24-METHYL-23-DEHYDROCHOLESTEROL: A NEW STEROL INTERMEDIATE IN C-24 DEMETHYLATION FROM THE NEMATODES *PANAGRELLUS REDIVIVUS* AND *CAENORHABDITIS ELEGANS*?

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ABSTRACT

Panagrellus redivivus produced 24-methyl-23-dehydrocholesterol as 4.0% of the 4-desmethylsterols when propagated in a medium containing campesterol as the dietary sterol. The re-examination of previous data revealed that Caenorhabditis elegans produced 1.8% 24-methyl-23-dehydrocholesterol when propagated in medium containing campesterol. 24-Methyl-23-dehydrocholesterol was not detected when the nematodes were propagated in medium containing 22-dihydrobrassicasterol or 24-methylenecholesterol. This may be a result of the greater efficiency of dealkylation of the latter two sterols. This is the first report of the natural occurrence of this sterol in a non-photosynthetic organism, and the first report in organisms that dealkylate 24-alkylsterols.

INTRODUCTION

The dealkylation and metabolism of campesterol ((24R)-24-methylcholest-5-en-3β-ol) by Caenorhabditis elegans have been previously reported [1-3]. However, similar studies with Panagrellus redivivus are lacking. During recent studies on the metabolism of campesterol by P. redivivus (unpublished data), we isolated 24-methyl-23-dehydrocholesterol (24-methylcholesta-5,23-dien-3β-ol), a sterol previously unidentified in nematodes. The re-examination of previous studies with C. elegans [1-2] revealed that this nematode also produces small quantities of this unusual sterol.

Reports of naturally occurring sterols possessing the unusual

C-23 double bond are rare. Cyclosadol (9β,19-cyclo-4,4,14α,24-tetramethylcholest-23-en-3β-ol) was first isolated from the germ oil of Zea mays [4]. Subsequently, 24-methyl-23-dehydrolophenol (4α,24-dimethylcholesta-8,23-dien-3β-ol) [5], 24-methyl-23-dehydrolathosterol (24-methylcholesta-7,23-dien-3β-ol), and 24-methyl-23-dehydrocholesterol (24-methylcholesta-5,23-dien-3β-ol) were isolated from the germ oil or coleoptiles of Zea mays [5,6]. Yeast was also previously reported to contain 24-methylcholesta-8,23-dien-3β-ol [7]. It was suggested that the 24-methyl-23-dehydrosterols might participate in the biosynthesis of the 24-methylsterols, via the isomerization of the C-24(28) double bond [5,6,8,9]. The isolation of 24-methyl-23-dehydrocholesterol from P. redivivus and C. elegans is the first report of the natural occurrence of this sterol in non-photosynthetic organisms, and the first report of its occurrence in organisms that dealkylate 24-alkylsterols.

EXPERIMENTAL

Nematode Cultures: Panagrellus redivivus was propagated axenically at 22° C in a semi-defined aqueous medium containing 30 mg/mL yeast extract, 30 mg/mL soy peptone, 10 mg/mL casein hydrolysate and 10 mg/mL dextrose [10]. All ingredients except the soy peptone were extracted (3x) with chloroform/methanol (2:1,v/v) to remove any exogenous sterols. A filter-sterilized stock solution of dichloromethane-extracted hemoglobin (0.5 mg/mL final medium concentration) was added after autoclaving. Campesterol was solubilized in Tween 80, filter-sterilized, and added to the medium after autoclaving at a final concentration of 10 µg/mL. The nematodes were acclimated through two subcultures in the above medium prior to introduction into mass culture.

Dietary Campesterol: The campesterol ((24R)-24-methylcholest-5-en-3β-ol), isolated from soybean sterols by fractional recrystallization from acetone, contained no impurities detectable by thin-layer

chromatography (TLC; silica gel 60, solvent system: hexane/ether, 8:2) but contained approximately 4% sitosterol ((24R)-24-ethylcholest-5-en-3β-ol) when analyzed by gas-liquid chromatography (GLC).

Sterol Isolation: Living nematodes were isolated from late logarithmic phase cultures by centrifugation and sucrose flotation [11]. Total lipids were isolated from lyophilized nematodes by homogenization (4x) with chloroform/methanol (2:1) in a Ten Broeck tissue grinder [12]. The neutral lipids were isolated by column chromatography through silica gel 60 (E. Merck, Darmstadt, FRG) by eluting with chloroform. These were fractionated on a 15 g silica gel 60 column (22 mm i.d.) by eluting with: 1) 100 mL hexane, 2) 50 mL hexane/benzene (1:1), 3) 50 mL hexane/benzene (2:8), 4) 50 mL benzene, 5) 50 mL chloroform, 6) 50 mL chloroform, 7) 50 mL ether and 8) 100 mL methanol. Fractions 1-3 (steryl esters) were saponified for 4 h in 4% KOH in methanol at 65° C. Fractions 6-7 (free sterols) were similarly treated as a control.

The sterols were isolated from the saponification products on a 7 g silica gel 60 column (1.0 cm i.d.) by eluting with 20 mL each of 0, 10, 12, 14, 16, 17, 20, 30 and 100% ether graded into hexane. The desmethylsterols eluted in the 20 and 30% ether fractions. The sterols were acetylated with pyridine/acetic anhydride (2:1) for 16 h at ambient temperature. The steryl acetates were separated on a 5 g 20% $AgNO_3$-Unisil column (1.0 cm i.d.) by eluting with 20 mL each of 0, 2, 3, 4, 5, 6, 7 and 100% ether graded into hexane.

Sterol Synthesis: The 24-methylcholesta-5,23-dien-3β-ol was synthesized by double bond migration with iodine [13]. A solution of 20 mg of 24-methylenecholesterol (ergosta-5,24(28)-dien-3β-yl acetate) and 4 mg of iodine in 3 mL of benzene was heated in a stoppered flask overnight at 65° C. The solution was diluted with hexane and washed with 1% $Na_2S_2O_3$ (2x), then water (2x). The hexane was dried over Na_2SO_4 and reduced to dryness under vacuum. TLC analysis on 20% $AgNO_3$-impregnated high performance silica plates developed in hexane/benzene/chloroform/acetic acid (110:65:25:0.5) indicated that very little of the starting material remained unreacted. GLC analysis showed three major peaks: 24-methylene-cholesteryl acetate (11.1%), 24-methyl-23-dehydrocholesteryl acetate (24-methylcholesta-5,23-dien-3β-yl acetate) (58.5%), and 24-methyl-24(25)-dehydrocholesteryl acetate (24-methylcholesta-5,24(25)-dien-3β-yl acetate) (30.4%)(Table 1). Separation and purification by argentation chromatography (20% $AgNO_3$-Unisil) yielded 2.7 mg of pure 24-methyl-23-dehydrocholesteryl acetate in the 4% ether fraction. The synthetic steryl acetate was further characterized by GLC, $AgNO_3$ TLC, melting point, ^1H NMR (Table 1), and GLC/MS. The ^1H NMR and mass spectral data are in agreement with those previously published [6]. Half of the fraction was saponified in 4% KOH in methanol for subsequent analysis by GLC/MS.

Table 1. Physical properties of steryl acetates

Steryl Acetate	mp °C	GLC RRT[a] DB-1	GLC RRT[a] OV-17	$AgNO_3$ TLC Rf	^1H NMR
Ergosta-5,24(28)-dien-3β-yl acetate	137-138	1.27	1.35	0.42	18-H(3H,s)0.68,19-H(3H,s)1.00,21-H(3H,d,j=6Hz)0.95,26,27-H(3H,3H,d,d,j=6Hz)1.03,1.03,28-H(2H,s)4.68,5-H(1H,m)5.35
24-Methylcholesta-5,24(25)-dien-3β-yl acetate	146-147	1.47	1.65	0.56	18-H(3H,s)0.67,19-H(3H,s)1.00,21-H(3H,d,j=6Hz)0.95,26,27-H(3H,3H,s,s)1.60,1.60,28-H(3H,s)1.60,5-H(1H,m)5.35
24-Methylcholesta-5,23-dien-3β-yl acetate	128-129	1.26	1.36	0.51	18-H(3H,s)0.67,19-H(3H,s)1.00,21-H(3H,d,j=6Hz)0.86,26,27-H(3H,3H,d,d,j=6Hz)0.96,0.96,28-H(3H,s)1.52,5-H(1H,m)5.35, 23-H(1H,t,j=7Hz)5.12

[a] RRT relative to cholesteryl acetate.

Instrumentation: GLC was performed isothermally on a glass column (2 mm i.d. x 2 m) packed with 2% OV-17 and a J & W DB-1 fused silica capillary column (0.25 mm i.d. x 15 m, 0.25 μm film) using a Varian model 3700 gas chromatograph equipped with a flame ionization detector. Retention times and areas were recorded on a Shimadzu C-R1B Chromatopac data processor. GLC/MS was performed at 70 eV on a Finnigan model 4510 equipped with a J & W DB-1 fused silica capillary column (0.32 mm i.d. x 30 m, 0.25 μm film) and interfaced with an Incos Data System. ^1H NMR was performed at 60 MHz at ambient temperature on a JEOL FX-60-Q Fourier transform instrument. Samples were analyzed in $CDCl_3$ with 1% TMS (tetramethylsilane) as an internal standard. All peaks are reported in ppm chemical shift from TMS. Melting points were determined on a Kofler block and are reported as corrected values.

RESULTS

Panagrellus redivivus metabolized more than 70% of the campesterol taken up from the medium. In our effort to characterize these metabolic products, we isolated a sterol component (4% of the 4-desmethylsterol) previously unidentified in nematodes.

The putative identification of the isolated sterol was accomplished by GLC and GLC/MS. This sterol had a relative retention time (RRT) to cholesterol of 1.26 ± 0.01 on DB-1 and 1.36 ± 0.02 on OV-17. The mass spectrum exhibited ions at m/z: 398(M^+, 11%), 383($M-CH_3$, 5%), 365($M-CH_3-H_2O$, 2%), 301 ($M-C_7H_{13}$, 7%), 299($M-C_7H_{13}$-2H, 14%), 283 ($M-C_7H_{13}-H_2O$, 52%), 271(M-side chain-2H, 100%), and 253(M-side chain-H_2O-2H, 6%), with other prominent ions at m/z: 227(7%), 215(27%), 213(13%), 159(37%), 133(56%), 81(46%), and 55(86%). The molecular ion (M^+) at m/z 398 indicated that this was a C_{28} diunsaturated sterol. The fragments at m/z 301($M-C_7H_{13}$), 299($M-C_7H_{13}$-2H), and 283($M-C_7H_{13}-H_2O$) suggested that one double bond was located

at C-23, which facilitated the allylic cleavage between C-20 and C-22 [6]. The RRTs and mass spectrum of the isolated sterol were identical to those of the synthetic 24-methyl-23-dehydrocholesterol.

The isolated sterol was acetylated and further characterized by GLC, $AgNO_3$ TLC (Table 1), and GLC/MS. The mass spectrum exhibited ions at m/z for the steryl acetate: 380(M-HOAc, 28%), 365(M-HOAc-CH_3, 3%), 296(M-144, 3%), 283(M-HOAc-C_7H_{13}, 34%), 259(M-181, 4%), 255(M-HOAc-side chain, 6%), and 253(M-HOAc-side chain-2H, 24%), with other prominent ions at m/z: 227(4%), 217(3%), 215(6%), 213(8%), 159(27%), 145(37%), 133(54%), 81(79%), 55(100%). The mass spectrum was identical to the spectrum of the synthetic steryl acetate and agreed with previously published spectral data [6]. These data characterized the isolated sterol as 24-methylcholesta-5,23-dien-3β-ol.

DISCUSSION

When Panagrellus redivivus was propagated in campesterol-containing medium, 4.0% of the free 4-desmethylsterols and 2.1% of the steryl ester fraction (2-3% of the total sterol) recovered from the nematodes was 24-methyl-23-dehydrocholesterol. This prompted us to re-examine earlier studies where Caenorhabditis elegans was also propagated in campesterol-containing medium [1,2]. Inspection of these data revealed that a previously unreported sterol component (1.8% of the free 4-desmethylsterols) from C. elegans was 24-methyl-23-dehydrocholesterol, based on GLC RRTs and mass spectral

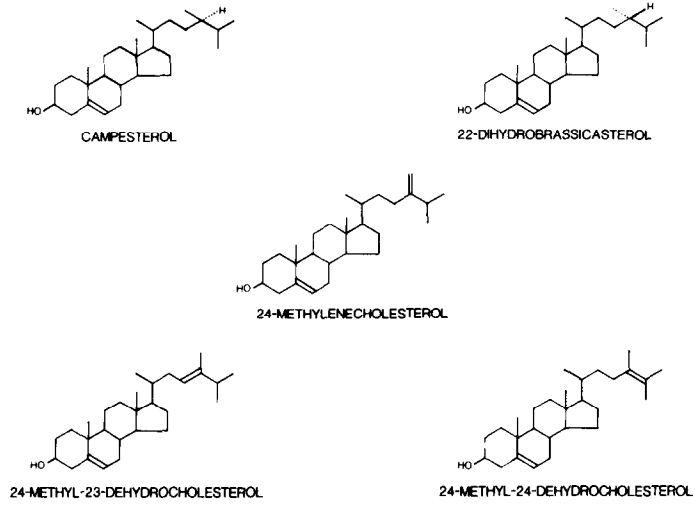

Figure 1. Structures of sterols.

data. No 24-methyl-23-dehydrocholesterol was detected in the steryl ester fraction.

The possibility existed that this was an artifact resulting from the isolation and separation procedures. The isomerization of the C-24(28) double bond to the C-24(25) double bond on silica gel columns has been reported [14]. However, the inability to detect isomerization to the C-23 double bond on silica gel in other work [14] or in our own work with 24-methylsterols and 24-methylenesterols indicated that this was a natural product. Our conclusions, supported by other investigators [5,6,13], is that the isomerization of 24-methylenecholesterol to a sterol containing the C-23 double bond was achieved only by chemical treatment.

It has been proposed that sterols possessing the C-23 double bond are involved in a minor pathway in the biosynthesis of the 24-methylsterols in Zea mays [6,8]. Recent specific labeling studies in Z. mays have demonstrated the obligatory passage through a 24-methylenesterol before isomerization to a 24-methyl-23-dehydrosterol [9]. In nematodes, the dealkylation of 24-alkylsterols proceeds via 24-alkylsterols $\longrightarrow$ 24-alkenesterols $\longrightarrow$ 24-desalkylsterols [1-3,15,16]. The re-examination of previous data [1,2] on the dealkylation of 24-ethylsterols did not reveal any sterols with a C-23 double bond. This is consistent with the proposed involvement of the C-23 double bond with the 24-methylsterols [6,8]. It is curious to note that no 24-methyl-23-dehydrocholesterol was detected when C. elegans was propagated in 24-methylene-cholesterol-containing medium. This may be a result of the more efficient dealkylation of 24-methylenecholesterol (>70%) when compared to campesterol (<45%)[2]. Similarly, no 24-methyl-23-dehydro-cholesterol was detected when C. elegans was propagated in medium containing 22-dihydrobrassicasterol ((24S)-24-methylcholest-5-en-3β-ol). Although the dietary sterol contained approximately 25% campesterol (24R-epimer), nearly 60% of the recovered sterols were 24-desalkylsterols, indicating that the 24S-methyl epimer is also more efficiently dealkylated [1]. Although the production of 24-methyl-23-dehydrocholesterol cannot be excluded, it may be obscured by the greater efficiency of dealkylation of these two dietary sterols.

The active participation of the C-23 double bond in the 24-methyl dealkylation pathway has not been demonstrated and its function is currently unknown. It is interesting to note that when propagated in campesterol-containing medium with N,N-dimethyldodecanamine (a C-24 sterol reductase inhibitor), C. elegans accumulates a greater percentage of sterols with a C-24 double bond [2]; however, no change in the percentage of 24-methyl-23-dehydrocholesterol (1-2%) has been found in this laboratory (unpublished data). The significance of this observation is currently unknown.

The intermediacy of 24-methylene sterols in sterol side chain methylation of sterols in plants, and in side chain demethylation in nematodes and insects, raises many intriguing questions with respect to the isomerization to the 24-methyl-23-dehydrosterols. Is the isomerization enzymatically catalyzed and is it reversible? Does the C-23 double bond actively participate in demethylation? Propagating nematodes in a 24-methyl-23-dehydrocholesterol- containing medium with a C-24 sterol reductase inhibitor may yield insight into the possible involvement of this sterol in the demethylation of 24-methylsterols.

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NOTE

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