Inhibition of *de novo* Fatty-Acid Biosynthesis in Isolated Chloroplasts by the Antibiotic Cerulenin and Its Synthetic Derivatives

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The antibiotic cerulenin was shown to be a potent dose-dependent inhibitor of *de novo* fatty-acid biosynthesis in intact isolated chloroplasts of different plants (measured as [^{14}C]acetate incorporation into the total fatty-acid fraction). Various chemical derivatives of cerulenin were synthesized and tested in the chloroplast assay-system of oat, spinach and pea. Modifications of the hydrocarbon chain of cerulenin (*e.g.* tetrahydro-cerulenin and its short-chain *cis*-2,3-epoxy-4-oxoheptanamide derivative) decreased the inhibitory activity of cerulenin, whereas variations of the epoxy-oxo-amide structural element led to a complete loss of inhibition potency. The results indicate that the naturally occurring antibiotic cerulenin is the most active specific inhibitor of *de novo* fatty-acid biosynthesis, but the formation of the hydroxylactam ring seems to be an essential requirement for the inhibitory activity. Those structural analogues of cerulenin, which can no longer form a hydroxylactam ring, do not possess any inhibitory capacity.

Introduction

In higher plants *de novo* fatty-acid biosynthesis is primarily or even exclusively bound to the compartment of plastids (chloroplasts, etioplasts, leucoplasts) [1]. This is in contrast to other organisms such as yeast, where the *de novo* fatty-acid biosynthesis is located in the cytoplasm and also in the mitochondria [2]. In the study of the potential effects of inhibitors of *de novo* fatty-acid biosynthesis of plants (*e.g.* herbicides, antibiotics) isolated chloroplasts are proved to be a very suitable test-system [3].

Cerulenin, a well-known antibiotic isolated from the culture filtrate of the fungi *Cephalosporium caerulens* [4] and of *Sarocladium oryzae* [5] is an inhibitor of 3-oxoacyl-ACP-synthases (also named β-ketoacyl-ACP-synthases [KAS]), the condensing enzymes of the fatty-acid synthetase complex. It blocks the fatty-acid synthetases from different sources like that of some bacteria [6], yeast [7, 8], *Euglena* [9], rat liver [9] and higher plants [10, 11]. Thiolactomycin, another antibiotic from the bacteria *Norcardia spec.*, also affects the 3-oxoacyl-ACP-synthase but is only active in the procaryotic type fatty-acid synthetase such as from *E. coli* [12] and higher plants [12–14]. In contrast to thiolactomycin, cerulenin binds covalently and irreversibly [6] to a cystein residue in the active center of the enzyme, as shown for *E. coli* [15]. The process of covalent binding takes some time to be completed, so that preincubation of isolated chloroplasts with cerulenin resulted in an enhanced inhibiting effect of this antibiotic [16, 11]. In higher plant chloroplasts three different condensing enzymes (KAS I, KAS II and KAS III) are known. KAS I is responsible for *de novo* fatty-acid synthesis up to a chain length of C16 and is strongly inhibited by cerulenin [10]. In contrast, KAS II (elongation from C16 to C18) is only slightly effected by cerulenin [10, 17]. KAS III, in turn, is involved in the synthesis of short chain fatty acids (C4 to C6) and is not inhibited by cerulenin [18].

Naturally occurring cerulenin has the absolute configuration 2*R*,3*S*,7*E*,10*E*-2,3-epoxy-4-oxo-dodeca-7,10-dienamide [19–21] as shown in Fig. 1A. Only this 2*R*,3*S*-enantiomer is physiologically active [22]. Also the epoxide group of cerulenin is essential for inhibition [6]. Cerulenin has a special keto-*cis*-epoxy-amide structure, which exists in protic solvents in an equilibrium with its cyclic isomer, the corresponding epoxylactam (Fig. 1) [23, 24]. The SH-group of the cystein residue in the active centre of the KAS I-enzyme binds

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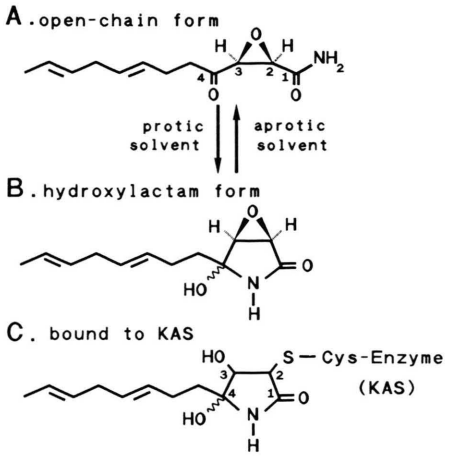


Fig. 1. Structure of cerulenin (A) and its diastereomeric hydroxylactam forms (B). The equilibrium between both forms can be shifted with either protic or aprotic solvents. C) Cerulenin as bound to the cystein-S of the target enzyme 3-oxo-acyl-ACP synthase (KAS). The numbering of the C-atoms of cerulenin is indicated.

covalently to the C2-atom of cerulenin as had been shown in *E. coli* [15]. It was shown for the yeast enzyme that cerulenin is bound to the cystein residue in the hydroxylactam form [25] (Fig. 1 C).

In order to study the specificity of the cerulenin structure we synthesized racemic cerulenin derivatives with variations either in the hydrocarbon chain or the keto-epoxy-amide structure. The effects of natural cerulenin and its synthetic derivatives on plant *de novo* fatty-acid biosynthesis were tested in the chloroplast assay-system of different plants in order to qualify the inhibitory specificity of cerulenin and related structural analogues.

Materials and Methods

Intact chloroplasts were isolated from seedlings of oat (*Avena sativa* L. var. Flämmingsnova), pea (*Pisum sativum* L. var. Lisa), and spinach (*Spinacia oleracea* L. var. Matador) and incubated with [1-^{14}C]acetate for 20 min in an iso-osmotic buffer-system as described in [27]. The incubation medium (0,5 ml) contained 300 mM sorbitol, 50 mM tricine, 50 mM phosphate (pH 7.9), 30 mM $NaHCO_3$, 2 mM ATP, 0.5 mM coenzyme A, 0.5 mM $MgCl_2$, 35 μM [1-^{14}C]acetate (37 kBq per assay) and chloroplasts with a chlorophyll content of about 50 μg. Depending on the plant source, incorporation of [^{14}C]acetate was 1% to 3% of the total radioactivity. The inhibitors were added as methanolic solutions, so that the final methanol content of the assay solution was 0.2%. These low amounts reduced the activity of the chloroplasts to only 95–97% of that of controls without methanol. The light intensity (light induced formation of ATP and $NADPH_2$ which is needed for fatty-acid biosynthesis) was 1400 μmol m^{-2} s^{-1}. Hydrolysis, acidification, extraction of fatty acids and measurement of the incorporated radioactivity was performed as described before [3].

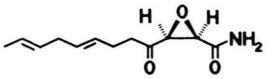
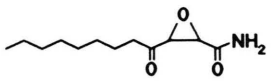
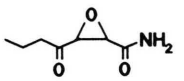
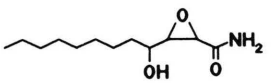
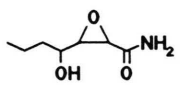
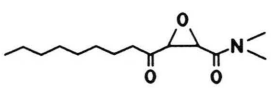
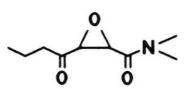
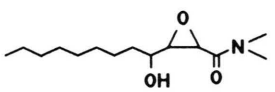
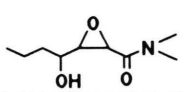
The synthesis of the cerulenin derivatives was performed according to the method of Jakubowski [26]. In the first step a 4-hydroxy-2-in-acidester was made by nucleophilic addition of the lithium salt of propiolic acid methylester to an aldehyde with the desired hydrocarbon chain. After partial hydrogenation (Lindlar's catalyst) and heating the corresponding butenolid was formed. Epoxidation with sodium hypochlorite led stereoselectively to the *cis*-epoxy-γ-butyrolactone. Conversion with ammonium hydroxide or dimethylamine led to the 2,3-epoxy-4-hydroxyamide or *N,N*-dimethyl-2,3-epoxy-4-hydroxyamide, respectively. Finally, the desired *cis*-2,3-epoxy-4-oxoamides were obtained by oxidation with Collins' reagent. This synthesis provided the racemic products only. An enantio selective synthesis of the cerulenin-type compounds was not realized in this work.

The ^{1}H-NMR spectra of tetrahydrocerulenin were measured using the Cryospec WM 250 instrument of Bruker, Karlsruhe.

Results and Discussion

The effect of cerulenin and its synthetic derivatives was tested using a well established test-system of isolated chloroplasts [27] monitoring *de novo* fatty-acid biosynthesis from [^{14}C]acetate. In Table I the chemical structures of the synthesized derivatives of cerulenin are shown together with their effects on the *de novo* fatty-acid biosynthesis of isolated chloroplasts from different plant species. In a concentration of 100 μM (with a 20 min preincubation time) the natural antibiotic cerulenin inhibited the incorporation of [^{14}C]acetate into the total fatty acid fraction by 65–80% depending on the chloroplast preparation. Between the chloroplasts of the monocotyledonous oat and the dico-

Table I. Percentage inhibition of *de novo* fatty-acid biosynthesis by cerulenin (100 μM) and the racemic cerulenin derivatives (200 μM) in the assay-system of chloroplasts isolated from oat, spinach and pea seedlings. Preincubation time of chloroplasts with active ingredients was 20 min. Mean of 6 determinations from 2 chloroplast isolations per plant.

Compound number and chemical name	Chemical structure	Inhibition of *de novo* fatty-acid biosynthesis
1: cerulenin (active 2 *R*,3 *S*-enantiomer)		strong inhibition (65–80%)
2: tetrahydrocerulenin (racemic)		moderate inhibition (40–50%)
3: *cis*-2,3-epoxy-4-oxoheptanamide (racemic)		weak inhibition (10–25%)
4: *cis*-2,3-epoxy-4-hydroxydodecanamide (racemic)		no inhibition
5: *cis*-2,3-epoxy-4-hydroxyheptanamide (racemic)		no inhibition
6: *N,N*-dimethyl-*cis*-2,3-epoxy-4-oxo-dodecanamide (racemic)		no inhibition
7: *N,N*-dimethyl-*cis*-2,3-epoxy-4-oxo-heptanamide (racemic)		no inhibition
8: *N,N*-dimethyl-*cis*-2,3-epoxy-4-hydroxydodecanamide (racemic)		no inhibition
9: *N,N*-dimethyl-*cis*-2,3-epoxy-4-hydroxyheptanamide		no inhibition

tolydonous pea and spinach plants significant differences were not observed.

Two of the synthetic cerulenin analogues *i.e.* tetrahydrocerulenin and the short chain 2,3-epoxy-4-oxoheptanamide (compounds **2** and **3** in Table I) were inhibitors of *de novo* fatty-acid biosynthesis in our chloroplast test-system. In both cases the inhibition was dose-dependent. Compound **2** gave a moderate inhibition (40–50%) and the short-chain derivative 2,3-epoxy-4-oxoheptanamide (compound **3**) a weak inhibition (10–25%). Cerulenin and the synthetic compounds **2** and **3** were tested in detail in oat chloroplasts with and without a preincubation (20 min) with the inhibitor before the [^{14}-C]acetate was added. Preincubation with cerulenin and its structural analogues resulted in an enhanced inhibitory effect as compared to the non-preincubated samples (Fig. 2). This was expected and had been observed before for cerulenin [11]. The reason may be that the formation of the covalent binding of the inhibitors to the cystein of the target-enzyme KAS I (Fig. 1C) takes several minutes to be completed. When comparing the effect of cerulenin (enantiomeric pure form) with its synthetic structural analogues one has to consider that the latter con-

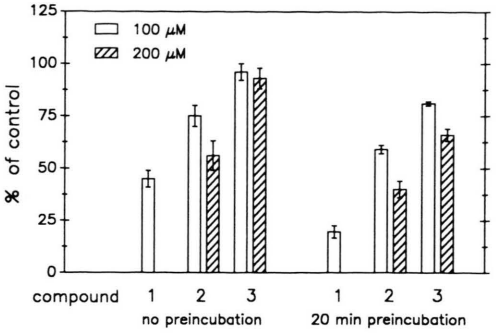


Fig. 2. Dose-dependent inhibition by cerulenin, tetrahydrocerulenin and 2,3-epoxy-4-oxoheptanamide (compounds **1, 2** and **3,** respectively) of *de novo* fatty-acid biosynthesis from [^{14}C]acetate in the oat chloroplast assay-system without or with a preincubation period of 20 min before adding [^{14}C]acetate. The synthetic compounds were applied as racemates. The incorporation rate of [^{14}C]acetate of the controls amounted to 1 to 3% of the applied radioactivity. Mean of 3 determinations with SD.

sisted of the racemate, from which presumably only the 2*R*,3*S*-enantiomer is active, as was shown in the case of cerulenin [22].

The concentrations for 50% inhibition of the *de novo* fatty-acid biosynthesis (I_{50}-values) were 50 µM (without) and 20 µM (with preincubation) for cerulenin. When only the presumable active enantiomers of cerulenin derivatives (2*R*,3*S*) are considered, the I_{50}-values of tetrahydrocerulenin were 120 µM without and 70 µM with preincubation. In the case of the short-chain compound 3 an I_{50}-value for the condition "without preincubation" cannot be given since the inhibitory activity without preincubation is very low; after preincubation an I_{50}-value of 130 µM was estimated for compound **3.** Concentrations higher than 250 µM could not be applied due to the limited solubility of the synthetic cerulenin compounds. The results indicate that changes and shortening of the hydrocarbon chain, which is not directly involved in the covalent bonding of cerulenin to the thiol group of the enzyme (Fig. 1 C), lead to a decrease of the inhibitory activity of the cerulenin molecule.

All other cerulenin derivatives (compounds **4** to **9**), where the epoxy-oxo-amide-structure of cerulenin was changed, were completely inactive and had no effect on *de novo* fatty-acid biosynthesis of chloroplasts. This was the case in the change from 4-oxo-amides to 4-hydroxyamides as well as the change of the amide residue to a *N,N*-dimethylamide. In these cases the formation of the hydroxylactam form (see Fig. 1 B) is blocked, whereas those derivatives with an intact epoxy-oxo-amide structure (compounds **1–3**) can form the hydroxylactam ring. With a simple kinetic ^{1}H NMR experiment we showed that the formation of the hydroxylactam form of tetrahydro-cerulenin in the protic solvent methanol is a rapid process, as shown in Fig. 3. Hydroxylactam formation of the natural antibiotic had been shown in 1981 *via* changes in the optical rotation of linearly polarized light [24]. The process of hydroxylactam formation using the kinetic ^{1}H NMR technique has been demonstrated in this investigation for the first time. It was followed *via* changes in the epoxide protons at C2 and C3 of the corresponding isomers. The equilibrium concentration of the lactam form is about 87% in deutero-methanol (Fig. 3).

In this respect it is of interest that the hydroxylactam form is more reactive against a nucleophilic attack at the epoxy-group at C2, which is the site of covalent binding of the inhibitor to the cystein-SH of the target-enzyme. Our observation that in chloroplasts only those cerulenin derivatives which can form the hydroxylactam ring are inhibitory is evidence that in plants the hydroxylactams are the biologically active agents, but not the open-chain cerulenin forms. This view is supported by investigations with a model substance [8] and by the isolation of a [^{3}H]cerulenin-cystein adduct from yeast [25]. Only on one occasion has it been

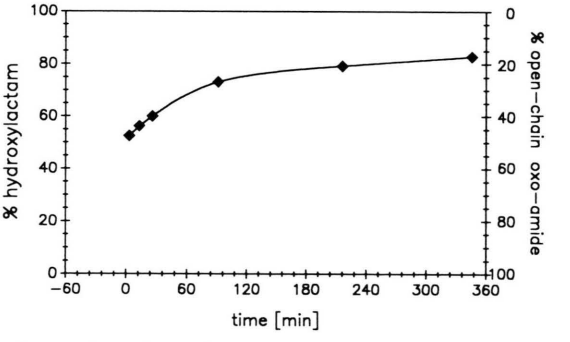


Fig. 3. Time-dependent formation of the hydroxylactam of tetrahydro-cerulenin from the open-chain form in the protic solvent methanol (^{1}H NMR in CD_3OD [400 MHz]). Each point is based on separate NMR spectra.

reported, that *N,N*-dimethyl-cerulenin, which cannot form a lactam ring, exhibited some modest inhibiting potency on *de novo* fatty-acid biosynthesis in certain sensitive organism [22]. In our opinion this work, however, requires repetition with a standardized biological test-system. The total loss of inhibiting potency with all derivatives, which could not form the corresponding hydroxylactams (compounds **4** to **9** in Table I), supports the conclusion that in plants and possibly also in all other cerulenin-sensitive organisms the hydroxylactam forms may represent the active components for inhibition. Though there is no final proof that only the hydroxylactam form can bind to the target protein, it is highly probable on the basis of the present results.

The natural crystallin cerulenin as well as the synthesized cerulenin analogues always represent a mixture of open-chain and hydroxylactam form, the relative proportions of which seem to depend on the protic properties of the solvent from which they were isolated [23, 24]. When the hydroxylactam is the biologically active inhibitor, which follows from our results, the open-chain form of cerulenin and tetrahydro-cerulenin must first be transferred to the hydroxylactam form before the latter can bind to and inhibit the target enzyme KAS I. The relatively long preincubation time of *ca.* 20 min, required for a better inhibition of the *de novo* fatty-acid biosynthesis assay-system of isolated intact chloroplasts, may be due to transport processes through the chloroplast envelope, to an establishment of the equilibrium of the open-chain / hydroxylactam form and the formation of the covalent C-S bonding of the inhibitor to the target-enzyme.

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[1] J. B. Ohlrogge, Proc. Natl. Acad. Sci. U.S.A. **76,** 1194–1198 (1979).
[2] J.-J. Bessoule, R. Lessire, M. Rigoulet, B. Guerinand, and C. Cassagne, FEBS Lett. **214,** 158–162 (1987).
[3] K. Kobek, M. Focke, and H. K. Lichtenthaler, G. Retzlaff, and B. Würzer, Physiol. Plant. **72,** 492–498 (1988).
[4] T. Hata, Y. Sano, A. Matsumae, Y. Kanio, S. Nomura, and R. Nugawara, Nippon Saiking Oku Zasshi **15,** 1075–1076 (1960).
[5] N. Sakthivel and S. S. Gnanamanickam, Current Science **55,** 988–989 (1986).
[6] G. D'Agnolo, J. Rosenfeld, and P. R. Vagelos, J. Biol. Chem. **250,** 5289–5294 (1975).
[7] A. Kawaguchi, H. Tomoda, S. Nozoe, S. Omura, and S. Okuda, J. Biochem. **92,** 7–12 (1982).
[8] H. Funabashi, I. Shigeo, and S. Omura, Tetrahedron Lett. **1983,** 2673–2676.
[9] D. Vance, I. Goldberg, O. Mitsuhoshi, K. Bloch, S. Omura, and S. Nomura, Biochem. Biophys. Res. Commun. **48,** 649–656 (1972).
[10] J. G. Jaworski, E. Goldschmidt, and P. K. Stumpf, Arch. Biochem. Biophys. **163,** 769–776 (1974).
[11] A. Feld, K. Kobek, and H. K. Lichtenthaler, Z. Naturforsch. **44c,** 976–978 (1989).
[12] I. Nishida, A. Kawaguchi, and M. Yamada, J. Biochem. **99,** 1447–1454 (1986).
[13] M. Focke, A. Feld, and H. K. Lichtenthaler, Z. Naturforsch. **45c,** 518–520 (1990).
[14] M. Kato, I. Nishida, A. Kawaguchi, and M. Yamada, Plant Cell Physiol. **28,** 851–855 (1987).
[15] S. Kauppinen, M. Sigaard-Andersen, and W. von Wettstein-Knowles, Carlsberg Res. Commun. **53,** 357–370 (1988).
[16] N. M. Packter and P. K. Stumpf, Arch. Biochem. Biophys. **167,** 655–667 (1975).
[17] T. Shikimata and P. K. Stumpf, Plant Physiol. **69,** 1257–1262 (1982).
[18] J. K. Jaworski, R. C. Clough, and S. R. Barnum, Plant Physiol. **90,** 41–44 (1989).
[19] H. Ohrui and S. Emoto, Tetrahedron Lett. **1978,** 2095–2098.
[20] J. R. Poughny and P. Sinary, Tetrahedron Lett. **1978,** 3301–3304.
[21] J. P. Vigneron and J. M. Blanchard, Tetrahedron Lett. **1980,** 1739–1742.
[22] A. A. Jakubowski, F. S. Guziec, M. Sagiura, C. Chan Tam, and M. Tishler, J. Org. Chem. **47,** 1221–1228 (1982).
[23] S. Omura, Meth. Enzymol. **72,** 520–532 (1981).
[24] T. Sano, S. Nomura, Y. Kamio, S. Omura, and T. Hata, J. Antibiotics **20,** 344–348 (1967).
[25] A. Funabashi, A. Kawaguchi, H. Tomoda, S. Omura, S. Okuda, and S. Iwasaki, J. Biochem. **105,** 751–755 (1989).
[26] A. A. Jakubowski, F. S. Guziec, J. Tishler, and M. Tishler, Tetrahedron Lett. **1977,** 2399–2402.
[27] K. Kobek, M. Focke, and H. K. Lichtenthaler, Z. Naturforsch. **43c,** 47–54 (1988).