NOVEL NUCLEAR METHYLATION OF STEROLS BY THE NEMATODE CAENORHABDITIS ELEGANS

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ABSTRACT

Caenorhabditis elegans possesses a unique sterol methylation pathway not reported to occur in any other organism and also removes the C-24 ethyl group of sitosterol (a plant sterol). This nematode produced substantial quantities of 4α-methyl-5α-cholest-8(14)-en-3β-ol and smaller amounts of lophenol from dietary cholesterol, desmosterol or sitosterol. When C. elegans was propagated in media containing sitosterol plus 25-azacoprostane hydrochloride (25-aza-5β-cholestane hydrochloride), an inhibitor of Δ^{24}-sterol reductase in insects, its 4α-methylsterol fraction largely consisted of equal amounts of 4α-methyl-5α-cholesta-7,24-dien-3β-ol and 4α-methyl-5α-cholesta-8(14),24-dien-3β-ol. Thus 25-azacoprostane hydrochloride inhibited both a Δ^{24}-sterol reductase and a Δ^{7}-sterol isomerase in C. elegans.

INTRODUCTION

The free-living nematode Caenorhabditis elegans has become an important tool for investigation of metazoan genetics, developmental biology, and biochemistry [1]. The nutritional requirement for sterol in nematodes as well as the lack of de novo biosynthesis has been well established [2-6]. Phytophagous insects also require dietary sterol; most produce cholesterol via removal of the side chain C-24 methyl and ethyl groups typical of C_{28} and C_{29} plant sterols [7]. We initially propagated C. elegans in a medium containing sitosterol (24α-ethyl-5-cholesten-3β-ol) to determine if it possesses a similar dealkylation pathway. Surprisingly, we found that C. elegans not only removed the substituent at C-24 but also converted a

substantial quantity of the dietary sitosterol to 4α-methylsterol derivatives.

EXPERIMENTAL

Nematode Culture: C. elegans was axenically propagated at 22° in an aqueous medium composed of 30 mg/ml soy peptone (Type IV, Sigma Chemical Co., St. Louis, MO), 30 mg/ml yeast extract (Difco Laboratories, Detroit, MI), 10 mg/ml dextrose (J. T. Baker Chemical Co., Phillipsburg, NJ), 10 mg/ml casein hydrolyzate (ICN Nutritional Biochemicals, Cleveland, OH), 0.5 mg/ml hemoglobin (Type I, from beef blood, Sigma), 1.25 μl/ml Tween 80 (Sigma) and 25 μg/ml sterol [8]. Yeast extract, dextrose and casein hydrolyzate were extracted with chloroform/methanol (2:1, v/v) and hemoglobin with chloroform to remove endogenous media component sterols.

Dietary Sterols: Sitosterol contained 1.5% campesterol (24α-methyl-5-cholesten-3β-ol) as an impurity; dietary cholesterol and desmosterol (5,24-cholestadien-3β-ol) contained no apparent impurities by gas-liquid chromatography (GLC) or thin-layer chromatography (TLC). [4-^{14}C]Sitosterol and [26-^{14}C]desmosterol were purchased from Amersham Corp., Arlington Heights, IL, and New England Nuclear, Boston, MA, respectively. After column chromatographic purification, the radiochemical purity of each exceeded 99% by TLC and GLC. Radiolabeled sitosterol and desmosterol were used at specific activities of 0.41 and 0.38 Ci/mol, respectively. The 25-azacoprostane hydrochloride (25-aza-5β-cholestane hydrochloride) was from our previous synthesis [9].

Synthetic Sterols: The 4α-methyl-5α-cholest-8(14)-en-3β-ol was synthesized via the following sequence of reactions [10]. An Oppenauer oxidation of 5α-cholest-7-en-3β-ol gave 5α-cholest-7-en-3-one, mp 142-143; Lit [10] mp 143-145°. This compound when treated with potassium, t-butyl alcohol and an excess of methyl iodide at room temperature gave a mixture of 4α- and 4β-methyl-5α-cholest-7-en-3-one (3:1) and 4,4-dimethyl-5α-cholest-7-en-3-one. The mixture was chromatographed over silica gel and the compounds were eluted from the column with 4% ether in hexane. The fractions were monitored by GLC on a DB-1 fused silica capillary column. Recrystallization from methanol of the combined fractions of the faster and slower eluting components from the column gave 4β-methyl-5α-cholest-7-en-3-one, mp 134-135°, $[\alpha]^{25}D$ +29°, and 4α-methyl-5α-cholest-7-en-3-one, mp 127-128°, $[\alpha]^{25}D$ +2°, respectively. Reduction of the 4α-methyl-5α-cholest-7-en-3-one gave a mixture of the 3β- and 3α-sterols, which were separable by TLC and column chromatography, though not by GLC. Recrystallization of the predominant sterol, after purification by column chromatography, gave the 4α-methyl-5α-cholest-7-en-3β-ol (lophenol), mp 148-150°, $[\alpha]^{25}D$ 0.0°; Lit [10] mp 146-147°.

The isomerization of 4α-methyl-5α-cholest-7-en-3β-ol in acetic acid and an atmosphere of hydrogen with pre-reduced platinum oxide catalyst gave, after filtering and recrystallization from dilute methanol, 4α-methyl-5α-cholest-8(14)-en-3β-ol, mp 149-151°,

$[\alpha]^{25}D$ +17°; Lit. [11] mp 160-163°, $[\alpha]^{25}D$ +19°.

Sterol Isolation: Living nematodes from logarithmic phase cultures were isolated by centrifugation and sucrose flotation [12]. Nematode neutral lipids [13] were separated on columns of silicic acid (Kieselgel 60, 70-230 mesh, E. Merck, Darmstadt, W. Germany) eluted with increasing amounts of diethyl ether in hexane. The resultant free sterol (containing diglyceride contaminants) and steryl ester fractions were separately saponified for 4 h in 4% KOH in methanol, and the saponification products were purified on similar silicic acid columns. Column fractions were monitored by thin-layer chromatography (TLC) on Anasil H chromatoplates (Analabs, North Haven, CT) developed with hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Acetates were prepared from free sterols via overnight reaction at room temperature with pyridine/acetic anhydride (3:1, v/v) and were separated by argentation column chromatography [14].

Instrumentation: GLC of both free sterols and steryl acetates was performed isothermally on packed glass columns (2 m x 2 mm i.d.) containing 2.0% SE-30 or 2.0% OV-17 stationary phases in a Varian 3700 gas chromatograph equipped with flame ionization detectors for analytical determinations and thermal conductivity detectors for preparative work. Compounds collected from the GLC effluent were dissolved in a toluene-based scintillation fluid containing 0.5% PPO and 0.03% dimethyl POPOP and were counted with a Packard 460 CD liquid scintillation system, with quench in each sample measured by external standardization. Sterols were further identified by GLC-mass spectrometry (GC-MS) with a Finnigan model 4510 instrument equipped with a 15 m x 0.32 mm J & W DB-1 fused silica capillary column (0.25 μm film). Data were collected and analyzed via an Incos Data System. The isolated sterols were also analyzed by PMR spectroscopy (60 mHz) with a JEOL FX-60-Q Fourier Transform instrument.

RESULTS AND DISCUSSION

When C. elegans was propagated in sitosterol-containing media, TLC analysis of saponification products from nematode lipid fractions revealed the presence of not only the expected 4-desmethylsterols but also component(s) which migrated similarly to a lophenol standard. Quantification by subsequent GLC indicated that the suspected 4-methylsterols were especially abundant in the steryl ester fraction (Table 1).

GLC also revealed two components (Table 2) with relative

Table 1. The 4-methylsterol content of Caenorhabditis elegans propagated with several different dietary sterols.

4-methylsterol content (%)	supplemented sterol: sitosterol	cholesterol	desmosterol	sitosterol plus 5 µg/ml of 25-aza-coprostane•HCl
dry wt	0.010	0.011	0.018	0.012
total sterol	7.5	6.4	5.4	6.5
free sterol	4.9	2.2	4.1	5.2
esterified sterol	24.7	24.7	12.2	13.8

retention times (RRT's) approximately 0.04 units greater than corresponding RRT's of steryl acetate derivatives (Table 3); such an increase is characteristic of 4-methylsterols [15]. GC-MS of the major 4-methylsterol revealed not only a molecular ion (M^+) at mass/charge (m/z) 400 (100%, relative intensity) and other fragments at m/z 385 ($M-CH_3$, 23) and 367 ($M-CH_3-H_2O$, 4) but also five fragments with side chain (C_8H_{17}) loss at m/z 287 ($M-C_8H_{17}$, 18), 269 ($M-C_8H_{17}-H_2O$, 8), 245 ($M-C_{11}H_{23}$, 15), resulting from D ring cleavage of the C-13,C-17 and C-14,C-15 bonds, 243 ($M-C_{10}H_{19}-H_2O$, 14), and 227 ($M-C_{11}H_{23}-H_2O$, 17) that were 14 mass units larger than typical nuclear fragments seen in 4-desmethylsterol spectra. The proton magnetic resonance (PMR) spectrum contained C-19 and C-18 methyl resonances at δ 0.73 ppm and 0.85 ppm, respectively, and thus indicated presence of a $\Delta^{8(14)}$ bond. Because the MS and PMR data were in agreement with literature data for 4α-methyl-5α-cholest-8(14)-en-3β-ol [16-18], the authentic compound was synthesized and was identical with the unknown by TLC, GLC, MS, and PMR behavior. Similarly, the minor unknown component was identical to lophenol in both GLC (Table 3) and MS properties [mass spectrum:

Table 2. Relative percentages of 4α-methylsterols in free sterol (FS) and steryl ester (SE) fractions from Caenorhabditis elegans fed different dietary sterols.

	supplemented sterol							
	sitosterol		cholesterol		desmosterol		sitosterol plus 5 μg/ml of 25-aza-coprostane•HCl	
	FS	SE	FS	SE	FS	SE	FS	SE
4α-methyl-5α-cholest-8(14)-en-3β-ol	86.4	94.5	96.3	94.6	91.8	86.9	4.1	1.6
4α-methyl-5α-cholest-7-en-3β-ol	13.6	5.5	3.7	5.4	8.2	13.1	8.8	2.2
4α-methyl-5α-cholesta-8(14),24-dien-3β-ol	-	-	-	-	-	-	35.3	45.7
4α-methyl-5α-cholesta-7,24-dien-3β-ol	-	-	-	-	-	-	46.6	49.2
4α-methyl-5α-cholestan-3β-ol	-	-	-	-	-	-	1.3	0.9
4α-methyl-24-ethyl-5α-cholestan-3β-ol	-	-	-	-	-	-	3.9	0.4

400(100%), 385(19), 367(3), 287(14), 269(61), 245(17), 243(9), and 227(19)].

The two 4α-methylsterols were present in about the same relative proportions regardless of whether the dietary sterol was sitosterol, cholesterol or desmosterol; the steryl esters were consistently rich in these compounds (Table 1). To determine if the compounds were possibly originating from the media, the fate of [4-^{14}C]sitosterol and [26-^{14}C]desmosterol was investigated in subsequent experiments. In each case, both of the resultant 4α-methylsterols isolated from C. elegans by preparative GLC had the same specific activity as the supplemented dietary sterol. These results indicate that C. elegans possesses mechanisms for methylation of desmethylsterols at C-4 and dealkylation of 24α-ethylsterols at C-24. These results also provide evidence for the lack of de novo sterol biosynthesis in this organism.

Subsequently, C. elegans was propagated in media containing ^{14}C-sitosterol plus 25-azacoprostane hydrochloride, a potent inhibitor of the Δ^{24}-sterol reductase in insects [9]. Under these conditions, the Δ^{7} and $\Delta^{8(14)}$ monoene sterols comprised merely a small fraction of the 4-methylsterols (Table 2). Instead, the major 4-methylsterols were identified as 4α-methyl-5α-cholesta-8(14),24-dien-3β-ol and 4α-methyl-5α-cholesta-7,24-dien-3β-ol by GC-MS and by observed GLC RRT's (Table 3) which were identical to calculated values. The mass spectrum of the former compound contained a base peak at m/z 69 (C_5H_9), a diagnostic fragment arising from cleavage of the C-22,C-23 bond of Δ^{24}-sterols [19], a M^+ at m/z 398(62%) and fragments at m/z 383 ($M-CH_3$, 31), 365 ($M-CH_3-H_2O$, 5),

287 [M-C_8H_{15} (side chain), 4], 285 (M-C_8H_{15}-2H, 11), 269 (M-C_8H_{15}-H_2O, 16), 245 (M-$C_{11}H_{21}$, 12), 243 (M-$C_{10}H_{17}$-H_2O, 13), and 227 (M-$C_{11}H_{21}$-H_2O, 16). The mass spectrum of the second major component included a base peak at 285 (M-C_8H_{15}-2H), indicative of cleavage of a Δ^7-sterol with side chain unsaturation [20], M^+ at m/z 398(13%), and fragments at 383(18), 365(2), 287(4), 269(7), 245(7), 243(4), 227(10), and 69(52). Also, an abundant peak at m/z 69 indicated side chain unsaturation to be at C-24. Both compounds were of the same specific activity as the dietary sitosterol. Thus, 25-azacoprostane hydrochloride inhibited the C. elegans Δ^{24}-sterol reductase and a Δ^7-sterol isomerase as well.

In addition, GLC (Table 3) and MS analyses of sterols from azacoprostane-treated nematodes revealed the presence of minor amounts of 4α-methyl-5α-cholestan-3β-ol and 4α-methyl-24-ethyl-5α-cholestan-3β-ol: m/z 430 (M^+, 56%), 415 (M-CH_3, 15), 397 (M-CH_3-H_2O, 4),

Table 3. Gas-liquid chromatographic relative retention times of 4α-methylsterols from Caenorhabditis elegans and their steryl acetate derivatives.

	Free Sterol		Steryl Acetate	
	SE-30	OV-17	SE-30	OV-17
4α-methyl-5α-cholest-8(14)-en-3β-ol	1.18	1.16	1.14	1.13
4α-methyl-5α-cholest-7-en-3β-ol	1.31	1.37	1.27	1.33
4α-methyl-5α-cholesta-8(14),24-dien-3β-ol	1.29	1.41	1.25	1.37
4α-methyl-5α-cholesta-7,24-dien-3β-ol	1.44	1.66	1.40	1.61
4α-methyl-5α-cholestan-3β-ol	1.20	1.18	1.16	1.15
4α-methyl-24-ethyl-5α-cholestan-3β-ol	1.95	1.90	1.88	1.84

Retention times are expressed relative to cholesterol or cholesteryl acetate for sterols or steryl acetates, respectively. GLC was performed isothermally on packed glass columns (2 m x 2 mm i.d.) containing 2.0% SE-30 or 2.0% OV-17 stationary phases.

247 (M-$C_{13}H_{27}$, 68), and 229 (M-$C_{13}H_{27}$-H_2O, 99). All major fragments from MS of the latter compound were 14 mass units larger than corresponding peaks in a spectrum of authentic 24α-ethyl-5α-cholestanol. The identity of 4α-methyl-5α-cholestan-3β-ol was confirmed by GC-MS of its acetate, which produced peaks at m/z 444 (M^+, 5%), 429 (M-CH_3, 2), 384 (M-CH_3COOH, 22), 369 (M-CH_3COOH-CH_3, 17), and 229 (M-$C_{11}H_{23}$-CH_3COOH, 100). These peaks were 14 mass units greater than corresponding ones for cholestanyl acetate.

It is easy for investigators to overlook the presence of 4α-methylsterols because of TLC cochromatography with fatty alcohols. The possibility that the 4α-methylsterols were of microbial origin was considered; however, attempts to isolate and culture such an organism failed. This is the first detection in nematodes of 4α-methylsterols, and it is likely that these sterols could be common to nematodes related to C. elegans. Although uncommon, 4α-methyl-5α-cholest-8(14)-en-3β-ol has been isolated from three dinoflagellates [18], an oyster [21], a tunicate [22], and a bacterium [23]. In no case, however, has biosynthesis of these or other 4α-methylsterols been reported to arise from a simple methylation of a 4-desmethylsterol precursor, a process which appears to be unique to C. elegans. The products of this process may prove to have interesting physiological roles indeed.

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