**Absolute Quantification of a Steroid Hormone that Regulates Development in Caenorhabditis elegans**

Tie-Mei Li,†,‡ Jie Chen,§ Xiangke Li,‡ Xiao-Jun Ding,‡ Yao Wu,‡ Li-Feng Zhao,‡ She Chen,‡ Xiaoguang Lei,‡,§ and Meng-Qiu Dong,†,§

†College of Life Sciences, Beijing Normal University, Beijing 100875, China
‡National Institute of Biological Sciences, Beijing, Beijing 102206, China
§College of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, China

**ABSTRACT:** Under favorable conditions, Caenorhabditis elegans larvae grow into reproductive adults after a series of molting cycles. When environmental conditions are harsh, they arrest as dauer larvae. Dafachronic acid (DA), a C. elegans steroid hormone, is required for reproductive development. Here, we report a mass spectrometry (MS) method for absolute quantitation of DA in C. elegans. The extraction of DA from C. elegans was optimized to achieve a recovery rate of greater than 83%. The MS sensitivity to DA increased 100-fold after carboxyl group derivatization with 2-picolylamine. High-resolution selected ion monitoring (HR-SIM) on a Q-Orbitrap mass spectrometer Q Exactive outperformed targeted-MS2 on the same instrument and selected reaction monitoring (SRM) on a triple-quadrupole mass spectrometer TSQ Quantum Discovery. With a limit of quantitation as low as 1 pg of DA, the HR-SIM method enables absolute quantification of endogenous DA during the reproductive development of C. elegans. We found that in wild-type (WT) worms, DA increases from 0.04 ± 0.02 ng/mg protein in the L1 larval stage to 1.21 ± 0.67 ng/mg protein in the L2 larval stage and decreases again after the L3 stage. In comparison, four genetic mutants that have a constitutive dauer-formation phenotype due to disrupted insulin, TGF-β, or cGMP signaling all have a very low DA level in the L2 stage (below 15% of the WT). These mutants are able to escape the dauer fate and most of them grow into fertile adults when supplied with exogenous DA. Therefore, a DA spike in the L2 stage is critical for the reproductive development of C. elegans.

Steroid hormones are signaling molecules that are synthesized from cholesterol. They are found in many organisms, from worms to mammals, and regulate a variety of vital physiological functions, including development, metabolism, and reproduction. The first steroid hormone identified in Caenorhabditis elegans is dafachronic acid (DA), a key molecule that helps determine whether C. elegans larvae grow into reproductive adults. Under favorable conditions, C. elegans undergo four larval stages (L1-L4) after hatching and develop into adults in 3 days. However, when environmental conditions are harsh, such as high temperature, starvation, or high population density, worms arrest at an alternative L3 stage in the form of dauer larvae (Figure 1). Dauer larvae exhibit many adaptive changes in morphology, behavior, and metabolism that help them survive under stressful conditions. Dauer larvae can live up to several months before recovering and developing into normal L4 larvae and subsequently adults when the environmental conditions improve.

Before the chemical structure of DA was determined, its receptor DAF-12 was identified as a common target downstream of several dauer regulatory pathways. Mutations in the daf-9 or daf-36 gene, each encoding an enzyme involved in DA synthesis (Figure 1), cause a constitutive dauer-formation phenotype (Daf-c) and impairment in gonad development. These data indicate that DA plays a decisive role in C. elegans larval development.

Despite its important role in the life history of C. elegans, DA is present in very small quantities. Motola et al. detected the presence of endogenous DA in C. elegans using normal phase fractionation and liquid chromatography–mass spectrometry (LC–MS), but this method required a lot of starting material from 20 to 100 million WT worms, which is too laborious to prepare for routine analysis. Subsequent studies measured DA levels by derivatizing the carboxyl group of DA with trimethylsilyldiazomethane, followed by gas chromatography/mass spectrometry (GC/MS) of the generated methyl ester. However, the sensitivity and precision of this method has not been reported.
To further study the function of DA and related regulatory mechanisms, a simple, accurate and sensitive quantitation method of DA is needed. In this study, we developed a LC–MS-based method for absolute quantification of DA in C. elegans. We evaluated the MS behavior of DA in both the positive and negative ion modes and found a derivatization method that increased detection sensitivity by 100-fold. We also compared three MS quantitation methods and found that high-resolution selected ion monitoring (HR-SIM) on a Q-Orbitrap instrument performed the best with excellent signal-to-noise ratios. Using HR-SIM, we measured the DA levels during larval development of WT C. elegans and in Daf-c mutants with defective insulin, TGF-β, or cGMP signaling. The results revealed that in WT but not the Daf-c mutant animals, there was a 10-fold increase of DA from the L1 to the L2 stage, suggesting that an elevated DA level in L2 larvae is critical for the C. elegans reproductive development.

**EXPERIMENTAL SECTION**

**Reagents and Chemicals.** HPLC grade chloroform, acetonitrile, and methanol were purchased from J. T. Baker (Center Valley, PA). Triphenylphosphine (TPP, CAS No. 603-35-0) was purchased from Sigma-Aldrich (St. Louis, MO). 2,2′-Dipyridyl disulfide (DPDS, CAS No. 2127-03-9) and 2-picolyamine (PA, CAS No. 3731-51-9) were purchased from J&K Scientific Ltd (Beijing, China). (2S)-Δ4-dafachronic acid and (2SR)-Δ2-dafachronic acid were gifts from Dr. Hans-Joachim Knöllker (Technische Universität Dresden, Germany). (2S)-Δ4-dafachronic acid (DA) and its triple-deuteron labeled version ([d₆]DA) were synthesized in-house. The chemical synthesis of DA and [d₆]DA will be published elsewhere. The identities of the synthesized molecules were confirmed by NMR and high-resolution mass spectrometry on a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). The biological activity of the synthesized DA was verified through a dauer rescue assay using the strain daf-9(dh6); dhEx24[pTG96(sur-S::gfp), T13C5(daf-9)]. DA is stable at the room temperature on NGM plates for at least 7 days.

**Worm Culture.** C. elegans strains N2 (WT), daf-2(e1368), daf-2(e1370), daf-7(ok3125), daf-11(k567), and daf-36(k114) were from Caenorhabditis Genetics Center (CGC); daf-9(dh6); daf-12(rl61rh411) and daf-9(dh6); dhEx24[pTG96(sur-S::gfp), T13C5(daf-9)] were kindly provided by Dr. Adam Antebi (Max Planck Institute for Biology of Aging, Cologne, Germany). Worms were maintained and cultured as described by Brenner. Briefly, to obtain synchronized animals, worms were cultured on high-growth (HG) plates seeded with Escherichia coli OP50 until gravid adults were obtained. The worms were then washed off plates with M9 buffer (3.0 g of KH₂PO₄, 6.0 g of NaHPO₄, 0.5 g of NaCl, and 1.0 g of NH₄Cl in 1 L of H₂O) and bleached with 30% sodium hypochlorite (NaOCl) containing 0.75 M KOH to obtain the eggs. After hatching, the synchronized L1 worms were seeded onto fresh HG plates with E. coli OP50 and incubated at the desired temperature until harvest. The WT L1, L2, L3, L4, and young adult worms were harvested 6, 23, 35, 47, and 54 h later at 20 °C. The Daf-c mutant L1s were cultured at 25 or 27 °C until the L2d stage (daf-2(e1368), 25 °C, 25 h; daf-2(e1370), 25 °C, 31 h; daf-7(ok3125), 25 °C, 31 h; daf-11(k567), 27 °C, 20 h), and WT L2s were cultured at the same temperatures (25 °C, 18 h or 27 °C, 16 h) to serve as controls. The harvested worms were aliquoted and stored at −80 °C until use.

**Dauer Rescue Assay.** The dauer rescue assay was adapted from Motola et al. Briefly, 3-cm plates containing 3 mL of normal-growth medium (NGM) were seeded with E. coli OP50 mixed with 3 μL of DA (200 μM) or ethanol (control). After the plates were dried, five adult worms of the genotype daf-9(dh6); dhEx24[pTG96(sur-S::gfp), T13C5(daf-9)] were placed on the plate to lay eggs overnight. Approximately 200 eggs were obtained on each plate before the mothers were removed. The plates were maintained at 27 °C. After 2.5 days, the developmental state of the worms was determined, and the worms were scored for dauer larvae, arrested L3 larvae, and adults.

**Lipid Extraction.** The lipid extraction method was adapted from the Folch method with modifications. Three volumes of extraction solvent (chloroform–methanol 2:1) were added to the worm pellet. The worms were homogenized with 1–2 volumes of glass beads using a FastPrep-24 (MP Biomedicals, Santa Ana, CA) homogenizer at 6.0 m/s, 20 s/pulse × 3 pulses with 5-min intervals on ice. The lysate was separated from the glass beads prior to the addition of another four volumes of extraction solvent. The samples were mixed thoroughly by vortexing for 1 min and then sonicated for 10 min. After

---

**Figure 1.** Biosynthesis of DA and the life cycle of C. elegans. Left panel, the DA synthesis pathway in C. elegans. DAF-36 catalyzes the formation of 7-dehydrocholesterol. DAF-9 catalyzes the formation of the carboxyl group in Δ⁴ and Δ²-DA. Right panel, the life cycle of C. elegans. Under favorable environmental conditions, WT worms develop into adults after four larval stages. When environmental conditions are harsh, C. elegans form dauer larvae, which is a diapause state alternative to L3. The dauer larvae recover and enter the L4 stage when environmental conditions improve. DA inhibits dauer formation.
centrifugation at 4000g for 10 min, the organic layer was isolated and dried under a low flow of high-purity nitrogen gas at 37 °C. The dried total lipids were weighed with a Mettler Toledo AB135-S balance.

**Derivatization of DA.** DA was reacted with 2-picolylamine (PA), and the amount of derivatization reagent and reaction time were optimized for the lipid samples. To 3–10 mg of dried lipids (from 0.3 to 1.0 mL packed worms), 1 mL of triphenylphosphine (10 mM in acetonitrile), 1 mL of 2,2′-dipyridyl disulfide (10 mM in acetonitrile), and 1 mL of PA (1 μg/μL in acetonitrile) were added successively and the mixture was incubated at 60 °C for 20 min. The reaction was stopped by adding 1 mL of methanol–acetic acid (99:1) and dried under nitrogen at 37 °C. The samples were reconstituted in 0.5 mL of acetonitrile–water (3:2), and the insoluble portion was removed by centrifugation at 14,000g for 10 min. The supernatant was used directly for MS analysis. The insoluble portion was dried under nitrogen and weighed. The difference between the weight of the total lipids and that of the insoluble fraction was used to estimate the lipid concentration in the supernatant. All samples were diluted 5-fold with acetonitrile–water (3:2), and 5 μL was used for the LC–MS analysis. Samples containing DA-PA are stable for 1 day at 4 °C or for 1 week at −20 °C. Samples containing DA-PA can be dried and stored at −20 °C or −80 °C for 8 months without reduction in MS signal.

**Protein Assay.** After lipid extraction, the protein pellet was reconstituted in 4% SDS. The protein concentration was determined using the BCA Protein Assay reagents (Pierce, Rockford, IL).

**Standard Solutions and Calibration Samples.** DA was dissolved in ethanol and serially diluted with ethanol to prepare standard stocks. For the SRM method, the stocks of DA were 10, 5, 2, 1, 0.5, 0.2, and 0.1 ng/μL, and the stock of [d₃]-DA was 2 ng/μL. For the HR-SIM method, the stocks of DA were 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 ng/μL, and the stock of [d₃]-DA was 0.5 ng/μL. For each calibration sample, 50 μL of the corresponding DA stock was spiked into 1 mL of daf-9(dh6);daf-12(rh61rh411) double mutant worms, which cannot produce DA. The daf-9;daf-12 double mutant was also used as the blank sample.

**SRM Analysis.** SRM analysis was performed on a triple quadrupole mass spectrometer with an ESI source (TSQ Quantum Discovery Max, Thermo Fisher Scientific) coupled online to a Surveyor autosampler and MS pump (Thermo Fisher Scientific). Samples (5 μL) were injected through the autosampler onto a C18 column (Hypersil Gold column, 50 mm long, 1 mm i.d., 3-μm particle size; Thermo Fisher Scientific). The mobile phase A consisted of 0.1% formic acid and 5 mM ammonium acetate in water, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The gradient was as follows: 0–0.5 min, 30% B; 1.5–2.0 min, 30%–100% B; and 2.0 min–4.0 min, 100% B. The flow rate was 200 μL/min.

**MS parameters for DA-PA and [d₃]-DA-PA analysis: spray voltage, 4000 V; sheath gas (nitrogen) flow rate, 35 arbitrary units; auxiliary gas (nitrogen) flow rate, 5 arbitrary units; ion transfer capillary temperature, 350 °C; Q1 and Q3 peak width, 0.7 m/z units; and collision gas (argon) pressure, 1.5 mTorr.** Data acquisition and analysis were performed using the LCquan software version 2.6.0 (Thermo Fisher Scientific).

**HR-SIM and t-MS2 Analyses.** High-resolution selected ion monitoring (HR-SIM) and targeted-MS2 (t-MS2) analyses were performed on a Q-Orbitrap mass spectrometer Q Exactive (Thermo Fisher Scientific) coupled to the same Surveyor autosampler and the MS pump as above. The column and the HPLC gradient were also the same as in the SRM analysis. The ion source settings were: spray voltage, 4000 V; capillary temperature, 320 °C; sheath gas pressure, 35 arbitrary units; auxiliary gas pressure, 10 arbitrary units; and heater temperature, 300 °C. The HR-SIM settings were as follows: resolution, 70,000; AGC target, 5E5; maximum injection time, 250 ms; MSX count, 2; isolation window, 4 m/z; and scan range, 490–520 m/z. The t-MS2 settings were as follows: resolution, 17,500; AGC target, 2E5; maximum injection time, 250 ms; isolation window, 2 m/z; scan range, 50–600 m/z; and collision energy, 35. The inclusion list was m/z = 505.3789 for DA-PA and 508.3977 for [d₃]-DA-PA. Data acquisition and analysis were performed with Xcalibur software version 2.2 SP1.48 (Thermo Fisher Scientific).

**Statistical Analysis.** The amount of DA was normalized to the amount of total proteins in each sample. For the ANOVA analysis of the DA levels in different developmental stages from L1 through young adulthood, which vary over 2 orders of magnitude, the DA concentration (ng/mg proteins) was log10-transformed so that the data conform to a normal distribution with equal variance. Following ANOVA, the Tukey’s HSD test was used to resolve pairwise differences among the means. To compare the mutant vs WT DA levels, paired Student’s t-test was performed.

## RESULTS AND DISCUSSION

**Optimization of MS Sensitivity to DA.** Both positive and negative ions of DA can be detected by mass spectrometry, but direct quantification of either proved to be inefficient (Figure S1 in the Supporting Information). The protonated positive ion (calculated m/z 415.3207) does not form readily because DA is a carboxylic acid (Figure 1), and it is surrounded by interfering ions of similar m/z (Figure S1A,B in the Supporting Information). Although selected reaction monitoring (SRM) combined with liquid chromatography (LC) using a phenyl column can separate DA from the major interference, the signal was unsatisfactory (Figure S1C,D in the Supporting Information). We encountered the same problems with the lithium adduct ion of DA (Figure S1A in the Supporting Information and data not shown). In the negative ion mode, the signal of deprotonated DA (calculated m/z 413.3056) was approximately 7-fold greater than that of protonated DA in the positive mode, and the signal of interference was much lower (Figure S1E in the Supporting Information). However, the negative DA ion is refractory to fragmentation. It remained intact unless the normalized collisional energy (NCE) was raised to a very high level; but once it started to fragment, it overfragmented and produced unstable MS2 spectra (Figure S1F in the Supporting Information).

To overcome these difficulties, we must chemically derivatize DA. Multiple derivatization strategies have been developed to improve LC–ESI-MS analyses of steroids. For example, for 3β-hydroxy-Δ^5 and 3β-hydroxy-5α steroids, oxidation of 3β-hydroxy to 3-oxo followed by derivatization using Girard P hydrazine increases the detection sensitivity by 1000-fold.

Similarly, derivatization of mono-oxosteroids with 2-hydrazino-1-methylpyridine improves the detection sensitivity by up to 3 orders of magnitude. In this study, we chose a simple procedure to block the carboxyl group of DA with 2-picolylamine (PA), which was shown to improve the LC–MS signal response of carboxylic acids by 9–158-fold. We found that 2-picolylamine improved the detection sensitivity by up to 3 orders of magnitude. In this study, we chose a simple procedure to block the carboxyl group of DA with 2-picolylamine (PA), which was shown to improve the LC–MS signal response of carboxylic acids by 9–158-fold. We found that 2-picolylamine improved the detection sensitivity by up to 3 orders of magnitude.
that in the positive ion mode, the derivatization product DA-PA had at least a 100-fold higher signal response than DA (Figure 2A). While this is a sizable improvement, it remains to be explored in the future whether derivatization at 3-oxo of DA is even better.

Fragmentation of DA-PA produced a distinct product ion of m/z 109.0760, corresponding to the PA moiety (Figure S2A in the Supporting Information). We tested three isoforms of DA, in descending order of biological potency, (25S)-Δ⁷-DA, (25S)-Δ⁴-DA, and (25R)-Δ⁷-DA, which differ from one another in the position of the carbon–carbon double bond or the chirality of C25. All three isoforms have the same MS/MS spectra and the same retention time in our LC system. Therefore, they were not distinguished from one another in this study. From this point on, (25S)-Δ⁷-DA was used to establish the quantification method and its deuterated form [d₃]-(25S)-Δ⁷-DA, simply referred to as [d₃]-DA, was synthesized as an internal standard (ISTD). In the MS2 spectrum of [d₃]-DA-PA, the dominant fragment ion is m/z 110.0817 (Figure S2B in the Supporting Information), which is protonated PA containing one deuterium atom from either C25 or C24 of [d₃]-DA (compared to Figure S2A in the Supporting Information). The observed mass of [d₃]-PA is no more than 6 ppm away from the theoretical mass. Both DA-PA and [d₃]-DA-PA produce a fragment ion of m/z 92.0495, which is the [PA + H]⁺ ion with a neutral loss of ammonia as inferred from the exact mass (Figures S2A,B in the Supporting Information).

Extraction and Derivatization of C. elegans DA. We compared two solvents chloroform–methanol (2:1) and hexane–isopropanol (3:2) for extraction of lipids from C. elegans and found that the former yielded a better recovery rate (≥83%) of spiked-in DA (Figure 2B,C). Using chloroform–methanol (2:1), approximately 10 mg of total lipids were extracted from 1 mL of packed worms (containing 30–40 mg of total proteins), which contained no more than 30 ng of endogenous DA. After the lipids were dried, derivatized with PA, and dried again, 20% of the lipids (by weight) including DA-PA were brought into solution with acetonitrile–water (3:2) and separated from the insoluble fraction. As such, derivatization not only increased the MS signal response of DA but also removed 80% (by weight) of the background lipids. This proved to be more effective and more reproducible than solid-phase extraction (SPE) with normal phase (silica), reverse phase (polystyrene-divinylbenzene), or ion exchange (ethylendiamine-N-propyl) columns that we had tried in order to reduce background complexity (data not shown). For optimal results, 3–10 mg of total lipids (extracted from 0.3 to 1.0 mL packed worms) were reacted with 1 mg of PA in 1 mL of acetonitrile at 60 °C for 20 min. This can be scaled down to 100 μL packed worms (corresponding to 20 000 adult worms),
in which case 0.1 mg of PA is sufficient for derivatizing the extracted lipids (Table 2).

**MS Quantification of DA-PA.** We compared three MS methods for quantitation of DA-PA, selected reaction monitoring (SRM) on a triple-quadrupole mass spectrometer (QqQ, TSQ Quantum Discovery Max), targeted-MS2 (t-MS2), and high-resolution selected ion monitoring (HR-SIM) on a quadrupole-Orbitrap (Q Exactive) mass spectrometer. For the traditional SRM method, the optimized SRM transitions were 505.3 → 109.1, 92.0 for DA-PA, 508.4 → 110.1, 92.0 for [d3]-DA-PA, with 109.1 and 110.1 being the quantified ions and 92.0 for qualification of the target compounds (Figure S2C in the Supporting Information). The Q1 peak width and the Q3 peak width were both 0.7 ms. For t-MS2, the parent ions 505.3789 (for DA-PA) and 508.3977 (for [d3]-DA-PA) were selected with a ±1.0 m/z window for high-resolution MS2 scans. To reconstruct the chromatographic peaks of the target compounds, the intensities of the product ions, m/z 109.0760 for DA-PA and 110.0823 for [d3]-DA-PA, were extracted with a mass tolerance of 10 ppm from the respective MS2 spectra (R = 17.500). In HR-SIM, full MS scans in the mass range 490–520 m/z were acquired at a resolution of 70,000, and the intensity of DA-PA (505.3789 m/z) and that of [d3]-DA-PA (508.3977 m/z) were extracted with a 10-ppm mass accuracy.

Among the three methods, HR-SIM achieved the highest sensitivity. Using HR-SIM, the signal-to-noise ratio (S/N 777) was 5 times higher than that of either SRM or t-MS2 (S/N 120–130) (Figure 2D–F). The S/N ratios of the extracted DA-PA chromatographic peaks from either the SRM or the t-MS2 data were likely compromised by a co-eluting ion of similar m/z that was also a PA derivative and thus generated the m/z 109.0760 product ion (PA) in MS2. The high resolution of the SIM method permitted the differentiation of the target compound from the majority of the interference in the full MS scan.

We further validated the HR-SIM method and, for comparison, the SRM method. The linear range of quantitation for DA is 1–200 pg with HR-SIM and 10–1000 pg with SRM (Figure S3 in the Supporting Information). The upper limit of quantitation was not explored further because the current values far exceed what is necessary for quantification of endogenous DA in *C. elegans*. The lower limit of quantification (LLOQ) was determined as the lowest amount of a given analyte that showed a relative standard deviation (RSD) of <20% among replicate injections and an accuracy of measurement within ±20%. As shown in Table 1, the intra- and inter-assay accuracy was within ±15% and the precision within 15% except for the interassay precision at the 2-pg level, which was 16.5% (Table 1).

**DA Levels in Worms with Impaired DA Synthesis.** Previous studies have identified a number of enzymes involved in DA synthesis in *C. elegans*.8–10,12,25 DAF-9, a cytochrome P450 in *C. elegans*, catalyzes the oxidation of the side-chain of DA (Figure 1 and refs 2, 8, and 9) and is therefore responsible for the formation of both Δ1- and Δ1-DA. Loss of daf-9 activity induces constitutive dauer arrest in a daf-12 dependent manner; daf-9 mutants form 100% dauers while the daf-9;daf-12 double mutants grow into adults.8,9 In agreement with the previous observation,2 we did not observe any DA signal in daf-9;daf-12 double mutant worms (Figure 3A). Therefore, daf-9;daf-12 worms of mixed developmental stages were used as the blank sample and as the matrix into which known amounts of DA were added in order to build a calibration curve for absolute quantitation of DA. DAF-36, a Rieske-like oxygenase, catalyzes the formation of a carbon–carbon double bond in the core ring-structure of Δ1-DA,10,11 but does not affect the synthesis of Δ1-DA (Figure 1). The DA level was greatly reduced in a daf-36 mutant compared to the WT (Figure 3A), demonstrating that our method was sensitive and specific. When normalized to the amount of total proteins, the DA level in the daf-36 mutant was 53% of that in WT.

**DA Levels during C. elegans Larval Development.** Using the HR-SIM method, we measured the DA level in WT *C. elegans* during development. We collected worms in the L1, L2, L3, and young adult stages (Figure 3B). In six biological repeats, we found a sharp, greater than 10-fold increase in DA at the L2 larval stage, from 0.04 ± 0.02 ng DA/mg protein at the L1 stage to 1.21 ± 0.67 ng DA/mg protein. This was followed by an equally dramatic decrease to 0.17 ± 0.06 and 0.08 ± 0.04 ng DA/mg protein when *C. elegans* reached the L4 and young adult stages, respectively (Figure 3C and Table S1 in Figure S4 in the Supporting Information). This is in keeping with a previous finding that the expression of daf-9 in the hypodermis is greatly increased in the L2 and L3 larval stages.26 The DA spike occurs in the L2 larval stage, a critical period during which worms choose between reproductive development and dauer formation. We propose that DA plays an important role in this process; high levels of DA in L2/L3 promote reproductive development whereas low levels of DA induce dauer formation.

**DA Levels in Daf-c Mutant Worms.** Shown in Figure 4A, genetic studies identified three major signaling pathways that regulate dauer formation in *C. elegans*, the insulin/IGF, TGF-β, and cGMP pathways.27,28 At 25 or 27 °C and with plenty of food, a condition under which WT animals grow into adults, most of the daf-2 (insulin receptor), daf-7 (TGF-β), or daf-11 (guanylyl cyclase) mutant animals form dauer larvae (the Daf-c phenotype). We cultured the daf-2 and daf-7 mutants at 25 °C and daf-11 at 27 °C to ensure >90% penetration of the Daf-c phenotype and harvested the mutant samples at the L2 stage along with WT L2s cultured under the same condition. We found that the daf-2, daf-7, and daf-11 L2 larvae all had very low

---

**Table 1. Precision and Accuracy of DA Quantitation Using a Triple-Quadrupole Mass Spectrometer and a Q-Orbitrap Mass Spectrometer**

<table>
<thead>
<tr>
<th>QC level</th>
<th>intra-assay (n = 3)</th>
<th>inter-assay (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spike-in amount (ng)</td>
<td>amount on column (pg)</td>
</tr>
<tr>
<td>SRM (QqQ)</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>HR-SIM (Q-Orbitrap)</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

**Analytical Chemistry**

Article

dx.doi.org/10.1021/ac402025c Anal. Chem. 2013, 85, 9281–9287
DA levels (Figure 4B), ranging from 4% to 15% of that in WT L2s. Therefore, when worms undergo reproductive development, the DA level is elevated during the L2 stage and when they prepare for dauer arrest, the DA level remains low. Previous studies suggest that DAF-12 acts downstream of insulin, TGF-β, and cGMP signaling to regulate dauer formation. 27,28 Our findings provide strong biochemical evidence that these signaling pathways regulate DAF-12 activity by down-regulating the synthesis of DA.

Further supporting the conclusion that these mutants form dauers because of greatly reduced DA levels, supplementation of exogenous DA rescued the Daf-c phenotype of daf-2(e1368), daf-2(e1370), daf-7(ok3125), and daf-11(ks67) (Figure 4C). The rescued mutants mostly grew into adults except for the daf-2(e1370) mutant, which arrested as L3s. These results are consistent with a previous report 2 and suggest that the e1370 allele causes additional defects that disrupt the development from L3 to L4. We conclude that the DA hormone is required for the reproductive development of C. elegans.

CONCLUSIONS

In this study, we developed a simple and sensitive method for DA quantification that requires relatively small amounts of worms (Table 2). Using derivatization and HR-SIM, we measured the C. elegans DA levels in different developmental stages and different Daf-c mutants. Our data provide direct biochemical evidence that during the L2 stage, DA is highly elevated to ensure the reproductive development of C. elegans. This method is applicable to studies of the DA synthesis pathway and the biological processes regulated by DA and DAF-12. It may also be used for the quantification of other small molecules with carboxyl groups.
Table 2. Recommended Amounts of C. elegans Samples for DA Quantitation

<table>
<thead>
<tr>
<th></th>
<th>L2</th>
<th>adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10^5 worms/15-cm plate</td>
<td>packed worms</td>
<td>total proteins</td>
</tr>
<tr>
<td></td>
<td>100 μL</td>
<td>5 mg</td>
</tr>
<tr>
<td>SRM</td>
<td>1 plate</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>1/10 SIM</td>
<td></td>
</tr>
</tbody>
</table>

*Estimated, may be used if only L2 samples are to be compared, otherwise 100 μL of packed worms should be collected for each sample. For comparison between different methods, ~1 mL of packed worms were used for each sample. We verified that 100 μL of packed worms worked just as well if the amount of extraction and derivatization reagents were reduced proportionally. *Below limit of quantification because of poor S/N.

# ASSOCIATED CONTENT

## Supporting Information
Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

### Corresponding Authors
*E-mail: leixiaoguang@nibs.ac.cn. Fax: 86-10-80704785.*
*E-mail: dongmengqiu@nibs.ac.cn. Fax: 86-10-80706053.*

### Notes
The authors declare no competing financial interest.

## ACKNOWLEDGMENTS
We thank Mei-Jun Zhang (NIBS, Beijing) for help with statistical analyses, Dr. Hans-Joachim Knölker (Technische Universität Dresden, Germany) for providing (25S)-Δ4- and (25R)-Δ4-dafachronic acid, and Dr. Adam Antebi (Max Planck Institute for Biology of Ageing, Cologne, Germany) for providing daf-9(Δ9Δ);daf-12(Δ61h411) and daf-9(Δ9Δ);ΔEx24. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (Grant P40 OD010440). The images of C. elegans life history are provided by Wormatlas (http://www.wormatlas.org). The Ministry of Science and Technology of China (973 Grants 2010CB835203 to M.D. and 2012CB837400 to X.L.), National Scientific Instrumentation Grant Program (2011YQ0900056 to M.D.), and the municipal government of Beijing funded this work.

## REFERENCES