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EFFECT OF AY-9944 ON STEROL BIOSYNTHESIS IN *CHLORELLA SOROKINIANA**

PEI-LU CHIU, GLENN W. PATTERSON† and SAMSON R. DUTKY‡

† Department of Botany, University of Maryland College Park, MD 20742;
‡ Insect Physiology Laboratory, Plant Protection Institute, Agricultural Research Service, U.S.D.A., Beltsville, MD 20705, U.S.A.

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Abstract—When *Chlorella sorokiniana* was grown in the presence of 4 ppm AY-9944 total sterol production was unaltered in comparison to control cultures. However, inhibition of sterol biosynthesis was shown by the accumulation of a number of sterols which were considered to be intermediates in sterol biosynthesis. The sterols which were found in treated cultures were identified as cyclolaudenol, 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol, 4α,14α-dimethyl-5α-ergosta-8,25-dien-3β-ol, 14α-methyl-9β,19-cyclo-5α-ergost-25-en-3β-ol, 24-methylpollinastanol, 14α-methyl-5α-ergost-8-en-3β-ol, 5α-ergost-8(14)-enol, 5α-ergost-8-enol, 5α-ergosta-8(14),22-dienol, 5α-ergosta-8,22-dienol, 5α-ergosta-8,14-dienol, and 5α-ergosta-7,22-dienol, in addition to the normally occurring sterols which are ergosterol, 5α-ergost-7-enol, and ergosta-5,7-dienol.

The occurrence of these sterols in the treated culture indicates that AY-9944 is an effective inhibitor of the $\Delta^8 \rightarrow \Delta^7$ isomerase and Δ^{14}-reductase, and also inhibits introduction of the Δ^{22}-double bond. The occurrence of 4α,14α-dimethyl-5α-ergosta-8,25-dien-3β-ol and 14α-methyl-9β,19-cyclo-5α-ergost-25-en-3β-ol is reported for the first time in living organisms. The presence of 25-methylene sterols suggests that they, and not 24-methylene derivatives, are intermediates in the biosynthesis of sterols in *C. sorokiniana*.

INTRODUCTION

AY-9944 (*trans*-1,4-bis-(2-chlorobenzylamino-methyl)cyclo-hexane dihydrochloride) was first synthesized by Ayerst Research Laboratories as a hypocholesterolemic drug inhibiting cholesterol biosynthesis [1]. It was reported to be an effective inhibitor of the 7-dehydrocholesterol Δ^7-reductase step in different animal tissues [1–5].

However, the effect of AY-9944 on a Δ^5-sterol containing alga, *Chlorella ellipsoidea*, is completely different from that in animal tissues. The biosynthesis of sterols was inhibited in the presence of 4 ppm AY-9944. Analysis of the sterols in treated cultures indicated that, rather than being a Δ^7-reductase inhibitor, AY-9944 appeared to be an extremely effective inhibitor of the $\Delta^8 \rightarrow \Delta^7$ isomerase and the Δ^{14}-reductase of this plant [6]. Investigation of the effects of AY-9944 on an organism not normally synthesizing Δ^5-sterols was also accomplished by Dickson and Patterson [7]. *Chlorella emersonii*, which synthesizes only Δ^7-sterols, was utilized. AY-9944 (20 mg/l.) was an effective inhibitor of the 14α-methyl removal and the second alkylation in the biosynthesis of sterol by *C. emersonii*. No accumulation of Δ^8 or $\Delta^{8,14}$-sterols was observed in this experiment. The larger accumulation of 14α-methyl sterols in *C. emersonii* compared to *C. ellipsoidea* was considered to be the effect of a greater drug concentration (20 mg/l. vs 4 mg/l.) in the former species [8].

The sterols of *Chlorella* have been studied extensively. Based on sterol composition, the genus *Chlorella* has been divided into three groups, namely Δ^5-, Δ^7- and $\Delta^{5,7}$-sterol containing algae [9]. *C. ellipsoidea*, *C. emersonii*, and *C. sorokiniana* were chosen as representatives respectively. In this paper the effect of AY-9944 on sterol biosynthesis in *C. sorokiniana* has been studied and compared with results obtained from previously studied *Chlorella* species.

RESULTS

Analysis of the sterol mixtures of control and AY-9944 treated cultures on an SE-30 column revealed large qualitative differences. The amount of total sterol per g dry weight of cells in the AY-9944 treated cultures was about the same as that of the control culture (Table 1). Relative retention times of sterol acetates of the control culture indicated a mixture similar to that previously reported [10]. After further purification and separation on the $AgNO_3$ column and Sephadex column, the sterols were identified as ergosterol, ergosta-5,7-dien-3β-ol and ergost-7-en-3β-ol. The GLC analysis of the sterols of the inhibited cultures showed a large decrease in ergosterol and a large quantity of sterols eluting just after ergosterol.

Alumina column chromatography separated the sterol mixtures into methyl sterols and desmethyl sterols. The RR_t's of these methyl sterols were obtained from the SE-30 column as free sterols, and further identification was achieved by combined GC–MS.

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Table 1. A quantitative comparison of sterols from control and AY-9944-treated cultures of *Chlorella sorokiniana*

Sterols	Control % of sample	Control μg/g dry wt	AY-9944-treated % of sample	AY-9944-treated μg/g dry wt
Cyclolaudenol			0.77	13.7
14α,14α-Dimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol			0 03	0.5
4α,14α-Dimethyl-5α-ergosta-8,25-dien-3β-ol			0 06	1.1
14α-Methyl-9β,19-cyclo-5α-ergost-25-en-3β-ol			0 45	8.0
24-Methylpollinastanol			3.03	53.9
14α-Methyl-5α-ergost-8-en-3β-ol			0.13	2.3
5α-Ergost-8(14)-en-3β-ol			6.57	116 8
5α-Ergost-7-en-3β-ol	15.0	254.8	14.50	257.8
5α-Ergost-8-en-3β-ol			28.80	512.1
5α-Ergosta-8(14),22-dien-3β-ol			0.29	5 2
5α-Ergosta-8,22-dien-3β-ol			2 46	43.7
Ergosta-5,7-dien-3β-ol	4.5	77.4	2 21	39.3
5α-Ergosta-7,22-dien-3β-ol			1 66	29 5
5α-Ergost-8,14-dien-3β-ol			14.67	260.8
Ergosterol	80.5	1371.0	20.80	369.8
Unknown A			0.44	7.8
Unresolved sterols			3.14	55.7
Total	100.0	1703.2	100.00	1778.0

Identification of methyl sterols

Methyl sterols were identified as cyclolaudenol, 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol and 4α,14α-dimethyl-5d-ergosta-8,25-dien-3β-ol. The identification of cyclolaudenol was based on the RR_t on the SE-30 column which is in agreement with the previously published data [11]. The MS of the isolated compound and authentic cyclolaudenol were indistinguishable. Cyclolaudenol was also isolated and identified by Chan and Patterson [10] from a triparanol treated culture of the same organism. It should be pointed out that since *Chlorella* sterols all have the 24β-configuration, the cyclolaudenol identified from *Chlorella* may have the opposite configuration at C-24 from the cyclolaudenol isolated from higher plants.

The identification of 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol was based primarily on its MS (see experimental) and was confirmed by GLC data. The GC–MS analysis of the free sterol indicated a 30-carbon sterol, with a cyclopropane ring and 9-carbon side chain, and only one double bond which was in the side chain. It also indicated the presence of 4- and 14-methyl groups. The RR_t of this sterol on the SE-30 column was 1.69 which upon repeated analyses was consistently lower than that of cycloeucalenol which had a RR_t of 1.75. Using the known retention characteristics of the Δ^{25} double bond [11], a theoretical RR_t of 1.71 is calculated for the proposed structure. This is in good agreement with the experimental value, GC and MS data for $\Delta^{24(28)}$ and Δ^{25} sterols are quite similar. Although the data presented here clearly point to the presence of the Δ^{25} isomer, at this point the presence of both isomers is a possibility.

The identification of 4α,14α-dimethyl-5α-ergosta-8,25-dien-3β-ol was based on GC–MS analysis (see experimental), which indicated the presence of two double bonds in the 30-carbon sterol and the occurrence of 4α, and 14α-methyl groups. The RR_t of this sterol on SE-30 was 1.48 for the free sterol, which was consistently lower than that of obtusifoliol [11]. The identification of both unknowns as Δ^{25} compounds rather than the more common 24-methylene sterol is supported by the work of Adler [12] who showed that all three deuteriums from CD_3-methionine were incorporated into the 24-methyl group of ergosterol in *C. sorokiniana*. Adler's data strongly supports our contention that Δ^{25} but not $\Delta^{24(28)}$ compounds are intermediates in the biosynthesis of ergosterol in this organism. Cyclolaudenol is thus envisioned as a precursor of ergosterol in this organism in place of 24-methylenecycloartanol. Sterols with the ethyl group at C-24 are not synthesized by *C. sorokiniana*.

Identification of desmethyl sterols

The desmethyl sterols, which were eluted from the alumina column in the 80–100% ether in hexane fraction, were acetylated and applied to the Sephadex column. Triene sterol acetates, diene sterol acetates and monoene sterol acetates were eluted, respectively. Further separation of sterol acetates was accomplished by using repeated $AgNO_3$ column chromatography. The desmethyl sterol acetates were identified by comparing the RR_t's obtained from four different GLC columns to previously published data [11]. Except for 14α-methyl-9β,19-cyclo-5α-ergost-25-en-3β-ol, all the desmethyl sterols which were identified were previously isolated and identified in triparanol-treated *C. sorokiniana* [10]. They were 24-methylpollinastanol, 14α-methyl-5α-ergost-8-en-3β-ol, 5α-ergost-8(14)-en-3β-ol, 5α-ergost-7-en-3β-ol, 5α-ergost-8-en-3β-ol, 5α-ergosta-8(14),22-dien-3β-ol, 5α-ergosta-8,22-dien-3β-ol, ergosta-5,7-dien-3β-ol, 5α-ergosta-8,14-dien-3β-ol, 5α-ergosta-7,22-dien-3β-ol, and ergosterol.

The identification of 14α-methyl-9β,19-cyclo-5α-ergost-25-en-3β-ol was accomplished by data from four GLC columns and GC–MS analysis. This sterol acetate was eluted together with 5α-ergosta-8,22-dienol and small amounts of 5α-ergosta-8,14-dienol from the $AgNO_3$ column. The MS (see experimental) showed the presence of a cyclopropane ring and a 14α-methyl group in a 29-carbon monounsaturated sterol. Since this sterol has not been isolated previously the RR_t of an authentic sample was not available. The RR_t's of this sterol on SE-30, QF-1, HI-EFF-8BP and PMPE were 1.45, 1.50, 1.64, 1.61, respectively, which agree with those calculated for the proposed compound by the method of Clayton [13].

DISCUSSION

A quantitative comparison of the sterols from control vs AY-9944 treated cultures is given in Table 1. AY-9944 obviously has a significant effect on sterol composition while apparently having no effect on total sterol production. The same phenomenon was also found in AY-9944 treated *Chlorella ellipsoidea* cultures [6]. But in AY-9944 treated *C. emersonii*, a 50% inhibition in total sterol production was recorded [7]. The different effects of AY-9944 on various *Chlorella* species may be caused by the different concentrations of AY-9944 which were used for these studies. Both *C. ellipsoidea* and *C. sorokiniana* were treated with 4 ppm AY-9944, while *C. emersonii* was treated with 20 ppm AY-9944, which the former two species do not tolerate. The study of AY-9944 treated *Ochromonas malhamensis* showed that AY-9944 at concentrations up to 50 ppm did not affect sterol production quantitatively or qualitatively [14]. It has been suggested that the effect of AY-9944 on sterol biosynthesis may

Table 2. Percentage* of different sterol structures occurring in control and AY-9944-treated *Chlorella sorokiniana*

Sterol structure	Control	AY-9944-treated
4α,4β,14α-Trimethyl		0.77
4α,14α-Dimethyl		0.09
14α-Methyl		3.60
9β,19-Cyclopropane		4.27
$\Delta^{8(14)}$		6.83
Δ^{8} (Desmethyl)		45.88
$\Delta^{8,14}$		14.67
Δ^{7}	100.0	39.17
$\Delta^{5,7}$	85.0	23.01
Δ^{22}	80.5	25.21
25-Methylene		1.31

* Percentage of total sterol.

involve interference in the formation of the active conformation of sterol carrier protein and perhaps also the binding of substrate to sterol carrier protein [15].

From previous studies with *C. ellipsoidea* and *C. emersonii*, it was found that AY-9944 interrupted the $\Delta^{8} \rightarrow \Delta^{7}$ isomerase step and the Δ^{14}-reductase step. These effects have been once more confirmed with *Chlorella sorokiniana*, since 45% of the accumulated sterols are $\Delta^{8(9)}$ and 14% are $\Delta^{8,14}$ sterol (Table 2). Some accumulation of 14α-methyl sterol indicated that AY-9944 may be an inhibitor of 14α-methyl removal. A great reduction in Δ^{22} sterol from 80 to 25% of total sterol may indicate another AY-9944 inhibition site, the introduction of the Δ^{22} double bond. Since Δ^{5} monounsaturated sterols are not normally produced in this organism, the inhibiting effect of AY-9944 on Δ^{7}-reductase cannot be examined. Generally, the effect of AY-9944 on sterol biosynthesis in *Chlorella sorokiniana* is very similar to its effect in *C. ellipsoidea* (which does produce Δ^{5} sterols) reported by Dickson [6].

From the accumulated sterols in AY-9944 treated cultures, the sterol biosynthetic pathway of *C. sorokiniana* can be visualized. In both triparanol and AY-9944 treated *C. sorokiniana* no 24-methylene sterols accumulated as intermediates. Isolation of cyclolaudenol and three other 25-methylene sterols, 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol, 4α,14α-dimethyl-5α-ergosta-8, 23-dien-3β-ol, and 14α-methyl-9β,19-cyclo-5α-ergost-25-en-3β-ol from AY-9944 treated *C. sorokiniana* suggest that Δ^{25} sterols replace $\Delta^{24(28)}$-sterols in the biosynthetic pathway in *C. sorokiniana*, and that the pathway after cyclolaudenol proceeds as follows. The removal of a 4-methyl produces 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol which is converted to 14α-methyl-9β,19-cyclo-5α-ergost-25-en-3β-ol by loss of the second 4-methyl group or it may be converted to 4α,14α-dimethyl-5α-ergosta-8,25-dien-3β-ol by the opening of the cyclopropane ring. Each of these sterols is eventually converted to 14α-methyl-5α-ergost-8-en-3β-ol and finally to ergosterol as pictured by Chan and Patterson [10]. Since the second alkylation in the sterol side chain requires a 24-methylene group [16], the presence of the Δ^{25}-double bond system in the sterols of *C. sorokiniana* prevents the second alkylation, and therefore C_{29} sterols (sterols with a 10-carbon side chain) are not synthesized. Work on *C. vulgaris*, [17], *C. sorokiniana* [12] and *Trebouxia* sp. [18] with CD_3-methionine supports the concept that 24-methylene sterols are not involved in the synthesis of C_{28} sterols in these organisms.

EXPERIMENTAL

Cell culture and sterol extraction. Control cells of *Chlorella sorokiniana* Shihira and Krauss, Indiana Culture Collection No. 1230, were grown for 12–14 days in carboys as described previously [10]. Inhibited cultures were grown under the same conditions as the controls except 4 ppm AY-9944 was added and sterilized with the inorganic cultural medium. A longer time of growth (20–22 days) was required to achieve a comparable quantity of plant material with the treated cultures. Free sterols were extracted by the method described by Chan and Patterson [10].

Sterol separation. The separation of methyl sterols from desmethyl sterols was achieved with a Grade II alumina column eluted with 50 ml vols of an increasing concn of Et_2O in *n*-hexane. Methyl sterols were primarily eluted in 40–70% fractions, while desmethyl sterols were eluted in 80–100% fractions. The methyl sterol fractions were combined and purified by TLC using Si gel HF-254 + 366 with the solvent system, C_6H_6–EtAc (9:1). Desmethyl sterols were acetylated and separated by column chromatography on Sephadex LH-20 [19,20]. Triene-, diene-, and monoene-sterol acetates were eluted in that order. Anasil B impregnated with 12% $AgNO_3$ [21] was used to accomplish further separation of those sterol acetates eluted from Sephadex LH-20. Identification and quantitation of sterols were achieved by GC–MS and GLC on four columns (SE-30, QF-1, HI-EFF-8BP, PMPE). MS were obtained as previously described [10].

Mass spectra. 4α,14α-Dimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol: *m/e* (rel int), M^{+}426(8), 411(17), 408(25), 393(24), 353(5), 333(5), 301(10), 300(11), 283(12), 281(17), 253(6), 245(3), 229(6), 218(10), 203(12), 201(11), 189(11), 175(21), 173(19), 163(20), 161(26), 159(18), 147(33), 133(31), 121(40), 109(57), 95(82), 81(66), 69(84), 55(100), 43(87), 41(85). 4α,14α-Dimethyl-5α-ergosta-8,25-dien-3β-ol: *m/e* (rel int), M^{+}426(30), 411(78), 393(14), 341(5), 327(6), 281(11), 269(13), 245(16), 233(9), 227(8), 215(10), 201(10), 175(12), 159(16), 147(18), 133(18), 123(35), 109(38), 95(62), 83(59), 81(53), 69(70), 57(69), 55(96), 43(100), 41(55). 14α-Methyl-9β,19-cyclo-5α-ergost-25-en-3β-ol: *m/e* (rel int), M^{+}412(7), 410(6), 397(13), 394(15), 379(16), 300(8), 287(8), 285(15), 269(19), 255(7), 245(7), 231(5), 215(9), 203(8), 201(8), 199(8), 187(9), 175(27), 163(30), 147(39), 133(31), 123(43), 121(41), 109(46), 107(44), 95(82), 81(64), 69(74), 55(100), 43(34), 41(46).

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