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THE INHIBITION OF CYTOPLASMIC ACETOACETYL-COA THIOLASE BY A TRIYNE CARBONATE (L-660,631)

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The compound L-660,631 (2-oxo-5-(1-hydroxy-2,4,6-heptatriynyl)-1,3-dioxolane-4 heptanoic acid), a natural product isolated from an Actinomycete culture, was found to inhibit rat liver cytosolic acetoacetyl-CoA thiolase, the first step in the cholesterol biosynthesis pathway, with an IC_{50} of 1.0 x 10^{-8} M. The inhibitor had no effect on other sulfhydryl containing enzymes of lipid synthesis such as HMG-CoA synthase, HMG-CoA reductase, and fatty acid synthase. When tested in cultured human liver Hep G2 cells the compound inhibited the incorporation of ^{14}C-acetate and ^{14}C-octanoate into sterols 56% and 48% respectively at 3 x 10^{-6} M with no effect on fatty acid synthesis. No noticeable effect was seen on fatty acid biosynthesis. This strongly suggests that the locus of inhibition of acetate incorporation into sterols found with this compound is the acetoacetyl-CoA thiolase step in the cholesterol biosynthesis pathway.

In the past few years a number of natural products from microorganisms have been found to inhibit some of the early steps of cholesterol biosynthesis in cells. Lovastatin and Mevastatin which were found in Aspergillus terreus (1) and Penicillium citrinum (2) respectively, inhibited the enzyme HMG-CoA reductase. Recently a β-lactone (L-659,699) which blocked the enzyme HMG-CoA synthase, the enzyme catalyzing the reaction immediately prior to HMG-CoA reductase, was isolated from both Fusarium sp. (3) and Scopulariopsis sp. (4). All of these inhibitors of the early steps in cholesterol biosynthesis also inhibited overall synthesis of sterols in cultured cells as well as in animals (1, 3).

It has recently been reported that a novel triyne carbonate independently isolated from an Actinomyctes culture (5) (L-660,631) or from Microbispora (6) (EV-22) inhibited the growth of Candida albicans (see figure 1). Furthermore, it was found that at the minimum inhibitory concentration

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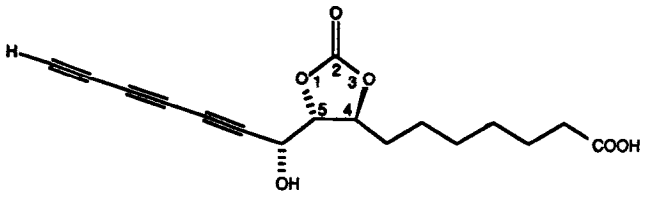


L-660,631

Figure 1. L-660,631: 2-oxo-5-(1-hydroxy-2,4,6-heptatriynyl)-1,3-dioxolane-4-heptanoic acid.

(MIC) for C. albicans the incorporation of ^{14}C-acetate into ergosterol in this organism was also inhibited (7). Whether inhibition of sterol synthesis was in fact the specific mechanism for inhibition of cellular growth was not further explored in these communications.

MATERIALS AND METHODS

Triyne carbonate (L-660,631) was obtained from Actinomycetes fermentation. The compound was isolated as described by Kempf et al (5). Acetyl CoA and acetoacetyl-CoA were purchased from Pharmacia-PL, and ^{14}C-acetyl CoA from ICN, and 1-^{14}C-octanoate from New England Nuclear.

Enzyme assays: HMG-CoA synthase was assayed as described by Greenspan et al (3), and HMG-CoA reductase by the method of Alberts et al (1). Fatty acid synthase was prepared and assayed by the method Alberts et al (8).

Acetoacetyl-CoA thiolase was assayed using either a spectrophotometric or a radioactive assay. The spectrophotometric assay followed the disappearance of the β-keto thioester, acetoacetyl-CoA, at 303 nm as described originally by Vagelos and Alberts (9) and modified to measure thiolase activity as described by Clinkenbeard et al (10). Thiolase can also be measured in the direction of the synthesis of acetoacetyl-CoA formation by coupling the synthesis of acetoacetyl-CoA to the production of HMG-CoA by addition of thiolase-free HMG-CoA synthase (3, 11). For this assay the incubation contained 44.4 μM ^{14}C-acetyl-CoA (4.5 μCi/μmole), 100 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 27.0 μg of HMG-CoA synthase (sp act. 10.3 nmoles/min/mg protein) and 31.5 μg of β-keto thiolase (sp. act. 4.02 nmole/min/mg)in a reaction volume of 100 ul. The enzymes and cocktail were preincubated with the compound for 5 min at 30°C, and the reaction was started by the addition of ^{14}C-acetyl-CoA. After 5 minutes the reaction was stopped by the addition of 50 μl of the reaction mixture to 100 μl of 6N HCl in a glass scintillation vial. The vial was heated for 1 hour at 95°C after which another 100 μl of 6N HCl was added and the heating continued for another hour. 1.0 ml of saline followed by 10 ml of scintillation fluid was added and the radioactivity of the sample determined.

Incorporation of ^{14}C-acetate or ^{14}C-octanoate into sterols and fatty acids in cultured Hep G2 cells was carried out as described in Greenspan et al (3).

RESULTS

The triyne carbonate, L-660,631, inhibited rat liver cytoplasmic acetoacetyl-CoA thiolase 50% at 1.0 x 10^{-8} M (Table 1). Other enzymes involved in lipid biosynthesis such as HMG-CoA synthase, HMG-CoA reductase,

TABLE 1

Inhibition of Rat Liver Lipogenic Enzymes by L-660,631

Enzyme	Concentration (M)	Inhibition (%)
β-ketoacyl-CoA thiolase[a]	1.0×10^{-8}	50
HMG-CoA synthase	1.5×10^{-4}	26
HMG-CoA reductase	1.3×10^{-4}	35
Fatty acid synthase	1.4×10^{-4}	34

[a]IC_{50} was determined by the radioactive assay. All other enzyme assays were performed as outlined in the Methods section of the text.

and fatty acid synthase were slightly inhibited by the compound at concentrations that were four-logs greater than that seen with thiolase (Table 1). Although the radioactive assay of the thiolase was used in this study, similar inhibition of the enzyme was seen when assaying the enzyme in the direction of acetoacetyl-CoA breakdown by the spectrophotometric assay.

A number of analogs of L-660,631 were prepared and assayed for their ability to inhibit the thiolase. The activities of these compounds are described in Table 2. Methylation of the carboxyl (L-663,992) or acylation of the hydroxyl (L-665,436) had only a minor effect on the activity. However, both methylation and acylation on the same molecule resulted in over a ten-fold reduction in activity (L-665,148), and these changes combined with changing the terminal H to a methyl caused a 50-fold loss (L-667,191).

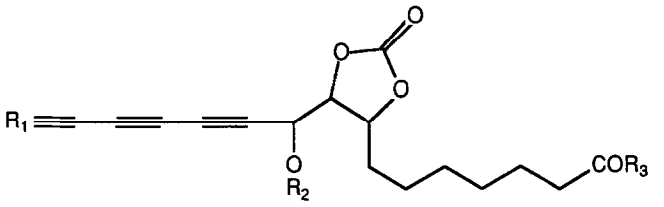
Table 3 examines the ability of L-660,631 to inhibit overall sterol biosynthesis in a cellular system. A concentration of 3×10^{-6} M inhibited the incorporation of radioactive acetate into sterols by 56% with no effect on fatty acid biosynthesis. Higher concentrations of L-660,631 were also without effect on fatty acid synthesis. The effect of the triyne carbonate on mitochondrial β-ketoacyl-CoA thiolase was determined by adding 1-^{14}C-octanoate to the cells as the radioactive substrate for sterol synthesis. In this case the results were similar to that found with ^{14}C-acetate, sterol synthesis was inhibited 48% with no effect on incorporation into fatty acids.

DISCUSSION

A number of studies have indicated that the triyne carbonate (L-660,631) inhibits the growth of filamentous fungi, yeast, and gram positive bacteria (6, 7). Furthermore, the studies of Onishi et al (7), showed that

TABLE 2

Inhibition of β-Ketoacyl-CoA Thiolase by Analogs of L-660,631



Compound ID. #	R_1	R_2	R_3	Inhibition of Thiolase ($IC_{50} X 10^{-8} M$)
660,631	H	H	—OH	1.0
663,992	H	H	$-OCH_3$	2.0
665,436	H	$-\overset{O}{\overset{\|}{C}}-CH_3$	—OH	0.9
665,148	H	$-\overset{O}{\overset{\|}{C}}-CH_3$	$-OCH_3$	15.0
667,191	CH_3	$-CH_3$	$-OCH_3$	56.0

The compounds were dissolved in DMSO and their effect on the β-keto thiolase was determined. Except as noted, compounds were preincubated with the β-keto thiolase and the reaction was started with the addition of CoA in the spectrophotometric assay as described in the Methods section.

TABLE 3

Inhibition of Cholesterol and Fatty Acid Synthesis from ^{14}C-Acetate and ^{14}C-Octanoate in Hep G2 Cells

L-660,631 ($x10^{-5}$ M)	Percent Inhibition			
	^{14}C-Acetate Incorporation		^{14}C-Octanoate Incorporation	
	Cholesterol	Fatty Acids	Cholesterol	Fatty Acids
0.3	56	0	48	0
1.5	83	6	79	2.2
3.0	--	--	86	6.9

Cells were grown in MEM containing 10% FCS then switched to serum-free Higuchi Media (16) for 48 hours. L-660,631 in DMSO and the appropriate radioactive substrate was added to 3 ml of media and the incubations continued for 3 hours. Sterols and fatty acids were extracted by the Bligh-Dyer method (17), saponified and chromatographed on silica gel thin layer plates in Petroleum Ether:Diethyl Ether:Acetic Acid (75:25:1). The areas corresponding to standard cholesterol and oleic acid were scrapped and counted in scintillation vials.

sterol synthesis in Candida albicans was inhibited at MIC concentrations of the compound, and suggested that the mechanism of inhibition of growth was by interfering with mevalonate synthesis in the organisms.

The studies described here suggest that growth inhibition of the organism was probably caused by specific inhibition of the cytoplasmic synthesis of acetoacetyl-CoA needed for sterol synthesis. The specificity of L-660,631 for cytoplasmic thiolase was particularly interesting from a mechanistic point of view, since several reports have indicated that a common feature in the mechanisms of β-ketoacyl-CoA thiolases (12), fatty acid synthase (13), and HMG-CoA synthase (14, 15) involved an initial trans-acylation with a sulfhydryl group in the active site of the enzyme to form an acyl-S-enzyme intermediate. And yet only the thiolase was affected by the compound. Like lovastatin and the β-lactone L-659,699 (3) the triyne carbonate, L-660,631, inhibited the incorporation of acetate into cellular sterols but not into fatty acids. The specificity of the inhibitor was further explored in the cultured Hep G2 cells by examining sterol and fatty acid synthesis from radioactive octanoate. Although only the mitochondrial β-ketoacyl-CoA is involved in the generation of ^{14}C-acetyl-CoA from 1-^{14}C-octanate the fact that incorporation of radioactivity from octanoate into fatty acids was not inhibited suggested that at least one of the mitochondrial thiolases and perhaps both were not affected by the compound. This lack of activity with the mitochondrial thiolase could be due to the specificity of the compound for the cytoplasmic enzyme or that it simply was not able to get into the mitochondria. This question was not further explored in this study.

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