Natural Products

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Maradolipids: Diacyltrehalose Glycolipids Specific to Dauer Larva in *Caenorhabditis elegans***

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In response to harsh environmental conditions, such as overcrowding or starvation, the nematode *C. elegans* interrupts and arrests its reproductive life cycle by forming a specific dauer (enduring) larva. Dauer larvae have very distinct metabolism, morphology, and enhanced stress resistance for surviving unfavorable environmental conditions. They express high amounts of stress-protective proteins such as the heat-shock protein Hsp90, superoxide dismutase, and catalase[1–3]. They also remodel their body surfaces—they build a dauer-specific cuticle and seal the pharynx with a cuticular block[4–6]. Although, the dauer larva formation pathway is biologically well investigated, there is not much information about the chemical means by which dauer larvae resist the various kinds of environmental stresses. Similar to some other organisms, lipids might play an important role in the adaptation process of dauer larvae to harsh conditions.

We asked whether the transition from reproductive stages to the dauer larva is associated with global changes in the lipid composition or metabolism. For this purpose we used a temperature-sensitive mutant of *daf-2(e1370)* that reproduces at 15 °C or 20 °C but forms dauer larvae at 25 °C[7]. Lipids were extracted from *daf-2(e1370)* worms grown at 20 °C and 25 °C (Figures 1 a and b, respectively) and separated by two-dimensional (2D) thin-layer chromatography (TLC) that resolved the major lipid classes: glycerophospholipids, ceramides, glycosphingolipids, fatty acids, sterols, etc. The plates were sprayed with the Molisch reagent, which specifically stains carbohydrate-containing lipids in purple and all other lipid classes in yellow-brown on the same TLC plates. As seen, there is a significant difference between reproductive L3 larvae and dauer larvae (compare Figures 1 a and b): In addition to two Molisch-positive (purple) forms of glucosylceramides (GlucCer), a spot that is visible exclusively on the TLC containing the dauer larvae is observed (arrowhead). This spot appeared to be specific to dauer larva; that is, it could not be detected either in the mixed population of wild-type worms grown at 20 °C and 25 °C, or in any other individual reproductive larval stages (L1 to L4, adults; not shown). Most importantly, dauer larvae obtained from starved plates of wild-type worms (N2) displayed a spot of comparable strength (see Figure 1 a (arrowhead) in the Supporting Information). Hence we conclude that the spot represents a genuine lipid component of the natural dauer larvae, which does not depend on the genetic background or temperature.

Mobility of the dauer-larva-specific lipid on TLC and its positive reaction to the Molisch reagent suggested that it might be a dauer-specific glycosphingolipid. To test this possibility, we isolated neutral glycolipids (NGL) from dauer larvae (see Figure 2 a in the Supporting Information). The dauer-larva-specific spot was indeed found in the glycolipid fraction (NGL, arrowhead). In contrast to glycosphingolipids, however, this lipid was susceptible to saponification (see Figure 1 b in the Supporting Information; compare patterns before and after saponification—the spot indicated by arrowhead is absent after the saponification). Our observation indicates that this lipid does not belong to the class of glycosphingolipids and must contain at least one ester bond (amide bonds of glycosphingolipids cannot be cleaved by saponification). On the basis of its occurrence exclusively in dauer larvae and its chemical dissimilarity to glycosphingolipids, we call this lipid maradolipid (from maradi, enduring/dauer in Georgian).

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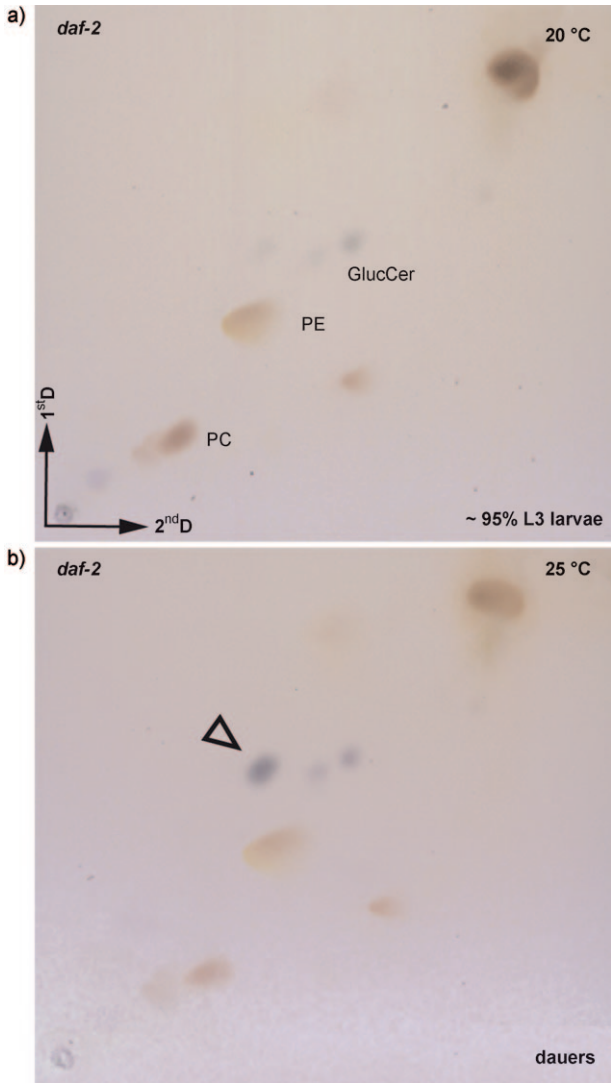

Figure 1. Dauer larvae contain a specific glycolipid(s) not belonging to the class of glycosphingolipids. The *daf-2* mutants were grown at 20 °C (a) or 25 °C (b). Lipids have been analyzed by 2D TLC methods. Note a specific spot in dauer larvae (b, arrowhead), not present in the reproductive larvae L3 (a). GlucCer = glucosylceramides, PC = phosphatidylcholine, PE = phosphatidylethanolamine.

Next we set out to identify the chemical structure of maradolipids. First, we investigated their sugar/glycan moiety. The NGL fraction mentioned above was saponified (see the Supporting Information) and the released sugar(s) were investigated by TLC analysis (see Figure 2b (left) in the Supporting Information). In this separation system, the sugar derived from the maradolipids runs as a disaccharide, having an R_f value identical to that of trehalose. Similar to trehalose (1-*O*-1′-diglucose that lacks free aldehyde groups), this sugar is a nonreducing sugar (see Figure 2b (right) in the Supporting Information). The identity of the sugar was determinately established with HPLC TOF/MS methods using a chiral column and synthetic compounds as reference standards. The retention time of the ion m/z 341 (ES^-) is identical to that of trehalose (molecular weight: 342 g mol^{-1}) and differs from other tested disaccharides (see Figure 2c in the Supporting Information).

A purified fraction of the maradolipids was subjected to shotgun lipidomics analysis on a LTQ-Orbitrap mass spectrometer in negative-ion mode. Maradolipids were detected as acetate adducts (Figure 2a) that, upon higher energy collisional fragmentation (HCD) produced acyl anions of the two fatty acid moieties, whose masses complemented the mass of the trehalose backbone (Figure 2b). As revealed by MS/MS analysis, the fatty acid composition of maradolipid species is highly heterogeneous (Figure 2c, and Figure 3 in the Supporting Information). About 40 mol % of the maradolipid fatty acid moieties are C15:0 and C17:0, which were previously identified as monomethyl branched-chain fatty acids (mmBCFAs)[8,9] (Figure 2c). This is remarkably different from the bulk fatty acid composition of glycerophospholipids and triacylglycerides in *C. elegans*[8]. Interestingly, approximately 66 % of maradolipids contain at least one mmBCFA moiety (Figure 2d).

The structure of the new class of glycolipids including the positions of the two fatty acid side-chains was identified using advanced 2D NMR spectroscopy. Supported by COSY, HSQC, HMBC, NOESY, and ROESY spectra, an almost complete assignment of the proton signals in the 500 MHz ^{1}H NMR spectrum (Figure 3a) has been achieved for the major component of the maradolipid mixture, which is 6-*O*-(13-methylmyristoyl)-6′-*O*-oleoyltrehalose (Figure 3d).

On the basis of the HMBC spectrum (Figure 3b), we assigned the glycolipids as 6,6′-di-*O*-acyltrehaloses. The complete superposition of the proton signals of the two glucose moieties confirms that the diacyltrehalose is a pseudosymmetric molecule. Moreover, in the HMBC spectrum the cross-peak between the 1s and 1s′ carbonyl groups and the 6b and 6b′ methylene protons (Figure 3b) emphasizes the connectivities of the two acyl side-chains which are located at the 6- and 6′-positions. The HSQC spectrum (Figure 3c) shows that the main components of the 6,6′-di-*O*-acyltrehalose derivatives have two different acyl side-chains with one of them being terminally branched (iso acyl side-chain of a mmBCFA). The second acyl side-chain of the major component in this mixture is an oleoyl side-chain (Figure 3d).

To the best of our knowledge, diacyltrehaloses have only been detected in prokaryotes and fungi[10] and have not been described for the animal kingdom until now. The specific structure of maradolipids resembles that of glycolipids from *Mycobacterium tuberculosis*, named cord factor[11]. The cord factor is a constituent of the outer lipid layer of the bacterial cell wall[12]. One of the presumable functions of the cord factor is to protect the bacteria from desiccation by stabilizing the physicochemical properties of the lipid layer and keeping it intact upon drying[13].

We have investigated the kinetics of the synthesis of maradolipids over the course of the development of dauer larva. L1 larvae of *daf-2(e1370)* were synchronized by starvation and then transferred to plates containing food at 25 °C. The lipid contents of developing worms were monitored by 2D TLC (see Figure 4a in the Supporting Information). Previously, it was described that the formation of the dauer larva is preceded by a morphologically distinct larval

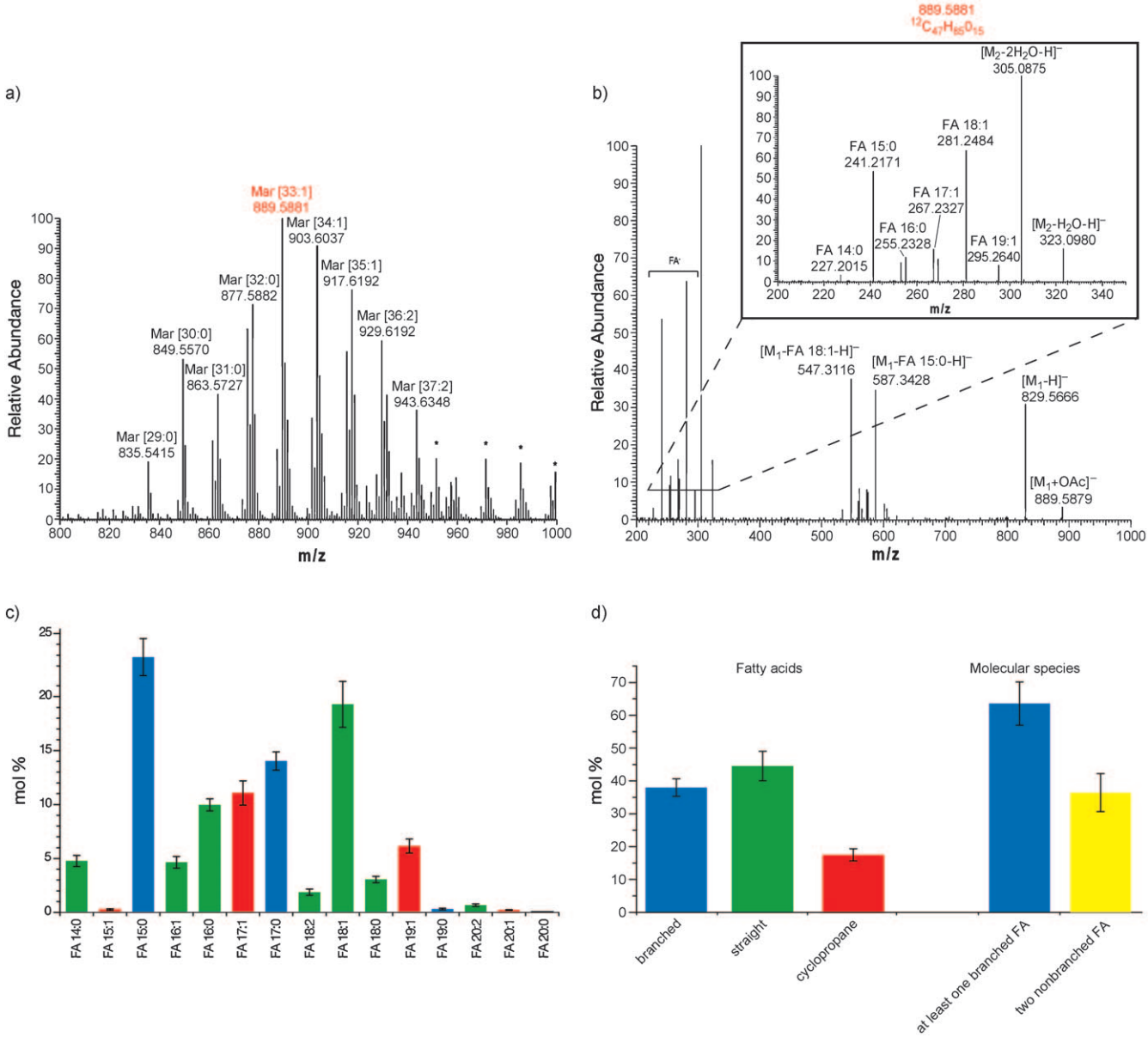

Figure 2. Maradolipids are 6,6′-di-*O*-acyltrehaloses with specific fatty acid composition. a) Mass spectrometric analysis of purified maradolipids on a LTQ-Orbitrap XL instrument in negative-ion mode. Maradolipids were detected as singly charged acetate adducts. The most abundant peaks are annotated by their *m/z* and by the total number of carbon atoms and double bonds in both fatty acid (FA) moieties of the corresponding maradolipid species. b) HCD fragmentation of maradolipid molecular ions revealed that each peak represents a mixture of several isobaric species that share the trehalose backbone and differ by the attached FA moieties. HCD MS/MS spectrum of the precursor ion with *m/z* 889.5881 (maradolipid 33:1) acquired at the normalized collision energy of 35%. Peaks corresponding to acyl anions of FA moieties are annotated by *m/z* and the number of carbon atoms and double bonds. Peak with *m/z* 323.0980 and 305.0875 corresponded to the trehalose moiety after loss of both FAs. Hence the analysis revealed that maradolipid 33:1 is a mixture of four isobaric species: maradolipid 15:0/18:1, maradolipid 16:1/17:0, maradolipid 16:0/17:1, and maradolipid 14:0/19:1. c) Bar diagram representing the abundance (mol%) of FA moieties of the maradolipid species. Blue bars: mmBCFAs; green bars: straight-chain FAs; red bars: cyclopropyl FAs. d) Relative abundance (mol%) of specific types of fatty acid moieties in maradolipids. Yellow bar: maradolipid species which contain straight and cyclopropane FAs, but contain no branched FAs.

stage (L2d, from L2-dauer)[14]. The latter can be additionally segregated into early and late L2d forms. The first sign of maradolipid synthesis was detected after about 32–36 hours. At this time point, larvae appear as a late L2d form (note that early L2d, collected after 26 h, display no maradolipids). The content of the maradolipids increases during dauer larva formation, reaching a plateau after about two days of morphologically identified dauer larvae. At this stage we determined the absolute content of maradolipids in dauer larvae. To build a calibration curve, we used synthetic 6,6′-di-*O*-myristoyltrehalose (C28:0; the synthesis will be published elsewhere) as a standard. The total lipid extract of *daf-2(e1370)* dauer larvae was subjected to HPLC TOF/MS analysis (see the Supporting Information). Of the masses on

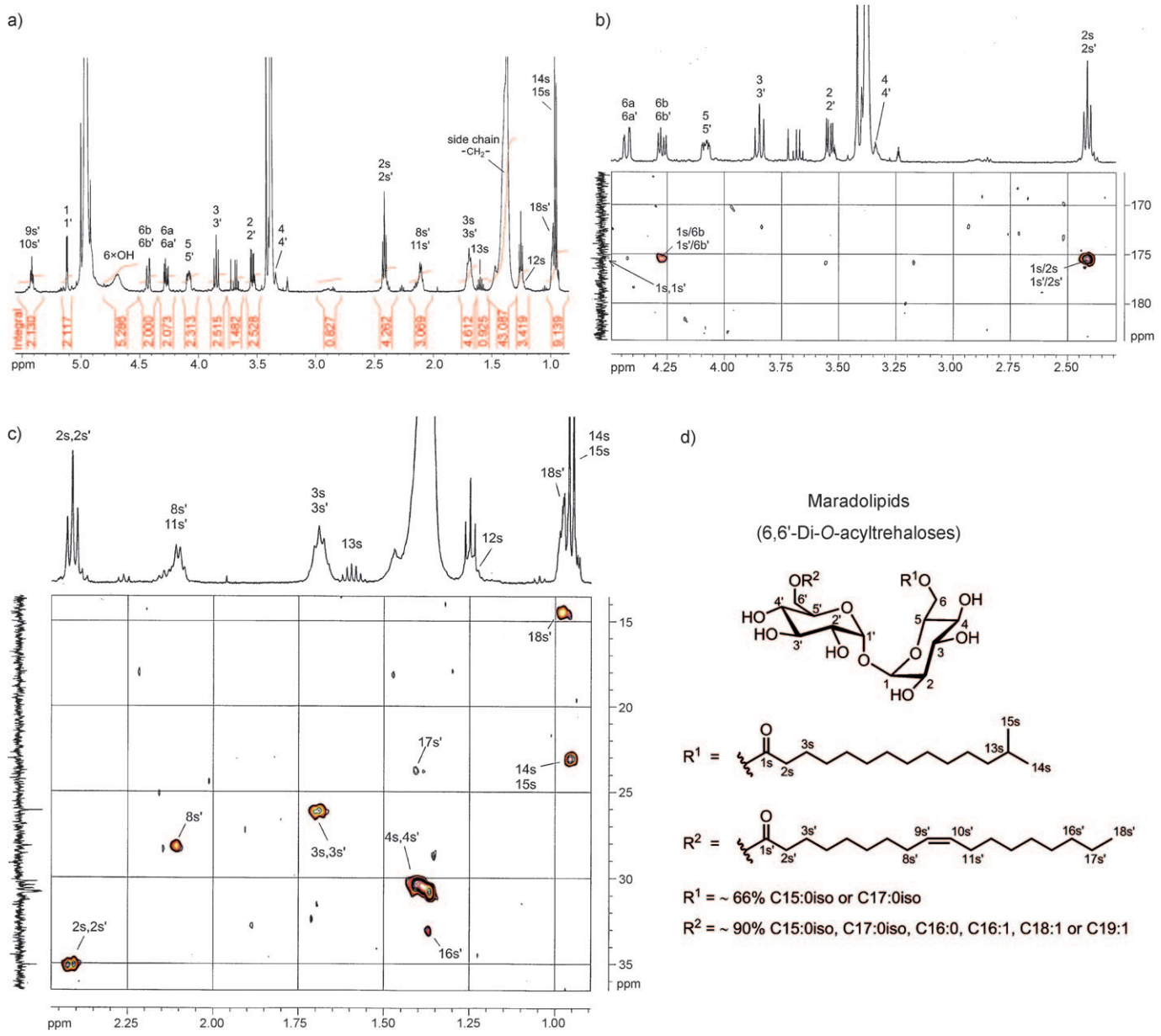

Figure 3. Maradolipid structure. a) ^{1}H NMR spectrum (500 MHz, [D$_4$]CH$_3$OH) of the maradolipid mixture with the assignment for the major component: 6-*O*-(13-methylmyristoyl)-6′-*O*-oleoyltrehalose. b) HMBC spectrum of the maradolipids ([D$_4$]CH$_3$OH). c) HSQC spectrum of the maradolipids ([D$_4$]CH$_3$OH). d) Structure of the major component of the maradolipids according to NMR data.

the TIC chromatogram (see Figure 5 in the Supporting Information), those ions corresponding to [*M*-H] ions of maradolipid species were extracted. The integrated area of the signals obtained by selected ion monitoring (SIM) chromatography was used to quantify the content, as correlated to a calibration curve. The quantification established that maradolipids in dauer larvae are present in the amount of about 10.2 μg (ca. 11.4 nmoles) per 10,000 animals. To relate absolute contents of maradolipids to other lipid classes we quantified the phospholipids in the same lipid extracts. We concluded that in the later phases of dauer, maradolipids constitute an abundant lipid class (about 6 mol% compared to the total dauer phospholipids).

To study the function of maradolipids, we established genetic conditions under which they are not synthesized or their amounts are reduced. First, we decided to inhibit the biosynthesis of trehalose. Two enzymes catalyze the first reaction of trehalose biosynthesis in *C. elegans*: TPS-1 and TPS-2 (trehalose phosphate synthases). There are deletion mutant strains of individual TPSs (*tps-1(ok373)* and *tps-2(ok526)*). A double-deletion strain was produced and crossed to *daf-2(e1370)* and *daf-7(e1372)* lines (*tps-2;daf-2; tps-1 and tps-2;daf-7;tps-1*, here abbreviated as *daf-2;*ΔΔ*tps* and *daf-7;*ΔΔ*tps*, respectively, see the Supporting Information). As shown for *daf-2;*ΔΔ*tps*, dauers of this strain contain neither maradolipids (see Figure 4b in the Supporting

Information) nor trehalose (see Figure 4d in the Supporting Information). We obtained the same results with *daf-7;*ΔΔ*tps* (not shown).

Second, we asked whether the absence of mmBCFAs would influence the synthesis of maradolipids. It was shown previously that *elo-5* is a long-chain fatty acid elongation enzyme, necessary for the production of mmBCFAs in *C. elegans*[9]. To decrease or inhibit the synthesis of mmBCFAs during dauer larva formation, we applied RNAi of *elo-5* on *daf-2(e1370)* and *daf-7(e1372)* (see the Supporting Information). Parental animals were subjected to RNAi at the L3–L4 stage and RNAi was continued in the progeny, which was incubated at the restrictive temperature (25 °C). Under these conditions, worms developed into regular dauer larvae without any obvious defects. The RNAi of *elo*-5 reduces the amount of maradolipids in *daf-2* significantly (see Figure 4c in the Supporting Information). RNAi of *elo*-5 in *daf-7* similarly reduces maradolipid abundance (not shown). Quantification of the maradolipids by using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) revealed that the reduction of maradolipids was about 80%. Indeed, when analyzing the individual maradolipids, almost no species with branched-chain fatty acids remained (not shown). In contrast, the levels of trehalose were identical to those of the control dauer larvae (see Figure 4d in the Supporting Information). Thus, we have produced conditions where the general role of trehalose and in particular that of maradolipids could be studied.

The high degree of accumulation of maradolipids in dauer larvae suggested that these lipids might be part of a dauer body structural unit (e.g. lipid membrane/film, cellular organelle, extracellular matrix, etc.). We wondered, whether depletion of trehalose/maradolipids could lead to some morphological changes in dauer larvae. Therefore, we performed electron microscopy (EM) of regular dauer larvae (*daf-2)*, dauer larvae missing trehalose (*daf-2*;ΔΔ*tps*), and dauer larvae with reduced maradolipids (*daf-2* fed with *elo-5*(RNAi)). To obtain high quality images, we used the cryosubstitution fixation technique (see the Supporting Information). The overall morphology of the dauers in all three cases is similar (Figure 4a, overview; details not shown). Also no significant changes in the structure of cellular organelles are detected. However, some dramatic differences are observed when the gut lumen is analyzed. Although EM analysis of dauer larvae has been performed in the past, the cryopreservation revealed structures not described before. On the surface of the gut of the control dauer larvae (*daf-2*), there is a dark, dense layer, into which microvilli are immersed (Figure 4b). Microvilli are much shorter than in reproductive larvae (3 to 4 times)[15] and covered quite often by spirals. The lumen of the gut is entirely filled with a compact multilamellar material. The dark layer was significantly reduced when maradolipids were depleted by *elo-5*-(RNAi) (Figure 4c); in the absence of trehalose, the dense layer could not be detected at all (Figure 4c). In contrast, lamellar structures in the lumen were present in all three types of dauer larvae. Taking into account that *elo-5*(RNAi) fed worms have the same amount of trehalose as worms grown on EV, it seems that these are maradolipids that are

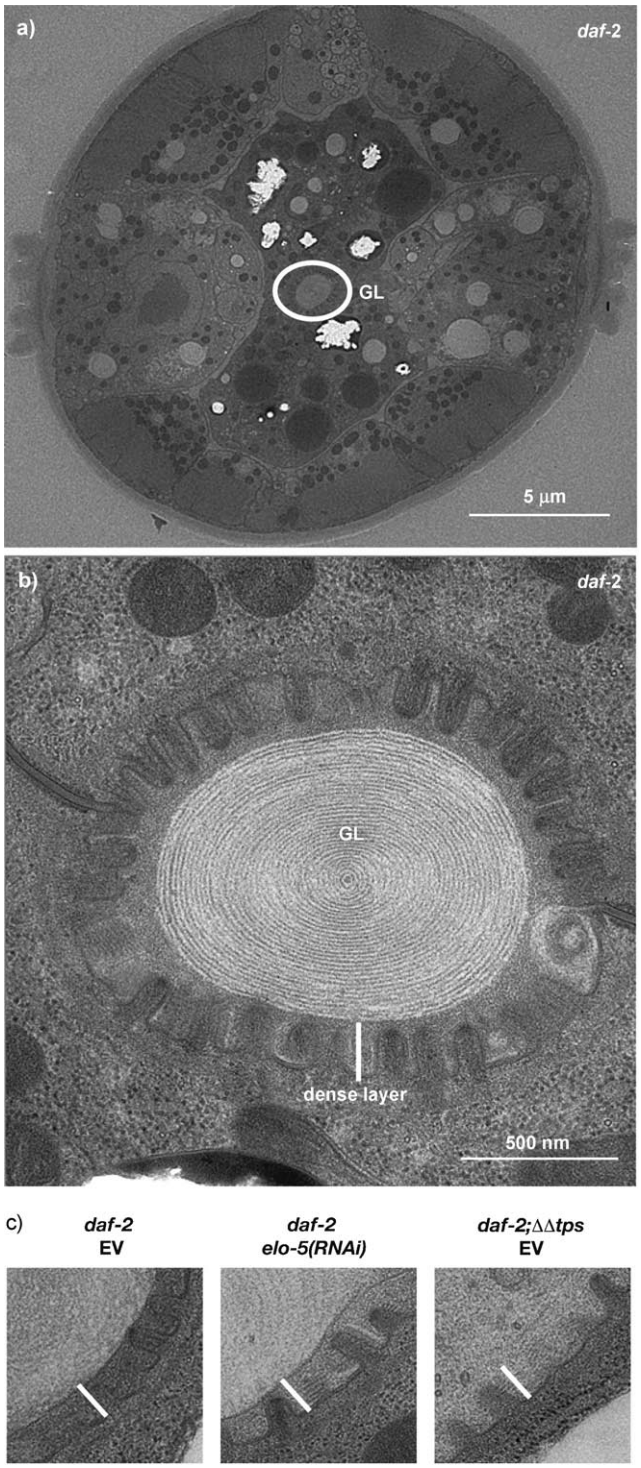

Figure 4. Maradolipids are required for the structuring of the gut lumen (GL). a) Electron micrograph of a cross-section of a *daf-2* dauer larva (low magnification). The organization of the body of the dauer larvae is characteristic, showing a dauer-larva-specific cuticle with alae and a reduced GL (encircled with white line) having almost no detectable microvilli. b) Electron micrograph of a GL of a *daf-2* dauer larva (high magnification). The lumen is covered with a dense layer in which microvilli are immersed (white bar). The lumen is entirely filled with lamellar structures. c) Electron microscopy shows reduction of the luminal dense layer in *daf-2* dauer larvae upon *elo-5*(RNAi). In *daf-2*;ΔΔ*tps* the layer is not present (white bars).

required for the morphology of the gut and in particular for the existence of an electron-dense layer on the apical surface. Most probably, this layer is formed by maradolipids themselves. This also implies that they should be effectively secreted by enterocytes. However, it should not be excluded that the effect of maradolipids on the structure of the gut is indirect.

In summary, we have shown that worms, upon transition from the reproductive larval stage to the dauer larval stage, produce a novel class of 6,6′-di-*O*-acyltrehaloses that we termed maradolipids. The exact localization and function of maradolipids has to be additionally investigated. However, they are involved in the structuring of the gut of the dauer larvae. We describe novel morphological characteristics of the dauer intestine: a dense layer, sealing the apical surface of the enterocytes, and multilamellar material, which fills the whole volume of the gut lumen. The chemical composition and the function of these structures are unknown and provide ground for extensive studies on dauer strategies to seal itself off from the external environment.

Experimental Section

A list of the chemicals and the biological material used in the study can be found in the Supporting Information.

Extraction of lipids and carbohydrates monitored by TLC analysis was done in the following way: worms were homogenized by freezing/thawing and extracted according to the method of Bligh and Dyer[16]. Organic and water phases were used separately for the recovery of the total lipids and sugars, respectively. Neutral glycolipid (NGL) fractions were obtained by flash chromatography of dauer larvae lipid extracts on a silica gel column (Kieselgel 60, 0.04–0.063 mm, Roth). After flushing the column with chloroform, a three-step elution was performed with 1) chloroform, 2) acetone/methanol (9:1, v:v), and 3) methanol. NGLs were always detected in the acetone/methanol fraction. Saponification was done as described previously[17]. Different (milder) basic hydrolysis conditions were used for the preparation of the carbohydrate moiety of maradolipids (see the Supporting Information) to prevent degradation of the saccharides. Analytical TLC techniques were performed on 10 cm HPTLC plates (Merck, Darmstadt, Germany) The following TLC eluents have been used; A: chloroform/methanol/H_2O (45:18:3, v:v:v); B: chloroform/methanol/32% ammonia (60:35:5, v:v:v; C: chloroform/methanol/H_2O (4:4:1, v:v:v). Total lipid extracts and NGL fractions (before and after saponification) were analyzed by 2D TLC methods in which systems A and B were used as the first and second running systems, respectively. 1D TLC analysis of the NGL fractions was performed with system B. For developing all sugar/hydrophilic fractions (including the deacylated maradolipid glycan residue; see the Supporting Information) system C was used. TLC plates were sprayed with the Molisch reagent. An aniline/diphenylamine reagent was used to detect the reducing sugars. Loading was corrected for the same volume of extract prepared from identical amount of larvae. Maradolipid purified fractions were obtained by preparative TLC methods of dauer NGL fractions on 20 cm TLC plates (Merck, Darmstadt, Germany) with system B.

Structural analysis by mass spectrometry was performed by shotgun analysis on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen). Lipids were dissolved in chloroform/methanol/2-propanol (1:2:4, v:v:v) containing 7.5 mM ammonium formate. The analyte was directly infused into the mass spectrometer at a flow rate of 200 nL/min using a robotic nanoflow ion source TriVersa (Advion BioSciences, Ithaca NY). Survey spectra were acquired in negative-ion mode at the target mass resolution of 100,000 (FWHM, full width at half maximum) on the Orbitrap analyzer. To acquire tandem mass spectra precursor ions were isolated within *m/z* window of 1.8 Da and fragmented in higher collision energy (HCD) mode at the normalized collision energy of 35%. MS/MS spectra were acquired on the Orbitrap analyzer with the targeted resolution of 30,000 (FWHM) in data-dependent acquisition mode[18]. Molecular species were identified and quantified using LipidX software developed in-house.

NMR spectra were acquired in $[D_4]CH_3OH$ on a Bruker DRX 500 instrument. An almost complete assignment of the ^{1}H NMR signals has been achieved for the major component of the maradolipids by the following 2D NMR spectra: COSY, HSQC, HMBC, NOESY, and ROESY.

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