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The Antibiotic Cerulenin, a Novel Tool for Biochemistry as an Inhibitor of Fatty Acid Synthesis

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INTRODUCTION

One of the most versatile uses of antibiotics is as potent drugs for clinical application. In recent years, attention has also been paid to agricultural uses of antibiotics, such as for feed additives for protecting plants and livestock against infectious diseases and for accelerating their growth. They are also used as food additives to retain freshness for an extended period.

The usefulness of antibiotics is not limited only to our daily needs, but also encompasses our research interests: they offer us remarkable experimental devices for biochemistry—novel biochemical tools, which have made a significant contribution to progress in this field (18).

Cerulenin, an antibiotic discovered by Hata et al. in 1960, was originally found as an antifungal antibiotic (30). Studies of its mode of action have revealed that it specifically inhibits the biosynthesis of fatty acids and sterols involving yeasts (55, 56). It should be particularly noted that such specificity of cerulenin has been used by investigators in various fields of biochemistry. In this connection, the present review deals with studies, which have hitherto been reported, on the production, isolation, structure, and mode of action of cerulenin and its application as a biochemical tool. Unfortunately, the instability of the antibiotic in the animal body prevents its use in therapy as an antimicrobial agent or as an antilipogenic agent.

PRODUCING STRAIN AND PRODUCTION OF CERULENIN

The cerulenin-producing strain has some unique characteristics: it forms aerial hyphae and conidiophore when applied on Czapek agar as shown in Fig. 1 and Fig. 2, respectively, and it forms bluish-green aerial mycelium on malt agar. It has thus been classified as genus *Cephalosporium* and accordingly named *Cephalosporium caerulens* (49).

The production of cerulenin was originally performed under cultural conditions of 27 to 32°C by using glucose and glycerol as carbon sources and peptone and corn steep liquor as nitrogen sources (50). Unfortunately, however, it was found that helvolic acid and steroidal antibiotics (Fig. 3) were produced predominantly over cerulenin under these conditions. Such difficulties precluded the usefulness of this method for efficient production of cerulenin. Meanwhile, because much attention began to be paid to its specific mode of action as an inhibitor of lipid biosynthesis, a method was needed to improve the yield of cerulenin. As a matter of course, the condition of fermentative production of the antibiotic that was originally reported was reexamined.

As a beginning step to do so, the antimicrobial activities of cerulenin and helvolic acid were reevaluated. Among the microorganisms listed, *Candida albicans* KF-1 and *Corynebacterium paurometablum* KB-121 were chosen as the most appropriate test microorganisms for the selective bioassay of cerulenin and helvolic acid, respectively (36).

Attempts were subsequently made to investigate the medium for the selective production of cerulenin. Although a previously employed production medium (50) that contained 6.0% glucose, 0.5% peptone, 0.3% NaCl, and 0.3% $CaCO_3$ afforded mostly helvolic acid (350 μg/ml) and a small amount of cerulenin (50 μg/ml), further study revealed that a more suitable medium for the efficient production of cerulenin should contain 1% glucose, 3% glycerol, 0.5% peptone, and 0.2% NaCl. By using this produc-

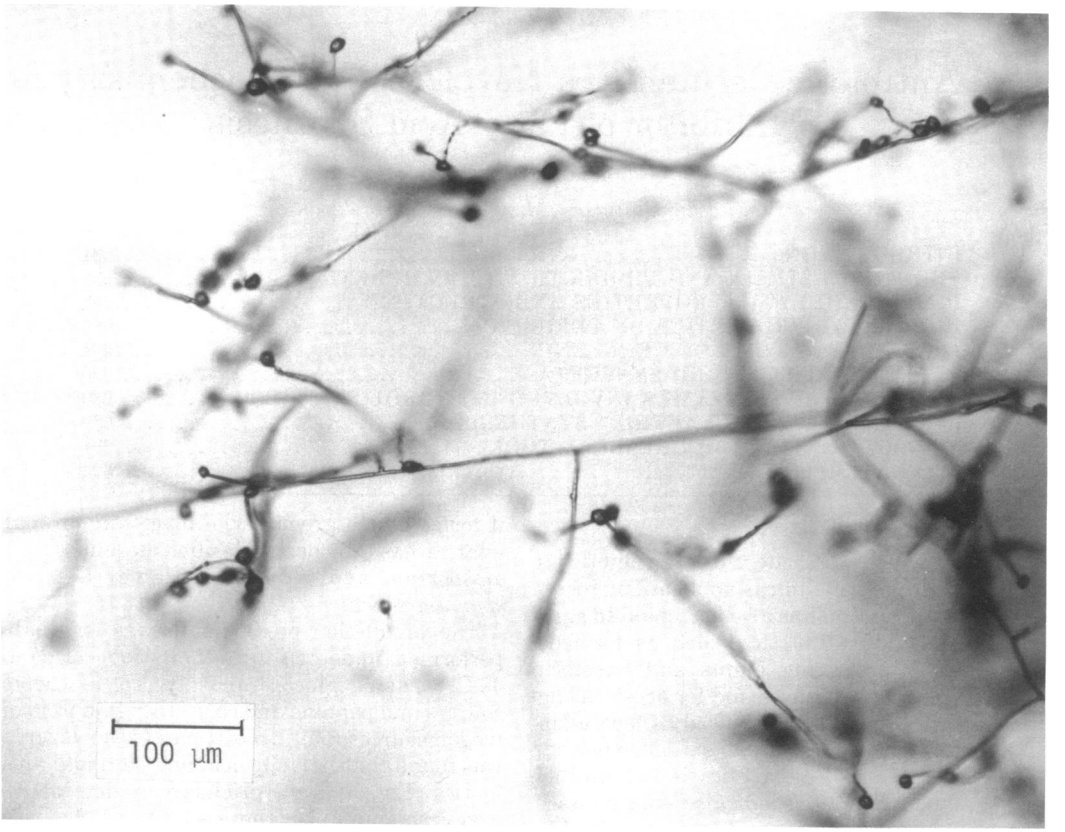


FIG. 1. *Aerial hyphae of C. caerulens, cultured on Czapek agar at 27°C for a week.*

tion medium, 250 µg of cerulenin per ml was obtained, with an almost negligible yield of helvolic acid. Thus, this improved method offered a simple procedure to produce and isolate a relatively large amount of the antibiotic at one time and in a short time.

To prepare ^{3}H-labeled cerulenin under these producing conditions, further studies were made with regard to the effect of the time of the [^{3}H]acetate addition and the glucose concentration in culture broth on the incorporation of [^{3}H]acetate into cerulenin. The results showed that the highest specific radioactivity (4.5×10^{-3} cpm/mg) of ^{3}H-labeled cerulenin was obtained by using the culture broth containing 2% glucose when [^{3}H]acetate (1 µCi/ml) was added 14 h after the initiation of fermentation. ^{13}C-labeled cerulenin was then prepared by adding the [^{13}C]acetate under these conditions. The nuclear magnetic resonance (NMR) spectrum of the ^{13}C-labeled cerulenin thus obtained suggested that its biosynthesis arises from six molecules of acetates. Therefore, the biosynthesis of cerulenin is closely related to that of fatty acids, which are catalyzed by fatty acid synthetase (4).

Helicoceras oryzae (K. Furuya and M. Shirasaka, Japan Kokai patent 45-21638), *Sartorya* sp. (T. Yamano, H. Yamamoto, and S. Itsumi, Japan Kokai patent 47-24156), *Acrocylindrium* sp. (Furuya and Shirasaka, patent 45-21638), etc., have been known as the producing strains of cerulenin other than *C. caerulens*. On the other hand, it is known that helvolic acid, which tends to be produced simultaneously with cerulenin, is produced by *Aspergillus fumigatus* mutant *helvola* (9), *Emericellopsis terricola* (62), and *Acrocylindrium oryzae* (15). It is of interest to note in connection with chemotaxonomy that one compound arises from various kinds of producing strains, as seen in this case.

PHYSICOCHEMICAL PROPERTIES AND STRUCTURE

It should be noted that the isolation and structural determination of antibacterial proto lanostan-type antibiotics, helvolic acid (62), helvolinic acid (63), and 7-desacetoxyhelvolic acid (63), and the pentaprenyl terpenoid antibiotic cepharonic acid (35), preceded those of cerulenin despite the fact that all of these anti-

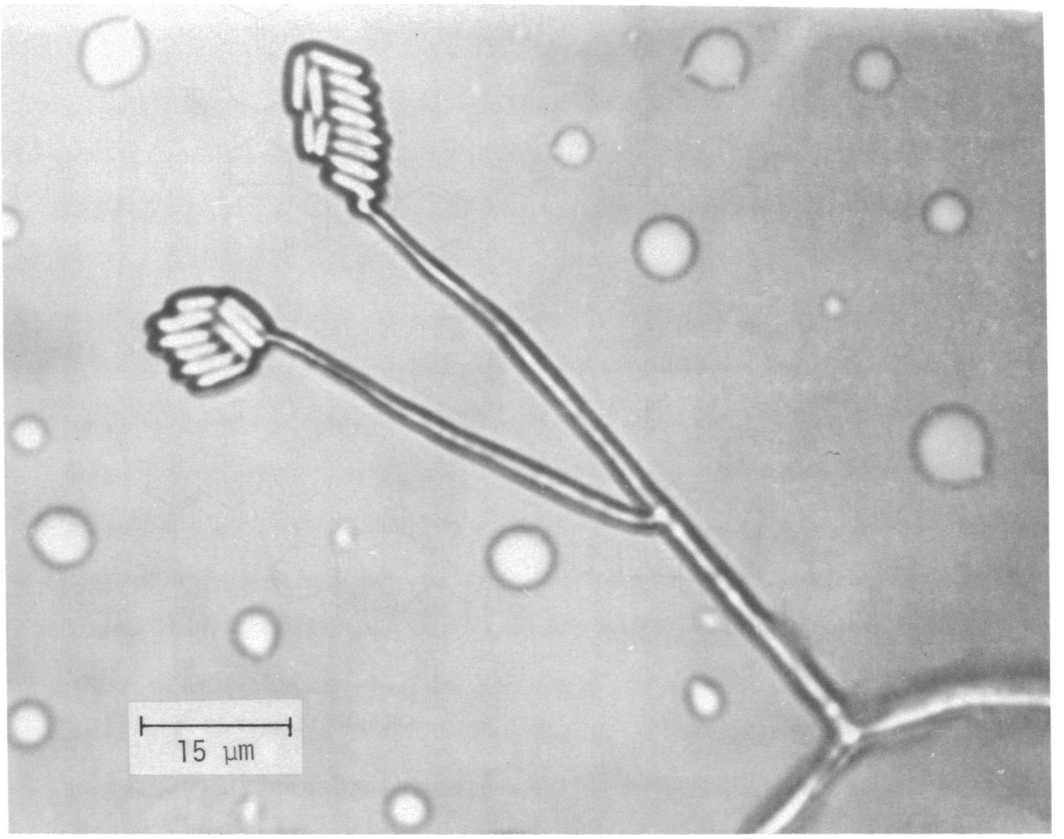


FIG. 2. *Conidiophore of C. caerulens, cultured on Czapek agar at 27°C for a week.*

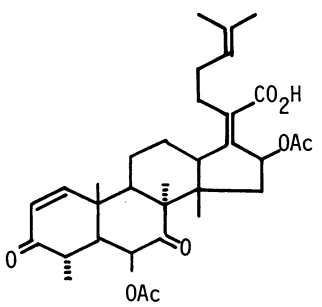
biotics are produced by the same producing strain (Fig. 3). This was mainly due to the fact that cerulenin is somewhat unstable in methanolic solutions and that it does not show a single spot on a thin-layer chromatography plate when eluted with some solvent systems containing methanol. It was thus extremely difficult to prove that it was a single compound. Notwithstanding, molecular weight and the molecular formula were finally determined for the acid by mass and NMR spectroscopic methods.

Table 1 shows the physicochemical properties of cerulenin (76). The infrared spectrum (Fig. 4) (3,425, 3,225, 1,663 cm^{-1}, KBr) and the evolution of ammonia upon alkaline hydrolysis are compatible with the presence of a primary amide moiety in cerulenin (63). It is also suggested by its infrared spectrum that there are *trans*-1,2-disubstituted olefin (969 cm^{-1}) and a carbonyl group (1,720 cm^{-1}). In agreement with the NMR, infrared, and mass spectra of the products obtained by the chemical transformations shown in Fig. 5 (63, 64), comparison of the optical rotary dispersion curve of hexahydrocerulenin with that of L-methyl malate reveals that the steric configuration of the two 1,2-disubstituted olefins are both *trans* and that the absolute configuration of the *cis*-epoxide is (2S,(3R) (63, 64). By the use of NMR, the solvent effect at 100 MHz further establishes the position of the two olefinic bonds. Thus it is unambiguously concluded that the structure of cerulenin is (2S),(3R) 2,3-epoxy-4-oxo-7,10-dodecadienoyl amide (3).

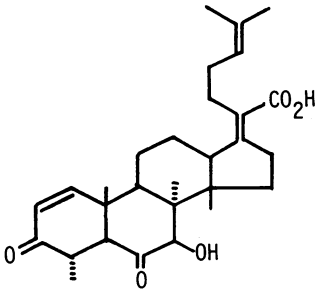
The fact that cerulenin is unstable in methanolic solutions is attributed to the structural transformation of the epoxide moiety located between the two carbonyl groups on C1 and C4 as evidenced by its CD spectrum (64).

BIOLOGICAL PROPERTIES OF CERULENIN

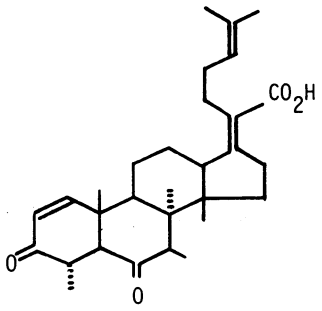
As indicated in Table 2 and Table 3, cerulenin possesses a remarkable growth-inhibitory action on various strains of mycobacteria, except *Mycobacterium tuberculosis, Nocardia*, and *Streptomyces*. A weak growth-inhibitory activity against other bacteria is also recognized. Furthermore, it inhibits significantly



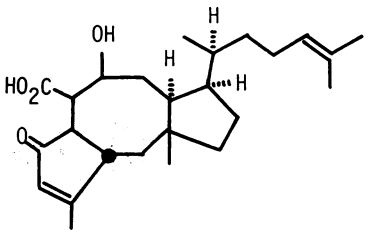
Helvolic acid (62)



Helvolinic acid (63)



7-Desacetoxy helvolic acid (63)



Cephalonic acid (35)

FIG. 3. *Products synthesized with cerulenin by C. caerulens.*

TABLE 1. *Physicochemical properties of cerulenin*

Property	Expt results
Molecular formula	$C_{12}H_{17}O_3N$ (mol wt 223.26)
mp	93-94°C
$[\alpha]_D^{26}$	−12° (C 1.0, $CHCl_3$)[a]
Solubility	Soluble in $CHCl_3$, CCl_4, EtOAc, benzene; slightly soluble in H_2O
Ultraviolet	End absorption in H_2O
Infrared	Fig. 2

[a] In a previous report (76), the optical rotation of cerulenin was reported to be $[\alpha]_D^{25}$ +63° (C 2.0, MeOH); however, it was reexamined in $CHCl_3$ because of the instability of cerulenin in MeOH.

the growth of yeastlike fungi, such as *Candida*, *Saccharomyces*, *Cryptococcus*, *Geotrichum*, etc., and *Trichophyton*, *Epidermophyton*, *Microsporium*, *Piricularia oryzae*, *Ophiobolus miyabeanus*, etc.

Cerulenin shows neither hemolytic action on various erythrocytes nor irritability on the conjugative mucous membrane of rabbits. Its toxicity to mice is, consequently, relatively low (51) (Table 4).

It has been found that cerulenin causes curling of the microorganisms that belong to filamentous fungi, such as *Trichophyton* and *Microsporium* (51). Among other derivatives of cerulenin in Fig. 5, the only one that exhibits antimicrobial activity is tetrahydrocerulenin (16, 66). This in turn suggests that the presence of the epoxide moiety is responsible for its salient antimicrobial activity.

ACTION MECHANISM OF CERULENIN

It has been reported that helvolic acid simultaneously produced with cerulenin inhibits the protein synthesis of *Escherichia coli* (22; Yamano et al., patent 47-24156). Studies of the action mechanism of cerulenin have begun with the intriguing observation that the antibiotic exhibits both antimicrobial and antifungal activity.

At the first period, the influence of cerulenin on the incorporation and the exogenous respiration of [^{32}P]orthophosphoric acid, ^{14}C-labeled amino acid, and [1-^{14}C]glucosamine into each biopolymer by using the intact cells of *Candida stellatoidea*, which are markedly sensitive to cerulenin, was investigated (55). Nevertheless, no significant effects were observed. Subsequently, a wide variety of biological substances were employed to investigate whether or not the antifungal activity of cerulenin was reversed by them. The antifungal activity of ceru-

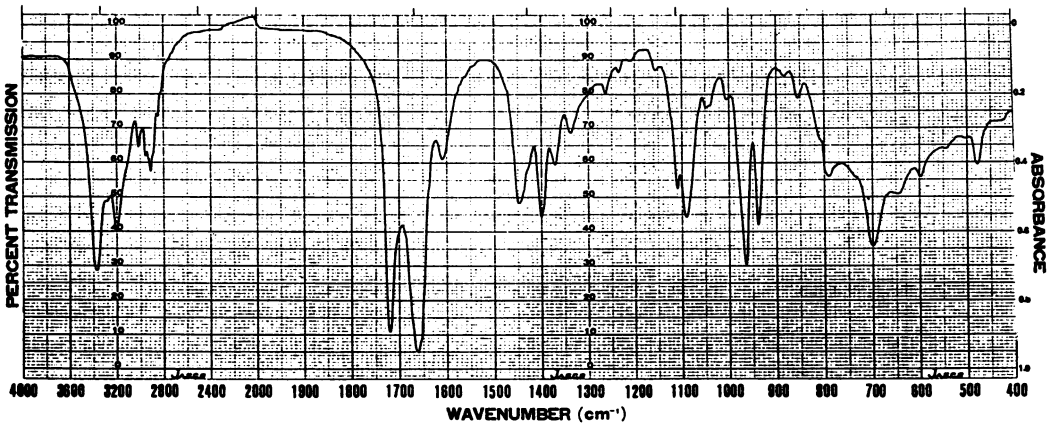


FIG. 4. *Infrared spectrum of cerulenin.*

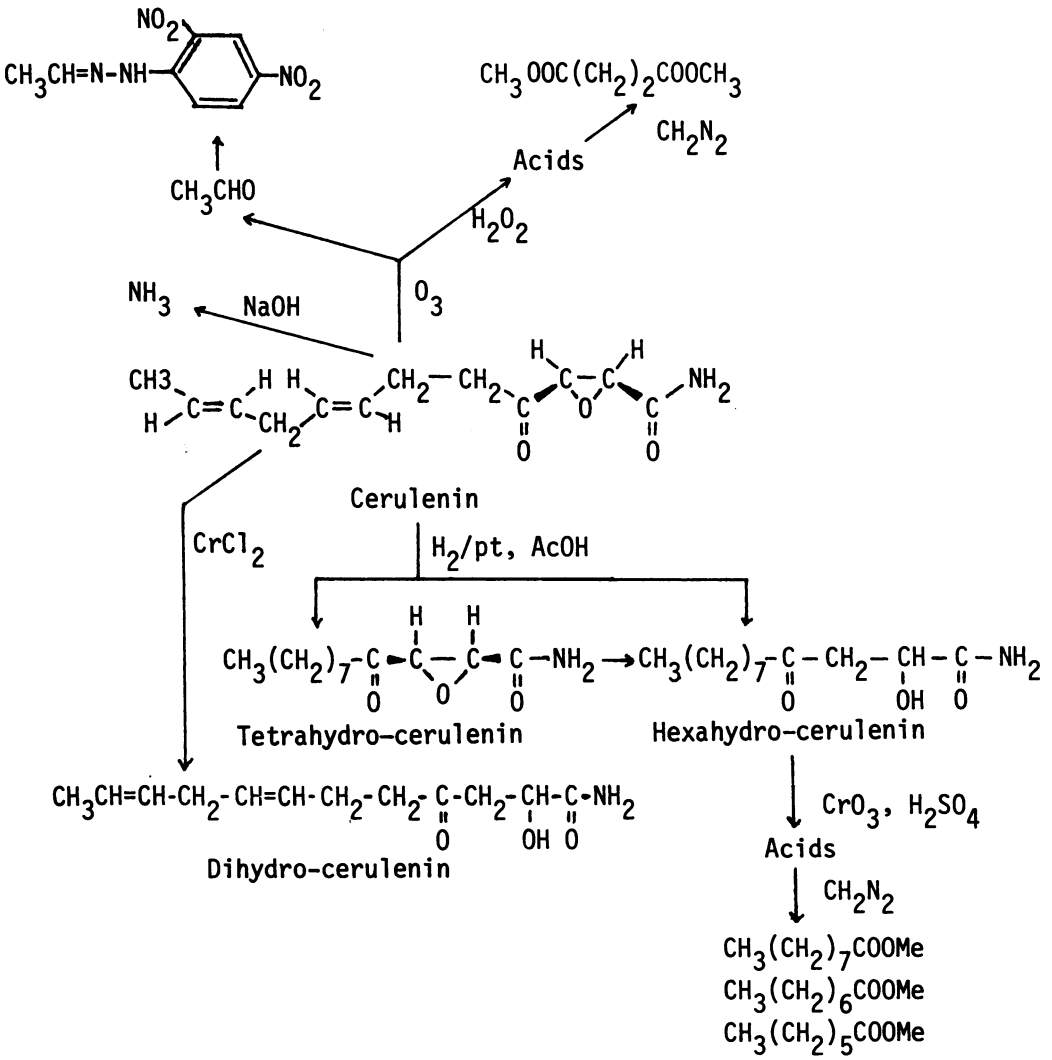


FIG. 5. *Chemical transformations of cerulenin (63).*

TABLE 2. *Antibacterial spectrum of cerulenin*

Test organism	MIC[a]	Medium[b]
Staphylococcus aureus FDA 209P	100	N
S. albus	100	N
S. citreus	12.5	N
Sarcina lutea, PCI 1001	25	N
Diplococcus pneumoniae, type 1	25	B
Streptococcus haemolyticus, Richard Cook NY-5	50	B
S. haemolyticus	100	B
S. haemolyticus	100	B
Escherichia coli, NIHJ	12.5	N
Klebsiella pneumoniae PCI 602	50	N
Proteus vulgaris OX 19	12.5	N
P. morganii	12.5	N
Salmonella typhosa	50	N
Shigella dysenteriae Shiga	25	N
S. flexneri E-20	50	N
S. sonnei E-33	50	N
Bacillus subtilis, PCI 219	12.5	N
B. anthracis	62.5	N
B. megaterium	50	N
Corynebacterium diphtheriae	50	B
Haemophilus influenzae	100	B
Mycobacterium ATCC 607	1.5	G
M. avium F	1.5	G
M. phlei	3.7	G
M. smegmatis	3.7	G
M. tuberculosis, $H_{37}R_v$	100	Y
M. tuberculosis, Aoyama B. Frankfurt	100	Y
M. tuberculosis	100	Y
Nocardia asteroides	1.5	G
N. coeliaca	1.5	G
Streptomyces griseus SN-15	3.1	G
S. lavendulae 22A	1.5	G

[a] Minimal inhibitory concentration, micrograms per milliliter.

[b] Media: N, Nutrient agar; B, 10% blood nutrient agar; G, 1% glycerol nutrient agar; Y, 1% Youman's media. Incubation was at 37°C for 48 h. Strains of *M. tuberculosis* were cultured for 4 weeks at 37°C, *Nocardia* and *Streptomyces* were cultured for 7 days at 27°C, and other strains were cultured for 48 h at 37°C.

lenin is markedly reversed by ergocarciferol, laurate, and oleate, but is not reversed by other fatty acids, such as myristate and stearate. Moreover, it can be concluded that the amount of intracellular ergosterol in growing cells of *C. stellatoidea* incubated in the presence of cerulenin appreciably decreases compared with that of the normal cells grown without cerulenin. It is thus suspected that cerulenin plays an important role as an inhibitor of biosynthesis of sterols and fatty acids. This is further supported by the experimental observation that cerulenin inhibits the incorporation of the [^{14}C]acetate into sterols and fatty acids. In the presence of 0.1 μg of cerulenin per ml, the incorporation of the [1-^{14}C]acetate into fatty acid and sterol fraction is inhibited by almost 50%, but its inhibitory effect is enhanced up to 98.8 and 97.2%, respectively, at a concentration of 2.5 μg of cerulenin per ml.

Because cerulenin does not inhibit the incorporation of [2-^{14}C]mevalonate into the nonsaponifiable fraction (NSF), it is suggested that cerulenin works in an early stage of the pathway between acetate and mevalonate in sterol and fatty acid biosyntheses. The action of avidin, which inhibits acetyl-coenzyme A (CoA) carboxylase in yeast, is then compared with that of cerulenin in a cell-free system of *Saccharomyces cerevisiae* (56). Expectedly, however, the action of cerulenin described earlier is distinctive in that avidin does not inhibit the incorporation of [1-^{14}C]acetate into NSF.

As well as [1-^{14}C]acetate, cerulenin markedly inhibits both the incorporation of [1-^{14}C]acetyl-CoA into fatty acid fraction and NSF and that of [1,3-^{14}C]malonyl-CoA into NSF. This result indicates that the antibiotic inhibits the stage of the postformation of malonyl-CoA and acetyl-CoA in fatty acid biosynthesis and the step of preformation of mevalonate in sterol biosynthesis.

EFFECT ON FATTY ACID SYNTHETASE

On the basis of the finding that the antibiotic cerulenin inhibits fatty acid and sterol

TABLE 3. *Antifungal spectrum of cerulenin*

Test organism	MIC[a] (μg/ml)
Acrocylindrium oryzae	12.5
Alternaria kikuchiana	50
Aspergillus flavus	100
A. fumigatus	50
A. fumigatus, 4 strains[b]	12.5–50
A. niger, 3 strains	100
A. terreus	100
A. ungius[b]	100
Botrytis cryptoneriae	12.5
Candida albicans	1.5
C. albicans, CA-7	0.8
C. albicans, 10 strains[b]	0.8–3.7
C. kruzei	6.25
C. tropicalis	0.8
C. stellatoidea	0.8
Cephalosporium caerulens	100
Cladosporium wernicke	25
Cryptococcus neogormans	1.5
Epidermophyton floccosum	0.2
Geotrichum sp.	3.7
Gibberella fujikuroi	50
G. saubinetii	50
Glomerella cingulata	12.5
Hormodendrum pedrosoi	50
Kloeckera apiculata	50
Microsporum gypseum, 2 strains[b]	12.5–25
M. canis	6.3
M. audouini	12.5
Mucor mucedo	1.5
Myxosporum rhois	6.25
Ophiobolus miyabeanus	1.56
Penicillium citrinum	50
P. chrysogenum	12.5
P. glaucum	12.5
P. notatum	12.5
Phialophora verrucasa	25
Piricularia oryzae, 3 strains	6.25
Pullularia pullulans	25
Rhizopus nigricans	25
Saccharomyces cerevisiae	0.8
S. sake	0.8
Sporotrichum schenckii	100
Trichophyton asteroides	6.2
T. asteroides, 2 strains[b]	3.7
T. mentagrophytes	12.5
T. interdigitale	12.5
T. purpureum	6.2
T. rubrum, 3 strains	6.2–3.1
T. violaceum	12.5
Trichoderma beigellii	12.5

[a] MIC, Minimal inhibitory concentration.
[b] From patient strain; fungi were cultured for 7 days at 27°C.

TABLE 4. *Toxicity of cerulenin*

Administrative route	Acute toxicity (LD_{50}) (mg/kg)[a]	Chronic toxicity (mg/kg per days)
Intravenous	154	40 × 14 days
Intraperitoneal	211	40 × 30 days
Subcutaneous	245	
Oral	547	

[a] LD_{50}, Mean lethal dose.

syntheses in cell-free systems (56), the effects of cerulenin on several fatty acid synthetases arising from diverse sources have been investigated (83). Three major types of synthetases have been chosen; type I synthetases, represented by the multienzyme complex (17) from rat liver, yeast, *Euglena gracilis*, *Corynebacterium diphtheriae*, and *Mycobacterium phlei*, are deactivated by 50 to 100% with 4 to 20 μg of cerulenin per ml. The activity of type II synthetase, which consists of a nonaggregated individual enzyme and shows a requirement for acyl-carrier protein (ACP) from *E. coli* and *Euglena*, are inhibited more than about 95% in the presence of only 4 to 8 μg of cerulenin per ml, whereas the type II enzyme system from *M. phlei* shows only 10% inhibition with 100 μg of cerulenin per ml. Type II systems from *E. gracilis*, which involves an ACP-dependent elongation of palmityl-CoA to stearate, show 77% inhibition with only 4 μg of cerulenin per ml. This extraordinary resistance of ACP-dependent synthetase from *M. phlei* against cerulenin still remains unexplained.

The activities of acyl-CoA transacylase, malonyl-CoA transacylase, and β-ketoacyl reductase, all of which come from the partial reaction of fatty acid synthetase (type II) from *M. phlei*, are not affected by the antibiotic in concentrations of even 100 μg/ml. On the other hand, condensing enzyme (β-ketoacyl thioester synthetase) was highly sensitive to cerulenin as assayed by measuring $^{14}CO_2$ fixation into malonyl-CoA in the presence of acetyl-CoA (83).

Because the β-ketoacyl-ACP synthetase has not been dissociated in active form from the fatty acid synthetase of multienzyme complex, the effect of cerulenin on the isolated enzyme component could not be evaluated in that system. The effect of cerulenin and its analogue on homogenous β-ketoacyl-acyl carrier protein synthetase from *E. coli* has been investigated (16). The synthetase has been isolated as a homogenous protein and studied in detail (1, 25, 72, 75). The reaction catalyzed by this enzyme proceeds in two partial reactions with an acyl-enzyme intermediate.

$$\text{RCO-S-ACP} + \text{HS-E} \rightleftharpoons \text{RCO-S-E} + \text{ACP-SH} \quad (1)$$

$$\text{RCO-S-E} + \text{HOOCCH}_2\text{CO-S-ACP} \rightleftharpoons \text{RCOCH}_2\text{CO-S-ACP} + \text{CO}_2 + \text{HS-E} \quad (2)$$

$$\text{Sum: RCO-S-ACP} + \text{HOOCCH}_2\text{CO-S-ACP} \rightleftharpoons \text{RCOCH}_2\text{CO-S-ACP} + \text{CO}_2 + \text{ACP-SH} \quad (3)$$

Thus, in reaction 1 the acyl group of an acyl-ACP is transferred to a sulfhydryl group of the enzyme to form an enzyme-thioester intermediate (25). In reaction 2, the acyl group of the enzyme thioester is condensed with malonyl-ACP to form a β-ketoacyl-thioester of ACP, CO_2, and the free enzyme. The overall reaction, reaction 3, represents the condensation reaction of fatty acid synthesis.

The homogenous β-ketoacyl-ACP synthetase was inhibited 50% by cerulenin at concentrations of less than 5 μM. The tetrahydrocerulenin also inhibited the enzyme, although substantially higher concentrations of inhibitor were required to reach similar inhibitory levels. The epoxide-free derivatives, dihydro- and hexahydrocerulenin, do not inhibit at even a concentration of 5.0 mM when tested under the similar conditions. The β-ketoacyl-ACP synthetase also catalyzes a number of model reactions that represent the component reactions and, therefore, the effect of cerulenin on each of these reactions was investigated. The activity of the fatty acyl transacylase (reaction 1), measured as the rate of [^{14}C]hexanoyl ACP synthesis, and that of the decarboxylation in the absence of fatty acyl-ACP are inhibited by cerulenin.

The possibility that a covalent complex was formed between acyl-ACP synthetase and cerulenin was tested. When substrate quantities of enzyme are treated with ^{3}H-labeled cerulenin and subjected to trichloroacetic acid precipitation, the resultant precipitate contains bound cerulenin. The amount of cerulenin bound to the enzyme is proportional to the extent of inhibition, and it is apparent that approximately 1 mol of cerulenin is bound to 1 mol of enzyme when inhibition of the enzyme approaches 100%. It has been established that inhibition of β-ketoacyl-ACP synthetase by iodoacetamide is due to the alkylation of one cysteine residue per mole of enzyme (25). The demonstration that inhibition by cerulenin is irreversible and that it accompanied the binding of 1 mol each of cerulenin and the enzyme suggests a highly specific interaction between the enzyme and the inhibitor.

According to Appleby's report on the effect of cerulenin on lipid synthesis in *Crambe abyssinica* tissues (12), the antibiotic is a potent inhibitor of fatty acid synthesis from [^{14}C]acetate in leaves and developing seeds of plants. A 1 or 2 μM concentration of cerulenin inhibits the uptake of [^{14}C]acetate into leaf lipids by 77 and 82%, and into seed lipids by 80 and 83%. The antibiotic inhibits equally the elongation of [1-^{14}C]oleate to erucic acid, which is the major fatty acid of the seed. There is no significant inhibition of fatty acid desaturation and acylation of lipids in either tissue.

Stumpf and his co-workers reported that the de novo synthesis by fatty acid synthetase from naturing safflower seeds and avocado mesocarp was fully inhibited by 10 μM cerulenin and that the enzymatic elongation from C16 to C18 was not affected by cerulenin (37). These findings led them to conclude that the enzymatic systems involved in the two condensation reactions are distinctly different from each other.

As previously demonstrated by Appleby, cerulenin inhibits the elongation of [1-^{14}C]oleic acid to erucic acid, whereas there is no effect of the antibiotic on the elongation from C16 to C18 with avocado mesocarp. This result indicates that there should be various kinds of elongation systems in plants.

EFFECT ON THE ENZYMES INVOLVED IN STEROL SYNTHESIS

The target of cerulenin on fatty acid biosynthesis was made clear. The location of the action of sterol biosynthesis has been well explored. Although it has been previously reported that cerulenin inhibits the incorporation of [^{14}C]acetyl-CoA, but not that of [^{14}C]mevalonate, into NSF in a cell-free system of yeast, three more steps still remained to be investigated (58), namely, the steps catalyzed by acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, and HMG-CoA reductase (Fig. 6), which is known as the rate-limiting enzyme of overall sterol synthesis in rat liver (74).

In a cell-free system of *S. cerevisiae*, cerulenin inhibits the incorporation of [^{14}C]acetyl-CoA into NSF by 67% at 9×10^{-6} M (2 μg/ml) (58). This inhibition is sufficient to account for the inhibition of overall sterol synthesis in vivo. On the contrary, the incorporation of [^{14}C]HMG-CoA or [^{14}C]mevalonate is not reduced up to concentrations of 2.3×10^{-4} M. This fact suggests that cerulenin has no effect on HMG-CoA reductase activity and on the subsequent steps of sterol synthesis. Therefore, the two catalytic steps, acetyl-CoA to acetoacetyl-CoA and then to HMG-CoA, must be affected by cerulenin.

The effect of cerulenin on the two enzymes, acetoacetyl-CoA thiolase and HMG-CoA synthase, has been tested. Cerulenin has no effect on acetoacetyl-CoA thiolase activity at a concentration of 1.8×10^{-3} M, but markedly inhibits HMG-CoA synthase activity. When the time of preincubation is reduced to 8 min from 30 min, the inhibition is also reduced to 21% from 71% in the presence of cerulenin, whereas no inhibition is observed without preincuba-

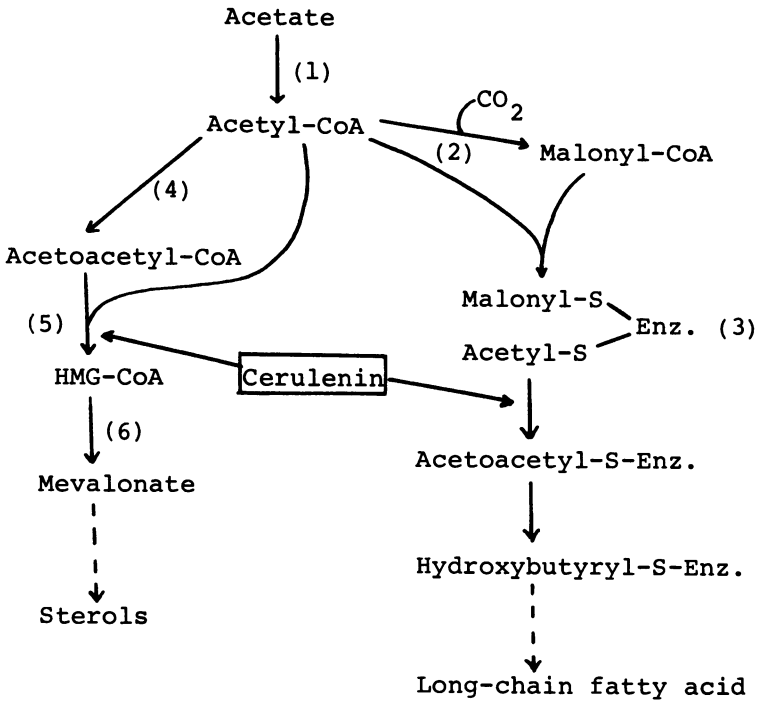


FIG. 6. *Targets of cerulenin in biosyntheses of sterol and fatty acid (16, 56, 58, 83). (1) Acetyl-CoA synthase; (2) acetyl-CoA carboxylase; (3) fatty acid synthetase; (4) acetoacetyl-CoA thiolase; (5) HMG-CoA synthase; (6) HMG-CoA reductase.*

tion. This therefore implies that the inhibition appears to be noncompetitive.

The fact that cerulenin specifically inhibits the activity of HMG-CoA synthase suggests that the antibiotic possesses distinctly different modes of action from that of iodoacetamide (81), which deactivates both HMG-CoA synthase and acetoacetyl-CoA thiolase.

The experimental observation that the cerulenin concentration required for 50% inhibition of HMG-CoA synthase activity (1.1×10^{-3} M) is higher than that required by inhibited incorporation of [^{14}C]acetyl-CoA into NSF by the cell-free system has not been explained.

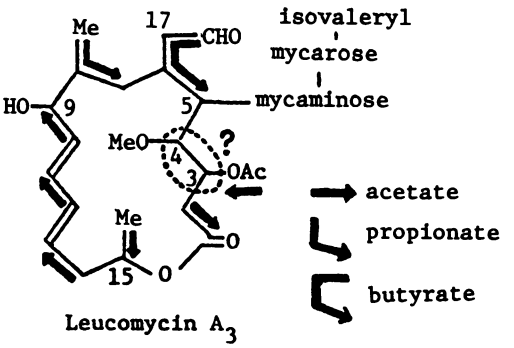


FIG. 7. *Biosynthesis of leucomycin-A_3 (69).*

INHIBITION OF "POLYKETIDE" SYNTHESIS

Shortly after the discovery that cerulenin specifically inhibited the biosynthesis of fatty acids, it was likewise expected that the antibiotic would have an inhibitory effect on the biosyntheses of macrolides (85), in which aglycone is produced by condensation of acetate, malonate, or methyl malonate. At first, the action of cerulenin on the biosynthesis of leucomycin-A_3 (Fig. 7), one of the 16-membered macrolides (69), was investigated (67).

The cerulenin added to the culture is metabolized by the leucomycin-producing strain and decreases as time goes on. However, the biosynthesis of leucomycin is fully inhibited until cerulenin is consumed completely. Some antibiotics are expected to be synthesized in vivo through a hypothetical intermediate "polyketide." Tetracycline (*Streptomyces aureofaciens*) (52), cycloheximide (*Streptomyces* sp. OS-786) (84), and LA-1 (*Streptomyces kitasatoensis* 4-4-2) (68) (Fig. 8) have been used as examples of such antibiotics and the effects of cerulenin on their production were studied (67). In each case it has been found that the production of the

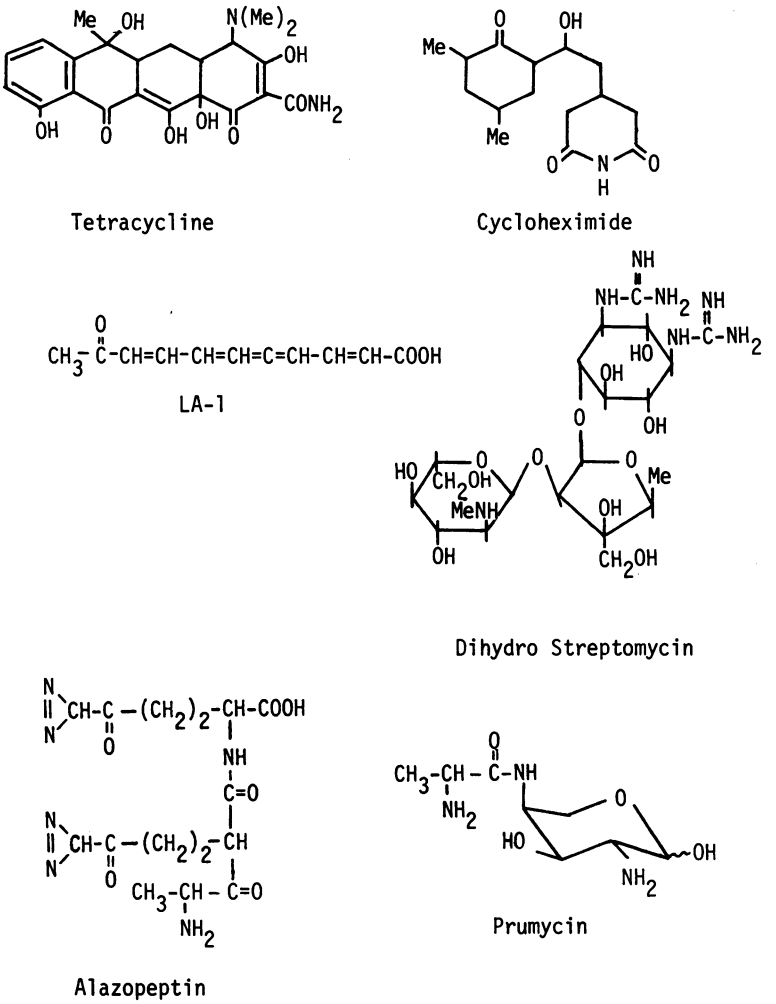


FIG. 8. *Antibiotics tested and the effect of cerulenin on their production.*

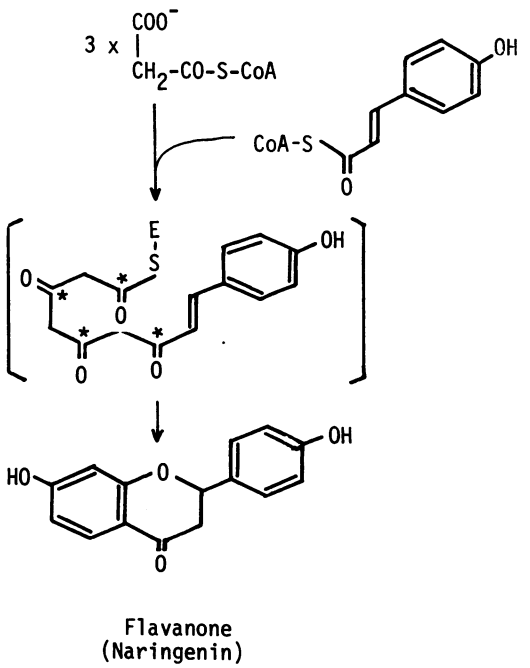
antibiotic, like that of leucomycin, is remarkably inhibited by cerulenin at a concentration of 10 to 20 μg/ml, i.e., 1/10 to 1/5 of the minimal inhibitory concentration against the organisms producing these antibiotics. On the other hand, even at a sufficiently high concentration that reduces glucose consumption (100 μg/ml) cerulenin does not inhibit the synthesis of dihydrostreptomycin (aminoglucoside antibiotic, *Streptomyces* sp. OS-3256) or prumycin (amino acid amino sugar antibiotic, *Streptomyces kagawaensis*) (65), neither of which is expected to be synthesized in vivo through the polyketide. The minimal inhibitory concentrations of cerulenin against the above organisms are 200 μg/ml for *S. kitasatoensis* 23-1 and *S. kitasatoensis* 4-4-2 and more than 200 μg/ml for the other organisms.

The mechanism of polyketide formation is considered to be the polymerization of acetate and malonate, which resembles the condensation of the two acids during the biosynthesis of a long-chain fatty acid. It is thought that the mechanism of polyketide formation lacks all or part of three reactions, hydrogenation, dehydration, and a second hydrogenation, which occur successively after condensation in fatty acid synthesis. The enzymatic reaction involving the first condensation step is common to both reactions. This implies that cerulenin inhibits the production of antibiotics synthesized in vivo through the polyketide.

The incorporation of labeled acetate and propionate into the aglycone moiety of the polyene macrolides nystatin (8), candicidin (44), amphotericin B (43), and lucensomycin (46) has been described (48). On the basis of this evidence it has been proposed that the polyene macrolide

antibiotics are synthesized via the polyketide pathway by a head to tail condensation of acetate and propionate, previously activated to malonyl-CoA and methyl malonyl-CoA, respectively, in a manner similar to that found in fatty acid biosynthesis. Using a phosphate-limited, resting-cell system of *Streptomyces griseus*, Martin and McDaniel showed that cerulenin specifically inhibits the biosynthesis of candicidin (48). Cells in which candicidin synthesis is completely inhibited for 10 h by the addition of 20 μg of cerulenin per ml remain capable of candicidin synthesis. After removal of the cerulenin by washing and suspension of the cells in fresh medium, candicidin synthesis resumes at a lower rate (70%) than that of the control.

Biosynthesis of flavonoids is an example for the application of the acetate rule to the synthesis of various aromatic ring structures (7, 45). Recently, flavonone synthase, the enzyme catalyzing the formation of the flavonone naringenin from *p*-coumaroyl-CoA and malonyl-CoA (Fig. 9), has been isolated and partially purified from fermentor-grown, light-induced cell suspension cultures of *Petroselium hortense* (40). The enzyme is strongly inhibited by cerulenin and further inhibited by the reaction products such as naringenin, CoA-SH, and several compounds reactive with sulfhydryl groups. Approximately 80% inhibition of the reaction catalyzed by flavonone synthase is achieved by 0.5 μg (5 μg/ml) of cerulenin. The close similarity between the mechanisms of chain elongation in fatty acid and flavonone synthesis is suggested by these results.



Flavanone
(Naringenin)

FIG. 9. *Biosynthesis of the flavanone naringenin (40).*

6-Methyl salicylic acid (Fig. 10) is one of the simplest aromatic metabolites derived from polyketide. Synthesis of 6-methyl salicylic acid using the cell-free system from *Penicillium urticae* is inhibited up to 60% by 100 μg of cerulenin. This is the same extent to which fatty acid biosynthesis is inhibited by cerulenin.

On the basis of these results, along with the effect of cerulenin on antibiotic production (67), it is possible, by examining the effect of cerulenin on the production of a natural product, at least one of microbial origin, to identify whether the product is synthesized through a polyketide intermediate. However, because the inhibitions of fatty acid and sterol syntheses take place simultaneously (60, 83), such an experiment requires caution. Among other things, the product should not be synthesized through mevalonic acid. Secondly, cerulenin must not be added until the organism is fully grown, as demonstrated by our experiment, to reduce the secondary effects of the inhibition of lipid synthesis as much as possible.

APPLICATION AS A BIOCHEMICAL TOOL

To control the fatty acid or lipid composition of bacterial membrane and to explore biochemical and biogenetic interference of fatty acid (or lipids), the following methods have been used: (i) unsaturated fatty acid and saturated fatty acid auxotrophs (13, 77, 78); (ii) temperature-sensitive mutants that require saturated and unsaturated fatty acid for growth (28, 29), and the mutants with temperature-sensitive phosphatidyl serine decarboxylase (32); (iii) temperature control of fatty acid composition (11, 47); and (iv) the application of 3-decynoyl-*N*-acetylcysteamine, an inhibitor of fatty acid synthesis in *E. coli* (38, 39).

Cerulenin has proved useful for studying the metabolism and the function of fatty acids in the cell membrane of bacteria (24) and yeast (5; J. Awaya, K. Otoguro, and S. Ōmura, unpublished data). Goldberg et al. demonstrated that cerulenin, which inhibits the growth of *E. coli*, specifically blocked lipid synthesis (24). I have reported that the cell growth of *S. cerevisiae* ATCC 12341, inhibited by cerulenin, is reversed by various exogenous fatty acids. Myristate ($C_{14:0}$), pentadecanoate ($C_{15:0}$), palmitate ($C_{16:0}$), and oleate ($C_{18:1}$) reverse effectively the inhibition of growth by cerulenin. When these cells are treated with pentadecanoate, over 90% of

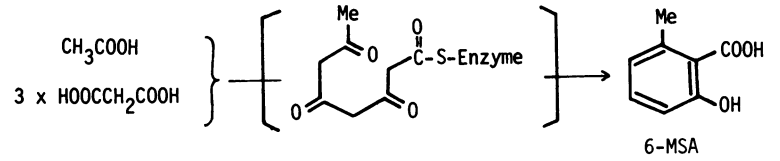


FIG. 10. *Biosynthesis of 6-methylsalicylic acid (6-MSA).*

native even-carbon fatty acids are replaced by odd-carbon fatty acid. Those found in the reversed cells treated with oleate are almost all unsaturated fatty acids. In both experiments, in the presence of myristate and pentadecanoate along with cerulenin, these added fatty acids are metabolized to yield fatty acids with an extended carbon chain and unsaturated fatty acids. This fact suggests that cerulenin inhibits neither elongation nor dehydrogenation in *S. cerevisiae*. Accordingly, it has been suggested from these experimental observations that cerulenin could serve as a convenient alternative to mutants for studying membrane biogenesis (5, 24; Awaya et al., unpublished data).

Sporulation of microorganisms is an attractive model for studies of cellular differentiation. Investigations have been made from biochemical viewpoints, such as those on nucleic acid, protein, carbohydrate, and intermediate metabolism (20, 21, 27). However, there seem to be fewer reports on lipid metabolism during sporulation, especially in yeast, in spite of well-documented lipid accumulation in sporulating cells (71). Esposito et al. (19) have found two remarkable periods of lipid synthesis during sporulation of *S. cerevisiae*, which have been confirmed by Illingworth et al. (34). Recently, Henry and Halvorson (33) have attempted a precise analysis of this process to show that much of the lipid synthesis is not specific to sporulation, and that, if the necessary diploids from the auxotrophs for saturated (77) and unsaturated fatty acids (82) are constructed, these strains could be used to determine the lipid requirement during sporulation.

The inhibition of sporulation in *S. cerevisiae* by cerulenin and its reversion by externally added fatty acid were studied (60). Both palmitate and oleate reverse the inhibition of sporulation by cerulenin. Pentadecanoate is the most effective among the saturated fatty acids even though fatty acids with additional carbons are not detected in the resulting fatty acid of *S. cerevisiae* G 2-2.

It was unknown whether lipid synthesis during the earlier stage of sporulation occurred independently of other sporulation-related metabolic changes or as an early part of sequential changes among the sporulation-related changes. If the former were the case, exogenous fatty acids should have rapidly reversed the inhibited sporulation in the cells pretreated with cerulenin. If the latter were true, exogenous fatty acids should have required several hours to reverse the inhibited sporulation in cells pretreated with the antibiotic and to reverse the inhibited sporulation upon their addition 12 h or more later to the sporulation medium. When cerulenin was added to the sporulation medium later than 12 h after the start of incubation, the marked inhibition disappeared. When the fatty acid fraction extracted from the sporulated yeasts was added to the cells pretreated with cerulenin for more than 6 h, sporulation became evident at 6 h after the addition of the fatty acid fraction. Therefore, sufficient synthesis of fatty acids required for sporulation was assumed to occur during the first 6 h in phase I of yeast sporulation.

Nunn and Cronan have performed the experiment using cerulenin to test in vivo if *rel* gene control of phospholipid synthesis is exerted solely at the level of fatty acid synthesis (57). Phospholipid synthesis, as well as ribonucleic acid synthesis, is under control of the *rel* locus in *E. coli* (23, 53, 70, 80). Amino acid starvation of stringent (*rel*$^+$) bacteria results in a two- to fourfold decrease in phospholipid synthesis (23, 53, 70, 80). In contrast, starvation of a relaxed (*rel*$^+$) strain results in decrease in phospholipid synthesis.

A requirement for an exogenous source of fatty acid for growth of strain CP78 (*rel*$^+$) and CP79 (*rel*$^+$) has been engendered by the addition of cerulenin. Addition of cerulenin in the presence of an appropriate fatty acid supplement results in an 87% decrease in the incorporation of [^{14}C]acetate into phospholipid (although growth continued). Under these conditions, amino acid starvation of strain CP78 (*rel*$^+$) results in a two- to fourfold inhibition of the rate of phospholipid synthesis. An identical experiment with strain CP79, the *rel*$^-$ derivative, showed no inhibition of phospholipid synthesis when starved for amino acid. This demonstrates that the inhibition by phospholipid synthesis observed in an amino acid-starved stringent strain cannot be due solely to an inhibition of fatty acid synthesis.

Attempts have been made, by using ceru-

lenin, to answer two questions as to how to stop overall fatty acid synthesis in *B. subtilis* (87). (i) Is it possible to prevent the accumulation of free fatty acids under the conditions where de novo phospholipid biosynthesis is blocked in these bacteria? (ii) Does the inhibition of lipid synthesis with cerulenin influence the rate of macromolecular syntheses in *B. subtilis*?

Although the bacteria ceased to grow upon inhibiting de novo fatty acid biosynthesis by the addition of cerulenin, they remained completely viable. Addition of 12-methyl tetradecanoate and palmitate to the culture medium of cerulenin-treated cells retains growth of the bacteria, albeit at a reduced rate. Although the de novo synthesis of all lipid components of the membrane is blocked, citrate-Mg^{2+} transport activity remains inducible and the induced cells do not lose this transport activity when treated with cerulenin. Shortly after the addition of cerulenin, the rate of ribonucleic acid synthesis drops rapidly and the rate of protein synthesis also decreases, but more slowly than the former. The rate of deoxyribonucleic acid synthesis remains almost unaffected.

Plasmid-mediated tetracycline resistance in *S. aureus* and enteric bacteria is usually inducible and appears to result from decreased uptake of antibiotic. Because plasmid-mediated tetracycline resistance probably results from the insertion of proteins into the membrane (42), it is of interest to know whether or not resistance can be expressed in the absence of lipid synthesis. Chopra has used cerulenin to resolve this problem (10). Addition of 125 μg of cerulenin per ml to growing cultures of UB_{4012} that contained plasmid pub_{111} (specifying resistance to tetracycline) resulted in virtually complete inhibition of fatty acid synthesis. Preincubation of UB_{4012} for 40 min with 5 μg of tetracycline per ml produced a marked fall in the subsequent inhibition of protein synthesis by tetracycline, compared with organisms grown initially in the absence of the drug. Addition of 125 μg of cerulenin per ml during preexposure of UB_{4012} to 5 μg of tetracycline per ml had no effect on the subsequent degree of inhibition of protein synthesis by tetracycline compared with organisms preincubated in the presence of tetracylcine, but without cerulenin. On the basis of these results, it was concluded that the induction of tetracycline resistance remained unchanged whether it appeared in the absence of lipid synthesis or under normal growth conditions.

The envelope of *E. coli* is composed of two membranes: the inner-most cytoplasmic membrane and the rigid peptidoglycan layer. Both membranes contain protein and phospholipid, which are synthesized either in the soluble cytoplasm or at the cytoplasmic membrane itself. In addition, the outer membrane contains lipopolysaccharide. Investigations of the assembly of the cytoplasmic membrane in glycerol auxotrophs have revealed that some proteins can be synthesized and incorporated into this membrane under conditions that do not permit the synthesis of lipids (54, 86). Assembly is not necessary for that of the cytoplasmic membrane. Thus, the protein, lipid, and lipopolysaccharide components must be translocated from the site of their synthesis inside the cell to the final location exterior to the cytoplasmic membrane and the peptidoglycan. It was of interest to know whether or not the insertion of a newly synthesized protein into the outer membrane requires simultaneous lipid synthesis. Cerulenin was used to demonstrate that a minor protein component of the outer membrane of *E. coli*, which serves as the receptor for the phage lambda, can be synthesized and inserted into the outer membrane during inhibition of lipid synthesis (73).

Marr and Ingraham (47) have reported that the fraction of unsaturated fatty acid in the envelope phospholipids of *E. coli* increases markedly as the growth temperature is decreased. This observation was confirmed by a large number of investigators, but the mechanisms regulating this alteration were not fully understood regardless of the considerable amount of work done in this field. There had been two major interpretations to explain such thermal regulation. One claimed that the enzymes catalyzing phosphatidic acid synthesis would be sensitive to temperature change (79) and the other suggested that a control at the level of fatty acid synthesis should also be required.

Cronan carried out experiments, a part of which involved the utilization of cerulenin, to verify that both fatty acid synthesis and phosphatidic acid synthesis accounted for one site of the thermal regulation of phospholipid fatty acid composition. When cerulenin-treated cultures are grown with a mixture of [^{3}H]palmitate and *cis*-[^{14}C]vaccenate at various temperatures in the presence of cerulenin, at least 85% of the fatty acid moieties in the phospholipid are derived from these exogenously supplied fatty acids. These phospholipids are found to contain different ratios of the two acids. These ratios depend directly on the temperature of growth.

Because cerulenin possesses a number of remarkable biological activities that have never been found in other antibiotics, it will more and more frequently be used as an unequivocal bio-

chemical tool not only for the studies of fatty acid biosynthesis, but also for the biosynthesis, structure, and functions of membranes produced by microorganisms.

Prospectively, the antibiotic will be used, as various other enzyme inhibitors have been, in a variety of ways, for biosynthetic studies of natural organic compounds produced via polyketide and malonate and for enzymes involved with biosynthesis.

Despite the fact that cerulenin inhibits the biosynthesis of sterol, its producing strain, *C. caerulens*, simultaneously affords sterol antibiotics as described earlier. Why this biosynthetic system of sterol strongly resists cerulenin still remains a problem for future investigation.

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ADDENDUM

After submission of this manuscript it was reported that Kamiryo et al., in a study of cerulenin, proved that acyl-CoA synthetase activity is required for repression of yeast acetyl-CoA carboxylase by exogenous fatty acid (T. Kamiryo, T. S. Parthasarathy, and S. Numa, Proc. Natl. Acad. Sci. U.S.A. 73:386-390, 1976).

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