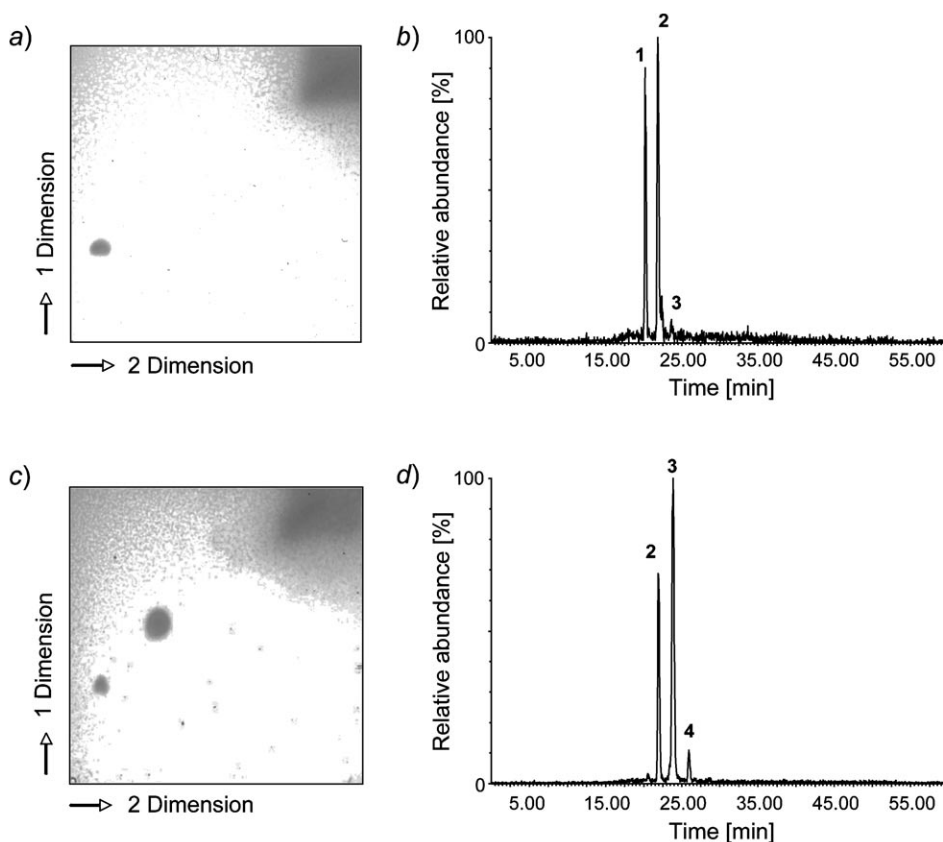


Fig. 1. a) *2D TLC of ascarosides isolated from* C. elegans. b) *HPLC/ESI-MS* (negative-ion mode) *of ascarosides isolated from* C. elegans. Selected ion traces: *m/z* 569 (**1**), 597 (**2**), and 625 (**3**). The ion at *m/z* 653 (**4**) was not detected. c) *2D TLC of ascarosides isolated from* A. suum. Fatty acids were also observed ($R_f$ 0.5 and 0.2 in 1D and 2D TLC, resp.). d) *HPLC/ESI-MS* (negative-ion mode) *of ascarosides isolated from* A. suum. Selected ion traces: *m/z* 597 (**2**), 625 (**3**), and 653 (**4**). The ion at *m/z* 569 (**1**) was not detected.

Absolute quantification of long-chain ascarosides was performed by HPLC/MS, using long-chain ascarosides isolated from *A. suum* as a standard. To achieve better linearity, an isocratic elution system was used. The area under the peak of known amounts of **3** was used to establish the calibration curve. Analyzing the ascarosides from synchronized populations of *C. elegans*, the amount of ascarosides was estimated to be *ca*. 150 pg per adult worm. When compared to the amount of phospholipids at the same developmental stage (*ca*. 15 ng per adult worm), long-chain ascarosides constitute *ca*. 1%. This is in contrast with what was observed in parasitic nematodes, where the ascaroside esters constituted up to 33% of all neutral lipids in the ovaries [9].

Interestingly, when normalized to the protein content, the amount of long-chain ascarosides reached its maximum at the L2 stage, thus indicating that they might be involved in the regulation of dauer larva formation, dauer larva being an alternative to

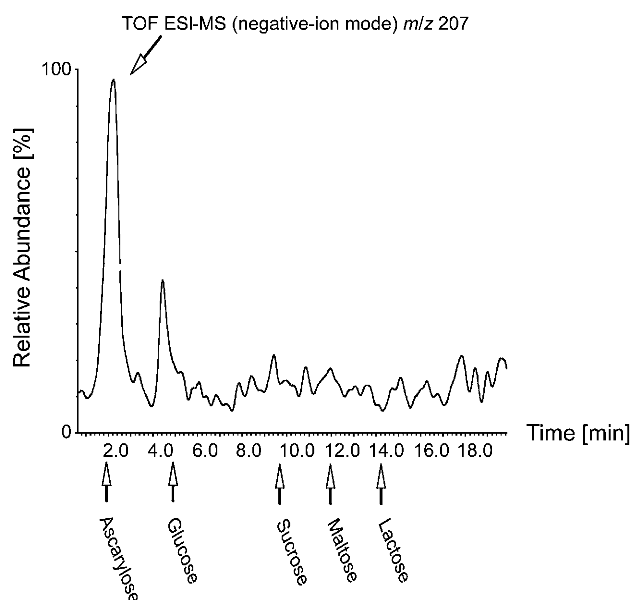


Fig. 2. *HPLC/ESI-MS* (negative-ion mode; mass chromatogram of *m/z* 207) *of the sugar residue obtained after acid hydrolysis of long-chain ascarosides isolated from* C. elegans. The $t_R$ of ascarylose and the reference carbohydrates in this system are indicated with arrows.

L3 under unfavorable conditions. To clarify whether long-chain ascarosides induce dauer formation similar to short-chain ascarosides (daumones) [5], the dauer-inducing activity was checked using the diol monoglycosidic ascarosides isolated from female *A. suum* with a recently described assay [10]. Long-chain ascarosides did not show any dauer-inducing effect at similar or higher concentrations than those of short-chain ascarosides (*Fig. 3,c*). These data show, that compounds **1** and **2** do not seem to be signaling molecules, and it is unlikely, that they are precursors for daumones [11].

Thus, long-chain ascarosides are present in *C. elegans* in all stages of development. Although their function still has to be elucidated, long-chain ascarosides do not seem to be involved in eggshell formation or dauer larva induction.

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## Experimental Part

*General.* All chemicals were purchased from *Sigma-Aldrich* (Germany), unless otherwise stated. Ascarylose was provided by the Department of Chemistry, TU Dresden (Organic Chemistry; Head, Prof. *H.-J. Knoelker*); the synthesis is described in [12].

*Animal Material.* Caenorhabditis elegans. Liquid cultures of mixed population N2 worms were grown according to *Wood* [13]. Homogenous populations were obtained as follows. Embryos of the wild-type Bristol N2 strain were obtained by treating the mixed population with 4% aq. NaOH soln. in household bleach (3–6% aq. NaClO), and were suspended in *M9* buffer for 48 h at 20°. Produced L1

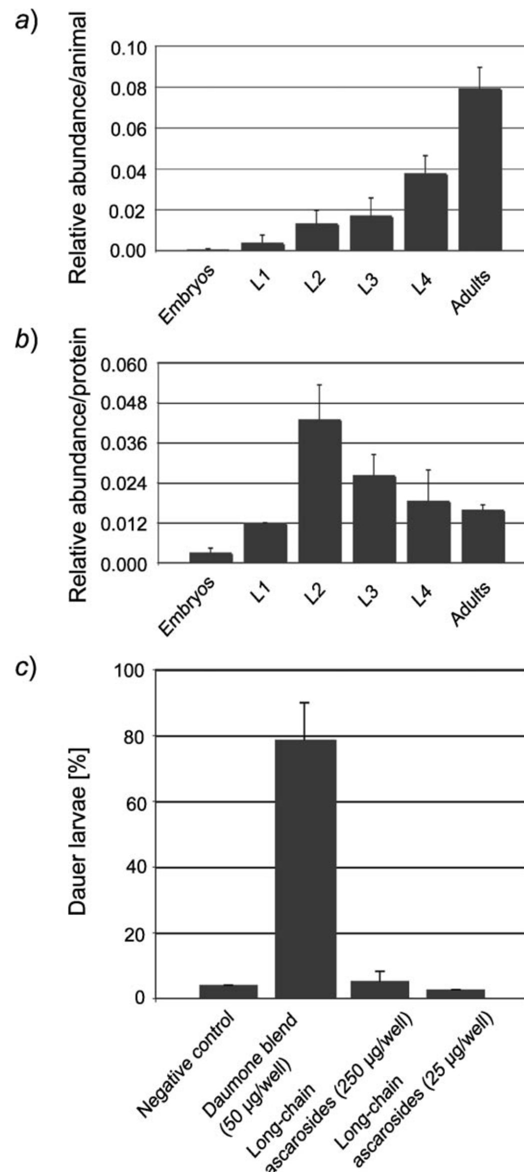


Fig. 3. *Relative abundance of ascarosides in the developmental stages of* C. elegans *normalized to* a) *the number of animals and* b) *the protein content.* c) *Effect of long-chain ascarosides isolated from* C. elegans *on the induction of dauer larva formation*. Data are presented as means ± SD.

were transferred to nematode growth medium (NGM) agar covered with bacteria, and the plates were incubated at 20°. The worms were washed off with *M9* buffer after 10, 20, 30, or 40 h of incubation, to obtain L2, L3, L4, or young adult homogenous populations, resp. Dauer larva were obtained using a *daf-7* mutant strain (*CGC*, UK) grown at 25°.

Ascaris suum. *Ascaris suum* were obtained from the Zoology Department of the TU Dresden (Head, *R. Entzeroth*). Females were dissected to excise the uterus, which was used for further procedures.

*Sample Preparation*. All samples were homogenized in a minimal amount of $H_2O$, and the extraction and sample pretreatments were performed according to *Matyash et al.* [14]. Before the extraction, an aliquot of D-glucosyl-$\beta$-1,1′-*N*-lauroyl-D-*erythro*-sphingosine (*AvantiLipids*) was added, to account for the treatment losses.

*TLC*. Prep. TLC was performed on silica-gel *60* HPTLC plates (20 × 20 cm; *Merck*) developed with $CHCl_3$/MeOH 9 : 1 (first dimension) and $CHCl_3$/MeOH 24 : 1 (second dimension). Anal. 2D TLC was performed in the same way, using silica-gel *60* TLC plates (10 × 10 cm; *Merck*). Bands were visualized by spraying the plates with 20% $H_2SO_4$ in EtOH and charring at 200°. For prep. purposes, the silica-gel was scraped with a scalpel, extracted with a tenfold amount of MeOH, and filtered. Initial solns. (10 ml) of packed *C. elegans* worms yielded *ca.* 0.5 mg of long-chain ascarosides.

O-*Ascarosylnonacosane-2,28-diol* (**1**). $t_R$ (*System 2*) 23.5 min. ESI-MS/MS (positive-ion mode) of *m/z* 588.427 ($[M+NH_4]^+$): 405.443 ($[M+H-2\,H_2O-C_6H_{10}O_3]^+$), 423.44 ($[M+H-H_2O-C_6H_{10}O_3]^+$), 441.461 ($[M+H-C_6H_{10}O_3]^+$).

O-*Ascarosyluntriacontane-2,30-diol* (**2**). $t_R$ (*System 2*) 29.5 min. ESI-MS/MS (positive-ion mode) of *m/z* 616.456 ($[M+NH_4]^+$): 433.472 ($[M+H-2\,H_2O-C_6H_{10}O_3]^+$), 451.469 ($[M+H-H_2O-C_6H_{10}O_3]^+$), 469.490 ($[M+H-C_6H_{10}O_3]^+$).

O-*Ascarosyltritriacontane-2,32-diol* (**3**). $t_R$ (*System 2*) 38.2 min.

*HPLC*. All HPLC separations were performed with a *Waters Alliance 2695* pump coupled to TOF MS. *System 1*. The detection and relative quantification of ascarosides was performed with an *Agilent XDB C8* RP column (4.6 × 150 mm, 5 μm) and gradient elution with 100% *Solvent A* (MeOH/0.1% aq. ammonium acetate 9 : 1) for 5 min, 0% *Solvent B* (MeOH/0.1% aq. ammonium acetate 99 : 1) to 100% *B* in 5 min, and 100% *B* for 35 min, followed by column equilibration with 100% *A* for 15 min. *System 2*. The absolute quantification of ascarosides was performed with an *Agilent XDB C8* reversed-phase column (3.0 × 150 mm, 3.5 μm,) eluted with MeOH/0.1% aq. ammonium acetate 9 : 1 for 45 min. For both systems, the flow rate was 0.3 ml/min, the column temp. was maintained at 40°, and the injection volume was 10 μl.

*HPLC Separation of Carbohydrates*. Carbohydrates were analyzed using an *Agilent* aminopropyl RP column (2.6 × 150 mm, 5 μm); elution was isocratic with MeCN/0.1% aq. ammonium acetate 85 : 15 for 20 min; the flow rate was 0.15 ml/min.

*MS Analysis*. MS Analysis was carried out on an *LCT* TOF mass spectrometer (*Waters Inc.*) with an ESI source. Instrument operation and data acquisition was carried out using MassLynx 4.1 software. $N_2$ was used as the cone and nebulizing gas. The ionization voltage was set to 3.0 kV. Acquisition was performed over the *m/z* range 100 – 1000. Tandem MS analysis was performed with a modified *QSTAR Pulsar i* quadrupole TOF apparatus (*MDS Sciex*) equipped with a *NanoMate HD* ion source (*Advion BioSciences*). The ionization voltage was set to 1.05 kV. The anal. quadrupole Q1 was operated under the unit resolution settings, and fragments of preselected masses were detected within the *m/z* range 100 – 1,000.

*Phosphate Assay*. The phosphate assay was performed according to [15].

*Protein Qunatification*. The amount of protein was determined with a *Micro BCA* protein assay kit (*Pierce*).

*Acidic Hydrolysis*. Acidic hydrolysis was performed according to [16].

*Saponification*. For the saponification, the dry sample was dissolved in a ten-fold amount of 20N aq. KOH soln./$H_2O$ 1 : 19, and the mixture was incubated for 1 h at 65°. Then, the phase separation was induced by adding $H_2O/CHCl_3$ 1 : 2. The lower phase was collected and dried in a vacuum centrifuge. The dry extract was used for further procedures.

*Dauer Larva Induction Assay*. The dauer larva induction assay was performed according to [9], with monoascarosyl alkanediol (25 or 250 μg per well) isolated from *A. suum*.

*Statistical Analysis*. Data are presented as means with standard deviation. The results were statistically analyzed by one-way analysis of variance (ANOVA), followed by a *Student*'s *t*-test ($P$ values $< 0.05$ were considered significant).

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