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Mechanism of Action of CM-55, a Synthetic Analogue of the Antilipogenic Antibiotic Cerulenin

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CM-55 is a synthetic analogue of the antibiotic cerulenin with the chemical structure of 2, 3-dodecenyl-4-oxo-dimethyl amide. This compound inhibited the growth of *Saccharomyces cerevisiae* ATCC 12341 and inhibited protein and lipid synthesis by 91 and 95%, respectively, at a concentration of 50 μg/ml (2.1×10^{-4} M). The inhibition of protein synthesis was associated with the partial reduction of ribonucleic acid synthesis and leucine transport. The mechanism of inhibition of lipid synthesis was further investigated in a cell-free extract of the yeast. CM-55 inhibited the incorporation of [^{14}C]acetyl Coenzyme A (CoA) into both fatty acid (FAF) and non-saponifiable fractions (NSF). However, it did not inhibit [^{14}C]malonyl CoA incorporation into FAF and only slightly inhibited [^{14}C]mevalonate incorporation into NSF. The activity of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthase was inhibited more strongly than the incorporation of [^{14}C]3-hydroxy-3-methylglutaryl CoA into NSF; this could account for the CM-55 inhibition of [^{14}C]acetyl CoA incorporation into NSF.

Cerulenin is an antibiotic found in the culture filtrate of *Cephalosporium caerulens* (8) and has the structure (2S)(3R)-2,3-epoxy-4-oxo-7,10-dodecadienoyl amide (Fig. 1) (1). It is the first antibiotic that attacks lipid synthesis as its primary action (9), and the action sites were determined to be fatty acid synthetase (14) and 3-hydroxy-3-methylglutaryl (HMG) Coenzyme A (CoA) synthase in yeast and β-ketoacylacyl carrier protein synthetase in *Escherichia coli*.

However, when cerulenin was used for treatment of *Candida albicans*-infected mice, it was hard to cure the animals of the infectious disease, and the drug was found to have been rapidly inactivated in serum (unpublished observations). As has been presented by D'Agnolo et al. (2), the epoxide ring at the 2-3 position of cerulenin is essential for the inhibition of β-ketoacylacyl carrier protein synthetase activity, but the two double bonds at the 7 and 10 positions are not necessarily essential, though they are important, for inhibitory activity. The integrity of the epoxide is also required for antimicrobial activity (11). Therefore, the rapid inactivation of cerulenin was assumed because of the degradation of the epoxide ring by mammalian epoxidase.

Efforts have been made to find more stable and active synthetic analogues of cerulenin. One of them, CM-55 (2,3-dodecenyl-4-oxo-dimethyl amide [Fig. 1]), lacks an epoxide function but has an antimicrobial spectrum quite similar to that of cerulenin (11). These characteristics led us to investigate its mode of action compared with that of cerulenin. Although CM-55 inhibited lipid synthesis of yeast as cerulenin does, we found quite different action mechanisms.

MATERIALS AND METHODS

Chemicals. Cerulenin was obtained from the culture broth of *C. caerulens* KF-140 by the methods previously reported (6). CM-55 was synthesized as previously reported (11). Fresh ethanol solutions of both drugs were prepared immediately before conducting each experiment. The final concentration of ethanol was always kept lower than 0.1%, and the same amount of ethanol was added to the control groups.

[1-^{14}C]sodium acetate, L-[^{14}C]leucine (uniformly labeled), DL-[2-^{14}C]mevalonic acid dibenzylethylenediamine salt, L-[1-^{14}C]-*N*-acetylglucosamine, [6-^{14}C]orotic acid [^{14}C]sodium bicarbonate and [6-^{3}H]thymidine were purchased from Daiichi Chemicals Co. Ltd., Tokyo. [1-^{14}C]acetyl CoA, [1,3-^{14}C]malonyl CoA, and [3-^{14}C]3-hydroxy-3-methylglutaric acid were purchased from New England Nuclear Corp., Boston. [3-^{14}C]CoA was synthesized by the methods of Goldfarb and Pitot (5). CoA, acetyl CoA, and acetoacetyl CoA were purchased from Sigma Co., St. Louis. Highest-quality sodium laurate, sodium palmitate, sodium oleate, and ergosterol were purchased from Kanto Chemicals Co. Ltd., Tokyo, and used without further purification.

Growth of yeast. *Saccharomyces cerevisiae* ATCC

CERULENIN

CM-55

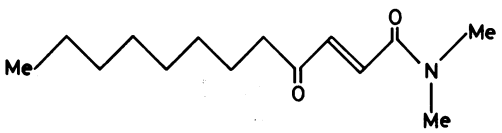

FIG. 1. *Structure of cerulenin and CM-55.*

12341 was grown on Kawaguchi's nutrient agar at 27 C for 48 h and transferred to semisynthetic liquid medium (7). After cultivation at 27 C for 48 h on a reciprocal shaker, an equal volume of fresh medium was added and shaking was continued further 1.5 h. Then a portion (0.3 ml) was transferred to a Monod-type tube containing 5 ml of fresh medium and incubated at 27 C in a water bath. Growth of cells was measured by the absorption at 620 nm.

Incorporation of radioactive precursors in vivo. To the cells growing exponentially, an indicated amount of CM-55 was added. After 15 min of incubation, radioactive precursors with each carrier substrate were added and incubation was continued for another 15 min. The incorporation was terminated by the addition of the same volume of 10% trichloroacetic acid. The cells were trapped on a membrane filter, washed with 20 ml of 5% trichloroacetic acid, dried in a hood, and transferred into a liquid scintillation counting vial.

For the experiments of [^{14}C]acetate incorporation into total lipid fraction, cells were acidified with perchloric acid, collected by centrifugation, and washed twice with 10 ml of 5% perchloric acid. Total lipids were extracted twice with 5 ml of ethanol-ether (1:1) at 45 C for 15 min. The extract was transferred to a counting vial and evaporated to dryness in a hood. Preblended scintillator [0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis-(5-phenyloxazolyl)-benzene in tolune] was added, and the radioactivity of the sample was determined.

Cellular uptake of [^{14}C]leucine and [^{14}C]acetate. The cells grown overnight in Kawaguchi's medium (7) were harvested and suspended in fresh medium. An inhibitor mixture as indicated in the legend of Fig. 5 was added to 5 ml of cell suspension and incubated for 15 min at 27 C; then 1 μCi of [^{14}C]leucine (5 μmol) or [^{14}C]acetate (0.5 μmol) was added, and the incubation was continued. At the indicated time after the ^{14}C addition, sample (0.2 ml) of the suspension was removed and the cells were trapped on a membrane filter, immediately followed by washing with 10 ml of ice-cold 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.0) containing 0.15 M NaCl and 0.5 mM $MgCl_2$. Radioactivites were measured as mentioned above.

Incorporation of radioactive precursors in vitro. A cell-free extract of *S. cerevisiae* was prepared as reported by Kawaguchi (7). Reaction mixtures and incubation conditions were the same as previously reported (10). The non-saponifiable fraction (NSF) was isolated by petroleum ether extraction after the reaction mixture was saponified with 15% KOH in 80% methanol at 60 C for 1 h. The residual aqueous layer was acidified with 2 N H_2SO_4, and then the fatty acid fraction (FAF) was extracted with petroleum ether.

The radioactivity of each fraction was determined by liquid scintillation counting.

Preparation and assay of acetyl CoA carboxylase. Acetyl CoA carboxylase (EC 6.4.1.2) was partially purified from *S. cerevisiae* ATCC 12341 according to the method of Sumper and Riepertinger (13) up to the step of first $(NH_4)_2SO_4$ fractionation. Enzyme activity was assayed by [^{14}C]bicarbonate fixation.

Preparation and assay of acetoacetyl CoA thiolase and HMG CoA synthase. Acetoacetyl CoA thiolase (EC 2.3.1.9) and HMG CoA synthase (EC 4.1.3.5) were partially copurified from baker's yeast (a generous gift from Oriental Yeast Co. Ltd., Tokyo) according to the method of Ferguson and Rudney (3) up to the step of 80% saturated $(NH_4)_2SO_4$ precipitation. Enzyme activities were assayed spectrophotometrically at room temperature as has been reported (10). Specific activities were 13.9 and 9.7 U/mg for acetoacetyl CoA thiolase and HMG CoA synthase, respectively.

RESULTS

Growth of S. cerevisiae. When cerulenin or CM-55 was added to a suspension of *S. cerevisiae* ATCC 12341 cells, growth was markedly inhibited by both of the agents at the same concentration, 5 μg/ml. However, differences were observed between the patterns of the yeast growth affected by the drugs (Fig. 2). These differences of inhibitory pattern suggest that CM-55 should have a different action mechanism(s) from cerulenin. This was also confirmed by an experiment (Fig. 3) showing that when a mixture of laurate, palmitate, oleate, and ergosterol was added to a culture containing cerulenin or CM-55, cerulenin did not inhibit cellular growth. However, CM-55 did not allow this kind of reversion.

Syntheses of macromolecules. Effects of CM-55 were surveyed on yeast deoxyribonucleic acid, ribonucleic acid, protein, polysaccharide, and lipid syntheses by a method of radioactive precursor incorporation (Fig. 4). When cells were pretreated for 15 min with CM-55, [^{3}H]thymidine and [^{14}C]*N*-acetylglucosamine incorporation into the acid-insoluble fraction was not or only slightly altered in a range of 2 to 50 μg of CM-55. [^{14}C]orotic acid incorporation was reduced to approximately 50% at a concentration of 50 μg/ml. The most remarkable inhi-

bition was observed in [^{14}C]leucine incorporation into the acid-insoluble fraction and [^{14}C]acetate incorporation into the total lipid fraction. The concentrations giving 50% inhibition were approximately 15 and 20 μg/ml for [^{14}C]leucine and [^{14}C]acetate incorporation, respectively. Therefore, it is conceivable that CM-55 suppresses the yeast growth by inhibiting mainly protein synthesis and lipid synthesis.

Cellular uptake of [^{14}C]leucine and [^{14}C]acetate. To investigate whether CM-55 inhibits protein and lipid synthesis directly or indirectly, cellular uptake of [^{14}C]leucine or [^{14}C]acetate was measured under conditions in which protein synthesis was inhibited with cycloheximide or lipid synthesis was inhibited by cerulenin. CM-55 at 50 μg/ml reduced the rate of uptake of [^{14}C]leucine to approximately 50% of control (Fig. 5a). Therefore, part of the inhibition of protein synthesis by CM-55 may be due to the reduction of leucine transport, although the 91% inhibition of [^{14}C]leucine incorporation cannot be accounted for by this reduction. On the other hand, CM-55 did not reduce the [^{14}C]acetate uptake (Fig. 5b), suggesting that CM-55 inhibits solely the enzymatic reaction(s) of lipid synthesis.

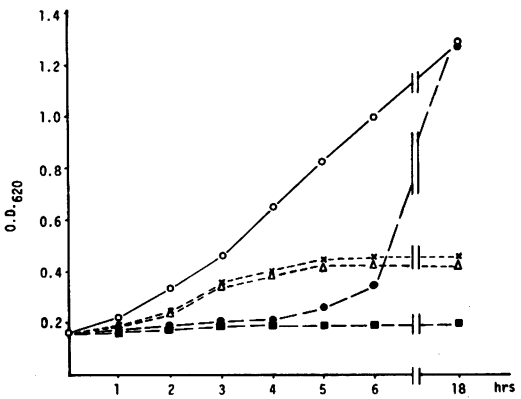

FIG. 2. *Effects of cerulenin and CM-55 on cellular growth of S. cerevisiae ATCC 12341. Symbols:* O, *control;* ×, *cerulenin at 5 μg/ml;* Δ, *cerulenin at 40 μg/ml;* ●, *CM-55 at 5 μg/ml;* ■, *CM-55, at 10 μg/ml or more.*

Inhibition sites of fatty acid and sterol synthesis. To clarify the action site(s) of CM-55 on lipid synthesis, a cell-free extract of the yeast was prepared and provided for incorporation studies of labeled substrates of each enzymatic step into the FAF or NSF. When [^{14}C]acetyl CoA was used as a precursor, both incorporation into FAF and NSF was inhibited by CM-55 (Fig. 6), and this inhibition was potent enough to account for the inhibition of [^{14}C]acetate incorporation into total lipid measured in vivo (Fig. 4). When [^{4}C]malonyl CoA was added to the reaction mixture, no inhibition was observed in the incorporation into FAF (Fig. 6a). This fact suggests that CM-55 inhibits specifically acetyl CoA carboxylase activity in overall fatty acid synthesis.

To confirm CM-55 inhibition of acetyl CoA carboxylase activity, the enzyme was partially purified by the method of Sumper and Riepertinger (13) up to the step after the dialysis of 35% saturated $(NH_4)_2SO_4$ precipitate. Acetyl CoA

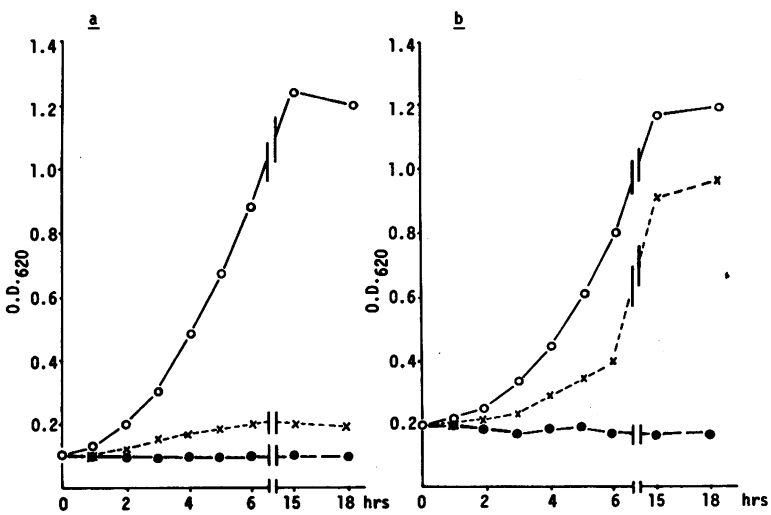

FIG. 3. *Reversion of inhibited growth of yeast by added lipid mixture. (a) Control culture. (b) To the medium, a lipid mixture consisting of sodium laurate, sodium palmitate, sodium oleate, and ergosterol was added to a final concentration of 40 μg/ml each. Symbols:* O, *control;* ×, *cerulenin at 40 μg/ml;* ●, *CM-55 at 10 μg/ml.*

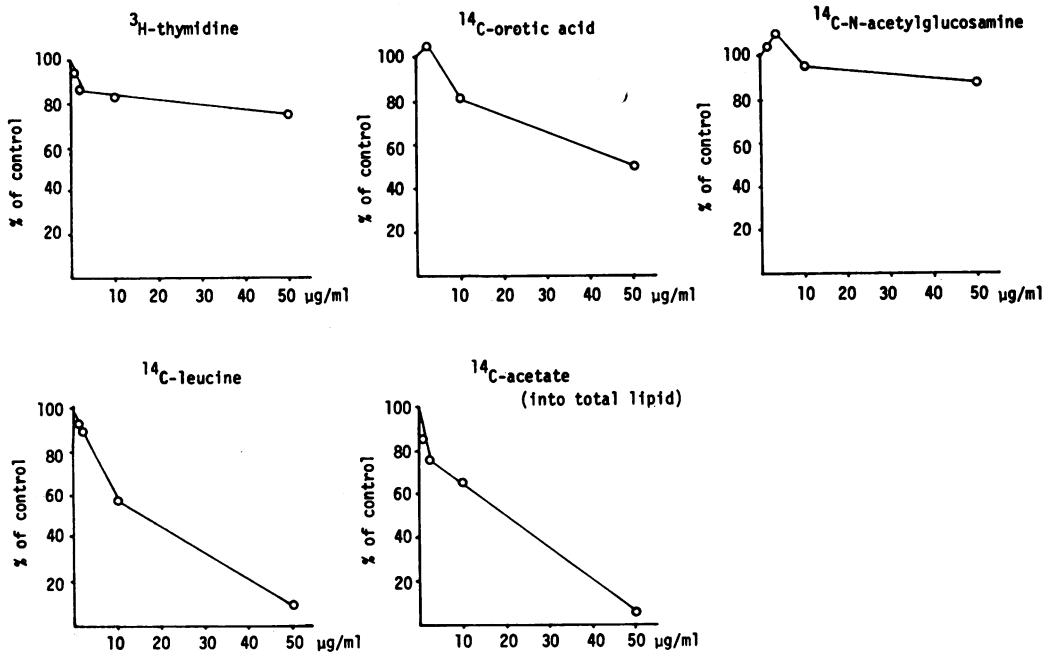

FIG. 4. *Effects of CM-55 on labeled precursor incorporation into macromolecules in yeast. Yeasts were preincubated with CM-55 for 15 min at 27 C, and then the labeled precursors were added to a final concentration of 0.2 µCi/ml (0.1 mM) for [^{3}H]thymidine and [^{14}C]acetate, and 0.5 µCi/ml (1 mM) for [^{14}C]leucine. Incubations were done for 15 min at 27 C. Except for [^{14}C]acetate incorporation into total lipid fraction, all the incorporations were into the acid-insoluble fraction.*

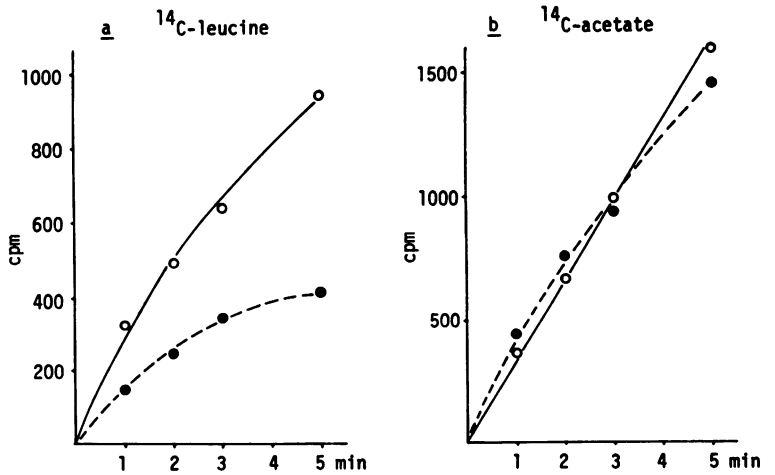

FIG. 5. *Effects of CM-55 on cellular uptake of [^{14}C]leucine and [^{14}C]acetate in yeast. Cells were preincubated with a mixture of inhibitors for 15 min, and then (a) [^{14}C]leucine or [^{14}C]acetate was added and a portion was sampled for ^{14}C determination at every minute. Added mixtures of inhibitors: (a) O, cycloheximide, 50 µg/ml; ●, cycloheximide + CM-55, 50 µg/ml each; (b) O, cerulenin, 50 µg/ml; ●, cerulenin + CM-55, 50 µg/ml each.*

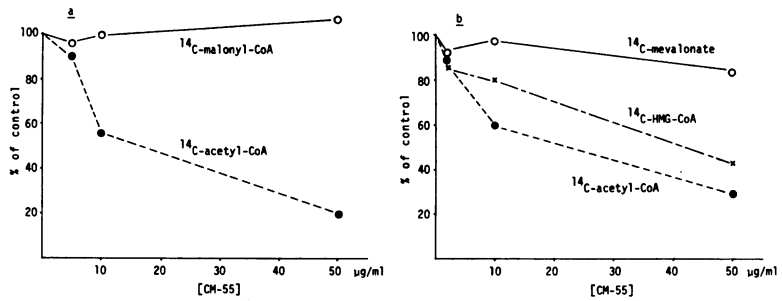

FIG. 6. *Effects of CM-55 on incorporation of labeled precursors of lipid synthesis in a yeast cell-free extract. (a) [^{14}C]acetyl CoA and [^{14}C]malonyl CoA incorporation into the fatty acid fraction. (b) [^{14}C]acetyl CoA, [^{14}C]HMG CoA, and [^{14}C]mevalonate incorporation into the non-saponifiable fraction.*

carboxylase activity was inhibited by CM-55 in a linear manner over a range of 0 to 350 µg/ml. However, the dose for 50% inhibition was 200 µg/ml, which is higher than the dose for 50% inhibition of [^{14}C]acetyl CoA incorporation into FAF. More precise investigations are required to elucidate this phenomenon.

Fig. 6b illustrates marked inhibition of [^{14}C]HMG CoA incorporation but slight inhibition of [^{14}C]mevalonate incorporation into NSF. Since HMG CoA reductase (EC 1.1.3.4) was generally thought to be a rate-limiting enzyme of overall sterol synthesis (12), the inhibition of [^{14}C]HMG CoA incorporation into NSF should most effectively contribute to the CM-55 inhibition of overall sterol synthesis. However, [^{14}C]acetyl CoA incorporation was inhibited by, for example, 70% at 50 µg/ml, whereas [^{14}C]HMG CoA incorporation was inhibited by 57% (Fig. 6b). This suggests that other steps, i.e., from acetyl CoA to HMG CoA, may be inhibited more potently by CM-55.

Inhibition of acetoacetyl CoA thiolase and HMG CoA synthase. Two enzymatic steps have been known in the path between acetyl CoA and HMG CoA, namely acetoacetyl CoA thiolase and HMG CoA synthase. When these enzymes were copurified and assayed (3,10), CM-55 markedly inhibited the activity of HMG CoA synthase and, to a lesser extent, the activity of acetoacetyl CoA thiolase (Fig. 7). Although HMG CoA synthase is not known as the rate-limiting enzyme of sterol synthesis, the inhibition (85% at 50 µg of CM-55 per ml) is enough to explain the inhibition of acetyl CoA incorporation into NFS (Fig. 6b).

DISCUSSION

This paper reveals the apparently different action mechanisms of CM-55 and cerulenin, though both agents showed almost the same antimicrobial spectrum (11). CM-55 delayed

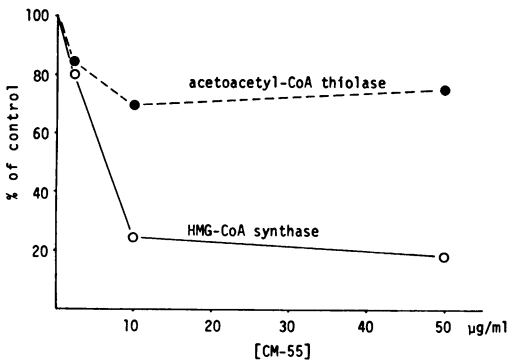

FIG. 7. *Effect of CM-55 on the activity of acetoacetyl CoA thiolase and HMG CoA synthase.*

the initiation of yeast cell growth, whereas cerulenin seemed to reduce the maximal growth of yeast (Fig. 2). This difference is thought to be at least partly derived from the difference of the inhibition of protein synthesis by each agent. In case of cerulenin, inhibition of protein synthesis was not observed in *Escherichia coli*, though ribonucleic acid synthesis was affected to a limited extent (25% inhibition) at a high concentration of cerulenin (4). In *Candida stellatoidea*, a slight inhibition was observed in protein synthesis, whereas ribonucleic acid synthesis was not affected (9). However, lipid synthesis was inhibited almost completely on both organisms under the same conditions. Therefore, cerulenin is thought to be specific for inhibition of lipid synthesis, which is supported by reversion of growth by the external lipid mixture (Fig. 3). On the other hand, CM-55 inhibited protein and lipid synthesis almost equally, and these inhibitions were associated with the reduction of ribonucleic acid synthesis (Fig. 4) and the amino acid transport (Fig. 5), resulting in no reversion of cell growth on addition of the exogenous lipid mixture (Fig. 3).

A typical difference in cerulenin and CM-55

action was also seen in the inhibition of lipid synthesis. Cerulenin has been known to specifically inhibit the condensing enzyme of fatty acid synthetase complexes (14), whereas CM-55 inhibited the activity of acetyl CoA carboxylase, the rate-limiting enzyme of overall fatty acid synthesis. In the path of sterol synthesis, CM-55 could affect three enzymes, acetoacetyl CoA thiolase, HMG CoA synthase, and HMG CoA reductase, whereas cerulenin is specific for HMG CoA synthase (10).

On the mechanism(s) of CM-55 action in protein synthesis, we tried only to clarify whether CM-55 inhibits protein synthesis by reducing membrane transport of amino acids, since marked inhibition of lipid synthesis by CM-55 may affect membrane transport of substrates necessary for protein synthesis. Although CM-55 inhibited leucine transport by approximately 50% (Fig. 5a), this was not enough to account for 91% inhibition of protein synthesis by the same concentration of CM-55. Therefore, further studies, especially in vitro, are required to clarify the mechanism(s) of CM-55 inhibition of protein synthesis in yeast.

All of these differences between cerulenin and CM-55 should be considered along with the differences in the essential partial structures for antimicrobial activities of these agents. Consideration of the structures of cerulenin and CM-55 indicates that the epoxide is required for cerulenin activity (2, 11), whereas dialkylation of the terminal amino is critical for the activity of CM-55 (11). Further studies of the mode of action of CM-55 will be necessary to establish structure-activity relationships in these molecules, although these differences in action mechanisms may provide a potent tool for studies of metabolic regulations, especially for regulation of lipid synthesis.

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