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# DAF-12-dependent rescue of dauer formation in *Caenorhabditis elegans* by (25S)-cholestenoic acid

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## Summary

**Population density, temperature and food availability all regulate the formation of the *Caenorhabditis elegans* dauer larva by modulating endocrine signaling pathways. The orphan nuclear receptor DAF-12 is pivotal for the decision to form a dauer or to undergo normal reproductive development. The DAF-12 ligand has been predicted to be a sterol that is metabolized by DAF-9, a cytochrome P450. Here we chemically characterize purified lipophilic nematode extracts and show that the ligand for DAF-12 contains a carboxyl moiety and is likely to be derived from a sterol. Using a candidate ligand approach we find that the C27 bile acid cholestenoic acid (5-cholesten-3β-ol-(25S)-carboxylic acid) promotes reproductive growth in dauer-constitutive mutants in a *daf-9*- and *daf-12*-dependent manner. Furthermore, we find that cholestenoic acid can act as a DAF-12 ligand by activating DAF-12 in a cell-based transcription assay. Analysis of dauer-rescuing lipophilic extracts from nematodes by gas chromatography–mass spectrometry indicates the presence of several regioisomers of cholestenoic acid that are distinct from $\Delta^5$-cholestenoic acid and are not present in extracts from *daf-9* mutants. These data suggest that carboxylated sterols may be key determinants of life history.**

**Key words: *C. elegans*; DAF-12; dauer; ligand; lipid; nuclear receptor.**

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## Introduction

A number of different genetic and developmental manipulations have implicated endocrine factors as major determinants of the lifespan of the nematode *Caenorhabditis elegans* (Kenyon, 2005). The orphan nuclear receptor DAF-12 lies at the convergence of an insulin and a transforming growth factor β (TGFβ) signaling pathway to direct programs of reproductive growth or entry into diapause (Antebi *et al*., 1998, 2000; Gerisch & Antebi, 2004; Mak & Ruvkun, 2004). DAF-12 also plays a complex role in the aging process. Most *daf-12* mutations either shorten lifespan or have no effect (Larsen *et al*., 1995; Gems *et al*., 1998), whilst others lead to increased lifespan (Fisher & Lithgow, 2006). *daf-12* mutants also interact with long-lived *daf-2* mutants to either suppress lifespan extension (class 1 *daf-2* mutants) or synergistically increase lifespan (class 2 *daf-2* mutants) (Larsen *et al*., 1995; Gems *et al*., 1998). A secondary endocrine system involving a lipophilic hormone has been proposed to link the transcriptional outputs of DAF-12 with the upstream signaling pathways through the cytochrome P450 DAF-9 (Tatar *et al*., 2003). The identity of the endogenous ligand produced by DAF-9 to act on DAF-12 has been elusive, but would provide an excellent tool to help further understand the mechanism of development, aging, and lifespan extension in *C. elegans*.

Under conditions of poor nutrition or overcrowding the nematode *C. elegans* is able to enter an alternate developmental stage in which the animal is nonfeeding, nonreproducing and stress resistant (Riddle, 1988). A genetic pathway for dauer formation has been defined by mutations causing inappropriate dauer formation (dauer constitutive – Daf-c) or failure to form dauers (dauer defective – Daf-d). Some Daf genes encode components of the insulin and TGFβ signaling pathways, which transduce environmental signals from sensory neurons to mediate the decision to proceed with normal reproductive growth or initiate dauer formation (Kimura *et al*., 1997; Riddle & Albert, 1997; Patterson & Padgett, 2000; Tatar *et al*., 2003). Most *daf-12* mutants are Daf-d and suppress dauer formation in Daf-c mutants from both the insulin and TGFβ signaling pathways indicating that DAF-12 occupies the terminal position in the dauer formation signaling pathway (Riddle & Albert, 1997).

A number of lines of evidence support the notion that the endogenous DAF-12 ligand is a steroid derived from cholesterol. Under laboratory conditions *C. elegans* must be supplemented with exogenous cholesterol since nematodes are unable to synthesize their own sterols (Chitwood, 1999). Cholesterol deprivation in wild-type animals leads to a variety of phenotypes including developmental arrest and reduced fertility (Shim *et al*., 2002; Merris *et al*., 2003). Additionally, weak alleles of *daf-9* that are thought to be compromised in their ability to synthesize the

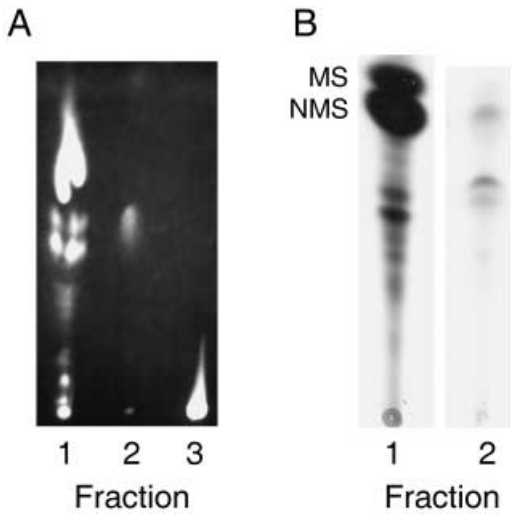



**Fig. 1** Thin-layer chromatography (TLC) of lipophilic extracts fractionated on an aminopropyl column. (A) Ether extract from *daf-12(m20)* worms. Fraction 1, neutral lipids; Fraction 2, fatty acids and carboxylated lipids; and Fraction 3, polar lipids. (B) Ether extract derived from *daf-12(m20)* worms grown on [4–$^{14}$C] cholesterol. Fraction 1, neutral lipids (MS = methylated sterols, NMS = nonmethylated sterols). Fraction 2, carboxylated sterols.

DAF-12 ligand often show an enhanced dauer phenotype under cholesterol deprivation (Gerisch *et al*., 2001; Jia *et al*., 2002). Finally, developmental delay is observed in *ncr-1* and *ncr-2* mutants in which lysosomal transport of sterols is compromised, while constitutive dauer formation occurs in *ncr-1;ncr-2* double mutants (Sym *et al*., 2000; Li *et al*., 2004).

Further evidence for the importance of sterols in development comes from the observation that sterol-deprived worms grown in the presence of a methylated sterol, lophenol, arrested as dauer larvae in a *daf-12*-dependent manner (Matyash *et al*., 2004). These lophenol-induced dauers could be rescued by the addition of exogenous cholesterol or by the addition of a nematode lipid extract, termed *gamravali* (Matyash *et al*., 2004). It was suggested that this extract contained a candidate DAF-12 ligand that was thought to be a polyhydroxylated sterol (Matyash *et al*., 2004). However, this extract was unable to rescue dauer formation in *daf-9* mutants as would be expected from a DAF-12 ligand (Entchev & Kurzchalia, 2005).

Nuclear receptors have undergone a tremendous expansion in *C. elegans* to 284 putative receptors compared to the 48 found in humans (Sluder *et al*., 1999). Some of these receptors are involved in neuronal development (Qin & Powell-Coffman, 2004), larval molting (Kostrouchova *et al*., 2001), and lipid metabolism (Van Gilst *et al*., 2005a,b). However, like all nuclear receptors in *C. elegans*, DAF-12 remains an orphan receptor. In order to identify the DAF-12 ligand we have previously isolated a nematode-derived lipophilic extract that possesses the activity predicted of a hormone produced by DAF-9 and acting on DAF-12 (Gill *et al*., 2004). This extract was able to rescue the dauer arrest of Daf-c mutants from the insulin and TGFβ signaling pathways as well as *daf-9* mutants, but did not alter the phenotype of a ligand insensitive *daf-12* mutant. Here we describe the chemical characterization of bioactivity in this extract and find that sterol acids exist in worms. Furthermore, we show that the sterol acid (25S)-cholestenoic acid can function as a DAF-12 ligand.

## Results

### Chemical characterization of lipophilic extracts

To characterize the lipid classes in dauer-rescuing lipophilic extracts we generated ether extracts from *daf-12(m20)* worms and performed fractionation on an aminopropyl bonded solid phase column. Neutral lipids, fatty acids and polar lipids were separated and eluted by changing the polarity and pH of the eluting solvent (Kaluzny *et al*., 1985). Thin layer chromatography (TLC) of an aliquot of these fractions indicated good separation between the fractions and their respective lipid classes (Fig. 1A). When fractions were tested for their ability to prevent dauer formation in the Daf-c mutant *daf-2(e1368)*, bioactivity was found to elute in the fraction corresponding to carboxyl-containing compounds. Methyl esterification of the lipophilic extracts also eliminated the bioactivity of the extract, confirming the importance of the carboxyl group (data not shown). Derivatization of extracts using acetic anhydride/pyridine, which acetylates hydroxyls and amines, also resulted in a loss of dauer-rescuing bioactivity (data not shown). However, an amine-specific reagent, disuccinimidylsuberate, had no effect on bioactivity, indicating that a hydroxyl moiety is also important for the activity of the lipophilic extract.

In order to determine if the bioactivity in this carboxyl-containing fraction could be derived from a sterol we fractionated nematode extracts derived from *daf-12(m20)* worms grown in the presence of $^{14}$C labeled cholesterol. TLC of these radio-labeled fractions indicated that the majority of radioactivity eluted in the neutral lipid fraction with major bands corresponding to methylated and nonmethylated sterols (Fig. 1B). However, a number of bands were also visible in the fraction containing carboxylated lipids indicating that worms are capable of adding a carboxyl group to a cholesterol precursor (Fig. 1B). This suggests that the bioactive component of the dauer-rescuing extracts could be a modified sterol containing a carboxylic acid group.

### Compound screens identify cholestenoic acid as a candidate DAF-12 ligand

Based on these observations we then took a candidate ligand approach and screened a number of sterol acids for their ability to rescue dauer formation in *daf-2(1368)* mutants. We found that none of the common C24 bile acids (cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid or $\Delta^5$-cholenic acid) were able to rescue the Daf-c phenotype of *daf-2(1368)* at 25 °C (data not shown). In contrast we found that (25S)-cholestenoic acid (5-cholesten-3β-ol-(25S)-carboxylic acid) was able to completely rescue the Daf-c phenotype of *daf-2(e1368)*,

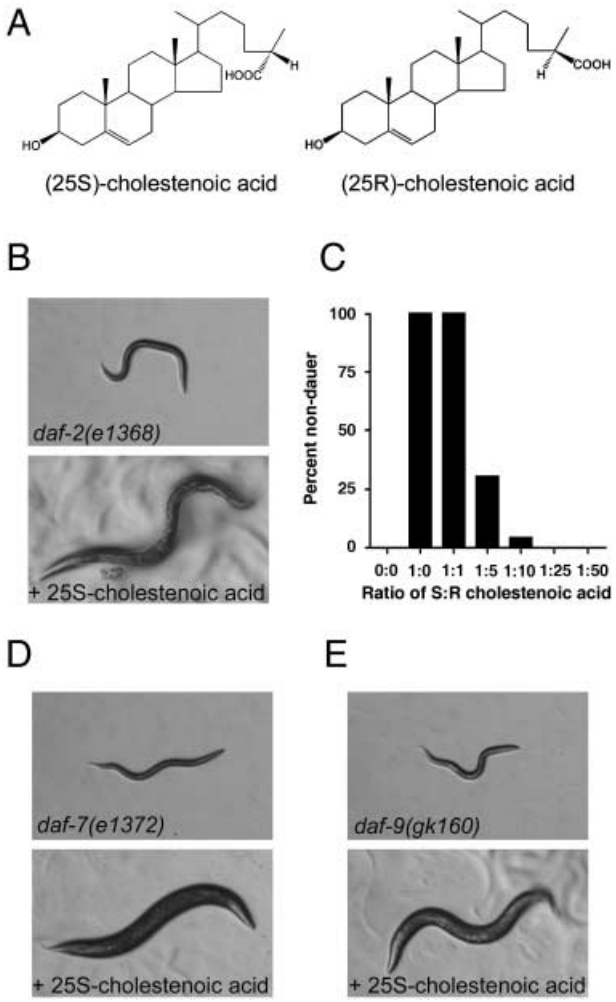



**Fig. 2** (25S)-cholestenoic acid rescues dauer formation in Daf-c mutants. (A) Structures of (25S) and (25R)-cholestenoic acid. (B) (25S)-cholestenoic acid prevents dauer formation in *daf-2(e1368)* mutants at 25 °C. (C) (25R)-cholestenoic acid competitively inhibits the ability of (25S)-cholestenoic rescue dauer formation in *daf-2(e1368)* mutants. (D) (25S)-cholestenoic acid rescues dauer formation in *daf-7(e1372)* mutants at 25 °C. (E) (25S)-cholestenoic acid rescues dauer formation in *daf-9(gk160)* mutants at 20 °C.

while the 25R stereoisomer had no effect (Fig. 2A,B and Table 1). Furthermore, in co-incubation experiments in which *daf-2(e1368)* animals were treated with 1 μM (25S)-cholestenoic acid, a dose sufficient to completely rescue the Daf-c phenotype, we found that excess (25R)-cholestenoic acid could abolish the rescue by the 25S diastereomer (Fig. 2C). This suggests that a high degree of selectivity is involved in this process.

(25S)-cholestenoic acid was also able to prevent dauer formation in other insulin signaling pathway mutants including the class 2 insulin receptor mutant *daf-2(1370)* and *age-1(hx546)* (encoding a phosphatidylinositol 3 kinase catalytic subunit) (Table 1). Rescue of dauer formation was also observed for mutants in the TGFβ pathway including *daf-7(e1372)* (Fig. 2D, Table 1) and in the TGFβ-like receptor mutant *daf-4(m63)* (Table 1). In addition, we found that (25S)-cholestenoic acid was able to rescue dauer formation in *daf-9(gk160)* mutants

**Table 1** Percentage dauer rescue of Daf-c mutants with (25S)-cholestenoic acid treatment

| Genotype | Temperature (°C) | Concentration of S-cholestenoic acid 0 | 1 μM | 5 μM | 10 μM | 25 μM | *N* |
|---|---|---|---|---|---|---|---|
| *daf-2(e1368)* | 25 | 0 | 94 | 99 | 100 | 100 | 727 |
| *daf-2(e1370)** | 25 | 0 | 100 | 100 | 100 | 100 | 624 |
| *age-1(hx546)*† | 27 | 0 | 66 | 99 | 100 | 100 | 1031 |
| *daf-7(e1372)* | 25 | 0 | 70 | 96 | 90 | 98 | 523 |
| *daf-4(m63)* | 25 | 0 | 33 | 47 | 93 | 95 | 596 |
| *daf-9(gk160)*‡ | 20 | 8 | 78 | 96 | 90 | 94 | 495 |
| *daf-12(rh273)*§ | 20 | 56 | 66 | 55 | 70 | 57 | 379 |

Worms were scored as dauer larvae or nondauer animals. Results are presented as percentage. *N* indicates number of animals scored over three or more independent assays.

*Nondauer animals developed into dark L3/L4 larvae similar to *daf-2(e1370); daf-12(m20)* double mutants described by Gems *et al*. (1998)

†Scored after 4 days, nondauer animals developed into L4 larvae and sterile young adults.

‡Partial dauers were transferred onto plates and scored 2 days later. Nondauer animals included L3 and L4 larvae and gravid adults. A fraction of animals of L3/L4 animals exhibited abnormal morphology such as vulval protrusion, often accompanied by a distended gut, and some demonstrated a moulting defect whereby the old cuticle was incompletely shed.

§Worms were scored after 5 days and the fraction developing into young and gravid adults was scored.

(Fig. 2E, Table 1) and importantly did not alter the phenotype of *daf-12(rh273)* mutants that are predicted to be ligand insensitive (Antebi *et al*., 2000) (Table 1). Taken together, these data demonstrate that (25S)-cholestenoic acid fulfills the genetic criteria required for the activity of a DAF-12 ligand.

## Cholestenoic acid interacts with DAF-12 in a luciferase reporter assay

To determine whether (25S)-cholestenoic acid could directly regulate the activity of DAF-12 we used a cell-based luciferase reporter assay. HEK 293T cells were cotransfected with a DAF-12 expression vector consisting of the DAF-12 hinge and ligand binding domains (DAF-12 H + LBD) fused to the yeast GAL4 DNA binding domain (DBD) and the pGL2 luciferase reporter construct containing the GAL4 upstream activating sequence (UAS). When transfected cells were incubated with cholestenoic acid we observed a dose-dependent increase in luciferase expression in the presence of the 25S stereoisomer but no change in luciferase with the 25R stereoisomer (Fig. 3A). Cells transfected with the luciferase reporter alone or the GAL4 DBD plus reporter did not respond to (25S)-cholestenoic acid (data not shown). Similarly cells expressing a mutant DAF-12 fusion protein (DAF-12 R564H H + LBD), which corresponds to the molecular defect in the ligand-insensitive mutant strain *daf-12(rh273)*, showed no response to either (25S) or (25R)-cholestenoic acid (Fig. 3B). Western blot analysis confirmed that the lack of transactivation through the mutant receptor was not simply due to a failure to express the mutant protein (data not shown).

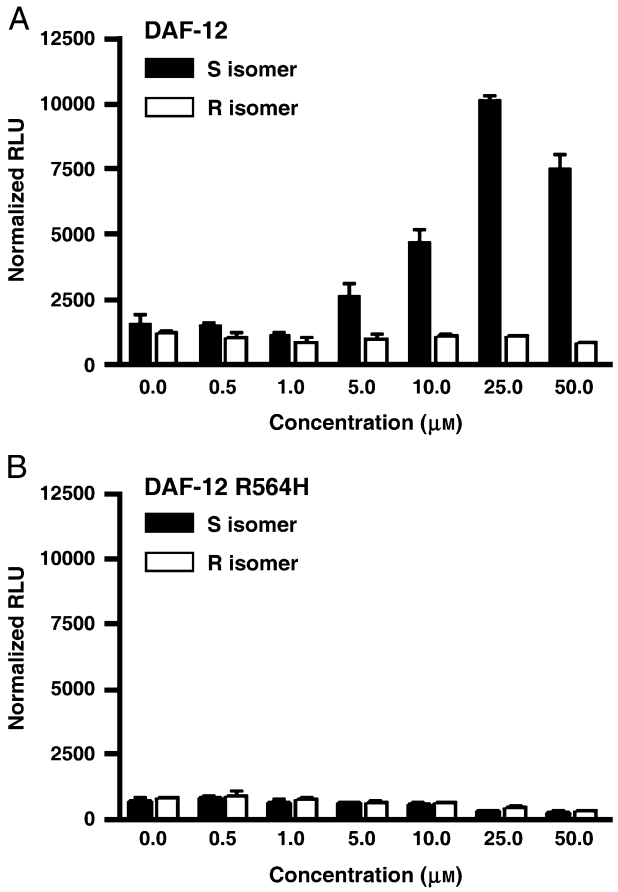



**Fig. 3** (25S)-cholestenoic acid activates wild-type DAF-12 but not the DAF-12 (R564H) mutant in a cell based reporter assay. HEK293 cells were transfected with a DAF-12 (A) or ligand insensitive DAF-12 (R564H) mutant (B) expression vector and treated with (25S) and (25R)-cholestenoic acid.

### Lipophilic extracts contain isomers of cholestenoic acid

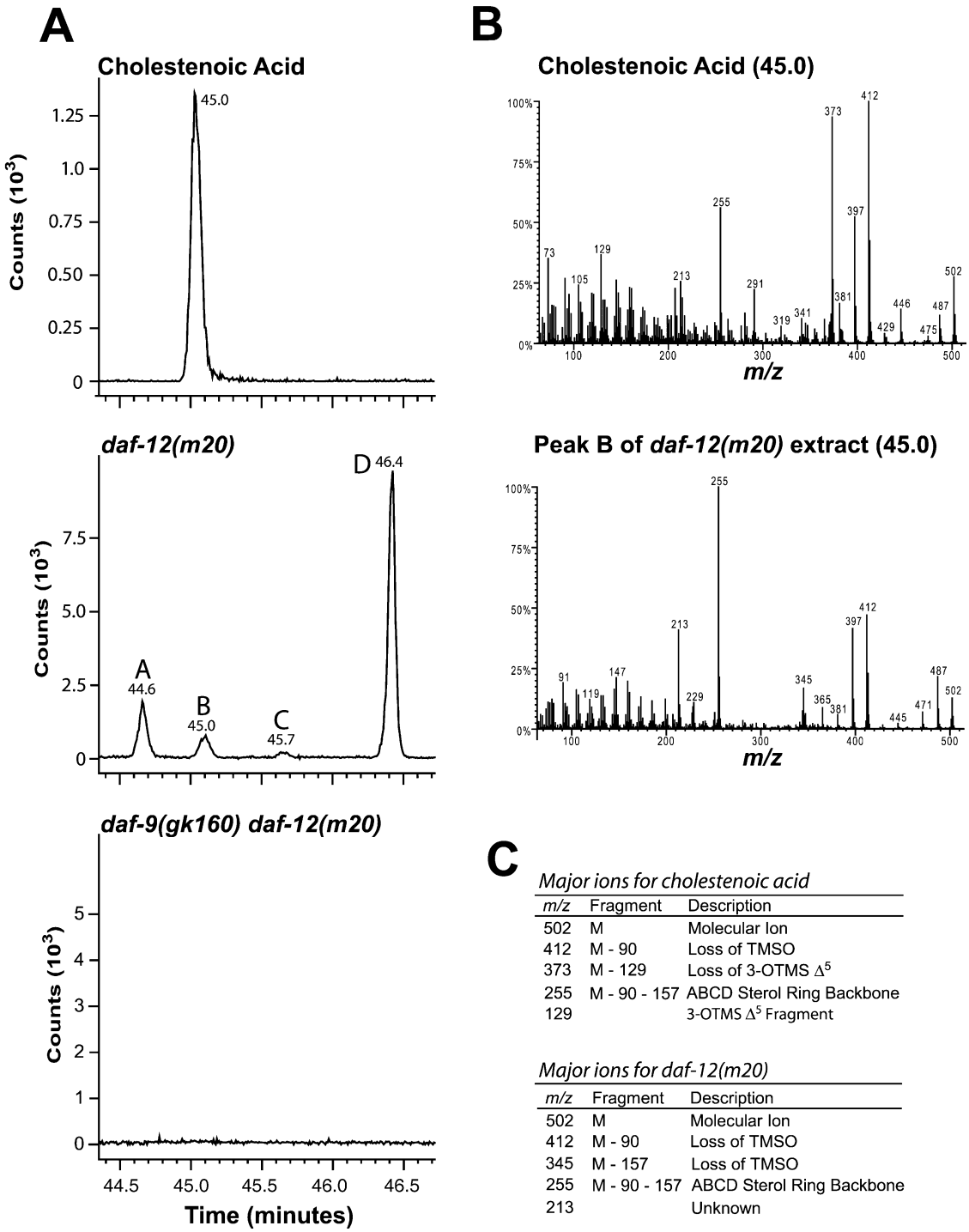
Given that (25S)-cholestenoic acid was able to rescue dauer formation and act as a DAF-12 ligand *in vitro*, we examined our lipophilic worm extracts to determine the existence of cholestenoic acid or related structures. We hypothesized that the sterol acid should be present in bioactive *daf-12(m20)* extracts but absent in *daf-9(gk160) daf-12(m20)* extracts that lack bioactivity. Using gas chromatography–mass spectrometry (GC-MS) we compared the double-derivatized (esterified and silylated) cholestenoic acid standard (5-cholesten-3β-ol-(25S)-carboxylic acid) with aminopropyl fractionated *daf-12(m20)* bioactive extracts and *daf-9(gk160) daf-12(m20)* extracts. The derivatized cholestenoic acid standard eluted at 45.0 min (Fig. 4A) with a base peak of *m/z* 412 (Fig. 4B). An extracted ion chromatogram (XIC) of *m/z* 412 reveals several peaks in the *daf-12(m20)* extract (at 44.6, 45.0, 45.7, and 46.4 min) but not even trace levels in *daf-9(gk160) daf-12(m20)* extracts (Fig. 4A). One of the peaks (B) in the *daf-12(m20)* extract has the same retention time as cholesteneoic acid (45.0 min), but does not have the same fragmentation pattern as the cholestenoic acid standard (Fig. 4B). The other *m/z* 412 XIC peaks have slight variations in fragmentation patterns from the *daf-12(m20)* extract peak B (data not shown), but they all lack the characteristic fragment ions of a sterol or bile acid with a $\Delta^5$ double bond (Fig. 4C). However, since the extract spectra share the molecular ion (*m/z* 502), loss of a single TMS-hydroxyl (*m/z* 412), and a sterol ring nucleus characteristic of having a single double bond (*m/z* 255) these compounds are very likely to be C27 mono-hydroxylated, monounsaturated cholestenoic acid regioisomers. However, $\Delta^5$-cholestenoic acid was not seen in the extracts suggesting that this structure may not be an endogenous ligand.

## Discussion

The identity of the endogenous ligand for the nuclear receptor DAF-12 has been the focus of a great deal of attention due to the role of the receptor in the regulation of dauer formation as well as the determination of adult lifespan (Tatar *et al.*, 2003). In this study we have determined that (25S)-cholestenoic acid acts as a DAF-12 ligand and that related structures exist in worms. Therefore, this class of chemicals may act as ligands *in vivo*.

### 25S-cholestenoic acid rescues dauer formation

We have previously identified worm extracts that were able to rescue dauer formation in Daf-c mutants and hypothesized that this was due to the presence of a DAF-12 ligand (Gill *et al.*, 2004). We have now performed further characterization to identify the chemical nature of this ligand. Based on amino-propyl fractionation we determined that the dauer-rescuing bioactivity eluted in the fraction containing carboxylated lipids and that worms are able to add carboxyl groups to a cholesterol precursor. This led us to consider bile acids as candidate ligands. The major biosynthetic pathway for bile acids in mammals involves a neutral pathway whereby modifications are made to the sterol backbone of cholesterol first, followed by oxidation and shortening of the alkyl chain (Chiang, 2002). However, none of the common C24 bile acids were able to rescue dauer formation in *daf-2(e1368)* worms. In contrast, the C27 bile acid (25S)-cholestenoic acid potently rescued dauer formation in *daf-2(e1368)* mutants as well as other Daf-c mutants from both the insulin-signaling and TGFβ-signaling pathways. In mammals, an alternate bile acid synthetic pathway involves the enzyme CYP27A1, which converts unmodified cholesterol to cholestenoic acid, with only 27-hydroxycholesterol as an intermediate (Chiang, 2002). Cholestenoic acid can be then further modified into the C24 monohydroxy bile acid cholenic acid (Javitt, 2000). In worms, it is possible that *daf-9*, a gene encoding a homolog of a sterol hydroxylase (Gerisch *et al.*, 2001; Jia *et al.*, 2002), may be involved in carboxylating a sterol substrate in a manner similar to CYP27A1 in worms. Interestingly we found that $\Delta^5$-cholenic acid had no effect on dauer rescue in *daf-2(e1368)* mutants (data not shown) suggesting that the extended alkyl chain is critical for DAF-12 binding.

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*Major ions for cholestenoic acid*

| *m/z* | Fragment | Description |
|---|---|---|
| 502 | M | Molecular Ion |
| 412 | M - 90 | Loss of TMSO |
| 373 | M - 129 | Loss of 3-OTMS $\Delta^5$ |
| 255 | M - 90 - 157 | ABCD Sterol Ring Backbone |
| 129 | | 3-OTMS $\Delta^5$ Fragment |

*Major ions for daf-12(m20)*

| *m/z* | Fragment | Description |
|---|---|---|
| 502 | M | Molecular Ion |
| 412 | M - 90 | Loss of TMSO |
| 345 | M - 157 | Loss of TMSO |
| 255 | M - 90 - 157 | ABCD Sterol Ring Backbone |
| 213 | | Unknown |

**Fig. 4** GC-MS identifies several cholestenoic acid isomers in worm extracts. (A) GC-MS extracted ion (*m/z* 412) chromatograms of cholestenoic acid standard and aminopropyl fractionated extracts of *daf-12(m20)* and *daf-9(gk160) daf-12(m20)*. (B) Comparison of the electron impact spectra of cholestenoic acid standard and peak B from the *daf-12(m20)* extract. (C) Summary of the major fragment ions for cholestenoic acid and *daf-12(m20)* mass spectra.

### 25S-cholestenoic acid transactivates DAF-12

Cholestenoic acid has been shown to be a naturally occurring ligand for the mammalian liver X receptor (Song & Liao, 2000), a nuclear receptor that is involved in cholesterol homeostasis, and based on sequence similarity is the closest mammalian homolog of DAF-12 (Mooijaart *et al.*, 2005). Our dauer rescue data suggest that cholestenoic acid can act as ligand for DAF-12. This is based on our chemical modification data in which methyl esterification of carboxyls completely abolished bioactivity

of extracts. Consistent with this was the observation that (25S)-cholestenoic acid could transactivate DAF-12 in a cell-based reporter assay. Furthermore, we have tested the liver X receptor oxysterol ligands (22R), 25, and 26-hydroxycholesterol (Janowski *et al*., 1996) and find that they do not rescue dauer formation in *daf-2(e1368)* (data not shown).

### Cholestenoic acid regioisomers exist in worms

Using GC-MS we subsequently detected the presence of several cholestenoic acid isomers or related structures in bioactive *daf-12(m20)* extracts that were not present in *daf-9(gk160) daf-12(m20)* extracts. The absence of these cholestenoic acid structures in *daf-9(–)* extracts suggests that these compounds could be products of DAF-9 enzymatic activity. The differences in retention time in conjunction with the similarity of the mass spectra suggest that these sterol acids are regioisomers. The most consistent peak is at the same retention time as the cholestenoic acid standard and shares the major ions of *m/z* 502, 412, and 255 which are typical of C27 monohydroxylated, monounsaturated bile acids (Setchell *et al*., 1998). However, the endogenous spectra are missing the major fragments at *m/z* 373, 291, and 129 which are characteristic of a $\Delta^5$ double bond (Setchell *et al*., 1998), suggesting that this sterol acid isomer is absent in worm extracts. The different retention times are consistent with positional changes in the location of the double bond in the ABCD ring. Identification and complete characterization of these cholestenoic acid isomers will require *de novo* synthesis of each possible unsaturation position as well as nuclear magnetic resonance spectroscopy. However, our GC-MS data is wholly consistent with the presence of C27 monounsaturated, monohydroxylated bile acids in the bioactive lipid fraction that are distinct from $\Delta^5$-cholestenoic acid. These nematode sterol acids are therefore prime candidates for endogenous DAF-12 ligands.

### Sterol metabolism in *Caenorhabditis elegans*

*Caenorhabditis elegans* requires sterols for development and it is unable to synthesize cholesterol or other sterols. Sterol deprivation leads to a number of developmental defects including growth arrest and diminished fertility (Matyash *et al*., 2001, 2004; Merris *et al*., 2003). Worms are able to utilize a number of different dietary sterols and it has been shown that the major sterol component of worms is 7-dehydrocholesterol, which can then be converted into lathosterol and the 4-methyl sterol lophenol (Chitwood, 1999). Sterol deprivation studies have suggested that lophenol as the sole sterol source cannot support reproductive growth indicating that this sterol is unlikely to be a precursor for sterol hormones in the worm (Matyash *et al*., 2004). In contrast, supplementation with lathosterol, but not cholesterol or 7-dehydrocholesterol, was able to rescue dauer formation in *daf-2(e1368)* but not *daf-9(gk160)* mutants (M. Gill, unpublished data) suggesting that lathosterol could be a precursor for Δ7-sterol acids in the worm.

### Is DAF-12 a sterol sensor?

Recent bioinformatic evidence suggests that DAF-12 is homologous to the mammalian liver X receptor (Mooijaart *et al*., 2005), which acts as a cholesterol sensor and binds oxidized derivatives of cholesterol (Janowski *et al*., 1996; Lehmann *et al*., 1997). Sensing of levels of cholesterol or a sterol metabolite by DAF-12 could provide a homeostatic mechanism by which programs of reproductive growth would be initiated only when the sterol availability was appropriate to support such growth. Conversely, under conditions of limited sterol availability such as population overcrowding and starvation, the absence of cholestenoic acid-like molecules would lead to DAF-12 promoting programs of dauer arrest. DAF-12 ligand synthesis is thought to take place in a pair of neuron-like cells in the head called the XXXL/R cells (Gerisch *et al*., 2001; Gerisch & Antebi, 2004; Mak & Ruvkun, 2004). During early development these cells are the principal site of expression of *daf-9* and are thought to act as neuroendocrine cells. Although the intestine is probably responsible for the majority of cholesterol uptake in the worm, cholesterol is also found in the amphid socket cells, the main chemosensory organ in the worm (Merris *et al*., 2003). Thus uptake of cholesterol by the amphid sensilla may provide a direct route by which cholesterol can reach the XXX cells, whereupon it can be metabolized into the DAF-12 ligand. The ability of worms to utilize a number of different dietary sterols supports the idea that multiple isomeric DAF-12 ligands exist.

## Conclusion

The identification of (25S)-cholestenoic acid as a ligand for the nuclear receptor DAF-12 prompts new approaches to delineating the role of this receptor in the regulation of life history and the determination of lifespan. The biochemistry of worms has only recently begun to be explored, and perhaps this finding will help in our understanding of how complex processes such as aging are regulated by small molecule endocrine effectors.

## Note added in proof

Sterol acids have also been identified as DAF-12 ligands by Motola *et al*. (2006).

## Experimental procedures

### Materials

5-cholesten-3β-ol-(25S)-carboxylic acid, 5-cholesten-3β-ol-(25R)-carboxylic acid (25R) and cholenic acid were purchased from Steraloids (Newport, RI, USA). 4–$^{14}$C-cholesterol was purchased from PerkinElmer (Wellesley, MA, USA). All other reagents and solvents were of the highest grade available.

### Nematode strains

The following nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health's National Center for Research Resources (NCRR): Bristol N2 (wild-type), DR1572[*daf-2(e1368) III*], CB1370[*daf-2(e1370) III*], TJ1052[*age-1(hx546) II*], CB1372[*daf-7(e1372) III*], DR63[*daf-4(m63) III*], VC305*[+/szT1[lon-2(e678)] I; daf-9(gk160)/szT1 X*], DR20[*daf-12(m20) X*], AA87[*daf-12 (rh273) X*]. GL216[*daf-9(gk160) daf-12(m20) X*] was constructed as previously described (Gill *et al*., 2004).

### Nematode culture and life history analysis

For routine culture worms were maintained at 20 °C on 5 cm nematode growth medium (NGM) agar plates carrying a lawn of *Escherichia coli* OP50. Dauer-formation assays were carried out as previously described (Gill *et al*., 2004). Lipophilic extracts were generated as previously described (Gill *et al*., 2004). Cholestenoic acid was resuspended in ethanol to a concentration of 20 mM. For dauer assays, 15 μL of a stock solution was added to 185 μL S-basal before being spotted onto a 3-cm Petri dish containing 3 mL NGM and 50 μL *E. coli*. The S-basal was allowed to cover the whole surface of the plate and left for 1 h prior to adding worms.

### Fractionation of lipophilic extracts on aminopropyl solid phase columns

Lipophilic extracts were dried under nitrogen and resuspended in chloroform. Fractionation was performed by the method of Kaluzny *et al*. (1985) on 500 mg aminopropyl cartridges (Supelco, Bellefonte, PA, USA). The column was equilibrated with hexane and extracts were applied to the column in 5 mL chloroform. Neutral lipids were eluted in 5 mL 2 : 1 (v:v) chloroform:isopropanol (Fraction 1), carboxyl containing groups were eluted with 5 mL 2% acetic acid in diethyl ether (v:v) (Fraction 2), and polar lipids were eluted with 2 mL methanol (Fraction 3). Thin layer chromatography samples (10 μL) were spotted onto high-performance silica TLC plates (Analtech Inc., Newark, DE, USA) and resolved with toluene:ethyl acetate:trimethyl borate 100 : 20 : 7.2 (v:v:v) (Pollack *et al*., 1971). Samples were dried with a hairdryer for 5 min, sprayed with primulin dye, and visualized with UV light (White *et al*., 1998).

### Generation of lipophilic extracts from worms grown on [4–$^{14}$C] cholesterol

A synchronous mass culture of worms was obtained by hypochlorite treatment of gravid adults and grown in 100 mL of S-media + *E. coli* OP50 supplemented with 10 μCi [4–$^{14}$C] cholesterol for 48 h at 25 °C. Worms were extracted as described by Merris *et al*. (2004) without the addition of NaOH, followed by aminopropyl SPE separation as described above.

### Plasmid construction

BS FLAG DAF-12 was constructed as follows. A full length (FL) DAF-12 cDNA was PCR amplified from worm cDNA using nested PCR (sequences available upon request). The final round of PCR added an amino terminal FLAG tag (N-Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C). The resulting PCR fragment was cloned into BS II SK + (Stratagene Corp., San Diego, CA, USA) as a KpnI-SacI fragment. The resulting cDNA was sequenced to verify sequence and identity. BS FLAG DAF-12 R564H was constructed as described above using a PCR fragment amplified from *daf-12(rh273)* cDNA. The wild-type and mutant DAF-12 hinge and ligand binding domain (H + LBD, amino acids 198–753) were PCR amplified from BS FLAG DAF-12 and BS FLAG DAF-12 R564H, respectively, to generate PCR fragments containing BamH1 and Not1 restriction sites at the 5′ and 3′ ends, respectively (primer sequences available upon request). These PCR fragments were cloned into pBIND (Promega Corp., Madison, WI, USA) as BamH1-Not1 fragments and sequenced to verify the sequences. The 5X Gal4 Promoter (pGL2 UAS vector) and the pCMV B-gal (BGal plasmid) have been described previously (Catron *et al*., 1995; Fisher *et al*., 1996).

### Transfection assay

HEK 293T cells were transfected and treated with cholestenoic acid according to a modification of the method of Song & Liao (2000). One day before transfection HEK 293T cells were seeded in a 24-well plate at a density of $4 \times 10^5$ cells well$^{-1}$ mL$^{-1}$ in 1 mL Dulbecco's Modified Eagle's Medium (DMEM, Mediatech, Inc., Herndon VA, USA) +10% fetal bovine serum (Mediatech, Inc.). The following day the medium was removed and replaced with 400 μL serum-free DMEM and the cells were transiently transfected using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA). 0.45 μg pBIND DAF-12 H + LBD, 0.45 μg pGL2 UAS and 0.1 μg pCMV β-galactosidase vectors were incubated with 2 μL Lipofectamine 2000 reagent before being added to each well in 100 μL DMEM. Transfected cells were incubated at 37 °C in a $CO_2$ incubator for 6 h before adding 500 μL DMEM + FBS. After incubation overnight the medium was aspirated and replaced with 1 mL DMEM +10% charcoal-stripped fetal bovine serum (Hyclone, Logan UT, USA). Treatments were added in 5 μL ethanol in triplicate. Following a further 24 h incubation cells were washed with PBS and lysed with 100 μL Passive Lysis Buffer (Promega Corp.). Luciferase levels were assayed using the Promega Luciferase Assay Kit and a Turner 20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA) and were normalized to β-galactosidase levels. Results were expressed as mean ± standard deviation for triplicate assays.

### GC-MS analysis

Worms were extracted and aminopropyl solid-phase fractionated as described above. For derivatization, samples were dried completely under $N_2$ and methyl esterified using 200 μL $BF_3$

+10% (w/w) methanol (Supelco) for 1.5 h at 100 °C. Samples were cooled and 200 μL water was added followed by two extractions with 600 μL hexane. The hexane was dried down thoroughly under $N_2$ and silylated with 250 μL BSTFA + TMCS, 99 : 1 (Supelco), at 75 °C for 2.5 h. Just before GC-MS analysis, samples were dried under $N_2$ and resuspended in 1 μL hexane for injection. GC-MS analysis was on a Varian 2100T ion-trap with a 3900 GC (Varian Inc., Walnut Creek, CA, USA) operating in splitless mode with a VF-5 ms capillary column (30 m × 0.25 mm i.d., 5% phenyl-95% methyl polysiloxine, 0.25 μm film thickness; Varian, Inc.). GC conditions: Injector was at 300 °C and pressurized to 40 p.s.i. for 0.8 min at the time of injection. Initial column temperature was 180 °C for 3 min and then ramped at 2.8 °C per min to 300 °C and held for 18 min. MS conditions: Electron impact ionization, scan time 0.53 s, and mass range 30–650 *m/z*.

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