AY-9944 Inhibition of Sterol Biosynthesis in *Chlorella emersonii*[1]

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ABSTRACT

When *Chlorella emersonii*, a green alga, was cultured in the presence of 20 ppm AY-9944, a number of sterols accumulated which appear to be intermediates of sterol biosynthesis in this organism. The sterols isolated include 14α-methyl-ergost-8-en-3β-ol, 14α-methyl 24S-stigmast-8-en-3β-ol, 14α-methyl ergosta-8,24(28)-dien-3β-ol and 4α, 14α-dimethyl 24S-stigmast-8-en-3β-ol. Smaller quantities of several other sterols were found in addition to the normally occurring Δ^7-ergostenol, chondrillasterol and Δ^7-chondrillastenol. Control cultures were found to contain, in addition to the normally occurring sterols, smaller quantities of most of the sterols isolated from AY-9944 inhibited cultures. AY-9944 is a specific inhibitor of Δ^7-reductase in cholesterol biosynthesis in animals. However, since *C. emersonii* terminates sterol biosynthesis one step prior to the Δ^7-reductase step, AY-9944 apparently inhibits sterol biosynthesis prior to this step in this organism. The accumulation of 14α-methyl sterols in treated cultures suggests that AY-9944 is an effective inhibitor of the 14α-methyl removal in *C. emersonii*.

INTRODUCTION

The hypocholesterolemic drug *trans*-1,4-bis-(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride (AY-9944) is a well known inhibitor of the reduction of 7-dehydrocholesterol to cholesterol in animals (1-3). Recently AY-9944 has been shown to be an inhibitor of sterol biosynthesis in algae (4,5). However it seems certain that rather than being a Δ^7-reductase inhibitor, AY-9944 is primarily an inhibitor of the reduction of the Δ^{14} double bond in the $\Delta^{8,14}$ sterol biosynthetic intermediates described in these reports.

Previous studies in this laboratory involved the use of *Chlorella ellipsoidea*, a green alga that normally produces only Δ^5 sterols. When such unpredicted intermediates were obtained with this alga, we felt some insight into the mechanism of inhibition by AY-9944 might be gained if the effect of AY-9944 was determined on an organism that does not normally carry out the Δ^7-reductase step at all, viz., an alga synthesizing only Δ^7 sterols. *Chlorella emersonii* is such an organism (6). The effects of AY-9944 on sterol synthesis in *C. emersonii* (if any) must therefore be on steps other than Δ^7-reductase.

EXPERIMENTAL PROCEDURES

Chlorella emersonii var. *emersonii*, Shihira and Krauss, Maryland Culture Collection No. 2 (Indiana Culture Collection No. 252) was cultured axenically in 15-1 carboys on basal inorganic medium supplemented with 0.5% glucose as previously described (5). AY-9944-treated cultures were grown in the same way as the controls except that at the time of inoculation AY-9944 was added to give a final concentration of 20 ppm. Cells grown at this level of AY-9944 produced ca. 50% the yield of control cultures. Sterols were extracted from freeze-dried cells with $CHCl_3/CH_3OH$ 2:1 v/v and after saponification were partially purified by digitonin precipitation as described by Doyle et al. (7).

Further purification and separation was accomplished using alumina and $AgNO_3$-impregnated silica gel and Anasil B column chromatography. Gas liquid chromatography (GLC) was carried out with a Glowall Chromalab Model A-110 gas chromatograph . These methods have been previously described (4,7).

RESULTS AND DISCUSSION

Both quality and quantity of sterols were markedly altered when *C. emersonii* was grown in the presence of 20 ppm AY-9944. Total sterol extracted from control and treated cultures was 2.3 mg/g and 1.7 mg/g dry weight, respectively. Relative retention times (RRT) of sterols from control cultures indicate that the major sterols are identical to those described by Patterson (6). They are thus identified as

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TABLE I

Quantitative Comparison of Sterols from Control and AY-9944-Treated Cultures of *Chlorella emersonii*

Sterols	Control		AY-9944-Treated	
	% of sample	μg/g dry wt	% of sample	μg/g dry wt
24-Methylene cycloartanol	0.3	6	tr[a]	tr
24-Dihydroobtusifoliol	0.8	19	0.2	2
4α, 14α-Dimethyl 24S-Stigmast-8-en-3β-ol	0.4	8	0.3	4
14α-Methyl-ergost-8-en-3β-ol	0.9	22	3.4	57
14α-Methyl 24S stigmast-8-en-3β-ol	0.4	8	9.8	165
14α-Methyl-ergosta-8,24(28)-dien-3β-ol	0.8	19	3.6	62
Obtusifoliol	0.2	5	tr	tr
Cycloeucalenol	0.0	0	tr	tr
Δ^7-Ergostenol	16.3	390	42.6	720
Δ^7-Chondrillastenol	8.6	200	9.8	165
Chondrillasterol	70.8	1650	29.2	500
5α-Ergosta-7,22-dien-3β-ol	0.5	12	0.4	7
Total	100.0	2339	99.3	1682

[a]tr = trace; indicates less than 1 μg/g dry wt.

Δ^7-ergostenol, chondrillasterol and Δ^7-chondrillastenol (Table I).

Obvious qualitative and quantitative differences in the initial gas chromatograms (on SE-30 columns) of treated cultures include the presence of five major peaks instead of three, the presence of one completely new major peak, a large increase in the Δ^7-ergostenol peak or a concommitent decrease in the chondrillasterol peak.

Alumina column chromatography separated the sterols into 4,4′-dimethyl, 4-methyl and 4-desmethyl fractions. Following conversion of these fractions into the acetates, the sterols were further separated and purified on $AgNO_3$-impregnated silica gel columns. Sterols thus separated were sufficiently pure for an accurate determination of the RRT of each sterol acetate on three GLC systems (3% SE-30, 1% QF-1, 3% HiEff-8BP) as previously described (5).

Positive identification of these sterols is based on comparative RRT data of free sterols and acetates with published values (8) and with authentic compounds. Identification was based on the characteristic movement of the compounds on alumina and $AgNO_3$-silica gel columns.

A quantitative comparison of control and treated sterols is presented in Table I. A more thorough check of sterols from control cultures revealed the presence of small amounts of many of the sterols found in the treated samples. Significant quantitative changes in sterols of the treated samples are as follows: production of the three major sterols was reduced from 96 to 82% of the total sterol; chondrillasterol was reduced by the greatest amount (1650 to 500 μg/g dry wt,) while Δ^7-ergostenol nearly doubled (390 to 720 μg/g dry wt); and there were large increases—as great as 20-fold—in the 14α-methyl Δ^8 sterols.

Obviously AY-9944 has a pronounced effect on sterol biosynthesis in *C. emersonii*. Accordingly AY-9944 must be more than a Δ^7-reductase inhibitor in this organism.

The point or points of this inhibition are not clear from the data; however the following possibilities are postulated. Although 20 ppm of AY-9944 was required in this study and only 5 ppm of triparanol was used by Doyle et al. (7), the result of AY-9944 inhibition is nearly identical to triparanol inhibition in *C. emersonii*. Considering the relatively large accumulation of 14α-methyl sterols (17.3% of total sterol in treated cultures, 3.8% in control cultures), the inhibitory activity of AY-9944 may be similar to that postulated for triparanol, i.e., inhibition of 14α-methyl removal.

The ratio of nine carbon to ten carbon side chains (0.25 and 1.0, in control and treated cells, respectively) is interesting. This is due in large measure to the great increase in Δ^7-ergostenol. This clearly provides the possibility that AY-9944 may be inhibiting the second alkylation reaction in the side chain. This large buildup of Δ^7-ergostenol otherwise cannot be explained. The increase in 24-methylene com-

pounds from 1.3% of total sterol (control) to 3.6% (AY-9944-treated) also suggests that AY-9944 may inhibit the second alkylation reaction.

In conclusion, when these data are compared with data previously reported (4,5) from AY-9944-treated *C. ellipoidea*, i.e., a large accumulation of $\Delta^{8,14}$ sterol dienes and lesser amounts of $\Delta^{8(9)}$ monoenes, the primary sites of inhibition by AY-9944 in sterol biosynthesis of *C. ellipsoidea* are postulated to be at steps 2 or 3 in the sequence shown below:

$$14\alpha\text{-methyl}\Delta^{8(9)}\text{sterols} \xrightarrow{1} \Delta^{8,14}\text{sterol dienes} \xrightarrow{2} \Delta^{8(9)}\text{ sterols} \xrightarrow{3} \Delta^{7}\text{ sterols}$$

However, in *C. emersonii*, at a much higher concentration of inhibitor (20 ppm vs. 4 ppm), inhibition is seen only at site 1 and at the second alkylation in the side chain. Side chain alkylation was not inhibited in *C. ellipsoidea* at 4 ppm AY-9944.

The differential qualitative and quantitative effect observed in accumulated sterols between these two organisms in response to AY-9944 is difficult to explain. That *C. emersonii* required five times the concentration of inhibitor to achieve the same level of growth inhibition as *C. ellipsoidea* indicates that the Δ^{14}-reductase of *C. emersonii* is relatively insensitive to AY-9944. Only at the higher level are the 14α-methyl removal and the second alkylation inhibited. The absence of any identifiable $\Delta^{8(9),14}$ intermediates in *C. emersonii* offers a second more exciting possibility—perhaps *C. emersonii* does not carry biosynthesis through this route at all.

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